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Poliovirus Infection and Expression of the Poliovirus Protein 2B Provoke the Disassembly of the Golgi Complex, the Organelle Target for the Antipoliovirus Drug Ro-090179

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Infection of Vero cells with poliovirus results in complete disassembly of the Golgi complex. Milestones of the process of disassembly are the release to the cytosol of the β-COP bound to Golgi membranes, the disruption of the *cis*-Golgi network into fragments scattered throughout the cytoplasm, and the disassembly of the stacked cisternae by a process mediated by long tubular structures. Transient expression of the viral protein 2B in COS-7 cells also causes the disassembly of the Golgi complex by a process preceded by the accumulation of the protein in the Golgi area. Vero cells infected for 3 h show no recognizable Golgi complexes at the ultrastructural level and display an enormously swollen endoplasmic reticulum (ER) with extensive areas of its surface heavily coated. Ro-090179 (Ro), a flavonoid isolated from the herb *Agastache rugosa*, provokes the specific swelling and disruption of the Golgi complex and strongly inhibits poliovirus infection. Ro provokes the swelling and the disruption of the stacked cisternae and *trans*-Golgi elements without affecting the *cis*-most Golgi cisternae much. Moreover, Ro inhibits the fusion of the Golgi complex with the ER in cells treated with brefeldin A and provokes the accumulation of the intermediate compartment membrane protein p58 into ERD2-positive Golgi elements but has no effect on the anterograde transport involved in protein secretion. Our results indicate that the secretory pathway and specifically the Golgi complex are preferential targets of poliovirus.

In spite of extensive studies (7, 8, 40), a detailed description of the mechanisms involved in the replication of the poliovirus genome and the assembly of poliovirus particles is still incomplete. The two processes are believed to begin with the targeting of one or more viral proteins to a receptor associated with intracellular membranes. The localization of the viral polyprotein 2BC and/or its by-product, 2C, to the surfaces of transport vesicles budding from the endoplasmic reticulum (ER) suggests that the receptor(s) may be an ER membrane protein (7). This localization and the recent observation that the viral proteins 3A and 2B block protein secretion at different levels (19) suggest that a set of viral proteins is targeted to organelles of the secretory pathway and disrupts their functioning. The observation that brefeldin A (BFA), a membrane perturbant that interferes with vesicle-mediated intracellular transport (23, 36, 39), strongly inhibits the synthesis of poliovirus RNA (29, 38) lends further support to that suggestion.

The massive proliferation of vesicles in the cytoplasm of poliovirus-infected mammalian cells (6, 12, 13, 18) and of yeast transformed with 2BC (3) suggests a cause-effect relationship between the binding of viral proteins to membranes of the secretory pathway and the uncontrolled proliferation of vesicles. Vesicle proliferation appears to be necessary for the replication of the viral genome, as indicated by the association of viral replicative complexes with rosettelike shells of vesicles isolated from infected cells (8, 15, 52, 57). The copurification of 2BC and its by-products with vesicle rosettes and the capacity of 2C to bind simultaneously to membranes and to poliovirus RNA (8, 9) support that hypothesis.

The use of selective inhibitors of specific steps of the poliovirus life cycle has helped in the elucidation of the different processes of viral infection and the proteins involved (14). A number of compounds are effective inhibitors of poliovirus genome replication, but only in a limited number of instances has the exact mode of action of these agents been elucidated (14). Flavonoids, such as 3-methylquercetin and Ro-090179 (Ro), are powerful and selective inhibitors of poliovirus RNA synthesis that do not affect cellular transcription (17, 26, 30, 31). They preferentially block plus-strand RNA compared to minus-strand RNA synthesis (16, 26), but a description of their mechanism of action is still lacking.

Here, we have further investigated the effects of poliovirus infection as well as of the expression of poliovirus proteins on the secretory pathway and specifically on the integrity of the Golgi complex. We observe that virus infection and the transient expression of the viral protein 2B promote the disassembly of the Golgi complex. The effect of the virus is inhibited by Ro, a flavonoid which we find acts specifically on the Golgi complex and inhibits phenomena associated with retrograde transport, such as the fusion of the Golgi complex with the ER in cells treated with BFA and the cycling of p58 between the ER and the Golgi, without affecting anterograde transport.

MATERIALS AND METHODS

Cell culture. Vero cells (African green monkey kidney), NRK cells (normal rat kidney), and COS-7 cells (simian virus 40-transformed African green monkey) were grown either on plastic petri dishes or glass coverslips, in Dulbecco modified Eagle medium (DMEM) containing 2 or 10% fetal calf serum (FCS), 2 mM glutamine, and antibiotics (50 U of penicillin/ml and 50 μg of streptomycin/ml) at 37°C in an atmosphere of 93% air, 7% CO2, with 85% humidity. When required, the cells were incubated with Ro, a gift of Roche Laboratories (H. Ishitsuka; Nippon Roche Research Center, Kanakora, Japan) BFA (Sigma), or the protein synthesis inhibitor cycloheximide (Sigma). Stock solutions of Ro and BFA were prepared in dimethyl sulfoxide at 10 μg/ml.

Virus infection and drug treatments. Vero cells grown in DMEM containing 2% FCS, 2 mM glutamine, and antibiotics were infected with poliovirus

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type 1 (Mahoney strain) at a multiplicity of 5 PFU per cell. Synchronized cell infections were performed by incubating the cells with the virus for 2 h at 4°C in media with 2% FCS and 10 mM morpholineethanesulfonic acid (MES), pH 7.4, washing the unbound virus with fresh DMEM, and then continuing the incubation in medium without MES for various periods of time.

Cell transfections. cDNAs encoding the viral protein 2BC (i.e., P2) and its by-products, 2C and 2B, as well as the cDNA of the protein 3A, were obtained by standard PCR cloning techniques from poliovirus cDNA (a gift of E. Wimmer, Stony Brook, N.Y.). The cDNAs were cloned in the expression vector pCEXV-3 and introduced into COS-7 cells by using lipofectins (Life Technologies) (22). The transfected cells were allowed to express the protein for periods of 16 to 48 h before their study by immunofluorescence microscopy.

Antibodies. The development of mouse monoclonal antibodies and rabbit polyclonal antibodies (pAb) to integral membrane proteins from the *cis*-Golgi network (CGN) (gp74), the stacked Golgi cisternae (SGC) (GMP_{c-1}), and the *trans*-Golgi network (TGN) (GMP_{t-1}) has been reported previously (2, 58). Specific rabbit pAb against the poliovirus proteins 2B and 2C were obtained by inoculation of the maltose binding protein-2B fusion proteins MBP.2B (3) and MBP.2C (43). Rabbit serum against 3A was obtained against the synthetic polypeptide. Rabbit pAb to the ER marker protein disulfide isomerase (PDI) was a gift of J. G. Castaño (Universidad Autónoma de Madrid, Madrid, Spain), and the pAb to the intermediate compartment marker p58 was a gift of J. Saraste (University of Bergen, Bergen, Norway). The rat monoclonal antibody YOL1/2 to tubulin was from J. V. Kilmartin (Addenbrooks Hospital, Cambridge, United Kingdom). Rhodamine- and fluorescein-conjugated rabbit anti-mouse immunoglobulin (immunoglobulin G [IgG] plus IgM plus IgA) antibodies were from Dako (Dakopatts, Glostrup, Denmark). Biotin-conjugated goat anti-rabbit immunoglobulin (IgG plus IgM plus IgA) antibody and fluorescein-conjugated avidin were from Boehringer (Mannheim, Germany).

Light microscopy. Single and double immunofluorescence microscopy studies were performed on either Vero, COS-7, or NRK cells grown on glass coverslips as described previously (4, 10, 33).

Electron microscopy. Ultrastructural studies were performed on thin cell sections processed as described previously (11).

Protein labeling and studies on protein synthesis and secretion. Metabolic labeling of NRK cells was performed as previously described (4). [^{35}S]methionine/[^{35}S]cysteine was from Amersham (Buckinghamshire, United Kingdom). The rates of protein synthesis and secretion were studied by measuring the radioactivity incorporated into total cellular trichloroacetic acid (TCA)-precipitable protein and into TCA-precipitable protein contained in 100 μl of medium.

Other procedures. The effects of Ro and BFA on the integrity of the Golgi complex and the egress of Golgi membrane proteins from the ER upon the removal of BFA were quantified by studying the changes in Golgi integrity in groups of 100 cells.

Other reagents. Paraformaldehyde was from Merck (Darmstadt, Germany), and glutaraldehyde (25%, in water) was from Fluka (Buchs, Switzerland).

RESULTS

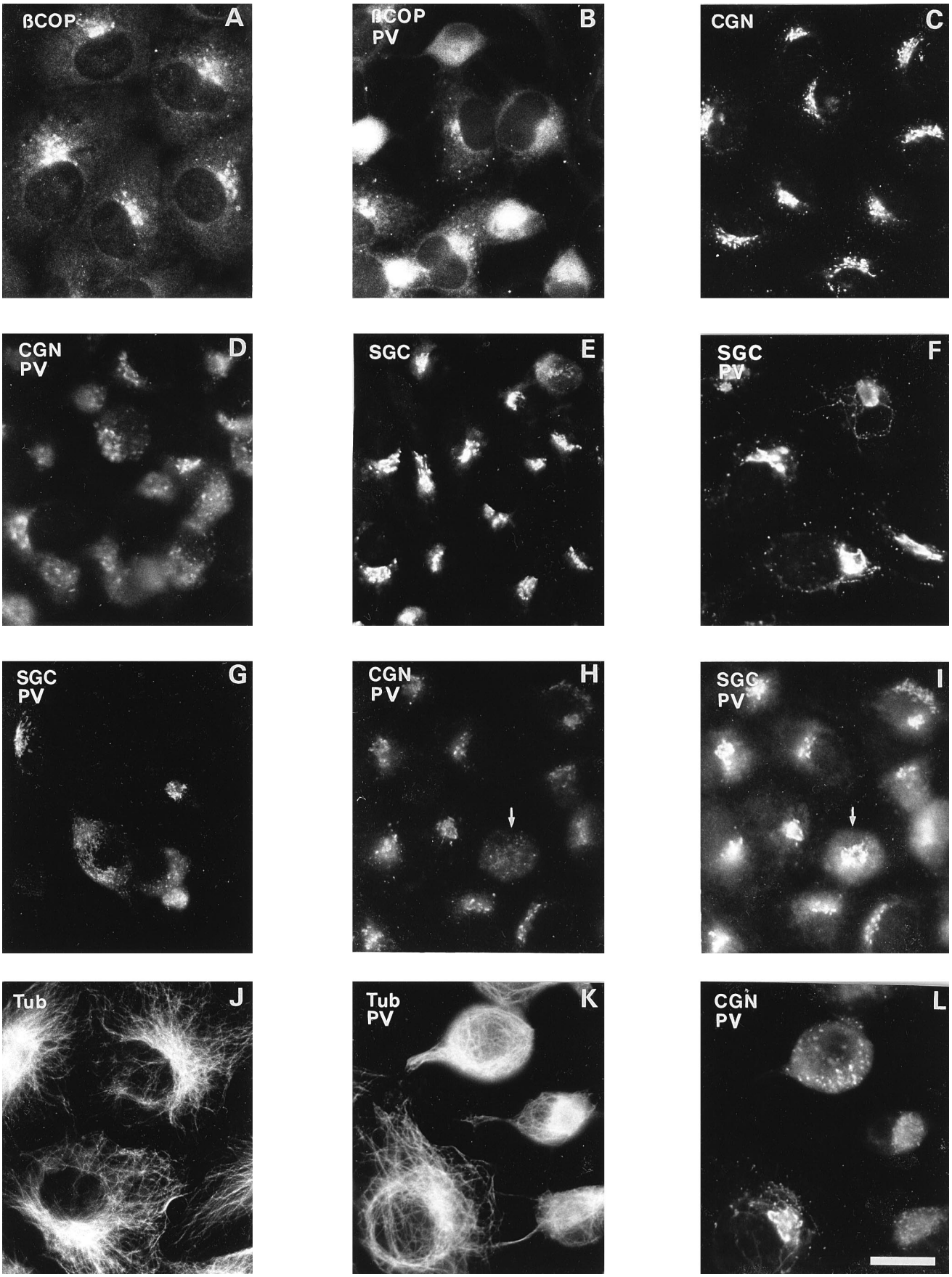
Poliovirus induces the disassembly of the Golgi complex. While studying the infection of Vero cells with poliovirus, we have observed that the virus disassembles the Golgi complex by a process that causes the β-COP bound to the Golgi complex as a component of the COPI coats to be released to the cytosol (compare Fig. 1A and B) and affects the different compartments of the organelle distinctly: the CGN is disrupted and its fragments are scattered throughout the cytosol (Fig. 1C and D), and the SGC is disassembled (Fig. 1E to G) by a process that occurs with the formation of long threadlike structures (Fig. 1F) and ends with the relocation of the SGC membrane marker GMP_{c-1} to a delicate reticulum spread throughout the cytoplasm and to a few punctate structures (compare Fig. 1E and G). Furthermore, we often observe that the disassembly of the CGN precedes the disruption of the SGC (compare Fig. 1H and I).

Although the disruption of the Golgi complex has been often associated with alterations of the cytoskeleton (55, 56) and cells infected with poliovirus show a profound remodelling both of intermediate filaments (34) and of microtubules (compare Fig. 1J and K), the Golgi complexes of cells infected with poliovirus are not disassembled as a result of the collapse of the network of cytoplasmic microtubules. This is indicated by the observation that cells in an intermediate stage of infection, with the microtubules collapsed in tangles around the nucleus, display intact Golgi complexes (Fig. 1K and L). Moreover, whereas in poliovirus-infected cells the disassembly of the SGC proceeds without fragmentation of the cisternae, in cells treated with drugs that disrupt microtubules, the cisternae were fragmented into large pieces (2, 53, 56).

The ultrastructural study of Vero cells infected with poliovirus confirms previous studies describing as the main phenotypic changes the enlargement of the perinuclear space, the enormous swelling of the ER, the disappearance of the Golgi complex, and the proliferation of vesicles, often decorated with electrodense membrane material (7, 52) (Fig. 2). We observe, in addition, that the disappearance of the Golgi complex is always preceded by the accumulation of a highly electrodense material inside the SGC and nearby vesicles, a material which does not accumulate in the lumen of the adjacent ER cisterna (Fig. 2A). Furthermore, with the progress of the infection, we note that the coating of ER membranes, usually restricted to the sites of vesicle budding, is spread over large areas of their surfaces (compare Fig. 2C and D).

Expression of the poliovirus protein 2B in COS-7 cells provokes the disassembly of the Golgi complex. According to a recent study, expression of the poliovirus proteins 2B and 3A in COS-7 cells blocks protein secretion (19). Cells transfected with 3A showed a blockage at an early stage of transport, as indicated by the inhibition of the processing of mannose oligosaccharide chains to complex carbohydrates and the retention of the secretory protein A1PI in the ER. In contrast, the expression of 2B appeared to block transport at a later step, as indicated by the retention of AP1PI molecules with complex mannose chains in the Golgi complex. These findings and the observation that poliovirus provokes the disassembly of the Golgi complex led us to study whether the expression of the viral proteins 2BC, 2B, 2C, and 3A affected the integrity of the Golgi complex. The experiments were performed by transfecting COS-7 cells with the cDNAs of the viral proteins for periods of 16 to 48 h and by simultaneously studying the expression and distribution of the viral proteins and the integrity of the Golgi complex by double immunofluorescence microscopy. As shown in Fig. 3, cells which did not express the viral protein 2B were not stained with the antibody against the protein (Fig. 3A) and displayed intact Golgi complexes arranged as fluorescent doughnut-shaped structures in the vicinity of the nucleus (Fig. 3B) (54). Moreover, among the cells that expressed 2B we distinguished two populations: cells that were faintly stained by the anti-2B antibody and displayed intact Golgi complexes

FIG. 1. Poliovirus attacks the Golgi complex by provoking the disruption of the CGN and the disassembly of the SGC. Vero cells grown to 80% confluence in DMEM containing 2% FCS were mock infected (A, C, and E) or infected with poliovirus (PV) at a multiplicity of 5 PFU/cell for 7 h (B, D, G, H, I, K, and L). Synchronous infections, performed to increase the percentage of infected cells with threadlike structures (F), were achieved by incubating the cells with the virus for 2 h at 4°C in DMEM with 2% FCS and MES and, after washing the free virus, continuing the incubation for 2 h at 37°C. Cells were fixed-permeabilized with methanol and stained with antibodies against β-COP (A and B), the CGN marker gp74 (C, D, H, and L), the SGC marker GMP_{c-1} (E, F, G, and I), or tubulin (J and K). The arrows in panels H and I point to a cell that displays a disrupted CGN and an intact SGC. The pairs of pictures H-I and K-L show cells double stained with two antibodies. Controls made by omitting the first antibodies showed no cell staining. Bars, 21 μm (A, B, J, K, and L) and 31 μm (C, G, E, H, and I).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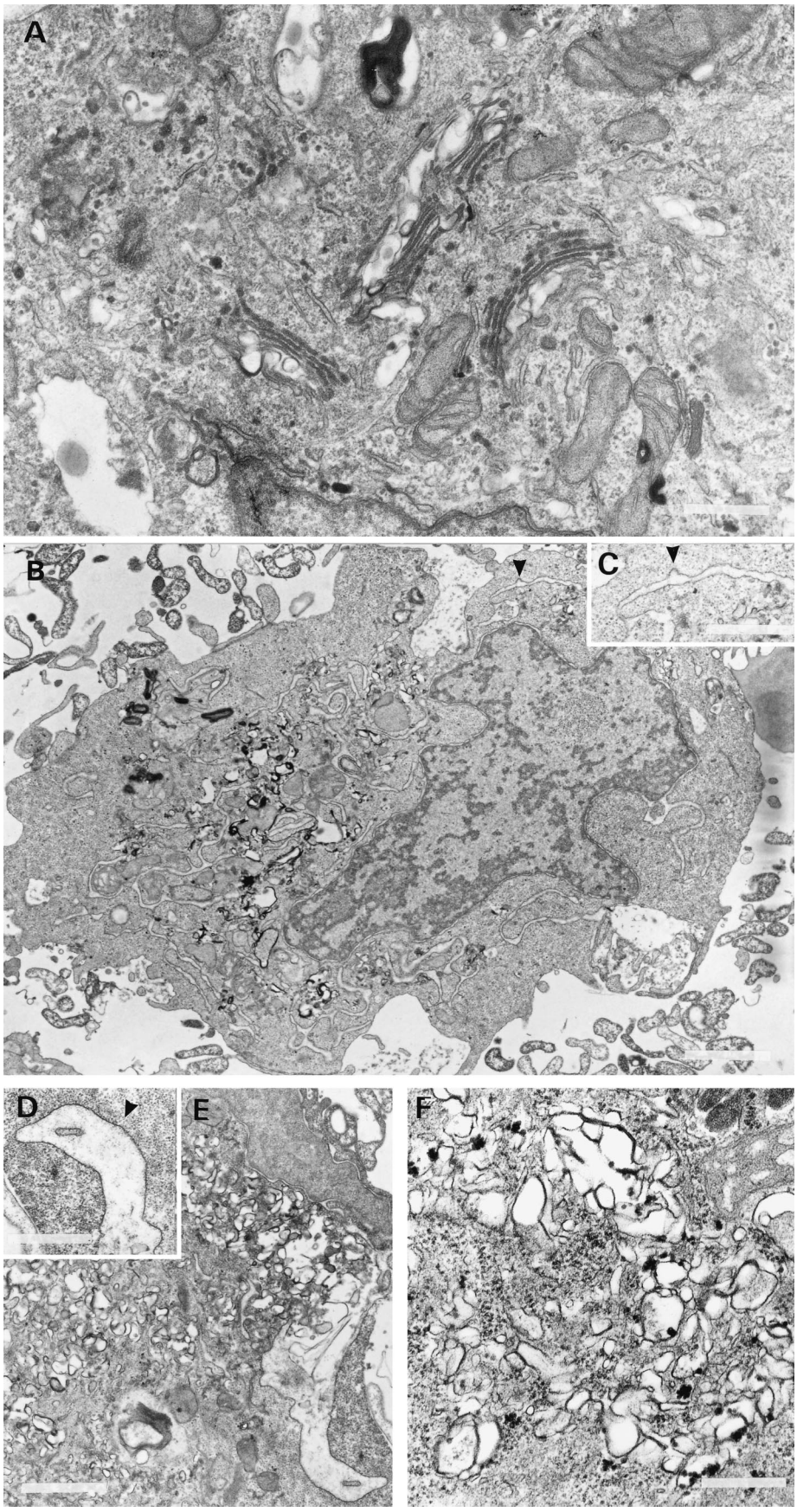


FIG. 2. Ultrastructural changes of cells infected with poliovirus. Vero cells were incubated with poliovirus at a multiplicity of infection of 5 PFU/cell for 2 h at 4°C in DMEM with 2% FCS and MES; the cells were then washed with warm DMEM, and the incubation was continued in DMEM containing 10% FCS for 2 (A), 3 (B and C), or 6 (D through F) h at 37°C. Coated ER membranes are marked with arrowheads. Bars, 0.4 (A), 1.32 (B), 0.88 (C), 1.13 (D), 1.32 (E), and 0.6 (F) μm.

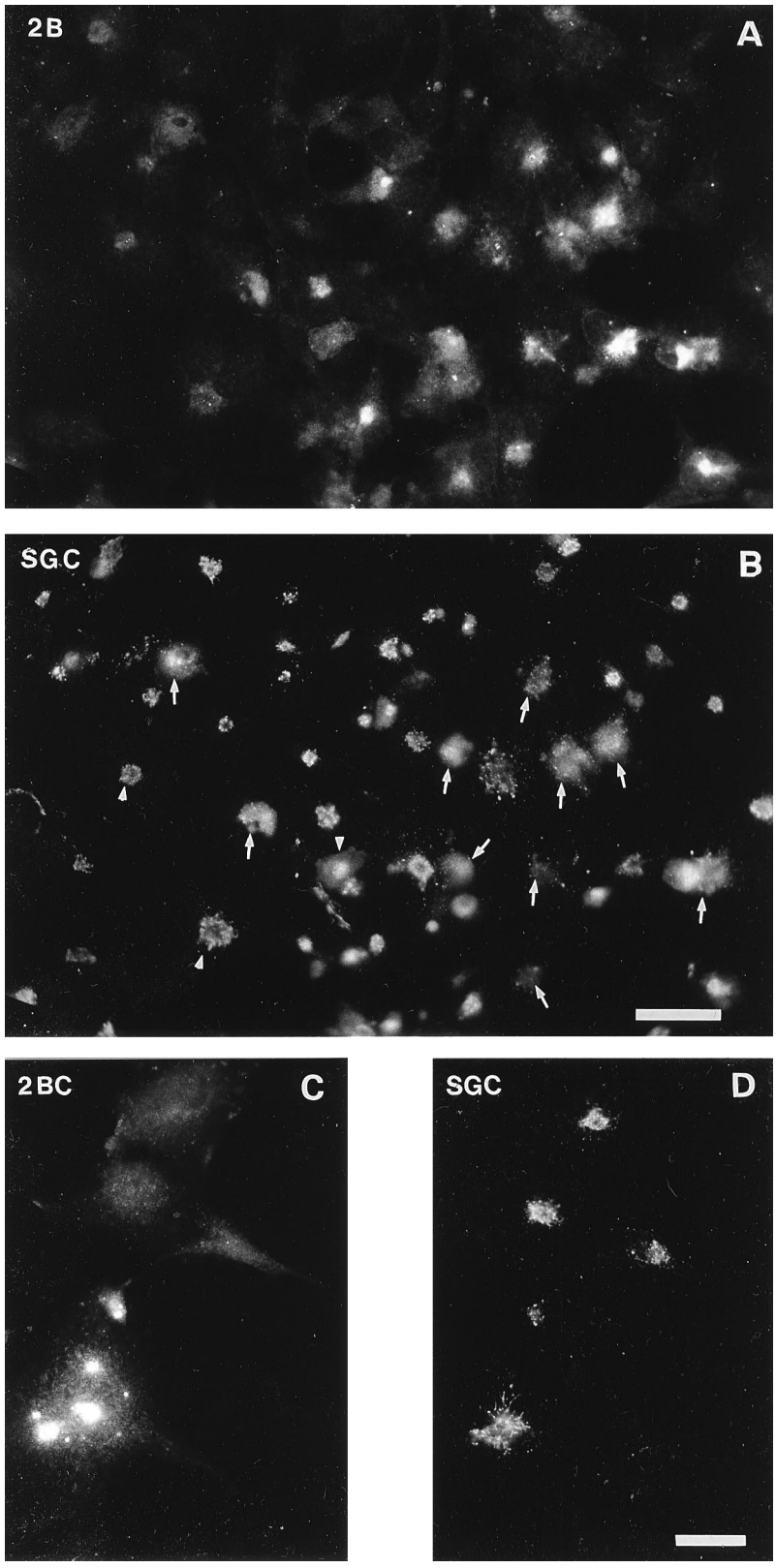


FIG. 3. Expression of the viral protein 2B promotes the disassembly of the Golgi complex in COS-7 cells. (A and B) COS-7 cells transfected with 2B for 16 h were fixed-permeabilized with cold methanol and double stained with antibodies against 2B (A) and the SGC protein GMP_{c-1} (B). Note that the cells that did not express 2B displayed intact doughnut-shaped Golgi complexes. Cells expressing low levels of 2B, as judged by their faint staining with the anti-2B antibody, showed intact Golgi complexes that were stained by both the anti-2B and the anti-GMP_{c-1} antibodies (arrowheads). Cells showing strong diffuse staining by the anti-2B antibody also showed diffuse staining with the anti-GMP_{c-1} antibody (arrows). (C and D) COS-7 cells transfected for 24 h with 2BC, simultaneously stained with antibodies against 2B (C) and GMP_{c-1} (D). Controls made by omitting the first antibodies showed no cell staining. Bars, 21 μm.

strongly stained by the anti-GMP_{c-1} antibody (Fig. 2A and B), and cells strongly stained by the anti-2B antibody in which the doughnut-shaped Golgi complexes were replaced by an area of diffuse fluorescence (Fig. 2A and B). Taken all together, these data indicate that following its accumulation in the Golgi area, 2B provokes the disassembly of the Golgi complex. With regard to the mechanism of disassembly, it is noteworthy that 2B did not provoke any alteration in microtubule architecture, a frequent cause of Golgi disruption (data not shown). In contrast with the effects of 2B expression, cells expressing 2BC (Fig. 3C and D), 2C, or 3A (data not shown) for periods between 16 and 24 h displayed intact Golgi complexes that were not stained by antibodies against the viral proteins. Only at 48 h after transfection did these proteins provoke alterations in the Golgi architecture, which may have been the result of unspecific cell damage, since these cells showed extensive vacuolization.

The flavonoid Ro inhibits the disassembly of the Golgi complex in cells infected with poliovirus or treated with BFA. Previous studies have shown that Ro, a natural flavonoid, is a strong inhibitor of poliovirus RNA replication (17). As shown in Fig. 4, Vero cells treated for 90 min with 10 μg of Ro/ml and then infected for 7 h with poliovirus in the presence of the drug remain flat and firmly attached to the culture dish and do not express the viral protein 2B (compare Fig. 4A and C), 2C, or 3A (data not shown), as revealed by immunoflorescence microscopy. In addition, Ro prevents the disassembly of the Golgi complex in the infected cells (compare Fig. 4B and D) but does not prevent the disassembly of the Golgi complex provoked by the expression of 2B (data not shown).

Since Ro inhibited the disassembly of the Golgi complex by poliovirus, we studied the possibility that Ro might also inhibit the capacity of BFA to disassemble the Golgi complex (36). In fact, there were similarities between the effects of BFA and the virus on the Golgi complex, since both disrupted the CGN and provoked disassembly of the SGC by a mechanism that involved the formation of long threadlike structures (2, 37). The experiment was also intended to clarify if Ro prevented the disassembly of the Golgi complex by inhibiting the poliovirus infection or by interfering with the cellular mechanisms of membrane transport. It can be seen in Fig. 5 that Ro did not prevent the primordial BFA effect, the redistribution of β-COP in cells incubated for periods of 5 (data not shown) to 15 min with 1 μg of BFA/ml (compare Fig. 5C and E) (20) but markedly inhibited the disassembly of the SGC (23, 36, 39) (compare Fig. 5D and F). The slowdown in the fusion between the Golgi complex and the ER suggested that Ro inhibits membrane traffic.

Ro does not inhibit protein secretion but provokes the accumulation of the intermediate compartment membrane protein p58 in Golgi elements housing the KDEL receptor ERD2. The disassembly of the Golgi complex by BFA is believed to result either from the unbalance of membrane traffic provoked by a block in the vesicle-mediated ER-Golgi transport (21, 28, 37) or, more likely, from the fusion between the Golgi and ER membranes that have lost their COPI coats (41, 47). Ro could act, therefore, by inhibiting either mechanism. The possibility that Ro might prevent or release the block of the anterograde ER-Golgi transport by BFA was studied as described in the legend to Fig. 6A. The results of secretion experiments performed with NRK cells show that Ro neither prevents nor releases the block of secretion by BFA. Moreover, the removal of BFA from cells continuously incubated in the presence of Ro was rapidly followed by a resumption of protein secretion (Fig. 6A). These conclusions were separately confirmed by the observation that Ro did not inhibit the secretion of thyroglobulin by FRTL-5 cells; this protein, which is synthesized in large amounts and continuously secreted by these thyroid cells, is an excellent indicator of the functional status of the secretory pathway. The lack of effect of Ro on the anterograde transport of proteins was confirmed by the study of the egress of Golgi membrane proteins from the ER of NRK cells pretreated with BFA (Fig. 6B). Previous studies have shown that Golgi membrane proteins incorporated into the ERs of cells treated with BFA leave the ER within minutes after the removal of BFA (1, 23, 36, 39). As shown in Fig. 6B, the rates of egress of Golgi membrane proteins from the ER are not affected by Ro. Moreover, the egress of the proteins occurs in the same orderly fashion, first the CGN proteins and then the TGN proteins, as in cells incubated without Ro (1) and ends with the accumulation of the Golgi membranes in the area that houses the Golgi complex next to the nucleus (Fig. 6C and D) (1).

Overall, these results indicate that Ro does not affect the anterograde transport of proteins, nor does it reverse the blockage of the secretory pathway by BFA. The incapacity to overcome the BFA effect agrees with the observation that Ro does not prevent the release of the β-COP bound to Golgi membranes upon the addition of BFA (Fig. 5).

The possibility that Ro might inhibit the retrograde transport of membranes from the Golgi complex to the ER was examined by studying its effect on the distribution of p58, an itinerant membrane protein which cycles continuously between the intermediate compartment and the CGN and accumulates in the latter upon inhibition of the retrograde transport by incubation of the cells at 20°C (45, 46, 49). The study was performed with NRK cells which reacted with the antibodies against p58. Confirming the results of previous studies, in cells incubated at 37°C, p58 was localized to a faint reticulum and vesicles spread throughout the cytoplasm (Fig. 7A), and in cells incubated at 20°C, p58 was localized to elongated elements located in the vicinity of the nucleus (Fig. 7B). Interestingly, the pattern of p58 distribution in cells treated with Ro at 37°C was similar to the protein distribution in cells incubated at 20°C (compare Fig. 7B and C). This and the colocalization of p58 with ERD2 (compare Fig. 7C and D), the itinerant KDEL receptor that cycles between the ER and Golgi complex but resides most of the time in the Golgi complex (35, 51), strongly suggest that Ro inhibits the transport of p58 from the Golgi complex to the ER. Ro may, thus, inhibit the disassembly of the Golgi complex by BFA by blocking the back transport of membranes from the Golgi complex to the ER.

Ro causes compartment-specific changes in the Golgi complex. The capacity of Ro to specifically inhibit phenomena associated with retrograde transport led us to study the effect of this inhibition on the integrity and organization of the Golgi complex. The study included examination of the effects of the drug on the three main parts of the organelle, the CGN, SGC, and TGN, and was performed by immunofluorescence microscopy on NRK cells. Large and flat, these cells had well-developed Golgi complexes and reacted with the battery of antibodies necessary to study the effects of Ro on the different Golgi compartments. As can be seen in Fig. 8, the response of the SGC and TGN to the drug was different from the response of the CGN. In cells incubated for 90 min with 10 μg of Ro/ml, the cisternal elements of the SGC and TGN become shorter and round, and their normal reticular structure is replaced with a few separated elements (compare Fig. 8A and C with E and F). In contrast, the CGN stained with antibodies to either gp74 (compare Fig. 8C and D) or ERD2 (data not shown) remains

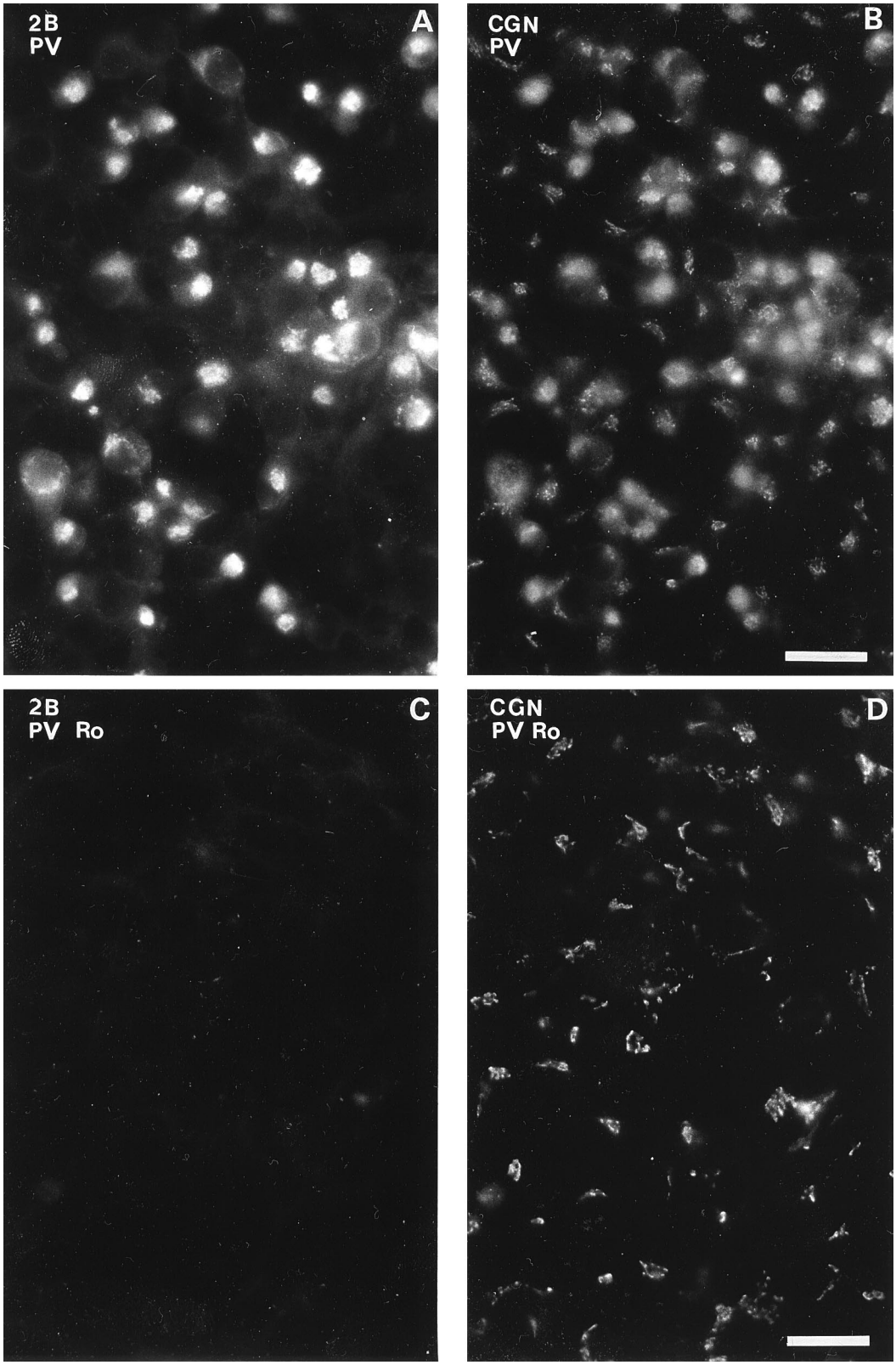
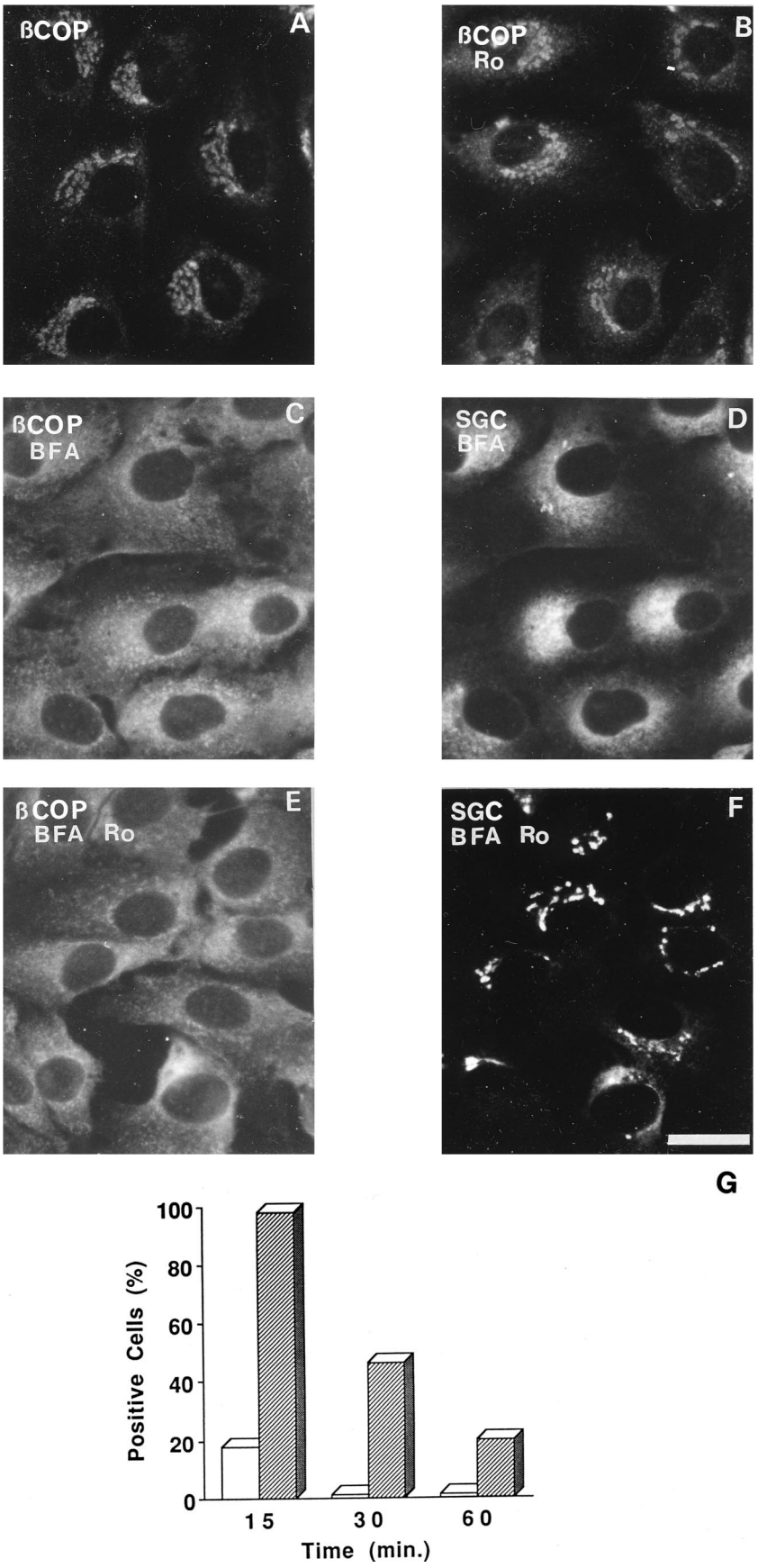


FIG. 4. Ro prevents the infection of Vero cells and the disruption of the Golgi complex by poliovirus. Vero cells incubated without Ro (A and B) or with 10 μg of Ro/ml for 90 min (C and D) were infected with poliovirus (PV) at a multiplicity of 5 PFU/cell, and the infections were continued without or with the drug. At the end of the 7 h of infection, the cells were fixed-permeabilized with cold methanol and double stained with antibodies against the viral protein 2B (A and C) and against the SGC marker GMP_{c-1} (B and D). Controls made by omitting the first antibodies showed no cell staining. Bars, 38 μm.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Positive Cells (%)
100
80
60
40
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0
15
30
60
Time (min.)

relatively intact. Interestingly, the rather intact CGN often appears to wrap the swollen SGC and TGN fragments (Fig. 8C and D). It is noteworthy that the disruption of the SGC and TGN is not provoked by changes in microtubule integrity or organization (55, 56), since cells treated with Ro display normal networks of cytoplasmic microtubules (Fig. 8G and H). Interestingly, the changes in Golgi integrity are accompanied by a reduction of the large area of ER exclusion that houses the Golgi complex in the vicinity of the nucleus (compare Fig. 8I and J with K and L) (1), probably as a consequence of the invasion and wrapping of the disjointed Golgi elements by the ER.

The changes in Golgi structure were also examined at the ultrastructural level by electron microscopy. These studies confirmed the swelling and fragmentation of the Golgi complex as well as the specificity of these effects, since the morphology of ER, mitochondria, and lysosomes, among other organelles, was normal (Fig. 9A). They also showed how the swelling begins at the rims of the cisternae (Fig. 9B), and they showed the frequent presence of coated vesicle buds in the swollen cisternae, a presence which agrees with the observation that Ro does not affect the vesicle-mediated secretion of proteins (Fig. 9B and C). Furthermore, they confirmed the immunofluorescence microscopy observation that the swollen fragments are often wrapped by intact cisternae (Fig. 9D), a phenomenon that underscores the unequal response of the different Golgi compartments to the drug, at the same time suggesting a physical link between them. Moreover, the study of serial sections of the Golgi complex revealed that the *cis*-most cisternae were almost intact (Fig. 9E to G), a result which agrees with the immunofluorescence microscopy observation that Ro does not affect the CGN.

Finally, the effects of Ro on the Golgi complex were concentration (Fig. 10A) and time dependent (Fig. 10B) and were fully reversed upon removal of the drug (Fig. 10C).

DISCUSSION

The interaction of viruses with intracellular membranes has been intensively studied. Enveloped viruses drag the membranes from organelles or from the plasma membrane after their assembly. Interestingly, poliovirus is not wrapped by any cellular membrane but interacts extensively with cellular membranes and provokes extensive changes in their organization (for a review see reference 15).

Newly synthesized poliovirus proteins appear to be targeted to membranes of the ER, and specifically to the budding sites of transport vesicles, where they assemble the machinery that replicates the viral RNA (7, 9). This interaction might profoundly alter the functioning of the secretory pathway. Ultrastructural studies of poliovirus-infected cells have shown important morphological alterations of both the Golgi complex and the ER (5). It is likely that the changes in the two organelles are related and that the accumulation of electrodense material in the SGC, the tremendous swelling of the ER lumen, and the extensive coating of ER membranes reflect a blockage of the ER-Golgi membrane transport. This possibility is suggested by the fragmentation of the CGN and the disassembly of the SGC by a process which involves the formation of long threadlike structures, two phenomena observed in BFA-treated cells and believed to reflect a blockage of ER-Golgi transport (36, 37). Blockage of membrane transport by poliovirus might involve the precursor 2BC, localized to vesicles budding from the ER (7), and/or the proteins 2B and 3A, which have been shown to block protein secretion (19). We observe that antibodies against 2B stain the Golgi of COS-7 cells expressing low levels of 2B, suggesting a physical association of the viral protein with the Golgi complex. Furthermore, we observe that the expression of high levels of 2B provokes the disassembly of the Golgi complex. These results and the observation that 2B provokes the intracellular accumulation of glycoproteins bearing complex carbohydrates (19) are not contradictory; they rather suggest that the virus may promote the fusion of the Golgi cisternae with the ER, an event which is followed by the processing of high-mannose to complex carbohydrates in the ER lumen (1). The disassembly of the Golgi by 2B could explain the enormous swelling of the ER in poliovirus-infected cells and the observation in our laboratory that expression of 2BC mutants in yeast induces swelling of the ER (3a).

The immunolocalization of viral replication complexes on the surface of the ER (6, 42, 52), the capacity of the protein 2C to bind simultaneously to membranes of replication complexes and to viral RNA (8, 9), the copurification of 2C, 2B, 3A, and 3AB with replication complexes embedded in vesicle rosettes (9), and the evidence that implicates the three first proteins in the assembly of replication complexes (7, 24, 25, 32, 50, 52) strongly suggest that the virus might use the membranes of the ER and the Golgi complex to assemble the replication complexes. The assembly of the replication complexes might, therefore, be at the origin of the interference of poliovirus with the functioning of the secretory pathway. The observation that Ro, an inhibitor of poliovirus RNA replication, provokes specific changes in the architecture of the Golgi complex (see below) is also consistent with this view. Furthermore, since the reaction of the human immune system to the virus depends, at least in part, on the presentation of viral antigens by major histocompatibility complexes (MHCs) at the cell surface, and the transport and loading of MHC I and MHC II depend on the operation of the secretory pathway and related endosomes, it is possible that, as suggested recently, the attack on the Golgi complex may be used by the virus to block the host immunoresponse (19).

The interaction of viral proteins with the transport machinery of the secretory pathway and the homology between the vesicle transport machineries which operate on different pathways of membrane transport (44) might explain both the broad dispersion of viral particles throughout the cell and the fast proliferation of vesicles derived from the ER, Golgi complex, and lysosomes (48). The apparent preference of poliovirus proteins for organelles of the secretory pathway might reflect only the higher concentration of targets for these proteins in a pathway with an intense traffic of transport vesicles. Separate studies have reported that the first poliovirus-induced vesicles are detected in the Golgi area and have tracked their

FIG. 5. Ro inhibits the disassembly of the Golgi complex by BFA but does not prevent the release of β-COP bound to Golgi membranes. Cells incubated for 90 min without Ro (A) or with 10 μg of Ro/ml (B) were stained for β-COP. Cells incubated for 15 min with 1 μg of BFA/ml were double stained for β-COP (C) and the SGC marker GMP_{c-1} (D). Cells incubated for 90 min with 10 μg of Ro/ml and then for 15 min with BFA (1 μg/ml) and Ro (10 μg/ml) were double stained for β-COP (E) and GMP_{c-1} (F). Controls made by omitting the first antibodies showed no cell staining. Bar, 25 μm. (G) Quantitation of the inhibitory effect of Ro. Cells preincubated for 90 min without Ro (□) or with 10 μg of Ro/ml (▨) were then incubated with the drug and 1 μg of BFA/ml for the indicated times, stained with the antibody against GMP_{c-1}, and scrutinized for SGC elements resistant to BFA. Cells displaying distinct SGC elements were scored as positive.

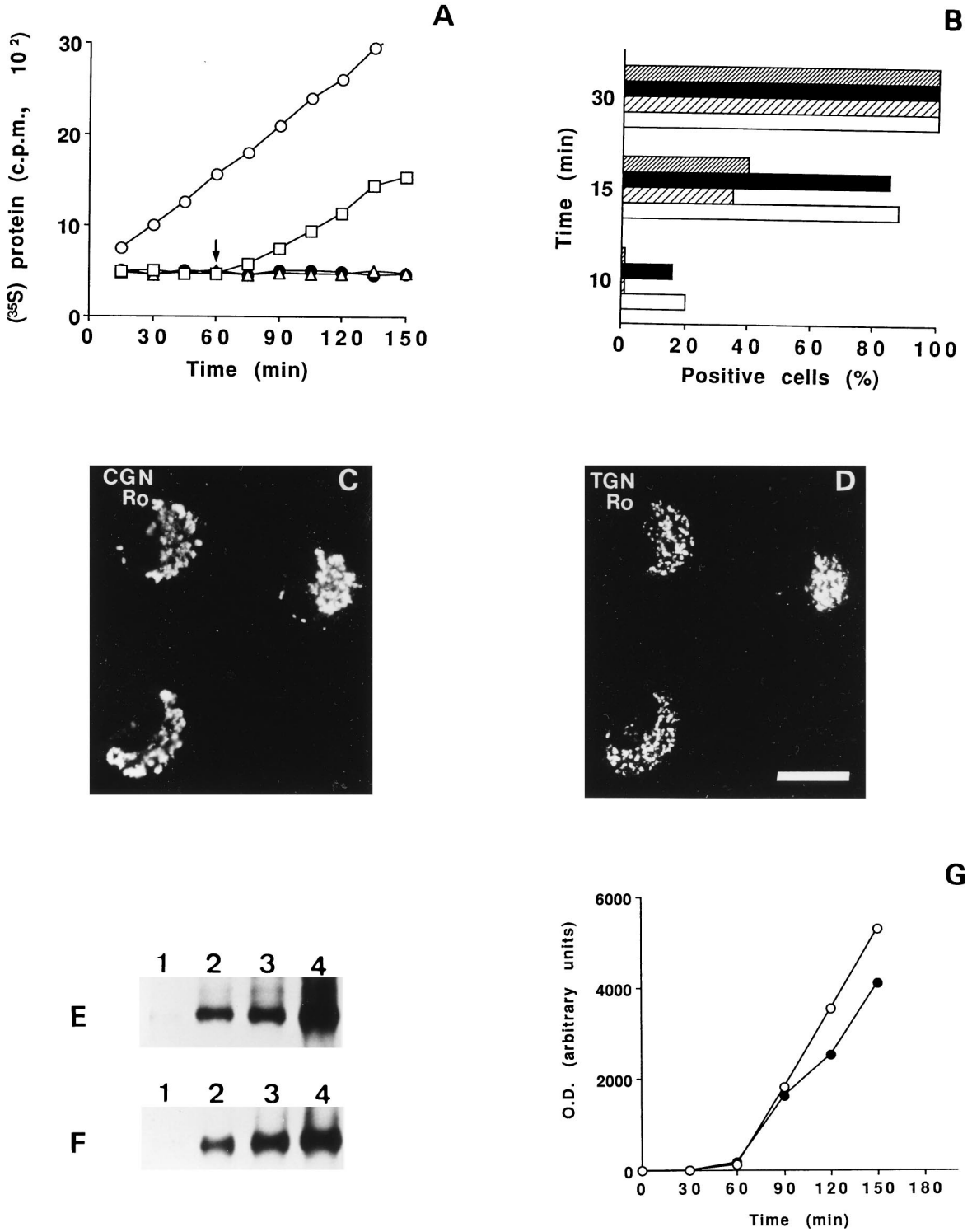

FIG. 6. The anterograde transport of soluble and membrane proteins is insensitive to Ro. (A) Protein secretion by NRK cells treated for 90 min with 10 μg of Ro/ml and pulse-labeled with [^{35}S]methionine/cysteine for 15 min (○), incubated for 60 min with 1 μg of BFA/ml before labeling (●), or incubated for 90 min with 10 μg of Ro/ml or for 60 min with 1 μg of BFA/ml before labeling (△ and □, respectively; arrow indicates the time of BFA removal). Levels of ^{35}S incorporated into newly synthesized TCA-precipitable secreted protein were measured in 100 μl of medium at the times indicated. (B through D) Egress of Golgi membrane proteins from the ERs of NRK cells treated with BFA. Cells treated for 30 min with 10 μg of BFA/ml were incubated for 60 min more without Ro (□ and ▨) or with 10 μg of Ro/ml (□ and ▨); then BFA was removed and the incubations were continued for the indicated times before counting of the cells that displayed distinct CGN (□ and ■) or TGN (▨ and ▨) elements as shown, respectively, in panels C and D. Bar, 25 μm. (E and F) Secretion of thyroglobulin by FRTL-5 cells preincubated without Ro (E) or with 10 μg of Ro/ml (F) for 90 min, pulse-labeled for 5 min with [^{35}S]methionine/cysteine, and chased for 60 min (lanes 1), 90 min (lanes 2) 120 min (lanes 3), and 150 min (lanes 4). (G) Plot of secreted thyroglobulin versus the time of the chase in a experiment performed in the absence (○) or presence (●) of Ro.

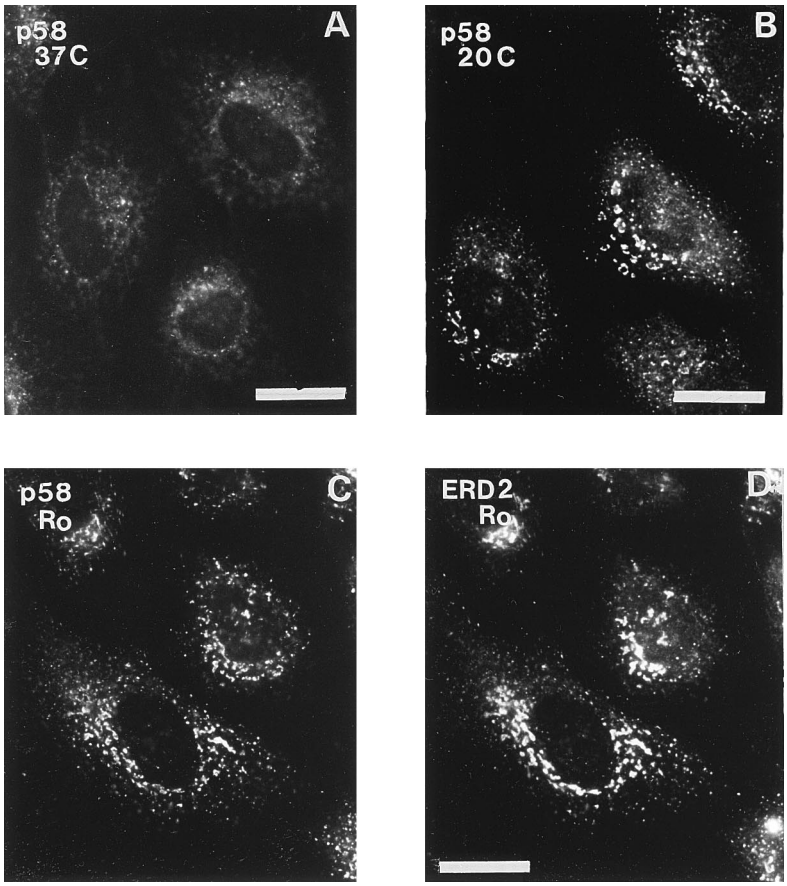

FIG. 7. Ro provokes the accumulation of p58 in ERD2 positive Golgi elements. (A and B) Distribution of p58 in NRK cells incubated at 37°C (A) or 20°C (B). (C and D) NRK cells incubated for 90 min with 10 μg of Ro/ml and double stained for p58 (C) and the KDEL receptor ERD2 (D). Controls made by omitting the first antibodies showed no cell staining. Bars, 25 μm.

origin to vesicles budding from the ER (6, 7). This and the presence of ER and Golgi markers in the vesicles that proliferate in the virus-infected cells (48) strongly suggest that the two organelles that constitute the secretory pathway are preferred targets of the virus. The proliferation of rosettelike vesicles and the observation that poliovirus promotes the synthesis of lipids indicate, however, that the membranes of an undetermined number of vesicles might be synthesized de novo (27).

Our studies show that the action of Ro, a strong inhibitor of poliovirus RNA synthesis (26), is restricted to the Golgi complex. The capacity of the drug to attack the Golgi complex at the same time that it inhibits the replication of the viral RNA is noteworthy. The effects of Ro on the Golgi complex, the binding of proteins that are involved in the assembly of the poliovirus replication complexes to vesicles involved in ER-Golgi transport (7), the recognized capacity of the viral proteins 2B and 3A to block the secretory pathway (19), and our observation that 2B provokes the disassembly of the Golgi complex once more strongly indicate that the Golgi complex is one of the main targets of the virus in the cell and suggest that the viral infection can be prevented by interfering with the attack of the virus on the Golgi complex. Ro does not prevent the disassembly of the Golgi complex by 2B in transiently transfected COS cells. While this observation appears to discard the possibility that the drug inhibits the viral infection by preventing the disassembly of the Golgi complex by 2B, it does not exclude the possibility that the drug may act on 2B, or on any one of the components of the viral replication complexes hosted in Golgi- or ER-derived membrane rosettes, and thus inhibit viral RNA replication. The mechanism by which Ro alters specifically the Golgi compartments *trans* to the CGN it is not known. Ionic perturbants, such as monensin, chloroquine, and ammonium chloride, slow down protein secretion, cause the rapid vacuolization of the Golgi complex, and induce the swelling of endosomes and lysosomes (53). These rather general effects are in contrast with the more restrictive effects of Ro, which in addition does not block protein secretion. The specific swelling of some of the Golgi elements in response to Ro suggests an effect of the drug on a membrane protein, probably a transporter, asymmetrically distributed through the Golgi compartments. The greater resistance of the CGN to Ro as well as its distinct reactions to poliovirus infection, BFA treatment, and energy depletion (unpublished data), are indicative of a compartment that has properties distinct from those of the rest of the Golgi compartments but that functions in tandem with these. Its different behavior may in fact reflect the privileged links of the CGN with the ER, as well as its location between the ER and the SGC. It is interesting that Ro inhibits two events that have been linked to the retrograde transport of membranes, the fusion of the Golgi with the ER in cells treated with BFA and the retrograde transport of the membrane protein p58. The failure of Ro to block the release of β-COP from the Golgi membranes and to release the inhi-

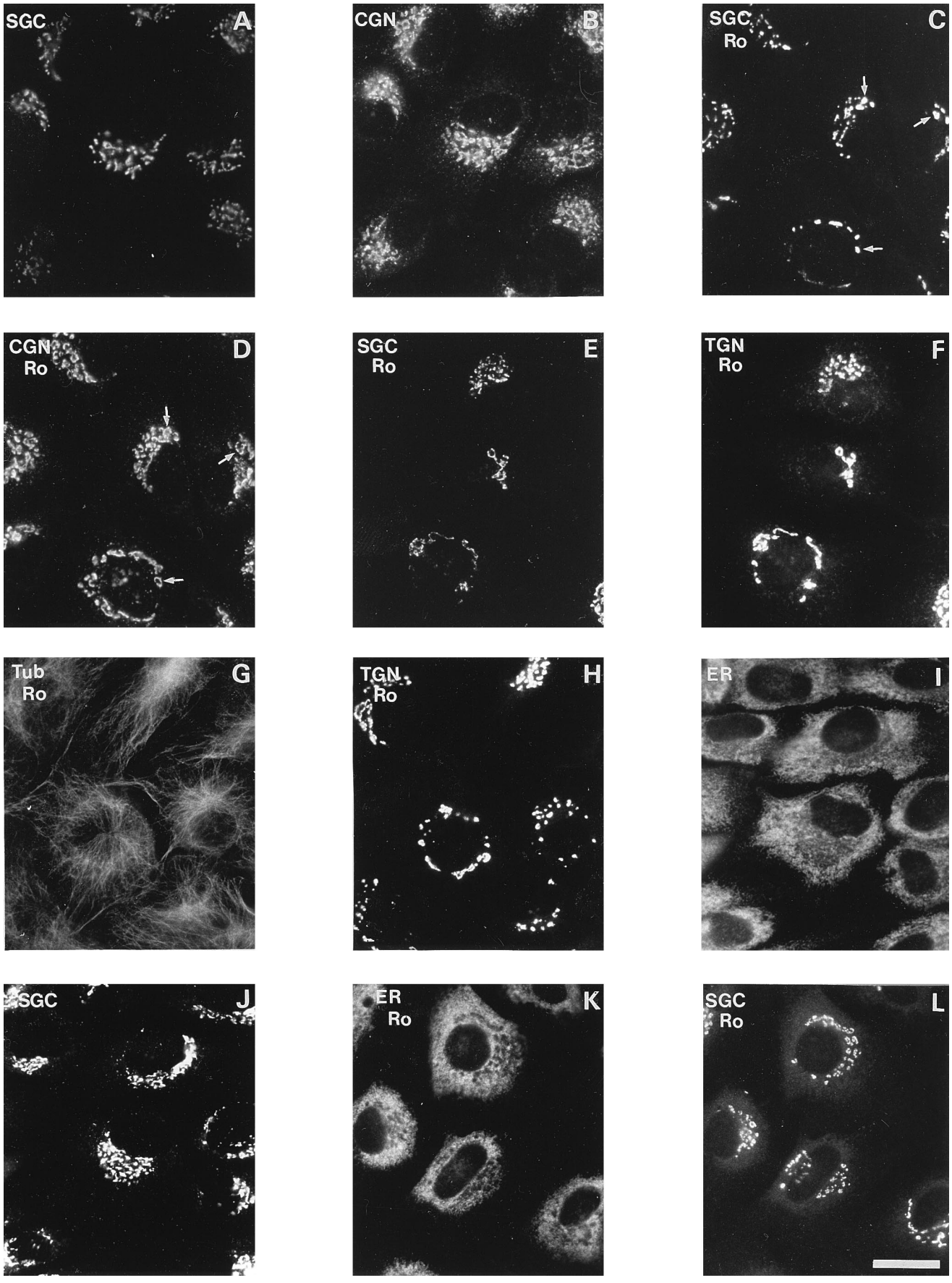
SGC
A
CGN
B
SGC
Ro
C
CGN
Ro
D
SGC
Ro
E
TGN
Ro
F
Tub
Ro
G
TGN
Ro
H
ER
I
SGC
J
ER
Ro
K
SGC
Ro
L

FIG. 8. Differential response of Golgi compartments to Ro. NRK cells incubated for 90 min without Ro (A, B, I, and J) or with 10 μg of Ro/ml (C through H, K, and L) double stained for the SGC marker, $GMP_{c\text{-}1}$ (A and C) and the CGN marker, gp74 (B and D); $GMP_{c\text{-}1}$ (E) and the TGN marker, $GMP_{t\text{-}1}$ (F); tubulin (G) and $GMP_{c\text{-}1}$ (H); and the ER marker PDI (I and K) and $GMP_{c\text{-}1}$ (J and L). Controls made by omitting the first antibodies showed no cell staining. Arrows in panels C and D show the rather intact CGN appearing to wrap the swollen SGC and TGN fragments. Bar, 25 μm.

bition of protein secretion in cells treated with BFA indicates that Ro does not inhibit the primordial BFA effect. Ro might inhibit the fusion of the Golgi complex with the ER by interfering either with the biogenesis of the vesicles and/or tubules that are thought to specifically mediate the retrograde transport or with the mechanisms involved in their recognition and/or fusion with ER membranes. We do not know if Ro specifically inhibits the retrograde transport between the Golgi and the ER or if the inhibition affects all the retrograde transport steps through the Golgi back to the ER. The normal rates of protein secretion and the abundance of coated vesicle buds in the swollen Golgi elements of cells treated with Ro are consistent with the resistance of the anterograde transport to the drug. The different sensitivities of the anterograde and retrograde transport systems to Ro agree with evidence suggesting that different molecular machineries operate in the two transport systems (47). Ro could, therefore, be a useful tool for study of the mechanism of retrograde membrane transport through the secretory pathway.

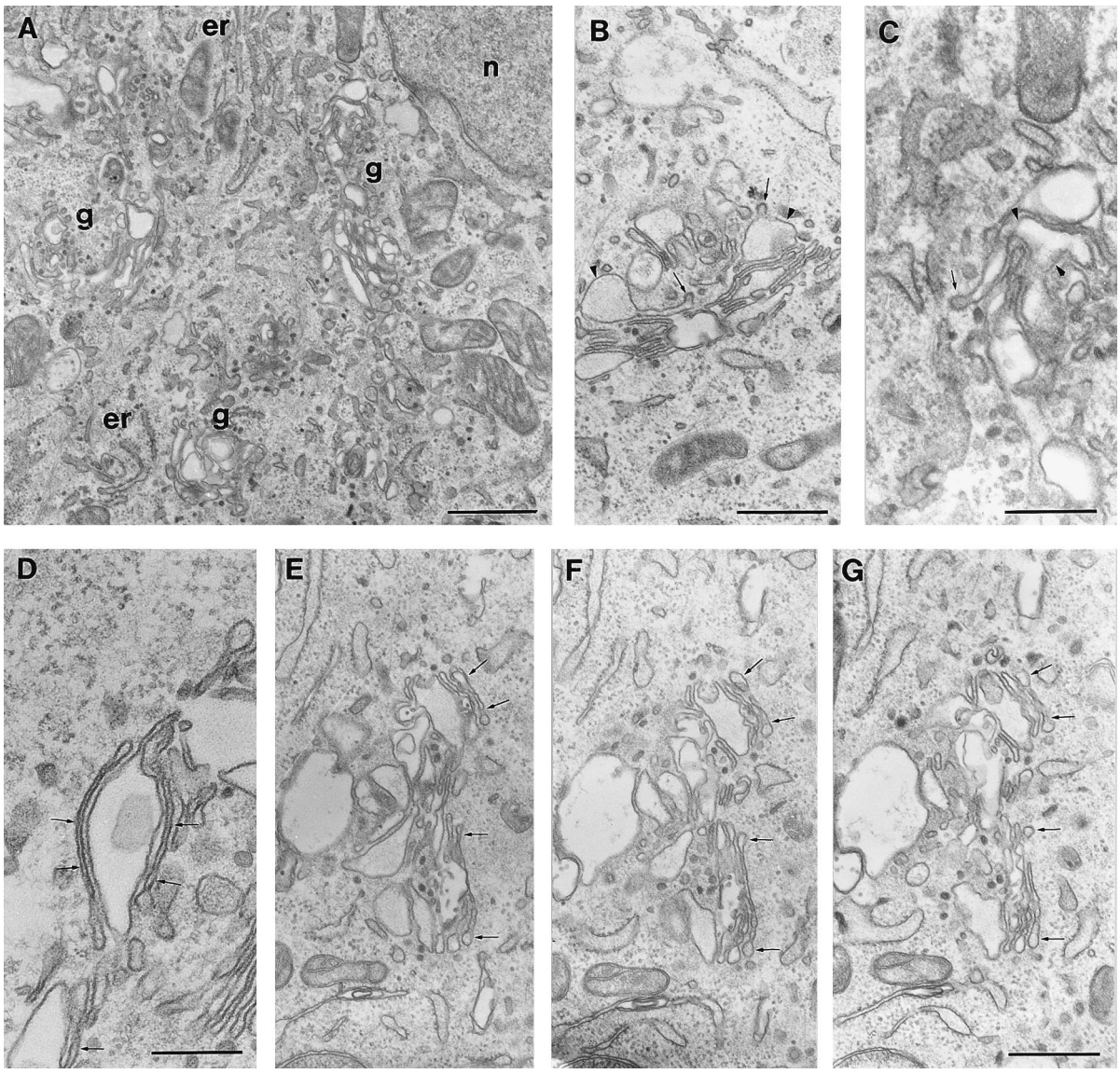

FIG. 9. Ultrastructural changes of the Golgi complex in cells treated with Ro. NRK cells were incubated for 45 min with 10 μg of Ro/ml. Note the disruption of the Golgi complex (g) and the separation of the fragments into clusters (A). Observe how the swelling of the cisternae begins at the rims (B; arrowheads), the frequent presence of coated buds on the swollen cisternae (B and C; arrows), how the swollen separate elements are often wrapped by intact cisternae (D; arrows), and the higher resistance of the *cis*-most cisternae to swelling (E through G; arrows). n, nucleus; er, endoplasmic reticulum. Bars, 0.6 μm (A, B, E, F, and G) and 0.4 μm (C and D).

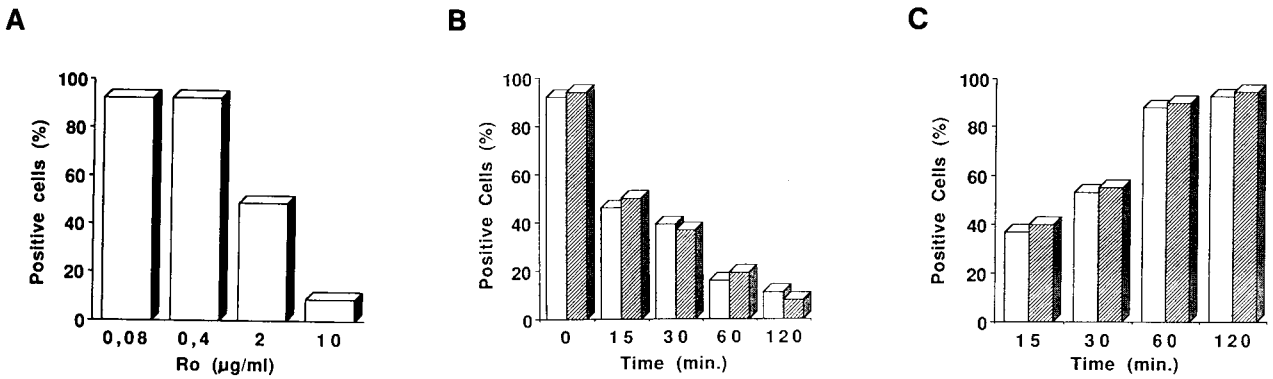

FIG. 10. The Ro effects on the Golgi complex are time and concentration dependent and are rapidly reversible. (A) Disruption of the SGC in cells incubated for 90 min with increasing concentrations of Ro. (B) Disruption of SGC (□) and TGN (▨) in cells incubated with 10 μg of Ro/ml for different times. (C) Recovery of the normal morphology of the SGC (□) and TGN (▨) in cells incubated for 90 min with 10 μg of Ro/ml and then with drug-free medium for the indicated times. Cells displaying intact SGC or TGN elements were scored as positive.

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