Effect of Lysosomotropic Amines on the Secretory Pathway and on the Recycling of the Asialoglycoprotein Receptor in Human Hepatoma Cells

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ABSTRACT We studied the intracellular transport of secretory and membrane proteins in the human hepatoma cell line HepG-2 infected with vesicular stomatitis virus. Cells were pulse-labeled in the presence of [^{35}S]methionine and chased in the presence of the lysosomotropic agent primaquine. At a concentration of 0.3 mM primaquine effectively inhibited the secretion of albumin and, to a lesser extent, that of orosomucoid and transferrin. The drug also prevented the budding of virus particles at the cell surface. The intracellular transport to the Golgi complex of the membrane protein VSV-G was not affected by primaquine as it acquires resistance to endo-β-*N*-acetylglucosaminidase H at the same rate as in control cells. Addition of primaquine at various times after the initiation of the chase period indicates that the effect of primaquine occurs just before secretion. In confirmation of the biochemical data, immunocytochemical localization of albumin in cells treated with NH_4Cl demonstrated that albumin accumulated in vesicles at the *trans* side of the Golgi complex. The effect of primaquine on secretion was also compared with its effect on receptor recycling. The dose-response characteristics of the effect of primaquine on receptor recycling are identical to those of the effects on protein secretion and virus budding. These results indicate that both processes involve the same transport mechanism, and/or that they occur via at least one identical intracellular compartment.

Secretory and plasma membrane proteins are transported from the rough endoplasmic reticulum (RER)[1] via the Golgi complex to the cell exterior. The transport rate between the RER and the Golgi complex differs for individual proteins and varies between 20 and 120 min. In rat and human hepatoma cells infected with vesicular stomatitis virus (VSV), albumin and the viral membrane protein VSV-G pass through the Golgi complex within 20 min after synthesis, whereas transferrin remains in the RER for almost 2 h before acquisition of the complex sugar configuration (29). After passage through the Golgi complex secretory and membrane (glyco)proteins rapidly (within a few minutes) reach the plasma membrane.

[1] *Abbreviations used in this paper:* ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; endo H, endo-β-*N*-acetylglucosaminidase; RER, rough endoplasmic reticulum; VSV, vesicular stomatitis virus.

Another intracellular pathway in which proteins are specifically targetted along specific routes is that of receptor-mediated endocytosis of extracellular ligands. The occurrence of receptor recycling has been inferred from studies of a variety of receptor-ligand systems in many different cells, i.e., the receptor-mediated systems for delivery of low density lipoprotein, transferrin, lysosomal enzymes, and asialoglycoproteins (ASGPs) in hepatocytes. During receptor-mediated endocytosis, a ligand binds to a specific cell surface receptor, and the receptor–ligand complex is internalized via a coated pit-coated vesicle pathway. Many ligands dissociate from their receptor in a prelysosomal, acidic compartment and allow the receptors to recycle back to the cell surface (1, 27). Neither the underlying mechanism nor the route for recycling of receptors back to the plasma membrane is known. From studies of the asialoglycoprotein receptor in a human hepa-

tocyte-derived cell line, hepatoma HepG2, it is known that a single receptor molecule can recycle from the cell surface into a compartment of uncoupling receptor and ligand (CURL) within the cell and back to the cell surface within 8 min (6, 9, 22). Acidification within (some of) the endocytotic structures may play a role in ASGP-receptor recycling (25, 33).

Both isolated endosomal and Golgi vesicles contain an H^+ pump that results in acidification of these compartments (11, 35, 36). Lysosomotropic agents such as the weak bases chloroquine, primaquine, and NH_4Cl raise the pH of acidic intracellular compartments. Studies following the fate of receptors and labeled ligands demonstrate that these agents interrupt recycling of some receptors with their consequent accumulation in an endosomal compartment (3, 12, 25). These observations suggest that the lysosomotropic amines inhibit the pathway by which the receptors return to the plasma membrane.

In the present study we have examined the effect of primaquine and NH_4Cl on the intracellular transport of secretory proteins and of the transmembrane glycoprotein VSV-G in human hepatoma HepG2 cells infected with vesicular stomatitis virus. In primaquine-treated cells the intracellular transport of secretory and membrane proteins is inhibited as compared with untreated cells. However, the degree of inhibition varies among the proteins examined. Both biochemical and immunocytochemical findings demonstrate that the block is localized at or just distal to the *trans* side of the Golgi complex. In addition we have compared the effect of primaquine on protein secretion with its effect on the recycling of the ASGP-receptor.

MATERIALS AND METHODS

Cells and Viruses: The human hepatoma cell line HepG2 (clone a16) was cultured in monolayer in Eagle's minimal essential medium containing 10% decomplemented fetal bovine serum (23). The Indiana serotype of VSV was originally obtained from C. Pringle (Institute of Virology, Glasgow, U.K.). Maintenance of the cells and virus infection were detailed earlier (23, 29).

Labeling of Cells: Almost confluent HepG2 cells grown on 35-mm petri dishes were pulse-labeled with [^{35}S]methionine (20–30 μCi/ml) (800–1,200 Ci/mmol; The Radiochemical Centre, Amersham U.K.), chased with unlabeled methionine, and lysed in 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline (PBS). Primaquine (0.3 mM) was added after the radioactive labeling and was generally present throughout the entire chase period.

Immunoprecipitation and SDS PAGE: Aliquots of the Triton X-100–soluble material were immunoprecipitated with either normal rabbit IgG (control) or affinity purified rabbit anti-rat ASGP-R, rabbit anti-human orosomucoid, rabbit anti-human albumin, rabbit anti-human transferrin (DAKOPATTS, Denmark), monoclonal anti-VSV-G (clone c-1, a-7, kindly provided by Dr. M. Pasternak, Massachusetts Institute of Technology). Gel electrophoresis was performed in 10% polyacrylamide gels in the presence of SDS. Immunoprecipitations were performed as previously described (28). After electrophoresis the gels were fluorographed and the fluorograms were scanned with a microdensitometer (E-C Apparatus Corp., St. Petersburg, FL) within the linear range of the film and the microdensitometer. Endo-β-*N*-acetylglucosaminidase H (endo H) digestion of immunoprecipitated proteins was performed as previously described (29).

Binding of ^{125}I-Asialoorosomucoid (^{125}I-ASOR) to Cell Surface: The binding of ^{125}I-ASOR to HepG2 cells at 4°C has been described (23); specific binding is defined as the difference of binding of ^{125}I-ASOR in the absence and presence of 100-fold excess unlabeled ASOR. Binding was assayed in Eagle's minimal essential medium containing 10 mM HEPES (pH 7.3) during a 2-h period.

Fixation and Immunocytochemistry: Cells attached to culture dishes were fixed in 1% acrolein in 0.1 M sodium phosphate buffer, pH 7.4, for 30 min and thereafter in 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 60 min at 4°C. The cells were then rinsed twice with the buffer, scraped off of the dish, and resuspended in 10% gelatin (37°C). After 2 min the suspension was centrifuged and the pellet was flattened at 0°C to provide a very thin layer of solidified gelatin containing the fixed cells. Cells were stored in 8% paraformaldehyde and 1 M sucrose at 4°C.

Cryosectioning, immunolabeling, staining, and embedding were carried out as detailed previously (9). Double-immunogold labeling was performed as described (10) with two sizes of monodisperse colloidal gold particles, prepared according to Slot and Geuze (26). Affinity purified antibodies were applied at a concentration of 0.05 mg/ml. Control sections were treated with affinity purified anti rat pancreatic amylase. Background labeling was negligible.

RESULTS

Effect of Primaquine on Secretion and Virus Production

To study the effect of primaquine on intracellular transport of secretory and membrane proteins HepG2 cells were infected with VSV and pulse-labeled in the presence of [^{35}S]-methionine. Fig. 1. shows that both viral proteins and hepatoma specific secretory proteins appear in the tissue culture medium by 40 min of chase. Except for membrane glycoprotein G, viral proteins are incorporated into virus particles after short chases (radioactive VSV-M and VSV-N (NS) are clearly present). After longer chases various hepatoma products are secreted, albeit at different rates. In the presence of 0.3 mM primaquine the secretory process is markedly inhibited. Essentially no radioactive labeled albumin and VSV-G are present in the media after 3 h of chase. In addition, the process of virus budding is substantially reduced, as seen by the amount of VSV-L in the media. Quantitative immunoprecipitation of specific proteins present in the cells after various chase times is shown in Fig. 2. The effect of primaquine on the secretion of individual proteins varies. Whereas albumin secretion is almost totally inhibited in the presence of 0.3 mM primaquine, the release of orosomucoid and transferrin is only delayed: half of the newly synthesized orosomucoid is normally secreted in <60 min, whereas in the presence of primaquine >120 min are required for secretion of 50% of this protein. A similar delay is observed for the secretion of α_1-antitrypsin (not shown).

Primaquine Does Not Interfere with Protein Transport between the RER and the Golgi Complex

We have used the enzyme endo H as an analytical tool for transport of VSV-G from the RER to the Golgi complex. During this movement from the RER and through the Golgi complex, *N*-linked oligosaccharides undergo a number of modification reactions, including removal of three glucose and six mannose residues, and the subsequent addition of acetylglucosamine, galactose, and sialic acid. The enzymes involved in these reactions are presumably present at specific sites along the transport route through the RER and the Golgi complex (4, 31). The acquisition of resistance to endo H is thought to take place in the middle of the Golgi stack. Fig. 3 shows that in the presence or absence of primaquine the membrane protein VSV-G acquires resistance to endo H. This indicates that primaquine neither affects the initial processing of *N*-linked oligosaccharides nor alters the time required to reach the processing sites in the Golgi complex. Both in the presence and absence of primaquine more than 90% of VSV-G becomes endo H-resistant within 20 min of chase.

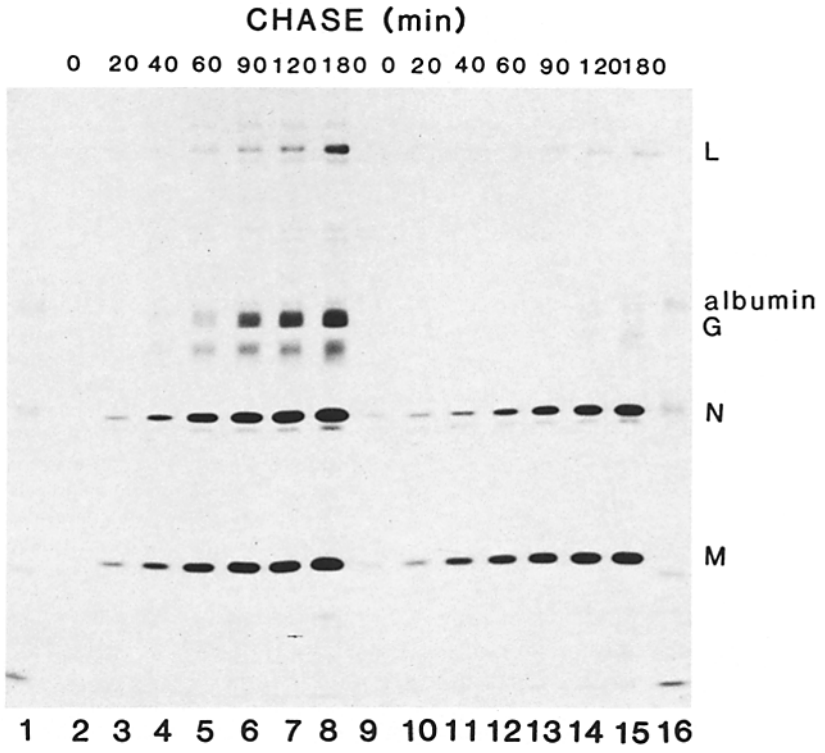

FIGURE 1 Time course of secretion of [^{35}S]-methionine-labeled proteins into the medium. HepG2 cells grown on 35-mm plates were infected with VSV, pulse labeled for 10 min, and then chased in the presence of 1 ml unlabeled chase medium. At the indicated times 10-μl samples were taken and analyzed on polyacrylamide gels. The 2,5-diphenyloxazole impregnated gel was dried and exposed to x-ray film for 3 d. Lanes *2–8* show patterns of control cells. Lanes *9–15* show patterns of cells chased in the presence of 0.3 mM primaquine. Lanes *1* and *16* show molecular weight standard proteins. The VSV proteins and albumin are indicated to the right of the figure.

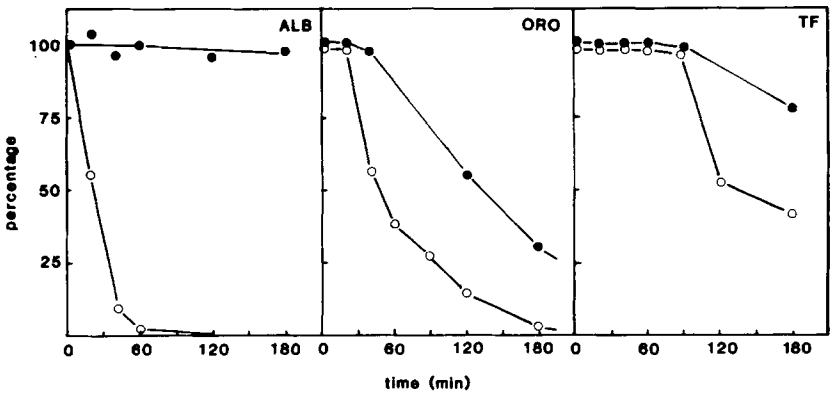

FIGURE 2 Effect of primaquine on protein secretion. VSV-infected cells were pulse-labeled and chased as described in the legend to Fig. 1. Proteins were immunoprecipitated from the lysed cells and analyzed on polyacrylamide gels, the gels were fluorographed, and the fluorograms were scanned. The relative amounts of radioactive protein are plotted as percentage of radioactivity initially present in the cells. O, control cells; ●, primaquine-treated cells. *ALB*, albumin; *ORO*, orosomucoid; *TF*, transferrin.

Effect of Primaquine Removal on the Appearance of Secretory and Membrane Proteins at the Cell Exterior

The transport rate of glycoproteins between the RER and the Golgi complex was probed by the susceptibility to the enzyme endo H. These studies demonstrate that primaquine does not interfere with either the kinetics or route of the (glyco)proteins in this portion of their intracellular transport. To dissect further the localization of primaquine action we determined the effect of removal of the drug on the transport rate of newly synthesized proteins. VSV-infected HepG2 cells were pulse-labeled in the presence of [^{35}S]methionine and then chased in the presence or absence of primaquine for 2 h (Fig. 4). In control cells (without primaquine) (Fig. 4, lanes *1–8*) secretory proteins and virus particles began to appear in the medium within 30 min. Whereas some proteins reached a plateau shortly thereafter, other proteins initially appeared only after 60 or 90 min and did not reach a plateau even after 2 h of chase. In the presence of primaquine very little radioactive protein became apparent in the medium after 2 h of chase (Fig. 4, lane *9*). However, removal of primaquine from the cells was followed by a rapid secretion of the labeled proteins: after 5 or 10 min most proteins are easily identified in the medium. Quantification of the secretory products shows that the kinetics of secretion and virus budding are completely different in control and primaquine-treated cells (Fig. 5). In the control situation individual proteins exhibit different rates of secretion, whereas after primaquine removal all secretory proteins leave the cells in synchrony. The effect of the removal of primaquine on protein secretion and on virus budding is again identical. Recovery of the cells after primaquine incubation for 2 h apparently is neither instantaneous nor complete, as it takes a considerable time for the proteins to be completely secreted. However, it is clear that incubation with primaquine does not interfere with the transport of membrane protein (i.e., VSV-G) or the secretory proteins through the intracellular site at which these proteins are retarded differently on route to the plasma membrane. This site presumably lies before the Golgi complex (14).

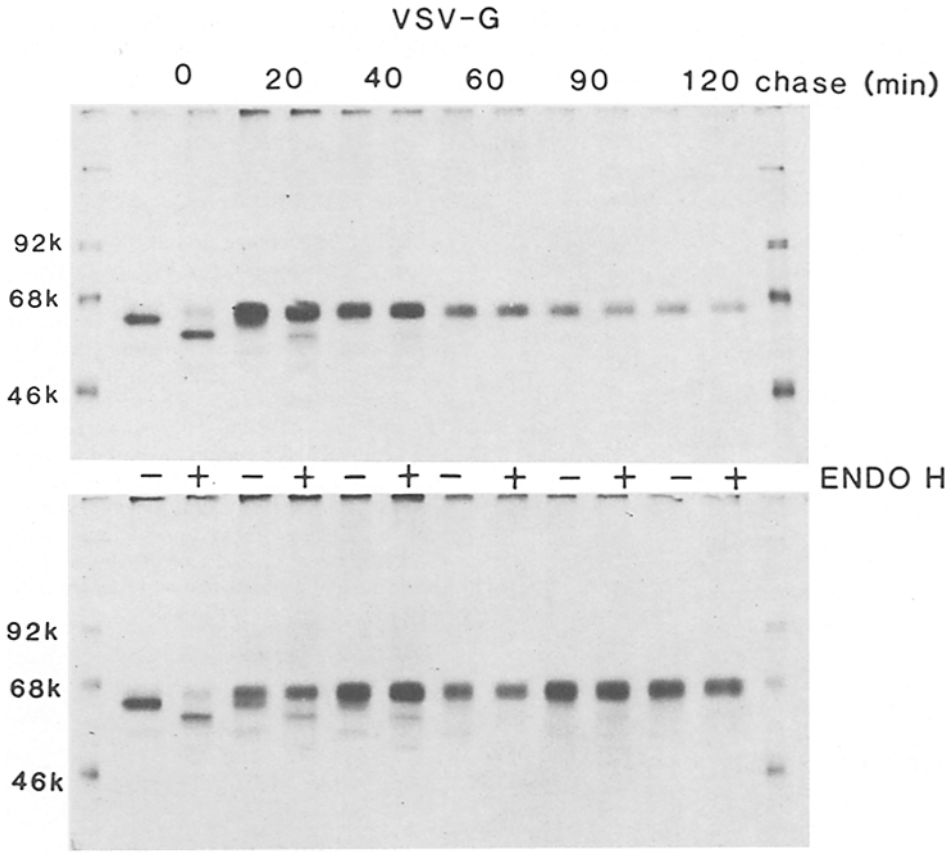

FIGURE 3 endo H digestion of VSV-G. Labeling and sample preparation were as described in the legend to Fig. 1. The chase times were as indicated in the figure. After immunoprecipitation each sample was digested for 18 h in the presence or absence of endo H before separation on gels as described in Materials and Methods. *Top*, control cells; *bottom*, primaquine-treated cells.

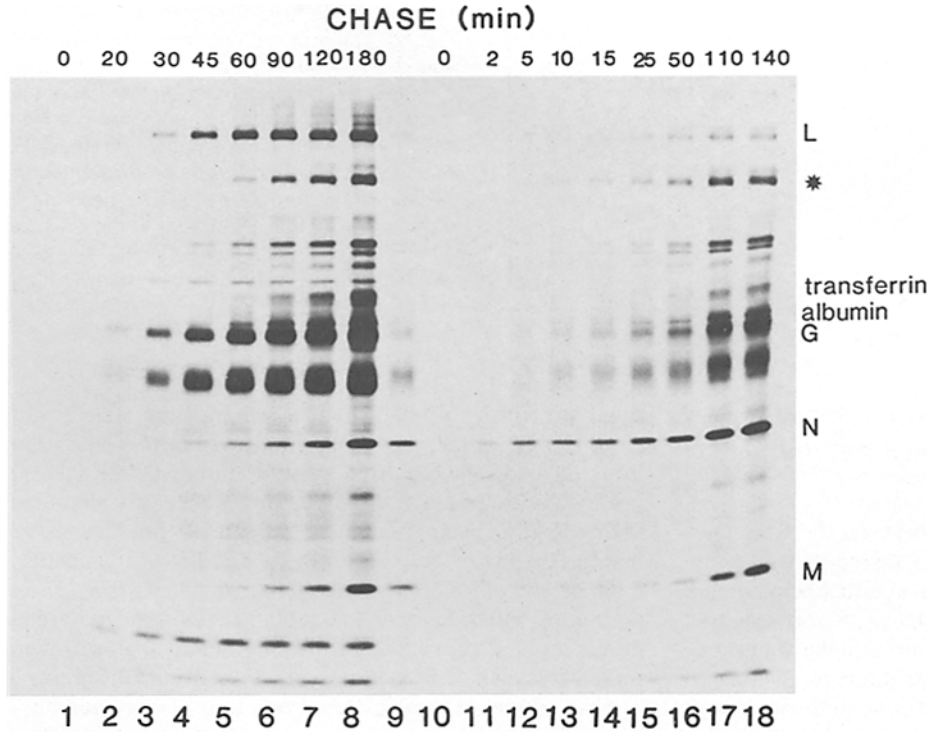

FIGURE 4 Effect of removal of primaquine on secretion. After VSV-infected cells were pulse-labeled for 10 min, the medium was replaced by 3-ml chase medium containing 50 μM unlabeled methionine and 0.3 mM primaquine. The cells were chased for 2 h at 37°C; then a 10-μl sample was taken for analysis by gel electrophoresis (lane 9). Thereafter the cells were put on ice and washed three times with 5 ml PBS (pH 7.4) at 0°C for 5 min each to remove primaquine from the cells. 3 ml prewarmed chase medium (without primaquine) was then added, and 10-μl samples were taken at the times indicated in the figure for analysis by gel electrophoresis (lanes *10–18*). In control cells (no primaquine added) 10-μl samples were taken at the indicated times after the pulse labeling (lanes *1–8*). The VSV proteins and albumin are indicated to the right of the figure. ★, see legend to Fig. 5.

Where Does Primaquine Affect the Transport of Secretory Proteins?

Thus far, the kinetic data localize the intracellular site of primaquine action to a position distal to both the RER and the *cis*-Golgi. To obtain a more precise picture of the primary effect VSV-infected HepG2 cells were pulse-labeled in the presence of [^{35}S]methionine and then chased for 2 h. At different times 0.3 mM of primaquine was added. Because the effect of primaquine on intracellular transport is almost instantaneous (1–2 min; reference 25) this experiment allows a direct determination of the time at which primaquine affects transport. The results are presented in Figs. 6 and 7. The addition of primaquine after 10 or 20 min of chase prevented

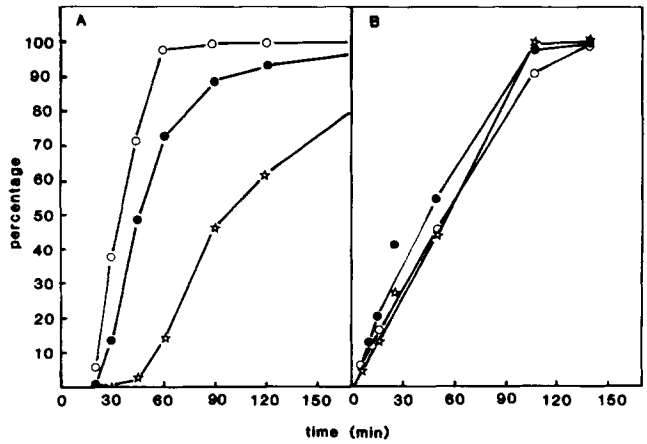

FIGURE 5 Time course of secretion after removal of primaquine. The proper exposure of the gel used in Fig. 4 (lanes *10–18*) was scanned and the amounts of labeled protein were quantitated and plotted in the figure. The kinetics of secretion of albumin and VSV-G were identical in Fig. 4; O, albumin and VSV-G; ●, VSV-L; ☆, protein indicated with a star in Fig. 4. *A*, control cells; *B*, cells chased in the presence of primaquine as described in the legend to Fig. 4.

secretion almost totally during the remaining 100 or 110 min of chase. However, primaquine addition after 30 min failed to alter the appearance of most secretory proteins in the medium. Quantitation of the disappearance from the cells of albumin, orosomucoid, and transferrin after immunoprecipitation and gel electrophoresis (Fig. 7) allows the determination of the precise time after the initiation of the chase period at which the drug maximally interferes with the intracellular transport. The time at which 50% of the pulse-labeled albumin has passed the intracellular site of primaquine action is 24 min; orosomucoid requires 70 min and transferrin 115 min to arrive at this point. There is a remarkable similarity between these values and the times required for the secretion of these proteins. As the transport of secretory proteins in hepatoma cells is dependent mainly on the time required for transport between the RER and the Golgi complex (29), it is clear that primaquine exerts its effect after the proteins have left the intracellular compartment where differences in transport rate arise.

Immunoelectron Microscopy

Immunocytochemistry of VSV-infected HepG2 cells makes it possible to characterize morphologically the site at which primaquine affects the intracellular transport. For these experiments we used NH_4Cl as the lysosomotropic amine, since primaquine inhibits protein synthesis substantially at the concentration required to inhibit secretion (0.3 mM). At a concentration of 10 mM NH_4Cl albumin synthesis is essentially unaffected, whereas its secretion is inhibited by 72%. By its immunogold labeling pattern in ultrathin cryosections we could localize the main concentration of albumin in control cells in the Golgi complex (Fig. 8*A*). In cells grown in the presence of NH_4Cl larger vacuoles occurred, often at the *trans* side of the Golgi complex, in which albumin appeared to accumulate. As we pointed out in the introduction, lysosomotropic agents such as primaquine and NH_4Cl not only interfere with intracellular transport of secretory and membrane proteins, but they also dramatically affect the receptor-mediated endocytosis. Thus, we have compared the effect of lysosomotropic agents on the cellular distribution of albumin with that of the ASGP receptor. From sections co-labeled for albumin and the ASGP-receptor it is clear that both labels are present in the same diluted vesicles distal to the Golgi complex (Fig. 8*B*). Because of this localization these vesicles are probably *trans*-Golgi reticulum or secretory vesicles. Both albumin and ASGP-receptor labeling is scarce over the Golgi complex. These results indicate that after 2 h of NH_4Cl treatment albumin accumulates in vesicles between the Golgi complex and the cell surface but not in the Golgi complex.

Comparison of the Drug Effects on Secretion and ASGP-Receptor Recycling

Studies of receptor-mediated endocytosis have shown that weak bases such as primaquine prevent the dissociation of

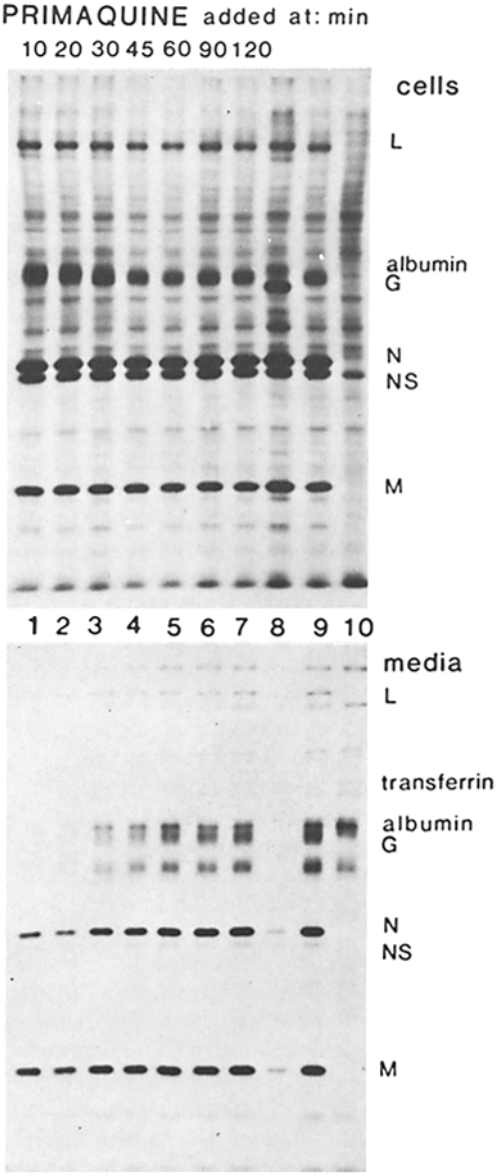

FIGURE 6 Effect of primaquine on intracellular transport. VSV-infected cells were labeled as described in the legend to Fig. 1 and chased for 120 min. At the indicated times primaquine was added to the chase medium (1 ml). The cells were lysed in 0.5 ml lysis buffer and 10 μl was used for gel analysis (*top*). 10-μl aliquots of the media were also applied on a gel (bottom; lanes *1–7*). Lane *8*, cells and medium after a 10-min pulse and a 10-min chase; lane *9*, the same as lane *7*, no primaquine added; lane *10*, the same as lane *7*, but uninfected cells were used. The VSV proteins and albumin are indicated to the right of the figure.

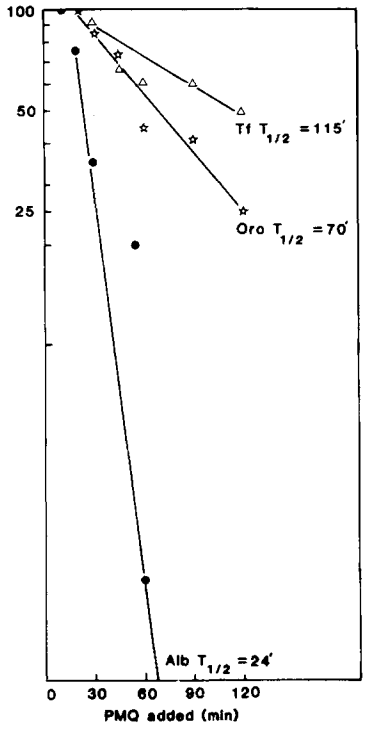

FIGURE 7 Effect of primaquine (*PMQ*) on secretion of albumin (*Alb*), orosomucoid (*Oro*), and transferrin (*Tf*). These three proteins were quantitatively isolated by immunoprecipitation from the cell lysates obtained from the experiment described in the legend to Fig. 6. After gel electrophoresis and fluorography the bands were scanned. The relative amounts of radioactivity are plotted in the figure.

ligand-receptor complexes within the endosomal compartment (CURL). This probably results from neutralization of the acid environment normally present in this compartment. In addition, in HepG2 cells in the absence of ligand primaquine also prevents the recycling back to the surface of the internalized ASGP receptor. However, this drug does not influence the rate at which the receptor enters the cell (25). This behavior has enabled us to compare the effect of primaquine on the intracellular transport of secretory proteins (and virus budding) with its effect on the return of recycling receptors to the the plasma membrane. VSV-infected HepG-2 cells were incubated at 37°C in the presence of various concentrations of primaquine for 2 h in order to reach equilibrium. Thereafter the cells were chilled on ice and the number of cell surface ASGP-receptor binding sites was determined using ^{125}I-ASOR. Since the number of receptors at the cell surface is determined by the concentration of primaquine, we can directly determine the dose-dependence of the return of receptor to the cell surface (Fig. 9*B*). Fig. 9*A* shows the dose-dependent effect of primaquine on protein secretion and virus budding. Primaquine affects both ASGP-receptor recycling and protein secretion in exactly the same way, supporting the notion that both processes make use of a common mechanism.

DISCUSSION

The present results demonstrate that lysosomotropic agents such as NH_4Cl and primaquine inhibit the intracellular transport of secretory and membrane proteins on route to the plasma membrane in a post-Golgi compartment. We have defined the site of primaquine action by three independent criteria: biochemically by the conversion of a "high mannose" to a "complex" sugar configuration of *N*-linked oligosaccharides as probed by sensitivity to endo H; kinetically by defining the actual intracellular transport rates; and morphologically by electron microscopic immunocytochemistry. The secretory transport route consists kinetically of at least two parts: from the RER to the Golgi complex and from the Golgi complex to the plasma membrane (21, 27). After synthesis in the RER (glyco)proteins are transported to the Golgi complex at different rates, presumably directed by interactions with components of the RER membranes. In some way these interactions induce differences in the transport rates for individual proteins, causing transport times to vary from 20 min for albumin to 2 h for transferrin (8, 14). The second portion of the secretory pathway (Golgi complex–plasma membrane) may be either "regulated" or "constitutive" (30). The regulated form of secretion occurs mainly in cells such as the pancreatic acinar cells, which after storage in granules secrete their proteins upon stimulation by calcium or cyclic AMP. The "unregulated" or "constitutive" pathway occurs in fibroblasts and macrophages and is insensitive to regulation by cyclic AMP or calcium. Hepatocytes and hepatoma cells secrete serum proteins as albumin, tranferrin, and α_1 antitrypsin in a constitutive manner. Pulse–chase experiments have shown that the transport from the Golgi complex to the plasma membrane is bulk-phase and that the time needed to reach the plasma membrane is relatively short (<5 min at 37°C) (14, 24, 29). Primaquine primarily affects the second portion of the transport route. Our experiments demonstrate that addition of the drug at the end of the pulse labeling causes considerable delay in the secretion of albumin, orosomucoid, and transferrin. Moreover, the process of virus budding is blocked almost instantaneously. Moore et al. demonstrated that chloroquine at a concentration of 0.2 mM diverts ACTH from a regulated to a constitutive pathway in AtT-20 cells. Whether this lysosomotropic agent also affects the rate of ACTH secretion is not clear (16).

The mechanism of action of the lysosomotropic agents has been the subject of intensive investigation (reviewed in reference 7). This class of drugs inhibits lysosomal protein degradation at low concentrations (5, 34) and neutralizes the acidic environment in lysosomes and endosomes (15, 18, 19, 32). Many of these actions may be a direct consequence of protonation within acidic intracellular compartments. Inhibition of transport by primaquine and NH_4Cl suggests that the secretory proteins pass through such a compartment. Furthermore, the kinetics of this inhibitory effect provides evidence for the intracellular localization of such a compartment. However, the inhibition of secretory transport is not identical for all proteins. Recently Oda et al. described the effect of NH_4Cl on protein secretion (17). Consistent with our results, NH_4Cl only partly inhibited α_1-antitrypsin and total secretion in rat hepatocytes. It is possible that some transport to the plasma membrane continues to occur at the primaquine concentration used. This possibility is consistent with the slow and continued accumulation of labeled virus particles in the tissue culture medium in the presence of primaquine (Fig. 1). It is not now clear why the inhibition is different for individual proteins.

We have compared the effect of primaquine on protein secretion to its effect on ASGP receptor movement and localization. As we reported previously primaquine induces a

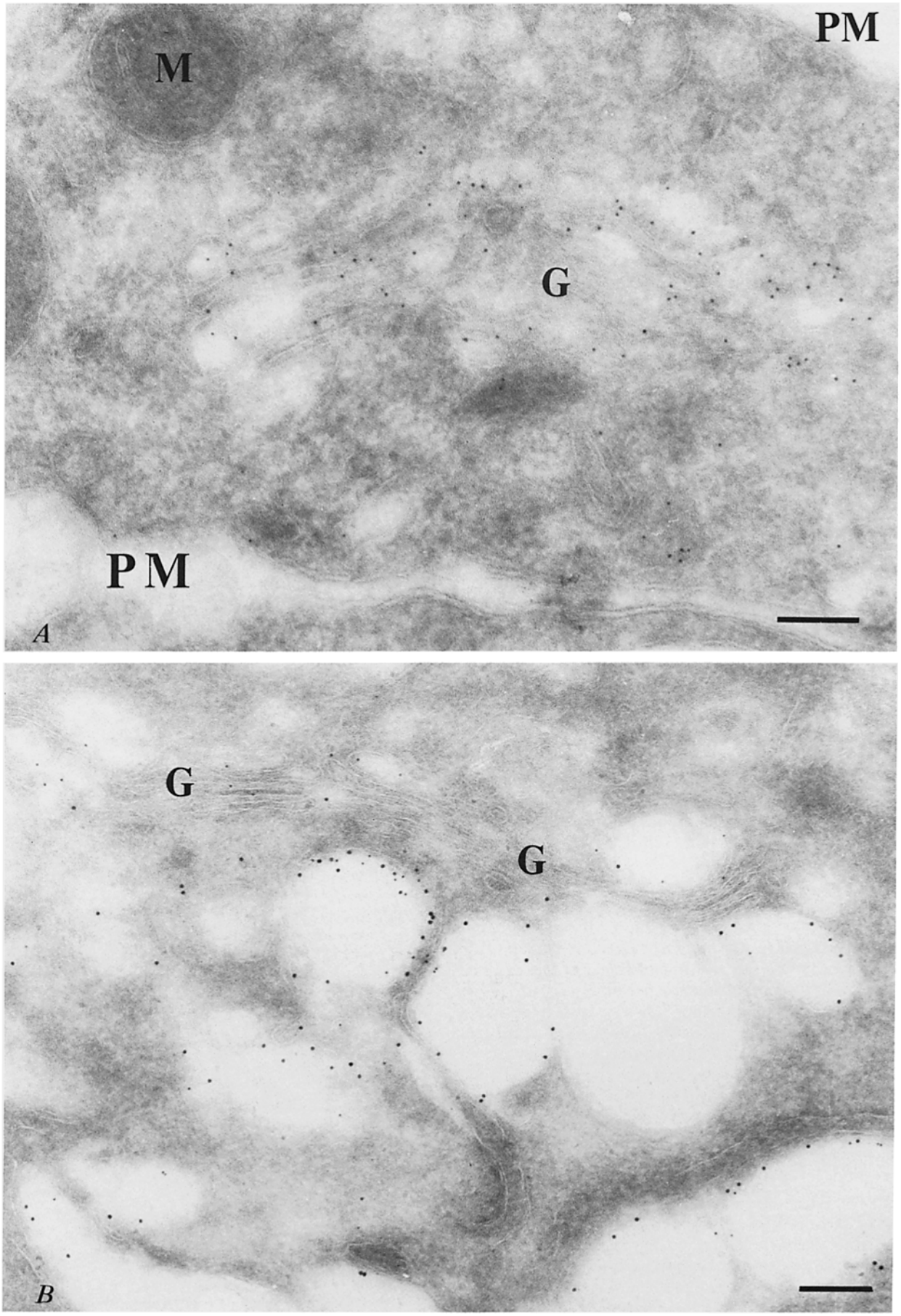

FIGURE 8 (*A*) Ultrathin cryosections of control cell, labeled for albumin with 6-nm gold particles. Albumin is predominantly located in the Golgi cisternae (*G*). *M*, mitochondrion; *PM*, plasma membrane. Bar, 0.2 μm. × 75,000. (*B*) Section of a cell incubated for 2 h with 10 mM NH_4Cl. Albumin is labeled with small (6 nm) and ASGP-receptor with large (9 nm) particles. Both albumin and the ASGP-receptor occur in vacuoles at the *trans* side of the Golgi system, sometimes clearly mixed together. Bar, 0.2 μm. × 62,500.

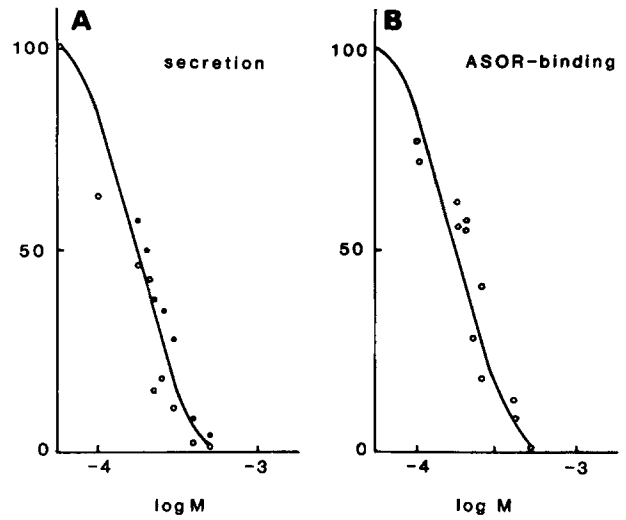

FIGURE 9 Comparison of the effect of primaquine on the secretion of proteins and binding of ^{125}I-ASOR to the HepG2 ASGP-receptor. Cells were pulse-labeled for 10 min and then chased for 120 min in the presence of various concentrations of primaquine as indicated in the figure. Albumin (○) and VSV-G (●) were quantitated by scanning of the fluorographed bands, and the results are expressed as the percentage of the amount secreted in the absence of the drug (*A*). For the binding studies HepG2 cells were incubated at 37°C for 120 min in the presence of various concentrations of primaquine. Thereafter binding of ^{125}I-ASOR was performed at 4°C in the absence and presence of excess unlabeled ASOR as described in Materials and Methods. The specific binding is plotted as a function of primaquine concentration (*B*).

rapid loss of ASGP receptor from the cell surface of HepG2 cells in a dose-dependent and reversible manner. This drug also prevents the return of the receptor to the cell surface whether or not ligand is present. However, primaquine has neither an effect on ligand binding to receptor nor on internalization of the receptor or the receptor–ligand complex (25). Thus by determining the amount of ASGP binding sites at the cell surface as a function of primaquine concentration it is possible to determine the effect of the drug on receptor return to the plasma membrane. Our results show that the primaquine concentrations used to block receptor recycling are identical to those required to achieve inhibition of intracellular transport of (glyco) protein destined for secretion. The effective concentration of the drug is similar to that reported for inhibition of receptor-mediated endocytosis of ligands such as low density lipoprotein, ASOR, and α_2-macroglobulin (2, 13, 25). The striking similarity in inhibition of the two processes suggests a common mechanism. In the case of receptor-mediated endocytosis acidification of the endosomal compartment appears to promote the separation of ligand and receptor as exemplified by studies on the receptors for ASGPs and low density lipoproteins. Morphological studies in hepatocytes also demonstrate that a physical segregation of receptor and ligand occurs in the same acidic compartment (CURL) (9). This sorting process is prevented by lysomotropic agents. Not only is the uncoupling of the receptor–ligand complex abrogated at neutral pH, but in addition the return of unoccupied receptors back to the cell surface is inhibited, resulting in intracellular accumulation of receptors. The precise underlying mechanism of this effect of the lysosomotropic agents remains unknown. Nonetheless, the observation that the constitutive secretion of serum proteins in these cells is inhibited in an identical manner suggests that bulk membrane transport to the cell surface is affected by primaquine. This idea is strengthened by the observation that virus budding is similarly inhibited by these agents, despite the fact that some of the nonintegral viral proteins are present and allow a modest degree of virus budding. Our results are consistent with a recent study of Robbins et al. on the effect of a single mutation in Chinese hamster ovary cells with respect to Golgi complex and endosomal function. They report a striking similarity between the alterations in both endocytotic and Golgi-associated steps of the maturation of viral proteins in a mutant cell line that exhibits a decreased ATP-dependent endosome acidification (20).

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