Formation of actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in stressed neurons

Barbara W. Bernstein, Hui Chen, Judith A. Boyle, and James R. Bamburg

Department of Biochemistry and Molecular Biology and the Molecular, Cellular, and Integrative Neuroscience Program, Colorado State University, Fort Collins, Colorado

Submitted 9 February 2006; accepted in final form 21 May 2006

Bernstein, Barbara W., Hui Chen, Judith A. Boyle, and James R. Bamburg. Formation of actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in stressed neurons. Am J Physiol Cell Physiol 291: C828–C839, 2006. First published May 31, 2006; doi:10.1152/ajpcell.00066.2006.—When neurons in culture are transiently stressed by inhibition of ATP synthesis, they rapidly form within their neurites rodlike actin inclusions that disappear when the insult is removed. Oxidative stress, excitotoxic insults, and amyloid β-peptide oligomers also induce rods. Immunostaining of neurites indicates that these rods also contain the majority of the actin filament. Actin-ADF/cofilin rods transiently retards decline of mitochondrial potential and ATP in neurites with rods is slower than in neurites without them. The ΔΨm was monitored with the fluorescent ion indicator mag-fura 2. Actin in rods is less dynamic than filamentous actin in other cytoskeletal structures. Because ΔΨm depends on cellular ATP and because ATP hydrolysis associated with actin filament turnover is responsible for a large fraction of neuronal energy consumption (~50%), the formation of rods transiently protects neurites by slowing filament turnover and its associated ATP hydrolysis.

Rods, containing actin-depolymerizing factor (ADF)-cofilin proteins (AC) and actin, appear within minutes in the axons and dendrites of cultured neurons and other cells when they are exposed to a wide variety of treatments, including injection of cofilin (50), ATP depletion, glutamate-induced excitotoxicity (48), and oligomers of β-amyloid peptide1–42 (41). They vary from needle- to sausage-shaped inclusions. They may disappear when the insult is removed but return in a reduced number of cells within 24 h without affecting cell survival. Rod reappearance may contribute to synaptic dysfunction and/or loss (32, 48), characteristic of neurodegenerative disease because, distal to the rod site, mitochondria are inactive and the neurite degenerates. It is not difficult to understand why these rods are damaging when they reappear and persist for days. They commonly achieve a width great enough to supplant all microtubules and inhibit axonal transport (29, 41, 48). Moreover, the rods are shown here stably to sequester essentially all AC within the neurite and thus are likely to impair numerous neurite functions, ranging from vesicle transport (11, 59) to regulation of ionic homeostasis (14, 46). These functions depend on dynamically regulated actin assembly.

The AC proteins are critical modulators of actin filament turnover (15, 57a, reviewed in Ref. 9). They accelerate turnover by enhancing depolymerization from the minus end of filaments and by severing them, thus creating plus ends for elongation and minus ends for preferential release of monomer actin (G-actin) complexed with AC. Regulation of these activities is complex. Acidification, binding to phosphatidylinositol 4,5-bisphosphate, tropomyosin binding to actin, and phosphorylation of AC all inhibit their activity. Inactivation by phosphorylation of the serine 3 residue of AC proteins occurs through TES (testicular) or LIM kinases (1, 4, 49, 68, 69, 73). Slingshot phosphatas (21, 53) and the haloside dehalogenase phosphatase chronophin (22) reactivate AC proteins. Another level of regulation, involving the protein 14-3-3, coordinates phosphorylation/dephosphorylation with the presence of filamentous actin (23, 51, 63). The complexity of AC behavior extends to promoting assembly of AC-actin filaments, rather than disassembly, if the AC-to-actin ratio exceeds a critical level (16, 19, 31, 74). Finally, these proteins also inhibit monomer nucleotide exchange (27, 52), often a rate-limiting step for actin reassembly.

All cell treatments that generate rods have in common the effect of elevating the concentration of AC/G-actin complex. Microinjecting AC protein and overexpressing AC-green fluorescent protein (GFP) fusion proteins do this directly. ATP depletion for 20–30 min generates rods in >80% of cultured hippocampal neurons (48). In this case, ATP depletion elevates AC/G-actin complex by increasing the dephosphorylated (i.e., active) AC fraction and by increasing the pool of ADP bound to actin (actinADP) (6). AC proteins bind actinADP with higher affinity than actinATP (15, 16). In neurons, AC dephosphorylation is complete within ~20 min after blocking ATP synthesis (48); similar AC activation has been reported in kidney cells (5) and in pancreatic endothelial cells (67). Three other rod inducers, glutamate, peroxide, and oligomers of Aβ1–42, also likely activate AC by reducing ATP levels. In these cases, mitochondrial generation of ATP probably is impaired either by elevating mitochondrial Ca2+ to abnormal levels (42) or by generating more reactive oxygen species.

Persistence of rods probably contributes to the development of dementia and its hallmark, the amyloid plaque. Staining of

*Address for reprint requests and other correspondence: B. W. Bernstein, Dept. of Biochemistry and Molecular Biology, 1870 Campus Delivery, Colorado State Univ., Fort Collins, CO 80523-1870 (e-mail: bwb@lamar.colostate.edu).*

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
brain tissue from patients with Alzheimer disease shows that >97% of dense core amyloid plaques have actin and/or AC rodlike inclusions nearby, but only ~55% of the inclusions are near amyloid plaques (48). Furthermore, concentrations of amyloid β-peptide as low as 10 nM will induce rods in cultured hippocampal neurons (41).

Even with the above findings, we still do not know whether rods are only an abnormal actin-AC aggregate and an early stage in the degeneration of neurites, eventually ending in cell death. The possibility exists that, because actin turnover can account for up to 50% of the ATP utilization in neurons (10), the initial sequestering of AC proteins into rods may be a temporary defense mechanism for stressed cells. The experimental approach we have taken to address this possibility (i.e., overexpression of a GFP chimera of Xenopus laevis XAC(wt) for rod generation) uniquely allows simultaneous real-time visualization of rod-like inclusions nearby, but only 97% of dense core amyloid plaques have actin and/or AC (49). Brain tissue from patients with Alzheimer disease shows that 97% of dense core amyloid plaques have actin and/or AC rod like inclusions nearby, but only ~55% of the inclusions are near amyloid plaques (48). Furthermore, concentrations of amyloid β-peptide as low as 10 nM will induce rods in cultured hippocampal neurons (41).

MATERIALS AND METHODS

Unless otherwise indicated, all nonfluorescent reagents were purchased from Sigma (St. Louis, MO) and all fluorescent reagents were from Molecular Probes (Eugene, OR).

Cell culture and viral infection. E18 rat hippocampal cells were acutely dissociated and plated on poly-d-lysine-coated no. 1 German glass coverslips (22 mm<sup>2</sup>) fixed to the bottom of drilled out 35-mm plastic dishes with aquarium sealant (Dow Corning). Sealant was cured for ~48 h before cells were plated to minimize leeching of acid into the growth medium. Cells were grown in 2 ml of neurobasal medium in B27 supplement (Invitrogen) and 200 mM glutamine at a density of 1.5 × 10<sup>4</sup> cells/120 mm<sup>2</sup> for 4 days. These were then infected at a multiplicity of infection of 50 with adenovirus for visualization of rods emerging in a fraction of neurites and for rod generation) uniquely allows simultaneous real-time monitoring of mitochondrial potential and ATP level in all neurites. The resulting data support rods being more than merely a stress-induced protein aggregate: the short-term effects of rod generation are beneficial.

Electron microscopy. Hippocampal neurons were cultured for 3 days on polylysine-coated, formvar-covered gold grids. Some cultures were infected at a multiplicity of infection of 250 with adenovirus for expressing XAC(wt)-GFP, and both infected and uninfected cells were ATP depleted (10 nM NaN<sub>3</sub>/6 mM 2-deoxyglucose, 20 min) 3 days postinfection. The ATP depletion medium was washed out, and the cells were left overnight to recover. The cells were then fixed in 2% glutaraldehyde in PBS for 45 min at room temperature, washed quickly two times with PBS, and placed in an 8% sucrose-PBS solution. These cells were plunge frozen in liquid ethane, transferred to a vial containing 1% osmium tetroxide and 0.1% uranyl acetate in acetone, which was cooled to ~196°C, and then warmed to ~90°C in a freeze-substitution chamber. The cells were held at this temperature for 3 days, allowed to warm to room temperature over a 24-h period, rinsed three to five times in 100% acetone, infiltrated with Epoxaraldite resin over 8–24 h, flat embedded, and polymerized at 60°C.

Serial sections (150–200 nm) were cut on a Reichert ultramicrotome, collected on formvar-coated slot grids, and viewed on a JEOL 2000 electron microscope.

Monitoring mitochondrial membrane potential. Cells, virally infected 2 days earlier, were examined for the initial appearance of rods before they were incubated for 15 min in 10 nM tetramethylrhodamine methyl ester (TMRM), a fluorescent dye whose uptake into mitochondria is a reversible function of mitochondrial membrane potential (ΔΨ<sub>m</sub>) (39, 56, 60). The TMRM-loaded culture was immediately transferred, without washing, to a 37°C, computer-controlled stage of an inverted Nikon Diaphot microscope fitted with a PlanApo objective, fluorescein filter cube, Texas red filter cube, a 0.5 neutral density filter, and computer-controlled phase and fluorescence shutters. A maximum of 30 min was spent recording positions of cells expressing XAC(wt)-GFP and capturing with a cooled charge-coupled device camera (PXL with 1400 Kodak chip; Roper Scientific, Tucson, AZ) three types of images: a phase image, a fluorescence image of the XAC(wt)-GFP, and a TMRM image. At ~30-min intervals, this sequence of image acquisition was repeated two more times (see Data analysis section).

Monitoring ATP and Ca<sup>2+</sup> levels. Because ATP has ~10-fold greater affinity for Mg<sup>2+</sup> than does ADP or AMP (25, 38), the measurable increase in free intracellular Mg<sup>2+</sup> concentration ([Mg<sup>2+</sup>]) is a reliable indicator of ATP decline (38). We have demonstrated in embryonic rat cortical neurons that the time course of ATP decline, following blockage of ATP synthesis, is identical when monitored either with a luciferase assay that measures ATP in cell extracts (48) or with mag-fura 2 in live cells (10).

To determine whether intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]) exceeds 1 μM and thus contributes to the mag-fura 2 observations, the experiments were repeated with [Ca<sup>2+</sup>]<sup>2-</sup> monitored through fura 2 and a fura 2 calcium imaging calibration kit. Cytoplasmic free Ca<sup>2+</sup> does not contribute significantly to mag-fura 2 intensity unless the concentration exceeds 1 μM (24). The Ca<sup>2+</sup>-magnifura 2 complex generates only ~2% of the mag-fura 2 signal when ion concentrations are close to physiological (100 nM [Ca<sup>2+</sup>]<sup>2-</sup> and 0.5 mM [Mg<sup>2+</sup>]<sup>2-</sup>). This estimate is based on the affinity of Ca<sup>2+</sup> for mag-fura 2 being ~1.000-fold greater than that of Mg<sup>2+</sup> and on the [Mg<sup>2+</sup>]<sup>2-</sup> being ~20,000-fold greater than that of [Ca<sup>2+</sup>]<sup>2-</sup>. If [Ca<sup>2+</sup>]<sup>2-</sup> reaches 1 μM and [Mg<sup>2+</sup>]<sup>2-</sup> reaches 1.5 mM, as they may under pathological or stress conditions, then Ca<sup>2+</sup> is estimated to generate ~8% of the mag-fura 2 signal.

Cells, cultured and virally infected as described above, were incubated for 25 min in either membrane-permeable mag-fura 2-AM (10 μM) or fura 2-AM (10 μM), the acetoxyethyl ester form of the ratiometric fluorescent dyes used to monitor [Mg<sup>2+</sup>]<sup>2-</sup> and [Ca<sup>2+</sup>]<sup>2-</sup>, respectively (26). The excess dye was removed and cells were washed three times with PBS before replacement of growth medium and the immediate transfer of the culture dish to a 37°C, computer-controlled stage of a microscope equipped as described above. A maximum of 30 min was spent recording positions of cells expressing XAC(wt)-GFP. For both mag-fura 2 and fura 2 experiments, four types of images were acquired: a phase image, a fluorescence image of the XAC(wt)-GFP, and two emission images. A fluorescein filter cube was used for XAC(wt)-GFP images. A computer-controlled excitation filter wheel (Ludl Electronics Products, Exton, PA) with 340 ± 7.5 nm and 380 ± 7.5 nm filters, a 400 nm dichroic mirror, and a 460 ± 25 nm bandpass emission filter were used for dye images. At ~30-min intervals, this sequence of image acquisition was repeated four times (see Data analysis section).

For the calibration of total ATP depletion and relative sensitivity of hippocampal neurons to inhibition of glycolysis and oxidative phosphorylation, the mag-fura 2 experiments described above were repeated but without virally infecting the cells. Time-lapse images were taken before and after the addition of inhibitors or their buffers.

Actin-AC rod stability. For all rod stability studies, hippocampal cells were cultured, virally infected, and transferred to the microscope...
stage as described above. For the latrunculin A studies, phase and XAC(wt)-GFP images of nonneuronal cells (probably glial cells) were taken before and ~30 min after addition of the reagent (final concentration 10 μM) (see Data analysis section).

For rod-stability studies using fluorescence recovery after photobleaching (FRAP) of XAC(wt)-GFP and actin-mRFP, a Zeiss LSM 5 Live confocal microscope with 488 nm and 532 nm diode lasers, LP 505 and LP 550 emission filters, and Alpha Plan ×100/1.45 oil objective was used. A 1-μm-wide stripe was photobleached with 80% laser power for 1 s in neurites with and without rods. Because the Zeiss LSM 5 Live is an x-direction slit-scanning system, it allows only rectangular areas orientated perpendicular to the microscope stage x-axis to be photobleached. Cells were selected that permitted a zone perpendicular to the long axis of areas of interest to be bleached. Fluorescence recovery images (exposure 100 ms, 15% laser intensity) were analyzed after background subtraction by measuring average pixel intensity of bleached area and correcting for unintended photobleaching by measuring an area of similar size immediately adjacent to the bleached area (MetaMorph version 6.1 software, Universal Imaging Corp., Downingtown, PA; and Microsoft Excel 2003).

Cofilin translocation to mitochondria. To detect cofilin translocation to mitochondria, cells, overexpressing XAC(wt)-GFP, were loaded with 1 μM MitoTracker red CM-H2XRos for 20–30 min, washed three times with PBS, and returned to growth medium. This dye is concentrated in active mitochondria, but, unlike TMRM, its binding is irreversible. Time-lapse fluorescence images of GFP were acquired every 45 min for 3 h as described above; accompanying MitoTracker red images were acquired with a Texas red filter cube.

Nondenaturing PAGE. The amount of actin displaced by latrunculin A from the ADF-MgATP-actin 1:1 complex was measured by nondenaturing gel electrophoresis. To form the starting complex, 5 μM MgATP-actin and 10 μM ADF were incubated in 2 mM Tris, pH 8.4, 0.5 mM DTT, 0.2 mM ATP, 0.21 mM MgCl₂, and 0.2 mM EGTA for 10 min on ice. A 2-to-1 molar ratio of ADF to actin was used to drive most of the actin into complex. Increasing concentrations of latrunculin A were incubated with the complex for 20 min to drive most of the actin into complex. Increasing concentrations of latrunculin A-actin mixtures that were then incubated for 1 h at room temperature with ADF. The proteins were then separated on a 7.5% nondenaturing polyacrylamide gel at 4°C and band densities were analyzed using 1D Phoretix gel analysis software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Data analysis. For the analyses of all images, measurements were taken with MetaMorph version 6.1 software (Universal Imaging) and transferred to either Microsoft Excel 2002 or MINITAB version 14 for statistical analysis. To monitor ∆Ψₘ, the background was subtracted before measurement of average TMRM intensity of each mitochondrial region with a line scan, a two-pixel-wide line drawn along the long axis of the region. Time-lapse imaging strongly suggested that individual mitochondria were identified, but we cannot be certain that some were not clusters. Infrequently, a large TMRM region splits into two pieces between time points. The line scan for each mitochondrial region at time point 1 was copied to images of the same cells at subsequent time points and repositioned as needed to compensate for movement.

To monitor intracellular ATP and Ca²⁺, background was subtracted before ratioing the two mag-fura 2 or fura 2 images and measuring the average ratioed pixel intensity along each neurite with a 2-pixel-wide line drawn from the cell body distally. Intensities of the line scan along the neurite were used to generate average curves of [Mg²⁺] increase (i.e., ATP loss) as a function of time and distance from the somata. Beyond 50 μm from the somata, neurites with old rods were too thin to measure. Analysis of covariance was performed with MINITAB version 14.1, using a general linear regression model (Minitab, Summerville, SC).

Lamellipodial and rod areas were measured by subtracting background and thresholding the structures of interest. MetaMorph software (Universal Imaging) was used to transfer measurements of the area of these structures to Microsoft Excel for statistical analysis.

Correlation coefficients, calculated with Microsoft Excel, were used to determine the colocalization of XAC(wt)-GFP and mitochondria stained with Mito Tracker red in neurites. The intensities of the two fluorophors in single-pixel line scans tracing the neurites were drawn in MetaMorph and exported to Excel for analysis. The correlation coefficient is shown in Eq. 1

\[ \rho_{xy} = \text{cov}(X,Y)/(\sigma_X)(\sigma_Y) \]  
(1)

and the covariance, \(\text{cov}(X,Y)\), is shown in Eq. 2

\[ \text{cov}(X,Y) = 1/n \sum_{i=1}^{n} (x_i - \mu_X)(y_i - \mu_Y) - 1 \leq \rho_{xy} \leq 1 \]  
(2)

RESULTS

Actin-AC rod formation transiently protects neurons from the somata. Beyond 50 μM M, the area of lamellipodia on the cell surface and the area of emerging rods increase (i.e., ATP loss) as a function of time and distance. Time-lapse images of GFP were taken before and after addition of latrunculin A, which displaced actin from the ADF-actin complex and formed the AC-actin complex through overexpression of XAC(wt)-GFP. Only fluorescently tagged AC allows the identification of emergent rods in live cell neurites. This identification is required for comparing an individual cell the Ψₘ and ATP level in neurites with and without emerging rods. Rods first appeared in hippocampal neurons ~60 h after adenoviral infection with constructs for XAC(wt)-GFP; adenoviral-mediated expression of GFP alone produced only diffuse fluorescence. Figure 2a shows a typical hippocampal cell at time point 1 with a few small rods generated through overexpression of an AC protein that elevates AC-actin complex levels. By time point 2 (~30 min later; Fig. 1b), many more rods have appeared.

Standard chemical fixation and electron microscopic visualization of neuronal rods, formed with endogenous proteins, showed that they are composed of wavy filaments (48). The filaments are straighter and more densely packed when prepared through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43). The ultrastructure of freeze-substituted rods, containing overexpressed XAC(wt)-GFP, is identical to rods produced through rapid freezing and freeze substitution, which seems to better maintain the filament alignment (Fig. 1c). This is not surprising because F-actin is sensitive to fragmentation by osmium (43).
and were used to determine the decline in $\Delta \Psi_m$. The same procedure was followed for mitochondria in neurites with no rods from the same cell.

Neurites fall into one of three categories: 1) those without rods at any time, 2) those with no rods at time point 1 but with $\geq 1$ rod at time point 2 (i.e., neurites with newly formed or “young” rods), and 3) those with $\geq 1$ rod at both time points 1 and 2 (i.e., neurites with more mature or “old” rods). Figure 3A shows that $\Delta \Psi_m$ is retained best in neurites that form rods between time points 1 and 2 (young rods). By time point 2, neurites with old rods show less retention of $\Delta \Psi_m$ than neurites with young rods. However, even neurites with old rods retain $\Delta \Psi_m$ more effectively than neurites with no rods. The greater decline in potential of mitochondria in neurites with no rods than neurites with young rods is not because of a difference in the $\Delta \Psi_m$ at time point 1; there is no difference (see Fig. 3B). However, between time points 2 and 3, the $\Delta \Psi_m$ drops faster in neurites with old rods than in neurites with no rods (see Fig. 3C). Hence, rod formation initially slows mitochondrial decline but with time accelerates it. Photobleaching is not responsible for the decline in TMRM intensity because we observed that the same number of exposures at 10-s intervals caused $<10\%$ intensity decrease. Neurites with old rods show a higher $\Delta \Psi_m$ at time point 1 than do neurites with young rods (see Fig. 3B). This finding is consistent with rods initially slowing the $\Delta \Psi_m$ drop because experiments were initiated soon after the first appearance of rods.

**Actin-AC rod formation transiently slows the rate of ATP loss.** Because it is reasonable to expect that the retention of $\Delta \Psi_m$ would accompany preservation of ATP levels, we monitored ATP levels in neurites with rods over a period of $\sim$90 min. Overexpression of XAC(wt)-GFP leads to rod formation $\sim$60 h after viral infection. The cells were then loaded with mag-fura 2. Figure 4A shows the GFP fluorescence image of neurons with young rods merged with a phase image. Time-lapse phase, GFP fluorescence, and mag-fura 2 dual excitation (340 and 380 nm) images were taken at $\sim$30-min intervals. The increase in $[\text{Mg}^{2+}]_i$, i.e., ATP decline, was derived from the ratioed mag-fura 2 images generated for each time point (Fig. 4b). Values of ratioed fluorescence intensity in a 2-pixel-wide line, drawn along the neurite from cell body distally (Fig. 4b), were transferred to an electronic spreadsheet.

It was earlier reported that rod length increased as expression of exogenous AC increased (48). Here, neurites were categorized according to the presence of rods and the apparent maturity of the rods, which was estimated from their size based...
neurites with old rods are actually high near the somata, suggesting that rod impedance of axonal transport may preserve ATP levels proximal to their location and deplete it in regions distal to them. No ATP decline in any neurite class is seen at 30- and 60-min time points. Analysis of covariance with a general linear regression model yielded the estimate of mean, SE, and significance of difference shown in Fig. 5B. Data from Fig. 5A are replotted in Fig. 5C to show the ATP percentage remaining ~90 min after the initiation of observation, assuming that a fourfold increase in [Mg<sup>2+</sup>]<sub>i</sub>, (the maximum change observed at 50 min) corresponds to total ATP depletion (Fig. 5D). Previously, our group (10) demonstrated that this mag-fura 2 assay accurately indicates the time course of total ATP depletion by combined NaN<sub>3</sub>-2-deoxyglucose inhibition of ATP synthesis. The inhibition of only glycolysis (2-deoxyglucose treatment) causes complete reduction of ATP (Fig. 5D) because the addition of NaN<sub>3</sub> to glycolysis-inhibited cells does not further reduce ATP. Moreover, glycolysis inhibition reduces ATP faster than does inhibition of oxidative phosphorylation (Fig. 5D). Thus embryonic rat hippocampal neurons, like chick sympathetic neurons (71), energetically rely primarily on glycolysis. The glycolytic generation of ATP may explain why neurite ATP levels do not follow more closely the decline in ΔΨ<sub>m</sub>.

Because Ca<sup>2+</sup> homeostasis is highly ATP dependent, it is conceivable that a dramatic decline in ATP, such as that which occurs in the more distal regions of neurites containing old rods, might raise [Ca<sup>2+</sup>]<sub>i</sub>, above 1 μM. If that were true, a significant fraction of the mag-fura 2 signal reported would reflect Ca<sup>2+</sup> increase rather than Mg<sup>2+</sup> increase and thus would not accurately reflect changes in ATP levels. To test for this possibility, we repeated the above described experiments but loaded cells with the Ca<sup>2+</sup> indicator fura 2 instead of mag-fura 2. The fura 2 data indicate that [Ca<sup>2+</sup>]<sub>i</sub>, in the distal regions of neurites with old rods does not exceed 600 nM, contributing <4% to the ATP drop calculated from the mag-fura 2 monitoring (Fig. 5, A and C).

Surprisingly, in neurites with old rods, [Ca<sup>2+</sup>]<sub>i</sub>, nearest the somata is high enough to generate ~8% of the mag-fura 2 signal in this region (Fig. 5E; see MATERIALS AND METHODS). Although mag-fura 2 intensity is much lower here than in distal regions, the high [Ca<sup>2+</sup>]<sub>i</sub> suggests that the true Mg<sup>2+</sup>-generated mag-fura 2 signal is even lower. The high [Ca<sup>2+</sup>]<sub>i</sub>, native to the cell body may reflect the high concentration of somal mitochondria. A fraction of them could be capable of retaining ATP levels but not capable of sequestering all the Ca<sup>2+</sup> released by failing ones.

**Actin-AC rods are stable structures.** Hydrolysis of ATP associated with actin filament turnover consumes a significant portion of the total ATP used by unstimulated neurons (10). We probed the idea that immediately after rod formation these inclusions may delay ATP loss by sequestering actin and the actin-dynamizing proteins ADF and cofilin (AC). To test this idea, we determined the stability of rods compared with other actin-based structures. Rods were generated as before by overexpression of XAC(Δt)-GFP, and cells were treated with either latrunculin A or subjected to FRAP analysis (30). Latrunculin
A is a membrane-permeable natural product that does not actively depolymerize filaments but rather passively sequesters actin monomers released by filaments undergoing turnover (18). It prevents monomer reassembly into F-actin (64). Analysis of rod area in images of nonneuronal cells, before and after latrunculin A addition (Fig. 6A), indicates that rods are more stable than the actin filaments within lamellipodia (Fig. 6B). With latrunculin A, rod area does not decline as does lamellipodial area (Fig. 6A and B), and rod intensity does not decline as does the intensity of rod-free neurites (Fig. 6C).

Our data from nondenaturing gels (Fig. 7) show that latrunculin A weakly competes with ADF in the ADF-actin complex. There is no difference in mobility of free actin and actin incubated with high concentrations of latrunculin A (presumed complex) under these nondenaturing gel electrophoresis conditions (data not shown). Hence, there is probably no difference between the mobilities of ADF-actin complex and ADF-actin-latrunculin A complex. Adding increasing amounts of latrunculin A to actin complexed with ADF results in the appearance of a band at the position of actin or latrunculin A-actin complex, demonstrating that latrunculin A competes with ADF for binding to G actin. With a $K_d = 19$ nM for actin-ADF and a $K_d = 200$ nM for actin-latrunculin A, lines were fit to the equations for the binding curves in Fig. 7B. Despite this 10-fold weaker affinity for actin, latrunculin A still promotes the collapse of F-actin-supported structures [such as lamellipodia (Fig. 6A and B)], even in these cells with overexpressed XAC(wt)-GFP.

We also examined rod stability by FRAP in neurons expressing either XAC(wt)-GFP or actin-mRFP. These data show that the bleached XAC(wt)-GFP rod zone remains unchanged for the period of observation (6 min; Fig. 8). During this period, neither fluorescence recovery nor any shift in position occurs during treadmilling, the process in which actin subunits are lost from the slow-growing end of rapidly turning-over filaments and added to the fast-growing end.

Actin-mRFP shows a faster recovery rate than does XAC(wt)-GFP when rods are photobleached (Fig. 8B). This is probably because, as indicated by immunofluorescence studies (48), rods sequester a larger fraction of the total neurite AC...
than total neurite actin. Actin, laterally surrounding rods, can contribute to the actin-mRFP recovery, but there is very little AC outside the rod to contribute to XAC(wt)-GFP recovery. The reduced recovery rate of actin in neurites with rods compared with neurites without rods reflects the extent to which actin is sequestered in the rod.

**AC translocation to mitochondria.** Translocation of dephosphorylated cofilin, capable of binding actin, was demonstrated to be both necessary and sufficient for triggering cytochrome c release and initiating apoptosis in staurosporine-treated neuroblastoma cells (17). Therefore, another potential mechanism for the observed protection of H9023 is the reduction of AC translocation to mitochondria in neurites with rods. To determine whether rods affect AC translocation to mitochondria, cells with rods, generated as above, were loaded with MitoTracker red, another fluorescent dye that is taken up only by active mitochondria. However, unlike TMRM, MitoTracker red is not released as the membrane potential drops. GFP and MitoTracker red images were taken at ~30-min intervals (Fig. 9). Discrete regions of concentrated XAC(wt)-GFP and rods were circled, and these markings were transferred from the GFP to MitoTracker red fluorescence images. When GFP fluorescence completely overlapped mitochondrial fluorescence, XAC(wt)-GFP was judged to be colocalized with a mitochondrion. Only 1% of the mitochondria in neurites with rods had colocalized regions of concentrated XAC(wt)-GFP. In contrast, 12% of the mitochondria in neurites without rods had colocalized XAC(wt)-GFP; the two experiments performed yielded the same percentages. The mere sequestering of AC in rods may be enough to reduce its translocation to mitochondria and help preserve H9004/H9023. To determine the extent of increased colocalization of XAC(wt)-GFP and MitoTracker red with time in nonrod neurites, we calculated the correlation coefficients at each time point. These coefficients support the idea that the two species are colocalized rather than the possibility that XAC(wt)-GFP increases uniformly throughout the neurite and randomly overlaps mitochondria. A random overlapping would also produce yellow mitochondria in merged images, but the correlation coefficient would remain close to zero with time due to the $\langle x_i - \mu_x \rangle$ term in Eq. 2 (see Data analysis...
above) rather than increase as observed. The \((y_i - \mu_y)\) term, representing irreversible MitoTracker red staining, remains relatively constant over time. Neurites without rods, judged to show XAC translocation to mitochondria, consistently showed an increase in correlation coefficient with time, whereas neurites with rods showed a decrease (Fig. 9). One would expect this correlation coefficient decrease if XAC is increasingly concentrated in rods over time.

**DISCUSSION**

Here, we have demonstrated that the formation of actin-AC rods in neurites initially slows \(\Delta \Psi_m\) rundown; however, after \(-60\) min, neurites with rods lose \(\Delta \Psi_m\) more quickly. In this study, rods were initially generated through overexpression of an AC chimera protein, XAC(wt)-GFP. Subsequent light microscopy probably accelerated rod generation because it produces reactive oxygen species, particularly in the presence of intrinsically fluorescent protein (20, 37, 42). Reactive oxygen species damage membranes, probably contributing to the mitochondrial potential decline observed and likely converting mitochondria from ATP producers, under normal conditions, into powerful ATP consumers in stressed cells. F1F0-ATPase is activated to prevent the collapse of the proton gradient (65). The resulting ATP drop exacerbates rod formation, generated initially through AC overexpression, but secondarily through dephosphorylation of AC. AC dephosphorylation contributes to abnormally high levels of AC-actin complex (48). However, those neurites with newly formed rods transiently show a reduced decline in ATP and \(\Delta \Psi_m\). Possible mechanisms for this short-term protective effect are discussed below.

Immunostaining shows that only a fraction of the total actin in neurites is confined to rods, but the majority of the AC protein appears so sequestered (48). This protein sequestering first appears energetically beneficial. However, prolonged sequestering probably accelerates the decline because many cel-
lular processes, including organelle transport and ionic homeostases, depend on actin dynamics (11, 36, 59). For example, the following ion regulatory proteins depend on actin dynamics for positioning or modulation of their activities: Na⁺/K⁺-ATPase, NHE1, Na⁺/Ca²⁺ exchanger, nonvoltage-dependent Na⁺ channels (14, 46), voltage-dependent Ca²⁺ channels (33), and NMDA receptors (38). Clearly, extended interruption in their function would produce conditions damaging to mitochondria, for example, elevated [Ca²⁺]. Although reduction in actin dynamics initially slows ATP decline by limiting actin-associated ATP hydrolysis (10), prolonged reduction in actin dynamics would perturb Na⁺/K⁺-ATPase activity, leading to intracellular accumulation of Na⁺ and reversal of the normal Ca²⁺ extrusion by the Na⁺/Ca²⁺ exchanger (75). Thus it is not surprising that, compared with neurites with no rods, neurites with young rods lose ATP more slowly and neurites with old rods lose ATP more rapidly.

The FRAP experiments herein support the possibility that rods can briefly preserve ATP levels by reducing the rate of ATP hydrolysis associated with filament turnover: actin-mRFP recovery in a neurite region containing a rod is one-third that of a neurite without rods. The stability of the XAC(wt)-GFP bleached zone (Fig. 8) in a rod region supports the idea that AC proteins, key drivers of filament turnover, are stably sequestered in rods. AC recovery being slower than actin recovery is consistent with AC being more effectively sequestered in rods (48). The AC sequestration by rods eliminates a major player in actin dynamics, blocking its normal ability to recycle continuously to filaments and promote turnover.

Latrunculin A efficiently disperses F-actin-supported structures in lamellipodia of these cells (Fig. 6, A and B) despite the fact that it competes only weakly with an AC protein for actin (Fig. 7) and these cells have an excess of exogenous AC protein. The observed lamellipodial collapse could be predicted from the well-documented ability of latrunculin A to sequester released actin subunits in other cells and prevent reassembly of those subunits. However, to the best of our knowledge, the XAC(wt)-GFP increase into rods is unique. Because rods do not recover XAC(wt)-GFP in the absence of latrunculin A (FRAP data), latrunculin A seems responsible for the addition. We speculate that a pool of latrunculin A-actin-AC complexes is built up from rapidly turning over F-actin. Because it cannot add to normal F-actin structures (hence the lamellipodial dispersion), it is free to add to rods. Thus less stable actin structures are supplying actin-AC-latrunculin A complex to the highly stable rods.

A somewhat similar result was reported with latrunculin B addition to mast cells. It dramatically increased the amount of actin translocated to the nucleus. This translocation is cofilin-dependent, and, as with latrunculin A, resulted in the accumulation of concentrated actin regions (55). However, it is also possible that latrunculin A enhances rod fluorescence through an induced conformational change of the actin-bound XAC(wt)-GFP or an alteration in the GFP environment. The fact that, over 60 min of observation, rods do not move (Fig. 4, f–h) is consistent with their being stable structures because often, if actin disassembly is followed by reassembly, the reassembled structure appears in a somewhat different location. The process of disassembly-reassembly is a major component of cell locomotion (66).

Another possible mechanism for the protective effects of rod formation reported here may derive from the inhibition of cofilin translocation to mitochondria observed in neurites with rods. The preservation of Ψm and ATP is only short-term after rod formation in cultured cells with overexpressed fusion protein because the rods in this experimental model are not reversible. However, it is conceivable that, if their presence were transient in whole animals, as they can be in culture (48), they could have long-term beneficial effects. Microischemia, generating only transient rods in whole animals, could, by sequestering AC, reduce the chances of triggering cytochrome c release and apoptosis.

Several other factors must be considered as potentially contributing to the observed transient reduction in mitochondrial decline associated with rod formation. They include the direct effects of actin, gelsolin, and amyloid precursor protein

---

Fig. 8. Fluorescence recovery after photobleaching (FRAP) analysis of XAC(wt)-GFP and monomeric red fluorescent protein chimera of actin (actin-mRFP) in neurites with and without rods. A: there is no recovery of XAC(wt)-GFP fluorescence in the bleached area of the rod. Arrow in image of whole cell shows bleached area in rod; arrows on the right indicate enlargement of that area immediately after and 6 min after bleaching. Scale bar = 10 μm. B: lack of recovery of XAC(wt)-GFP fluorescence in neurites with rods and a faster recovery of actin-mRFP fluorescence in the neurrite without rod than in the neurite with rod. Third-order polynomial equation was fitted to full time span of data; linear equation was fitted to first 80 s of data.
on mitochondria. Translocation of the amyloid precursor protein to mitochondria is deleterious and depends on fast axonal transport of vesicles containing it (2). Rods probably inhibit this transport and may initially slow the damaging delivery to mitochondria. However, eventually, the impedance of axonal transport by rods spanning the neurite is likely to result in a build up of vesicles carrying amyloid precursor protein and its proteases, β-secretase and presenilin (57, 61). Thus, with time, rod blockage of axonal transport may promote the accumulation of amyloid precursor protein and its proteolytic fragment Aβ-amyloid_{1–42} peptide (29, 40, 41).

In addition, the rod sequestering of AC may preserve ATP levels and ΔΨ_m by increasing the amount of gelsolin free to bind to mitochondria. Gelsolin is a well-characterized actin filament severing and plus-end capping protein with known AC interdependencies (70). Its binding to mitochondria inhibits the voltage-dependent anion channel of the mitochondrial permeability transition pore and protects against apoptosis (35). Some part of the transient beneficial effect of overexpressed AC rod formation may be through enhancement of gelsolin binding to mitochondria.

Transient neuronal stress, such as occurs with microischemia, is a common medical event with serious consequences, including increasing the likelihood of long-term neurodegeneration seen in cerebrovascular dementia and Alzheimer disease (34, 72). In this study, we have tried to determine whether rods, when occurring only transiently, might play a beneficial role in medical conditions like microischemia. Here, we have found that rod appearance has an initial beneficial energetic effect on neurites compared with neurites of the same cell in

---

**Fig. 9.** Presence of rods inhibits translocation of XAC(wt)-GFP to mitochondria. Neurites without rods in a are an example of the increased colocalization with time of XAC(wt)-GFP and mitochondria; b exemplifies the typical absence of this phenomenon in neurites with rods. Arrowheads indicate mitochondria with increased XAC(wt)-GFP presence in a and mitochondria without XAC(wt)-GFP in b. Arrows indicate neurites whose line scans at 2 time points are plotted below each pair of images. Correlation coefficients (cc) of the distribution of these fluorophors have been calculated from single-pixel-wide line scans tracing these neurites. MT red, MitoTracker red. Scale bar = 5 μm.
which rods do not appear. Is this beneficial effect on neurites sustained? Because rod generation through AC overexpression is not reversible and because overexpression is presently the only way to follow rod formation in live cells while monitoring mitochondrial potential and ATP level, it is unfortunately not possible to test for the long-term benefit. However, brief ischemia in whole animals does protect against the damage of longer ischemia-reperfusion, a phenomenon termed “ischemic preconditioning” (7, 54). Short-term rod appearance may be a contributing factor in this preconditioning. It would be interesting to know whether such preconditioning generates transient rods. The findings of this study do suggest that the brief appearance of actin-AC rods may have a pivotal protective long-term role because the extent of cell death in a neuronal population depends on the degree of ΔΨm decline experienced (3). Beyond a critical point, mitochondrial recovery is no longer possible. Transient rod formation may reduce the number of mitochondria that cross the line and are irreversibly impaired.

ACKNOWLEDGMENTS

We appreciate the technical assistance of Mary Morphew in the Boulder Laboratory for 3D Fine Structure (supported by National Institutes of Health Grant P41 RR-00592), Jennifer Whitesell and Alisa Shaw for production of mRFP-actin adenovirus, and Troy Tholen (Zeiss, Thornwood, NY). Present address of H. Chen: Department of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205.

GRANTS

This work was supported in part by grant IIRG-01-2730 from the Alzheimer’s Association and by National Institutes of Health Grants GM-35126 and NS-40371.

REFERENCES


35. Kisuno H, Shimizu S, Koya RC, Fujita H, Kamada S, Kuzumaki N, and Tsujimoto Y. Human gelsolin prevents apoptosis by inhibiting...

**ACTIN RODS TRANSIENTLY PROTECT NEURONS**

C839