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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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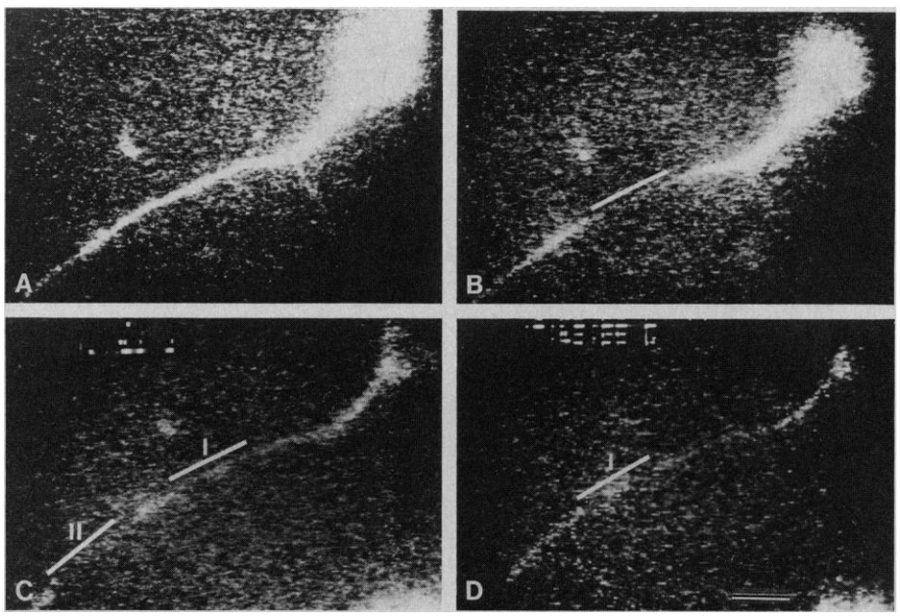

Fig. 1. A PC12 cell injected with DTAF-labeled tubulin (**A**) before, (**B**) immediately after, (**C**) 22 minutes after, and (**D**) 82 minutes after being locally photobleached by exposure to an unattenuated fluorescein excitation source. The original band in (B) (white bar) splits into two separate bands [I and II in (C)] that move out the process at different rates. In (B) the bleaching is incomplete and nonuniform across the aperture; this is even more apparent in (C) and (D), where the middle of the slower moving band is significantly brighter than the edges. Calibration bar, 10 μm.

for the cells to attach, but not to grow processes longer than two cell diameters), these cells were microinjected with DTAF-labeled tubulin at a micropipette concentration of 5 mg/ml (*13*). The cells were then returned to the incubator for 15 to 18 hours to regenerate their processes. In control experiments, cells were injected with bovine serum albumin (BSA) (5 mg/ml) labeled with fluorescein isothiocyanate (*13*) and treated identically to cells injected with DTAF-labeled tubulin.

A PC12 cell injected with DTAF-labeled tubulin with suitable processes was selected by visual observation of fluorescein fluorescence with a Zeiss IM microscope equipped for epifluorescence with a 100-W tungsten-halogen illuminator. After selection, the fluorescence of the cell was videotaped at approximately ×100 (faceplate magnification) with a DAGE ISIT 66 camera, a Panasonic NV8050 video cassette recorder, and tungsten-halogen illumination to minimize global photobleaching (Fig. 1A). Thereafter, the field aperture of the microscope was closed down, and a 100-W mercury arc lamp was switched into the excitation path. The neurite was locally photobleached for 5 minutes, with the mercury arc lamp filtered through the 450- to 490-nm fluorescein excitation filter. Electron microscopic studies of bleached regions of neurites revealed that such treatment did not detectably alter the ultrastructure of the neurite (Fig. 2). This lack of effect on cytoplasmic ultrastructure was also the case in Saxton and co-workers' studies of photobleached mitotic cells (*14*). Furthermore, time-lapse videomicrographic observations of neurites before and after photobleaching indicated that exposure to the beam did not disrupt vesicular transport in the neurite. After photobleaching, the tungsten illuminator was switched in and the aperture opened up. The cell was again videotaped immediately after bleaching (Fig. 1B) and at various times thereafter to follow the fate of the bleached region with time. (In each videotaping session, I recorded an out-of-focus image for use as a background image.) Observation of subsequent images revealed that the bleached region was not static with respect to the cell body, but moved slowly out the neuritic process with little or no spreading (Fig. 1, C and D). Analysis (see below) revealed that the bleached regions moved out the neuritic process at one of two distinct slow rates. In particularly favorable images (Fig. 1C), I could discern two different bleached spots, each of which is translated with respect to the original bleach site.

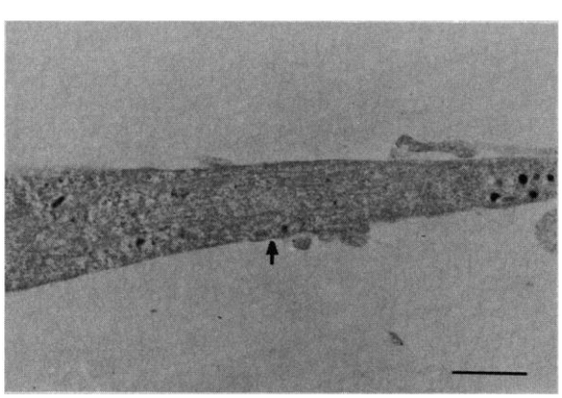

Fig. 2. Electron micrograph of a neurite of a PC12 cell injected with DTAF-labeled tubulin and fixed immediately after being photobleached for 5 minutes. The left boundary of the bleached region, as determined from the video record, is indicated by the arrow. The ultrastructure within the bleached region is indistinguishable from that outside it; unbleached neurites in the culture also have similar ultrastructure. Calibration bar, 1 μm.

The results of experiments on control cells, injected with fluorescein-labeled BSA, present a distinct contrast to those with DTAF-labeled tubulin–injected cells. In cells injected with BSA, it was not even possible to generate a locally bleached region of the neurite with the mercury arc lamp; I could only achieve a general reduction of fluorescence intensity in the neurite (Fig. 3). The contrast between the behavior of fluorescent tubulin and that of fluorescent BSA in these experiments suggests that tubulin is not freely diffusible in the cells as is the BSA, which has been shown to have a diffusion constant in excess of 10^{-8} cm^2/sec in a variety of cell types (*15*).

The analysis of videotaped fluorescence recovery data proceeded in a number of stages. First, because the data were taken with an intensified television camera at very low light levels, there was a significant amount of uncorrelated noise in each video frame; to minimize the effect of this variability, I averaged the data from 16 video frames with a Hughes Image Σ video processor. The variable input contrast and brightness of this processor were adjusted on the first image of each photobleach series, so that the image filled the processor's dynamic range. The controls were not readjusted for the subsequent images of the series. Next, the image was digitized with a PCVISION image digitizer-framestore, and stored on the hard disk of an IBM PC-AT. The corresponding background image was then averaged, digitized, multiplied by a factor of 0.75, and subtracted from the image of the neuron. (The factor of 0.75 was found to give adequate background subtraction for visual observation, without dropping pixels of the original image that had low values because of statistical fluctuations in the detection system.) The contrast of the resulting image was "stretched" to fill the entire range of intensities available to the output

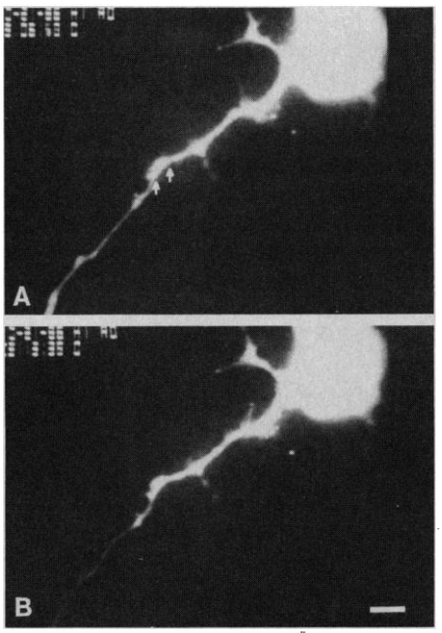

Fig. 3. A PC12 cell injected with fluorescein-labeled BSA that was photobleached in a protocol similar to that used for cells labeled with DTAF-labeled tubulin injections. In the image taken before bleaching (**A**) the boundaries of the bleach aperture are illustrated by arrows. In the image taken after bleaching (**B**) no local photobleaching is apparent, although a global reduction in fluorescence intensity has occurred. The reduction in intensity is more visible distal to the bleached region because the process is thinner there. The signal-to-noise ratio in this image is considerably higher than in the DTAF-labeled tubulin images because the BSA was more heavily labeled than the DTAF-labeled tubulin. Calibration bar, 10 μm.

display. Finally, the distance between the trunk of the neurite (equivalent to the axon hillock of a nerve) and the beginning and end of the bleached region was measured along the length of the process; these values were averaged to give separations from the trunk to the bleach spot for each time point. These separations were plotted as a function of the time since the end of photobleaching to give rates of movement of the bleached region (Fig. 4). In cases where two separate bleach spots were visible within the image, the distance of each from the neurite trunk was measured separately and treated as separate data points. The data were assigned by inspection to one of two rate groups, and a regression line was fit to each group separately. To establish that the assignment of the low-lying points to the wrong rate group would not have changed the interpretation of the data, the regression analysis was performed again with the two lowest data points assigned to the other rate group; this treatment changed the slopes of the regression lines by less that 25%.

Analysis of multiple photobleach experiments in regenerating PC12 cells revealed that DTAF-labeled tubulin moved out the neurite at one of two transport rates, corresponding to 0.31 ± 0.07 (SEM) and 2.3 ± 0.4 mm per day (Fig. 4). The former (slower) rate is comparable to the fastest rate at which the neurites of these cells will regenerate (*16*). From visual comparison, it appears that more material moves at the faster rate than at the slower. The data do not give any indication of the form of tubulin (polymer or dimer) carried in each class, but only show that it is not freely diffusing. It has been suggested that tubulin polymerizes at the growing tip of the neurite (*17*); my results would indicate that if this is the case, the microtubule proteins involved must have been transported in a complex, not as freely diffusing subunits. My data also indicate that the majority of DTAF-labeled tubulin in these cells is transported, and that there is little, if any, tubulin in a stationary pool in these cells.

In these experiments, I have demonstrated that in cultured neuron-like PC12 cells, tubulin is transported slowly and coherently out regenerating processes. This behavior is distinct from that observed for fluorescein-labeled microtubule associated proteins (MAPS) in NG108-15 cells (*18*) or for mitotic spindles (*14*), but is consistent with the behavior and rates of transport in neurons in vivo. Furthermore, I am able to detect tubulin moving in two different rate classes, a behavior that is similar to that of tubulin transport in peripheral neurons in the whole animal. Due to the resolution of the optical technique, I am able to measure transport within a time scale of hours and to fully resolve the a and b subclasses of the slow component. In addition to demonstrating that slow neuroplasmic transport occurs in these cells, I have developed a method that should be generally applicable to any regenerating neuron in tissue culture and possibly to any neuron in tissue culture. There are numerous possibilities for experimental manipulation with this technique.

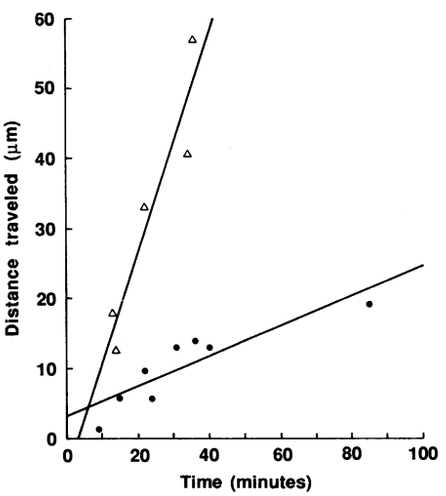

Fig. 4. Rates of movement from five different DTAF-labeled tubulin photobleach experiments. The data are assigned by inspection as falling into two classes, fitting rates of movement of 1.56 ± 0.3 (SEM of the regression coefficient) and 0.21 ± 0.05 μm/min. These rates are equal to 2.3 ± 0.4 and 0.31 ± 0.07 mm/day. The data have not been constrained to pass through the origin, and the y intercepts are −4.9 and 3.3 μm, respectively. The assignment of the proximal points does not critically affect the resulting slopes because reassigning the points at $x = 9$ minutes and $x = 15$ minutes to the fast rate group, both separately and together, does not change the slope by more than 24%.

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