Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 234-240

DYNEIN ATPASE IS INHIBITED SELECTIVELY IN VITRO BY ERYTHRO-9-[3-2-(HYDROXYNONYL)]ADENINE*

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Received November 30,1981

Summary-Dynein (ATP phosphohydrolase, EC 3.6.1.3) extracted from sea urchin sperm tails was inhibited by erythro-9-[3-2-(hydroxynonyl)]adenine in a dose-dependent fashion; at the 50% inhibitory concentration, 0.23 mM, twelve other ATP-metabolizing enzymes were not significantly affected. Actomyosin and myosin ATPase activities were enhanced 1.5- to 2-fold by millimolar concentrations of erythro-9-[3-2-(hydroxynonyl)]adenine. Enzyme kinetic analysis supported a model of linear mixed-type inhibition, which suggests that the binding site for erythro-9-[3-2-(hydroxynonyl)]adenine on dynein is remote from the ATPase active site. As a selective inhibitor in vitro, erythro-9-[3-2-(hydroxynonyl)]-adenine appears to offer a biochemical criterion for identifying dynein isozymes in tissue extracts.

Dynein ATPases, the microtubule-associated force-transducing enzymes of cilia and flagella, appear to comprise a structurally heterogeneous group of isozymes (1,2). Evidence has also been reported of cytoplasmic dyneins which may power microtubule sliding in mitosis (3-5). At present, no unambiguous biochemical criterion exists for identifying cellular ATPases as dynein-like (1); identification is based primarily on structural criteria, especially association with microtubules in the cell (2). We report here evidence that the potent adenosine deaminase inhibitor erythro-9-[3-2-(hydroxynonyl)]adenine (EHNA) (6), shown recently to arrest sperm motility by reversibly blocking dynein ATPase activity (7), acts as a selective inhibitor of partially purified sea urchin

*This investigation was supported by NSF Grant PCM 7905611 and NIH Grant RR 09085-03 to SMP, NIH Grant HD-13541 to CWB, and a grant from the Medical Research Council of Canada to CG. †To whom correspondence should be addressed. §Dr. Bouchard's present address is: Centre Hospitalier de Bicetre, 78, rue du General-Leclerc, 94270 Le Kremlin-Bicetre, France. Abbreviation: Erythro-9-[3-2-(hydroxynonyl)]adenine, EHNA.

0006-291X/82/010234-07$01.00/0

sperm flagellar dynein, displaying minor and/or non-specific effects on twelve other partially purified ATP-metabolizing enzyme activities. Our results suggest that EHNA may be useful in distinguishing dynein isozymes from other ATPases in vitro, thus facilitating their purification and, ultimately, functional analyses of their role(s) in cell motility.

MATERIALS AND METHODS

Biochemicals: Biochemicals were from Sigma except phosphoenolpyruvate, $NADP^+$, and glucose-6-phosphate dehydrogenase from Boehringer-Mannheim, EHNA from Burroughs-Wellcome, n-nonanol and nonylamine from Aldrich, n-dodecanol from ICN, and [γ-^{32}P]ATP (2000 Ci/mmol) and [2-^{3}H]ATP (24 Ci/mmol) from Amersham.

Enzyme preparations and assay conditions: Dynein 1 was prepared by demembranating sea urchin (L. pictus) sperm with 0.1% Triton X-100, isolating the flagellar axonemes and extracting them with 0.6 M KCl, as described (7). This procedure gives a kinetically homogeneous preparation of dynein ATPase activity with a K_m of 59 µM (8); the ATPase activity was shown to sediment at 11.3S, and the 11.3S ATPase displayed a prominent A-band on SDS gel electrophoresis, characteristics indicative of dynein 1 (data not shown) (1,2). Dynein ATPase activity was assayed in 20 mM Tris-HCl, pH 8.1, 0.15 M KCl, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM ethylenediaminetetraacetic acid (EDTA) at 16°C (TKMDE) (7). Actomyosin prepared from rabbit skeletal muscle as described (9) was assayed in either 50 mM Tris-HCl, pH 9.0, 0.1 M KCl, 5 mM $MgCl_2$, 1 mM ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) at 25°C (Mg^{++}-ATPase) or in the same buffer except that 0.01 mM $CaCl_2$ was added and EGTA was omitted (Ca^{++}-activated Mg^{++}-ATPase) (9). Myosin (Sigma) was assayed in either 50 mM Tris-HCl, pH 8.0, 0.1 M KCl, 5 mM $MgCl_2$ at 20°C (Mg^{++}-ATPase) (10); 20 mM Tris-HCl, pH 7.5, 0.25 M KCl, 10 mM $CaCl_2$ at 25°C (Ca^{++}-ATPase) (11); or 20 mM Tris-HCl, pH 7.5, 0.5 M KCl, 1 mM EDTA at 25°C (EDTA-ATPase) (11). Both actomyosin and myosin contained less than 1% impurities as judged by SDS gel electrophoresis (9,12). Sub-mitochondrial membrane particles were prepared from rat liver (13,14) and assayed in TKMDE at 25°C; their ATPase activity was inhibited 95% by 5 µg/ml oligomycin. Ca^{++}-dependent ATPase protein prepared from sarcoplasmic reticulum (15) was kindly donated by Dr. Gary Bailin, CMDNJ-N.J. School of Osteopathic Medicine; it was assayed in 50 mM Tris-HCl, pH 6.8, 80 mM KCl, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, 30°C, with and without 1 mM EGTA (15). Dog kidney (Na,K)ATPase (Sigma) was assayed in 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 25 mM KCl, 1 mM DTT, 25 mM $MgCl_2$ at 37°C (16); it was inhibited 84% by 10^{-4} M ouabain. ATPase activities were assayed on the basis of the liberation of [^{32}Pi] from [γ-^{32}P]ATP (7). Rabbit muscle D-fructose-6-phosphate kinase (Sigma), yeast hexokinase and rabbit muscle adenylate kinase (Boehringer-Mannheim) were assayed spectrophotometrically in TMKDE at ambient temperature by means of coupled enzyme assays, as described (17). Adenylate cyclase prepared as the particulate fraction from rat cerebral cortex (18) was kindly donated by Dr. Charles Brostrom, CMDNJ-Rutgers Medical School; it was assayed in the presence of Ca^{++} and calmodulin, on the basis of conversion of [^{3}H]ATP to [^{3}H]cAMP (18). The Ca^{++}-dependent ATPase, (Na,K)ATPase, D-fructose-6- phosphate kinase and adenylate kinase preparations contained less than 10% and hexokinase less than 30% impurities as judged by SDS polyacrylamide gel electrophoresis (19-23).

Other methods: Protein concentration was determined by the method of Lowry et al (24) using bovine serum albumin as a standard. Nucleotide extinction coefficients were from Cohn (25). The extinction coefficient for adenine was used to determine EHNA concentration. SDS polyacrylamide gel electrophoresis was performed essentially as described (26) except that a 5%-15% linear acrylamide gradient was used. Sample loadings were 150 µg. Relative concentrations of components were estimated visually after staining with Coomassie Brilliant Blue and destaining overnight in 5% methanol, 7.5% acetic acid.

TABLE 1

Effects of EHNA on the Specific Activities of ATP-Metabolizing Enzymes

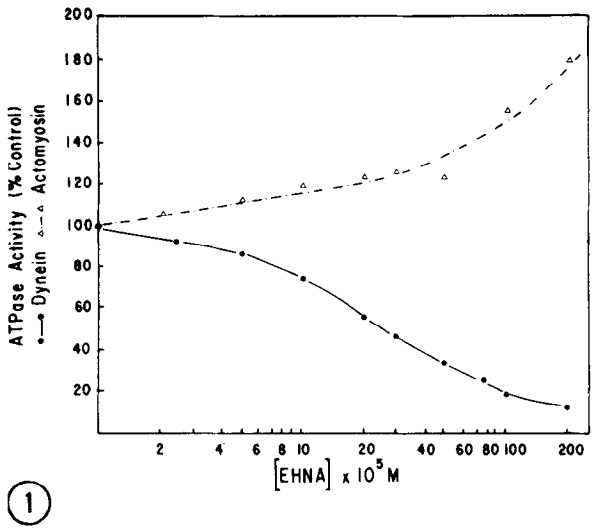
Enzyme	Specific Activity[a] (μmoles/min/mg protein)	Activity in Presence of 0.23 mM EHNA (% Control)
Dynein	0.094	50
Mg^{++}-actomyosin	0.009	105
Ca^{++}-activated Mg^{++}-actomyosin	0.018	122
Mg^{++}-myosin	0.018	110
Ca^{++}-myosin	0.163	100
EDTA-myosin	0.069	108
Oligomycin-sensitive mitochondrial ATPase	0.150	90
Ouabain-sensitive (Na,K)ATPase	0.008	102
Ca^{++}-dependent sarcoplasmic reticulum ATPase	0.290	101
Adenylate cyclase	0.00015	100
Hexokinase	11.70	100
Phosphofructokinase	13.30	100
Adenylate kinase	112.0	100

[a]Determined at 0.1 mM [γ-^{32}P]ATP, except adenylate cyclase, which was determined at 0.025 mM [^{3}H]ATP.

RESULTS

Selective inhibition of dynein 1 ATPase: Dynein 1 ATPase activity isolated as the 0.6 M KCl extract of sea urchin sperm axonemes was inhibited by EHNA in a dose-dependent fashion; the 50% inhibitory concentration was 0.23 mM EHNA (Fig. 1). Twelve other partially purified ATP-metabolizing enzyme activities were not significantly affected by EHNA at a concentration of 0.23 mM (Table 1). The Ca^{++}-dependent sarcoplasmic reticulum ATPase, (Na,K)ATPase, mitochondrial ATPase and adenylate cyclase were inhibited 30-50% by 1-2 mM EHNA (data not shown); this finding suggested that, at millimolar concentrations, EHNA may have a non-specific, detergent-like effect on membrane-bound enzymes. Ca^{++}-activated Mg^{++}-actomyosin ATPase activity was stimulated progressively by increasing EHNA concentrations (Fig. 1). Similar stimulatory effects of EHNA on the Mg^{++}-actomyosin, Mg^{++}-myosin and Ca^{++}-myosin ATPase activities were also observed (data not shown).

Kinetic model of EHNA action on dynein 1: Earlier we reported that EHNA acted as a mixed-type inhibitor of dynein ATPase activity (7). We distinguished



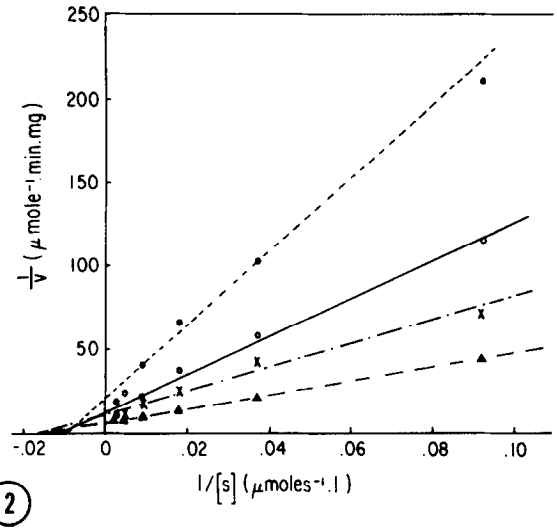


Figure 1: Effects of increasing EHNA concentrations on dynein and actomyosin ATPase activities. *——* Dynein; Δ— · —Δ Ca^{++}-activated Mg^{++}-actomyosin.

Figure 2: Effects of increasing doses of EHNA on the ATP-concentration dependence of dynein ATPase activity. Plots were fitted by the method of least squares. ▲– – –▲ 0.00 mM EHNA; x— · —x 0.064 mM EHNA; o——— o 0.12 mM EHNA; ●– – –● 0.24 mM EHNA.

among various kinds of mixed-type inhibition (27,28) by analyzing Lineweaver-Burke plots obtained at three different EHNA concentrations. The intersection of the plots to the left of the ordinate (Fig. 2), and the linearity of two replots: slope versus inhibitor concentration, and $\frac{1}{v}$-axis intercept versus inhibitor concentration (data not shown), indicated a kinetic model of linear mixed-type inhibition, or a mixture of partial competitive inhibition (affinity of dynein for ATP reduced 2.8-fold (calculation not shown)) and pure, non-competitive inhibition (27,28). The reversibility of EHNA inhibition was confirmed directly by demonstrating that inhibition was reduced by dilution consonant with the data in Fig. 1 (data not shown). This kinetic model suggests that EHNA does not bind to the dynein ATPase active site, but to a separate site on dynein, exerting its inhibitory effect by an allosteric mechanism (27). The K_I for EHNA was estimated at 74 μM (27).

Structure of the EHNA binding site: Structural analogs of the adenine and the 2-nonanol moieties of EHNA inhibited dynein ATPase activity only slightly. Mixing adenine with either nonanol or nonylamine resulted in an approximately two-fold potentiation of inhibition (Table 2), suggesting

TABLE 2

Inhibition of Dynein ATPase by Analogs of EHNA

Analog(s)[a]	% Inhibition of Dynein ATPase Activity
Adenine	7
Adenosine	20
Isopentenyl adenosine	10
n-nonanol	18
nonylamine	29
n-dodecanol	3
Adenine + n-nonanol	49
Adenine + nonylamine	57
Adenine + n-dodecanol	13

[a]Concentration of all analogs was 1 mM. Concentration of [γ-^{32}P]ATP was 0.1 mM in all assays.

that the EHNA-binding site contains two regions, one for the adenine and one for the 2-nonanol portion of EHNA, which interact synergistically to produce inhibition of ATPase activity.

DISCUSSION

Our findings suggest that EHNA is a selective inhibitor of dynein ATPase activity *in vitro*, binding at a unique, predominantly hydrophobic site on the dynein particle. A selective inhibitor of dynein ATPase activity has not been reported previously. Vanadate, a potent dynein inhibitor (29, 30), is known also to inhibit the (Na,K)ATPase (31) and myosin ATPase (32). We conclude from our data that inhibition of enzyme activity by EHNA provides a reliable criterion for identifying dynein-like ATPases *in vitro*. Our previous demonstration that the dynein ATPase activity of rat sperm tails is sensitive to EHNA, indicating evolutionary conservation of the EHNA binding site (7), supports the feasibility of using EHNA sensitivity as a generic, *in vitro* marker for dynein-like ATPases.

It is clear that use of EHNA as a probe for the physiological role(s) of putative cytoplasmic dyneins *in vivo* (3-5) is severely limited by a combination of two factors: its low potency as a dynein inhibitor (Fig. 1; Table 1), and its high potency as an inhibitor of adenosine deaminase (6). Through its effect on adenosine metabolism, EHNA could block protein carboxylmethylation, which has been implicated in at least two forms of

cytoplasmic motility, exocytosis and chemotaxis (33), and which also, by modifying calmodulin (34), could conceivably regulate a host of Ca^{++}-dependent cellular functions (35).

From our kinetic data (Fig. 2) we conclude that the EHNA-binding site on dynein is remote from the ATPase active site. Because recent structural studies have suggested that dynein may contain up to ten subunits (36,37), it is possible that the EHNA-binding site and the ATPase active site could reside on separate subunits within the dynein particle.

REFERENCES

1. Warner, F.D. and D.R. Mitchell (1980) Int. Rev. Cytol. 66, 1-34.
2. Gibbons, I.R., E. Fronk, B.H. Gibbons and K. Ogawa (1976) In Cell Motility, R. Goldman, T. Pollard and J. Rosenbaum (eds.), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., Book C, pp. 915-932.
3. Pratt, M.M. (1980) Devel. Biol. 74, 363-378.
4. Hisanaga, S.-I. and H.Sakai (1980) Develop., Growth and Differ. 22,373-384.
5. Pratt, M.M., T. Otter and E.D. Salmon (1980) J. Cell Biol. 86, 738-745.
6. Schaeffer, H.J. and C.F. Schwender (1974) J. Med. Chem. 17, 6-8.
7. Bouchard, P., S.M. Penningroth, A. Cheung, C. Gagnon and C.W. Bardin (1981) Proc. Natl. Acad. Sci. USA 78, 1033-1036.
8. Penningroth, S.M., A. Cheung, K. Olehnik and R. Koslosky (1982) J. Cell Biol., in press.
9. Bailin, G. (1975) Biochim. Biophys. Acta 400, 167-177.
10. Chock, S.P. and E. Eisenberg (1979) J. Biol. Chem. 254, 3229-3235.
11. Bailin, G. and M. Barany (1972) J. Biol. Chem. 247, 7815-7821.
12. Weeds, A.G. and G. Frank (1972) CSHS Quant. Biol. 37, 9-17.
13. Hogeboom, G.H. (1955) Methods Enzymol. 1, 16-18.
14. Keilley, W.W. (1955) Methods Enzymol. 2, 593-595.
15. Bailin, G. (1980) Biochim. Biophys. Acta 623, 213-224.
16. Josephson, L. and L.C. Cantley (1977) Biochemistry 16, 4572-4578.
17. Secrist, J.A., III, J.R. Barrio, N.J. Leonard and G. Weber (1972) Biochemistry 11, 3499-3506.
18. Brostrom, C.O., M.A. Brostrom and D.J. Wolff (1977) J. Biol. Chem. 252, 5677-5685.
19. MacLennon, D.H., C.C. Yip, G.H. Iles and P. Seeman (1972) CSHS Quant. Biol. 37, 469-477.
20. Mannsbach, A.B., E. Skriver and P.L. Jorgensen (1979) In (Na,K)-ATPase Structure and Kinetics, J.C. Skoll and J.G. Norby (eds.), Academic Press, N.Y., pp. 3-13.
21. Hess, B., D.B. Wetlaufer, L. Bornmann, T. Reed and N. Tamaki (1975) In Enzymes-Electron Transport Systems. P. Desnuelle and A.M. Michelson (eds.), North-Holland Publishing Co., Amsterdam, Oxford, pp. 49-62.
22. Callaghan, O.H. (1957) Biochem. J. 67, 651-657.
23. Schmidt, J.J. and S.O. Colowick. (1973) Arch. Biochem. Biophys. 158, 458-470.
24. Lowry, R.W., N.J. Rosenbrough, A.L. Farr and R.J. Randall (1951) J. Biol. Chem. 193, 265-275.
25. Cohn, W.E. (1957) Methods Enzymol. 3, 724-743.
26. Laemmli, U.K. (1970) Nature 227, 680-685.
27. Segel, I.H. (1975) Enzyme Kinetics, pp. 170-208, John Wiley and Sons, New York.
28. Dixon, M. and E.C. Webb (1964) Enzymes, 2nd Ed., pp. 324-331, Academic Press, New York.

29. Gibbons, I.R., M.P. Cosson, J.A. Evans, B.H. Gibbons, B. Horick, K.H. Martinson, W.S. Sale and W.-J.Y. Tang. (1978) Proc. Natl. Acad. Sci. USA 75, 2220-2224.
30. Kobayashi, T., T. Martensen, J. Nath and M. Flavin (1978) Biochem. Biophys. Res. Comm. 81, 1313-1318.
31. Cantley, L.C., Jr., L.G. Cantley and L. Josephson (1978) J. Biol. Chem. 253, 7361-7368.
32. Goodno, C.C. (1979) Proc. Natl. Acad. Sci. USA 76, 2620-2624.
33. Gagnon, C. and S. Heisler (1979) Life Sci. 25, 993-1000.
34. Gagnon, C., S. Kelly, V. Manganiello, M. Vaughan, C. Odya, W. Strittmacher, A. Hoffman and F. Hirata (1981) Nature, in press.
35. Means, A.R. and J.R. Dedman (1980) Nature 285, 73-77.
36. Piperno, G. and D.J.L. Luck (1979) J. Biol. Chem. 254, 3084-3090.
37. Bell, C.W., E. Fronk and I.R. Gibbons (1979) J. Supramol. Struct. 11, 311-317.