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DEPRESSION OF HUMAN SPERM MOTILITY BY INHIBITION OF ENZYMATIC METHYLATION*

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Abstract—Alteration of membrane fluidity during enzymatic methylation of membrane phosphatidylethanolamine (PE) and neutralization of negative charges of membrane proteins due to methylation of carboxyl groups may contribute to sperm motility. Therefore, enzymatic phospholipid methylation and carboxymethylation, and the consequences of their inhibition on motility, were studied using human sperm. These studies gave the following results. Human sperm homoganates contained two phospholipid *N*-methyltransferases (PMT) which converted PE to phosphatidylcholine (PC) in the presence of *S*-adenosylmethionine (SAM). The first PMT converted PE to phosphatidyl-*N*-methylethanolamine (PME). In had a K_m of 4.0 μM and a pH optimum of 8.0. The second PMT converted PME to phosphatidyl-*N*,*N*-dimethylethanolamine and PC. It had a K_m of 71 μM and a pH optimum of 10.0. Spermatozoa also contained protein carboxymethylase (PCM) and methyl aceptor protein (MAP). The intracellular levels of *S*-adenosylhomocysteine (SAH), an inhibitor of SAM-mediated methylations, were increased by adding adenosine (100 μM), L-homocysteine thiolactone (L-HCT, 10 μM), and erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA, 10 μM), an inhibitor of adenosine deaminase, to human sperm ejaculates that had been diluted with sodium phosphate buffer at pH 7.4 and 25°. The motility index of each sperm suspension was determined every hour for 4 hr. In the presence of the mixture of adenosine, L-HCT and EHNA, the motility index was depressed by 57%. Under similar conditions, phospholipid methylation was depressed by 48%. Similar experiments were also conducted in the presence of 3-deazaadenosine (Deaza, 80 μM), a selective inhibitor of SAH hydrolase. In the presence of adenosine and L-HCT, Deaza depressed the motility index by 60% and phospholipid methylation by 86%. The potencies of SAH in the inhibition of phospholipid methylation and protein carboxymethylation in sperm homogenates had the following order: PMT I > PCM > PMT II. These observations indicate that the PMT system and/or the PCM–MAP system play a significant role in the regulation of human sperm motility.

Enzymatic methylations, in the presence of *S*-adenosyl-L-methionine as a methyl donor, may play significant roles in cellular functions of spermatozoa. Membrane phospholipid methylation and carboxymethylation may influence sperm motility. Membrane phospholipid methylation has been implicated in transient changes of membrane fluidity and lateral oscillation of one bilayer against the other during biological signal transfer [1, 2]. Changes in membrane fluidity may influence fusion capacity of spermatozoa [3], lateral mobility of proteins on the sperm surface during capacitiation [4], and initiation of bending waves and their propagation along the tail during sperm motility. However, enzymatic phospholipid methylation in spermatozoa has not been demonstrated before now.

Enzymatic protein carboxymethylation has been demonstrated in rat spermatocytes and spermatids [5]. It is also involved in the control of the direction of rotation in bacterial flagella [6, 7] and the regulation of locomotion in rabbit leucocytes [8] and human macrophages [9]. Therefore, there has been speculation that carboxymethylation is associated with sperm motility [5]. Carboxymethylation has been shown to be involved in the exocytosis of secretory vesicles [10–13]. There are no known secretory products for germ cells, but the genesis of the acrosome is analogous to a secretory granule. Therefore, it has been suggested that carboxymethylation could be involved in acrosomal function [5].

The above observations have prompted us to investigate the occurrence of membrane phospholipid methylation and carboxymethylation. Our studies indicate that human spermatozoa contain two phospholipid *N*-methyltransferases (PMT) which convert membrane phosphatidylethanolamine (PE) to phosphatidylcholine (PC) by stepwise methylation. They also contain protein carboxymethylase (PCM) and methyl acceptor protein (MAP). Inhibition of these enzymatic methylations with *S*-

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adenosylhomocysteine (SAH) depressed the sperm motility index.

MATERIALS AND METHODS

Materials. Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) was supplied by the Burroughs Wellcome Co. (Research Triangle Park, NC) L-Methionine, L-homocysteine thiolactone (L-HCT) and adenosine were obtained from the Sigma Chemical Co. (St. Louis, MO). *S*-Adenosyl-L-methionine (SAM) and *S*-adenosylhomocysteine (SAH) were purchased from Boehringer-Mannheim (Indianapolis, IN). Deazaadenosine (Deaza) was supplied by the Southern Reserach Institute (Birmingham, AL).

Several phospholipids were collected for indentification of the methylated phospholipids. Phosphatidylethanolamine, phosphatidyl-*N*-methylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine were obtained from the Grand Island Biological Co. (Grand Island, NY). These phospholipids were derived from egg phosphatidylcholine by the exchange of bases in the presence of phospholipase D. Synethetic β,γ-dipalmitoyl-α-phosphatidylethanolamine and its *N*-methylated derivatives were obtained from the Calbiochem-Behring Corp. (La Jolla, CA).

S-Adenosyl-L-[methyl-^{3}H]methionine (SAM) (64 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA). This preparation of SAM was diluted with 100 mM Tris–glycylglycine buffer to obtain concentrations lower than 1.0 μM. Unlabeled SAM was added to labeled SAM to obtain concentrations higher than 2 μM. L-[Methyl-^{14}C]methionine (50 Ci/mmole) was supplied by the Amersham Corp. (Arlington Heights, IL). All other reagents were of analytical grade.

Spermatozoa. Human ejaculates were collected from normal healthy volunteers, age 20–35 years, who maintained celibacy for 48 hr prior to collection. Each ejaculate was collected in a plastic cup and allowed to liquify at room temperature (25°). An aliquot (0.01 ml) was used for counting cells using a Spencer Bright-line hemacytometer. These ejaculates contained 173 ± 32 million cells/ml and had an average motility index of 1114 ± 91 as described below. For motility studies, each human ejaculate was kept separate and was handled as described in the section on motility index. For enzymatic studies, samples were pooled and cells were separated from the plasma by centrifugation at 1985 *g* for 10 min in a Sorvall RC-2B refrigerated centrifuge. The cells were washed thrice with isotonic saline by centrifugation. The final pellet from each ejaculate was sonicated in the incubation medium for 15 min at 4° using a model 431A cup horn and a model 225R sonicator (Heat-Systems-Ultrasonics, Inc.) with continuous pulse and 60% duty cycle. The resulting sonicate was used as the source of enzymes.

Rat spermatozoa were collected directly from epididymis and prepared as previously described [14]. Frozen bull spermatozoa were obtained from the Tennessee Artificial Breeding Association, washed, and sonicated as described for human spermatozoa.

Assay for phospholipid N-methyltransferases. These enzymes were estimated by assaying ^{3}H-methyl groups transferred from [^{3}H]SAM to the endogenous phosphatidylethanolamine in the membrane. The procedures were minor modifications of those described by Hirata and Axelrod [1] and Sastry *et al.* [15, 16].

The total volume of the reaction medium (150 μl) was composed of a mixture of three aliquots (60 μl, 60 μl, and 30 μl) in Tris–glycylglycine buffer (50 mM) at pH 8. The first aliquot contained the membrane preparation (100 μg protein); the second was Tris–glycylglycine buffer, and the third aliquot (30 μl) contained the substrate, [^{3}H]SAM. The reaction was started by the addition of the third aliquot to a mixture of the first two in a 6 ml tube. The reaction medium was incubated at 37° for 30 min. The reaction was stopped by the addition of 600 μl of trichloroacetic acid reagent (12.5%) which was kept at 4°. The supernatant fraction was siphoned off, and the pellet was extracted with 3 ml of chloroform–methanol (2:1, v/v). The chloroform/methanol extract was washed twice by shaking with 2 ml of 0.1 M KCl in 50% methanol. The aqueous phase was aspirated each time and rejected. One milliliter of the chloroform phase was transferred to a counting vial, and the solvent was evaporated to dryness under a heat lamp at 80°. The residue of phospholipids was dissolved in 10 ml of Aquasol (Amersham Corp.), and the total radioactivity was measured. The remaining chloroform phase was dried over anhydrous Na_2SO_4 and used to determine the ratios of ^{3}H-methylated phospholipids in the chloroform extracts by the method described below.

The chloroform extracts of phospholipids (> 5000 dpm) were concentrated under a stream of nitrogen and were chromatographed on silica gel G plates (Uniplate, Analtech Inc., Newark, DE). Chromatograms were developed in a solvent system comprised of chloroform–propionic acid–*n*-propyl alcohol–water (1:2:2:1, by vol.). The solvent was allowed to move 6.5 inches, and 0.25 inch sections were transferred to counting vials. Aquasol was added, and radioactivity was counted. Authentic samples of phospholipids were chromatographed simultaneously, and their spots were visualized by iodine vapor. The radioactivities in the TLC peaks corresponding to phosphatidyl-*N*-methylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine and phosphatidylcholine were determined and expressed as ratios of the total radioactivity on the TLC plates. These ratios were used to estimate the quantities of the three methylated phospholipids formed during the enzymatic reactions.

Assay for protein carboxymethylase (PCM) and methyl acceptor protein (MAP). In the assay for PCM, protein carboxyl groups were methylated with [^{3}H]SAM (2.4 μM) according to the general scheme described by Diliberto *et al.* [12, 13]. In this assay, albumin (20 mg/ml) was used as MAP. Protein ^{3}H-methyl esters were hydrolyzed, and [^{3}H]methanol was recovered and counted. For the assay of endogenous MAP, albumin was omitted in the above assay.

The total volume of reaction medium (50 μl) was composed of a mixture of three aliquots in sodium

phosphate buffer (pH 6.0). The first aliquot (20 μl) contained the enzyme preparation (protein, 11 μg/incubation tube); the second aliquot (10 μl) contained phosphate buffer with or without methyl acceptor protein (1 mg of albumin); and the third aliquot (20 μl) contained [^{3}H]SAM. The reaction was started by the addition of the third aliquot to a mixture of the first two in a 6 ml tube. The reaction medium was incubated for 15 min at 37°. To stop the reaction and to hydrolyze the protein methyl esters, 250 μl of 0.5 M borate buffer (pH 10) in 1% methanol and 3 ml of isoamylalcohol–toluene (2:3), which was kept at 4°, were added to the reaction mixture. The tubes were capped and allowed to stand for 15 min. The mixture was gently vortexed, allowed to stand for another 15 min, and then centrifuged for 10 min at 3090 *g*. One milliliter of the organic layer was added to 10 ml of Aquasol and counted (A). One milliliter of the organic layer was pipetted into an empty counting vial and evaporated at 80°. Ten milliliters of Aquasol was added to the residue and counted (B). The difference between A and B gave [^{3}H]methanol formed. A boiled enzyme preparation was used as a blank.

Protein determinations. Protein content in the sonicates was established according to the method of Lowry *et al.* [17].

Determination of the motility index of human spermatozoa. The motility indexes of human spermatozoa in the absence and presence of various enzymatic inhibitors, pharmacological agents, and their mixtures were determined. Each ejaculate was subdivided into samples of 0.4 ml each and placed in plastic tubes. Each tube contained an average of (69 ± 13) × 10^6 sperm cells. The pharmacological agents were dissolved in phosphate buffered saline (276 mOsM, pH 7.4). Solutions (0.1 ml) containing the pharmacological agent were added to the tubes containing the sperm samples. Equal volumes of the phosphate buffer were added to the control tubes. The tubes were kept at laboratory temperature. At various times, the motility index of each sperm sample was determined as described below. One drop of each sperm sample (100–200 cells) was placed on a microscope slide, and the motile behavior of spermatozoa was observed using an Olympus Inverted Research Microscope (model IMT) equipped with a turret condenser (for long working distance) and an Hitachi–Shibaden closed circuit TV system consisting of a camera (model HV-62) and a monitor (model VM-910). The viewing surface of the monitor (9 in. diagonal) was divided into twenty rectangles by incorporating a grid into the objective. Observations of the behavior of spermatozoa were made by two or three individuals watching the TV monitor simultaneously and were graded by a double-blind technique. Two variables were measured for each sperm sample: (1) the percentage of spermatozoa showing movement, and (2) the average degree of motility estimated on a scale of 0 to 4. Grades were designated to sperm samples showing the following motility patterns: 0 for spermatozoa standing still; 1 for movement in place; 2 for circular movement or very sluggish movement across the field; 3 for progressive movement with wide, slow, whiplash action of the tails; and 4 for rapid progressive movement with rapid whiplash action of the tails. To determine the index of sperm motility, the percentage of spermatozoa showing motility in each sample was multiplied by the square of the value for degree of motility. The graded score for motility was squared because it was the variable which was significant for capacitation. Further details of this method have been described by Sastry *et al.* [18]. Ejaculates from the same group of volunteers were used to test all inhibitors. Each pair of control and drug-treated samples was from the same ejaculate.

Statistics. Results are expressed as means and standard errors wherever possible. The significance of the difference between the mean values was calculated by Student's *t*-test. A result was considered significant if its P value was less than 0.05.

RESULTS

Phospholipid methylation in human sperm sonicates and identification of methylated phospholipids. Sonicates of human spermatozoa were examined for their abilities to incorporate ^{3}H-methyl groups into phospholipids after incubation with [methyl-^{3}H]SAM. The identity of ^{3}H-methylated phospholipids formed after the sperm sonicates were incubated with [methyl-^{3}H]SAM was determined by TLC. There were three distinct peaks of radioactivity co-chromatographing with PME, PMME and PC in the products when sperm sonicates were incubated with a SAM concentration of 2 μM (Fig. 1). As the SAM concentrations were increased to 200 μM, the relative proportions of PME and PMME decreased and the proportion of PC increased. The three major

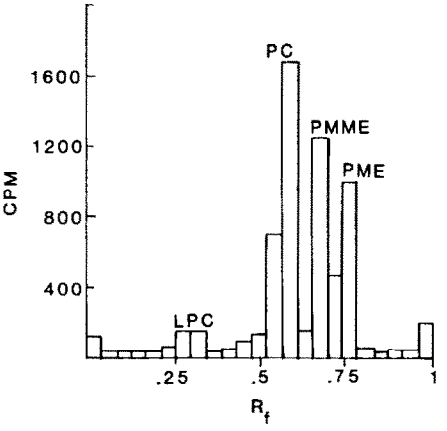

Fig. 1. Separation of methylated phospholipids formed when human spermatozoal homogenates were incubated with various concentrations of [^{3}H]*S*-adenosyl-L-methionine (SAM), by TLC on silica gel G plates in propionic acid–*n*-propyl alcohol–chloroform–water (2:2:1:1, by vol.). The three major phospholipids, phosphatidylcholine (PC), phosphatidyl-*N,N*-dimethylethanolamine (PMME), and phosphatidyl-*N*-methylethanolamine (PME), appear in the "finger print" region of R_f values 0.4 to 0.8. The figures were not corrected for differences in specific activities. The peaks for PME, PMME and PC were not corrected for different numbers of ^{3}H-methyl groups (1, 2 and 3 respectively) incorporated into them. Therefore, this figure should be considered qualitative.

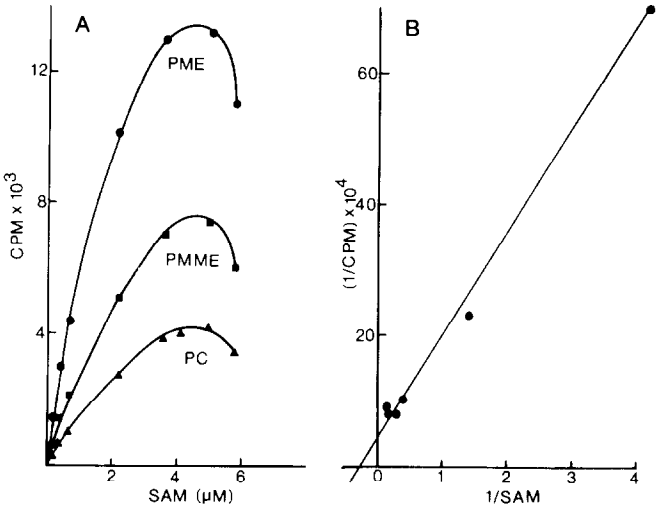

Fig. 2 (A) Formation of methylated phospholipids when human spermatozoal homogenates were incubated with various concentrations of [^{3}H]SAM (0.2 to 6 μM). The major product formed was PME. The curves show molar relationships of the three products formed. The total counts in PMME were divided by 2 and those in PC were divided by 3. PME Includes PME and PME converted to PMME and PC. Similarly, PMME contains PMME and PMME converted to PC. (B) The reciprocal of the initial linear rate for the formation of PME is plotted as a function of the reciprocal of SAM concentration in μM. K_m for PME formation: 4 μM.

phospholipids, PME, PMME and PC, appeared always in the "finger print" region of R_f values 0.4 to 0.8. At concentrations higher than 200 μM, a very prominent peak for PC and only minor peaks for PME and PMME were observed in the finger print region.

The R_f values for PME, PMME and PC were within 0.04 units on different TLC plates. The relative order of their R_f values (PME before PMME before PC) remained the same on every TLC plate. PME, PMME and PC were also identified by high performance liquid chromatography as described by Sastry *et al.* [16].

Effects of low SAM concentrations on phospholipid methylation in human spermatozoa. The enzymes that convert PE to PME in liver and adrenal microsomes have low K_m values [15, 19]. To determine whether sperm enzyme resembles enzymes from other sources, human sperm sonicates were incubated with low concentrations of SAM (0.2 to 6 μM), and the methylated phospholipids were separated by TLC (Fig. 2A). The formation of all three methylated phospholipids increased up to 5 μM SAM. Significant inhibition was observed at 6 μM. This may have been due to accumulation of PME in the biomembrane leaflet, which inhibits the first step in the transmethylation. Liposomes of exogenous dipalmitoyl-PME inhibited total phospholipid methylation in liver microsomes; they did not inhibit solubilized PMT II [15]. Substrate inhibition of step 1 in sperm sonicates could be overcome by increasing the SAM concentration to threshold levels (30–600 μM) for the formation of PMME and PC (see following section). Increasing the SAM concentrations increases the rate of conversion of PME to PMME and PC and, therefore, prevents product inhibition due to PME.

It is difficult to determine an accurate K_m for SAM in the conversion of PE to PME under the above conditions. The apparent K_m was 4 μM (Fig. 2B). It has been reported that Mg^{2+} activates the enzymatic formation of PME from PE. Therefore, formation of PME was studied using dialyzed sperm homogenates and a low SAM concentration. Enzymatic formation of PME was not activated by Mg^{2+} (0.4 to 10 mM) in sperm sonicates.

Effects of high SAM concentrations on phospholipid methylation in spermatozoa. To evaluate whether more than two enzymes were involved in the stepwise methylation of PE, sperm sonicates were incubated with 5–640 μM SAM, and the PC formed was measured. When ^{3}H-methyl groups incorporated into methylated phospholipids were plotted as a function of SAM concentration, a typical rectangular hyperbola was obtained (Fig. 3A). No significant inflection points were observed in the curve. The K_m for the formation of PC was 71 μM (Fig. 3B). The K_m for the formation of PMME (75 μM) was not significantly different from that of PC. Mg^{2+} (0.4 to 10 mM) did not activate the formation of PC.

Enzymatic formation of PME and PC as a function of pH. To evaluate conditions for the formation of PME and PC, the incorporation of ^{3}H-methyl groups into methylated phospholipids was measured using SAM concentrations of 2 and 200 μM and pHs between 6 and 11. At 2 μM, the major product formed was PME, part of which was converted to PMME and PC. At 200 μM, the major product formed was PC. The pH optimum for the formation of ^{3}H-methylated phospholipids was about 8 at 2 μM SAM (Fig. 4A) and 10 at 200 μM SAM (Fig. 4B).

Phospholipid N-methyltransferase activities in spermatozoa. The optimum conditions (K_m for SAM

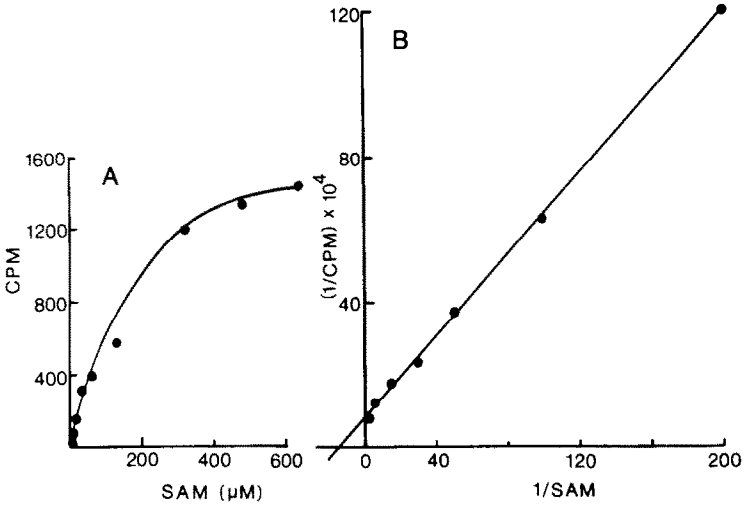

Fig. 3. (A) Formation of phosphatidylcholine (PC) when spermatozoal sonicate was incubated with various concentrations of [^{3}H]SAM (5–640 μM). (B) Reciprocal for the linear rate of incorporation of ^{3}H-methyl groups into PC versus the reciprocal of the SAM concentration in μM. K_m for the formation of PC: 71 μM.

and pH optimum) for the formation of PME and PC by human sperm sonicates were different, suggesting that there are two PMT enzymes in spermatozoa. PMT I activity was measured by the PME formation at a low SAM concentration (1.1 μM). PMT II was measured by the formation of PC at a high SAM concentration (201.1 μM) (Table 1).

Human spermatozoa contained both PMT enzyme activities. No detectable PMT activity was found in cell-free semen. Rat and bull spermatozoa contained lower levels of both enzymes than human spermatozoa.

PCM and MAP in human spermatozoa. The enzyme, PCM, esterifies free carboxyl groups of proteins with [^{3}H]SAM. Human spermatozoa had an MAP content of about 3.5 pmoles of [^{3}H]methanol formed/mg protein. The MAP content of seminal plasma was about 14% of that in spermatozoa (Fig. 5).

Human albumin was shown to be a substrate for PCM, although it was a weaker substrate than the endogenous MAP [12, 13]. Human spermatozoal PCM was capable of methylating human albumin. The total methanol formed in the presence of albumin was about 8 pmoles/mg protein. This indicates that PMC was not saturated with endogenous MAP, and the addition of exogenous albumin saturated PME and increased the [^{3}H]methanol formed.

Influence of methionine, adenosine, L-*HCT, EHNA and their combination on the motility index of human spermatozoa.* Incubation of cells with L-methionine (100 μM), adenosine (100 μM), L-HCT (10 μM) and EHNA (10 μM) increased the intracellular concentrations of SAH, an inhibitor of

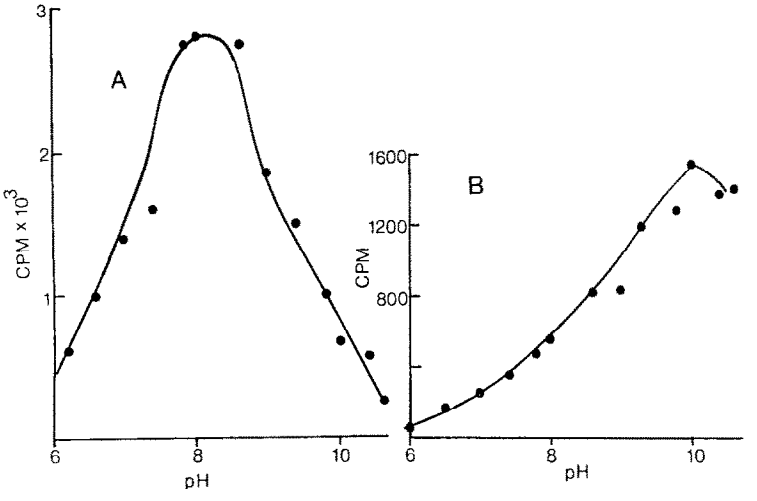

Fig. 4. Incorporation of ^{3}H-methyl groups into phospholipids when human spermatozoal homogenates were incubated with [^{3}H]SAM as a function of pH. (A) SAM concentration: 2 μM; major product formed: PME. (B) SAM concentration: 200 μM; major product formed: PC.

Table 1. Phosphatidylethanolamine *N*-methyltransferase activity in spermatozoal homogenates*

Source of spermatozoal cells	[^{3}H]PME formed at 1.1 μM SAM† [pmoles·(mg protein)$^{-1}$·(30 min)$^{-1}$]	[^{3}H]PC formed at 201.1 μM SAM‡ [pmoles·(mg protein)$^{-1}$·(30 min)$^{-1}$]
Human ejaculate	3.81 ± 0.35§	23.42 ± 2.29§
Rat epididymis	0.32 ± 0.009	1.07 ± 0.08
Frozen bull sperm	0.23 ± 0.007	2.53 ± 0.52

* The three methylated phospholipids which contained ^{3}H-methyl groups, phosphatidyl-*N*-methylethanolamine (PME), phosphatidyl-*N,N*-dimethylethanolamine (PMME) and phosphatidylcholine (PC), were separated by TLC and identified by comparing their R_f values with those of standards. Each value is the mean ± S.E. from six values.

† [^{3}H]PME formed at low concentrations of *S*-adenosyl-L-methionine (SAM) gives an estimate of phospholipid-*N*-methyltransferase I (PMT I) activity.

‡ [^{3}H]PC formed at high concentrations of SAM gives an estimate of phospholipid *N*-methyltransferase II (PMT II) activity.

§ The means for human spermatozoa were higher than the corresponding means for rat and bull spermatozoa, $P < 0.01$.

enzymatic methylations [9]. Incubation of human ejaculates at 21° for 4 hr with L-methionine or adenosine alone did not depress the motility index significantly (Fig. 6, compare bars 1, 2 and 3 from left to right, $P > 0.1$). L-HCT and EHNA depressed the motility index by about 25 and 30% respectively (Fig. 6, compare bars 1, 4 and 5, $P < 0.05$). The greatest inhibition of the motility index (57%) was observed when ejaculates were incubated with a mixture of adenosine, L-HCT and EHNA, when formation of the highest concentrations of intracellular SAH was expected (Fig. 6, compare bars 1 and 7, $P < 0.01$). The motility index was not depressed any further by increasing EHNA concentrations to 80 μM.

The combination of adenosine, L-HCT and EHNA decreased the number of cells with motility (38%, $P < 0.05$) as well as the graded score for motility (14%, $P < 0.05$).

Influence of adenosine, L-HCT, EHNA, and their combination on phospholipid methylation in spermatozoa. The formation of PME, PMME, and PC was measured in the presence of adenosine (100 μM), L-HCT (100 μM) and EHNA (10 μM) individually, and in combination, using sonicated sperm cells and SAM (1 μM). Neither adenosine nor EHNA depressed the formation of methylated phospholipids, PME, PMME, or PC (Fig. 7, compare bars 1, 2 and 4 from left to right in each set, $P > 0.1$). L-HCT alone depressed the formation of methylated phospholipids by about 20–35% (Fig. 7, compare bars 1 and 3 in each set, $P < 0.05$). The highest depression (40–48%) in the formation of methylated phospholipids was observed when all three, adenosine, L-HCT and EHNA, were present in the reaction medium (Fig. 7, compare bars 1 and 5 in each set, $P < 0.01$). These are conditions under which the phospholipid methylation inhibitor, SAH, accumulates in the tissues [9].

Influence of adenosine, L-HCT, Deaza, and their combination on the motility index of human spermatozoa. 3-Deazaadenosine is an inhibitor of

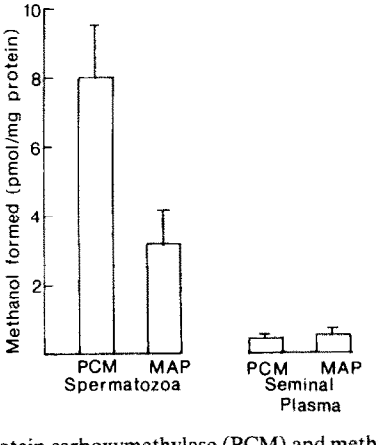

Fig. 5. Protein carboxymethylase (PCM) and methyl acceptor protein (MAP) in human spermatozoa. Human sperm ejaculates were centrifuged at 1475 *g*. The cell pellet was washed thrice with saline. The sperm cells were sonicated at 4° in water. The sonicate was added to an equal volume of buffer at pH 6.0 and aliquots were analyzed for PCM. Human albumin (20 mg/ml) was used as the exogenous MAP.

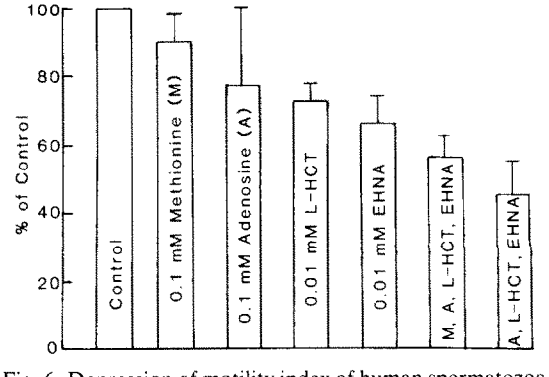

Fig. 6. Depression of motility index of human spermatozoa by incubation with methionine (M), adenosine (A), L-homocysteine thiolactone (L-HCT) and erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA). The motility index was determined according to the following formula: Motility index = % motile cells × graded score for motility (0 to 4). The motility index was determined after 4 hr of incubation and expressed as a percentage of the control as described by Sastry *et al.* [18]. Each vertical bar is a mean from eight to sixteen values. Vertical lines on the bars represent S.E. The P values are given in the appropriate places in the text.

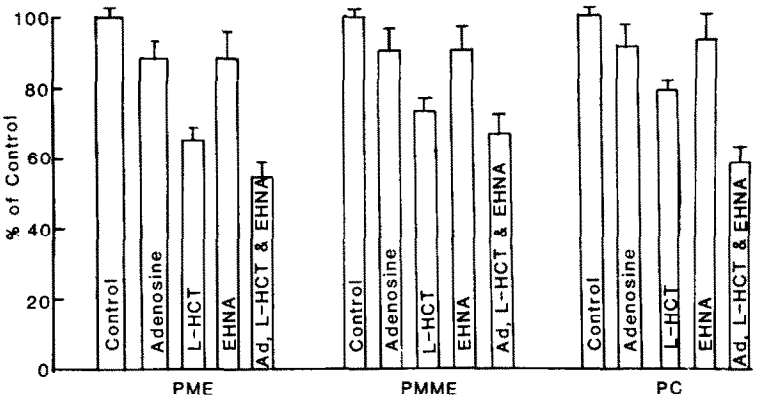

Fig. 7. Inhibition of phospholipid methylation by incubation with adenosine (Ad), L-homocysteine thiolactone (L-HCT) and erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA). Washed sonicated human sperm cells were incubated at 37° with labeled SAM (1 μM) in the presence and absence of Ad, L-HCT and EHNA. Methylated phospholipids formed in the presence of the inhibitors were expressed as percentages of the corresponding controls. Each bar is a mean from four values. The vertical lines on the bar represent S.E.

SAH hydrolase, and treatment of cells with Deaza results in the elevation of SAH, an inhibitor of enzymatic methylations [20]. Incubation of human ejaculates with adenosine (100 μM), L-HCT (10 μM) and Deaza (80 μM) should increase SAH levels in spermatozoa and inhibit enzymatic methylations. Therefore, the effects of Deaza in the presence and absence of adenosine and L-HCT on human sperm motility index were studied. Adenosine alone did not depress the motility index (Fig. 8A, compare bars 1 and 3 from left to right, $P > 0.01$). The slight depression (15–25%) in the motility index due to Deaza alone was not significant (Fig. 8A, compare bars 1 and 2, $P > 0.01$). L-HCT alone depressed the motility index by about 30–35% (Fig. 8A, compare bars 1 and 4, $P < 0.05$). The greatest depression of the motility index (60%) was observed in the presence of a mixture of all three compounds (Fig. 8A, compare bars 1 and 5, $P < 0.01$).

The combination of adenosine, L-HCT and Deaza decreased the number of cells with motility (30%, $P < 0.01$) as well as the graded score for motility (24%, $P < 0.01$).

Influence of adenosine, L-HCT, Deaza, and their combination on phospholipid methylation in spermatozoa. The formation of PME, PMME, and

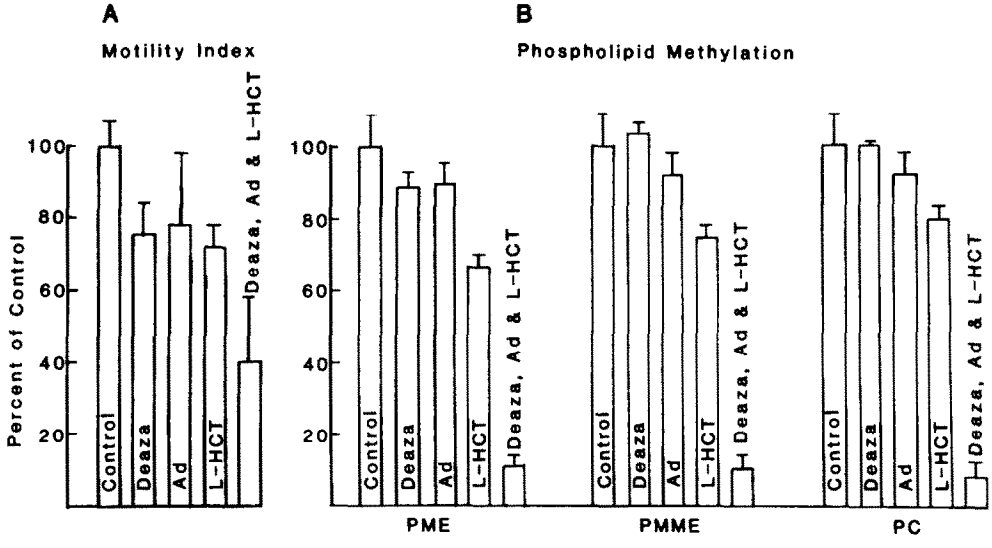

Fig. 8. (A) Depression of motility index of human spermatozoa by incubation with adenosine (Ad), 3-deazaadenosine (Deaza), L-homocysteine thiolactone (L-HCT) and their combination. The motility index was determined as described in the legend to Fig. 6. Each bar is the mean ± S.E. from four to eight determinations. The P values are given at appropriate places in the text. (B) Inhibition of phospholipid methylation by incubation with Ad, L-HCT and Deaza. Washed sonicated human sperm cells were incubated at 37° with labeled SAM (1.1 μM) in the presence and absence of Ad, L-HCT and Deaza. Methylated phospholipids formed in the presence of the inhibitors were expressed as percentages of the corresponding controls. Each bar is a mean from four values. The vertical lines on the bars represent S.E. The P values are given at appropriate places in the text.

Table 2. Inhibition of phospholipid methylation and carboxymethylation in human spermatozoa

Product measured*	SAM concn (μM)	SAH I_{50}† (M)
PME‡	1.2	$(5.9 \pm 0.19) \times 10^{-7}$ (M1)§
	201.2	$(2.5 \pm 0.16) \times 10^{-6}$
PMME‖	1.2	$(6.0 \pm 0.20) \times 10^{-7}$
	201.2	$(2.8 \pm 0.17) \times 10^{-6}$
PC‖	1.2	$(5.9 \pm 0.19) \times 10^{-7}$
	201.2	$(2.7 \pm 0.17) \times 10^{-6}$ (M2)§
Methanol (no exogenous MAP)	2.4	$(1.3 \pm 0.11) \times 10^{-6}$ (M3)¶
Methanol (in presence of exogenous MAP)	2.4	$(1.1 \pm 0.12) \times 10^{-6}$ (M4)¶

* Source of enzymes: human sperm homogenates.
† Mean ± S.E. from four to six determinations.
‡ Product of PMT I. Endogenous PE was the substrate.
§ M1 gives I_{50} for inhibiting PMT I and M2 gives I_{50} for inhibiting PMT II under optimum conditions. The difference between M2 and M1 was significant at $P < 0.01$.
‖ Products of PMT II. Substrate was PME formed by PMT I.
¶ The differences M3–M1, M4–M1, M2–M3, and M2–M4 were significant at $P < 0.01$. The difference between M3 and M4 was not significant at $P < 0.05$.

PC was measured in the presence of adenosine (100 μM), L-HCT (100 μM) and Deaza (80 μM) individually, and in combination using sperm cells and SAM (1.1 μM). Neither adenosine nor Deaza alone significantly interfered with phospholipid methylation (Fig. 8B, compare bars 1, 2 and 3 from right to left in each set, $P > 0.1$). L-HCT alone decreased phospholipid methylation by about 20–30% (Fig. 8B, compare bars 1 and 4 in each set, $P < 0.05$). The greatest decrease (85–90%) in phospholipid methylation was observed when spermatozoa were incubated by a combination of adenosine, L-HCT, and Deaza (Fig. 8B, compare bars 1 and 5 in each set, $P < 0.001$). These are the conditions under which the phospholipid methylation inhibitor, SAH, accumulates in the tissue [20].

Inhibition of phospholipid methylation and carboxymethylation by SAH. SAH inhibited both PMT enzymes (Table 2). To evaluate the selectivity of SAH as an inhibitor of SAM-mediated methylations, I_{50} values for inhibition of enzymes PMT I, PMT II and PCM were studied. SAH inhibited all three enzymes. SAH was found to be four to five times more potent for inhibiting PME formation at a low SAM concentration than PC formation at a high SAM concentration. These observations indicate that SAH was more effective in inhibiting PMT I than PMT II.

SAH inhibited PCM equally with or without MAP, albumin, in the bath. It was about two times more potent for inhibiting PMT I than PCM. It was about two to three times less potent for inhibiting PMT II than PCM.

DISCUSSION

This is the first report to describe the occurrence, properties and significance to motility of PMT in human spermatozoa. The data in this report suggest that there are at least two PMT enzymes in human spermatozoa. The first enzyme, PMT I, converted membrane bound PE to PME. It had a low K_m for SAM of 4 μM and a pH optimum of about 8.0. The second enzyme, PMT II, converted PME to PMME and PC and had a pH optimum of about 10.0. The K_m for SAM (71 μM) for the formation of PC was not significantly different from that of PMME (75 μM). Mg^{2+} had no effect on PMT I or PMT II in human spermatozoa.

Direct evidence for the existence of two PMTs in human spermatozoa will not be available until the two enzyme activities are separated. In view of the small quantities of human spermatozoa available for these studied and the low specific activities of these enzymes in spermatozoa as compared to rat liver, no attempts were made to separate these enzyme activities from the spermatozoal membranes. In previous investigations from our laboratories, PMT II from rat liver microsomes was solubilized and separated from PMT I [15]. This solubilized PMT II utilized exogenous PME to form PMME and exogenous PMME to form PC. The K_m values and pH optima for rat liver PMTs were not significantly different from those of human spermatozoal PMTs. The possibility that the PMME and PC are formed by two different enzymes instead of by one PMT has not been definitely excluded by the present studies on spermatozoa or previous studies on rat liver [15].

S-Adenosyl-L-homocysteine is a potent inhibitor of SAM-mediated methylation. It does not enter cells efficiently. Its concentration in the cells was increased by incubating spermatozoa with adenosine, L-HCT and EHNA, an inhibitor of adodeaminase [9] (Fig. 7). Conditions which increased SAH concentrations inhibited both phospholipid methylation and sperm motility. Sperm motility and phospholipid methylation were also inhibited when sperm cells were incubated with adenosine (100 μM), L-HCT (100 μM) and 3-deazaadenosine (80 μM), an inhibitor of *S*-adenosyl-L-homocysteine hydrolase. It is tempting to speculate that phospholipid methylation plays a significant role in sperm motility. Con-

ditions which increased intracellular levels of SAH did not depress sperm motility completely. The motility index was depressed by only 57–60%. Phospholipid methylation was depressed by 40–90% under the above conditions. These results indicate that SAM-mediated methylation plays only a regulatory role in sperm motility and does not initiate motility.

The concentration of EHNA used in the present investigation (10 μM) depressed the motility index by 25% after 4 hr of incubation. This concentration of EHNA alone did not inhibit dynein ATPases [21]. According to Bouchard *et al.* [21], values for inhibiting dynein ATPases from sea urchin sperm tails and rat sperm tails by EHNA were 0.23 and 1.0 mM respectively. According to their investigations, the inhibition of dynein ATPase was not significant at 10 μM EHNA [21]. They used 0.5 to 6.8 mM EHNA to inhibit dynein ATPase and to immobilize mammalian spermatozoa. These EHNA concentrations were 50–680 times higher than the concentration of EHNA (10 μM) in the present studies. At 10 μM, EHNA is a potent inhibitor of ado-deaminase and, therefore, has a sparing effect on cellular adenosine [9] and, therefore, cellular SAH. PMT I was very sensitive to inhibition by SAH (I_{50}, 0.59 μM). Therefore, the small depression of motility index by 10 μM EHNA could be explained by inhibition of phospholipid methylation *in situ*, which could not be measured by the present techniques. This depression of the motility index in the presence of 10 μM EHNA was enhanced in the presence of L-HCT and adenosine. These conditions increased the intracellular levels of SAH by several-fold [9] which inhibited PMT and PCM.

Human spermatozoa also contained PCM and MAP. This work supports earlier work that found PCM and MAP to occur in human spermatozoa [5]. SAH inhibited PCM as well as phospholipid methylation. Its potency for inhibiting the three methylating enzymes in sperm homogenates had the following order: PMT I > PCM > PMT II. There was only a 2-fold difference in I_{50} values of SAH for inhibiting PMT I and PCM. These studies do not eliminate a role for PCM in sperm motility. There is a deficiency of PCM in immotile spermatozoa from infertile men [22]. There have been no studies on PMT enzymes in immotile spermatozoa from infertile men. The relative roles of PMT and PCM systems cannot be assessed until selective inhibitors of these systems become available.

The spermatozoan tail consists of an axoneme composed of a 9 + 2 pattern of microtubules surrounded by a portion of cell membrane. A sliding microtubule hypothesis has been postulated for the generation of waves of bending in spermatozoa [23, 24]. Microtubules do not change in length, but they slide during motility of spermatozoa of all species including human [25, 26]. This microtubular sliding should be coordinated with the cell membrane. Bending and wave formation during sperm motility make it necessary for the two bilayers of the cell membrane to undergo localized lateral microoscillation against one another. Changes in membrane fluidity facilitate the lateral movement of bilayers [1, 27]. Transient accumulation of PME in the membrane bilayer increases membrane fluidity [1, 16]. The occurrence of two PMTs in spermatozoa suggests that these enzymes may regulate transient membrane fluidity changes during sperm motility.

There is substantial evidence to indicate that sperm motility is regulated by acetylcholine. Spermatozoa contain acetylcholine, choline acetyltransferase, acetylcholinesterase, cholinergic receptor, and contractile proteins [14, 28–30]. Inhibition of acetylcholine synthesis depresses sperm motility [18]. Extracellular Ca^{2+} is necessary for sea urchin sperm motility [31]. Acetylcholine is known to depolarize smooth muscle and skeletal muscle membranes and to cause Ca^{2+} influx in skeletal and smooth muscle cells [32, 33]. Acetylcholine may cause analogous effects in spermatozoa. In the plasma membrane of ram spermatozoal tails, two systems capable of transporting Ca^{2+}, Na^{+}/Ca^{2+} antiporter, and Ca^{2+}–Mg^{2+} ATPase, have been demonstrated [34, 35]. Although there is no direct evidence that phospholipid methylation influences these processes in sperm tails, it has been demonstrated that phospholipid methylation affects Ca^{2+} efflux in erythrocytes [36]. The efflux of Ca^{2+} is regulated by Ca^{2+}-ATPase. This enzyme is enclosed by an annulus of phospholipids which are necessary for its function [37]. The maximum increase in ATPase activity was achieved at a low concentration of SAM, when PME accumulated in the membrane. Accumulation of PMT in the erythrocyte membrane increases fluidity [1]. Increased membrane fluidity increases ATPase activity in sarcoplasmic reticulum [38]. All of these observations are consistent with the suggestion that phospholipid methylation may play a significant role in Ca^{2+} entry and exit from the cytoplasm of the sperm cell and thereby influence sperm motility.

The presence of Ca^{2+} in the medium is required for the acrosome reaction to take place [39, 40]. Lanthanum ions, which block transmembrane Ca^{2+} movement, inhibit the acrosome reaction in spermatozoa [41]. Ionophore A23187, which transports Ca^{2+} across biomembranes, facilitates an acrosome reaction in sea urchin sperm [37]. Inhibitors of enzymatic methylation, Deaza (200 μM) and homocysteine thiolactone (250 μM), inhibit the acrosome reaction [42]. It is possible that phospholipid methylation facilitates entry of Ca^{2+} which is required for an acrosome reaction to occur. Although there is no direct evidence with sperm heads, there is a direct relationship between phospholipid methylation and Ca^{2+} entry into mast cells [2, 43].

Increased levels of SAH in the sperm cell inhibit not only phospholipid methylation and carboxymethylation but also nucleic acid methylation. It is suggested that there is modulation of RNA transcription or translation, thus affecting protein synthesis. However, ejaculated human spermatozoa are already highly differentiated and highly motile, and it is doubtful that new protein synthesis which will affect sperm motility takes place.

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