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18. This analysis used the Jupp-Mardia circular correlation coefficient (*17*).
19. The Y-maze has been described by A. O. D. Willows [in (*10*)]. *Tritonia* is strongly rheotactic and will locomote into water currents in the maze. Water flow into each arm was 1000 ± 50 ml/min in all Y-maze experiments reported here.
20. Nudibranchs in experiment 1 (conducted in May and June of 1984) were tested in small groups to facilitate rapid data collection (many animals do not move in the 1-hour time period allotted). In experiment 2 (April to June of 1985), animals were tested one at a time; after each trial, the walls and floor of the Y-maze were scrubbed thoroughly with a nylon bristle brush to reduce the possibility that residual chemical cues or slime trails would influence subsequent trials. Throughout both experiments we alternated between trials in the geomagnetic field and rotated or reversed field (a trial under one field condition was followed by a trial in the other).
21. We had few data points from new moon trials (Fig. 2), but predicted westward movement at new moon because (i) at full moon the animals seem to orient approximately eastward (for example, Figs. 1A and 2); (ii) Fig. 2 suggests that the mean angle of orientation rotates at an essentially constant rate through 360° over the course of the lunar month.
22. Both choices could be outside the range of "preferred" directions (resulting in no preference) or the animals might turn south because this is closer to west than east is.
23. Since experiments in the circular tank were conducted at essentially the same time each solar (24-hour) day and therefore at different times in the lunar (24.8-hour) day, it is hypothetically possible that the relationship in Fig. 2 reflects variation attributable to a lunar daily rhythm rather than a lunar monthly rhythm. Y-maze experiments were conducted continuously for 120 hours at new and full moon, however, and there was no apparent variation in response over the course of the lunar day. At present the data are thus most consistent with the circa-lunar rhythm hypothesis (but other possibilities cannot be ruled out).
24. *Tritonia* feeds on sea pens and sea whips (pennatulacean cnidarians), which are often found in patches [C. Birkeland, *Ecol. Monogr.* **44**, 211 (1974)]. A circular pattern of movement could keep nudibranchs in an area of high food concentration, or a gradual outward spiraling from the last known prey item might enhance search effectiveness.
25. Numerous marine invertebrates have reproductive rhythms related to lunar phase. All of our nudibranchs were trawled from a location near Olga, Orcas Island, Washington, where the nearest body of land is almost directly eastward. Eastward movement at the time of full moon could facilitate reproduction by bringing dispersed groups of animals together on shoals at a specific point in the lunar month. Westward movement around the time of new moon could represent an offshore migration back to deeper water.
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31. We thank T. Quinn, J. Palka, W. Wright for critically reading the manuscript; M. Njegovan for technical assistance during preliminary experiments; and S. Adolph for assistance with the statistical analyses. Supported by NIH grants 18658-01 and 22974-01 to A.O.D.W., GM 07108-10 (instructional training grant) to K.J.L., and funding from the Western Society of Malacologists to K.J.L.

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Myosin Rod Phosphorylation and the Catch State of Molluscan Muscles

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"Catch" is a prolonged state of tension in molluscan smooth muscles shown by mechanical measurements to be associated with the level of protein phosphorylation. Myosin isolated from these muscles is unusual in being phosphorylated in the rod portion by an endogenous kinase, like certain nonmuscle myosins. These findings suggest that the myosin rod is a target for phosphorylation and that this reaction may control the transition from catch to relaxation.

CERTAIN MOLLUSCAN SMOOTH MUSCLES display a specialized stretch-resistant state called "catch" in which tension is maintained for long periods. In the catch state, although adenosine triphosphate (ATP) is present, the myosin cross-bridges are attached to actin but cycle very slowly [for review, see (*1*)]. Catch muscles are characterized by distinctive thick filaments containing a large core of paramyosin and an outer layer of myosin (*2–4*). Aspects of the packing of the paramyosin core are understood (*3, 5–7*). In contrast, the organization of myosin in the thick filaments of catch muscle is not yet known, although the myosin array in a related molluscan non-catch muscle has been visualized (*8*). The myosin of both catch and the more rapidly relaxing striated adductor muscles of molluscs is directly activated by the binding of Ca^{2+} to the head portion of the molecule (*9, 10*). Several hypotheses for the mechanism of catch involve phosphorylation of paramyosin, with possible effects on paramyosin-paramyosin and paramyosin-myosin interactions (*11–14*). We report that the maintenance of catch is associated with dephosphorylation and also show that the rod portion of a catch muscle myosin can be phosphorylated in vitro. These findings suggest new possibilities for the molecular basis of catch.

We have confirmed the reports of Cornelius (*15, 16*) that detergent-skinned fiber preparations of the anterior byssus retractor muscles (ABRM) of *Mytilus edulis* display mechanical properties similar to those of the intact muscle (Fig. 1). Contraction is activated by addition of 10^{-6} *M* Ca^{2+}, producing an "active state." The muscle is locked into catch by removal of Ca^{2+}, and relaxation from catch is obtained by the addition of cyclic adenosine monophosphate (cAMP). This cyclic nucleotide, however, does not inhibit development of active tension by Ca^{2+}. This permeable fiber preparation therefore allows the direct investigation of the role of second messengers in the contractile process.

We have studied the mechanical response of these muscles to the catalytic subunit of cAMP-dependent protein kinase [for review see (*17*)]; to ATP-γ-S, a nonhydrolyzable ATP analog often used as a substrate for kinases (*18*); and to NaF, an inhibitor of phosphatases. Skinned ABRM fiber bundles preincubated with the catalytic subunit of the cAMP-dependent kinase develop active tension comparable to the control (Fig. 2), but removal of Ca^{2+} produces rapid relaxation instead of locking the muscle into catch. This finding suggests that the cAMP-dependent kinase is the target protein of cAMP in this system.

Relaxation from the catch state can also be induced by addition of 20 m*M* NaF (Fig. 3). The presence of NaF does not affect activation by Ca^{2+}, but it inhibits the development of catch even after its removal by extensive washing.

When ATP-γ-S is substituted for ATP during catch, rapid relaxation is observed

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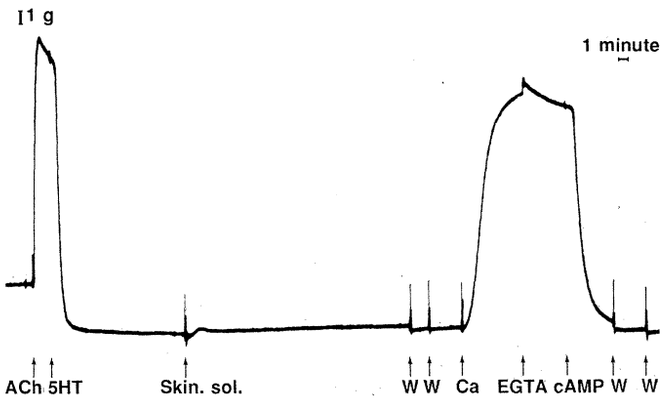

Fig. 1. Typical trace of tension development in the ABRM before and after chemical skinning. A freshly dissected ABRM fiber bundle (2 to 3 mm in diameter) was mounted horizontally with one end fixed to a rigid support and the other attached to a tension transducer connected with a Gould 2400 recorder. Changes of bathing medium are indicated by arrows. The muscle bundle, kept in artificial seawater at room temperature, was activated by $5.5 \times 10^{-5}M$ acetylcholine (ACh). Relaxation was produced by $5.5 \times 10^{-5}M$ serotonin (5HT). The bathing solution was then replaced by a skinning solution (Skin. sol.) containing 20 m*M* EGTA, 8 m*M* $MgCl_2$, 5 m*M* ATP, 50 m*M* Tes [2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid] at *p*H 7.0, and 0.1% saponin. After ~20 minutes, the bundle was washed (indicated by W) with a solution containing 3 m*M* $MgCl_2$, 1 m*M* ATP, 0.5 m*M* EGTA, and 50 m*M* Tes at *p*H 7.0. Activation was induced by the addition of 0.5 m*M* $CaCl_2$ to the wash solution (*p*Ca, ~5.0). Active contraction was stopped with a solution (EGTA) containing 3 m*M* $MgCl_2$, 1 m*M* ATP, 20 m*M* EGTA, and 50 m*M* Tes at *p*H 7.0 for quick removal of Ca^{2+}. The muscle was then in the catch state, characterized by very slow tension decay. Relaxation from catch was achieved by direct addition of $5.5 \times 10^{-5}M$ cAMP.

with or without addition of cAMP, the response being faster when the cyclic nucleotide is present (Fig. 4A). Once the fiber bundle has been treated with ATP-γ-S, however, it cannot be locked into catch, although active tension is produced in the presence of Ca^{2+} (Fig. 4B). Both NaF and ATP-γ-S thus appear to prevent the development of the catch state in skinned ABRM preparations, although the properties of these two compounds are very different.

Taken together, these results appear to indicate that phosphorylation of specific proteins—either kinases or contractile proteins—may be part of the mechanism controlling the catch state. The observation that both NaF and ATP-γ-S produce the same effect might be explained by assuming that during catch both phosphatase and kinase activities are present, with the phosphatase predominating. Maintenance of catch would therefore be related to a state of dephosphorylation. Blocking the phosphatase by NaF, or thiophosphorylating the target proteins by using ATP-γ-S as a nonhydrolyzable substrate for the kinase, would result in a relative increase in kinase activity and therefore in relaxation. A different response to ATP-γ-S is observed in vertebrate smooth muscles where, in the presence of Ca^{2+}, ATP-γ-S produces a long-lasting activation that results from irreversible phosphorylation of the myosin light chains (*19, 20*). In the ABRM, the state of phosphorylation appears to have no direct effect on the development of active tension, implying that light chain phosphorylation does not trigger activity (*21*).

In parallel with these studies, we have begun to characterize the phosphorylation of the contractile proteins of the ABRM. Myosin isolated from the ABRM by standard methods (*22*) contains a tightly bound endogenous kinase (Fig. 5). When this myosin is incubated with labeled ATP, phosphate is incorporated into the heavy chain. Addition of myosin heavy chain kinase from *Dictyostelium* increases the rate of phosphorylation of the ABRM myosin. In contrast, myosin prepared from the striated adductor muscle of scallop (a myosin-regulated noncatch muscle) does not contain endogenous kinase and is not phosphorylated by the myosin heavy chain kinase from *Dictyostelium* (Fig. 5). This finding indicates that phosphorylation of the heavy chain may be characteristic only of myosins from those muscles that display catch. Digestion of the phosphorylated ABRM myosin with papain indicates that the site or sites phosphorylated by the endogenous kinase are located in the rod portion of the molecule (Fig. 6). These findings demonstrate that the rod portion of myosin may be a target for phosphorylation during the contractile cycle of catch muscles.

Previous theories of catch have focused on the role of paramyosin phosphorylation. According to one view, a direct interaction between thick filaments via phosphorylated

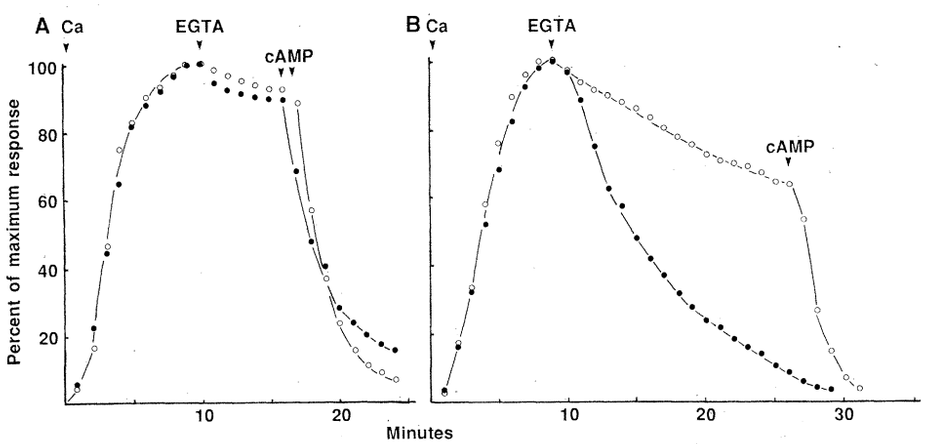

Fig. 2. Effect of the catalytic subunit of cAMP-dependent kinase on the ABRM contraction cycle. Tension is expressed as percentage of the maximum response in the presence of Ca^{2+}. Changes of the bathing medium are indicated by arrows. (**A**) Two fiber bundles (○ and ●) from the same animal, skinned at room temperature under the same conditions, were put through a control contraction cycle as described in Fig. 1. (**B**) Tension developed in response to calcium in the two bundles after overnight incubation at 4°C in relaxing medium (○) and with 0.25 μ*M* catalytic subunit (●). The presence of cAMP-dependent kinase inhibited catch completely, whereas cAMP was required to accelerate relaxation in the control. After storage, the catch state in the control was somewhat less pronounced than in the fresh preparation [see (A)].

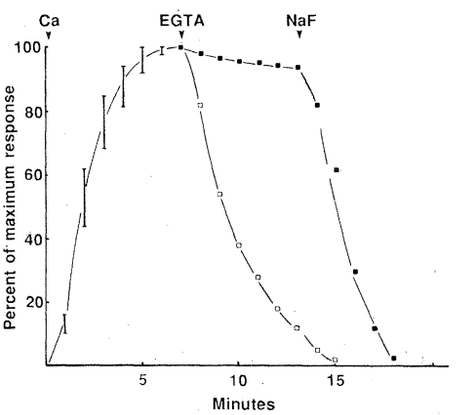

Fig. 3. Response of the ABRM to NaF. Tension is expressed as percentage of the maximum response in each succeeding cycle. Tension developed during activation is expressed as an average of subsequent cycles. Bars represent maximum distribution. During the first cycle of contraction, the catch state was released by the addition of 20 m*M* NaF (■). After the NaF was washed out, the bundle was again induced to contract by addition of Ca^{2+}. Upon removal of Ca^{2+} (marked EGTA) (□), the bundle relaxed rapidly without going into the catch state.

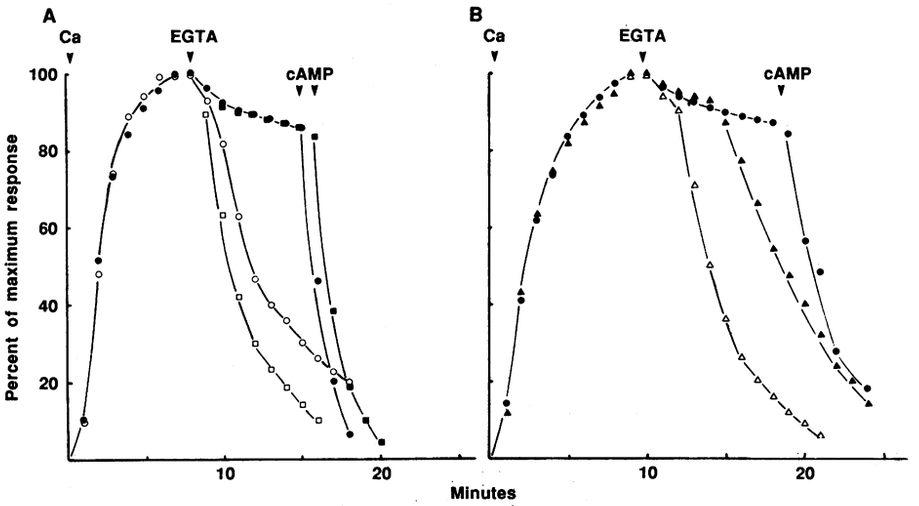

Fig. 4. ABRM response to ATP-γ-S. Tension is expressed as a percentage of the maximum response in each cycle. (**A**) The same fiber bundle underwent consecutive cycles of contraction. During the first cycle (●) the catch state was released by the addition of 5.5×10^{-5} *M* cAMP; in the following cycle (○) cAMP was present in the high EGTA buffer used to remove Ca^{2+}, and catch did not develop. In the third cycle (■), relaxation of catch was induced by a solution containing cAMP and 0.5 m*M* ATP-γ-S. In the cycle following the one with ATP-γ-S, however, upon removal of Ca^{2+}, the bundle relaxed rapidly, although cAMP was not present (□). The bundle appears to have lost the property of developing catch. (**B**) The ABRM fiber bundle was relaxed with cAMP during the first cycle as control (●). During the second cycle of contraction, the bundle was put into catch in a bathing medium with high EGTA where ATP has been replaced by 0.5 m*M* ATP-γ-S (▲). An apparent catch state was developed for 3 to 4 minutes and was followed by a rapid relaxation although cAMP was not present in the bathing medium. Removal of Ca^{2+} during the next contraction cycle (△), although performed in the presence of ATP, induced the bundle to relax without development of the catch state.

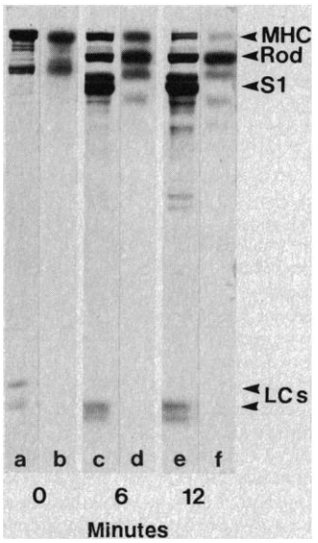

Fig. 6. Phosphorylated ABRM myosin digested with papain: 12.5% polyacrylamide SDS-PAGE under reducing conditions (a, c, and e) and corresponding autoradiograph (b, d, and f). ABRM myosin (3 mg/ml), labeled by the endogenous kinase as indicated in Fig. 5, was dialyzed against 40 m*M* NaCl, 1 m*M* EDTA, 1.5 m*M* $MgCl_2$, 1.5 m*M* $CaCl_2$, and 10 m*M* sodium phosphate buffer, *p*H 7.0. Digestion with papain at 15 μg/ml was performed at room temperature for 6 and 12 minutes. Myosin heavy chain, rod, S1, and light chains (LCs) are indicated. The autoradiograph shows that upon digestion, the labeled phosphate is bound to the rod fragment. Note that the extra band present in the myosin preparation is cleaved by papain and runs behind the S1 band.

paramyosin would increase muscle stiffness (*13, 23*). Achazi (*11, 12*) has suggested that phosphorylation of paramyosin could affect its interactions with myosin to decrease cross-bridge cycling. Cohen (*14*) proposed a special organization of thick filaments of catch muscle in which specific strong interactions between paramyosin and the rod portion of myosin would depend on the state of phosphorylation of these proteins.

In contrast to previous findings, our results with both NaF and ATP-γ-S suggest that dephosphorylation is associated with the catch state and that a phosphorylation step mediated by cAMP is related to the relaxation of catch. Our studies do not reveal the state of phosphorylation of paramyosin, but we have found that myosin from the ABRM is associated with an endogenous heavy chain kinase that phosphorylates the molecule. We do not yet know whether this kinase also phosphorylates paramyosin.

These results thus suggest new possibilities for the molecular basis of catch. Phosphorylation of the myosin heavy chain near the carboxyl terminal end of the rod has been described previously in nonmuscle systems such as *Acanthamoeba* (*24*), *Dictyostelium* (*25*), and calf brain (*26*). This phosphorylation appears both to diminish filament formation (myosin-myosin interactions) and to decrease actin-activated adenosinetriphosphatase rates. Our results reveal an analogous phosphorylation in the rod region of a muscle myosin. Correspondingly, we suggest that in catch muscles, changes in intermolecular interactions (myosin-myosin or myosin-paramyosin) which influence the rate of cross-bridge cycling could be controlled by phosphorylation of either or both of these contractile proteins. Preliminary studies in vivo indicate, in fact, that several proteins, including the myosin heavy chain and paramyosin, are phosphorylated during the contractile cycle. The phosphorylation of the myosin heavy chains appears to correlate with the relaxation effect of cAMP, whereas that of paramyosin seems to be independent of the state of muscle contraction. Determination of the precise target protein (or proteins) and the conformational effects produced by phosphorylation should reveal the molecular basis for control of catch.

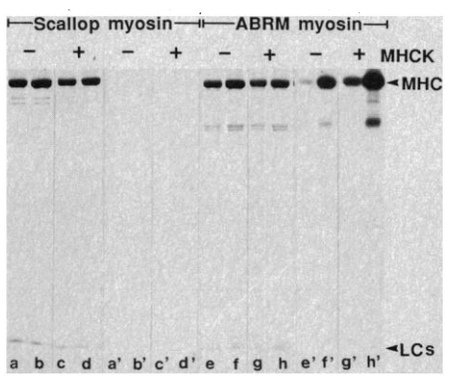

Fig. 5. Myosin heavy chain phosphorylation revealed by reducing gel electrophoresis [SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide] and corresponding autoradiograph. Myosins from scallop striated adductor (used as control) and from ABRM were incubated at room temperature for 15 and 60 minutes without (a and b) and (e and f), and with (c and d) and (g and h) myosin heavy chain kinase (MHCK) from *Dictyostelium* in 40 m*M* NaCl, 1 m*M* $MgCl_2$, 0.1 m*M* EGTA, 0.5 m*M* dithiothreitol, and 20 m*M* Tes at *p*H 7.5. The reaction was started by addition of a mixture of Mg-ATP and [γ-^{32}P]ATP to a final concentration of 0.5 m*M* and stopped by addition of boiling sample buffer for SDS-gel electrophoresis (2% SDS, 2% mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, and 50 m*M* tris-Cl, *p*H 6.8). The corresponding autoradiographs are labeled with primes. The data show that the myosin heavy chain (MHC) from ABRM was phosphorylated by both the endogenous kinase (e', f') and the *Dictyostelium* kinase (g', h'), whereas the scallop myosin heavy chain was not phosphorylated. ABRM myosin shows a contaminant (running slightly slower than paramyosin) that was labeled during the phosphorylation assay. Degradation of the myosin heavy chain may account for this band. LCs, light chains.

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Slow Transport of Tubulin in the Neurites of Differentiated PC12 Cells

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In order to study the rate and form of tubulin transport in cultured neuronal cells, the fluorescence recovery after the photobleaching of a fluorescent tubulin analog has been followed within the neuritic processes of differentiated PC12 cells. In these cells, as in peripheral axons, tubulin is transported in coherent, nondiffusing waves at two different slow rates that are within the range of the slow components a and b of axonal transport measured in vivo. Finally, it appears that most, if not all, of the tubulin analog is moving out these processes. Thus, slow neuroplasmic transport in cultured neuron-like cells is a good model of axonal transport, in which experimental manipulations of the system can be performed that would be difficult in the whole animal.

DURING AXONAL TRANSPORT, MACROMOLECULAR constituents traverse the length of the axon. Because the axon has no protein synthetic machinery (*1*), all axonal proteins must be synthesized in the cell body and moved out the axon. Materials are transported along the axon at various discrete rates that range from 400 mm per day to 0.2 mm per day, and each rate group has a definitive composition (*2*). The materials transported in the fastest anterograde rate groups, as well as those transported in the retrograde direction, are largely contained in vesicles; this fact has allowed these components to be visualized optically, both in the living axon (*3*) and in extruded axoplasm (*4, 5*). This capability has enabled the detailed analysis of the process and identification of the molecular components involved in fast axonal transport (*5, 6*). In contrast, soluble and filamentous structural proteins are transported in the slowest classes of axonal transport, but their transport cannot be easily visualized by optical methods alone. For these reasons, slow axoplasmic transport has been studied primarily by radioisotope localization techniques, in which a radiolabeled amino acid is taken up by the cell body and incorporated into proteins that are detected distally after transport out the axon. These methods have proved invaluable in defining the characteristics of slow neuroplasmic flow—coherent waves of material with defined composition and logarithmically decreasing boundaries that move out the axonal process (*7*) and provide materials for the growth and maintenance of the axon (*8*). However, these techniques are limited in that they measure an average behavior in a population of processes.

I have used fluorescence recovery after photobleach (FRAP) with fluorescein-labeled tubulin to follow slow neuroplasmic transport in cultured neuron-like cells. Tubulin was labeled with a fluorophore to form an analog that can compete effectively with native tubulin for assembly into polymer. This material will "stain" the microtubule by incorporation, either in vitro or after microinjection into living cells (*9*). It was microinjected into cells of the PC12 rat pheochromocytoma line that were rapidly extending neurites and resemble regenerating peripheral neurons in their properties (*10*). After 15 to 18 hours, when maximal incorporation into the microtubules should have been achieved, a region of the neurite was photobleached by exposure to an unattenuated mercury arc lamp filtered to 450 to 490 nm. The subsequent movement of this bleached band out the neurite was then followed under attenuated illumination. Using this methodology, I have established that in PC12 cells, as in axons in vivo, tubulin is transported slowly and coherently (nondiffusively) out the neurite. As is the case for peripheral neurons in vivo (*11*), tubulin transport in PC12 cells occurs in two rate groups. These two groups move at rates of 2.3 and 0.31 mm per day (at 35°C); these values are similar to those measured in vivo for the slow components a and b of axonal transport—the rate groups in which most cytoskeletal components are carried.

Microtubule proteins from bovine brain were labeled as the polymer with dichlorotriazinylamino fluorescein (DTAF) at a 50:1 molar excess and purified by cycling in 1*M* glutamate (*9*). The final pellet was depolymerized in 100 m*M* MES buffer (*p*H 6.8) and frozen in 10 μl aliquots for later use. The labeled tubulin could be incorporated into the interphase microtubules of PtK2 epithelial cells after microinjection and remained fibrillar for up to 18 hours.

In preparation for transport experiments, PC12 cells were grown in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal calf serum, on tissue culture dishes coated with rat tail collagen. They were treated for 5 days with 2.5*S* nerve growth factor (1000 ng/ml) (*12*). Under these conditions, the cells extend long neuritic processes, which will regenerate rapidly after being sheared off (*10*). For a given experiment, cells with processes were aspirated from the culture dish, thereby shearing off their neurites, and plated onto a polylysine-collagen–coated Bionique culture chamber. After 2 to 4 hours (sufficient time

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