

Effect of Caffeine and Reduced Temperature (20°C) on the Organization of the Pre-Golgi and the Golgi Stack Membranes

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Abstract. In the present study we have dissected the transport pathways between the ER and the Golgi complex using a recently introduced (Kuismanen, E., J. Jääntti, V. Mäkiranta, and M. Sariola. 1992. *J. Cell Sci.* 102:505–513) inhibition of transport by caffeine at 20°C. Recovery of the Golgi complex from brefeldin A (BFA) treatment was inhibited by caffeine at reduced temperature (20°C) suggesting that caffeine inhibits the membrane traffic between the ER and the Golgi complex. Caffeine at 20°C did not inhibit the BFA-induced retrograde movement of the Golgi membranes. Further, incubation of the cells in 10 mM caffeine at 20°C had profound effects on the distribution and the organization of the pre-Golgi and the Golgi stack membranes. Caffeine treatment at 20°C

resulted in a selective and reversible translocation of the pre- and *cis*-Golgi marker protein (p58) to the periphery of the cell. This caffeine-induced effect on the Golgi complex was different from that induced by BFA, since mannosidase II, a Golgi stack marker, remained perinuclearly located and the Golgi stack coat protein, β -COP, was not detached from Golgi membranes in the presence of 10 mM caffeine at 20°C. Electron microscopic analysis showed that, in the presence of caffeine at 20°C, the morphology of the Golgi stack was altered and accumulation of numerous small vesicles in the Golgi region was observed. The results in the present study suggest that caffeine at reduced temperature (20°C) reveals a functional interface between the pre-Golgi and the Golgi stack.

TRANSPORT inhibitors have been important tools in revealing the existence of functionally distinct compartments within the cell. Site-specific perturbation of the endomembrane architecture often results in the accumulation of transported proteins and membranes at specific steps along the transport pathways. In many cases the use of inhibitors has made it possible to identify functions and operating components resident in the sites of question.

Reduced temperature has been widely used to dissect transport steps in the exocytotic transport pathway (for review see Saraste and Kuismanen, 1992). Specifically, incubation at 15°C blocks the transport at the pre-Golgi level (Saraste and Kuismanen, 1984) and has led to the characterization of p58, a resident protein in the pre- and *cis*-Golgi compartments (Saraste et al., 1987). The depletion of cellular ATP has been shown to inhibit distinct steps in vesicular transport (Balch et al., 1986; Tartakoff, 1986). The important role of calcium in vesicular traffic between the ER and the Golgi compartment has become evident from *in vitro* studies (Beckers and Balch, 1989; Sambrook, 1990). Also, the perturbation of intracellular Ca^{2+} -levels blocks the movement of secretory proteins out from the rough ER (Lodish and Kong, 1990). In addition, lowered extracellular pH has been reported to arrest the transport of influenza virus hemagglutinin at the pre-Golgi level (Matlin et al., 1988). The central role of GTP in the vesicular transport has become evident from *in vitro* studies where the nonhydrolyz-

able GTP-analogue, GTP- γ -S, has been used to inhibit ER-to-Golgi transport both in mammalian and yeast cells (Beckers and Balch, 1989; Ruohola et al., 1988). Recent localization of the small GTP-binding proteins rab1b and rab2 to the membranes responsible for the ER-to-Golgi transport has further emphasized the role of GTP as a regulator of ER-to-Golgi membrane traffic (Chavrier et al., 1990; Plutner et al., 1991). Interestingly, there is also evidence for the participation of trimeric GTP-binding proteins in the regulation of membrane traffic (Stow et al., 1991; Barr et al., 1991), thus revealing a link between signal transduction and the processes in the intracellular membrane traffic (for review see Balch, 1992; Barr et al., 1992). Okadaic acid, a phosphatase inhibitor, has been shown to inhibit ER-to-Golgi transport (Davison et al., 1992) and result in the fragmentation and dispersal of the Golgi apparatus (Lucocq et al., 1991), further linking mechanisms of signal transduction and the endomembrane traffic.

Brefeldin A (BFA)¹ (Härri et al., 1963) has been a frequently used transport inhibitor in mammalian cells. BFA was first reported to block the transport of vesicular stomatitis virus G protein (Takatsuki and Tamura, 1985). Later it has been shown that BFA treatment results in the fusion of the Golgi membranes with the ER (Doms et al., 1989;

1. *Abbreviations used in this paper:* BFA, brefeldin A; Endo H, endoglycosidase H; man II, mannosidase II; SFV, Semliki Forest virus.

Lippincott-Schwartz et al., 1989, 1990, 1991a; Strous et al., 1991). The almost immediate effect of BFA is to detach at least two of the coat components— β -COP (Donaldson et al., 1990) and a 200-kD protein (Narula et al., 1992)—from the Golgi stack, allowing Golgi membranes to form tubular extensions along microtubules that finally fuse with the ER (Lippincott-Schwartz et al., 1990). This effect of BFA has been proposed to reveal a normally occurring retrograde membrane traffic from the Golgi apparatus to the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1989; Klausner et al., 1992). BFA has also been reported to have effects on the organization of the endocytic compartment; TGN and endosomes form large tubular structures after a BFA treatment (Wood et al., 1991; Lippincott-Schwartz et al., 1991b; Reaves and Banting, 1992).

We have previously shown that 10 mM caffeine affects the intracellular transport of Semliki Forest virus (SFV) membrane glycoproteins (Kuismanen et al., 1992). In this study we have characterized the temperature-dependent effect of caffeine on the distribution and organization of the Golgi complex membranes. At 20°C caffeine is shown to translocate p58, a marker for the pre- and *cis*-Golgi compartments, to the periphery of the cell. In contrast, under the same conditions a Golgi stack marker, mannosidase II (man II), remains perinuclearly located. The redistribution of membranes induced by caffeine is clearly different from that induced by BFA. Further, caffeine at reduced temperature did not inhibit the retrograde movement of Golgi stack membranes observed in BFA-treated cells. Taken together the results in the present study suggest that 10 mM caffeine at reduced temperature can be used to dissect the interface between the *cis*-Golgi and the Golgi stack.

Materials and Methods

Materials

BFA was either a kind gift from Professor A. Takatsuki (University of Tokyo, Tokyo, Japan) or purchased from Epicentre Technologies, Madison, WI. Caffeine was purchased from Sigma Chem. Co., St. Louis, MO, and all other chemicals, if not otherwise stated, were from Sigma Chem. Co. or Merck, Darmstadt, Federal Republic of Germany.

Antibodies

The antibodies were kind gifts from the following persons: polyclonal antibodies to β -COP (EAGE) (Duden et al., 1991) were from Drs. R. Duden (EMBL, Heidelberg, Germany) and T. Kreis (University of Geneva, Geneva, Switzerland); polyclonal antibodies to man II were from Drs. M. Farquhar (University of California, San Diego, CA) and K. Moremen (Massachusetts Institute of Technology, Cambridge, MA); monoclonal antibodies to man II (135-kD protein) were from Drs. B. Burke (Harvard Medical School, Boston, MA) and G. Warren (Imperial Cancer Research Fund, London, UK) (Burke et al., 1982); polyclonal antibodies to p58 were from Dr. J. Saraste (University of Bergen, Bergen, Norway) (Saraste and Svensson, 1991); and polyclonal antibodies to SFV glycoproteins E1 and E2 were from Dr. J. Peränen (University of Helsinki, Helsinki, Finland).

TRITC- and FITC-conjugated secondary antibodies to rabbit or mouse IgG were purchased from Dako A/S, Glostrup, Denmark and peroxidase-conjugated Fab fragments to rabbit IgG were purchased from Biosys, Compiegne, France.

Cell Culture and Virus Infections

BHK-21 cells were grown on 35-mm-diam tissue culture dishes in DMEM (Gibco BRL, Roskilde, Denmark) in the presence of 10% FCS (Gibco BRL), 100 IU/ml of penicillin, and 100 μ g/ml of streptomycin (Penstrep)

(Gibco BRL). For immunofluorescence experiments the cells were grown on 12-mm-diam microscope coverglasses. Incubations in temperatures other than 37°C were carried out in water baths in NaHCO₃-free MEM (Gibco BRL) supplemented with 20 mM Hepes and Penstrep as described earlier (Kuismanen and Saraste, 1989). The temperature-sensitive mutant ts-1 of SFV (SFV ts-1) was used for all virus infections (Keränen and Kääriäinen, 1975; Saraste et al., 1980). Before infections, the cells were washed once with PBS and twice with serum-free MEM (Gibco BRL). Infections were carried out for 1 h at 37°C before the cells were shifted to the restrictive temperature 38.5°C (Kuismanen et al., 1992). Depending on the experiment, the cells were then shifted to the appropriate chase temperature as indicated in the text.

Pulse-labeling Experiments and Immunoprecipitations

For [³⁵S]methionine pulse-labeling experiments, the BHK-21 cells were infected as described above. Before labeling, the cells were grown for 30 min in a methionine-free MEM (Gibco BRL) whereafter they were pulsed for 10 min with 200 μ Ci of [³⁵S]methionine/dish. After the appropriate chase times, the cells were washed once with ice-cold PBS followed by solubilization for 15 min on ice with NET buffer (20 mM Tris, pH 8.8, 1 mM EDTA, 1% Triton X-100, 0.4 M NaCl, and 1 mM PMSF).

Immunoprecipitations and endoglycosidase H (Endo H) analysis were carried out as described earlier (Kuismanen et al., 1992). The samples were analyzed under reducing conditions in a 10% SDS-PAGE according to Laemmli (1970). The gels were prepared for fluorography using AmplifyTM (Amersham, Aylesbury, UK). The film used for autoradiography was Fuji RX 100 (Fuji Photo Co. Ltd., Tokyo, Japan).

Immunofluorescence

At the end of the incubations, the cells were fixed for 30 min at room temperature with 3% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by three washes with PBS. The cells were then permeabilized for 30 min with 0.05% Triton X-100 in PBS at room temperature followed by three washes with PBS containing 0.1% BSA. The primary antibodies were always incubated for 30 min at 37°C, whereafter the cells were washed with PBS containing 0.1% BSA. Incubation conditions for the second antibodies were the same as for the primary antibodies. Finally, the cells were washed and mounted in 75% glycerol, pH 8.5, on microscope objective glasses and viewed with Zeiss Lab 16 fluorescence microscope with 100 \times oil immersion objective. Film used for photography was TMAX 400 ASA (Eastman Kodak Co., Rochester, NY).

The quantitation of the immunofluorescence experiments was performed by photographing random fields of the coverglasses, counting the cells in the photographs, and by observing the distribution of man II in the counted cells. For every time point at least 150 cells were counted.

Electron Microscopy

To study the intracellular distribution of p58, the cells were fixed with a paraformaldehyde-lysine-periodate fixative (McLean and Nakane, 1974). The immunoelectron microscopy procedure followed was essentially that described by Brown and Farquhar (1989).

In conventional electron microscopy the cells were fixed for 1 h at room temperature with a fixative containing 2% glutaraldehyde and 3% paraformaldehyde in 100 mM cacodylate buffer, pH 7.4, and postfixed with reduced 1% OsO₄ for 1 h on ice. All samples were embedded in LX-112 (Ladd Research Industries Inc., Burlington, VT) and were viewed with a Jeol JEM-1200EX transmission electron microscope operated at an acceleration voltage of 60 kV.

Results

Temperature Dependence of the Caffeine-induced Transport Inhibition

We have previously shown that at 20°C 10 mM caffeine blocks efficiently the movement of newly synthesized SFV glycoproteins out from the ER (Kuismanen et al., 1992). Normally, in the absence of caffeine at 20°C, protein transport occurs to the level of the *trans*-Golgi (Matlin and Simons, 1983; Saraste and Kuismanen, 1984). The exact

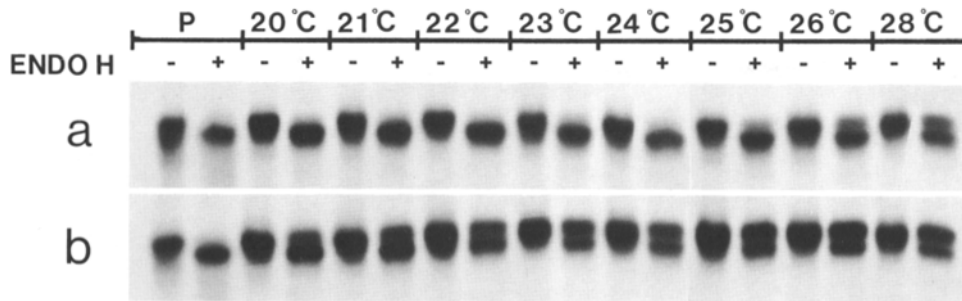


Figure 1. Determination of the temperature threshold allowing SFV E1 to reach the Golgi in the presence (a) or absence (b) of 10 mM caffeine. BHK-21 cells were infected with SFV ts-1 temperature-sensitive mutant, labeled with [³⁵S]methionine and chased in the presence or absence of 10 mM caffeine at 20, 21, 22, 23, 24, 25, 26, and 28°C for

3 h. The cells were then solubilized, immunoprecipitated with monospecific polyclonal antibodies to SFV E1, treated with Endo H, and run in a 10% SDS-PAGE under reducing conditions. From *a* it can be observed that SFV E1 remains Endo H sensitive in the presence of 10 mM caffeine up to 24°C. E1 becomes gradually resistant to Endo H treatment at 25°C and at higher temperatures, indicating the arrival of E1 to the Golgi stack. In *b* the sensitivity of E1 to Endo H treatment in the absence of caffeine at different temperatures is shown. In the absence of caffeine, E1 becomes Endo H resistant already at 20°C (*b*). *P*, pulse.

temperature threshold that allows SFV E1 membrane glycoprotein to reach the Golgi stack in the presence of caffeine was analyzed by following the maturation of SFV E1-linked glycans to the Endo H-resistant form. We also carried out immunofluorescence experiments to analyze the exact temperature that allows SFV glycoproteins to exit the ER. These experiments together should further reveal whether caffeine at reduced temperature could arrest the ER-to-Golgi traffic in possible intermediate steps.

BHK-21 cells were infected with SFV ts-1, shifted to the restrictive temperature, labeled at 3 h 45 min after infection with [³⁵S]methionine for 10 min and chased for 3 h at 20, 21, 22, 23, 24, 25, 26, and 28°C with or without 10 mM caffeine. E1 glycoproteins were then immunoprecipitated and analyzed for their sensitivity to Endo H. In the presence of 10 mM caffeine, SFV E1-glycoproteins remained Endo H sensitive up to 24°C (Fig. 1 *a*). At 25°C and at higher temperatures E1 reached the Golgi stack as indicated by the increasing amount of Endo H-resistant form of E1 (Fig. 1 *a*). In control cells (Fig. 1 *b*), E1 reached the Golgi stack already at 20°C as indicated by the maturation of the proteins to the Endo H-resistant form. In Fig. 1 *b*, it can be seen that E1 does not become completely resistant to Endo H treatment even at 28°C. This, however, is a constant finding for SFV ts-1 and has been reported and discussed earlier (Saraste and Kuismanen, 1984; Gahmberg et al., 1986).

Since the acquisition of Endo H resistance indicates only that E1 has reached the Golgi stack, we next studied whether SFV glycoproteins can exit the ER at temperatures between 20°C and 25°C, and whether they are observed to accumulate in intermediate elements between the ER and the Golgi compartment. BHK-21 cells were infected with SFV ts-1 for 1 h, shifted to restrictive temperature (38.5°C), and grown there for 4 h. The cells were then shifted to 20, 21, 22, 23, 24, 25, and 28°C, and chased for 2 h in the presence or absence of 10 mM caffeine. To be able to follow the movement of presynthesized E1 and E2, the chase mediums contained 50 µg/ml of cycloheximide. In cells incubated at 20, 21, 22, and 23°C in the presence of caffeine, the SFV glycoproteins remained in a reticular structure (Fig. 2, *b*, *c*, *d*, and *e*, respectively) identical to the ER labeling observed in the control cells fixed at the restrictive temperature (38.5°C) (Fig. 2 *a*). At 24°C, only a few cells showed perinuclear vesicular labeling (Fig. 2 *f*, arrow) in addition to ER labeling. At 25°C, the number of cells displaying a perinuclear pattern of labeling increased significantly compared with the

situation at 24°C (Fig. 2 *g*). As was previously reported (Kuismanen et al., 1992), the SFV glycoproteins accumulated in perinuclear structures at 28°C in the presence of caffeine (Fig. 2 *h*). In control cells, the glycoproteins were transported to the *trans*-Golgi region already at 20°C as described earlier (Matlin and Simons, 1983; Saraste and Kuismanen, 1984), and at higher temperatures increased labeling of the plasma membrane was observed (data not shown).

These biochemical and morphological experiments suggest that in the presence of 10 mM caffeine, SFV E1 glycoproteins are retained in the ER up to 23°C, whereas at 25°C they reach the Golgi stack. The experiments may further indicate that SFV glycoproteins are able, at least in some degree, to escape to peripheral vesicular structures already at 24°C when still possessing clearly Endo H-sensitive glycans. However, to further clarify this issue, morphological colocalization experiments with a marker to the pre-Golgi membranes will be necessary.

The Effect of Caffeine and Reduced Temperature on the Membrane Traffic from the ER to the Golgi Region

The observed inhibition of ER-exit of SFV glycoproteins could be a result of not only the inhibition of membrane traffic, but also other factors, such as inhibition of proper folding and oligomerization, known to control the ER-exit of individual proteins (Rose and Doms, 1988; Helenius et al., 1992). To test the effect of 10 mM caffeine and 20°C on the movement of cellular proteins, we followed the recovery of Golgi membranes from BFA treatment using as a marker man II, a resident Golgi membrane protein. BFA has recently been shown to cause the redistribution of man II and other resident Golgi proteins as well as Golgi membranes to the ER in a reversible manner (Doms et al., 1989; Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989, 1990, 1991a; Strous et al., 1991). BHK-21 cells were treated for 90 min with 2 µg/ml of BFA at 37°C to ensure complete translocation of man II to the ER. To follow the movement of man II out of the ER, BFA was removed with several washes of preconditioned medium (20°C) in the presence or absence of 10 mM caffeine. Thereafter, the cells were chased for 60, 120, and 180 min at 20°C with (Fig. 3, *d*, *f*, and *h*, respectively) or without (Fig. 3, *c*, *e*, and *g*, respectively) caffeine, and then fixed and prepared for immunofluorescence. In Fig. 3 *a*, the distribution of man II is shown before any drug treatments. In cells treated for 90 min with BFA at 37°C, man

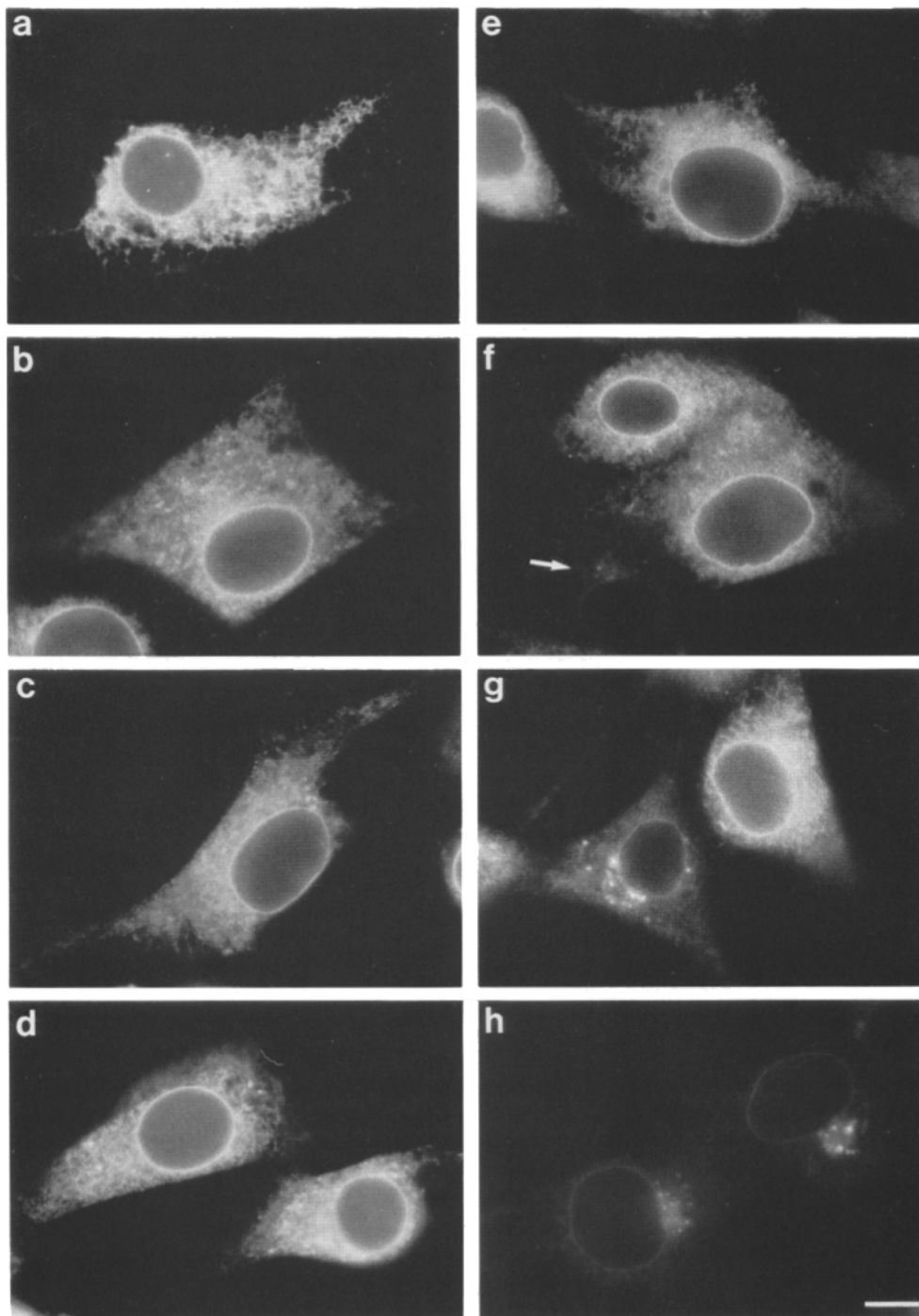


Figure 2. The exit of SFV membrane glycoproteins E1 and E2 from the ER in the presence of 10 mM caffeine at different temperatures. BHK-21 cells infected with SFV ts-1 were incubated for 120 min in the presence of 10 mM caffeine and 50 μ g/ml of cycloheximide at 20 (b), 21 (c), 22 (d), 23 (e), 24 (f), 25 (g), and 28°C (h), fixed and processed for immunofluorescence. a shows the distribution of glycoproteins at the restrictive temperature 38.5°C. At temperatures 20, 21, 22, and 23°C (b, c, d, and e, respectively), SFV glycoproteins can be found in the ER. At 24°C (f), a few cells possess also vesicular labeling (f, arrow). At 25°C, in more than half of the cells, SFV glycoproteins can exit the ER and accumulate to perinuclear structures (g). At 28°C virtually all cells show perinuclear localization of E1 (h). Bar, 20 μ m.

II is translocated efficiently (in 99% of the cells) to the ER (Fig. 3 b). As the cells were incubated for 60 min in a caffeine-free medium at 20°C (Fig. 3 c), man II appears in peripheral and more central elements (in 47% of the cells), and also in the ER. After a 120-min chase in caffeine-free medium at 20°C, the ER was efficiently emptied and man II was found in a few peripheral elements, but was mainly perinuclearly located (Fig. 3 e) (in 95% of the cells). After 180 min in caffeine-free medium, man II was found perinuclearly located with no detectable labeling in the ER (Fig. 3 g) (in 98% of the cells). When the cells were chased

after the removal of the BFA in the presence of 10 mM caffeine at 20°C for 60 min, man II failed to exit the ER (Fig. 3 d) (in 98% of the cells). After a 120- (Fig. 3 f) or 180-min (Fig. 3 h) chase in the presence of caffeine, man II was still located in the ER (in 94 and 96% of the cells, respectively). These results show that 10 mM caffeine and 20°C inhibited efficiently the movement of man II, a resident Golgi membrane protein, out from the ER. This may further suggest that caffeine at reduced temperature inhibits the recovery of the Golgi from the BFA treatment and thus inhibits the membrane traffic from the ER to the Golgi complex.

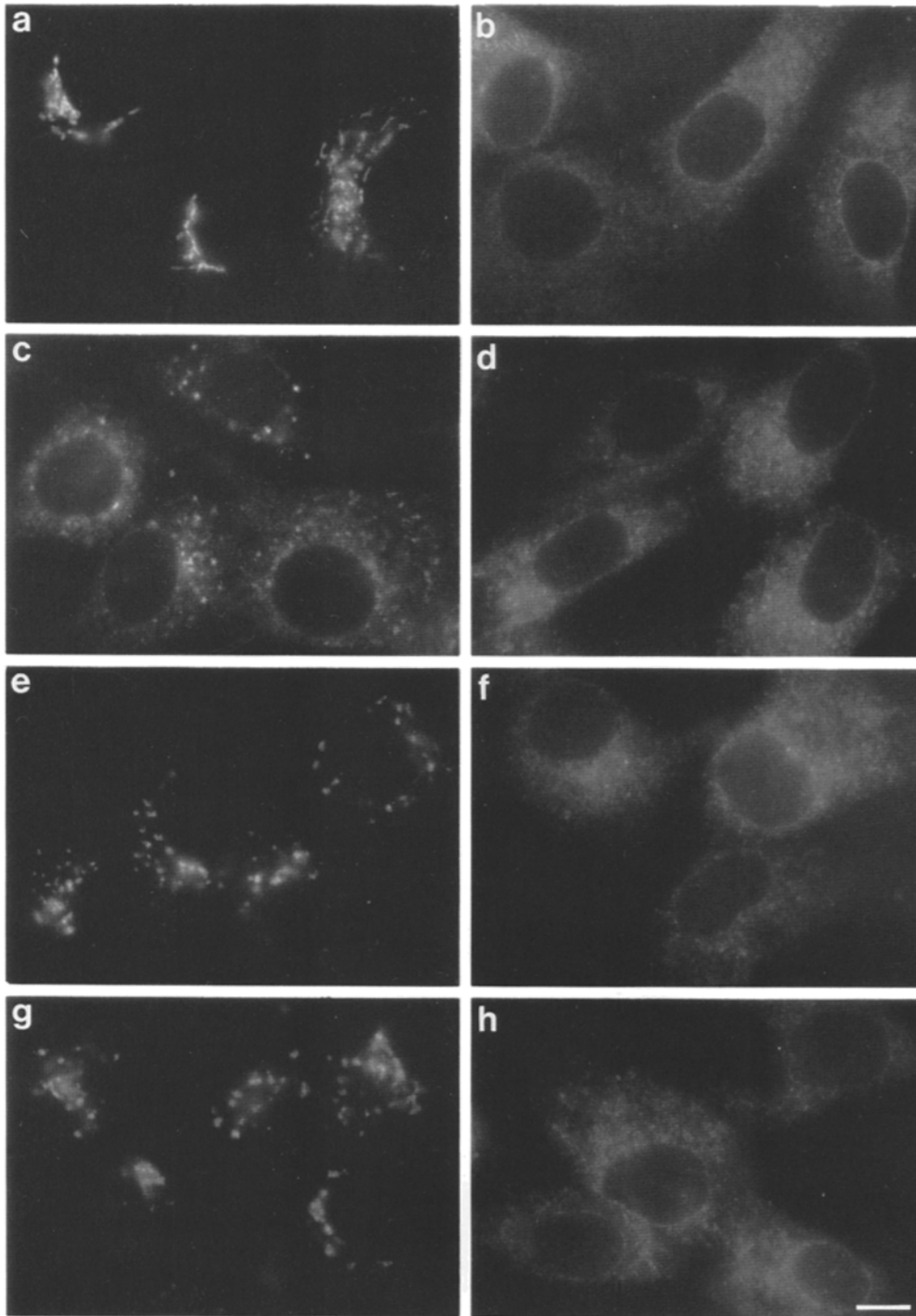


Figure 3. 10 mM caffeine at 20°C inhibits the movement of man II out from the ER. *a* shows the normal distribution of man II in BHK-21 cells. BHK-21 cells were treated with BFA (2 μ g/ml) at 37°C for 90 min (*b*) or treated with BFA for 90 min followed by washout with a medium supplemented with or without 10 mM caffeine at 20°C. The cells were then chased for 60, 120, or 180 min at 20°C in the absence (*c*, *e*, and *g*, respectively) or presence of 10 mM caffeine (*d*, *f*, and *h*, respectively), fixed, and labeled with antibodies to man II. Incubation of the cells in the presence of caffeine at 20°C inhibits the exit of man II from the ER after the removal of the BFA (*d*, *f*, and *h*). In control cells at 20°C already 60 min after the BFA removal, man II can be seen to be located in peripheral elements and in some degree in the ER (*c*). After 120 min, man II is mainly in perinuclear elements (*e*). After a 180-min chase in a caffeine-free medium at 20°C man II is located perinuclearly (*g*). Bar, 20 μ m.

The BFA-induced Retrograde Transport Is Not Inhibited by Caffeine and Reduced Temperature

Since caffeine at 20°C inhibited the ER-to-Golgi membrane traffic, it was of great interest to study whether 10 mM caffeine would also inhibit the Golgi-to-ER traffic. Recently, the fungal antibiotic BFA has been used to study the retrograde transport mechanism from the Golgi complex to the ER (Doms et al., 1989; Lippincott-Schwartz et al., 1989, 1990, 1991a; Fujiwara et al., 1988). We therefore studied the effect of 10 mM caffeine on the BFA-induced redistribution of Golgi membranes at 20°C using man II as a marker. BHK-

21 cells were treated for 60, 120, or 180 min with 5 μ g/ml of BFA and 50 μ g/ml of cycloheximide at 20°C in the presence or absence of 10 mM caffeine. In Fig. 4 *a* the distribution of the Golgi marker man II is shown before any drug treatments. When the cells were treated with 5 μ g/ml of BFA for 90 min at 37°C, a characteristic ER staining pattern was observed (Fig. 4 *b*). When the cells were treated with 5 μ g/ml of BFA for 60 min at 20°C (Fig. 4 *c*) in 60% of the cells man II was found in a perinuclear localization, and in 89% of the cells, man II was found to be translocated partly or completely to the ER. After a 120-min chase at 20°C in the

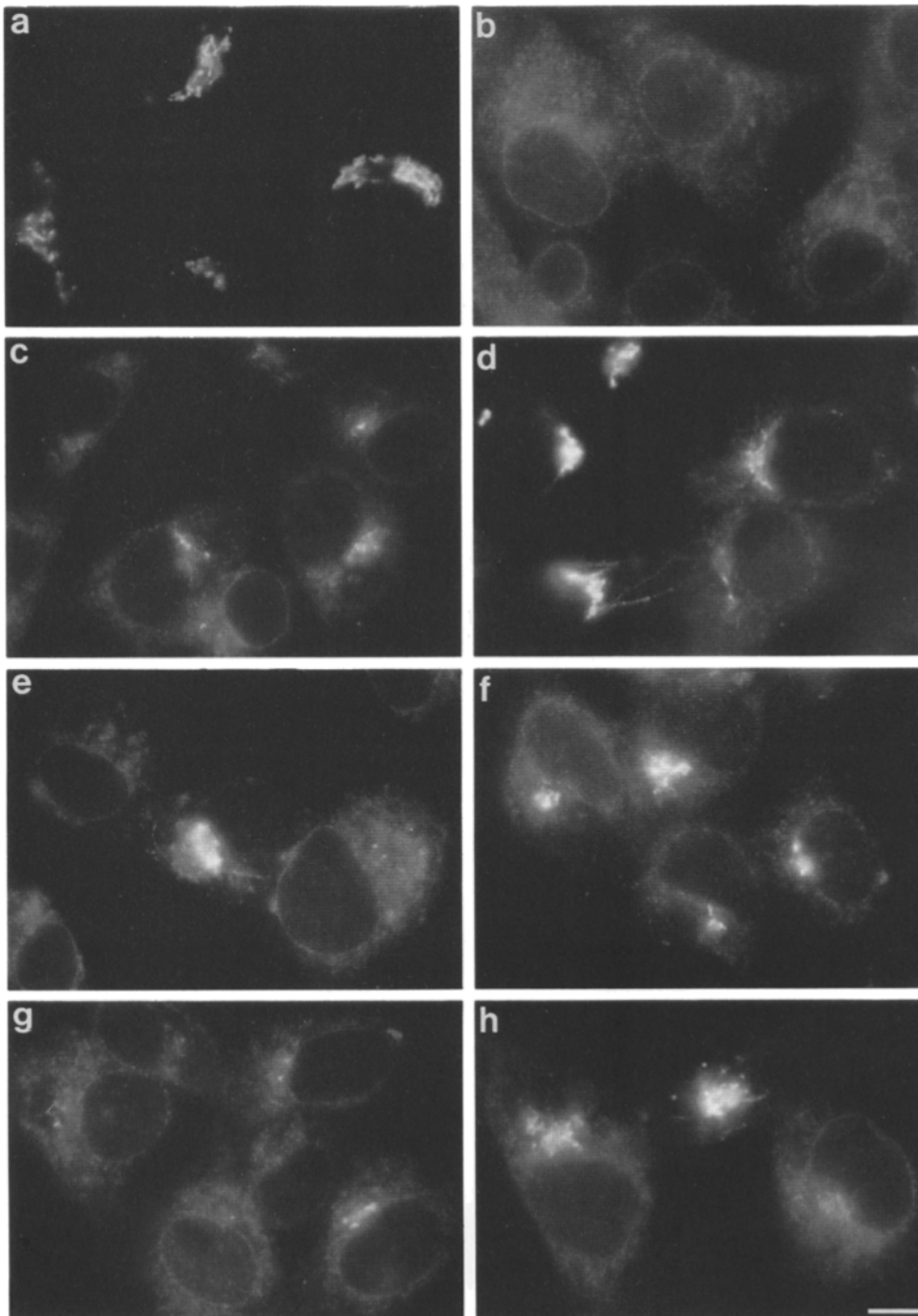


Figure 4. The BFA-induced recycling of man II from the Golgi complex to the ER is not inhibited by caffeine at 20°C. BHK-21 cells were incubated with BFA (5 µg/ml) at 37°C (*b*), with BFA (5 µg/ml) and 10 mM caffeine at 20°C for 60, 120, and 180 min (*d*, *f*, and *h*, respectively) or with BFA (5 µg/ml) only at 20°C for 60, 120, and 180 min (*c*, *e*, and *g*, respectively), fixed, and processed for immunofluorescence microscopy. In all cases 50 µg/ml of cycloheximide was supplemented to prevent further synthesis of man II. Normal distribution of man II in BHK-21 cells (*a*). In *b* man II is observed to label the ER after a 90-min BFA treatment at 37°C. In caffeine-treated cells at 20°C the BFA-induced translocation of man II to the ER is not inhibited (*d*, 60 min; *f*, 120 min; and *h*, 180 min). When compared with the control cells (*c*, 60 min; *e*, 120 min; and *g*, 180 min) the BFA-induced disappearance of the perinuclear Golgi labeling of man II seems to happen more slowly in the caffeine-treated cells than in the control cells. Bar, 20 µm.

presence of BFA only (Fig. 4 *e*), in 41% of the cells man II had a perinuclear localization and in 97% of the cells it was found only or partly in the ER. As the chase was continued to 180 min at 20°C in the presence of 5 µg/ml of BFA (Fig. 4 *g*), man II was still found in 47% of the cells in perinuclear elements and in 97% of the cells only or partly in the ER. When the cells were treated with 5 µg/ml of BFA supplemented with 10 mM caffeine at 20°C for 60 min (Fig. 4 *d*), man II was found in the ER in 68% of the cells and in the perinuclear localization in 92% of the cells. After a 120-min chase in the presence of caffeine, man II was located partly

or completely in the ER in 91% of the cells and was perinuclearly located in 75% of the cells (Fig. 4 *f*). When the chase was carried out for 180 min (Fig. 4 *h*), man II was found in the ER in 96% of the cells and perinuclearly located in 76% of the cells. These results show that, after 60 min in the presence of caffeine, 5 µg/ml of BFA is able to translocate man II to the ER 0.76-fold slower compared with control cells. After 120 min, however, there is no significant difference between caffeine-treated and control cells in the number of cells showing label in the ER. However, in caffeine-treated cells the disappearance of the perinuclear labeling of

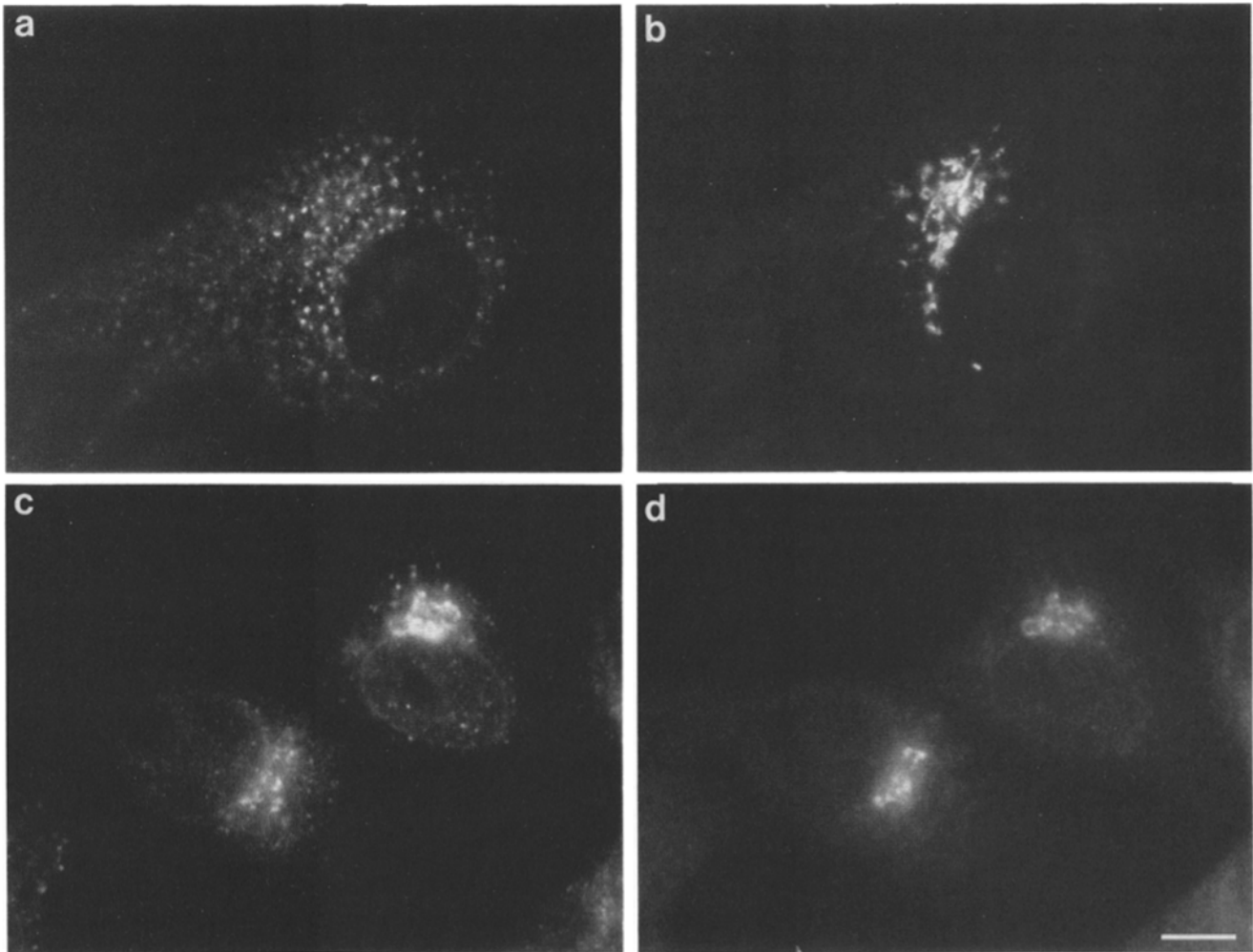


Figure 5. Caffeine-induced translocation of p58 to the periphery of the cells. BHK-21 cells were grown for 30 min at 20°C in the presence (a and b) or absence (c and d) of 10 mM caffeine, fixed, and double-labeled with antibodies to p58 (a and c) and man II (b and d). The distribution of p58 can be seen to change dramatically after a caffeine treatment at 20°C. In a, p58 can be found scattered through the cytoplasm in contrast to control cells at 20°C (c), where p58 is concentrated in the perinuclear region of the cell in addition to some labeling in peripheral vesicles. When the localization of p58 is compared with the localization of man II in caffeine-treated and in control cells incubated at 20°C, p58 can be seen to colocalize only randomly with man II in caffeine-treated cells (a for p58 and b for man II), but to colocalize a great deal in control cells (c for p58 and d for man II). Also, the localization of man II seems not to be affected by the caffeine treatment at 20°C (b). Bar, 20 μ m.

man II seems to occur more slowly than in the control cells at the 60-min time point by a factor of 1.5, at the 120-min time point by a factor of 1.8, and at the 180-min time point by a factor of 1.6. Thus, these results indicate that caffeine does not inhibit the effect of BFA, but it does appear to slow it down somewhat. Interestingly, it seems that during a 180-min chase at 20°C both in caffeine-treated and in control cells the BFA-induced translocation of man II reaches a steady-state situation after a 120-min incubation, whereafter no significant changes in the distribution of man II in the cells seems to occur.

These results suggest that even though caffeine inhibits the movement of membranes from the ER to the Golgi region, it does not inhibit the BFA-induced retrograde movement of Golgi membranes to the ER at 20°C.

Caffeine-induced Reversible Translocation of a cis-Golgi Marker at 20°C

Since 10 mM caffeine at 20°C did not prevent the BFA-

induced retrograde movement of Golgi membranes to the ER, it was of interest to study the distribution of the pre-Golgi and the Golgi membranes under conditions where ER-exit was inhibited. When the anterograde but not the retrograde membrane traffic between the ER and the Golgi is inhibited by caffeine, two things could happen to the Golgi membranes: all the Golgi membranes would recycle to the ER as in the case of BFA or part of the Golgi would do so.

As markers for Golgi membranes we used p58 protein, known to be localized in various cell types in the pre-Golgi elements and in *cis*-Golgi (Saraste et al., 1987; Saraste and Svensson 1991; Lahtinen et al., 1992), and a 135-kD integral Golgi stack membrane protein (Louvard et al., 1982; Burke et al., 1982) which has recently been shown to be identical with man II (Baron and Garoff, 1990). BHK-21 cells were treated for 30, 60, or 120 min with or without 10 mM caffeine at 20°C, fixed, and labeled with a polyclonal antibody to p58 and a monoclonal antibody to man II (135 kD). Surprisingly, after 30 min at 20°C in the presence of

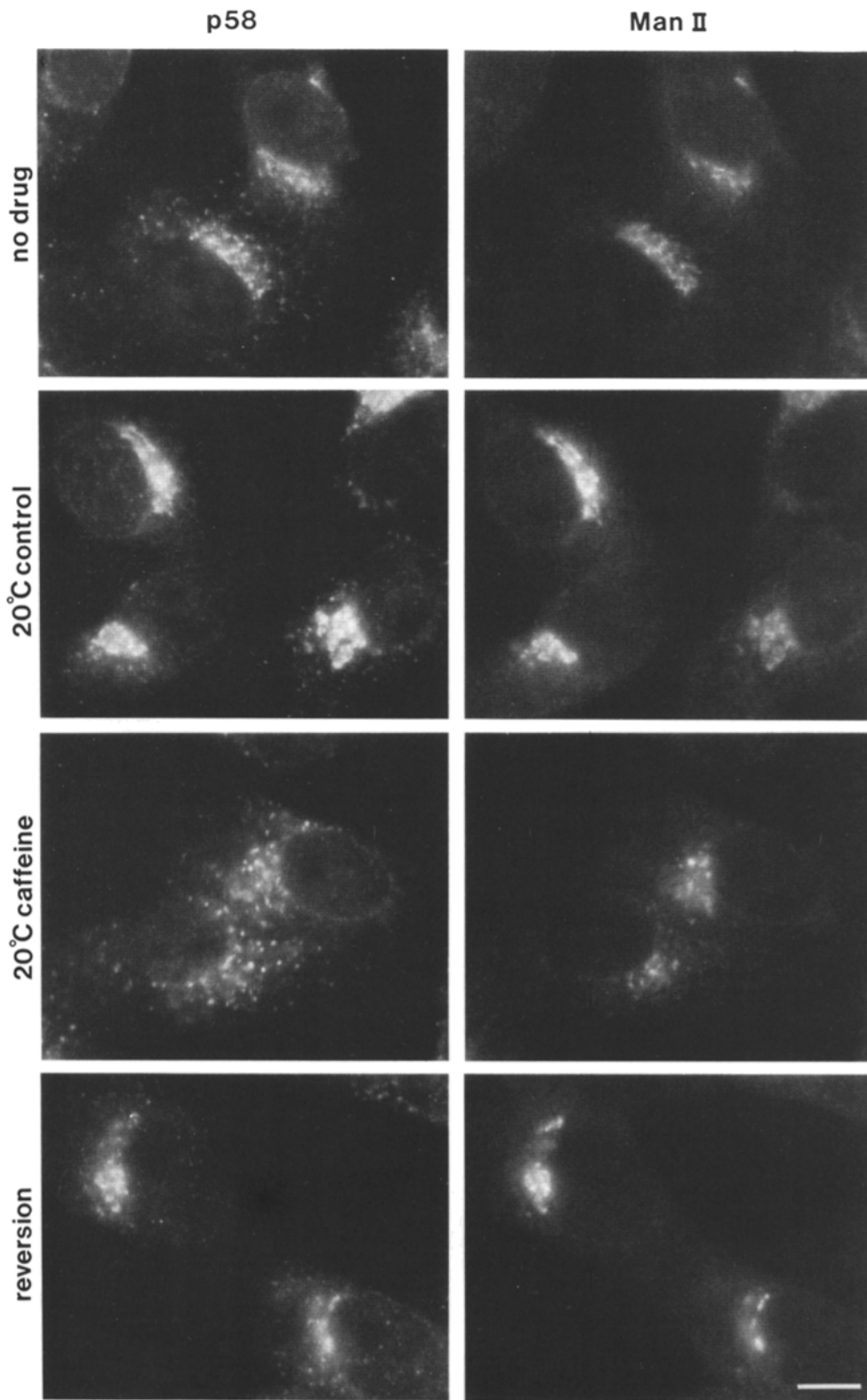


Figure 6. The caffeine-induced translocation of p58 to the periphery of the cells is reversible. BHK-21 cells were incubated in the presence (*20°C caffeine*) or absence (*20°C control*) of 10 mM caffeine at 20°C for 60 min. Cells incubated at 20°C with caffeine were further incubated 60 min without caffeine at 20°C (*reversion*), fixed, and double-labeled with antibodies to p58 and man II. *no drug* shows the normal distribution of p58 and man II in BHK-21 cells. In *reversion*, the perinuclear localization of p58, similar to that in *20°C control*, is restored. Bar, 20 μ m.

caffeine, p58 was found scattered throughout the cytoplasm (Fig. 5 *a*) whereas man II still remained concentrated in a perinuclear localization (Fig. 5 *b*). Incubation for an additional 30 or 90 min showed similar distribution of p58 and man II (data not shown) (see also Fig. 6 for a 60-min incubation). In control cells, incubated for 30 min at 20°C, p58 and

man II (Fig. 5, *c* and *d*, respectively) were found to colocalize largely, except for the peripheral vesicles positive only for p58. Fig. 5, *a* and *c*, demonstrates that p58 is translocated away from its normal perinuclear localization at 20°C in the presence of 10 mM caffeine. In caffeine-treated cells, the colocalization of p58 (Fig. 5 *a*) and man II (Fig. 5 *b*) oc-

curred only rarely. Even though the majority of the p58 protein seemed to move to the periphery of the cell in the presence of caffeine at 20°C some p58-positive vesicles still colocalized with the perinuclear elements also labeled with antibodies to man II. This could partly be due to an apparent colocalization of not the same but closely adjacent structures. These results suggest that 10 mM caffeine at 20°C induces a translocation of p58-positive, pre-Golgi, and *cis*-Golgi membranes to the periphery of the cells leaving man II-positive membranes perinuclearly located.

To study the reversibility of the caffeine-induced translocation of p58, BHK-21 cells were treated first with 10 mM caffeine at 20°C for 60 min, followed by several washes with noncaffeine medium. After incubation for an additional 60 min in the absence of caffeine at 20°C, the localization of p58 was compared with that at normal temperature (37°C), and to that at 20°C without caffeine. In addition, the localization of p58 was compared with that of man II in the same cells. In untreated cells, p58 is located perinuclearly with some labeling in small peripheral vesicles and ER as reported earlier (Saraste and Svensson, 1991) (Fig. 6, *no drug*). In cells treated with caffeine at 20°C for 60 min (Fig. 6, *20°C caffeine*), the bulk of p58 becomes distributed throughout the cytoplasm while man II still remains perinuclearly located. On the contrary, in control cells without caffeine at 20°C, p58 was concentrated to the Golgi area and colocalized with man II (Fig. 6, *20°C control*). The normal localization of p58 was restored when the cells maintained for 60 min in the presence of 10 mM caffeine at 20°C were further incubated for 60 min at 20°C in a caffeine-free medium (Fig. 6, *reversion*). The observed reversible translocation of pre- and *cis*-Golgi marker protein to the periphery of the cell and the unaltered distribution of a Golgi stack marker protein suggest that caffeine at reduced temperature can be used to manipulate pre- and *cis*-Golgi elements without affecting the localization of Golgi stack membranes.

Electron Microscopy of the Cells Treated with Caffeine at 20°C

Because of the effective inhibition of membrane traffic out of the ER at 20°C and the translocation of p58 to the periphery of the cell in the presence of caffeine, it was of great interest to study whether the morphology of the Golgi stack is affected under the conditions used. BHK-21 cells were incubated for 120 min in the presence or absence of 10 mM caffeine at 20°C, fixed, and processed for electron microscopy. In caffeine-treated cells the morphology of the Golgi stack was altered. The Golgi regions of the cells were filled with small (60–70-nm-diam) vesicles (Fig. 7 *a*). In addition, some tubular membranous structures, possibly representing remnants of the Golgi stack, were also observed (Fig. 7 *a*, *arrow*). On the contrary, control cells incubated for 120 min at 20°C possessed typical Golgi stacks (Fig. 7 *b*). Taken together, caffeine at 20°C does not only inhibit the ER-exit of proteins and translocate p58 to the periphery of the cells but also induces a change in the morphology of the Golgi stack.

To study the distribution of p58 in the morphologically altered Golgi region in caffeine-treated cells, we performed immunoelectron microscopy experiments. In control cells, maintained at 20°C for 60 min, p58 was found in *cis*-Golgi and also in cytoplasmic tubulovesicular elements (Saraste et

al., 1987; Saraste and Svensson, 1991) (Fig. 8 *b*, *arrows*). When the cells were incubated for 60 min at 20°C in the presence of 10 mM caffeine the morphology of the Golgi stack was altered as described above (Fig. 7 *a*). Only a few p58-positive vesicles could be found in the Golgi area (Fig. 8 *a*, *arrows*), thus supporting the immunofluorescence finding where the majority of the p58 seemed to get translocated to the periphery of the cells while some of it remained also located near the nucleus.

β-COP Is Not Detached from the Golgi in the Presence of Caffeine

It has been shown that the pre-Golgi markers p58 in normal rat kidney cells (Saraste and Svensson, 1991) and p53 (Schweizer et al., 1988) in M1 cells (Lippincott-Schwartz et al., 1990) get translocated to the periphery of the cell after a BFA treatment. The distribution and morphology of p58-positive elements in BHK-21 cells after incubation at 20°C in the presence of 10 mM caffeine resembled that observed earlier in BFA-treated cells. Even though incubation at 20°C in the presence of caffeine did not result in the movement of a resident Golgi stack marker to the ER (see Fig. 5 *b*), it did cause profound effects on the organization of the Golgi complex (see Fig. 7 *a*). β-COP, a protein known to be associated with the pre-Golgi and the Golgi stack membranes (Duden et al., 1991) is released rapidly from the Golgi membranes in cells treated with BFA (Donaldson et al., 1990). We therefore tested whether caffeine would have an effect on the localization of β-COP. BHK-21 cells were incubated at 20°C in the presence or absence of 10 mM caffeine, fixed, and processed for immunofluorescence microscopy. In Fig. 9 *a*, the distribution of β-COP is shown in cells before any treatments. β-COP has a strong perinuclear localization and also the labeling of cytoplasmic vesicular elements is observed (Fig. 9 *a*). When cells were incubated at 20°C for 60 min in the absence of caffeine (Fig. 9 *b*), the distribution of β-COP was essentially the same as in control cells without any treatments (Fig. 9 *a*). A 60-min incubation at 20°C with 10 mM caffeine did not have any detectable effect on the distribution of β-COP (Fig. 9 *c*). Because of the strong perinuclear labeling of β-COP, possible changes in the distribution of pre- and *cis*-Golgi elements were undetectable in the experiment. This result nevertheless suggests that BFA and caffeine affect the membranes of the Golgi complex through different mechanisms.

Discussion

Recent work by many laboratories has shown that the transport between the ER and the Golgi is mediated by transport vesicles. These transport vesicles appear to exit throughout the ER and to concentrate perinuclearly along the microtubules *in vivo* (Saraste and Svensson, 1991). Only a few markers for these intermediate elements are known. Two membrane proteins, p58 (Saraste et al., 1987) and p53 (Schweizer et al., 1988), are localized to the intermediate elements and the *cis*-Golgi. In addition, the KDEL receptor, responsible for the retention of soluble ER proteins (Pelham, 1988; Pelham, 1989), is likely to be located at least in some degree to these membranes (Lewis and Pelham, 1990; Lewis et al., 1990). There is accumulating knowledge on the com-

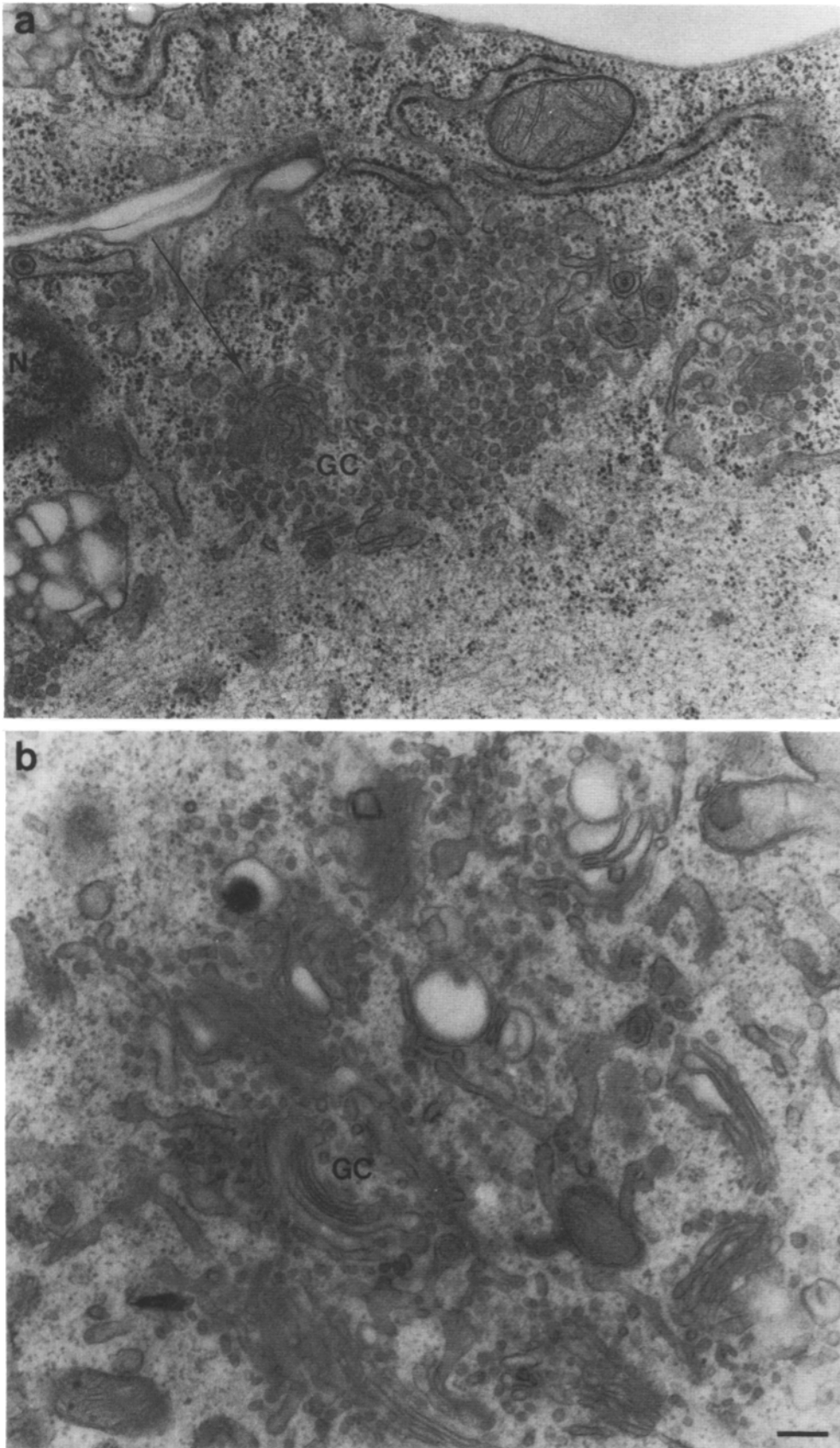


Figure 7. Morphology of the Golgi stack in cells treated with caffeine at 20°C. BHK-21 cells were grown for 120 min at 20°C in the presence (a) or absence (b) of 10 mM caffeine, fixed, and processed for electron microscopy. In caffeine-treated cells the morphology of the Golgi stack is vesicularized (a) in contrast to control cells where the Golgi stack morphology is unaffected (b). Arrow in a shows remnants of the Golgi stack. GC, Golgi complex; N, nucleus. Bar, 200 nm.

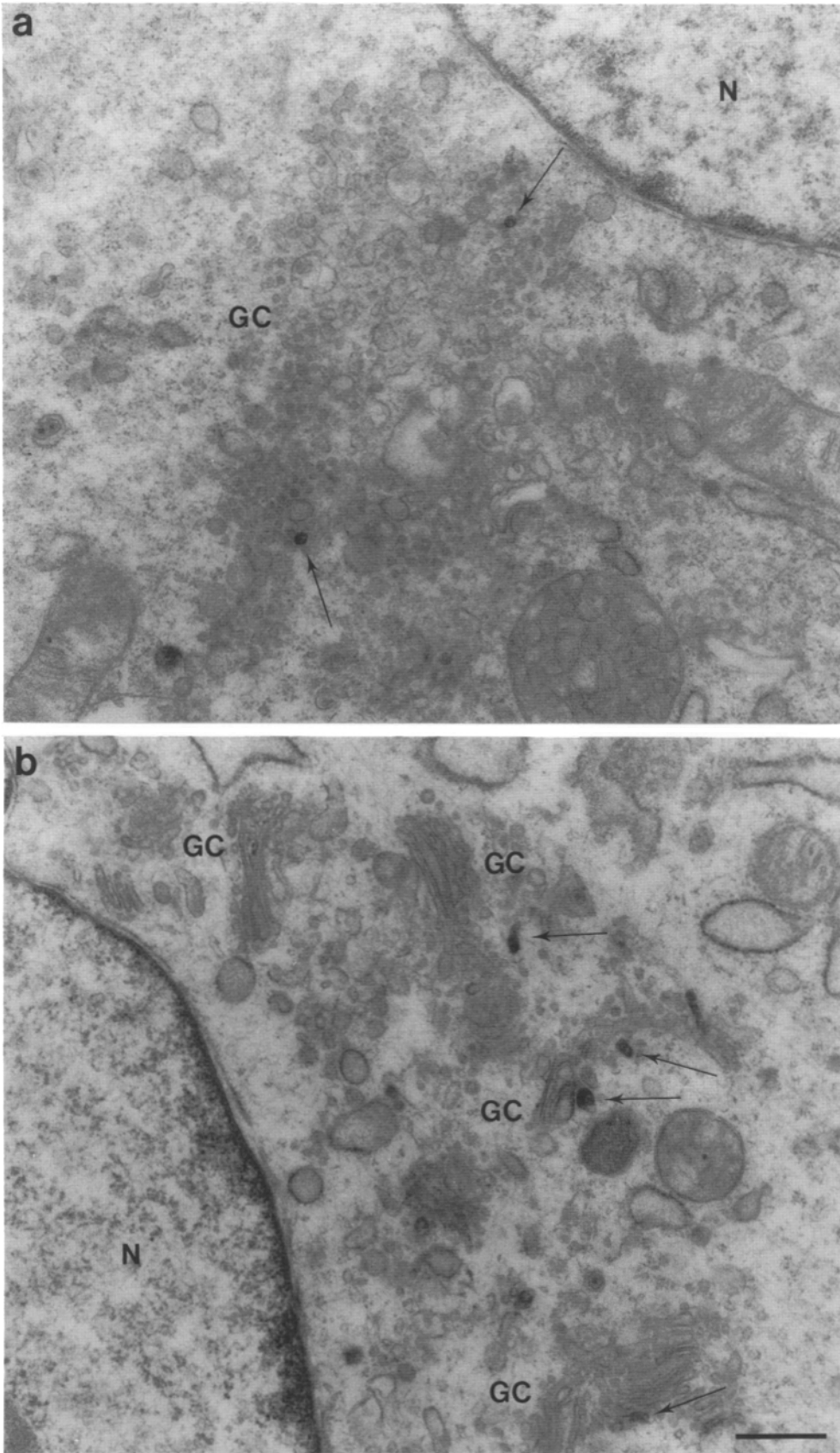


Figure 8. Localization of p58 in the Golgi area in cells treated with caffeine at 20°C. BHK-21 cells were incubated at 20°C with (a) or without (b) 10 mM caffeine, fixed, and processed for immunoperoxidase electron microscopy with antibodies to p58. The alteration of the Golgi morphology in caffeine-treated cells (a) can be observed. Arrows mark the p58-positive structures found in the Golgi region both in caffeine-treated (a) and in control cells (b). In caffeine-treated cells (a) the amount of p58-labeled membranes seems to be lower compared with that observed in control cells (b). Bar, 500 nm.

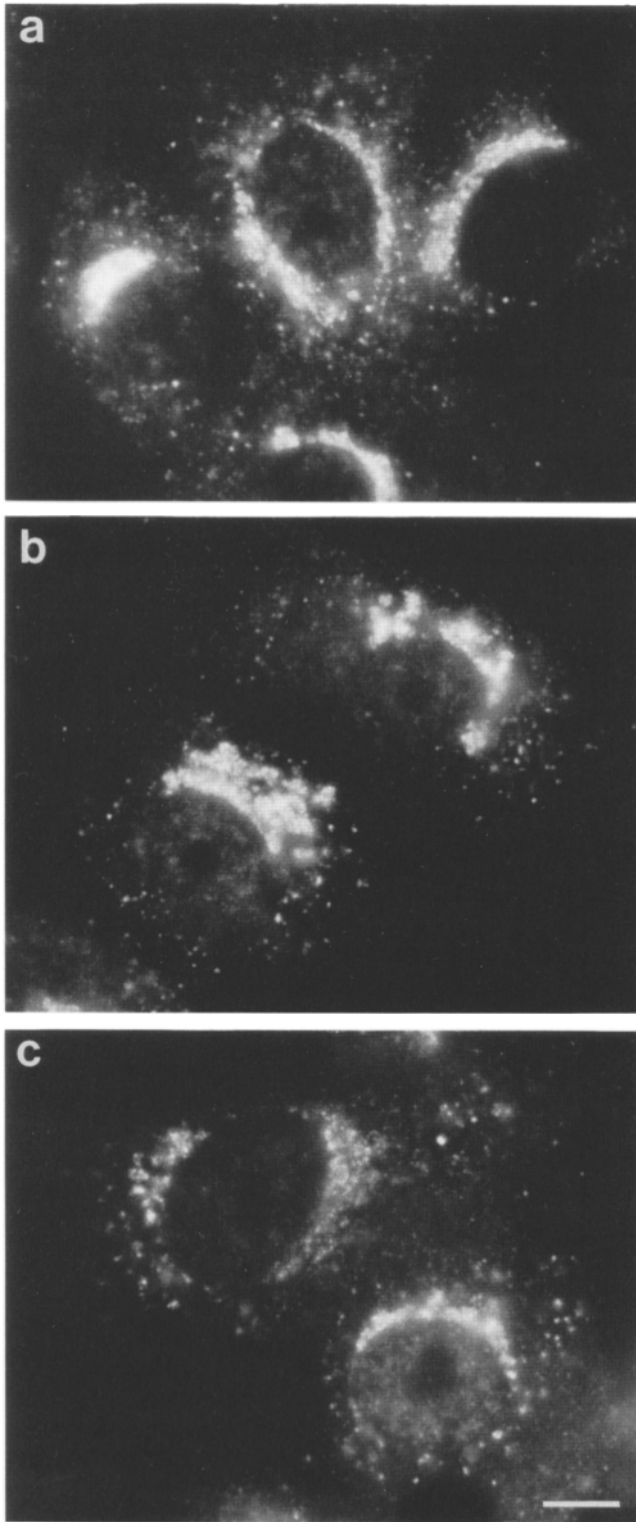


Figure 9. Effect of caffeine at 20°C on the localization of β -COP in BHK-21 cells. BHK-21 cells were grown for 60 min in the presence (c) or absence (b) of 10 mM caffeine at 20°C, fixed, and processed for immunofluorescence with antibodies to β -COP. *a* shows the normal distribution of β -COP in BHK-21 cells. Caffeine at reduced temperature does not seem to affect the distribution of β -COP (c) compared with that in control cells incubated only at 20°C (b) or to that observed in cells without any treatment (a). Bar, 20 μ m.

plexity of the molecular machinery involved in the ER-to-Golgi transport (for review see Rothman and Orci, 1992), based on work with yeast mutants (Novick et al., 1980) and cell-free transport assays (Fries and Rothman, 1980; Balch et al., 1984). In studies concerning the organization of the anterograde and the retrograde membrane traffic, the reversible effect of BFA on the organization of the Golgi complex has provided important new information (Pelham, 1991; Klausner et al., 1992). Our previous report (Kuismanen et al., 1992) and the results in the present study suggest that caffeine is a new potential tool to dissect steps in the exocytic pathway.

Caffeine is known to inhibit the enzyme phosphodiesterase (Butcher and Sutherland, 1962), thus resulting in elevated levels of cAMP inside the cells. Forskolin, which is known to elevate cAMP levels inside cells, did not affect the movement of SFV glycoproteins (data not shown). More recently caffeine has been shown to release Ca^{2+} ions from the intracellular stores other than the inositol triphosphate-sensitive store (Burgoyne et al., 1989). Also the temperature dependence of the caffeine-induced inhibition of membrane traffic follows interestingly well the conditions used in studies of the GTP-mediated Ca^{2+} release (Gill et al., 1986). If the effect of caffeine on the membrane traffic and on the calcium release are in fact related, this could indicate that the ability of the membranes in the ER-to-Golgi interface to recycle escaped, resident ER proteins back to the ER might be regulated by calcium.

Since, on the one hand, caffeine is known to affect the control mechanisms preventing cells in the S-phase to proceed in the mitotic cycle (Schlegel and Pardee, 1986; Downes et al., 1990), and, on the other hand, the onset of mitosis involves complex cascades of phosphorylations and dephosphorylations controlling the progress of cell division (Lewin, 1990), it could be possible that caffeine acts through the activation or deactivation of some kinase or phosphatase. Also, it has recently been reported that okadaic acid, a phosphatase inhibitor, affects the ER-to-Golgi traffic (Davidson et al., 1992) and the morphology of the Golgi apparatus (Lucocq et al., 1991).

In the present study, we have dissected the compartmental organization of the ER-to-Golgi interface using the transport inhibition exerted by 10 mM caffeine at 20°C (Kuismanen et al., 1992). The temperature threshold inhibiting the exit of SFV glycoproteins from the ER was found to be 23–24°C. At 25°C, SFV E1 reached the Golgi stack as indicated by the Endo H analysis. Some variation, however, was observed in the temperature dependence between individual cells close to the threshold temperature. Therefore, in other experiments, 20°C in the presence of caffeine was used. To study whether the transport of cellular proteins is also affected, man II was translocated with BFA to the ER, whereafter BFA was washed away and the movement of man II was followed in the absence or presence of 10 mM caffeine at 20°C. As was the case with virus proteins, man II was unable to exit from the ER in the presence of 10 mM caffeine at 20°C during a 180-min chase. In contrast, during a 180-min chase in the control cells, ER was efficiently emptied and man II accumulated perinuclearly in the absence of caffeine at 20°C. It is therefore suggested that caffeine affects the movement of membranes between the ER and the Golgi complex. It is, however, necessary to follow the movement of other cellular

proteins and, for example, lipids between the ER and the Golgi in the presence of caffeine at reduced temperature to obtain a clear picture of this matter. We have also tested that the ER-exit inhibition by caffeine functions in SFV-infected normal rat kidney cells (data not shown), indicating that the observed effect is not specific for one cell type.

An interesting question was whether caffeine and reduced temperature would inhibit also the BFA-induced retrograde movement of the Golgi membranes. In the presence of 10 mM caffeine at 20°C, BFA was able to redistribute at least part of the cellular man II to the ER with nearly similar kinetics than with BFA only at 20°C. Interestingly, caffeine did seem to slow down the rate by which BFA treatment results in the disappearance of any recognizable Golgi complex by a factor of ~ 1.6 . Nevertheless, already after a 60-min chase in the presence of caffeine at 20°C, 68% of the cells showed labeling in the ER and long tubular structures emanating from the Golgi complex. These results show that caffeine does not inhibit the BFA-induced retrograde Golgi-to-ER transport, although the traffic in the opposite (ER-to-Golgi) direction, is inhibited. Also, since caffeine at 20°C did not inhibit the BFA-induced retrograde movement of Golgi membranes, known to be inhibited by the GTP- γ -S (Tan et al., 1992), it seems unlikely that the effect of caffeine would be due to caffeine mimicking GTP- γ -S.

Electron microscopy study of the Golgi structure revealed a vesicularization of the Golgi stack in the presence of 10 mM caffeine at 20°C. These small vesicles remained as tight clusters at the perinuclear site where the Golgi complex is normally localized. This result is in agreement with the immunofluorescence observations with man II. Since the perinuclear localization of the Golgi complex in the cell is dependent on the integrity of the microtubules (Thyberg and Moskalewski, 1985; Kreis, 1990), it is unlikely that caffeine at 20°C would act through disrupting the microtubular network. This is further supported by the fact that also in caffeine-treated cells the BFA-induced tubular, microtubule-dependent structures (Lippincott-Schwartz et al., 1990) were able to form. One possible explanation for the caffeine-induced vesicularization of the Golgi stack could be that when the transport from the ER to the Golgi complex is inhibited, while the vesicular recycling in the Golgi stack is still occurring, the Golgi stack ultimately becomes vesicularized.

Localization of marker proteins of the pre-Golgi membranes and the Golgi stack demonstrated an interesting, differential effect of caffeine on the distribution of these membrane compartments. The p58-positive pre- and *cis*-Golgi membranes lost their normal perinuclear localization while the localization of a Golgi stack protein, man II, remained unaffected. The normal distribution of p58 was restored when caffeine was removed. When the localization of p58 was studied with immunoelectron microscopy in caffeine-treated cells at 20°C, the typical tubulovesicular p58-positive elements (Saraste and Svensson, 1991) were absent from the Golgi region and p58 was found only to be localized in a few small vesicles associated with the clusters of vesicles in the Golgi region. Even though immunoperoxidase technique is by no means a quantitative method, when compared with the control cells, the number of p58-positive membrane elements in the Golgi region seemed to be lower in caffeine-treated cells at reduced temperature. The statement that p58 is translocated away from the perinuclear lo-

calization in caffeine-treated cells is based on the results obtained in the immunofluorescence experiments, where distribution of the bulk of p58 can be observed. Overall, the immunoelectron microscopic finding is in agreement with the immunofluorescence result in Fig. 5 *a*.

As suggested by the immunofluorescence experiments, the bulk of the p58 seems to be translocated away from the perinuclear localization in caffeine-treated cells. These results suggest that caffeine and reduced temperature are able to induce a selective retrograde translocation of pre- and *cis*-Golgi membranes while the localization of the Golgi stack membranes remains unaffected. This was further confirmed by the unaffected localization of β -COP protein. Possible explanation to the observed loss of the perinuclear labeling of p58 could be that in the presence of caffeine the pre- and *cis*-Golgi elements are induced to move toward the periphery of the cell. Another possibility is that as the membrane traffic from the ER is inhibited and at least the BFA-induced retrograde movement of membranes is not inhibited, p58 could finally be recycled to the ER, being unable to exit from there. Whatever the solution turns out to be, caffeine seems to make it possible to manipulate the membranes operating in the ER-to-Golgi traffic in a novel way. Interestingly, Lewis and Pelham (1992) have recently shown that, by overexpressing the ligand for the KDEL receptor, the bulk of the receptor is translocated to the ER.

To obtain a more detailed picture of the effect of caffeine and reduced temperature on the membranes operating between the ER and the Golgi complex, it is necessary to study whether the distribution of other markers for these membranes such as p53 (Schweizer et al., 1988), Corona virus E1 glycoprotein (Machamer et al., 1990), and rab2 (Chavrier et al., 1990), is also affected. Caffeine at reduced temperature on the one hand inhibited the exit of proteins from the ER and on the other hand induced a selective translocation of pre- and *cis*-Golgi membranes to peripheral locations; this is in contrast to the BFA action which seems to translocate unselectively all Golgi membranes from their normal perinuclear location (Lippincott-Schwartz et al., 1989, 1990, 1991a; Doms et al., 1989; Fujiwara et al., 1988; Strous et al., 1991). Further, caffeine did not detach β -COP from the Golgi membranes nor did it translocate man II to the ER. Thus, the effect of caffeine on the organization of the membranes between the ER and the Golgi complex is clearly different from that induced by BFA. The different effect of BFA and caffeine on the Golgi complex would indicate that the Golgi complex is composed of two subsets of homotypic membranes differentially sensitive to caffeine treatment. Whether this is also reflected in the differences of the coat structures of these membranes remains to be seen.

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