The glycoprotein of VSV accumulates in a distal Golgi compartment in the presence of CCCP

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Summary

The post-translational modifications of the G protein of vesicular stomatitis virus, described in the preceding paper, indicate that its transport is arrested by carbonylcyanide m-chlorophenylhydrazone (CCCP) in or near the trans-Golgi. Immuno-fluorescence microscopy of BHK-21 cells infected with vesicular stomatitis virus and treated with CCCP shows an accumulation of G protein in the Golgi area. In the same cells, the morphology of wheat germ agglutinin (WGA)-staining structures in the perinuclear region is aberrant. Using anti-BiP antibody, there is no obvious change in the structure of the endoplasmic reticulum. Electron microscopy reveals that the aberrant structures in the perinuclear region result from dilation of Golgi cisternae and accumulation of large vacuoles near the Golgi stack. The appearance of these aberrant structures is dose-dependent and they disappear after the protonophore is removed. The vast majority of the vacuoles accumulate on the trans side of the Golgi stack. A small fraction of them contain the marker enzyme thiamine pyrophosphatase (TPPase). By immunoelectron microscopy, most of the vacuoles contain G protein. We conclude that most of the Golgi-associated vacuoles are derived from a distal Golgi transport compartment, possibly the trans-Golgi reticulum, and that CCCP reversibly inhibits the transport of newly synthesized G protein through this distal compartment.

Key words: CCCP, monensin, trans-Golgi, vacuoles, immunolocalization.

Introduction

Export of membrane and secreted proteins entails their orderly transfer between organelles of the secretory apparatus. The first major transfer is from the endoplasmic reticulum (ER) to the Golgi complex, and the second is between the Golgi complex and the final destination of the proteins. It is during these two transitional transport steps that exported proteins are concentrated (Salpeter & Farquhar, 1981; Quinn et al. 1984), sorted (reviewed by Farquhar, 1985; Pfeffer & Rothman, 1987), and their transport kinetics regulated (Fries et al. 1984; Lodish et al. 1983; Scheele & Tartakoff, 1985). As with any active process, these transitional steps require ATP and are temperature sensitive (Jamieson & Palade, 1968; Balch et al. 1986; Matlin & Simons, 1983; Tartakoff, 1986).

Relatively little is known about the membrane compartments that mediate the transitions from the ER to the Golgi complex and from there to the plasma membrane. Transitional elements are thought to represent structures through which proteins exit from the ER (Palade, 1975), but there is as yet no direct demonstration of such a role for transitional elements. Likewise, the complicated system of membrane vesicles and tubular elements, known as the trans-Golgi reticulum (TGR; Willingham & Pastan, 1984), the trans-Golgi network (TGN; Griffiths & Simons, 1986) or GERL (Novikoff, 1964), is thought to represent the structure where protein traffic exits the Golgi stack (Griffiths & Simons, 1986). There is evidence that a variety of proteins are sorted and packaged into vesicles in the TGR. Condensing vacuoles, which give rise to secretory granules, bud from this part of the Golgi (Hand & Oliver, 1977; Jamieson & Palade, 1968; Novikoff et al. 1977). Lysosomal hydrolases and the mannose 6-phosphate receptor have been localized to its clathrin-coated regions (Geuze et al. 1985). Proteins destined for plasma membrane accumulate in this organelle when their transport is blocked at 20°C (Griffiths et al. 1985; Saraste & Kuismanen, 1984).

To date, the TGR is defined almost exclusively by microscopy. Given its probable role in protein transport, the purification and characterization of the TGR is of great interest. This is difficult, however, because the architecture of the TGR is complicated. It contains...
numerous tubular elements and a variety of vesicles. Some of these vesicles are smooth, some are clathrin-coated, and others bear a different, unidentified coat (Griffiths et al. 1985; Orci et al. 1986). Some of the structures in the region are endocytic (Marsh et al. 1984), while others probably mediate ER-to-Golgi and intra-Golgi traffic. Only a subpopulation is involved in mediating traffic from the Golgi to plasma membrane. Although the TGR contains sialyltransferase and acid phosphatase (Hand & Oliver, 1984b; Novikoff, 1964; Roth et al. 1985), there are at present no markers that are unique to this compartment. For the time being, the TGR and other distal compartments are best defined by the passage of transported proteins.

The protonophore CCCP inhibits the transport of a variety of membrane and secreted proteins at two stages (Tartakoff & Vassalli, 1979; Fries & Rothman, 1980; Godelaine et al. 1981; Argon & Milstein, 1984; Kabacencell & Atkinson, 1985; Argon et al. 1989; Burkhardt & Argon, 1989). The post-translational modifications of the arrested proteins indicate that these two stages correspond to the transitions between the ER and Golgi and the Golgi and the plasma membrane. Because of the specificity of its action, CCCP provides a means of identifying the membrane compartments that correspond to these transport stages, by determining where the arrested proteins accumulate.

The fact that CCCP acts specifically at both transport transitions might indicate a common underlying mechanism. However, because CCCP inhibits two transport stages, the analysis of either one alone is complicated. In the preceding paper (Burkhardt & Argon, 1989), we described a system in which the G protein of vesicular stomatitis virus (VSV) is largely arrested at the late transitional step. The G protein arrested in the presence of CCCP bears sialylated complex oligosaccharides but does not appear on the cell surface. We interpret this to indicate transport of G protein as far as the trans-Golgi.

In this paper, we take advantage of the fact that G protein is refractory to arrest at the first CCCP-sensitive stage, in order to characterize the site of the late transport arrest by microscopy. We show that in the presence of CCCP, G protein indeed accumulates in the trans-Golgi region and provide evidence that the site of accumulation is the TGR. In addition, we describe the disruption of Golgi structure by CCCP, and show that this structural alteration is related to the arrest of protein transport.

Materials and methods

Cell culture, virus infections and drug treatments

BHK-21 cells were grown as described in the preceding paper (Burkhardt & Argon, 1989). The IgD-producing hybridoma B1-8.δ (Neuberger & Rajewsky, 1981) was grown in RPMI-1640 supplemented with glutamine, penicillin, streptomycin, and 5% foetal calf serum. Viral infections were as described (Burkhardt & Argon, 1989). Infections of cells with VSV and treatments with CCCP, monensin and cycloheximide were done as described in the preceding paper (Burkhardt & Argon, 1989).

Light microscopy

Fluoresceinated anti-G (DTAF-H14D5) was prepared as described (Burkhardt & Argon, 1989). Wheat germ agglutinin (WGA) was directly conjugated to rhodamine-B-isothiocyanate (TRITC) by a modification of the procedure described by Godding (1976). WGA binds to glycoproteins with complex carbohydrates and therefore serves as a stain for the Golgi complex, distal exocytic compartments, the plasma membrane and endocytic compartments (Virtaanen et al. 1980). To mark the endoplasmic reticulum, we used a rat monoclonal antibody (MAb) against the resident ER protein BiP (Bole et al. 1986) and a generous gift from Dr David Bole, Yale University). This MAb was detected with MAR18.5, an anti-rat k light-chain MAb (Lanier et al. 1982), tagged with rhodamine as described for WGA. BHK cells were grown to subconfluence on sterile coverslips. After infection and treatment with CCCP, coverslips were rinsed with PBS to remove serum proteins, fixed for 1 h in fresh 2% paraformaldehyde, 0.1% glutaraldehyde in PBS, and washed three times with 50 mM-ammonium chloride in PBS. For immunofluorescence, cells were permeabilized by incubation for 10 min in 1% Triton X-100, 0.25% gelatin, PBS. For direct labelling, coverslips were incubated for 1 h with DTAF-H14D5 or TRITC-WGA, both at 10 μg ml⁻¹ in 0.25% gelatin/PBS, and rinsed six times with the same buffer. For indirect labelling, coverslips were incubated with undiluted anti-BiP culture supernatant, rinsed with gelatin/PBS, and incubated for an additional hour with TRITC-MAR18.5 at 10 μg ml⁻¹. Double-labeling regimes in either order gave the same results as with each reagent separately. Following labeling, coverslips were rinsed briefly in water, mounted on slides with mounting medium containing 2.5% DABCO (Poly-science, Warrington, PA) to minimize fading, and examined with a Zeiss IM35 inverted microscope equipped with epifluorescence optics. Photographs were taken using T-MAX 400 (Kodak, Rochester, NY).

Electron microscopy

Subconfluent BHK cells were washed with balanced salt solution (BSS) and removed from culture dishes by digestion with 50 μg ml⁻¹ proteinase K for approximately 5 min at 4°C. Phenylmethylsulphonyl fluoride was added to 40 μg ml⁻¹. Cells were pelleted and resuspended in a small volume of BSS, and fixed in suspension at room temperature with 2% glutaraldehyde in 150 mM-sodium cacodylate, pH 7.4, 0.01% CaCl₂. After 10 min, the cells were centrifuged and fixed for an additional hour as a pellet. The fixed cell pellets were embedded in 1 % agar and postfixed for 1 h at 4°C with 2% osmium tetroxide, 1% potassium ferrocyanide, 150 mM-sodium cacodylate, pH 7.2. Following washes with sodium cacodylate and sodium acetate, the agar blocks were stained en bloc with 1% uranyl acetate, 0.2 M-sodium acetate, pH 5.2, for 1 h at room temperature. Following washes with sodium acetate and water, the samples were dehydrated through a graded ethanol series and embedded in EMBED 812 (EM Sciences, Fort Washington, PA). Silver sections were contrasted with uranyl acetate and lead citrate, and observed with a Philips EM300 electron microscope at 80 kV.

Enzyme cytochemistry

Thiamine pyrophosphatase (TPPase) cytochemistry was done using the method of Novikoff & Goldfischer (1961), as modified by Hand & Oliver (1984a). Cells washed with BSS were fixed for 10 min in suspension with 1% paraformaldehyde, 1% glutaraldehyde, 0.05% CaCl₂, 0.1 M-sodium cacodylate, pH 7.2. Cells were then washed with cacodylate buffer containing 7% sucrose, embedded in agar, and stored overnight at 4°C.
After several washes in Tris–maleate buffer, agar blocks were incubated in complete reaction mix (2 mM-TPP–HCl, 3.6 mM-maleic acid, 5 mM-MnCl₂, 5% sucrose, 0.1 M-Tris–maleate, pH 7.2) for 1 h at 37°C with shaking. The specificity of this reaction was demonstrated by omission of substrate. The reaction mixture was replaced after 30 min of incubation. Following the reaction, cells were washed several times and treated with 1% ammonium sulphide for light microscopy. They were then postfixed with 2% osmium tetroxide, 1% potassium ferrocyanide, 7% sucrose, 0.1 M-sodium cacodylate, pH 7.2, washed with cacodylate, sucrose and water, and stained en bloc for 1 h with 0.5% aqueous uranyl acetate. Dehydration and embedding was as described above.

**Immunoelectron microscopy**

Immunoelectron microscopy was done by the ultrathin frozen-sectioning procedure of Tokuyasu (1980), as modified by Griffiths et al. (1983b). Cells were washed with BSS and fixed for 10 min at room temperature with 4% paraformaldehyde, 150 mM-Pipes, pH 7.2, followed by overnight fixation in 3% paraformaldehyde in the same buffer. Cells pellets were cryoprotected by infiltration with 2:1 M-sucrose in PBS, and frozen in liquid nitrogen. Sections were cut at -100°C using a Reichert Ultracut E equipped with an FC4E cryoultramicrotome. Sections collected on a loop containing 2:3 M-sucrose in PBS were transferred to Formvar-coated grids and floated onto 5% FCS in PBS as a blocking step. After washing with PBS, grids were incubated on 5 μl drops of H14D5 at 50 μg/ml (diluted in 5% FCS in PBS) for 30 min at room temperature, and washed on several drops of PBS for a total of 15 min. Grids were then incubated on drops of appropriately diluted protein A–colloidal gold (gift from Dr G. Griffiths, EMBL, Heidelberg), and washed again on PBS for 30 min. After a brief water wash, grids were incubated for 10 min at 4°C on drops of 2% methyl cellulose, 0.3% uranyl acetate. Grids were then looped out, excess methyl cellulose was removed, and the grids were dried in a desiccated chamber.

**Results**

**G protein is present in the Golgi region of CCCP-treated cells**

The post-translational modifications borne by CCCP-arrested G protein predict that most of it has reached the Golgi region. The perinuclear distribution of G protein is present in the Golgi region of CCCP-treated cells. The post-translational modifications borne by CCCP-arrested G protein predict that most of it has reached the Golgi region. We used immunofluorescence microscopy to localize the intracellular distribution of G protein, surface G was then postfixed with 2% osmium tetroxide, 1% potassium ferrocyanide, 7% sucrose, 0.1 M-sodium cacodylate, pH 7.2, washed with cacodylate, sucrose and water, and stained en bloc for 1 h with 0.5% aqueous uranyl acetate. Dehydration and embedding was as described above.

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**CPC disrupts WGA-positive structures**

In the course of the co-localization studies, we observed that CCCP treatment alters the structure of some organelles. The reticular anti-BiP staining pattern is not altered significantly by CCCP treatment (Fig. 1, top panel), implying that ER structure is not grossly affected. Staining with TRITC–WGA, however, reveals significant effects of CCCP treatment on cell structure (Fig. 2). The typical pattern of Golgi staining with WGA shows compact perinuclear staining in addition to finer punctate staining that is probably due to endocytic structures. Instead, CCCP-treated cells exhibit large, disperse, WGA-positive patches that are often distributed far from the nucleus (Fig. 2B). This suggests that CCCP treatment disrupts the structure of the Golgi complex or other distal transport organelles. In addition, WGA staining of CCCP-treated cells is invariably more intense than that of control cells, perhaps because CCCP causes the accumulation of cellular glycoproteins in the Golgi region.

It should be noted that the disruption of WGA-positive structures was consistently less severe in VSV-infected cells than in uninfected cells (compare Fig. 1, lower right panel with Fig. 2B). The reason for this 'stabilization' is unknown.

Like the inhibition of G protein transport, the morphological alteration induced by CCCP treatment can be reversed by washing out the protonophore. To quantify the reversibility, we scored the disruption of WGA-
Fig. 1. Localization of G protein by double-label immunofluorescence. VSV-infected BHK cells were either not treated or treated for 60 min with CCCP (20 μM). Fixed and permeabilized cells were then double-labelled with DTAF-anti-G and anti-BiP/TRITC-anti-κ (top panel), or with DTAF-anti-G and TRITC-WGA (bottom panel). The reactivity due to cell surface G was reduced by incubation with unlabelled anti-G prior to permeabilization. Staining of the nuclear envelope is marked with an arrowhead.
stained structures, by determining the percentage of cells displaying large, WGA-positive patches >0.5 of a nuclear diameter away from the nucleus. Samples that were treated with 20 μM-CCCP for 90 min contained 84% such cells (n = 495). Only 8% of untreated cells had this distribution (n = 391). If treated cells were incubated for one hour in the absence of CCCP, this fraction decreased to 30% (n = 217), showing that the effect is reversible.

Specific disruption of Golgi ultrastructure
The morphological effects of CCCP treatment were defined further by analysis at the ultrastructural level. As predicted by the immunofluorescence results, we observed that CCCP alters the ultrastructure of the Golgi complex without affecting the structure of the ER. With the exception of the expected changes in mitochondria (Hackenbrock, 1968) other organelles appear normal. As compared to untreated controls (Fig. 3A), BHK cells treated for 90 min with CCCP exhibit both dilation of Golgi cisternae and pronounced accumulation of large vacuoles in the Golgi area (Fig. 3B). In these cells, some vacuoles are distributed further from the Golgi region, accounting for the patchy fluorescence that we observe with WGA labelling.

The morphological effects of CCCP are not limited to BHK cells. Under conditions where CCCP arrests the secretion of immunoglobulin by hybridoma cells (Argon & Milstein, 1984), we observe a similar disruption of Golgi ultrastructure (Fig. 4A and B). As in BHK cells, the Golgi cisternae are dilated and vacuoles accumulate in the Golgi region. Another feature of CCCP treatment is that the Golgi cisternae usually remain in close apposition, keeping the stack structure largely intact. In this respect the effects of CCCP are distinct from those of monensin, which under similar conditions completely disrupts the structure of the Golgi stack (compare Fig. 4B and C). As compared with BHK cells, the CCCP-induced vacuoles in hybridoma cells are more closely confined to the Golgi region. This makes it more apparent that the vacuoles accumulate preferentially on one side of the Golgi stack (see below).

The disruption of the Golgi complex by CCCP is not due to the accumulation of transported proteins. If hybridoma cells are treated with cycloheximide to block protein synthesis, and then treated with CCCP, the disruption of Golgi ultrastructure is the same as with CCCP alone (Fig. 4D). Treatment with cycloheximide alone does not alter Golgi structure (data not shown).

In order to characterize the dose-dependence of CCCP's effects on Golgi structure, B1-8.6 hybridoma cells were treated for 90 min with CCCP at concentrations ranging from 0–100 μM, and with 10 μM-CCCP for times up to 4 h, and analysed by electron microscopy. The severity of Golgi disruption was scored according to an arbitrary scale, as described in the legend to Fig. 5. As shown in Fig. 5A, the primary effect on Golgi structure is probably the dilation of Golgi cisternae, because it is evident already at 1 μM-CCCP. The vacuolization near the stack is already maximal at 5 μM-CCCP, and the disruption increases in severity over the range of 5–100 μM. Even at the highest doses and the longest times of CCCP tested (100 μM for 90 min or 10 μM for 4 h), the Golgi stack remains intact. Although the cisternae become quite dilated and contorted, adjacent cisternae retain their close apposition.

As shown in Fig. 5B, the disruption of Golgi structure by CCCP occurs very quickly. Abnormal stacks are visible by 5 min, and the disruption is maximal by 30 min of treatment. Over the course of 4 h, the Golgi disruption does not change significantly, but by 4 h the cells begin to show signs of toxicity, including condensation of chromatin and dilation of the ER and nuclear envelope. These results suggest that the Golgi complex is very sensitive to the effects of CCCP, since the disruption occurs with as little as 2 μM-CCCP, and within as few as 5 min.
Fig. 3. Electron micrographs of the disrupted Golgi region in CCCP-treated BHK cells. A. Untreated cells; B. cells treated with 10 μM-CCCP for 90 min. n, nucleus; m, mitochondrion; l, lysosome; er, endoplasmic reticulum; mvb, multivesicular body; v, CCCP-induced vacuoles; arrowheads, coated vesicles. Bar, 0.5 μm.
The CCCP-induced vacuoles are on the trans side of the Golgi stack

As noted above, the CCCP-induced vacuoles accumulate preferentially on one side of the Golgi stack. Since the site of vacuole accumulation has important implications for the effects of CCCP on protein transport, we used cytochemistry to determine whether the vacuoles accumulate on the cis or the trans side of the Golgi stack. B1-8.δ cells were untreated, or treated for 90 min with 10 μM-CCCP, and the reaction product of thiamine pyrophosphatase was used to mark the trans side of the Golgi stack (Novikoff & Goldfischer, 1961; Hand & Oliver, 1984a). As an additional means of establishing Golgi polarity, we used the cluster of small, smooth vesicles that is often seen on the cis side of the Golgi stack, away from the TPPase-positive cisternae (Fig. 6, arrows). In control cells (Fig. 6A), TPPase reactivity is present in the last one or two cisternae of the Golgi stack; the TGR is usually unreactive.

When CCCP-treated cells are assayed histochemically for TPPase (Fig. 6B), it can be seen that the vacuoles accumulate preferentially on the TPPase-positive, or trans, side of the Golgi stack (Table 1). Of the 53 Golgi stacks counted (one Golgi counted per cell), none exhibited the vacuoles only in the cis-Golgi; 83% had vacuoles exclusively or predominantly in the trans-Golgi. In 11/53 Golgi complexes, one or more vacuoles were

<table>
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<th>Side of Golgi</th>
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<td>Only trans</td>
<td>15</td>
<td>28</td>
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<td>Mostly trans</td>
<td>29</td>
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<td>Cis + trans</td>
<td>6</td>
<td>11</td>
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<td>Mostly cis</td>
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<td>Only cis</td>
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<td>Total</td>
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*Only Golgi complexes that satisfied the following criteria were scored: they showed a clearly polarized TPPase reaction product in the stack and they were sufficiently isolated from other stacks to score the association of CCCP-induced vacuoles.
†Golgi complexes, where more than 3/4 of the associated vacuoles were on the trans side of the stack.
‡Golgi complexes, where the associated vacuoles were equally prominent on both sides of the stack.

Table 1. Distribution of CCCP-induced vacuoles
Fig. 5. Dose dependence of Golgi disruption. B1-8.6 cells were treated for 90 min with various concentrations of CCCP (A), or with 10 μM-CCCP for various times (B), and analysed by electron microscopy. Golgi morphology was scored as follows: category 1, normal morphology (•); category 2, dilated cisternae without vacuoles (○); category 3, dilated cisternae with vacuoles in the Golgi region (▲); category 4, dilated cisternae with dispersed vacuoles (□). Samples were coded and analysed randomly. Fifty stacks were scored for each data point.

Discussion

We show here that the protonophore CCCP causes distinct ultrastructural changes in the Golgi complex of treated cells. Together with the data presented in the companion paper (Burkhardt & Argon, 1989), a straightforward relation emerges between the biochemical and morphological effects of CCCP: it arrests G protein transport in the compartment whose structure it disrupts.

The apparent absence (or leakiness) of the early CCCP-sensitive stage in this system (Burkhardt & Argon, 1989) makes it possible to ask where G protein accumulates when arrested at the late CCCP-sensitive stage. Immunofluorescence confirms the prediction from the post-translational modifications of G protein, that it is enriched in the Golgi region. That other glycoproteins also accumulate in the Golgi regions of CCCP-treated cells is indicated by the increased intensity of WGA labelling, even in uninfected cells.

The Golgi complexes of CCCP-treated BHK cells do not appear normal, even at the light-microscope level. They seem vesiculated, and are no longer strictly perinuclear. As seen by electron microscopy (EM), the Golgi cisternae are dilated, and large vacuoles accumulate in the Golgi region. This morphology is characteristic not only of BHK cells, but also of myeloma cells under conditions where CCCP inhibits the secretion of Ig (Argon & Milstein, 1984). The alteration of Golgi structure is dependent on the dose of CCCP. At lower drug concentrations, which produce only partial inhibition of Ig secretion (Argon & Milstein, 1984), only dilated cisternae are observed. Doses of CCCP that produce complete inhibition of transport correlate with the appearance of both dilated disternae and Golgi-associated vacuoles. No other morphological changes are observed at higher doses, unless toxic doses are reached.

Surprisingly, we have detected no effects of CCCP on ER structure by either light or electron microscopy. At all the doses tested (below the toxic dose of 100 μM) there is no dilation or vesiculation of ER elements. Moreover, we could not detect any paucity of transitional elements similar to that reported by Tartakoff (1986) in dinitrophenol (DNP)-treated pancreas cells. This is true even in myeloma cells, where the export of Ig from the ER is blocked by CCCP (Tartakoff & Vassalli, 1979; Argon & Milstein, 1984).
Fig. 6. Thiamine pyrophosphatase activity in CCCP-treated cells. B1-8.5 cells were untreated (A), or treated for 90 min with 10 μM-CCCP (B), and reacted for TPPase. Note the presence of large CCCP-induced vacuoles (v) on the TPPase-positive side of the Golgi stack. Arrowheads, cis-Golgi vesicles. Bar, 0.1 μm.

Because the CCCP-induced vacuoles seem to underlie the inhibition of protein transport, we wanted to identify the compartment(s) from which they are derived. Most of the CCCP-induced vacuoles are WGA-positive, as seen by immunofluorescence (Fig. 1) and by immunoelectron microscopy (data not shown). In addition, most of the CCCP-induced vacuoles are positive for ricin communis agglutinin I binding (not shown), indicating that their glycoproteins contain galactose (Virtaanen et al. 1980). These lectin bindings show that most of the vacuoles are derived from compartments distal to the mid-Golgi (Roth & Berger, 1982). The use of a more specific marker for the trans-Golgi, thiamine pyrophosphatase (TPPase) activity (Hand & Oliver, 1984b; Novikoff & Goldfischer, 1961), confirmed this conclusion. The vast majority of the vacuoles accumulate on the TPPase-positive, or trans, side of the Golgi complex. Most of the TPPase reactivity remains in the Golgi stack after treatment with CCCP, consistent with the observation that the stack remains intact. The CCCP-induced vacuoles themselves are seldom positive for TPPase, making it unlikely that they derive from the trans-Golgi cisternae. Taken together, this marker analysis suggests that the Golgi-associated, CCCP-induced vacuoles represent dilated post-Golgi structures.

We have also examined the distribution of another distal Golgi marker, acid phosphatase reactivity (Novikoff, 1964; Hand & Oliver, 1984b). However, the levels of acid phosphatase in the Golgi complexes of BHK and myeloma cells (even in the absence of CCCP) were insufficient to determine unequivocally whether the vacuoles contain this marker.

We cannot at present exclude the possibility that some of the CCCP-induced vacuoles are derived from other transport compartments. Few, if any, of the vacuoles would be expected to come from Golgi-proximal compartments (such as the transition from the ER to the cis-Golgi), because most label with lectins specific for terminal sugars, and because most contain G protein that bears complex glycans. We also expect that few, if any, of the vacuoles are endocytic structures. We consider this unlikely for three reasons. First, in our hands the...
Fig. 7. EM immunolocalization of G protein in CCCP-treated BHK cells. Ultrathin frozen sections of infected BHK cells were labelled with anti-G followed by protein A–colloidal gold. A. Untreated cells; B–D, cells treated for 90 min with CCCP. G, Golgi stack; m, mitochondrion; n, nucleus; pm, plasma membrane; mvb, multivesicular body; arrowhead in A, budded virion. Arrows in C and D point to G protein-containing vacuoles that are clearly distinct from the Golgi stack. Vacuoles that are not clearly bounded by a membrane, and may therefore be a drying artifact, are marked by *. Bar, 0.5 μm.
internalization of $^{[35]}	ext{S}\text{T}ra$transferrin bound to its surface receptor is not inhibited by CCCP (Wiest & Argon, unpublished). Second, the CCCP-induced vacuoles do not contain VSV particles, only G protein, while endosomal structures in VSV-infected cells often contain virions. Third, immunolabelling of myeloma cells treated with CCCP shows that the vacuoles contain high concentrations of immunoglobulin (Burkhardt, Dul & Argon, unpublished data), and in these cells the endocytic traffic of immunoglobulin is negligible (Pernis, 1985).

The ultrastructural alteration caused by CCCP is reminiscent of the effects of another transport inhibitor, monensin. Both drugs inhibit protein transport by disrupting a compartment related to the Golgi complex, thereby trapping the exported proteins in the altered compartment. The effects of the two ionophores differ, however, in that monensin disrupts the architecture of the Golgi stack (Tartakoff & Vassalli, 1977; and Fig. 4), while CCCP causes vacuolization at its periphery, without breaking up the stack itself. These differences are consistent with the biosynthetic stages inhibited by the two drugs: monensin inhibits mid-Golgi processing (Griffiths et al. 1983a) and disrupts the Golgi structure, whereas CCCP inhibits late processing events (Argon et al. 1989; Burkhardt & Argon, 1989), and disrupts peripheral Golgi elements. Quinn et al. (1983) showed that when the transport of Semliki Forest virus (SFV) glycoprotein is arrested in monensin-included vacuoles, incomplete virions bud into the disrupted Golgi membranes. In contrast, intracellular budding of VSV is observed in CCCP-induced vacuoles although they contain high concentrations of G protein. It will be interesting to examine the ability of SFV to bud into the CCCP-induced vacuoles. The dilation of the CCCP-sensitive compartment, as well as the accumulation of viral glycoproteins within it, provide experimental means for its isolation and future biochemical characterization.

As a protonophore, CCCP neutralizes acidic compartments (Poole & Okhuma, 1981). Trans-Golgi elements have been shown to be mildly acidic (Anderson & Pathak, 1985). Thus, the selective effect of CCCP on particular membranes in the Golgi region may reflect pH differences among Golgi subcompartments. Indeed, other weak bases also cause dilation of trans-Golgi elements (Geuze et al. 1985; Thorens & Vassalli, 1986). CCCP arrests transport at two stages, both of which have been previously shown to require ATP, but dilates only one compartment. It may therefore be argued that the disruption of trans-Golgi elements results from pH effects rather than ATP depletion. Whether the accumulation of the arrested proteins in this compartment results directly from pH or ATP perturbation remains to be rigorously tested.

A likely interpretation of our data is that the compartment marked by sensitivity to CCCP is the distal compartment known as the trans-Golgi reticulum (Willingham & Pastan, 1984; Griffiths & Simon, 1986). The vast majority of the dilated structures are associated with the trans side of the stack, as expected for the TGR. The analyses of marker enzymes and lectin binding are also consistent with this interpretation. Another supporting observation is the occasional finding of clathrin-like coats on portions of the CCCP-induced vacuoles. One defining characteristic of the TGR is its high clathrin content, as compared with other Golgi elements (Friend & Farquhar, 1967; Orri et al. 1985; Willingham et al. 1981).

Finally, the most important criterion for defining the TGR is the state of maturation of the proteins contained in it. The vast majority of CCCP-arrested G protein bears ER and cis-Golgi modifications, such as N-glycosylation and acylation with palmityte (Burkhardt & Argon, 1989). Its glycans contain galactose and are partly sialylated (Burkhardt & Argon, 1989). This is the expected phenotype of a TGR-arrested protein (Fuller et al. 1985; Griffiths et al. 1985). In this paper, we show that the arrested G protein indeed accumulates in the CCCP-induced vacuoles. Thus, it seems likely that by alkalinizing the TGR elements, CCCP causes their swelling so that G protein accumulates in them and is unable to complete its transport to the plasma membrane.

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