***erythro*-9-[3-(2-Hydroxynonyl)]adenine is an inhibitor of sperm motility that blocks dynein ATPase and protein carboxylmethylase activities**

(axonemal movement/dynein arms/demembrated spermatozoa)

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ABSTRACT Protein carboxylmethylase (S-adenosyl-L-methionine:protein O-methyltransferase, EC 2.1.1.24) is believed to be involved in the regulation of sperm motility. To test this hypothesis, we investigated the effects of *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) which, in combination with adenosine and homocysteine thiolactone, inhibits protein carboxylmethylase activity in monocytes. This group of compounds inhibited sea urchin sperm motility. Unexpectedly, EHNA alone inhibited the motility. This observation was confirmed in intact spermatozoa from rats, rabbits, and humans. EHNA also inhibited the motility of demembrated, reactivated sea urchin and rat spermatozoa from which protein carboxylmethylase had been extracted. In these preparations, motility was restored by ATP. These observations suggested that EHNA arrests sperm motility by inhibiting the axonemal dynein ATPase on which motility depends. Kinetic analysis demonstrated that EHNA produced mixed inhibition of both the axonemal ATPase and the partially purified dynein 1 from sea urchin sperm tails, as well as the axonemal ATPase of rat sperm tails. These observations also provide evidence for the similarity of the active site of the dynein ATPase in sea urchin and rat spermatozoa.

The sliding microtubule hypothesis of flagellar and ciliary motility is based on evidence that armlike projections containing dynein ATPase "walk along" neighboring microtubule fibers in the axoneme in response to the binding and hydrolysis of ATP (1-4). The mechanisms used by the spermatozoon to regulate microtubule sliding are of interest because they may be important in the control of cell motility in general. Various factors have been identified as possible physiological regulators of sperm motility, including Ca^{2+} (5-9), cyclic AMP (10-13), catecholamines (14-15), and a protein factor (16). Recently, the suggestion was made that protein carboxylmethylation may be involved in the regulation of sperm motility (17, 18). To test this hypothesis, we investigated the effects of *erythro*-9-[3-(2-hydroxynonyl)]adenine (EHNA) on sea urchin and mammalian spermatozoa. This compound is a potent inhibitor of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) (19) which, in combination with adenosine and homocysteine thiolactone, inhibits protein carboxylmethylase (S-adenosyl-L-methionine:protein O-methyltransferase, EC 2.1.1.24) by increasing the intracellular concentration of S-adenosyl-L-homocysteine (20). This latter compound is a competitive inhibitor of protein carboxylmethylase, which blocks the movement of human monocytes (20). The results of the present study demonstrate that EHNA also inhibits motility in all species of spermatozoa examined. In addition to its effect on protein carboxylmethylase, EHNA also inhibits dynein ATPase. The arrest of sperm motility is secondary to this latter effect.

MATERIALS AND METHODS

Materials. L-[³⁵S]Methionine (550 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), L-[methyl-³H]methionine (15 Ci/mmol), and [side chain-2-¹⁴C]tryptamine bisuccinate (50 Ci/mol) were obtained from New England Nuclear. [γ -³²P]ATP (>2000 Ci/mmol) was purchased from Amersham. EHNA was obtained from Burroughs Wellcome (Research Triangle Park, NC), artificial sea water (Instant Ocean) was from Aquarium Systems (East Lake, OH), and ATP (vanadium-free), chloramphenicol, and Triton X-100 were from Sigma. Ham's F12 nutrient mixture and Dulbecco's modified Eagle's medium were purchased from GIBCO.

Sperm Motility. Sea urchins (*Strongylocentrotus purpuratus*) were purchased from Pacific Biomarine Laboratories (Venice, CA). Sperm were collected in 3 ml of artificial sea water after injection of 2-3 ml of 0.5 M KCl. The concentrated sperm were stored on ice and used within 3 hr. Experiments were performed at 16°C, except that samples were inspected at ambient temperature (21°C) with a darkfield light microscope.

Native sea urchin sperm were made motile by diluting concentrated sperm 1:250 with artificial sea water. To test the effect of drugs on demembrated, reactivated sea urchin sperm, rigor wave sperm prepared in rigor buffer (20 mM Tris-HCl/0.15 M KCl/6 mM MgCl_2 /1 mM dithiothreitol, pH 8.1, adjusted at 21°C) were reactivated by addition of ATP (21, 22). The threshold of reactivation was defined as the concentration of ATP needed to reinduce motility in 80-100% of a sample of rigor sperm at a flagellar beat frequency of 0.5-0.8 Hz (determined with a stopwatch).

Rat spermatozoa were obtained from cauda epididymis and rabbit and human spermatozoa from ejaculates. Spermatozoa were collected and washed in F12/modified Eagle's medium, 1:1 (vol/vol), at 30°C as reported (17). Rat tails were isolated as described (17). Demembration and reactivation of rat sperm were performed in extraction medium (0.1% Triton X-100/0.2 M sucrose/25 mM potassium glutamate/1 mM MgSO_4 /1 mM dithiothreitol/20 mM Tris-HCl, pH 7.9) as described for hamster and guinea pig spermatozoa (13). Experiments were conducted at 30°C, except that samples were inspected at ambient temperature (21°C) with an inverted microscope (Unitron).

Samples of sea urchin and mammalian sperm were scored in the microscope as being immotile if no flagellar movement was detected on scanning at least three fields of cells (approximate sperm concentration was 25 cells per microscope field). Cells were scored as being motile if a majority were beating. In this

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

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report, intact motile spermatozoa will be termed "native" and demembrated motile sperm will be termed "reactivated."

Enzyme Assays. Protein carboxylmethylation was measured in native mammalian or sea urchin spermatozoa. Motile rat spermatozoa were suspended in methionine-free F12/modified Eagle's medium and incubated in 0.2 ml at 37°C with L-[methyl-³H]methionine (10 μ Ci) in the presence or absence of EHNA. Motile sea urchin spermatozoa were incubated in 1 ml of sea water at 16°C with L-[methyl-³H]methionine (10 μ Ci) in the presence and absence of EHNA. The incubation was stopped at various times by addition of 15% (wt/vol) trichloroacetic acid. After centrifugation (10,000 $\times$ g for 10 min), the protein pellet was washed four times with 15% trichloroacetic acid containing 1 mM unlabeled methionine. Radioactive methanol produced from the alkaline hydrolysis of protein [³H]methyl esters was extracted, and radioactivity was measured as described (23).

Mitochondrial protein synthesis was measured by incubating motile native spermatozoa with L-[³⁵S]methionine (10 μ Ci) in 1 ml of methionine-free F12 medium. The incubation was stopped with 15% trichloroacetic acid. The mixture was centrifuged (10,000 $\times$ g for 10 min), and the protein pellet was washed four times with 15% trichloroacetic acid containing 1 mM unlabeled methionine. The last pellet was solubilized with 1 M NaOH and neutralized, and radioactivity was measured in a liquid scintillation spectrometer.

ATPase activity was measured by quantitating the liberation of ³²P_i from [γ -³²P]ATP, as described (24), in the following preparations: (i) sea urchin sperm demembrated in rigor buffer containing 0.1% Triton X-100, pelleted at 850 $\times$ g for 2 min, and washed twice to remove mitochondrial ATPase; (ii) crude dynein I was extracted with 0.6 M KCl from the axonemes prepared from demembrated, twice-washed sea urchin sperm from which heads were removed by sonication followed by differential centrifugation (25); and (iii) rat sperm tails demembrated with 0.1% Triton X-100, pelleted at 15,600 $\times$ g for 4 min, and washed twice to remove mitochondrial ATPase contamination.

Protein concentrations were measured by the method of Lowry *et al.* (26) with bovine serum albumin as a standard.

RESULTS

Effects of Inhibitors of Protein Carboxylmethylation on Motility of Native Sperm. The effects of inhibitors of protein carboxylmethylation on the motility of native sperm from sea urchin are presented in Table 1. The combination of EHNA, L-homocysteine thiolactone, and adenosine abolished motility in these sperm. EHNA alone also inhibited motility. This latter observation was confirmed in rat sperm (Fig. 1) and with rabbit and human sperm (data not shown). The EHNA concentration required for instantaneous immobilization of mammalian spermatozoa was approximately 2- to 3-fold higher than that needed

Table 1. Effect of carboxylmethylation inhibitors on movement of intact sea urchin spermatozoa

Additions	Sperm motility	
	0 min	35 min
EHNA + adenosine		
+ L-homocysteine thiolactone	0	0
L-Homocysteine thiolactone	+	+
Adenosine	+	+
EHNA	0	0

Sperm motility was scored as 0 (no motile cells) or + (majority of cells motile). The concentrations used were 2 mM for EHNA and 1 mM for the other compounds. No effect was observed at lower concentrations.

FIG. 1. Relationship between dose of EHNA and time of incubation necessary to immobilize 100% of rat spermatozoa. Aliquots containing intact rat spermatozoa were incubated in F12/modified Eagle's medium with various concentrations of EHNA, and motility was measured as a function of time. Controls containing no EHNA were motile at all times. These data represent one of three identical experiments.

to produce a similar effect in sea urchin sperm (Table 2). Sea urchin spermatozoa arrested by 2 or 3 mM EHNA regained motility immediately when diluted 1:10, but those from mammals did not (Table 2).

The irreversibility of EHNA-induced inhibition of intact mammalian spermatozoa suggested that this compound may act as a general cytotoxic agent. To test this possibility, we studied the effect of EHNA on protein synthesis (Fig. 2). No direct correlation was found between inhibition of protein synthesis and motility; EHNA concentrations (0.4–1.0 mM) capable of inhibiting motility (Fig. 1) had little or no effect on protein synthesis (Fig. 2).

Inhibition of Protein Carboxylmethylation in Spermatozoa by EHNA. The concentration of EHNA (2.5 mM) causing immediate arrest of motility in sea urchin sperm also suppressed protein carboxylmethylation activity for up to 6 hr (Fig. 3A). In rat spermatozoa, EHNA (5 mM) caused the instantaneous arrest of motility while inhibiting protein carboxylmethylase activity only 20% over the first 15 min and 80% after 90 min (Fig. 3B). Cycloleucine, an inhibitor of methionine of adenosyltransferase (ATP:L-methionine S-adenosyltransferase, EC 2.5.1.6) (27), also inhibited protein carboxylmethylation in rat spermatozoa (Fig. 3B) but did not have a dramatic effect on rat sperm motility (data not shown). This finding suggested that inhibition of sperm motility by EHNA may be due to a mechanism other than the inhibition of protein carboxylmethylation.

Effect of EHNA on Sperm Motility and ATPase Activity. EHNA inhibited the motility of demembrated, reactivated sea urchin spermatozoa, suggesting that this inhibitor acted directly on a protein of the flagellar axoneme. The arrest of motility induced by increasing concentrations of EHNA was reversed by corresponding increases in ATP concentration (Fig. 4). These observations suggested that EHNA inhibited the axonemal dynein ATPase activity on which motility depends (28–31). When this enzyme activity was examined in demembrated,

Table 2. Minimal concentration of EHNA required to immobilize 100% of intact spermatozoa instantaneously

Spermatozoa	EHNA, mM	Reversible by dilution*
Sea urchin	1.75	Yes
Rat	4.4	No
Rabbit	6.4	No
Human	6.8	No

* Immobilized cells were diluted 1:10 with artificial sea water (sea urchin) or F12/modified Eagle's medium (mammalian) and inspected in the darkfield microscope for recovery of motility.

FIG. 2. Incorporation of [^{35}S]methionine into proteins of native rat spermatozoa. Motile rat spermatozoa were incubated with L-[^{35}S]methionine, and the radioactivity in the trichloroacetic acid precipitate was determined. (A) Protein synthesis with (■) and without (●) chloramphenicol (50 $\mu\text{g}/\text{ml}$), an inhibitor of mitochondrial protein synthesis. (B) Protein synthesis measured in the presence of various concentrations of EHNA (○, 0.4 mM; △, 1 mM; ◇, 2.5 mM; +, 5 mM) or in the presence of chloramphenicol (50 $\mu\text{g}/\text{ml}$).

reactivated sea urchin sperm, a 50% inhibition was produced by 0.12 mM EHNA in the presence of 0.1 mM ATP (Fig. 4).

Sea urchin sperm tails contain at least three intracellular ATPases: dynein 1, dynein 2, and a mitochondrial ATPase (32–34). Dynein 1 is believed to be the major force-transducing ATPase located in the dynein arms (28, 29, 31). Because dynein 1 represents about 85–90% and dynein 2 about 10–15% of the ATPase activity in sea urchin sperm axonemes (33, 34), the results in Fig. 4 showing that up to 98% of the ATPase activity of demembrated sea urchin sperm was inhibited by EHNA suggest that both dynein 1 and dynein 2 are affected by EHNA.

EHNA inhibition of the dynein ATPase activity of demembrated sea urchin sperm axonemes was compared before and after solubilization with 0.6 M KCl (34, 35). Fig. 5 shows that EHNA acted as a mixed inhibitor of both the axonemal ATPase activity and the partially purified dynein 1 ATPase activity (Fig. 5A and B). These data, together with those in Fig. 4, provided evidence that EHNA blocks motility by acting as a mixed inhibitor of the dynein 1 ATPase activity in the flagellar axoneme. Inhibition of dynein 2, whose role in motility is not understood, was not tested.

EHNA also completely blocked the motility of demembrated, reactivated rat spermatozoa, and the block was overcome by further addition of ATP. A correlation was established, sim-

FIG. 3. Inhibition of protein methylation by EHNA in native sea urchin and mammalian spermatozoa. (A) Motile intact sea urchin sperm were incubated at 16°C with L-[methyl- ^3H]methionine with (●) and without (○) 2.5 mM EHNA. (B) Mammalian spermatozoa were incubated at 37°C with (●) and without (○) 5 mM EHNA. Cycloleucine (△) (12.5 mM) was used as a control because it inhibits methionine adenosyltransferase in mammalian cells. The reactions were stopped by precipitation with trichloroacetic acid, and the radioactive methanol was extracted and measured. Data are presented as the mean of duplicate samples.

FIG. 4. Effect of EHNA on the dynein ATPase activity and the ATP concentration required for reactivation in preparations of demembrated sea urchin spermatozoa. Demembrated rigor wave sea urchin spermatozoa were incubated at 16°C with increasing concentrations of EHNA. The minimal concentration of ATP required to reactivate the spermatozoa at each EHNA concentration was determined (○). In a separate preparation, the axonemal dynein ATPase activity was measured by using [γ - ^{32}P]ATP (0.1 mM) in the presence of increasing concentrations of EHNA at 16°C (●). Data are presented as the mean of duplicate samples.

ilar to that in demembrated, reactivated sea urchin sperm (Fig. 4), between the dose of EHNA and the concentration of ATP required to overcome the inhibitory action of EHNA and reach a "threshold" of reactivation identical to that defined for sea urchin sperm (data not shown). EHNA inhibited the ATPase activity of purified, demembrated, washed rat sperm tails. Mixed inhibition was observed (Fig. 5C) similar to that in sea urchin sperm axonemes (Fig. 5A). Possible contamination of rat sperm tails by mitochondrial ATPase was ruled out by demonstrating that successive washes of demembrated rat sperm tails resulted in a progressive increase of EHNA-sensitive ATPase activity and a concomitant decrease of monoamine oxidase activity (data not shown).

The ATPase of demembrated rat sperm tails had a K_m of 57 μM , which was similar to that of demembrated, washed sea urchin sperm tails (69 μM) and to the value of 50 μM reported by Gibbons and Fronk (35) for *Colobacentrotus atratus*. The sea urchin sperm tail ATPase was inhibited by 55% with 0.25 mM EHNA at 100 μM ATP (Fig. 4) and the rat sperm tail

FIG. 5. Mixed inhibition of axonemal dynein ATPase activity by EHNA. All curves were fitted by the least-squares method. Each point is the mean of duplicate samples. (A) Hydrolysis of [γ - ^{32}P]ATP in the presence (●) and absence (■) of 0.25 mM EHNA. Demembrated, washed sea urchin sperm were incubated for 10 min at 16°C, and the liberation of $^{32}\text{P}_i$ was determined. (B) ATP hydrolysis by partially purified dynein 1 from sea urchin sperm in the presence (●) and absence (■) of 0.18 mM EHNA. Experimental details were the same as for demembrated sea urchin sperm. (C) ATP hydrolysis by purified demembrated rat sperm tails in the presence (●) and absence (■) of 1 mM EHNA. The incubation was at 37°C.

ATPase was inhibited by 55% with 1.0 mM EHNA at the same ATP concentration (Fig. 5C), suggesting that the rat sperm tail ATPase is about 25% as sensitive to EHNA as the sea urchin sperm tail ATPase.

DISCUSSION

Previous studies showing that the oriented motility of bacteria and leukocytes depended on protein carboxymethylation suggested that protein carboxymethylase might have a role in sperm motility. The data presented here indicate that EHNA antagonized the enzyme activity in mammalian as well as in sea urchin sperm. In addition, EHNA unexpectedly was also found to inhibit sperm flagellar dynein ATPase activity. Several observations, reported here, strongly suggest that inhibition of the dynein ATPase rather than inhibition of protein carboxymethylase activity represents the mechanism by which EHNA blocked motility. EHNA inhibited the movement of demembranated, reactivated sea urchin and rat spermatozoa, and this inhibition was reversed by addition of ATP (Fig. 4). Further, protein carboxymethylase is extracted from spermatozoa during demembration because it is a detergent-soluble enzyme (17). Enzyme kinetic measurements demonstrated that the axonemal ATPase and partially purified dynein 1 ATPase from sea urchin sperm tails, as well as the axonemal ATPase from demembranated rat sperm tails, all showed a similar pattern of mixed inhibition by EHNA (Fig. 5). These data do not exclude the possibility that protein carboxymethylase activity has a role in sperm motility. To determine this, further studies are required with other blockers of protein carboxymethylase activity.

The axonemal ATPases of mammalian and sea urchin spermatozoa had similar K_m values as well as similar patterns of mixed inhibition by EHNA. These observations represent biochemical evidence for an evolutionary conservation of the active site of the dynein ATPase. The reason for the irreversibility of EHNA action in native mammalian sperm is not known (Table 2). One possible explanation is a greater permeability of the mammalian sperm membrane to the influx than to the efflux of EHNA. The observation that low concentrations of EHNA block mammalian sperm motility after long lag times (Fig. 1) appears consistent with the notion of a slow accumulation of EHNA within the flagellum until a concentration is reached at which the dynein ATPase is sufficiently inhibited to prevent motility. By contrast, long incubation times do not enhance the ability of low EHNA concentrations to block native sea urchin sperm motility (data not shown), and the inhibitory effect of EHNA on motility is instantly reversed by dilution (Table 2), suggesting that the sea urchin sperm flagellar membrane is freely permeable to EHNA in both directions.

Preliminary data suggest that EHNA may be a relatively specific inhibitor of the dynein ATPase with little effect on either the myosin or the Na^+, K^+ -ATPase.^{||} The relative specificity of EHNA as a dynein ATPase inhibitor, coupled with its ability to cross the cell membrane, could make this compound an important tool for probing the role of putative cytoplasmic dynein(s) (36–39) in mitosis, axoplasmic flow, and other forms of cytoplasmic motility.

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