erythro-9-[3-(2-Hydroxynonyl)]adenine is an effective inhibitor of cell motility and actin assembly
(dynein ATPase/stress fibers/cyttoplasmic extracts/gellation)

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Communicated by Gunther S. Stent, June 18, 1984

ABSTRACT erythro-9-[3-(2-Hydroxynonyl)]adenine (EHNA) has been reported previously to be an agent that arrests sperm motility by inhibiting the axonemal dynein ATPase activity and has been used to probe the involvement of putative cytoplasmic dyneins in mitosis and intracellular organelle transport. We report here that EHNA profoundly and reversibly affects several actin-dependent processes, both in vivo and in vitro. It induces dramatic changes in actin organization in cultured cells, inhibits cell translocation, blocks actin-dependent cytoplasmic streaming, interferes with actin-dependent gelation of cytoplasmic extracts, and inhibits actin assembly. Just as the cytochalasins, EHNA appears to be a highly effective inhibitor of actin-based motility, whose effects in complex biological systems should be interpreted with caution.

Analysis of the function of cytoskeletal components greatly profits from the use of specific inhibitors. Prime examples include colchicine, which depolymerizes microtubules and inhibits their assembly, and the cytochalasins, which decrease the rate of actin assembly and interfere with actin network formation. Such inhibitors, of which colchicine and cytochalasin are just two of the most widely known, have become invaluable tools in research on cell motility. Recently, an inhibitor of dynein ATPase activity, erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA), became available, which, unlike vanadate, another potent dynein inhibitor (1), can easily cross the cell membrane and thus can be applied to living cells (2). Its potential usefulness in the search for "cytoplasmic" dyneins involved in motility phenomena, such as intracellular transport, was immediately realized. Thus, in addition to its use in the study of the properties of ciliary and flagellar dynein (2, 3), EHNA has been employed as a probe for the possible involvement of dynein-like ATPases in chromosome movement in mitosis (4), particle transport in chromatophores (5, 6), and organelle movements along axons (7, 8). Inhibition of these forms of motility by EHNA has been taken as an indication for the involvement of a cytoplasmic, possibly microtubule-associated, dynein ATPase activity.

In the course of studies on intracellular organelle movements using EHNA, we noted effects of this compound apparently unrelated to a possible action on dynein-like molecules. Here we report that EHNA interferes with the functional organization of actin in vivo and in vitro. It profoundly affects actin filament organization in cultured cells, reversibly interferes with actin-dependent forms of motility, and inhibits gel formation of cytoplasmic extracts and actin assembly in vitro. Next to the cytochalasins, it is probably one of the most effective inhibitors of actin-based motility.

MATERIALS AND METHODS

Cells. African green monkey kidney cells (strain BSC-1) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (GIBCO). For experiments, cells were seeded on coverslips and were used in the exponential growth phase. Mouse 3T3 cells were grown in the same medium and were passaged every 3 days. Primary cultures of fish epidermal cells were obtained as follows: scales removed from the flanks of Midas cichlids or angelfish were incubated with collagenase (Worthington) in Ring er's solution for 20–40 min and the epidermal cell layer was peeled off. These pieces of skin tissue were then allowed to attach to coverslips in the presence of Ringer's solution supplemented with 0.5% amphibian culture medium (GIBCO). Within 30–60 min, cells emigrate from the explant. Such cultures can be kept for several days; for the experiments described here, cells were used within 2–5 hr of explantation. Nitella was obtained from Carolina Biological (Burlington, NC) and was kept in pond water under a 12-hr light/dark regime. For experiments, several intermedial cells were removed from the stock culture and were placed in 35-mm plastic Petri dishes, where the cells were allowed to equilibrate for 1–2 hr in the presence of pond water supplemented with 0.2% dimethyl sulfoxide. The rate of streaming was determined with a Zeiss laboratory microscope using an ocular micrometer.

Cytoplasmic Extracts. High-speed cytoplasmic extracts (HSE) from Xenopus laevis eggs were prepared essentially as described (9). Mature, ovulated, dejellied eggs were first washed with a medium of 250 mM sucrose/200 mM NaCl/35 mM imidazole chloride/4 mM EGTA/2 mM MgCl2/1 mM dithiothreitol/0.5 mM benzamine-HCl/0.3 mM phenylmethylsulfonyl fluoride, pH 6.5. Washed eggs were packed by centrifugation at 300 × g for 5 min, supernatant medium was removed, and the packed eggs were then centrifuged at 12,000 × g for 45 min. This disrupts the eggs and stratifies their contents. The middle viscous layer was removed and centrifuged twice at 150,000 × g for 3 hr. The resultant clear supernatant is the HSE. All procedures were carried out at 2–4°C. Waves of gelation and contraction of HSE, prepared in the presence of phosphatase inhibitors, were monitored by time-lapse video recording in cuvettes containing 0.2 ml of extract (9).

For determination of viscosity, extracts were diluted 1:2 at 4°C in 250 mM sucrose/100 mM NaCl/20 mM imidazole chloride, pH 7.0/0.5 mM dithiothreitol. Dimethyl sulfoxide, EHNA, cytochalasin B, or adenosine was added to this diluted extract just prior to warming. Viscosity was measured at 25°C in a falling-ball apparatus according to MacLean-Fletcher and Pollard (10).

Abbreviation: EHNA, erythro-9-[3-(2-hydroxynonyl)]adenine.

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Polymerization of Actin. Chicken skeletal muscle actin, provided to us by R. C. Strohman, was prepared according to the procedure of Spudich and Watt (11). Polymerization of G-actin was induced by the addition of 50 mM KCl/1 mM MgCl₂/1 mM ATP. Actin polymerization was monitored by determining the kinematic viscosity at high shear rates in an Ostwald capillary viscometer. The specific viscosity, \( \eta_s \), was calculated according to \( \eta_s = (\eta - \eta_0) / \eta_0 \), where \( \eta \) is the viscosity of the sample, and \( \eta_0 \) is the viscosity of the buffer solution.

**Microscopy.** Cell behavior and motility was monitored in a Zeiss photomicroscope III equipped with phase-contrast optics. For fluorescence microscopy, cells were fixed with 1% glutaraldehyde in a buffer of 60 mM Pipes/25 mM Hepes/2 mM MgCl₂/10 mM EGTA (PHEM buffer; see ref. 12) for 10 min, washed twice with phosphate-buffered saline (PBS), and exposed to 1% sodium borohydride in PBS/NaCl twice for 5 min each. Coverslips were then incubated sequentially with rhodamine-phalloidin (20 min), a monospecific rabbit anti-tubulin antibody (40 min), and a fluorescein-labeled goat anti-rabbit antibody (40 min). After thorough rinsing with PBS/NaCl, coverslips were mounted in 90% glycerol/10% PBS/NaCl supplemented with 1 mM phenylenediamine and viewed in a Zeiss photomicroscope III equipped with epifluorescence.

**Materials.** Adenosine, cycloleucine, cytochalasin B, phenylmethanesulfonyl fluoride, and p-phenylenediamine were from Sigma. Rhodamine-phalloidin was a generous gift of Theodor Wieland (Max-Planck-Institut für Medizin, Heidelberg). The rabbit anti-tubulin antibody was kindly provided by Marc Kirschner (University of California, San Francisco). EHNA was supplied by Burroughs Wellcome (Research Triangle Park, NC).

**RESULTS AND DISCUSSION**

Treatment of African green monkey kidney cells (Fig. 1) or 3T3 cells (not shown) with 1 mM EHNA, a concentration known to inhibit effectively dynein ATPase activity (3), induced a dramatic alteration of the organization of F-actin, as seen by staining with rhodamine-labeled phalloidin. Within 60 min, all stress fibers were disassembled and replaced by punctate and needle-shaped aggregates. Even though in the process the cell shape might be distorted substantially, the integrity of microtubules was unaffected (Fig. 1d). Because EHNA also is a potent inhibitor of protein carboxymethylation (13, 14), two other such inhibitors, adenosine (2 mM) and cycloleucine (10 mM), were also employed and were found not to induce significant changes in actin organization of cultured cells (Fig. 1a).

Hence, EHNA does not cause actin disassembly by inhibiting protein carboxymethylation. The effects induced by EHNA were fully reversible within 90–120 min (Fig. 1e).

To test whether EHNA interferes with cell movement, fish epidermal cells in culture were used. These cells move by virtue of actin-containing lamellipodia at speeds of up to 1 \( \mu \)m/sec (15). The lamellae contain a highly ordered arrangement of actin filaments but are free of microtubules, intermediate filaments, and other major cellular components, including the nucleus, mitochondria, and endoplasmic reticulum, which all are concentrated in the bulbous cell body at

![Fluorescence micrographs of BSC-1 cells stained with rhodamine-phalloidin](image)

**Fig. 1.** Fluorescence micrographs of BSC-1 cells stained with rhodamine-phalloidin (a–c and e) and antibody against tubulin (d). (a) Cells from a culture treated with 10 mM cycloleucine for 60 min. The morphology and stress fiber organization of these cells is indistinguishable from that of untreated cells. (b) Cell treated with 1 mM EHNA for 30 min. The shape of EHNA-treated cells becomes irregular, and stress fibers disassemble. (c and d) Cell treated with 1 mM EHNA for 60 min. All stress fibers are disassembled, and many punctate or needle-shaped actin-containing structures are visible. The microtubule system of this double-labeled cell (d) is apparently unaffected. (e) Cell treated with 1 mM EHNA for 1 hr and then recovered in EHNA-free medium for 2 hr. Normal cell morphology and actin organization is completely restored. (×450.)
the cell posterior (15). Lamellae are extremely susceptible to
the action of cytochalasins, being withdrawn within a few
seconds of their exposure to the agent (not shown). Thus,
they may serve as an excellent model system to study the
action of actin-perturbing agents in vivo. Addition of 1–2 mM
EHNA to fish epidermal cells induced lamellar retraction
within a few seconds, frequently causing the lamellae to tear
away from the cell body (Fig. 2). Lamellar withdrawal was
followed by cell rounding and extension of bleb-like fiaccid
protrusions. This state was maintained as long as the agent
was present. Upon removal of the agent by washing, re-ex-
tension of lamellae was initiated within 1 min (Fig. 2 f–h).
Full lamellar extension was achieved within 3–5 min, at
which time normal cell locomotion resumed. The extent and
time course of EHNA-induced changes in lamellar organiza-
tion and function are concentration-dependent, being maxi-
mal at 2 mM; 0.2–0.4 mM constitute threshold concentra-
tions at which lamellae are visibly affected (they “shrink”
and undergo convulsions) but remain more or less extended.

Another cellular phenomenon known to require the integ-
ity of actin filaments is rotational streaming in the green
alga *Nitella*. In internodal cells, endoplasm streams unidirec-
tionally at rates of 30–100 μm/sec along bundles of actin fila-
ments anchored in the stationary ectoplasm (16, 17). To test
whether EHNA influences this actin-dependent intracellular
transport system, internodal cells were exposed to 1 mM
EHNA (Fig. 3). Within about 5 min of addition of EHNA,
streaming gradually slowed down until, at 15 min, movement
ceased in most of the cells (14 of 18 in our experiments).
Streaming fully recovered within 15–20 min after EHNA was
removed; streaming resumed very slowly after about 3 min
and gradually accelerated until normal rates of movement
were achieved.

To test whether EHNA inhibits actin-dependent processes
in vitro, we studied cytoplasmic extracts of *X. laevis* eggs
(9). These extracts will gel when warmed to room tempera-
ture and slowly contract over a period of 8–12 hr. EHNA
was found to inhibit gel formation completely at concentra-
tions of 1 and 2 mM, as determined by falling-ball viscometry
(10) (Fig. 4). In the presence of 0.5 or 0.25 mM EHNA, gela-
tion was delayed and the apparent viscosity of the resultant
gel was reduced. These effects were similar to those pro-
duced by 0.01 and 0.05 μM cytochalasin B. Adenosine at 2
mM had no effect.

Extracts stained with rhodamine-phalloidin to visualize
filamentous actin showed a network of long fibers in an ap-
parently random orientation in control preparations (Fig.
5a). If samples of the same extracts were prepared with 1
mM EHNA added just prior to warming, no such network
formed, and the fluorescent image showed mostly punctate
staining and only a few filamentous strands (Fig. 5b). Thus,
EHNA disrupts the organization of F-actin in these extracts.

When extracts are prepared under conditions that increase
protein phosphorylation, including that of myosin light
chains, gel contraction is accelerated and several additional
gels form and contract (9). In the presence of 1 or 2 mM
EHNA, however, no waves are initiated. This would be ex-
pected since at these concentrations of EHNA, gelation is
completely inhibited, and formation of a gel is a prerequisite
for subsequent contraction. EHNA at 0.25 and 0.5 mM re-
duces the number of waves by >50% by increasing the dura-
tion of contraction of each gel formed (not shown). This sug-
gests that contraction *per se* is not inhibited, in accord-
cance with the observation that myosin ATPase activity is not im-
paired by EHNA (3).
To determine whether EHNA influences actin polymerization in vitro, we employed high-shear Ostwald viscometry to follow the time course of assembly of "conventional" (i.e., not gel-filtered) chicken skeletal muscle actin (18). EHNA inhibited actin assembly in a concentration-dependent manner (Fig. 6), with essentially no assembly occurring at 2 mM. An unusual time course of viscosity development was observed at 0.25 mM EHNA, at which assembly initially proceeded at a rate comparable to that of control preparations, but after 4 min declined until a stable, but lower, plateau level was reached. Two millimolar adenosine (Fig. 6) or 10 mM cycloleucine (not shown) did not interfere with the development of viscosity in these actin preparations. Negative staining demonstrates that in the presence of 2 mM EHNA, preparations of actin prepared under polymerizing conditions are essentially free of filaments but contain clumps of amorphous material (not shown). Fewer filaments than in control preparations were observed at 1 mM EHNA; at still lower concentrations, filaments were present, but it is not possible to determine with certainty whether their number or length (or both) might be reduced compared to control preparations.

EHNA also reduced the viscosity of actin solutions po-

Fig. 5. Fluorescence micrographs of extract fixed with 1.2% formalin and stained with rhodamine-phalloidin. (a) Control extract 1 hr after warming to room temperature. (b) Extract in the presence of 1 mM EHNA for 1 hr. (x550.)

Fig. 4. Effect of EHNA and cytochalasin B on gelation of X. laevis egg extracts, as determined by falling-ball viscometry. Apparent viscosity (napp) is plotted against time. Additions were made at 0 min, as marked; the crosses stand for all experimental conditions. No gel formed in 0.1 or 1 μM cytochalasin B. Under normal conditions, a gel forms within 10 min. Gelation is inhibited by 1 and 2 mM EHNA and by concentrations of cytochalasin B > 0.1 μM. Lower concentrations of EHNA and cytochalasin B delay gelation and reduce its apparent viscosity. ■, Two millimolar adenosine; ○, 0.2% dimethyl sulfoxide; □, 0.01 μM cytochalasin B; ▽, 0.25 mM EHNA; ○, 0.05 μM cytochalasin B; □, 0.5 mM EHNA; ○, 1 mM EHNA; □, 2 mM EHNA.

Fig. 6. Effect of EHNA on the polymerization of conventional actin as determined by Ostwald viscometry. Twelve micromolar conventional, depolymerized chicken skeletal muscle actin was induced to polymerize by the addition of 50 mM KC1/1 mM MgCl2/1 mM ATP (19). Other additions, as marked, were also made at the same time. The mixture was then quickly loaded into the viscometer. ■, Two millimolar adenosine; ○, 0.2% dimethyl sulfoxide; ▽, 0.25 mM EHNA; □, 0.5 mM EHNA; ○, 1 mM EHNA; □, 2 mM EHNA.
lymerized in the absence of the compound when added after polymerization. Actin was allowed to assemble in the presence of 1 mM ATP in a test tube and EHNA (1 or 2 mM) or 0.2% dimethyl sulfoxide was added to the assembled actin and gently mixed. The mixture was then introduced into an Ostwald viscometer. EHNA, but not dimethyl sulfoxide, reduced the viscosity of the F-actin solution, which, however, did not decline to the same level as that obtained with comparable mixtures to which EHNA had been added prior to actin assembly (Fig. 7).

The ATP analog EHNA was first described as an inhibitor of protein carboxymethylation (13) and was later shown to arrest the motility of spermatozoa due to an inhibition of the axonemal dynein ATPase activity (2). EHNA is a relatively specific inhibitor of this ATPase, with little effect on several other ATP-metabolizing enzymes (3). In particular, myosin ATPase activity is not only unaffected but even slightly enhanced by EHNA. This relatively high degree of specificity led to the suggestion that EHNA could be used as a probe for the putative participation of cytoplasmic dyneins in several forms of cytoplasmic motility. Indeed, subsequent studies showed that EHNA blocks certain forms of microtubule-based motility (4–8). These observations were taken as indirect evidence for the involvement of a cytoplasmic dynein-like ATPase.

The results reported here provide evidence that EHNA is also an effective inhibitor of actin-dependent cellular processes, affecting both the organization (Figs. 1 and 4) and function (Figs. 2 and 5) of cytoplasmic actin. The effects produced by EHNA and the speed with which it acts in the systems tested are reminiscent of the cytochalasins, even though the mechanism of action of both agents is probably different. It may well be that the effects observed in living cells are a consequence of EHNA’s inhibitory action on actin assembly or induction of disassembly (or both). One possible explanation for this is that it interferes with the ATP binding site on actin since EHNA is, after all, an ATP analog. However, its precise mechanism of action is not understood and will require more detailed work.

Though it seems clear that EHNA is indeed an inhibitor of dynein ATPase activity in vitro (3), our results indicate that this is by no means its only biochemical action. Hence, considerable caution is necessary in the interpretation of the effects produced by EHNA in complex systems—such as intact or permeabilized cells or cell extracts—and a diversity of control experiments is required in such experiments.

We thank Richard Strohman for generously providing chicken skeletal muscle actin and Marc Kirschner for a generous gift of tubulin antibody. The helpful comments of Gunther Stent on the manuscript are greatly appreciated. This study was supported by National Institutes of Health Grant GMS 31041.