Targeting of Secretory Vesicles to Cytoplasmic Domains In AtT-20 and PC-12 Cells

Linda Matsuuchi, Kathleen M. Buckley, Anson W. Lowe, and Regis B. Kelly

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448

Abstract. Organelles are not uniformly distributed throughout the cytoplasm but have preferred locations that vary between tissues and during development. To investigate organelle targeting to cytoplasmic domains we have taken advantage of the mouse pituitary cell line, AtT-20, which, when induced to extend long processes, accumulates dense core secretory granules at the tips of the processes. During mitosis, these secretory granules accumulate along the plane of division. Protein synthesis is not mandatory for such redistribution of secretory granules. To explore the specificity of the redistribution we have used transfected AtT-20 cells that express the immunoglobulin kappa light chain. While the endogenous hormone ACTH is found in secretory granules, the kappa chain is a marker for organelles involved in constitutive secretion. By immunofluorescence, kappa also accumulates at the tips of growing processes, and along the midline of dividing cells, suggesting that the redistribution of vesicles is not specific for dense-core secretory granules. Since there is evidence for selective organelle transport along processes in neuronal cells, the rat pheochromocytoma cell PC-12 was transfected with DNA encoding markers for regulated and constitutive secretory vesicles. Again regulated and constitutive vesicles co-distribute, even in cells grown in the presence of nerve growth factor. We suggest that at least in the cells studied here, cytoskeletal elements normally carry exocytotic organelles to the surface; when the cytoskeletal elements coalesce in an extending process, exocytotic organelles of both the constitutive and regulated pathway are transported nonselectively to the tips of the cytoskeletal elements where they accumulate.

UNDERSTANDING how a cell acquires its distinctive morphology requires knowledge of how proteins are targeted to different organelles and how organelles are targeted to different regions of the cytoplasm. The mouse pituitary cell line, AtT-20, has already been useful for understanding the sorting of secreted proteins into secretory vesicles (for review, see Kelly, 1985). In this study, AtT-20 cells were used to explore mechanisms by which organelles accumulate in specific regions of the cytoplasm.

Cells of the mouse pituitary line, AtT-20, extend long processes when grown in the presence of 8-Br-cAMP. Using immunofluorescence to detect ACTH, a hormone secreted by AtT-20 cells, we found that the tips of processes were packed with hormone (Kelly et al., 1983; Burgess et al., 1985). The tips also contained dense-core granule membrane proteins (Buckley and Kelly, 1985). Electron microscopy demonstrated a high concentration of dense core granules (Kelly et al., 1983), shown by immunoelectron microscopy to contain ACTH (Burgess et al., 1987; Tooze and Tooze, 1986). Accumulation of dense-core secretory granules in regions of AtT-20 cells offered an opportunity to ask what might cause targeting of organelles to cytoplasmic domains, a phenomenon found commonly in differentiated cells, especially neurons.

The AtT-20 cell line has two pathways of protein secretion. Proteins that enter the regulated pathway are stored in dense-core secretory granules until the granules are triggered to fuse with the plasma membrane by an external signal. Proteins that exit constitutively are contained in secretory vesicles that release their contents by exocytosis without external stimulation. By DNA transfection, we have generated cell lines that secrete the immunoglobulin light chain, kappa, exclusively by the constitutive pathway (Matsuuchi, L., and R. B. Kelly, unpublished observations). The level of kappa chain expression is high enough to be readily detected by immunofluorescence, and so can be used to determine where in the cell the regulated and the constitutive secretory vesicles are targeted. The transfected cell lines have revealed that constitutive secretory vesicles also accumulate in cytoplasmic regions associated with the ends of microtubules. In the cells studied here, organelle location in the cell could be explained by a transport mechanism along microtubules that does not distinguish between regulated secretory granules and constitutive secretory vesicles. It also appears likely that lysosomes and mitochondria are transported by the same or a similar mechanism.

Materials and Methods

Materials

The MPC-11 kappa and the S107 kappa expression vectors were constructed from genomic clones for the MPC-11 kappa (obtained from B. Van Ness, University of Iowa, Iowa City, IA) or the S107 kappa (obtained from S. Mor-

rison, Columbia College of Physicians and Surgeons, New York, NY) and pRSVCAT (Moore and Kelly, 1985) using standard procedures. The antibiotic G418 (Gentecin) was obtained from Gibco (Grand Island, NY). 8-Bromo-adenosine 3′-5′ cyclic monophosphate, dibutryl-cAMP, methyl (5-[2 thienly carbonyl]-1H benzimedazol-2-yl) carbamate (nocodazole), and 1,4-diazabicyclo (2.2.2) octane (DABCO) were from Sigma Chemical Co. (St. Louis, MO). Nerve growth factor (7S) from mouse submaxillary gland was from Calbiochem/Behring Diagnostics (La Jolla, CA). Laminin was a generous gift from Deborah Hall and Louis Reichardt (University of California, San Francisco, San Francisco, CA). Paraformaldehyde was from J. T. Baker Chemical Co. (Phillipsburg, NJ). *Trans*-^{35}S label was from ICN Radiochemicals (Irvine, CA). Rabbit anti-mouse kappa chain antisera was from Miles Labs, Inc. (Elkhart, IN) and from ICN Immunobiologics (Irvine, CA). Goat anti-rabbit-FITC and sheep anti-rabbit-rhodamine were from Cooper Biomedical (Malvern, PA). Biotinylated goat anti-rabbit and biotinylated horse anti-mouse immunoglobulin light and heavy chain were from Vector Labs (Burlingame, CA). Strepavidin-FITC was from either Bethesda Research Laboratories (Gaithersburg, MD) or from Molecular Probes (Eugene, OR). Guinea pig anti-porcine insulin was from Linco Research, Inc. (Eureka, MO). Rabbit anti-guinea pig IgG-FITC was from E. Y. Labs (San Mateo, CA).

Cell Culture and Transfection

The AtT-20 cell lines expressing mouse immunoglobulin kappa light chain (MPC-11 kappa or S107 kappa) were generated by DNA-mediated transfection of expression vectors containing genomic kappa light chain genes and the Rous Sarcoma virus (RSV) long terminal repeat as a promoter-enhancer element (Matsuuchi, L., and R. B. Kelly, unpublished observations). Cells were maintained in DME-H21 containing 10% FCS, penicillin (100 U/ml), streptomycin (100 U/ml) and 0.25 mg/ml of the antibiotic G418, under an atmosphere of 15% CO_2.

PC12 cells, a rat pheochromocytoma cell line (Greene and Tischler, 1976) were obtained from the University of California, San Francisco tissue culture facility. PC12 cells expressing rat proinsulin were obtained from Erik Schweitzer, University of Wisconsin (Madison, WI). Cells were maintained in DME supplemented with 10% FCS, 5% horse serum, penicillin, and streptomycin, under an atmosphere of 10% CO_2.

Cell transfections were performed as follows. PC12 cells were co-transfected with two plasmid expression vectors, pSV2neo and pRSVΔ5′ MPC-11 k-1 essentially as described (Schweitzer and Kelly, 1985). The amounts of plasmid DNA were adjusted such that 3×10^6 PC12 cells received a mixture of 20 μg pSV2neo and 100 μg of pRSVΔ5′MPC-11 k-1 DNA. Transfected cells were first selected in media containing 0.5 mg/ml G418 (active drug) for one week at which point the concentration of drug was decreased to 0.25 mg/ml. Stable clones were recovered, grown to mass culture and tested for the production of kappa light chain by in vivo labeling followed by immunoprecipitation with specific anti-kappa antisera.

Metabolic Labeling

Cells were labeled with radioactive cysteine and methionine as described (Moore and Kelly, 1985). 5×10^6 cells were washed with PBS and then with DME-H21 medium depleted of cysteine and methionine but supplemented with 2% FCS and penicillin/streptomycin. Cells were then incubated for 5 h in depleted DME-H21 containing 100–200 μCi *Trans*-^{35}S-label. The culture media was removed and cell extracts were prepared by lysing cells in a detergent buffer, as described previously (Burgess and Kelly, 1985). 5 μl of rabbit anti-mouse kappa antisera and prewashed Staph A cells (Pansorbin, Calbiochem, San Diego, CA) were used to recover the labeled kappa chain. Samples were analyzed on SDS-polyacrylamide gels.

Immunofluorescence

AtT20 cells were grown on poly-D-lysine-coated glass coverslips in either DME-H21 as described under cell culture or in medium containing 5 mM 8-Bromo-cAMP for 3–4 d to promote the extension of processes. Cells were rinsed in PBS, fixed for 20 min at room temperature in 3% paraformaldehyde (wt/vol), pH 7.4, containing 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, quenched for 5 min in 50 mM NH_4Cl and permeabilized in PBS containing 2% gelatin and 0.2% Triton X-100 (PGT). Primary antibodies were absorbed with formalin fixed, non-transfected AtT-20 cells before use. Antibodies were clarified by centrifugation and by filtration through 0.45 μm Spin-X microfilters. Coverslips were incubated for 30 min at room temperature on a 10–15 μl drop of antibody. Coverslips were rinsed in PGT buffer and incubated with a 10–15 μl drop of the secondary antibody. Coverslips were washed sequentially in PGT, PBS, and distilled H_2O, mounted in 90% glycerol, 7.5% PBS, and 2.5% DABCO (Johnson et al., 1982). The cells were visualized and photographed using a Nikon Diaphot TMD inverted scope equipped with a Nikon FE 35-mm camera. Similarly, biotinylated antibodies were incubated with the coverslips for 30 min at room temperature followed by a 30 min incubation with strepavidin-FITC.

To detect intracellular kappa chain, cells were incubated with preabsorbed rabbit anti-mouse kappa antisera (diluted 1:10). To detect intracellular ACTH, cells were incubated with an affinity purified rabbit anti-ACTH antisera (diluted 1:10). Fluorescein-conjugated goat anti-rabbit antibodies (diluted 1:300) were used to detect the primary antibody. For anti-kappa/anti-ACTH double immunofluorescence, the kappa chain was detected with a biotinylated horse anti-mouse immunoglobulin heavy and light chain antibody (diluted 1:40) followed by strepavidin-FITC (BRL; diluted 1:50, Molecular Probes; diluted 1:100). The ACTH was detected with the rabbit anti-ACTH described above (diluted 1:60) and sheep anti-rabbit-rhodamine (diluted 1:400).

PC12 cells were also grown on poly-D-lysine-coated glass coverslips either in medium as described under cell culture or in medium containing 25 ng/ml of nerve growth factor and 1 mM dibutryl-cAMP to induce neurite outgrowth. Intracellular insulin was detected with a guinea pig anti-insulin antibody (diluted 1:400) followed by a rabbit anti-guinea pig IgG (diluted 1:100). Intracellular kappa chain was detected with preabsorbed (with non-transfected PC12 cells) rabbit anti-mouse kappa chain antisera (diluted 1:10) followed by goat anti-rabbit antibody (diluted 1:50–1:100) and strepavidin-FITC (diluted 1:50).

Electron Microscopy of AtT20 Cells

To examine the ultrastructure of processes extended by AtT-20 cells chronically exposed to cAMP, cells were plated on poly-D-lysine-coated glass coverslips and grown for one week in DME-H21 containing 5 mM 8-Bromo-cAMP and 10% horse serum. Cultures were fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer and processed for electron microscopy as described (Buckley and Landis, 1983). Mitotic cells were chosen at random from cells grown in DME-H21/10% horse serum without cAMP or nocodazole. Cultures were sectioned in a plane parallel to the coverslip.

Enrichment of Mitotic Cells

Mitotic cells were enriched for those in telophase as described (Tooze and Burke, 1987). Cells were grown on poly-D-lysine-coated coverslips in DME-H21 containing 10% FCS and penicillin/streptomycin. Cells were incubated in medium containing 40 ng/ml nocodazole for 2–3 h to accumulate metaphase cells. The nocodazole containing medium was removed, the cells were washed, and fresh medium was added. Samples were fixed immediately or at 30-, 60-, or 90-min intervals after the removal of nocodazole and stained for intracellular kappa chain or ACTH as described in the immunofluorescence section. Nuclei were stained for 10 min at room temperature in 2 μg/ml bisbenzamide (Hoechst nuclear stain).

Process Extension in AtT-20 Cells in the Presence of Cyclohexamide

AtT-20 cells were washed off tissue culture plates with calcium-magnesium free PBS containing 5 mM EGTA. The cells were collected by centrifugation and resuspended in methionine and cysteine deficient DME-H21 medium containing one-fifth normal medium and either 10 μm cyclohexamide (final concentration) or an equal volume of PBS. For the morphological studies of process extension, AtT-20 cells were plated on laminin-coated plates at a concentration of 131,000 cells per cm^2. Photographs of the cells were taken at hourly intervals. At each time point 500 cells were counted and the number of cells containing processes greater than one cell body length was assessed. Tissue culture plates were coated with laminin by incubating them overnight at 4°C in PBS containing 20 μg/ml laminin and plates were rinsed with PBS before plating cells. For immunofluorescence, plastic coverslips were coated with laminin and the cells were stained with antibodies as described above.

To monitor the extent of protein synthesis, TCA-precipitable labeled proteins were monitored. Cells were plated in laminin-coated 24-well plates at 206,000 cells/cm^2 with 10 μCi/well of *trans*-^{35}S label with or without cyclohexamide as described. At each time point, the medium was aspirated and the cells solubilized with 1 ml per well of NDET buffer (Burgess et al., 1985). The extract was spun at 12,000 *g* for 10 min, the supernatant collected, and phenylmethylsulfonyl fluoride (PMSF) and iodoacetamide

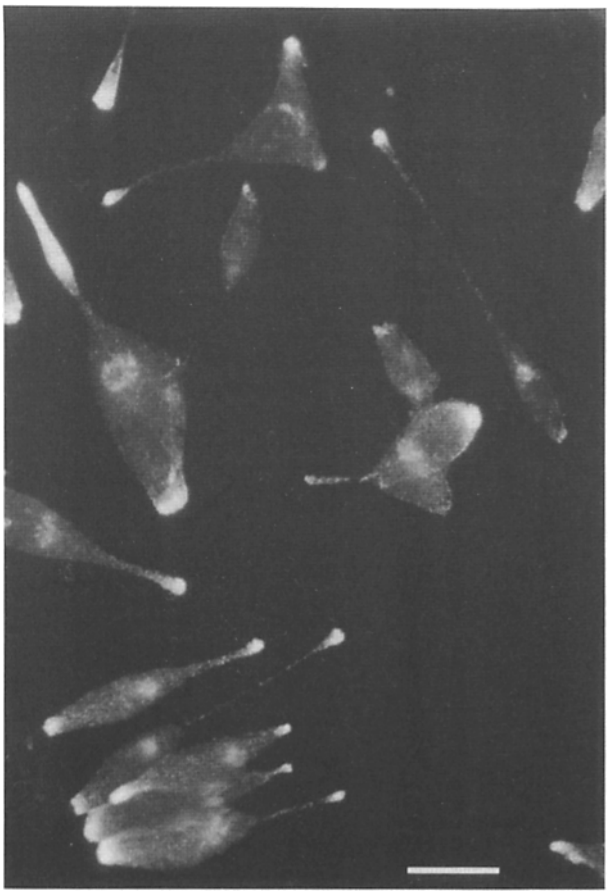

Figure 1. AtT-20 cells grown on laminin. Unipolar processes containing ACTH can be seen when AtT-20 cells are grown on laminin-coated glass coverslips. A juxtanuclear Golgi region containing immunoreactive ACTH as well as an accumulation of ACTH at the pole opposite the process is also visible. Cells were stained with rabbit anti-ACTH antibody and goat anti-rabbit FITC. Bar, 24 μm.

added to a final concentration of 0.3 mg/ml. 200 μl of the extract was added to an equal volume of 10% TCA in PBS and incubated on ice for 15 min. The precipitated material was collected on Whatman GF/B glass filters and counted with Ecolite (Westchem, San Diego, CA) scintillation fluid.

Results

Process Extension is Associated with Accumulation of Secretory Granules

Under normal growth conditions the mouse pituitary cell line, AtT-20, has an pleiotropic morphology similar to that of pituitary corticotrophs. When cells are grown in 8-Bromo-cAMP, process extension is stimulated, the cells increase in size and become flatter, and the tips of the processes accumulate the endogenous hormone, ACTH (Kelly et al., 1983; Buckley and Kelly, 1985; Burgess et al., 1985; Tooze and Burke, 1987). When process extension was induced in AtT-20 cells in ways that avoid prolonged exposure to a stimulus such as cAMP, the tips of processes still accumulated ACTH. For example, if AtT-20 cells were grown on the extracellular matrix component laminin, they developed a unipolar shape within 3–6 h. In most cases the Golgi apparatus, visualized as juxtanuclear ACTH staining, was oriented towards the long process (Fig. 1). The hormone accumulated at the tip of the long process, but also at the opposite pole of the cell. Even in the absence of cAMP or laminin, some cells in the population extended long processes that accumulated ACTH at their tips. This is especially noticeable when cells were plated at low density. We have isolated clonal variants of AtT-20 cells that extend long processes in normal media even in the absence of 8-Bromo-cAMP or extracellular matrix components. The regulated secretory pathway was unaltered in such clones (not shown). Taken together the data imply that stimulation of secretion with cAMP is not essential for process development or the distribution of ACTH-containing vesicles at process tips. Whether AtT-20 processes are single or multiple, thick or thin, branched or unbranched, they can accumulate secretory granules containing ACTH at their tips.

Under normal growth conditions, secretory granules, detected by their electron dense cores, are distributed throughout the cytoplasm, although they preferentially line up along the plasma membrane. After process extension was induced, electron microscopy confirmed the presence of dense core granules at the tips of processes (Fig. 2; Kelly et al., 1983). At the tips the secretory granules filled the cytoplasmic space.

Protein Synthesis is not Required for Vesicle Redistribution

If process extension required de novo protein synthesis, then blocking protein synthesis should inhibit process formation. About one-half of the AtT-20 cells in a culture dish could extend processes as early as 3–6 h after cells were plated on a laminin substrate. To determine if protein synthesis was essential for process extension, cells were plated on laminin in the presence or absence of cyclohexamide (10 μM). Using incorporation of [^{35}S]methionine into acid-precipitable material as a measure of the extent of protein synthesis, inhibition was greater than 95% after a 2-h exposure to cyclohexamide. Despite almost complete inhibition of protein synthesis, process extension could still occur. The number of cells extending processes was reduced, but was still ~50% of the number extending processes in the absence of cyclohexamide treatment (Fig. 3). Process extension in the absence of protein synthesis has been observed in neuroblastoma cell lines (Seeds et al., 1970) and in isolated sensory ganglion growth cones (Shaw and Bray, 1977; Baas et al., 1987). By immunofluorescence the ACTH-containing secretory granules could also redistribute to the tips of processes even in the absence of protein synthesis (data not shown). The induced change in morphology should probably be considered a rearrangement or redistribution of preexisting organelles and cytoskeleton, at least in its early stages.

Constitutively Secreted Proteins also Accumulate at the Growing Tips

We next asked if there was selectivity in the targeting mechanism. In AtT-20 cells, regulated secretory granules detected by their hormone content accumulate at the tips of processes. If accumulation were selective for regulated secretory vesicles, secretory vesicles carrying constitutively secreted protein would be absent from the ends of processes. To identify constitutive secretory vesicles, we used a transfected AtT-20 cell line that expressed an immunoglobulin kappa light chain.

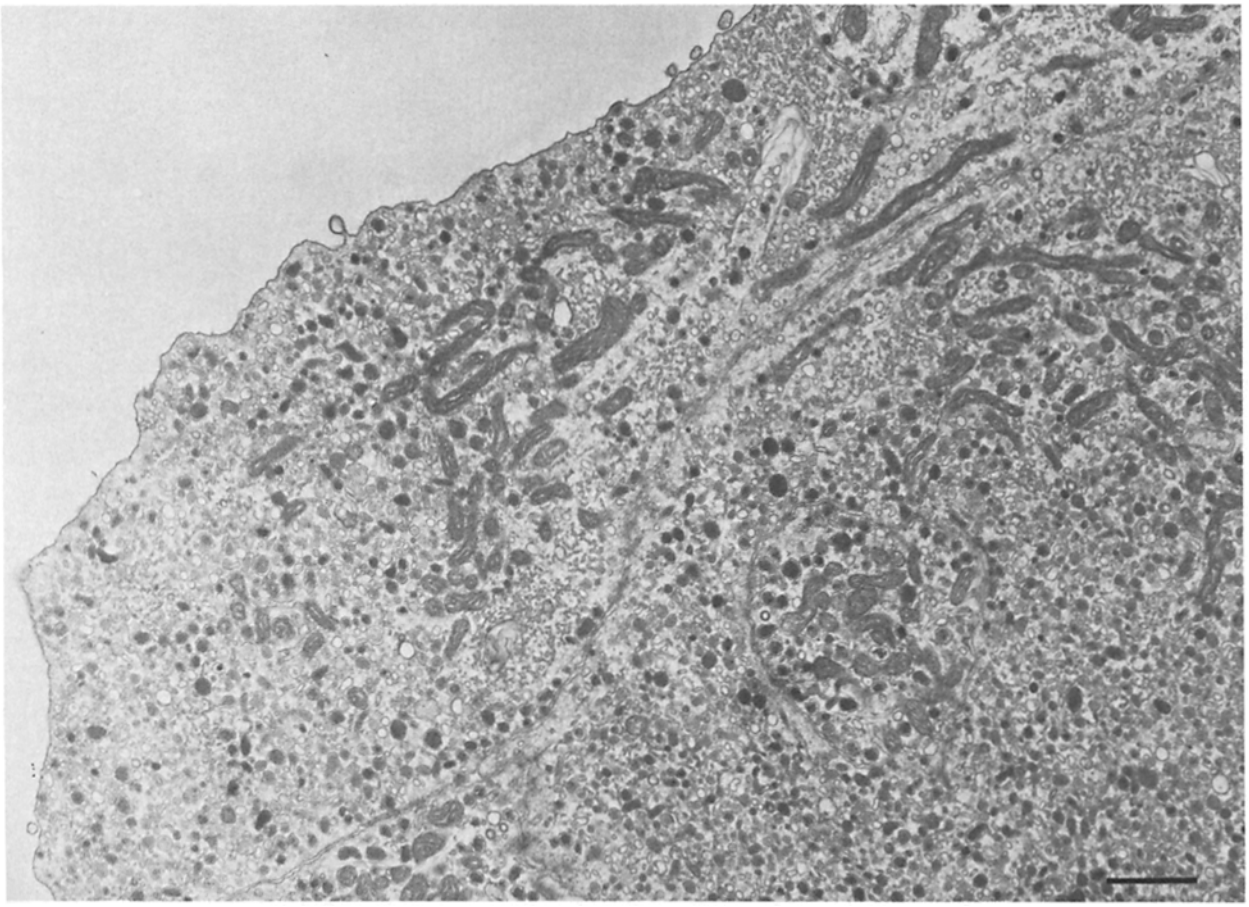

Figure 2. Accumulation of organelles in processes. Dense core granules and other small clear vesicles can be seen filling the cytoplasmic space at the end of a process. Mitochondria can also be seen streaming into the process. Bar, 1 μm.

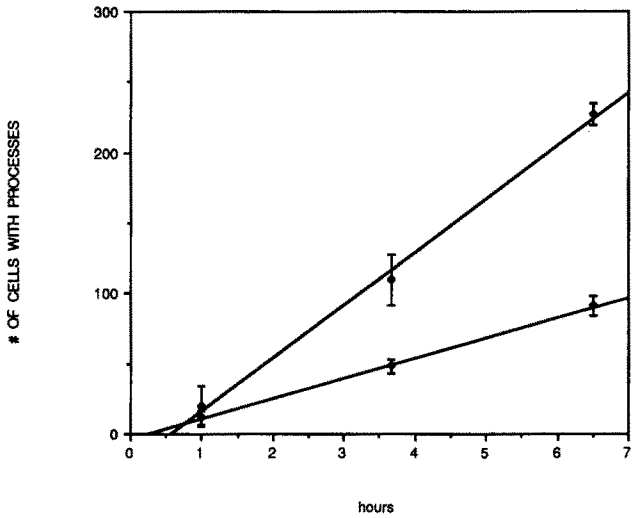

Figure 3. Quantitation of the number of cells with processes after cyclohexamide treatment. AtT-20 cells were treated with either 10 μm cyclohexamide or PBS and plated onto laminin-coated tissue culture dishes. At the times indicated dishes were photographed and numbers of cells containing processes per 500 cells in the photos were counted. In parallel cultures protein synthesis was inhibited 95%. (◇), no cyclohexamide; (◆), 10 μM cyclohexamide.

The secretion of kappa chain from cell lines expressing both low and high levels of light chain has been examined by pulse and chase experiments and in both cases kappa chain was secreted rapidly from the cell with a time course comparable to secretion from antibody secreting lymphoid cells. In addition, conditions that result in a fourfold stimulation in the release of ACTH from hyperexpressing cells resulted in no stimulation of the secretion of kappa chain. These results suggest that kappa chain was not stored in regulated secretory vesicles and that the majority if not all kappa chain was probably all secreted via the constitutive pathway. To ask if dense-core vesicles containing ACTH and constitutive vesicles containing kappa light chain accumulated in different regions of the cell, kappa expressing AtT-20 cells (clone AtT20/pRSV-MPC-11k) were induced to form processes. Kappa chain, detected by immunofluorescence, accumulated at the tips of processes and at a perinuclear region which we identified as Golgi by parallel staining with wheat germ agglutinin (not shown).

The number of processes containing ACTH or kappa chain was quantified by counting the fraction of processes with detectable immunofluorescent staining for each. No significant difference was observed. 87 cells containing 166 processes were counted and 79% had detectable ACTH. 117 cells con-

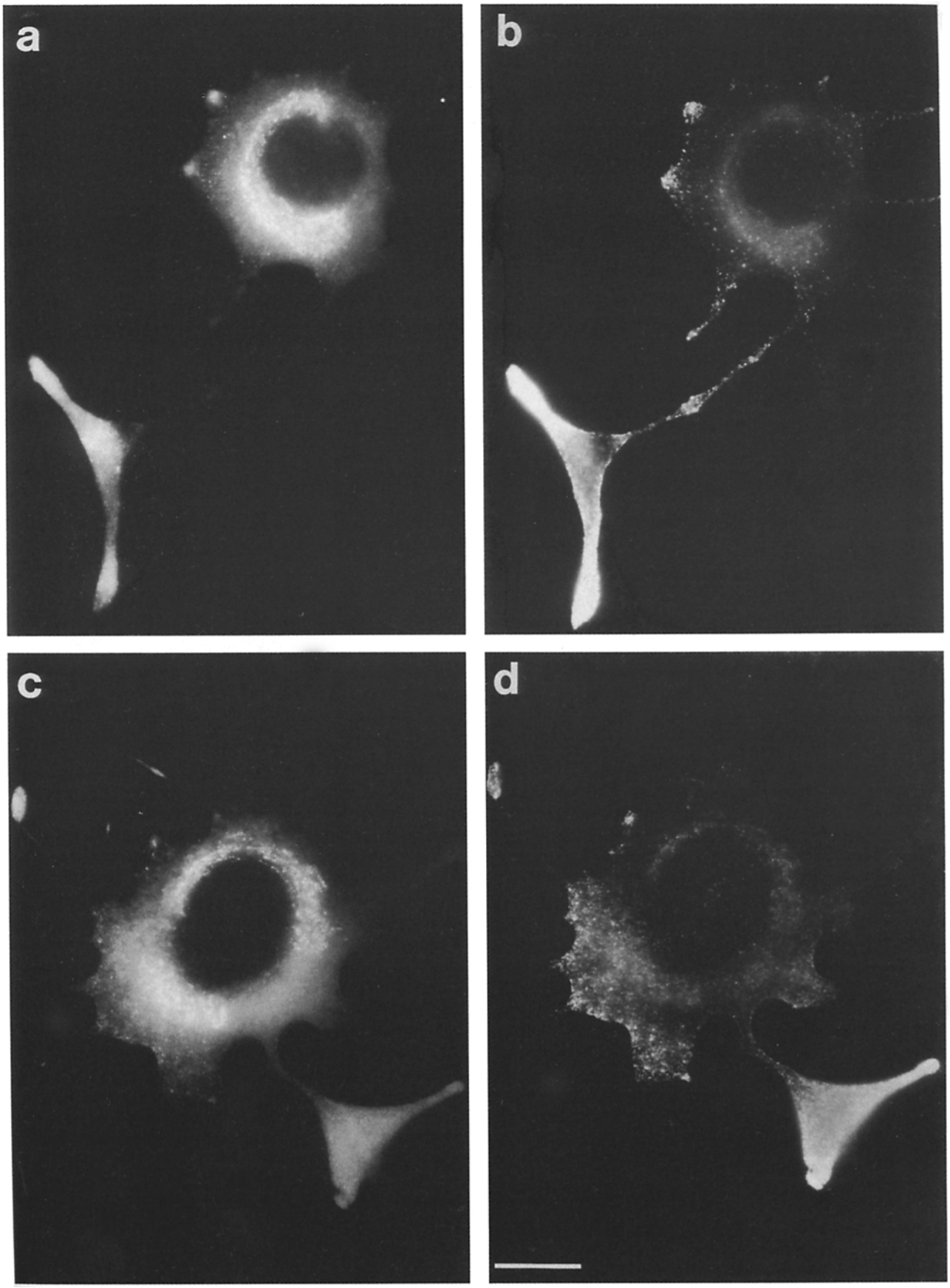

Figure 4. Double immunofluorescence of transfected AtT-20 cells expressing the secreted MPC-11 kappa light chain and the endogenous hormone, ACTH. Cells were grown on poly-D-lysine–coated coverslips in the presence of 5 mM 8-Br-cAMP for 4 d. Cells were stained with rabbit anti–ACTH and biotinylated horse anti–mouse heavy and light chain. The antigen–antibody complexes were detected with sheep anti–rabbit-rhodamine and strepavidin–FITC. (*a* and *c*) anti–kappa; (*b* and *d*) anti–ACTH.

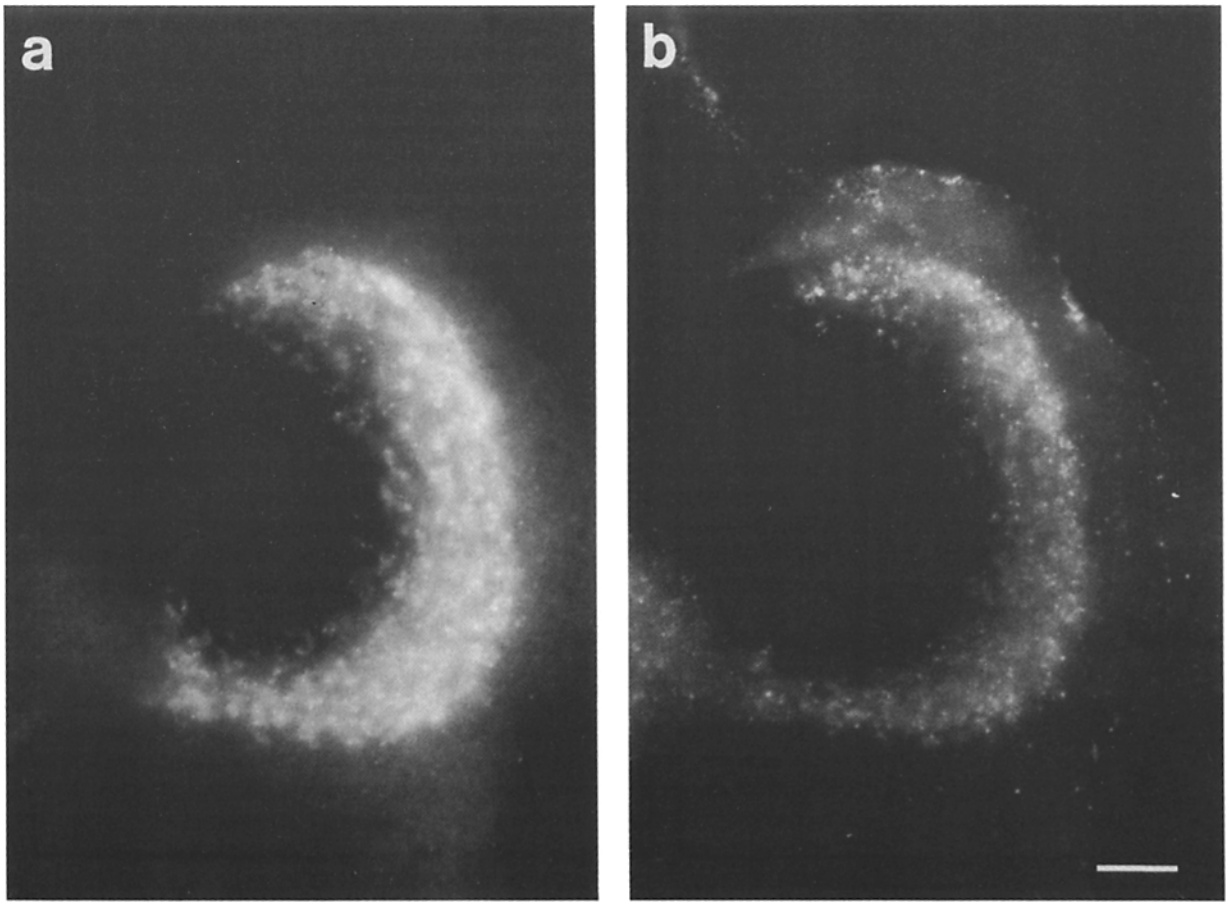

Figure 5. Double immunofluorescence of the Golgi region of a transfected AtT-20 cells expressing the secreted MPC-11 kappa light chain and ACTH. Immunofluorescence was performed as described in Fig. 4. (*a*) anti-kappa; (*b*) anti-ACTH. Bar, 6 μm.

taining 314 processes were counted and 75% had detectable kappa chain. Moreover, by comparing cells double stained by immunofluorescence for kappa chain and for ACTH, kappa chain clearly colocalized with ACTH to the ends of processes (Fig. 4). In controls using only the secondary antibodies, no cells had detectable staining. Non-transfected AtT-20 cells had no detectable staining with anti-kappa antibodies nor was there detectable spillover of the fluorescein fluorescence into the rhodamine channel and vice versa. This suggests that constitutive secretory vesicles accumulate at the tips just as readily as secretory granules.

A noticeable difference between the distribution of the kappa light chain and ACTH in the cells (Fig. 4) was the relatively higher concentration of kappa in the Golgi region. This could be due to the preference of the anti-ACTH antibodies for the mature form, found in mature and immature secretory vesicles, to the precursor form, proopiomelanocortin, found in the Golgi. If anti-ACTH antibodies preferentially detect secretory granules that contain mature ACTH, then the distribution of kappa and ACTH immunofluorescence would be expected to differ in the Golgi region. This was indeed found in many cases (Fig. 5). The anti-kappa antibodies had a reticular distribution while the anti-ACTH antibodies showed a more punctate pattern. If longer exposures were taken, ACTH staining could be seen in all the reticular regions that express kappa. This is consistent with a model in which kappa and the precursor to ACTH, proopiomelanocortin, are together in the majority of the Golgi regions, but segregate during the formation of immature granules. Proteolytic processing of proopiomelanocortin to ACTH occurs in immature granules (Tooze and Tooze, 1986).

The codistribution of kappa-containing vesicles and ACTH-containing vesicles could also be seen before process extension. Cells grown in the absence of cAMP take several days to flatten and to extend short processes. At 24 h after plating, cells were rounded and had small protuberances. Both ACTH and kappa were seen in the protuberances (Fig. 6). Co-distribution of kappa and ACTH did not require cAMP stimulation or the presence of long processes.

Secretory vesicles, mitochondria, and lysosomes all seem to accumulate at the ends of processes. Mitochondria were abundant in process tips as shown in Fig. 2 (also in Kelly et al., 1983). Because organelles were crowded at the tips, it was difficult to identify lysosomes with confidence. Along the length of processes, mitochondria, coated vesicles, and electron dense structures that were probably lysosomal organelles could be readily identified (Fig. 7). Lysosomes, identified by immunofluorescence using an antibody directed to an integral protein of lysosomal membranes (lamp2) (Chen et al., 1985) accumulated at process tips (Schroer, T. A., and R. B. Kelly, unpublished results).

Not all organelles, however, accumulate in processes.

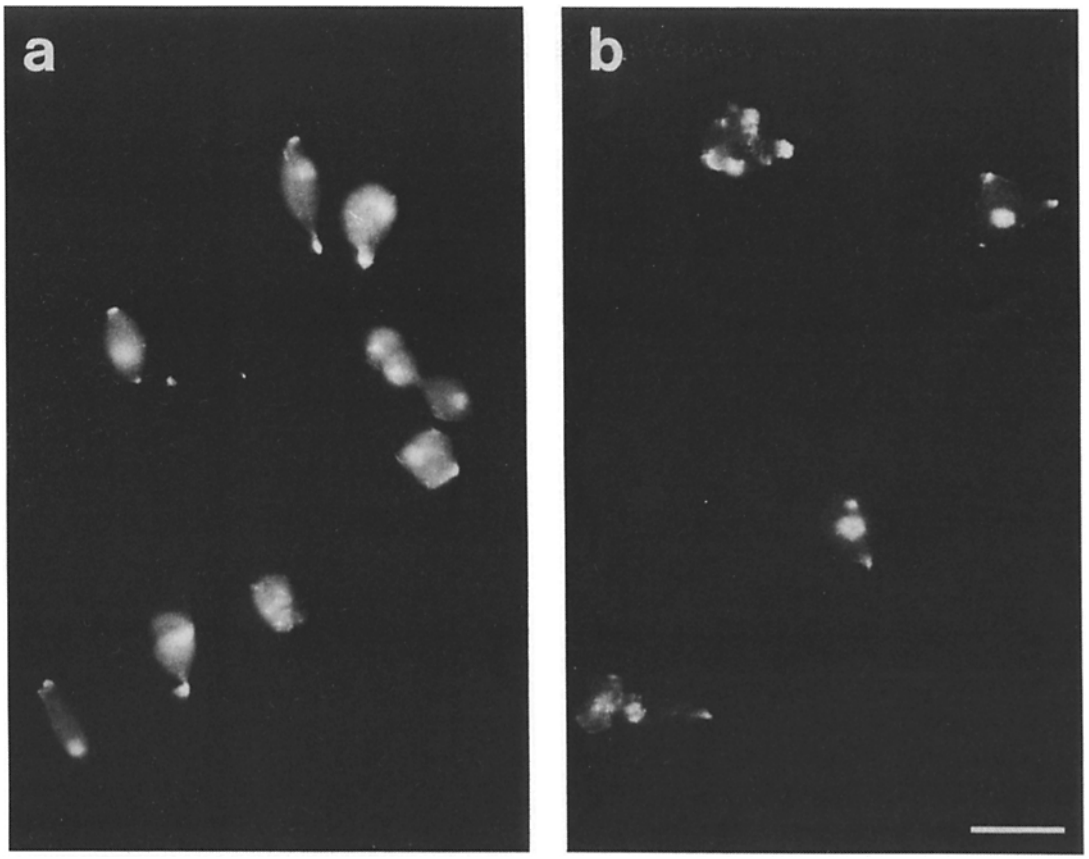

Figure 6. AtT-20 cells grown without cAMP. Cells were plated on glass coverslips in medium without cAMP. After 24 h, cells were fixed and stained for immunofluorescence as described. (*a*) Rabbit anti-mouse kappa; (*b*) rabbit anti-ACTH. Goat anti-rabbit FITC was used to develop both. Bar, 20 μm.

When AtT-20 cells were transfected with a expression vector encoding a kappa light chain that is not secreted, the kappa chain accumulated in the endoplasmic reticulum (Matsuuchi, L., and R. B. Kelly, unpublished results). The localization of the non-secreted kappa light chain by immunofluorescence verified that endoplasmic reticulum did not accumulate in the tips of processes (not shown).

Association of Secretory Granules with Microtubules during Cell Division

Another striking redistribution of secretory granules occurred during cell division. In electron micrographs of an AtT-20 cell during cell division, microtubules could be seen in the midbody, a structure that occurs during cytokinesis. Microtubules interdigitate in this region and dense core secretory granules were seen to accumulate here, lined up along some of the microtubules (Fig. 8). An identical finding has been made recently by Tooze and Burke (1987). The accumulation is not specific for dense-core secretory granules since smaller, less dense vesicles could also be seen lining up in rows between the layers of microtubules (Fig. 8; see also Tooze and Burke, 1987). Accumulation of ACTH-containing secretory vesicles in the region between dividing nuclei was also seen by immunofluorescence. Cells captured in mitosis by exposure to nocodazole were identified with Hoechst nuclear stain. In all cases, the ACTH, detected by immunofluorescence, was concentrated along the plane of division between the nuclei (Fig. 9 *B*). These data are consistent with the hypothesis that ACTH-containing secretory granules associate with the minus ends of microtubules in the Golgi region and move in an anterograde direction to the plus ends (Tooze and Burke, 1987).

When kappa secreting cells were arrested in cell division, kappa chain was also found at the plane of division between the nuclei (Fig. 9 *E*; Fig. 10 *B*). Transport to the plane of division is clearly not selective for ACTH-containing secretory granules in dividing cells. We cannot be certain from these experiments whether the kappa chain was exclusively in constitutive secretory vesicles or also in Golgi-derived vesicles. We do know, however, that membrane fragments of the endoplasmic reticulum do not distribute in this manner during mitosis. When the AtT-20 cell line that expresses the non-secreted kappa light chain was examined during mitosis, the kappa chain was distributed uniformly throughout the cytoplasm (Fig. 10 *E*). The distribution of ACTH under these conditions was the same as described in Fig. 9 *B*.

Distribution of a Constitutive Marker in PC-12 Cells

Adult neurons have a striking accumulation of synaptic vesicles at the nerve terminal. To test the selectivity of transport in a cell line with more neuronal characteristics, the rat

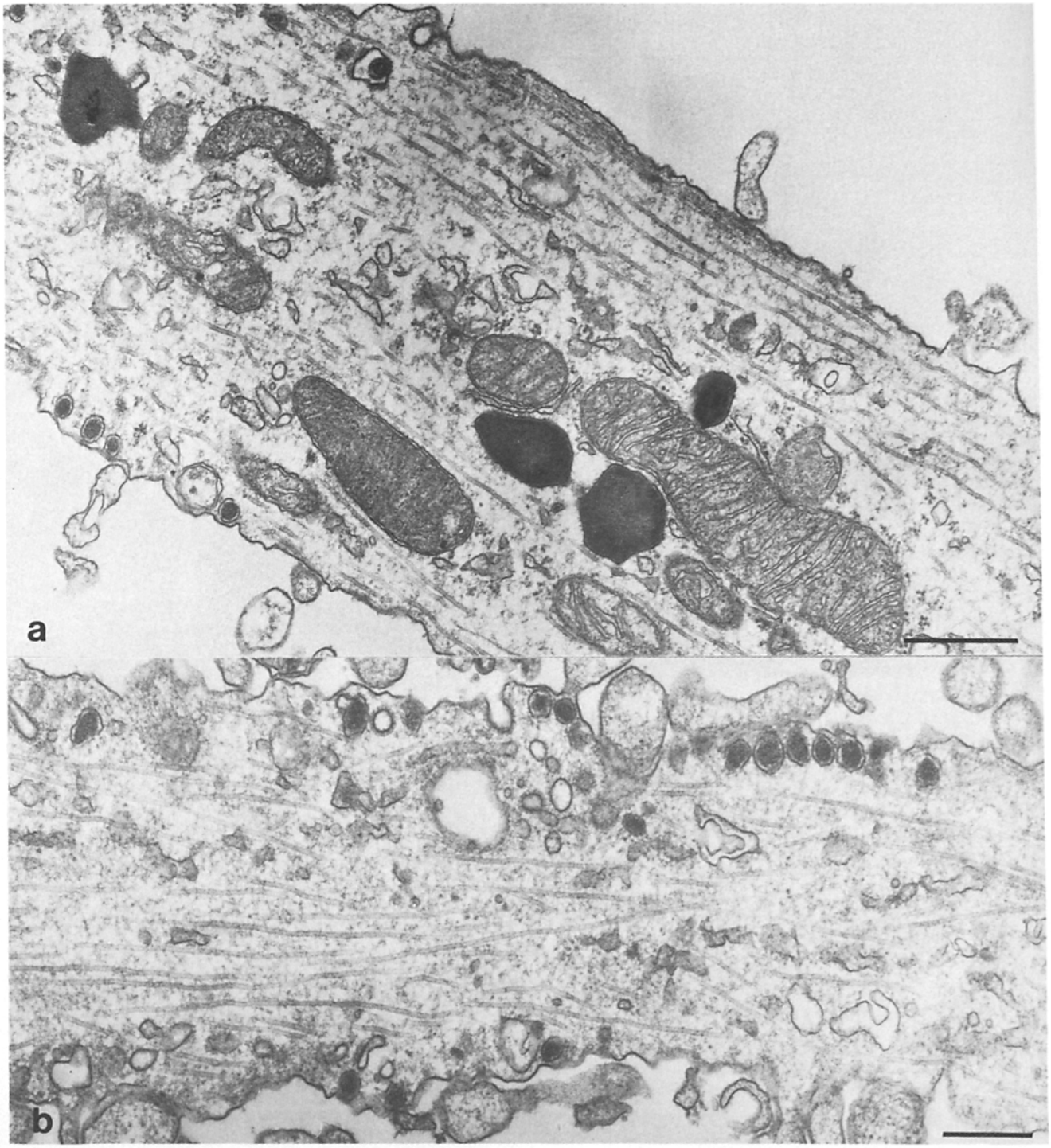

Figure 7. Sections of AtT-20 processes. Microtubules and dense core secretory granules can be seen lining the edge of the processes. Mitochondria and lysosomal organelles are visible (*a*) as well as budding viruses and coated pits and vesicles (*b*). Bar, 0.5 μm.

pheochromocytoma cell line PC-12, was used. To observe regulated secretory vesicles, PC-12 cells were transfected with DNA encoding the regulated secretory protein, proinsulin; to detect organelles involved in constitutive secretion another clone was developed that expressed the MPC-11 kappa light chain. Secretion of kappa chain was not stimulated with high potassium under pulse-chase conditions that led to a threefold stimulation of the release of norepinephrine (not shown). When these cells were grown in the presence of nerve growth factor and dibutryl-cAMP to stimulate the extension of neurites, proinsulin and kappa chain immunoreactivity were found at the ends of processes (Fig. 11) and in varicosities distributed along their lengths. Under the conditions used non-transfected PC-12 cells did not stain

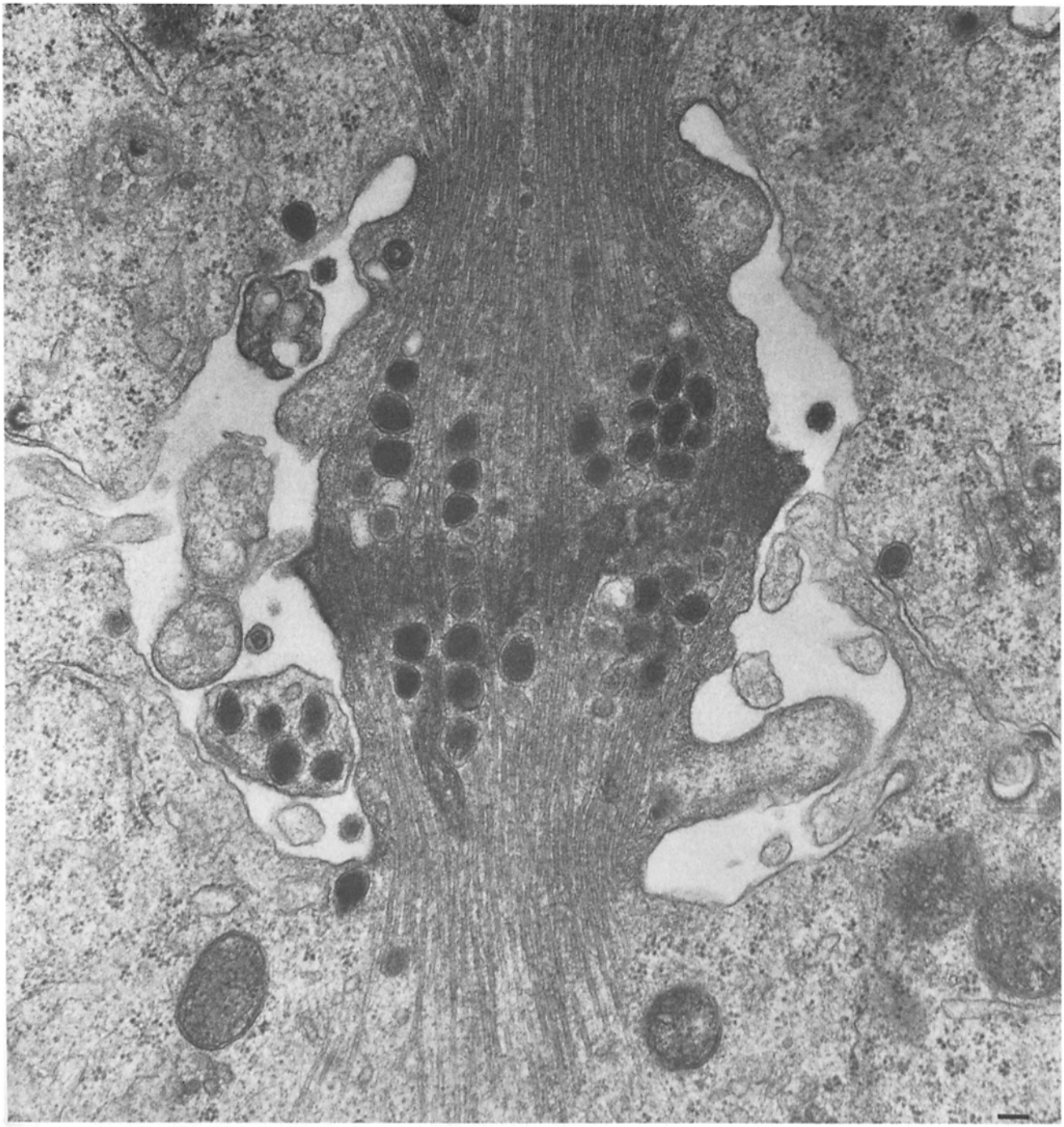

Figure 8. Mitotic AtT-20 cell. Microtubules are interdigitated during mitosis, probably late telophase, dense core granules and smaller, less dense vesicles are lined up between the layers of microtubules. Bar, 0.1 μm.

with anti-kappa antibodies and the secondary antibodies alone did not stain either the wild-type PC12 cells or the transfected PC-12 cells. We conclude that transport of constitutive exocytotic vesicles to the end of processes occurs even in a neuronal-like cell line. Either the processes extended by PC-12 cells are not a model for axons of mature neurons in vivo, or both regulated and constitutive secretory vesicles can be axonally transported.

Discussion

In secretory cells with regulated secretion, the secretory vesicles are not uniformly distributed throughout the cytoplasm but cluster in a cytoplasmic domain close to their site of exocytosis. Zymogen granules are close to the apical membrane in exocrine cells and synaptic vesicles are close to the active zones of the presynaptic plasma membrane. In

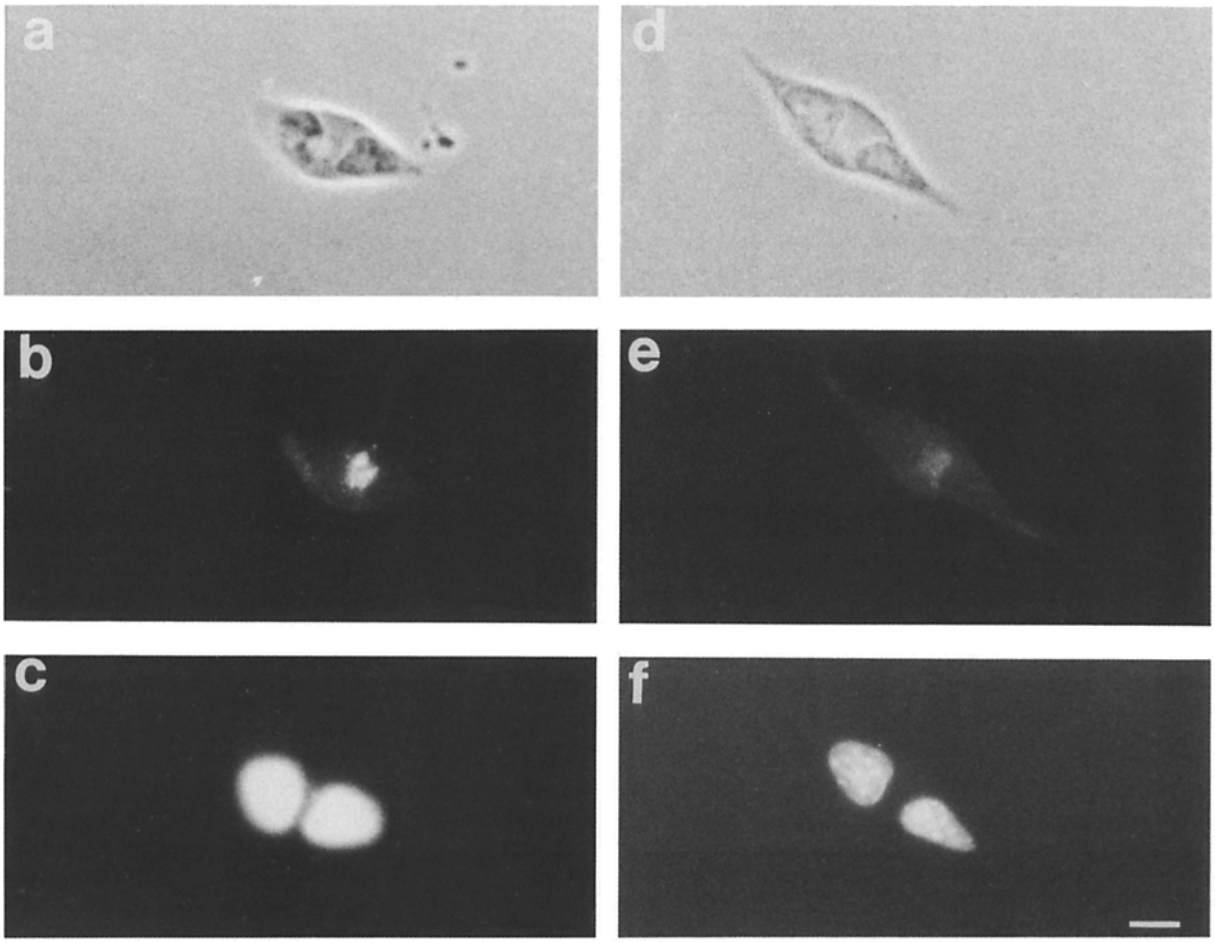

Figure 9. Immunofluorescence of AtT-20 cells expressing kappa light chain and ACTH, after release from a nocodazole block. ACTH and kappa light chain can be seen accumulating at the plane of division during mitosis. Cells were blocked with nocodazole for 2 h and fixed and stained for immunofluorescence at 60 min after the removal of the nocodazole block. (*a* and *d*) Phase; (*b*) anti-ACTH; (*e*) anti-kappa; (*c* and *f*) Hoechst nuclear stain. Bar, 18 μM.

the latter case organelle transport is thought to be due to kinesin-mediated movement along microtubules from the site of synthesis in the cell body to the plus ends of microtubules near the nerve terminal (Vale et al., 1985). The data of Tooze and Burke (1987) and the data presented here on the accumulation of secretory vesicles (Figs. 2 and 7–10) in regions of AtT-20 cytoplasm are most readily explained, as the authors suggest, by a similar orthograde transport to the tips of microtubules. A feature of our data (Fig. 8) that can also be noted in the work of Tooze and Burke (1987) is the positioning of granules along only certain preferred microtubules at the plane of division. These images are highly reminiscent of the lines of vesicles along axonal microtubules when transport is inhibited by a cold block (Miller et al., 1987). Following the suggestion of Miller and his collaborators that this selectivity distinguishes "transporting" microtubules from "architectural" microtubules we shall refer to those microtubules involved in organelle transport as transport microtubules.

Although the role of transport microtubules in organelle movement is now unequivocal, there is less certainty about how selectivity of organelle transport is achieved. In the epithelial cell line, Madin–Darby canine kidney (MDCK), vesicles containing apical or basolateral membrane proteins are thought to fuse only with their respective membrane domains (for review see Simons and Fuller, 1985). Presumably, in neurons, vesicles containing dendritic proteins fuse only with plasma membranes of the dendrites, and not the plasma membrane of the nerve terminal. Selectivity of vesicle insertion could result from selective recognition by the vesicles of the correct domain of the plasma membrane, from selective microtubule transport of the vesicles from the Golgi region to the appropriate region of plasma membrane, or from both. To examine selectivity of organelle transport we used the presence of two secretory pathways in AtT-20 or PC-12 cells and the convenient soluble protein markers for vesicles of each pathway. We have demonstrated that transport of secretory organelles is not selective for regulated secretory granules but occurs with constitutive vesicles as well. Even mitochondria and lysosomes were found to accumulate at the ends of processes. Three alternatives present themselves: we may have failed to detect selective transport in the cell lines we have chosen to study; or they may lack a mechanism for selective transport present in mature neurons or epithelial

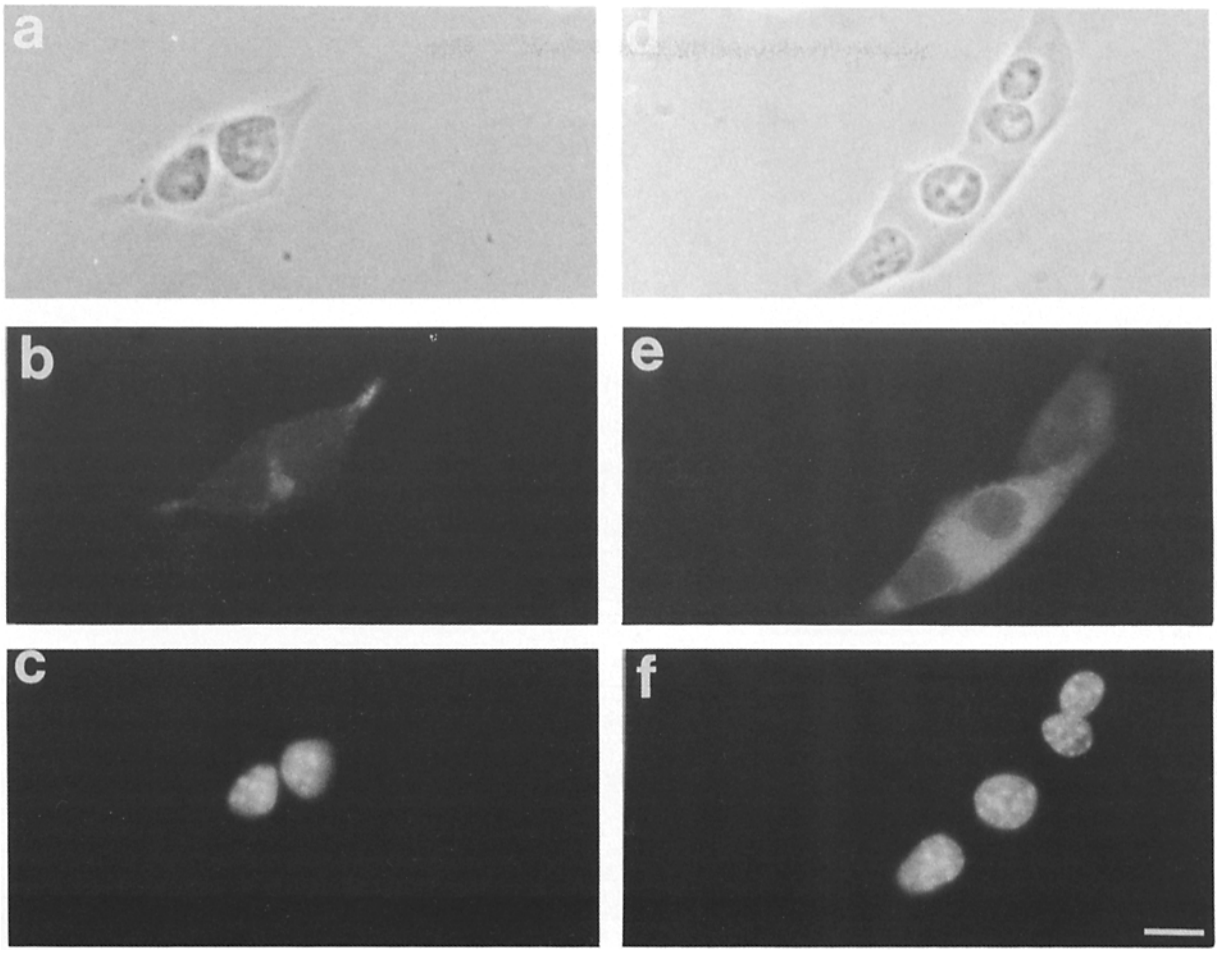

Figure 10. Immunofluorescence of AtT-20 cells after a nocodazole block as described in Fig. 9. (*a* and *d*) Phase; (*b* and *e*) anti-kappa; (*c* and *f*) Hoechst nuclear stain. (*a–c*) AtT-20 cell expressing the secreted MPC-11 kappa light chain. (*d–f*) AtT-20 cell expressing the non-secreted S107 kappa light chain. Bar, 18 μM.

cells; or transport is nonselective in all cells. Thus nonselective microtubules may contribute to the speed at which new vesicles find their cytoplasmic docking sites, but contribute nothing to the selectivity. Secretory vesicles may be transported to all regions of the cell but only selectively recognize and fuse to the appropriate domains of the plasma membranes. Those vesicles that are not retained may be transported away from the inappropriate site and distributed throughout the cell until they find the correct membrane domain. As far as we are aware compelling evidence for or against selective transport of membrane vesicles along microtubules in epithelia or neurons is lacking. Pharmacological evidence suggests that microtubule disruption inhibits selective apical insertion of membrane proteins in MDCK cells (Rindler et al., 1987). There is also tantalizing evidence in neurons for a non-selective mechanism of microtubule transport. Axonal microtubules in vitro are found to be capable of transporting mitochondria and other large organelles at the same speed and even along the same microtubule as smaller vesicles (Schnapp et al., 1985).

The constitutive secretory pathway, which we can now identify using kappa light chain as a marker is believed to be the pathway used by cells to externalize membrane proteins as well as soluble secreted proteins. It is appropriate therefore to compare these results with data on selective insertion of membrane proteins in cells that lack processes (for review, see Singer and Kupfer, 1986). In migrating fibroblasts, for example, the Golgi complex is oriented towards the growing edge, where membrane proteins are selectively inserted. Selective insertion is disrupted by disrupting the cytoskeleton (Rogalski et al., 1984). Selective secretion of cytotoxic factors by T lymphocytes at the point of contact with target cells appears also to be associated with orientation of the cytoskeleton towards the target (Kupfer et al., 1986*a*, *b*). The simplest model to explain the various morphologies is to assume that transport microtubules capable of carrying membrane vesicles to the plasma membrane need not be uniformly distributed over the cytoplasm. If the transport microtubules converge on one edge of a cell, the addition of membrane at that edge is a growing edge. If the transport microtubules are focused into one or a few small points on the plasma membrane, vesicles accumulate at localized domains under the plasma membrane (Fig. 6).

In this limited selection of examples the generation of

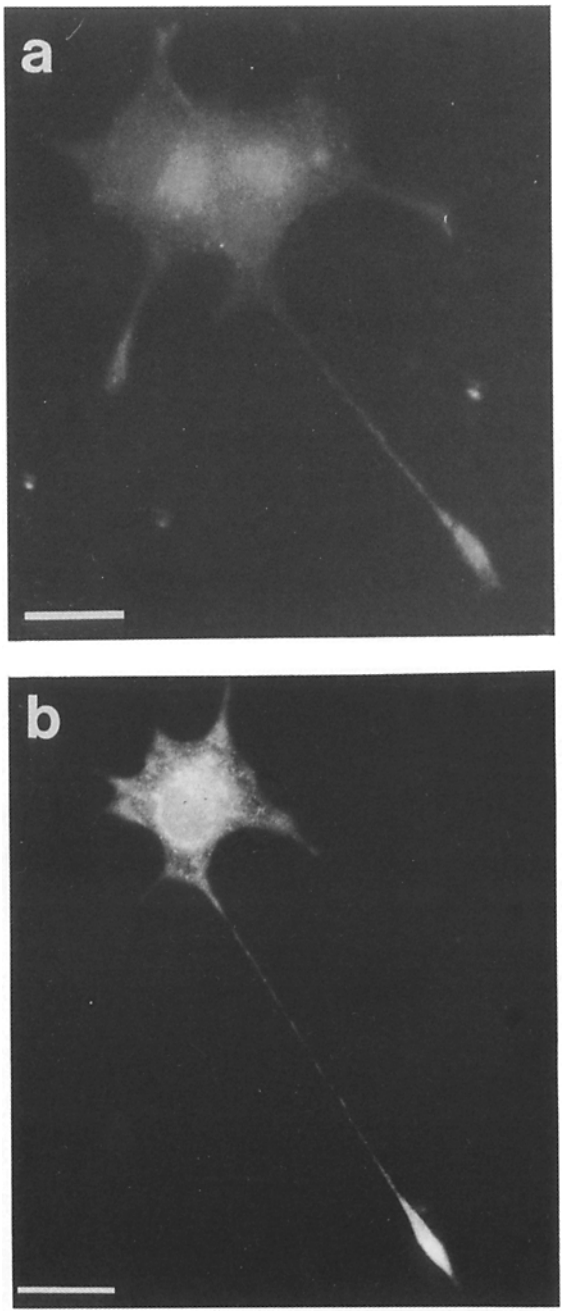

Figure 11. Immunofluorescence of PC12 cells expressing either proinsulin or kappa light chain. PC12 cells were stimulated to extend neurites by treatment with 1 mM dibutyryl cAMP and 10 ng/ml nerve growth factor. Cells were incubated with either rabbit anti-mouse kappa antibody and goat anti-rabbit-FITC (*a*) or guinea pig anti-insulin and rabbit anti-guinea pig-FITC (*b*). Bar (*a*) 20 μm; (*b*) 12 μm.

polarity defined as an asymmetry in organelle distribution may be explained by nonselective microtubular transport of membrane vesicles carrying newly-synthesized membrane proteins from the Golgi complex to localized regions of the cell cytoplasm. It remains to be seen if polarized cells in vivo may have a microtubule transporting system that discriminates between organelles or if specific organelle accumulation such as that of synaptic vesicles in nerve terminals is due to selective docking with retention at membrane domains rather than selective transport to them.

The authors thank Leslie Spector for typing this manuscript and the members of kelly lab for their advice during the preparation of this manuscript.

This work was supported by grants from the National Institutes of Health (NIH) to R. B. Kelly (NS16033 and AM33937). L. Matsuuchi was supported by a New Investigator Research award from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDKD) (AM35895) and A. Lowe by a Physician-Scientist Award (AM01458).

Received for publication 17 September 1987, and in revised form 3 November 1987.

References

Baas, P. W., L. A. White, and S. R. Heidemonn. 1987. Microtubule polarity reversal accompanies regrowth of amputated neurites. *Proc. Natl. Acad. Sci. USA.* 84:5272–5276.

Buckley, K. M., and R. B. Kelly. 1985. Identification of a transmembrane glycoprotein specific for secretory vesicles of neural and endocrine cells. *J. Cell Biol.* 100:1284–1294.

Buckley, K. M., and S. C. Landis. 1983. Morphological studies of synapses and varicosities in dissociated cultures of sympathetic neurons. *J. Neurocytol.* 12:67–92.

Burgess, T. L., C. S. Craik, and R. B. Kelly. 1985. The exocrine protein trypsinogen is targeted into secretory granules of an endocrine cell line: studies by gene transfer. *J. Cell Biol.* 101:639–645.

Burgess, T. L., C. S. Craik, L. Matsuuchi, and R. B. Kelly. 1987. In vitro mutagenesis of trypsinogen: role of the aminoterminus in intracellular protein targeting to secretory granules. *J. Cell Biol.* 105:659–668.

Chen, J. W., T. L. Murphy, M. C. Willingham, I. Pastan, and J. T. August. 1985. Identification of two lysosomal membrane glycoproteins. *J. Cell Biol.* 101:85–95.

Greene, L. A., and A. S. Tischler. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA.* 3:2424–2429.

Johnson, G. D., R. Davidson, K. McNamee, G. Russell, D. Goodwin, and E. J. Holbrow. 1982. Fading of immunofluorescence during microscopy: a study of the phenomenon and its remedy. *J. Immunol. Methods.* 55:231–242.

Kelly, R. B. 1985. Pathways of secretion in eukaryotes. *Science (Wash. DC).* 230:25–32.

Kelly, R. B., K. B. Buckley, T. L. Burgess, S. S. Carlson, P. Caroni, J. E. Hooper, A. Katzen, H. P. H. Moore, S. R. Pfeffer, and T. A. Schroer. 1983. Membrane traffic in neurons and peptide secreting cells. *Cold Spring Harbor Symp. Quant. Biol.* 48:698–705.

Kupfer, A., S. J. Singer, and G. Dennart. 1986*a*. On the mechanism of unidirectional killing in mixtures of two cytotoxic T lymphocytes. Unidirectional polarization of cytoplasmic organelles and membrane-associated cytoskeleton in the effector cell. *J. Exptl. Med.* 163:489–498.

Kupfer, A., S. L. Swain, C. A. Janeway, Jr., and S. J. Singer. 1986*b*. The specific direct interaction of helper T cells and antigen-presenting cells. *Proc. Natl. Acad. Sci. USA.* 83:6080–6083.

Miller, R. H., R. J. Lasek, and M. J. Katz. 1987. Preferred microtubules for vesicle transport in lobster axons. *Science (Wash. DC).* 235:220–222.

Moore, H. P. H., and R. B. Kelly. 1985. Secretory protein targeting in a pituitary cell line: differential transport of foreign secretory proteins to distinct secretory pathways. *J. Cell Biol.* 101:1773–1781.

Rindler, M. J., I. E. Ivanov, and D. D. Sabatini. 1987. Microtubule-acting drugs lead to the non-polarized delivery of the influenza hemagglutinin to the cell surface of polarized Madin-Darby Canine Kidney Cells. *J. Cell Biol.* 104:231–241.

Rogalski, A. A., J. E. Bergmann, and S. Singer. 1984. Effect of microtubule assembly status on the intracellular processing and surface expression of an integral protein of the plasma membrane. *J. Cell Biol.* 99:1101–1109.

Schnapp, B. J., R. O. Vale, M. P. Sheetz, and T. S. Reese. 1985. Single microtubules form squid axoplasm support bidirectional movement of organelles. *Cell.* 40:455–462.

Schweitzer, E. S., and R. B. Kelly. 1985. Selective packaging of human growth hormone into synaptic vesicles in a rat neuronal (PC-12) cell line. *J. Cell Biol.* 101:667–676.

Seeds, N. W., A. G. Gilman, T. Amano, and M. W. Nirenberg. 1970. Regulation of axon formation by clones lines of a neural tumor. *Proc. Natl. Acad. Sci. USA.* 66:160–167.

Shaw, G., and D. Bray. 1977. Movement and extension of isolated growth cones. *Exptl. Cell Res.* 104:55–62.

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

Singer, S. J., and A. Kupfer. 1986. The directed migration of eukaryotic cells. *Annu. Rev. Cell Biol.* 2:337–365.

Tooze, J., and B. Burke. 1987. Accumulation of adrenocorticotropin secretory granules in the midbody of telophase AtT-20 cells: Evidence that secretory granules move anterogradely along microtubules. *J. Cell Biol.* 104:1047–1057.

Tooze, J., and S. A. Tooze. 1986. Clathrin-coated vesicular transport of secretory proteins during the formation of ACTH-containing secretory granules in AtT-20 cells. *J. Cell Biol.* 103:839–850.

Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell.* 42:39–50.