Journal of Cell Science 106, 955-966 (1993)
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Regulation of vesicle transport in CV-1 cells and extracts

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SUMMARY

To investigate the regulation of microtubule-dependent vesicle motility, we have studied the effects of pharmacological agents on the frequency and velocity of small vesicle movements in intact CV-1 cells. Nocodazole, but not cytochalasin B or D, abolished vesicle movements, indicating that these movements were microtubule and not actin-dependent. The frequency of vesicle movements was stimulated maximally sixfold by okadaic acid from a resting value of 1.6 movements/min per μm^2 in serum-starved CV-1 cells. Other activators in decreasing order of effectiveness are fetal calf serum, dibutyryl cAMP, cholera toxin, genistein, A23187, and trental. On the other side, taxol inhibited vesicle movements by a factor of four. The activators, okadaic acid, fetal calf serum, and dibutyryl cAMP, also increased vesicle velocity and run length, while taxol decreased vesicle velocity. Although modulation of the frequency of vesicle movements over a >20-fold range was observed, under all conditions the fraction of vesicles moving inward versus outward did not significantly change. Only in the case of taxol was the distribution of microtubules altered within this same time period. Both inward and outward microtubule-dependent vesicle movements therefore appear to be coordinately regulated. The enhanced vesicle motility elicited by fetal calf serum in intact cells correlated with in vitro measurements of vesicle motility and velocity on purified microtubules using microtubule affinity-purified motors and carbonate-washed vesicles from cells treated with fetal calf serum. This suggests that the amount of vesicular intracellular membrane traffic is coordinately regulated with microtubule-dependent motor activity.

Key words: vesicle motility, microtubule, membrane traffic, cAMP, okadaic acid, serum

INTRODUCTION

The concept of membrane recycling was proposed to account for the high rates of extracellular fluid and plasma membrane uptake in cultured cells. Since in cultured cells, the influx of membrane can equal the surface area of the cell in <1-2 hours, it is clear that recycling rather than degradation and replacement of the internalized membrane is the major membrane trafficking event (Steinman et al., 1983). Endocytic vesicles move inward to the perinuclear region for sorting of contents either to the lysosomal network for degradation, or to the *trans*-Golgi network for recycling. Exocytic vesicles are then transported outward to the plasma membrane to maintain plasma membrane area at a steady state. Although numerous studies support the concept of membrane recycling (for reviews see Steinman et al., 1983; Gruenberg and Howell, 1989a; Kaplan and Ward, 1990; Kelly, 1990; Schroer and Sheetz, 1991b), the molecular mechanisms by which membrane traffics inward and outward are unclear. There is, however, an increasing body of evidence suggesting that membrane trafficking is accelerated by microtubule (MT)-dependent transport (reviewed by Kelly, 1990; Schroer and Sheetz, 1991b).

In support of the involvement of MTs in membrane trafficking, tubular lysosomes collapse in the presence of an injected mAb against kinesin (Hollenbeck and Swanson, 1990). Fusion of apical and basolateral endosomes in permeabilized Madin-Darby canine kidney cells can also be inhibited by depletion of MT-dependent motors from the supernatant (Bomsel et al., 1990). Other MT-dependent membrane traffic includes movement of endocytic vesicles from the cell periphery inward (Herman and Albertini, 1984; Matteioni and Kreis, 1987; DeBrabander et al., 1988), transport of endosomes from the apical to the basolateral surface of polarized epithelia (Achler et al., 1989; Eilers et al., 1989), exocytic traffic from the *trans*-Golgi network to the plasma membrane (Kreis et al., 1989), extension of *trans*-Golgi membranes along MTs (Cooper et al., 1990), and recycling of membrane into the ER from a salvage compartment (Lippincott-Schwartz et al., 1990).

MT-dependent transport should be tightly regulated since it appears to be involved in maintenance of membrane compartments and trafficking of vesicles between compartments. Two MT-dependent motor proteins have been identified as candidates for the intracellular motors powering vesicle transport: kinesin, a plus end-directed motor (Vale

et al., 1985) and cytoplasmic dynein, a minus end-directed motor (Paschal et al., 1987). The minimum molecular complex responsible for vesicle motility includes kinesin and/or cytoplasmic dynein and a motor receptor on the vesicle (for reviews see Vale, 1987; Schroer and Sheetz, 1991b). An accessory factor, dynactin (Schroer and Sheetz, 1991a; Gill et al., 1991), and a membrane-bound receptor, kinectin (Toyoshima et al., 1992), have recently been implicated in the vesicle motor complex. A new class of cytoplasmic linker proteins (CLIPs) have also been implicated in binding of endocytic vesicles to microtubules (Rickard and Kreis, 1990; Pierre et al., 1992). Each of these components is a possible point for regulation of vesicular motile activity. Additionally, the production of new vesicles could be used by the cell to regulate the level of motility. In the first case, changes in cellular vesicle motility should be correlated with changes in the soluble motor-associated proteins, and in the second case, with changes in the concentration and composition of cellular vesicles.

Regulation of the direction of vesicle transport may be an important mechanism for the cell to control the distribution of membranous compartments within the cell. Although unidirectional movements of vesicles have been observed under special circumstances, the CV-1 cells chosen in this study do not exhibit the unidirectional movements elicited by hormonal or other changes that have previously been described (Rodzial and Haimo, 1986; Lynch et al., 1986; Heuser, 1989; Parton et al., 1991). If a cell used vesicular transport for secretion and membrane tubule formation for endocytosis, outward transport of all vesicles involved in trafficking would result. Since video-enhanced differential interference contrast (DIC) microscopy can only follow movements of vesicles and not diffusion through membrane tubules, an asymmetry of vesicle transport could belie the existence of a tubular pathway particularly in balanced transport processes. Transport through membrane tubules has been invoked in the transport of material to the ER-Golgi transition compartments (Klausner et al., 1992), some of the endocytic transport (Bomsel et al., 1990) and in some of the *trans*-Golgi transport events (Cooper et al., 1990). The modulation of vesicular traffic could thus involve switching from a vesicular to a membrane tubule mechanism of transport.

In this paper, we examine the regulation of MT-dependent vesicle transport in cultured cells by looking at changes in vesicle transport activity. Our approach has involved the exposure of CV-1 cells to a selection of pharmacological agents that target intracellular kinases and phosphatases and examination of the effects of these agents on different parameters of MT-dependent vesicle motility. We show that several parameters of MT-dependent small vesicle movement (frequency, velocity and run length) can be increased or decreased significantly (*P* 0.05) giving over a twenty-fold range of transport activity. Both inward and outward MT-dependent vesicle transport are coordinately stimulated up to sixfold without changes in the distribution or density of peripheral MTs. The stimulation of vesicle motile activity in intact treated cells correlates with stimulation of in vitro vesicle motile activity driven by MT affinity-purified motors and vesicles from treated cells. The greater stimulation of motile activity exhibited by the soluble fractions indicates that the in vivo changes in the MT-dependent motor protein-associated fraction cause the changes in vesicle motility in vivo.

MATERIALS AND METHODS

Chemicals

Cholera and pertussis toxins were obtained from List Biologicals or from Gibco-BRL. Okadaic acid was obtained from Gibco-BRL. 7 -deacetyl-7 -(-*N*-methylpiperazino)-butyryl forskolin dihydrochloride and 1,9-dideoxy forskolin were purchased from Calbiochem. Genistein was obtained from ICN. Trental was purchased from Hoechst-Roussel Pharmaceuticals. Taxol was obtained from the National Cancer Institute or from Calbiochem. All other hormones and drugs used in perfusion studies were from Sigma.

Cell culture

CV-1 cells (African green monkey kidney cells from ATCC) were maintained at 5% CO_2 in a humidified air atmosphere in MEME (no phenol red) supplemented with 20 mM HEPES, 1 mM glutamine, 10% fetal calf serum (v/v), 40 units/ml penicillin and 40 μg/ml streptomycin, and passaged with trypsin-EDTA. For in vitro vesicle motility assays, CV-1 cells were split 3 days prior to preparation of extracts and grown at 37°C in roller bottles (Corning, 850 cm^2) filled with 5% CO_2. Tissue culture grade HEPES was obtained from Sigma. All other reagents were from Gibco-BRL.

For perfusion studies, CV-1 cells were plated at low density on 20 mm × 40 mm #1 glass coverslips that were previously treated by washing three times in 50% methanol/50% ethanol and baking for several hours. The CV-1 cells were incubated for 18-24 hours at 37°C in a 5% CO_2 incubator. At this time, the old medium was replaced by fresh medium containing 1% bovine serum albumin (Fraction V, Sigma) substituted for the serum, and the cells were incubated for 12-18 hours at 37°C in a 5% CO_2 incubator before use. Cells depleted of serum in this manner are defined as our controls.

Cell treatments

CV-1 cells grown on coverslips were exposed in a Petri dish for 60 minutes at 37°C in conditioned serum-free medium containing the drug. For nocodazole treatment, MTs were depolymerized by incubation of the cells on ice for 30 minutes, followed by the addition of either 1 μg/ml or 2.5 μg/ml nocodazole. The cells were incubated for the indicated time and then placed in the 5% CO_2 incubator at 37°C for 30 minutes prior to observation of vesicle movements with the video-enhanced DIC microscope.

Extraction, fixation and visualization of MTs in CV-1 cells by immunofluorescence

The following protocol was modified from Osborn and Weber (1982). CV-1 cells were plated and treated with the drugs of interest as described above and then rinsed twice in calcium-free PBS followed by a brief incubation in PMEE (35 mM Pipes, pH 7.4, 5 mM $MgSO_4$, 1 mM EGTA, 0.5 mM EDTA) containing 0.5% Triton X-100 (Pierce) for 15 seconds. The cells were then fixed in 0.1% glutaraldehyde/2% formaldehyde for 15 minutes, rinsed in PBS, and permeabilized in methanol (−20°C) for 6 minutes followed by acetone (−20°C) for 1 minute. The cells were rinsed sequentially in PBS containing 50 mM NH_4Cl, PBS containing 1% bovine serum albumin (Fraction V, Sigma) and 0.1% Tween-20 (Sigma), and PBS. Cells were then incubated with a rat anti-tubulin antibody (YOL1/34, Accurate Chemical and Scientific

Corp., Westbury NY, 1/75 dilution) for 30 minutes at 37°C, rinsed 3 times in PBS, and incubated with fluorescein-5-isothiocyanate-conjugated goat anti-rat antibody (Cappel, 1/50 dilution) for 30 minutes at 37°C. The cells were then rinsed three times in PBS and mounted in 90% glycerol containing 100 mM DTT. Visualization was with an Zeiss Axiophot (×100 Neofluar objective) equipped with a Photometrics Star 1 CCD camera.

In vitro assay for vesicle motility

Extracts were prepared from CV-1 cells grown to confluence in roller bottles. Procedures were based on those previously described (Schroer and Sheetz, 1991a). The cell pellet was resuspended at a 1:1 ratio (v/v) in Buffer A. Buffer A is composed of PMEE , a protease inhibitor cocktail and 1 mM DTT. A Balch cell press (8.006 mm ball) obtained from the European Molecular Biology Laboratory was used to lyse the cell suspension in 10 passes (Balch and Rothman, 1985).

The lysed cell suspension was centrifuged at 4°C in a Fisher microcentrifuge (Model 59A) at a setting of 2.5 for 15 minutes. The supernatant from this first low speed spin (S1) was then centrifuged in a Beckman TL100 ultracentrifuge (TLS55 rotor) at 166,000 ***g*** for 30 minutes at 4°C. The supernatant from this high speed spin (S2) was incubated with 20 μM taxol and 1 mM GTP for 15 minutes at 37°C, and centrifuged in an airfuge at 20 psi for 5 minutes to remove endogenous polymerized MTs. This supernatant fraction (S3) was subjected to a MT affinity-purification step for isolation of a MT-dependent motor fraction (Schroer and Sheetz, 1991a). The MT affinity-purification step was modified by the addition of 3 units/ml apyrase along with 1 mM AMP-PNP during the MT-binding step.

The pellet from the high speed spin (P2) was resuspended in 2 volumes of Buffer A. Carbonate-washed vesicles were prepared by addition of 1 M sodium carbonate, pH 11.3, to the P2 at a final concentration of 0.1 M and incubation for 10 minutes on ice prior to 2-fold dilution in Buffer A. The preparation was then centrifuged through a 15% sucrose cushion in Buffer A in a TLS55 rotor at 166,000 ***g*** for 30 minutes at 4°C.

Phosphocellulose affinity-purified tubulin (5.4 mg/ml) prepared according to the method of Williams and Lee (1982) was polymerized in Buffer A containing 1 mM GTP and 20 μM taxol for 15 minutes at 37°C. Acid-washed coverslips were coated with a uniform coating of a 0.01% solution of poly-L-lysine by spinning the poly-L-lysine solution on to the coverslip on a spinner platform (Inoué, 1986). Formation of a flow chamber was as described above. Polymerized MTs were diluted 1:10 or 1:20 in Buffer A containing 20 μM taxol, and 25 μl of the diluted mixture was flowed into the chamber and incubated at room temperature for 5 minutes. The flow chamber was then rinsed with Buffer A containing 20 μM taxol before reaction mixtures containing motors (S3 or MT affinity-purified fractions), vesicles (P2 or carbonate-washed P2), and 1 or 10 mM ATP were flowed in and visualized by video-enhanced DIC microscopy. When MT affinity-purified fractions were assayed, additional casein (Sigma, from bovine milk, 5% solution) and $MgSO_4$ were added to the reaction mixture to final concentrations of 150 μg/ml and 5 mM, respectively.

DIC microscopy

Cells were observed by video-enhanced differential interference contrast (DIC) microscopy as described previously (Schnapp, 1986). A Zeiss inverted microscope equipped with fiber optic illumination and a ×100/1.3 NA objective was attached to a DAGE VE-1000 camera with a newvicon tube and a Hamamatsu/Argus 10 Image processor. Data were recorded on S-VHS cassettes. An air curtain incubator maintained the microscope stage at 37°C. All intact cell and in vitro assays were at 37°C.

Data analysis

For analysis of vesicle movements in intact cells, a 12.25 μm^2 region of each cell was observed either for two minutes, or for the time required to observe 30 movements. Only movements of vesicles 300 nm in diameter that moved 1 μm were counted. Multiple control and treated CV-1 cells (10-15 cells per plate) were observed and differences in vesicle movements were evaluated for statistical significance at the P 0.05 level by using a *t*-test for independent samples with equal variances. The *t*-test was also used to evaluate significant differences in sample populations for the following parameters: vesicle velocity in intact cells, vesicle run length in intact cells, total vesicles per unit area in intact cells, and the radius of intact cells. For in vitro vesicle motility assays, measurements included the total number of vesicle movements (1 μm in length) per minute per field and the vesicle velocity.

Calculation of total membrane turnover for control and treated cells

To estimate the total membrane area being transported inward within the cells we assumed that inward-moving vesicles were generated randomly within the cell and moved to the cell center. The average distance a vesicle would move was then the radius of a circle encompassing half of the membrane area ($r/\ 2\ \ 2r/3$). Each vesicle movement was for the average run length; therefore, the total number of inward movements per cell per minute times the average run length was divided by $2r/3$ to give the average number of complete (resulting in membrane fusion) inward vesicle movements per minute. The rate of inward membrane movement (μm^2 of membrane per minute) then is the product of the area per vesicle and the number of complete vesicle movements per cell:

$$\text{Rate}\ (\mu m^2 min^{-1}) = (0.07\ \mu m^2) \times (N/2 \times d \times\ \ r^2/12.25\ \mu m^2)/(2r/3),$$

where N is the number of vesicle movements/min/12.25 μm^2, d is the average run length and r is the radius of the cell. The average vesicle (assuming diameter = 150 nm) has a membrane area of 0.07 μm^2.

RESULTS

Nocodazole experiments

CV-1 cells were selected for study of MT-dependent vesicle movements because of their flat and highly spread morphology. Since the cells are <1 μm thick at the periphery, small vesicle movements are easily seen with the video-enhanced DIC microscope. Images of CV-1 cells at low and high magnification are shown in Fig. 1, with representative vesicles (300 nm in diameter) marked by arrows in Fig. 1B.

To verify that these small vesicles were moving in a MT-dependent manner, CV-1 cells were exposed to the microtubule poison, nocodazole (DeBrabander et al., 1986). Cells were cooled to induce depolymerization of cellular MTs, and were then exposed to different concentrations of nocodazole for varying lengths of time. The results are shown in Fig. 2. At a high dose of nocodazole (2.5 μg/ml), virtually all directed movements of small vesicles ceased after 5 minutes. At a lower concentration of nocodazole (1 μg/ml), inhibition of small vesicle movements was complete after 45 minutes. The nocodazole-induced inhibition was fully reversible (data not shown). In addition, immuno-

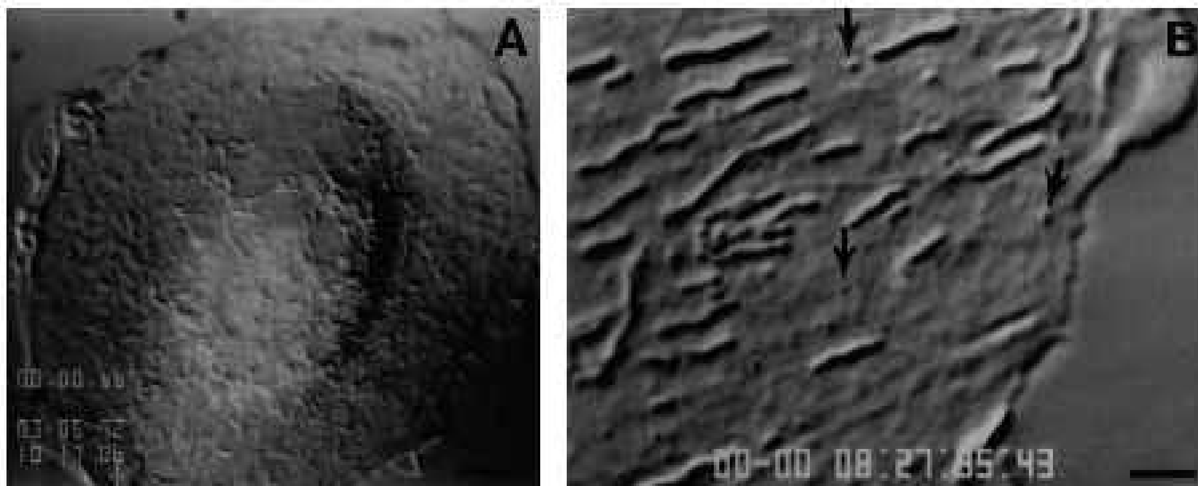

Fig. 1. CV-1 cell morphology. (A) Low magnification image of a CV-1 cell (bar, 5 μm). (B) High magnification image of a CV-1 cell (bar, 1 μm). Small vesicles typical of those included in the assay are marked by arrows.

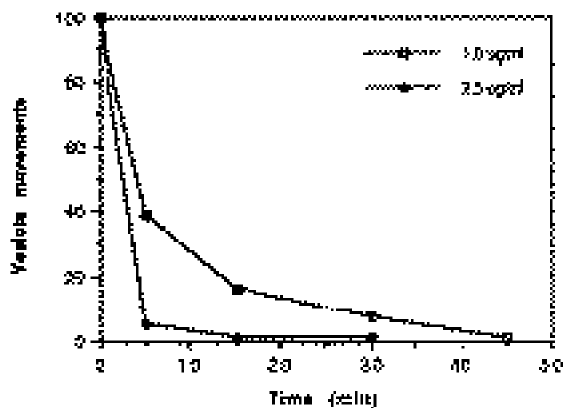

Fig. 2. Effect of nocodazole on vesicle movements in CV-1 cells. Movements are expressed as a percentage relative to the movements observed in control cells. Cells were cooled for 30 minutes at 4°C and then treated with nocodazole at either 1 or 2.5 μg/ml at 4°C for the indicated times. The cells were then incubated at 37°C for 30 minutes and analyzed at 37°C by video-enhanced DIC microscopy.

fluorescence experiments verified that nocodazole treatment under these conditions caused MT depolymerization (data not shown).

Although nocodazole experiments suggested that the small vesicle movements were MT-dependent, the potential contribution of actin was assessed by analysis of vesicle movements after cytochalasin treatment. CV-1 cells were treated with cytochalasins B or D for 60 minutes at 37°C either at 10 μM or 1 μM. Observation of these cells at 10 μM cytochalasin B or D revealed that considerable retraction and thickening of the cell lamellae had occurred, but that the small vesicles inhibited by nocodazole treatment continued to move in a directed manner. Numerous vesicle movements were observed at 10 μM of either cytochalasin, although measurement of the frequency of movements in treated versus control cells was not directly comparable because of changes in cell thickness. At 1 μM of either cytochalasin, the frequency of vesicle movements relative to control was 220 ± 70% for cytochalasin D and 133 ± 27% for cytochalasin B. At this lower dose, the cells were only slightly retracted at the periphery. These experiments demonstrate that the vesicles in our system were moving in a MT-dependent, and not an actin-dependent manner, and even suggest an inhibitory role for actin-dependent processes.

Serum stimulates the frequency of MT-dependent vesicle movements. Our initial observations showed that the presence of serum in the medium stimulated MT-dependent vesicle movements, which led to difficulty in observing stimulation over the high basal frequency of motility in serum-treated cells. In order to obtain a rigorous analysis of pharmacological agents on vesicle movement, CV-1 cells were serum-deprived prior to treatment with agents of interest. Multiple treated and untreated cells were observed on separate plates and compared statistically by a difference in means test to eliminate potential problems with individual cell variation.

The serum-free CV-1 cells remained stable (adhered to the cover slip, no changes in morphology) for at least 24 hours after removal of serum. When CV-1 cells exposed to serum for the entire growth period were compared to control (serum-free) CV-1 cells, the frequency of MT-dependent vesicle transport in the serum-treated cells was stimulated by almost fourfold (364%, s.d.=19). In order to ensure that the numbers of moving vesicles did not vary in individual cells, the frequency of vesicle movements was measured in 3-4 different unit areas per individual cell. Control CV-1 cells exhibited a mean percent deviation in vesicle movements/unit area of 22.1% ± 2.7% (n=10 cells) and serum-treated CV-1 cells exhibited a mean percentage deviation in vesicle movements/unit area of 14.7% ± 3.1% (n=10 cells). Thus, there are only slight regional variations in the level of transport throughout these cells.

Okadaic acid and dibutyryl cAMP dramatically stimulate MT-dependent vesicle movement in CV-1 cells. Over 25 pharmacological agents targeting protein kinases, phosphatases, and other regulatory pathways were assayed for effects on MT-dependent vesicle movements. The drugs

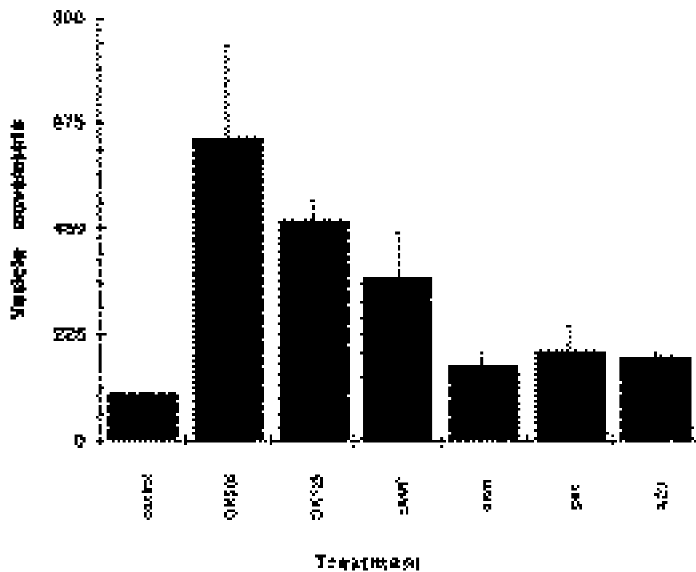

Fig. 3. Percent increase or decrease in the number of vesicles moving in CV-1 cells after exposure to the following treatments for 60 minutes at 37°C. Movements are expressed as a percentage relative to the movements observed in control cells. Concentrations of the drugs were: A23187, 50 nM; dibutyryl cAMP, 10 mM; okadaic acid, 500 nM or 125 nM; genistein, 100 μg/ml; trental, 10 μg/ml; serum, 10% v/v. All compounds listed here consistently elicited significant stimulation at the *P* 0.05 level. Results represent at least three experiments with 10-15 cells/experiment. Bars represent a s.d. calculated from the sample means from each experiment.

eliciting significant (*P* 0.05) stimulation of the frequency of vesicle movements are shown in Fig. 3. These results are expressed as the percentage increase over the frequency of vesicle movements seen in control cells (100%); for reference, these cells exhibited 20 movements per minute within the area of observation (s.d.=17, s.e.m.=1.1), corresponding to 1.6 movements/min per μm^2.

Okadaic acid is a potent inhibitor of serine/threonine phosphatases, particularly 1 and 2A (Bialojan and Takai, 1989; Cohen et al., 1990; Haystead et al., 1989). Treatment of CV-1 cells with okadaic acid induced the greatest stimulation of the frequency of vesicle movements; the frequency was stimulated the most at 500 nM (641%, s.d.=195) although a submaximal dose of 125 nM okadaic acid was also effective (468%, s.d.=40). The frequency of movements in the presence of 500 nM okadaic acid was reduced to <7% of control by coadministration with 1 μg/ml nocodazole (data not shown). Okadaic acid concentrations of 500 nM are less than those reported to inhibit some membrane trafficking pathways by causing entry into M-phase (Tuomikoski et al., 1989; Warren, 1989; Pypaert et al., 1991). Although a dose of 1 μM okadaic acid caused the cells to round up, 500 nM okadaic acid caused only minor changes in cell morphology characterized by slight retraction and thickening of the peripheral region.

Dibutyryl cAMP elevates intracellular cAMP levels, activating cAMP-dependent protein kinase, which catalyzes serine/threonine protein phosphorylation. Dibutyryl cAMP (346%, s.d.=92) significantly stimulated the frequency of vesicle movements to the same extent as serum (364%, s.d.=19). Exposure of the CV-1 cells to dibutyryl cGMP at 10 mM did not elicit a stimulatory response (data not shown). Trental also significantly increased the frequency of MT-dependent vesicle movements. Like dibutyryl cAMP, trental elevates intracellular cAMP, but the mechanism appears to involve inhibition of intracellular phosphodiesterases (Bessler et al., 1986). Of the other pharmacological agents assayed, genistein and A23187 repeatedly and significantly increased the number of moving vesicles within CV-1 cells. A23187 (calcium ionophore) elevates intracellular calcium, a major second messenger. The isoflavone, genistein, is a tyrosine kinase inhibitor (Akiyama et al., 1987).

Table 1. Stimulation of adenylate cyclase and effects on frequency of vesicle movements in CV-1 cells

Compound	Movements per minute
Control	22 ± 2.4
Cholera toxin*	58 ± 7.6
Pertussis toxin	11 ± 1.8
Forskolin	32 ± 5.8
1,9-dideoxyforskolin	23 ± 7.3
7 -deacetyl-7 -(-*N*-methylpiperazino) -butyryl forskolin dihydrochloride	14 ± 3.8

*Denotes values significant at *P* 0.05 (*n* 10 cells) when sets of treated cells are compared to the appropriate controls. Units are movements/min per 12.25 m^2.

Concentrations are as follows: cholera toxin, 1 μg/ml; forskolin, 50 μM; 1,9-dideoxyforskolin, 100 μM; 7 -deacetyl-7 -(-*N*-methylpiperazino)-butyryl forskolin dihydrochloride, 25 μM. Errors are s.e.m.

Activation of adenylate cyclase by cholera toxin stimulates the frequency of vesicle movement. Because of the dramatic increase in the frequency of vesicle movements after treatment with drugs that influence either cAMP levels or serine-threonine protein phosphorylation (dibutyryl cAMP, okadaic acid, trental), other compounds that affect intracellular cAMP levels were evaluated (Table 1). Cholera toxin constitutively activates G_s, a GTPase that stimulates adenylate cyclase, ultimately inducing cellular protein phosphorylation by cAMP-dependent protein kinase (Middlebrook and Dorland, 1984; Casey and Gilman, 1988). It elicited a significant (*P* 0.05) stimulation of the frequency of vesicle movements observed (58 ± 7.6 movements/minute for cholera toxin-treated, 22 ± 2.4 movements/minute for control). Treatment of CV-1 cells with pertussis toxin, which uncouples adenylate cyclase from G_i, an inhibitory GTPase (Hewlett et al., 1983), had no significant effect on the frequency of movements. Three forskolin derivatives - two that activate adenylate cyclase

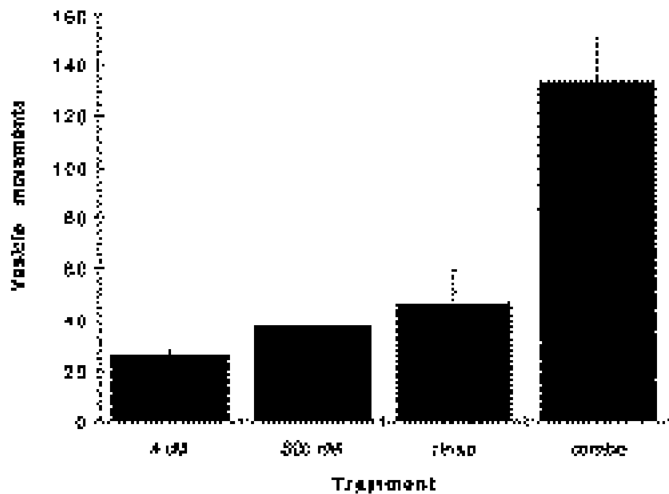

Fig. 4. Effects of taxol on CV-1 cells. Movements are expressed as a percentage relative to the movements observed in control cells. All exposures were for 60 minutes at 37°C. Taxol was added to conditioned media from a 4 mM stock in dimethyl sulfoxide to a final concentration of 4 µM or 500 nM. Rinse refers to a 60 minute exposure to 4 µM taxol followed by a 60 minute exposure to taxol-free conditioned media. Combo refers to the treatment of cells with 4 µM taxol and 500 nM okadaic acid. Results are averages of analysis of two different assays of 10 cells each with the agent of interest. Bars represent a s.d. calculated from the sample means from each experiment.

(forskolin and 7 -deacetyl-7 -(-*N*-methylpiperazino)-butyryl forskolin dihydrochloride) - and one that does not (1,9-dideoxy forskolin) (Laurenza et al., 1987) - were also assayed but failed to elicit a significant effect on the frequency of vesicle movements.

Treatment of CV-1 cells with taxol

In light of the observation that nocodazole inhibited vesicle movements, we investigated the effect of the MT-stabilizing drug, taxol (Schiff et al., 1979), on vesicle transport. Taxol treatment significantly reduced the frequency of vesicle movements (Fig. 4) to 26% ± 1% after 4 µM taxol and 37% (± 0%) after 500 nM taxol. Taxol-treated cells were also characterized by changes in morphology, specifically, a slight retraction of peripheral cytoplasm accompanied by an apparent redistribution of some perinuclear vesicles like the Golgi apparatus to the cell periphery.

Cells that were rinsed and incubated in fresh media following treatment with 4 µM taxol for 60 minutes exhibited a frequency of vesicle movement that was 47% ± 13% of control, indicating that treatment was partly reversible under these conditions (Fig. 4). Interestingly, taxol-induced inhibition was blocked by coadministration of okadaic acid; after 60 minutes at 500 nM okadaic acid and 4 µM taxol, the frequency of vesicle movements was 133% ± 18% of control. Cells treated with a combination of taxol and okadaic acid still exhibited the characteristic changes in morphology described for taxol treatment alone.

Effects of drugs on vesicle directionality

In control cells, we observed that 51% ± 2% of all moving vesicles move outward to the cell periphery and the remainder move to the cell interior. These numbers suggested the existence of a steady state of MT-dependent vesicle transport, irrespective of the contribution of membrane flow through tubular vesicles. Each pharmacological agent that altered the frequency of MT-dependent vesicle transport was evaluated to see if the steady-state relationship of inward and outward MT-dependent movements was maintained. The results are shown in Fig. 5. Directionality was not significantly (P 0.05) altered by any of the agents that stimulated or inhibited the frequency of vesicle transport. In all cases, the diameters of the vesicles moving inward and outward were in the same size range. Inward and outward MT-dependent vesicle movements therefore appear to be regulated in concert.

Evaluation of run length and vesicle velocity in control and treated CV-1 cells

The treatments that elicited the greatest stimulation and inhibition of the frequency of vesicle movements were selected for further analysis of run length and vesicle velocity (Table 2). After treatment with serum (2.64 ± 0.18

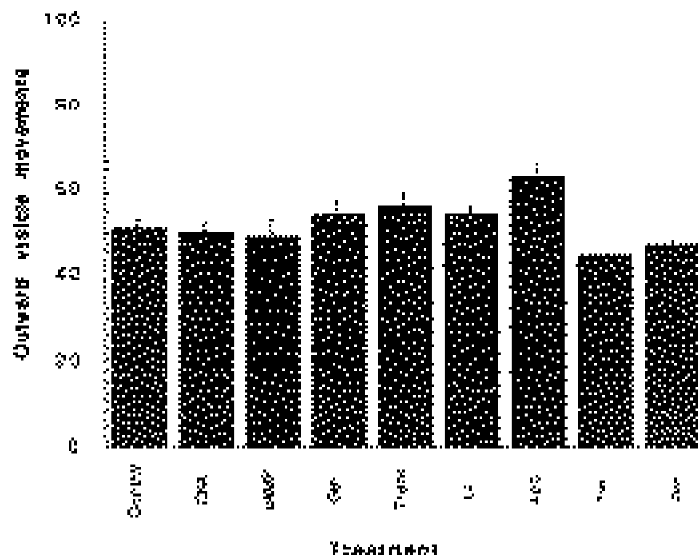

Fig. 5. Percent of total vesicle movements directed outward. Movements are expressed as a percentage relative to the movements observed in control cells. Error bars represent the s.d. within the sample population (n 10). Concentrations are the same as those listed in Fig. 2 except for taxol, which was 500 nM. None of the differences in the percentage of outward movements in each sample were significant at P 0.05.

Table 2. Vesicle velocity and run length in control and treated CV-1 cells

Treatment	Velocity (μm/s)	Run length (μm)
Control	2.10 ± 0.13	2.58 ± 0.17
Serum	2.64 ± 0.18*	3.34 ± 0.22
Taxol	1.24 ± 0.15*	1.95 ± 0.15
Okadaic acid	2.71 ± 0.17*	3.64 ± 0.42*
dbcAMP	4.10 ± 0.25*	4.71 ± 0.45*

*Denotes significant differences from control cells at the *P* 0.05 level.
Concentrations are as follows: serum, 10%; taxol, 4 μM; okadaic acid, 500 nM; dibutyryl cAMP, 10 mM. *n*=35 for control measurements and *n*=25 for all other measurements. Errors are s.e.m.

Table 3. Comparison of vesicles/μm^2 and mean cell radius in control and treated CV-1 cells

Treatment	Vesicles/μm^2	Mean cell radius (μm)
Control	0.75 ± 0.04	24.5 ± 1.7
Serum	0.71 ± 0.06	25.9 ± 1.8
Taxol	0.75 ± 0.07	23.5 ± 1.2
Okadaic acid	1.16 ± 0.11*	23.7 ± 1.8
dbcAMP	0.76 ± 0.06	27.8 ± 2.6

*Denotes differences significant at the *P* 0.05 level.
Concentrations are as follows: serum, 10%; taxol, 4 μM; okadaic acid, 500 nM; dibutyryl cAMP, 10 mM. Errors are s.e.m. and *n*=10 cells.

μm/s), dibutyryl cAMP (4.71 ± 0.45 μm/s), or okadaic acid (2.71 ± 0.17 μm/s), vesicle velocities were significantly (*P* 0.05) higher than control cells (2.10 ± 0.13 μm/s). Vesicle velocities in taxol-treated cells (1.24 ± 0.15 μm/s) were significantly lower than in control cells.

When the run lengths of vesicles were compared in control cells versus cells treated with dibutyryl cAMP, okadaic acid, serum, or taxol, differences were also noted. Although only dibutyryl cAMP (4.1 ± 0.25 μm) and okadaic acid (3.64 ± 0.42 μm) significantly (*P* 0.05) increased the run length, vesicles in serum-treated cells also exhibited increased run length (3.34 ± 0.22 μm) relative to control (2.58 ± 0.17 μm). Taxol reduced the run length (1.95 ± 0.15 μm) although this difference was not significant at the *P* 0.05 level. These data illustrate a possible coordinate stimulation or inhibition of the frequency of MT-dependent vesicle movements, vesicle velocity, and run length.

Evaluation of total vesicles/unit area and mean radius in control and treated CV-1 cells

To evaluate whether treatments that significantly altered the frequency or velocity of vesicle movement or run length had effects on the total number of cellular vesicles or the cell size, the mean cell radius and the total number of vesicles (moving and unmoving sampled in a single frame) per unit area were measured under the conditions of treatment. The results are shown in Table 3. We were able to compare cells treated with different pharmacological treatments since the control cells for each experiment exhibited the same values for these measurements (within standard error). In addition, the number of vesicles/unit area did not significantly differ when measured in different regions within the same cell. Okadaic acid elicited approximately a 50% increase in the total number of vesicles/unit area from 0.75 ± 0.04 (control) to 1.16 ± 0.11 (okadaic acid), which was significant (*P* 0.05). No other treatments elicited any significant changes in the total number of vesicles/unit area.

When the mean radius was compared in control and treated cells, no significant differences were observed. Video-enhanced DIC microscopy did reveal a slight thickening in the peripheral regions of taxol-treated and okadaic acid-treated cells but this difference was not manifested in a significantly reduced radius. These cell populations were characterized by great variability in cell shape and cell size, so minor differences would not be discernible.

Distribution of cellular MTs in control and treated CV-1 cells

To ascertain if the drugs that significantly altered MT-dependent vesicle transport changed the distribution or density of MTs, we visualized MTs by immunofluorescence in control and treated CV-1 cells. Representative immunofluorescence micrographs of cells comparable in shape and size to those analyzed for the frequency of MT-dependent vesicle movements are shown in Fig. 6. These studies revealed that the MTs in CV-1 cells exist in a radial array with the ends at the periphery. None of the drugs that stimulated MT-dependent vesicle transport (Fig. 6B, serum, D, dibutyryl cAMP, E and F, okadaic acid) induced significant alterations in MT distribution or density (Fig. 6A, control). In okadaic acid-treated cells (500 nM), the thickened ridge at the edge of the cell (that is evident with DIC microscopy) stained heavily for tubulin (Fig. 6E). No evidence for looping of the MTs was seen in stimulated or control cells, indicating that MT structure was not responsible for the coordinate regulation of inward and outward MT-dependent vesicle movements observed over a sixfold range.

In contrast to the radial array of MTs in control and stimulated cells, taxol treatment resulted in the formation of a more random array of MTs (Fig. 6C). The polar orientation of these MTs is unknown. The randomized distribution and greater MT density may inhibit vesicle transport activity, vesicle velocity and run length directly but an indirect effect seems more likely. Interpretation of the balanced inward and outward vesicle movements following taxol treatment is also more complex. Taxol-treated cells exhibited a higher intensity of labeling, indicating the presence of a higher density of MTs (data not shown). Since taxol causes substantial alterations both in the density and distribution of cellular MTs, regions of mixed polarity may be generated. In addition, the apparent taxol-induced redistribution of some perinuclear vesicles to the cell periphery suggests an alteration in cell polarity. These issues make the directionality of vesicle movements in taxol-treated cells difficult to resolve.

Reconstitution of vesicle movements on MTs

To see if the changes in vesicle motility in intact CV-1 cells were reflected in vitro, vesicle motility in extracts from control and serum-treated CV-1 cells were compared in vitro at 37°C (Fig. 7). When high speed supernatant (S) and vesi-

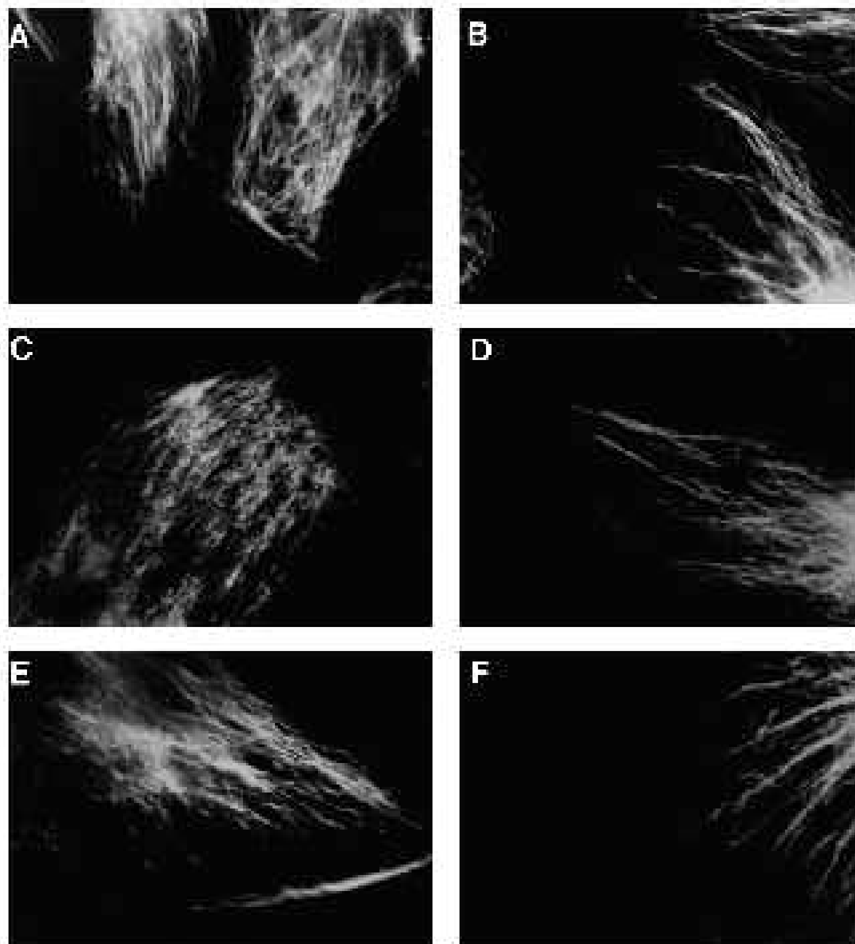

Fig. 6. Tubulin immunofluorescence in control and treated CV-1 cells. Treatments are as described in Materials and Methods. (A) Control cells. (B) Serum-treated cells. (C) Taxol (4 μM) treatment. (D) Dibutyryl cAMP (10 mM) treatment. (E) Okadaic acid (500 nM) treatment. (F) Okadaic acid (125 nM) treatment.

cle fractions (V) from serum-treated and control cells were assayed for vesicle motility (vesicle movements/min/field), the extracts and vesicles from serum-treated cells exhibited the most activity and the extracts and vesicles from control cells exhibited the least activity (Fig. 7A). When extracts and vesicles from different sources were assayed together, both combinations, S(−)/V(+) and S(+)/V(−), exhibited intermediate activity (Fig. 7A), although the activity of the preparation containing the soluble fraction from serum-treated cells, S(+), was higher.

Soluble fractions were subjected to MT affinity-purification, which yielded a partially purified motor fraction (M) that promoted vesicle motility on MTs. The protein composition of the MT affinity-purified samples from control and serum-treated cells is shown in the gel in Fig. 8. Vesicles were treated by carbonate-washing, which removes associated membrane proteins. Assay of these samples was also at 37°C. Carbonate-washed vesicles (WV) showed no motility when assayed without a soluble source (S or M) of a MT-dependent motor even when supplemented with casein (data not shown). When MT affinity-purified fractions and carbonate-washed vesicles from serum-treated and control cells were compared, the activity in the samples from serum-treated cells was again the highest and the activity in the controls was again the lowest (Fig. 7B). MT affinity-purified fractions and carbonate-washed vesicles from different sources [M(−)/WV(+) and M(+)/WV(−)] exhibited intermediate activity (Fig. 7B), although the activity of the M(+) fraction was again the highest regardless of the source of vesicle. Treatment of CV-1 cells with dibutyryl cAMP prior to lysis also generated a MT affinity-purified motor sample that had higher activity than controls (data not shown). These data suggest that most of the enhanced activity observed in the intact cells is a result of regulation of the motor complex, but that there is also a contribution by a target(s) on the vesicle.

Finally, when the in vitro velocity of carbonate-washed vesicles was measured with MT affinity-purified motors from either source, the velocity of vesicles driven by M(+) fractions was significantly higher than the velocity of vesicles driven by M(−) fractions, regardless of the source of vesicle (Table 4). Exposure of cells to serum therefore appears to activate factors in the vesicle motor complex that affect both the frequency and velocity of vesicle movements.

DISCUSSION

We have found major changes in the level of MT-dependent vesicle transport, velocity, and run length in intact cells, which are elicited by drugs that target cellular kinases and phosphatases. The drugs that we have identified mod-

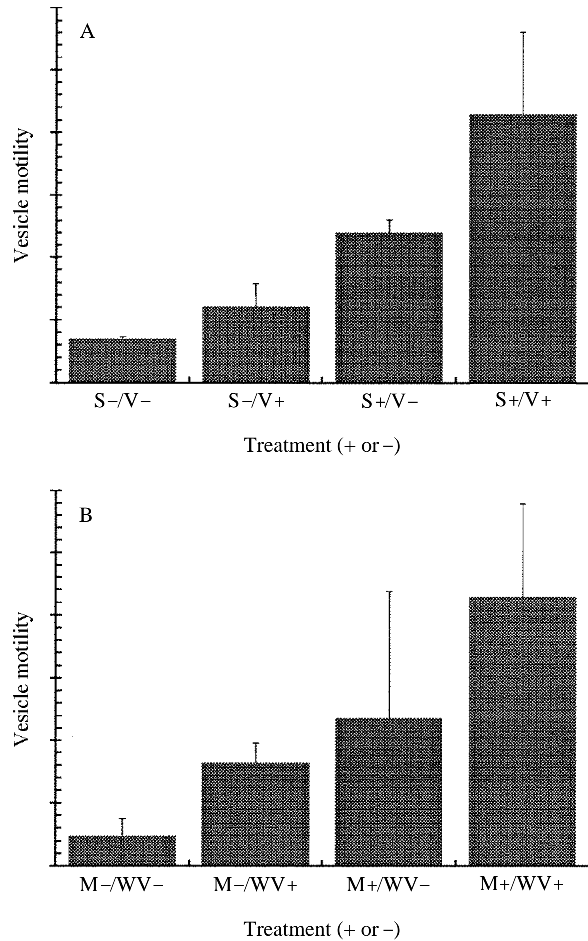

Fig. 7. In vitro vesicle motility of samples prepared from control (−) or serum-treated (+) CV-1 cells. The number of vesicle movements/minute per field at 37°C was measured to evaluate vesicle motility for samples adjusted to give the same concentration of protein and vesicles. (A) Vesicle motility with high speed supernatant (S) and membrane vesicle (V) fractions. (B) Vesicle motility with MT affinity-purified motors (M) and carbonate-washed membrane vesicles (WV). Error bars represent s.e.m.

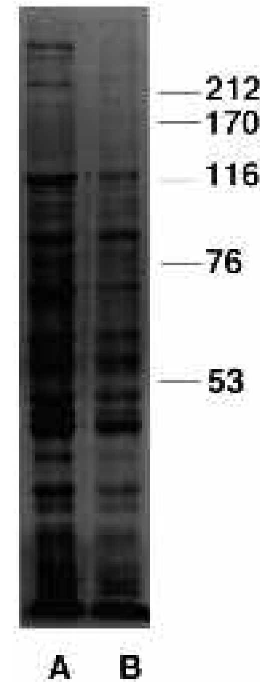

Fig. 8. MT affinity-purified motors from control and serum-treated CV-1 cells visualized after electrophoresis on a 7.5% acrylamide gel and staining with Coomassie Blue. The loading of the protein was less in the serum lane and densitometry of the kinesin bands was used to adjust the dilutions for motility assays (the kinesin band intensity relative to total protein content was the same in both samples). Lane A, MT affinity-purified motors from control cells [M(−)]. Lane B, MT affinity-purified motors from cells treated with serum [M(+)].

Table 4. Velocity of vesicle movements in vitro at 37°C

Samples	Velocity (μm/s)
M(−)/WV(−)	1.40 ± 0.13
M(−)/WV(+)	1.42 ± 0.09
M(+)/WV(−)	1.74 ± 0.07
M(+)/WV(+)	1.88 ± 0.02

Assays contain MT affinity-purified motors (M) and carbonate-washed vesicles (WV) from control (−) or serum-treated (+) CV-1 cells.

*Differences in vesicle velocities with MT affinity-purified motors from control [M(−)] cells versus MT affinity-purified motors from serum-treated cells [M(+)] are significant, regardless of the source of vesicles. Errors are s.e.m.

ulate the frequency of vesicle transport over a 20-fold range. Equivalent numbers of inward and outward movements were observed over >sixfold stimulation of the frequency of vesicle movements, without accompanying changes in the distribution or density of cellular MTs. Since no major changes in cell size or in the total number of vesicles were observed, these results suggest that cellular kinases alter vesicle motile activity.

Although actin and MT-based motility may have some common roles in vesicle transport, the small vesicle movements are MT-dependent as shown by several observations. First, vesicle movements were blocked by nocodazole treatment but mildly stimulated by cytochalasin treatment. Second, the frequency of vesicle transport was sensitive to MT redistribution by taxol. Third, the in vitro activity (as measured by a MT-dependent vesicle motility assay) was correlated with in vivo levels of motility.

The most potent activator of MT-dependent vesicle transport was okadaic acid (500 nM), which elevated the frequency of vesicle movement by >sixfold and also significantly increased the vesicle velocity and run length. Since okadaic acid inhibits serine/threonine protein phosphatases, its effect on MT-dependent vesicle transport may be due either to inhibition of protein phosphatases or to hyperphosphorylation by protein kinases. Okadaic acid also inhibits fusion of vesicles from the endoplasmic reticulum with the Golgi (Davidson et al., 1992), which might be expected to create additional small vesicles in treated cells. We observed an okadaic acid-induced increase in the mean total vesicles/unit area of only 50% (Table 3), so that the increased number of vesicles is an unlikely explanation for the >600% increase in vesicle motility. However, the rela-

tive increase in vesicle number corresponds to the relative frequencies of vesicle movement in okadaic acid-treated cells versus dibutyryl cAMP or serum-treated cells. We therefore suggest that roughly the same level of activation is achieved with okadaic acid, dibutyryl cAMP and serum, but that okadaic acid-treated cells have a higher concentration of vesicles.

Okadaic acid also potentiates the activity of the cdc2 protein kinase that controls events leading to cell division (Nurse, 1990), and has been used extensively at high concentration to trigger M-phase in various cells. Membrane trafficking in mitotic cells is almost completely halted (Tuomikoski et al., 1989; Warren, 1989; Pypaert et al., 1991). This inhibition can be mimicked in vitro with extracts of eggs in M-phase (Allan and Vale, 1991). Inhibition of endocytosis of fluid-phase fluorescent markers was observed in cells treated with 1 μM okadaic acid to induce a mitotic state (Lucocq et al., 1991), and as previously discussed we see corresponding changes in morphology indicative of a shift to M-phase in CV-1 cells at 1 μM but not at 500 nM okadaic acid.

Several studies have previously demonstrated that either hormones or pharmacological agents can accelerate the rates of movement of internalized receptors through the pathway (Buys et al., 1984; Buys et al., 1987; Wiley and Kaplan, 1984). A common theme linking several of the agents that stimulate the frequency of MT-dependent vesicle movements (dibutyryl cAMP, cholera toxin, trental, okadaic acid), and vesicle velocity and run length (dibutyryl cAMP, okadaic acid) is activation or potentiation of phosphorylation by cAMP-dependent protein kinase. Genistein and A23187 also stimulate the frequency of MT-dependent vesicle movements, possibly through a different regulatory pathway. The stimulation that we observe as a result of protein phosphatase inhibition, or elevations in cAMP or calcium, indicates that protein phosphorylation is a common mechanism for regulation of MT-dependent vesicle transport. It is not clear from our investigations in intact cells whether these drugs share common or separate targets.

The size of the moving vesicles in the CV-1 cells correlates with earlier measurements from in vivo and in vitro studies (Steinman et al., 1976; Steinman et al., 1983; Gruenberg et al., 1989b). The basal frequency of vesicle transport in control cells corresponds to the inward movement of a membrane area equivalent to the plasma membrane in approximately two hours (Table 5). Since these measurements in control cells faithfully reproduce earlier estimates for the turnover of plasma membrane in cultured cells (Steinman et al., 1983), they suggest that we are observing a significant fraction of the endocytic traffic within the cell in our assay. After stimulation with serum, okadaic acid, or dibutyryl cAMP, the rate of total membrane flux was increased to <30 minutes for inward movement of the total membrane surface area (Table 5). Remarkably, taxol treatment slowed the flux of detectable movements to >9 hours (Table 5), which is perhaps related to the dispersion of the Golgi apparatus and could also involve a transition to a membrane tubular mechanism of transport.

Our in vitro data strongly suggest that elements of the vesicle motor complex are activated by treatment with serum, which contains multiple growth factors that stimulate numerous cellular kinases and phosphatases. Activation of the frequency of MT-dependent vesicle transport in intact cells by serum and activation of the purified vesicle motor complex from serum-treated cells are directly correlated. The stimulation by serum of vesicle velocity was also correlated in vivo and in vitro. Our results also indicate that activation occurs predominantly in the motor fraction, and to a lesser extent on the vesicle.

Table 5. Total membrane movement inward in control and treated CV-1 cells

Treatment	Flux of inward membrane (μm^2/min)	Time for PM equivalent (min)
Control	34	111
Serum	176	24
Taxol	6	542
Okadaic acid	299	12
dbcAMP	245	20

*This represents an estimation of the total flux of membrane moving inward assuming the total length of such vesicle movements before membrane fusion is on average $2r/3$ and the average vesicle diameter is 150 nm. PM, plasma membrane.

Our data support a model of coordinate regulation of vesicle transport in both the inward and outward legs of the endocytic and exocytic pathways, involving regulation of MT-dependent motors and not the number of vesicles. There is no evidence for the involvement of membrane tubules, since our measurements of membrane flux are in agreement with previous measurements of the amount of membrane transport and both directions of transport are matched in activity. The marked decrease in vesicle transport with taxol could signal a conversion to a membrane tubule transport mechanism. The major differences that we observe in cellular vesicle motile activity following treatments that activate cAMP-dependent and other protein kinases cannot be accounted for by increases in intracellular vesicle concentration, which indicates that fission is not the primary target of regulation under these conditions. Our isolation of ‘activated’ MT affinity-purified motors from treated cells further substantiates the model that we propose. Although some intracellular membrane trafficking clearly does not involve MT-dependent motors, our studies indicate that modulation of motor activity is correlated with transport. The consistency of the directionality of movements is expected in order for the integrity of the various membrane compartments to be maintained, but it is remarkable that directionality is maintained over a six-fold range. Elucidation of the molecular mechanisms governing the simultaneous activation of inward and outward movement in the MT-dependent motors will be a critical next step.

The authors thank R. Michael Alvarez for his assistance in statistical analysis, Ron Sterba for expert technical assistance, and Terry O’Halloran, Denise Wayne, Richard Walker, Scot Kuo and Jim McIlvain, Jr for a critical reading of the manuscript. Hanry Yu is also thanked for his assistance with figure preparation. The project was supported by grants GM 36277 and NS 23345 to

M.P.S.; S.H.-A. was supported by a postdoctoral fellowship from the Muscular Dystrophy Association.

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(Received 19 May 1993 - Accepted, in revised form, 4 August 1993)