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A Study of cAMP Binding Proteins on Intact and Disrupted Sperm Cells Using 8-Azidoadenosine 3′,5′-Cyclic Monophosphate

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The photoaffinity probe (^{32}P)8-N_3 cAMP was used to label the cAMP binding proteins in washed ejaculated human sperm. Three saturable binding proteins were photolabeled in both intact and disrupted cells with apparent molecular weights of 55,000, 49,000 and 40,000 daltons corresponding to the regulatory subunits of type II and type I cAMP-dependent protein kinase (cAMP-PK) and to an endogenous proteolytic product of the regulatory subunits, respectively. Photoincorporation in the three proteins could be totally blocked by preincubating the cells with cAMP. Cell-free seminal plasma was found to be free of detectable (^{32}P) 8-N_3 cAMP-binding proteins. The 8-N_3 cAMP was also effective in stimulating endogenous cAMP-PK activity in intact and disrupted sperm. A substantial amount of (^{32}P) 8-N_3 cAMP binding to types I and II regulatory subunits and cAMP-PK activity was detected on washed intact cells. Intact cells bound 1.80 pmol of (^{32}P) 8-N_3 cAMP/mg protein and had cAMP-PK activity of 824 units/10^8 cells. Disrupted cells bound 3.95 pmol (^{32}P) 8-N_3 cAMP/mg protein and had a cAMP-PK activity of 2,206 units/10^8 cells. The data presented support the concept of two classes of cAMP receptors being differentially available to externally added (^{32}P) 8-N_3 cAMP and proteases. Cellular membrane integrity and membrane sidedness are discussed as possible explanations for the observation reported.

Key words: membrane sidedness, regulatory subunits, ejaculated sperm, photoaffinity

Abbreviations: 8-N_3 cAMP, 8-azidoadenosine 3′,5′-cyclic monophosphate cAMP, cyclic AMP; SDS, sodium dodecyl sulfate.

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Adenosine 3′,5′-cyclic monophosphate (cAMP) appears to be present in mammalian spermatozoa [1], and in many spermatozoan environments including seminal plasma [2,3] and the female reproductive tract [3]. Although the utilization of extra- or intracellular cAMP by sperm in vivo has not been documented, it is well known that the addition of cAMP analogues or phosphodiesterase inhibitors in vitro increases respiration [4,5] and can induce or maintain motility [4–6]. Researchers have speculated that cAMP metabolism may be important in sperm development and maturation [7], capacitation [8], and the acrosome reaction [9], since these events have been associated either with changes in nucleotide level [1,8,9], or with increases in respiration [10] or motility [7]. cAMP is known to mediate the activity of a class of protein kinases in which the inactive holoenzyme consists of a multimer of two regulatory subunits and two catalytic subunits. The regulatory subunits bind cAMP and in turn release the catalytic subunits, which become enzymatically active [11]. Researchers have identified cAMP-dependent protein kinases in reproductive tissues such as the testis [12,13], vas deferens [13], and spermatozoa [14,15]. In addition, cAMP-stimulated phosphorylation has been reported in rat epididymal [16,17] and ejaculated human [17,18] sperm. Recently Brandt and Hoskins [19] reported a relationship between cell motility and the cAMP-dependent phosphorylation of a protein in bovine epididymal sperm, but other functions of cAMP-dependent protein kinases in sperm metabolism are unknown, as are further relationships between kinases and the cAMP-mediated physiological events mentioned above. Externally facing ATPases [20,21] and protein kinases [16,17] have been reported in sperm and other cells [22–24]. Stephens et al [25] have speculated that one of the multiple forms of spermatozoan phosphodiesterases may also be external. This leads to the obvious question concerning the membrane sidedness of cAMP-binding proteins of sperm and their comparison to the known cAMP receptor sites of cAMP-activated types I and II kinases.

The use of 8-azidopurine nucleotide analogues as photoaffinity probes has been highly productive in the investigation of nucleotide-related phenomena of numerous biological systems. These analogues, 8-N_3 cAMP, 8-N_3ATP, 8-N_3 GMP, and 8-N_3 GTP, have been used to label the catalytic and regulatory subunits of cyclic nucleotide-stimulated protein kinases [26,27], tubulin [28], (Na^+ + K^+) and (Ca^{2+} + Mg^{2+}) ATPases [29,30], and F-ATPases [31]. The present study concerns the characterization of the cAMP receptors in washed ejaculated human sperm and presents evidence that the major cAMP receptors represent the regulatory subunits of cAMP-dependent protein kinases. We also present data indicating that a portion of these binding proteins and cAMP-stimulated protein kinase activity can be found on the outer surface of the spermatozoa as they are isolated, using standard techniques.

MATERIALS AND METHODS

Sperm Preparation

Semen was collected after 48 h abstinence, by masturbation, and allowed to liquefy for 30–45 min at 20°. The sperm were separated and washed three times by centrifugation (620g for 10 min, 20°C) using Krebs Ringers phosphate buffer (NaCl, 120 mM; NaH_2PO_4, 16 mM, KCl, 5 mM; $CaCl_2$, 0.4 mM; KH_2PO_4, 1 mM; $MgSO_4$, 1 mM; pH 7.5). For protein kinase assays sperm were washed in STE

buffer (sucrose, 340 mM, Tris, 5 mM; EDTA, 1 mM; pH 7.5). Cell condition was determined throughout the washing procedures by their ability to exclude Eosin Y in a 0.5% aqueous solution. Incorporation of the dye is generally thought to indicate dead cells. A maximum of 30% of the sperm incorporated the dye after three washes. When necessary, intact sperm were brought to 4°C by submerging the tube containing the sperm in a 20°C water bath and refrigerating it for 1.5 h. The sperm were either (a) gently resuspended in five volumes of the appropriate buffer and passed through loosely packed glass wool; (b) hypotonically disrupted by resuspension in five volumes of 10 mM phosphate buffer (K_2HPO_4, 5 mM; KH_2PO_4, 5 mM; ph 7.5) followed by sonication (Bronwill Biosonik, power 70, tune +4) for two 30-sec periods separated by 30 sec on ice; or (c) sonicated without hypotonic treatment. Protein concentrations were determined by BioRad protein assay (BioRad).

Photoaffinity Labeling

(^{32}P)8-N_3 cAMP was synthesized by a previously reported procedure [26]. Unless otherwise noted, the equivalent of 40 μg of protein from intact or disrupted sperm in Krebs Ringers phosphate buffer, pH 7.5, was incubated in porcelain planchets with (^{32}P)8-N_3 cAMP for 5 min at 4°C or 37°C in a total volume of 75 μl. The analogue solution was then photolyzed for 10 min at the incubation temperature using a UVS-54 Mineralite lamp at a distance of 1 cm [26].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Photolabeled sperm solutions were heated in sealed tubes at 90°C for 5 min with a protein solubilizing media (1 μl/μg protein) containing sucrose, 25%; Pyronin Y, 2.5 mg%; SDS (sodium dodecyl sulfate), 2.5%; dithiothreitol 23 mg/ml; β-mercaptoethanol, 40 μl/ml; EDTA, 2.5 mM; and Tris, 2.5 mM (pH 8.0). A 10.0–13.5% linear gradient polyacrylamide slab gel and a 4% acrylamide stacking gel, both containing 0.1% SDS, were prepared as previously described [26]. Electrophoresis, autoradiography, and quantification of (^{32}P)8-N_3 cAMP incorporation were done as previously described [26]. Molecular weights of (^{32}P)8-N_3 cAMP-incorporating proteins were estimated by comparison of a log molecular weight versus a migration distance plot of commercial protein standards (BioRad).

Protein Kinase Assay

Protein kinase activity was determined at 37°C , pH 6.8, in a reaction mixture (total volume 0.2 ml) containing final concentrations of sucrose, 204 mM; $MgCl_2$, 3.6 mM; K_2HpO_4, 1.5 mM; KH_2PO_4, 1.5mM; mixed histones, 2.22 mg/ml; (^{32}P)-ATP, 80 μM; and where added, either cAMP or 8-N_3 cAMP, 3.25 μM. The assay was started by the addition of 0.04 ml sperm solution containing 5×10^5 to 5×10^6 cells, and stopped after 5 min by transferring 65 μl of the reaction mixture into cold 10% TCA (trichloroacetic acid). Following incubation on ice for 30 min, the solutions were passed through glass fiber filters (Whatman GF/C), which were subsequently washed with 10 ml cold TCA, dried, and analyzed, using liquid scintillation spectrometry. cAMP-dependent and independent (^{32}P)-PO_4 incorporation was computed by subtraction of a blank, containing sperm but no histone. All experiments were repeated at least three times.

RESULTS

Photoaffinity Labeling of Ejaculated Human Sperm

Washed intact sperm incorporated over 90% of the total bound (^{32}P)8-N_3 cAMP into three proteins with apparent molecular weights (± 5%) of 55,000, 49,000 and 40,000 daltons, as determined by SDS-PAGE (Fig. 1). Photoincorporation could be blocked by preincubating the cells with low levels of cAMP (Fig. 2), indicating that these were cAMP-specific receptors. Nonspecific binding of the analog was negligible. Sperm-free seminal plasma did not contain detectable (^{32}P)8-N_3 cAMP-binding proteins (Fig. 4). Comigration of photolabeled sperm proteins were identical, as analyzed by this method. The 40,000 Mr protein is the primary endogenous proteolytic product of the regulatory subunits [26].

Intact cells photolabeled at 4°C incorporated 1.80 ± .38 (SEM) pmol (^{32}P) 8-N_3 cAMP/mg protein (Table I). Thirty-seven percent of the label was found in the 55,000 Mr protein, 49% in the 49,000 Mr protein, and 14% in the 40,000 Mr protein. Freeze-thawing, sonication, or hypotonic treatment of these cells resulted in a significant increase (Student's t-test; $P < 0.01$; $df = 10$) in total analogue binding to 3.66 ± 1.33 (SEM) pmol/mg sperm protein. Binding proportions in disrupted cells were 18% in the 55,000 Mr protein, 60% in the 49,000 Mr protein, and 23% in the 40,000 Mr protein. In comparison with photoincorporation in whole cells, this represented an actual decrease in binding of about 5% in the 55,000 Mr protein, and increases of 150% and 230% in the 49,000 Mr and 40,000 Mr proteins, respectively. Intact and disrupted cells photolabeled at 37°C showed a similar relationship to cells labeled at 4°C, but total analogue incorporation increased about 25%. Photolabeling at 37°C also shifted the binding in individual subunits, decreasing the relative amount of incorporation in the 55,000 Mr protein, and increasing the 40,000 Mr protein binding.

Although mammalian sperm contain several forms of phosphodiesterases [25], no hydrolysis of 8-N_3 cAMP was observed when intact or disrupted cells were incubated with the analog for 1 h at 4°C or 37°C. Under identical conditions, disrupted cells degraded a maximum of 2.4×10^7 moles of (^{3}H)cAMP to (^{3}H)cAMP/10^8 sperm as determined by thin-layer chromatography.

Dependence of Photoincorporation on (^{32}P)8-N_3 cAMP Concentration

Figure 3 illustrates that the incorporation of (^{32}P)8-N_3 cAMP by the 55,000 and the 49,000 Mr proteins of disrupted sperm was dependent on the concentration of the analogue in the reaction mixture and displayed saturation effects. Sonically disrupted sperm were incubated for 5 min at 4°C with concentrations of (^{32}P) 8-N_3 cAMP between 0.01 μM and 0.5 μM. Both receptor proteins appear to be maximally photolabeled at analogue concentrations between 75 and 150 nM.

Susceptibility of (^{32}P)8-N_3 cAMP-Binding Proteins of Intact Sperm to Washing

Intact sperm were repeatedly washed by centrifugation and resuspension without a detectable loss of (^{32}P)8-N_3 cAMP-binding ability (Fig. 4). In fact, photoincorporation in the 40,000 Mr protein increased, whereas a significant decrease in the 55,000 Mr receptor occurred with repeated washing, indicating increased proteolysis stimulated by centrifugation or washing.

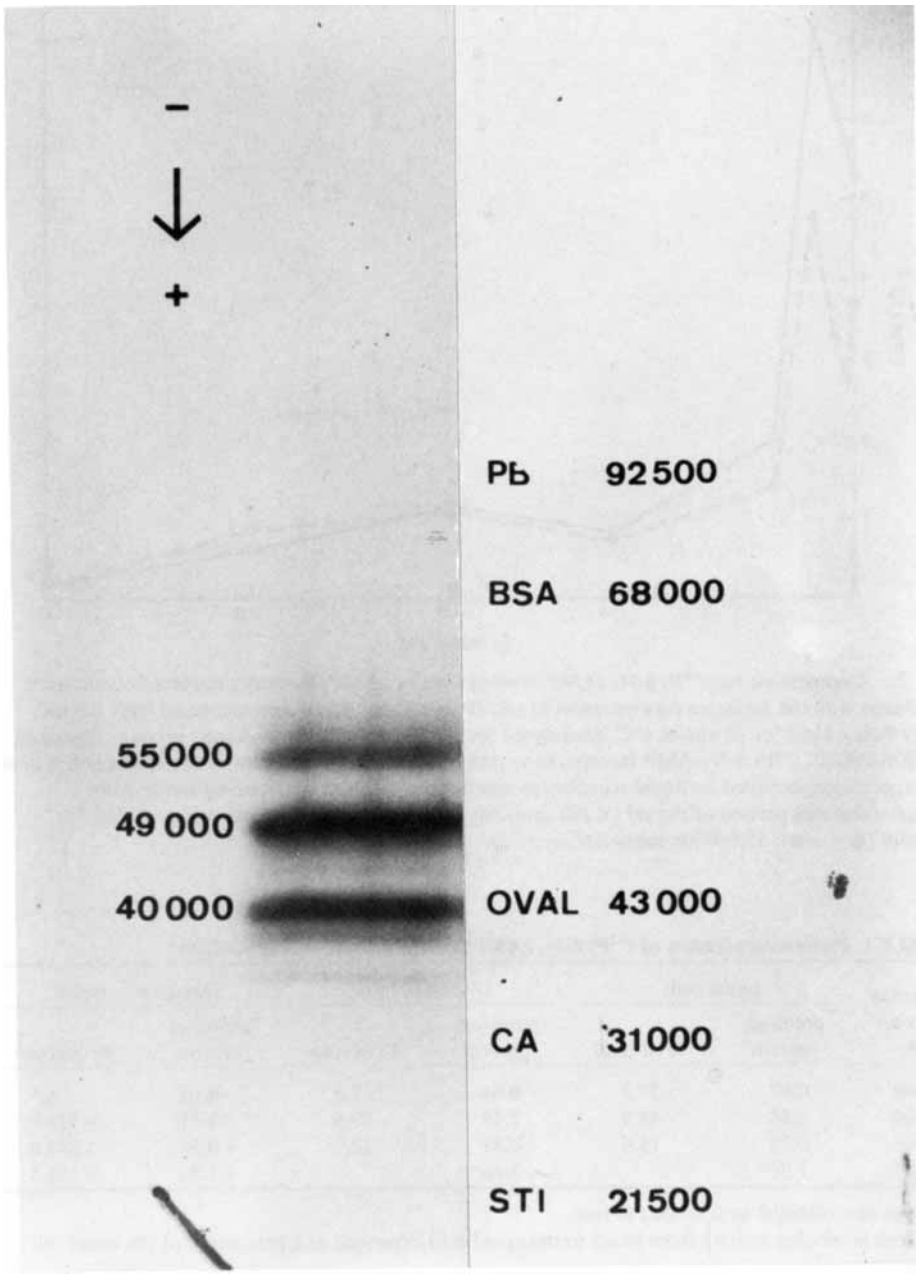


Fig. 1. Photolabeling of ejaculated human sperm with (^{32}P) 8-N_3 cAMP. Washed sperm were sonically disrupted then incubated with 100 nm (^{32}P)8-N_3cAMP for 5 min at 4°C, photolysed for 10 min, solubilized, and their proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The autoradiograph from the dried slab gel is shown with the relative mobilities of the molecular weight standards. Pb = Phosphorylase b, BSA = Bovine Serum Albumin, OVAL = ovalbumin, CA = Carbonic Anhydrase, STI = Soybean Trypsin Inhibitor.

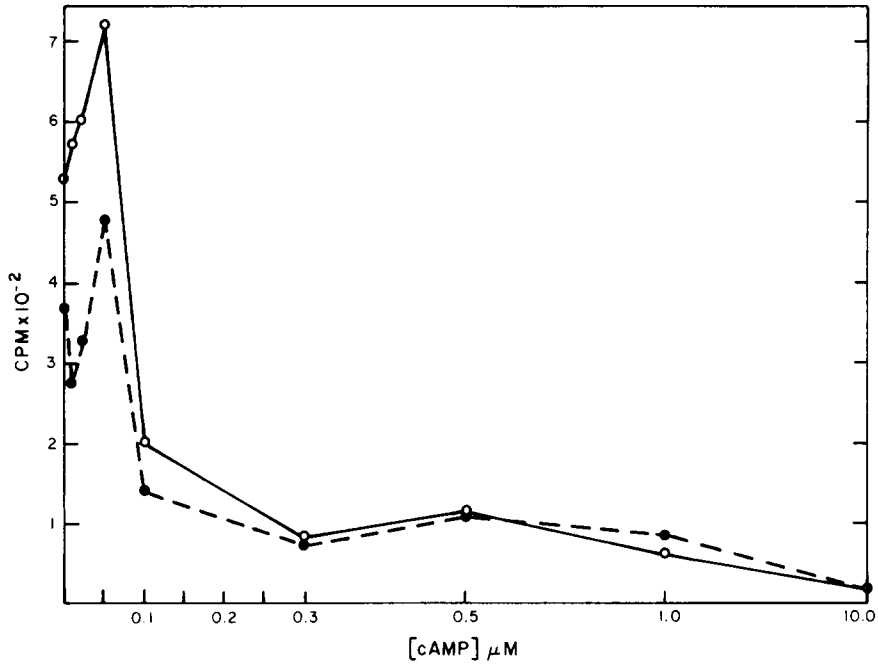


Fig. 2. Competition for (^{32}P) 8-N_3 cAMP binding sites by cAMP. Sonically disrupted sperm were incubated with the indicated concentration of cAMP at 4°C for 5 min then incubated with 100 nM (^{32}P) 8-N_3 cAMP for 10 min at 4°C, photolysed for 10 min, solubilized, and their proteins separated by SDS-PAGE. (^{32}P) 8-N_3cAMP incorporating proteins were sliced from the dried gel and radioactive incorporation quantified by liquid scintillation spectroscopy. A blank containing a slice from a nonphotolabeled portion of the gel (< 100 cpm) has been subtracted from each value. 49,000 Mr subunit (●– –●), 55,000 Mr subunit (○——○).

TABLE I. Photoincorporation of (^{32}P) 8-N_3 cAMP Into Intact and Disrupted Cells*

Receptor protein M_r	Intact cells		Disrupted cells		Disrupted – Intact	
	pmol/μg protein	% of total	pmol/μg protein	% of total	pmol/μg protein	% change[a]
55,000	0.67	37.2	0.64	17.5	−0.03	−4.5
49,000	0.88	48.9	2.19	59.8	+1.31	+148.9
40,000	0.25	13.9	0.83	22.7	+0.58	+232.0
Total	1.80[b]		3.66[b]		+1.86	+103.3

*Sperm photolabeled as described in text.
[a]Change in binding activity from intact to disrupted cells expressed as a percentage of the intact cell value.
[b]Means significantly different (Student's t-test $P < 0.01$; df = 10).

In contrast, intact cells which were first photolabeled, then washed, showed a dramatic decrease in the amount of label seen on the autoradiograph. In Figure 5, lane 1 represents proteins from intact photolabeled cells. Lanes 2 and 3 represent proteins from intact cells that were first incubated with (^{32}P)8-N_3 cAMP, photolyzed and then centrifuged (lane 2), or centrifuged before photolysis (lane 3). Lanes 4 and

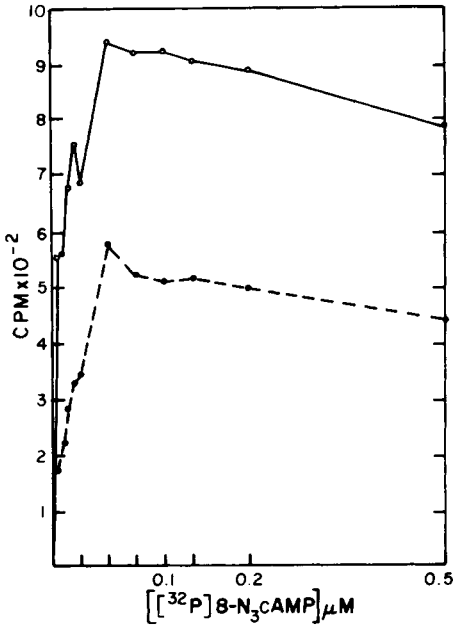


Fig. 3. Concentration dependence of 8-N_3 cAMP labeling. Sonically disrupted sperm were incubated with the indicated concentrations of (^{32}P) 8-N_3cAMP for 5 min at 4°C, photolysed for 10 min, and their proteins separated by SDS-PAGE. The dried gel was sliced and radioactive incorporation into the photolabeled proteins quantified by liquid scintillation spectroscopy. A blank containing a slice from a nonphotolabeled portion of the gel (< 100 cpm) has been subtracted from each value. 49,000 Mr subunit to (○——○), 55,000 Mr subunit (●– – ●).

5 represent the supernatants from the washings of the cells electrophoresed in lanes 2 and 3. UV irradiation was eliminated as the cause of binding protein dissociation from the cell because labeled receptors were also found in the supernatant fraction of (^{32}P)8-N_3 cAMP-incubated cells which were washed before photolysis (Fig. 5, lanes 3 and 5). Reduction of labeling in the washed intact cells and the recovery of the major labeled proteins were susceptible to removal by centrifugation after binding cyclic mucleotide, and therefore suggests that they were exposed to the external environment.

Trypsin Degradation of (^{32}P)8-N_3 cAMP-Binding Proteins

To investigate the location of the cAMP-binding proteins further, intact photolabeled cells were subjected to trypsin proteolysis. Over a period of 30 min at 4°C , a progressive proteolysis of the photolabeled 55,000 and 49,000 Mr proteins and a concomitant increase in lower molecular weight degradation products were observed (Fig. 6A). The 40,000 Mr-binding protein, although a degradation product, is not a tryptic product but is also proteolysed by this treatment. An analysis of the Coomassie blue stained gel used to produce this autoradiograph indicated little detectable degradation of the bulk of the cellular proteins during the incubation period.

Figure 6B is an autoradiograph in which intact sperm were first incubated with (^{32}P)8-N_3 cAMP at 4°C for 1 min to decrease possible transmembrane

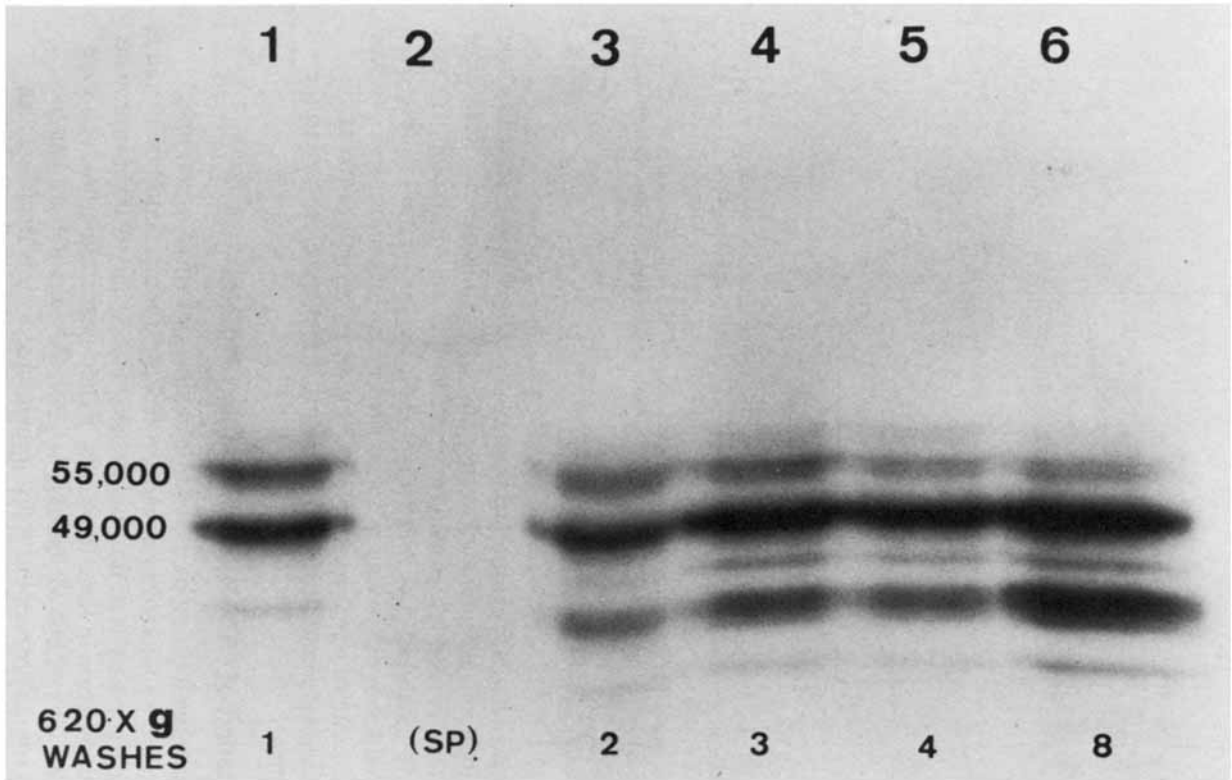


Fig. 4. Effect of centrifugation on the ability of proteins from intact sperm to bind (^{32}P) 8-N_3 cAMP. Ejaculated sperm were centrifuged at 620g and resuspended in 5 ml of fresh buffer at 20°C the indicated number of times before incubation with 100 nM (^{32}P) 8-N_3 cAMP at 20°C for 5 min and photolysis for 10 min at 20°C. Seminal plasma (lane 2) was collected from the first centrifugation, recentrifuged at 18,000g for 15 min at 4°C to remove remaining cells and photolabeled with 100 nM (2P) 8-N_3 cAMP. Proteins were then separated by SDS-PAGE, and the above autoradiograph was produced from the dried gel.

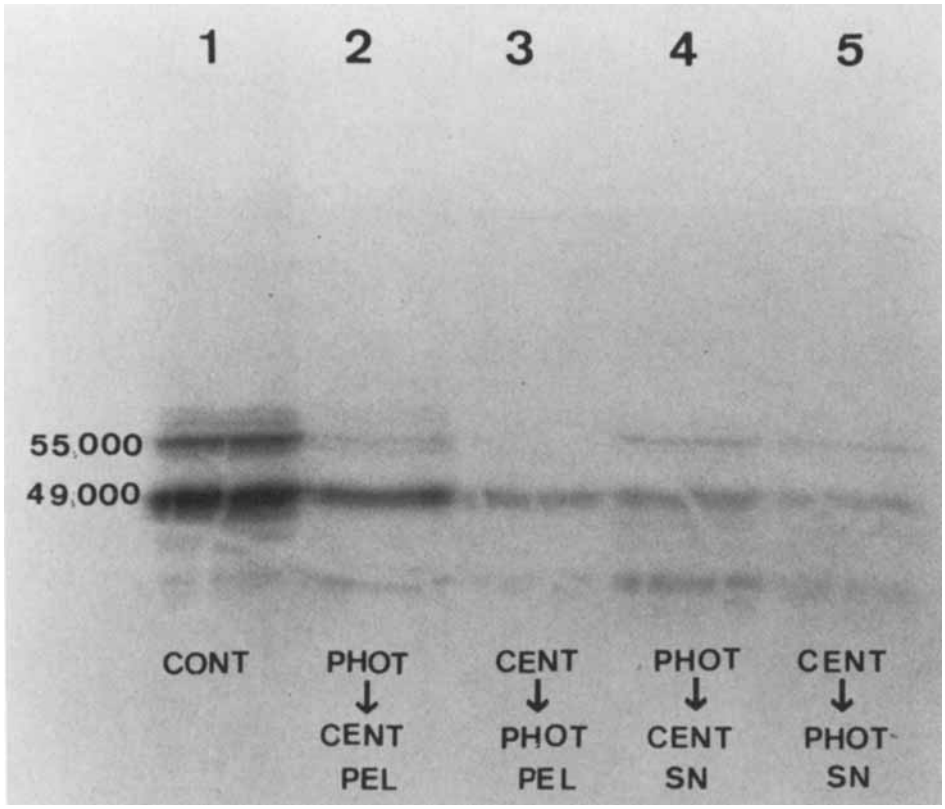
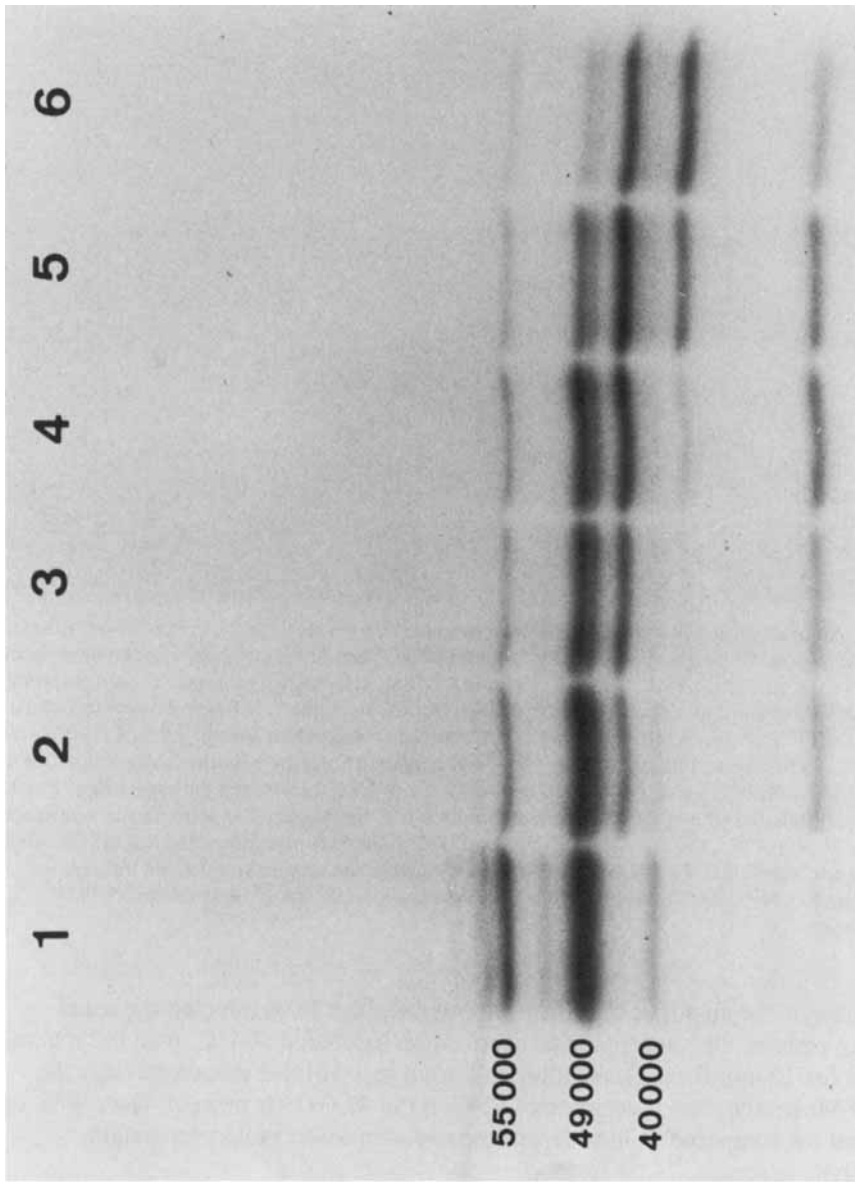


Fig. 5. Autoradiograph of a dried SDS-polyacrylamide slab gel showing the effects of centrifugation on (^{32}P) 8-N_3 cAMP binding proteins after photolabeling. Lane 1: Whole washed sperm photolabeled with 100 nM (^{32}P) 8-N_3 cAMP, 5 min, 4°C. Lane 2: Whole cells treated as in lane 1, then suspended in buffer and centrifuged at 620g, 15 min, 20°C, after photolysis. Lane 3: Whole cells were incubated with 100 nM (^{32}P) 8-N_3 cAMP, 5 min, 4°C, but were then resuspended and centrifuged at 620g, 15 min, 20°C before being photolysed. Lane 4: The supernatant fraction from the centrifugation of cells in lane 2 was recentrifuged at 18,000g, 10 min, 4°C, the pellet discarded and the supernatant fraction proteins precipitated with 10% PCA (percloric acid) at 0°C for 30 min. The solution was centrifuged at 18,000g, 30 min, 4°C , and the pellet dissolved in PSM. Lane 5: A procedure identical to that carried out between lanes 2 and 4 was used to harvest proteins from the supernatant derived through the treatment of cells in lane 3 except that the sperm-free supernatant was photolysed before PCA precipitation.

migration of the analogue and then photolyzed (lane 1), producing the usual labeling pattern. The photolabeled sperm were incubated at 4°C with 0.2 mg/ml trypsin for 15 min (lane 2), resulting in a total loss of label associated with the 55,000 Mr protein and a decrease of 95% in the 49,000 Mr protein. Over 90% of the label (as compared to lane 1) was recovered in lower molecular weight proteolytic products.

Photolabeled, trypsinized cells, represented in lane 2, were further treated with Soybean Trypsin Inhibitor (0.32 mg/ml) and rephotolabeled with 100 nM (^{32}P)8-N_3 cAMP (lanes 3 and 4). This produced only a small amount of photoincorporation in the 49,000 and 55,000 Mr-binding proteins (25% of lane 1), indicating that the majority of accessible binding proteins were degraded by trypsinization.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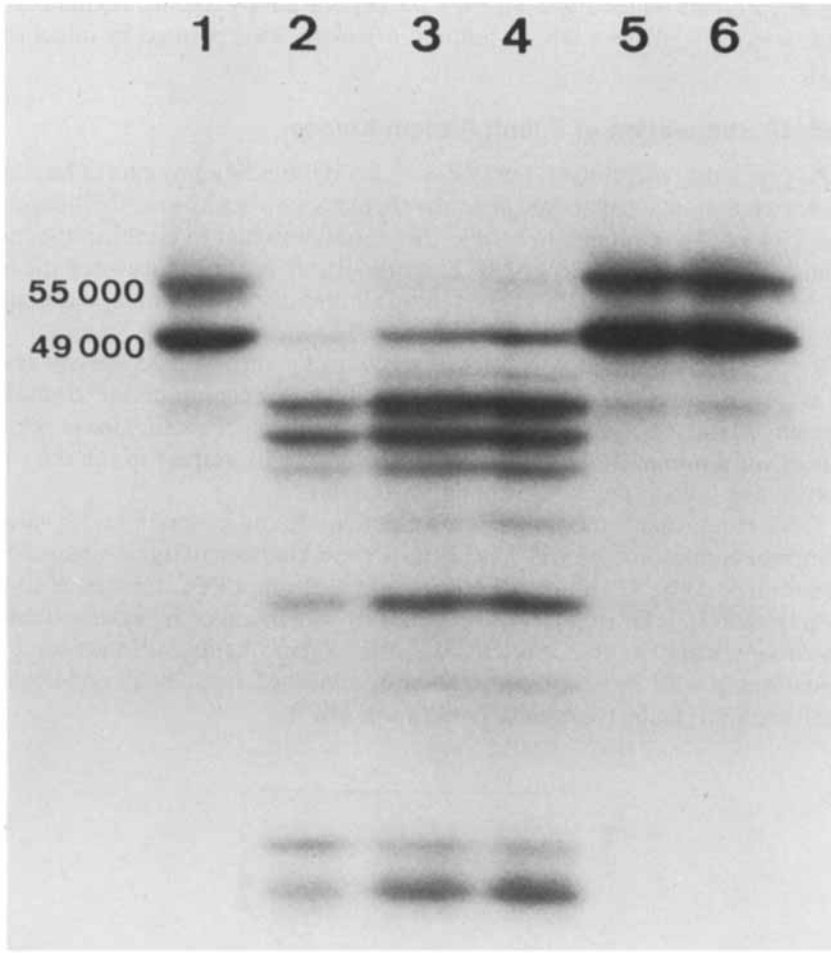

Fig. 6. Effect of trypsin treatment of photolabeled whole sperm. A) Lane 1: Whole washed sperm incubated with 100 nM (^{32}P) 8-N_3 cAMP for 1 min, 4°C, then photolysed for 10 min, 4°C. Lanes 2-6: Whole sperm photolabeled as in lane 1, then exposed to trypsin (0.2 mg/ml) for the indicated times. PSM was added and the samples heated to 90°C for 5 min to stop the reaction. Low molecular weight degradation products were allowed to run off of the gel to improve resolution. B) Lane 1: Washed whole cells photolabeled for 1 min, 4°C , with 100 nM (^{32}P) 8-N_3 cAMP. Lane 2: Cells treated as in lane 1 then incubated with trypsin (0.2 mg/ml) for 5 min, 4°C. Lanes 3 and 4: Cells treated as in Lane 2 then incubated with trypsin inhibitor (0.32 mg/ml and 100 nM (^{32}P) 8-N_3 cAMP for 1 min (lane 3) and 5 min (lane 4) at 4°C and rephotolysed. Lanes 5 and 6: Cells treated as in lane 2 then trypsin inhibitor (0.32 mg/ml) was added and then cells disrupted by hypotonic treatment. The cells were then rephotolabeled with 100 nM(^{32}P) 8-N_3 cAMP for 1 min (lane 5) and 5 min (lane 6) at 4°C, and rephotolysed.

However, when photolabeled, trypsinized cells (from the same pool as in lane 2) were treated with Soybean Trypsin Inhibitor, hypotonically disrupted, and rephotolabeled, a large increase of photoincorporation occurred in the 55,000 and 49,000 Mr proteins (lanes 5 and 6). Photoincorporation by the two regulatory subunits was 65% greater than the amount originally incorporated by intact cells (lane 1).

8-N_3 cAMP Stimulation of Sperm Protein Kinase

As previously mentioned, the (^{32}P)8-N_3 cAMP-binding proteins in human sperm correlate in molecular weight to the regulatory subunits of cAMP-dependent protein kinases. To substantiate further this correlation and to establish the biological mimicry of cAMP by 8-N_3 cAMP in spermatozoa, we have measured the activity of cAMP-dependent protein kinases in intact and disrupted human ejaculated sperm.

In all situations tested, 8-N_3 cAMP stimulated protein kinase activity as effectively as cAMP (Fig. 7). Therefore, to eliminate the problem of cAMP degradation by phosphodiesterases, 8-N_3 cAMP was used in all assays. Protein kinase activity was linear for 5 min at 37°C (Fig. 7) and was linear with respect to the cell concentrations used in the assay (unpublished data).

Table II represents the protein kinase activity found in sperm under various experimental conditions. cAMP-dependent protein kinase activity in intact sperm was determined to be 824 units/10^8 cells (1 unit = 1 pmol PO_4 transferred to substrate/min at 37°C). Disruption of these cells by sonication or hypotonic treatment increased kinase activity to 2,206 units/10^8 cells. Preincubation of intact or disrupted sperm with Protein Kinase Inhibitor abolished virtually all cAMP-dependent and independent kinase activity (Table II).

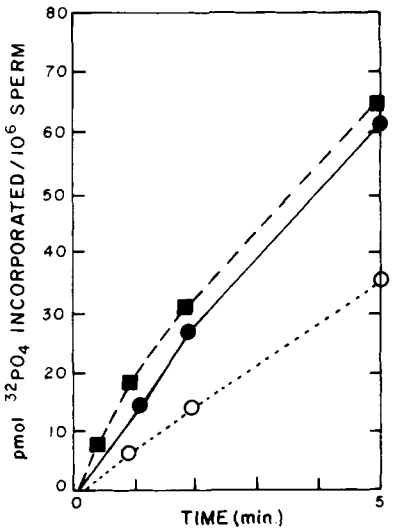

Fig. 7. Results of cAMP-dependent protein kinase assay performed as described at 37°C, pH 6.8, with cAMP or 8-N_3 cAMP. Aliquots of the assay mixture were added to cold 10% TCA at the indicated times, allowed to precipitate for at least 30 min at 0°C, then filtered as described. A sample containing no histone was taken at each time point, and that acitivity was substracted to derive the displayed activities + 8-N cAMP (■ – – ■), + cAMP (●——●), – cAMP (○ – – ○).

TABLE II. Protein Kinase Activities of Sperm Assayed Under Various Conditions Described in Text

Sperm treatments	Sperm protein kinase activities ± SD[a]		
	cAMP-dependent	cAMP-independent	No histone
Intact cells	824 ± 650[b]	226 ± 145[c]	138 ± 66[d]
Disrupted cells	2,206 ± 646[b]	467 ± 220[b]	175 ± 36[d]
Intact cells plus protein kinase inhibitor	1	0	Not done

[a]Human ejaculated sperm protein kinase activities expressed as pmoles $^{32}PO_4$ incorporated/min 10^8 cells as explained in Methods.
[b]Student's t-test $P < 0.001$, df = 15.
[c]$P < 0.01$, df = 15.
[d]Not significantly different at $P < 0.05$.

DISCUSSION

It is of prime importance to biologists to ascertain the membrane sidedness of nucleotide protein receptors in all cells. This is particularly true in sperm, where cAMP, generally considered a second messenger, may be acting as a first messenger. The data presented herein support the hypothesis that human spermatozoa, as isolated by widely used techniques, contain cAMP-activated protein kinases in two distinct cellular locations. Washed intact sperm contain the typical intracellular cAMP receptors for types I and II protein kinases (ie, regulatory subunits of approximately 49,000 and 55,000 molecular weight) which are unavailable to (^{32}P) 8-N_3 cAMP and trypsin. Intact cells also contain a population of types I and II kinase regulatory and catalytic subunits, which are readily available to both the photoprobe and trypsin. We feel that our data leave little doubt as to the existence of externally available cAMP-dependent holoenzyme. This raises the question of how and why such a situation exists. Possibilities are that (a) the washing techniques or cellular aging causes membrane damage [21,32] inducing leakage of soluble intracellular enzymes, which preferentially adhere to the external membrane instead of partitioning into the seminal fluid; or (b) this kinase sidedness occurs in situ and is important in the regulation of spermatozoan function.

Possibility (a) is very important from a technical standpoint because the cells are prepared by widely used standard procedures. Some cell damage is observed after washing. However, this percentage was always at least 10% lower than the amount of cAMP-dependent protein kinase accessible in intact cell preparations. If physical damage or aging generated this effect, (^{32}P)8-N_3 cAMP will be of great use in monitoring cellular integrity as affected by isolation and washing techniques or aging. Experiments designed to test this hypothesis are currently in progress.

Possibility (b), that the protein kinases are located on the external face of the sperm membrane in situ, is intriguing because of the multitude of cAMP-associated physiological phenomena in sperm. These include, in part, cell development and maturation [7], capacitation [8], and the acrosome reaction [8,9]. Significantly, each of these events also involves the cell membrane [33–35].

Experimental evidence supporting the concept of external protein kinases in human sperm is based on the ability to photolabel kinase regulatory subunits on intact cells, and several supporting observations can be made from these experiments.

First, although cell disruption results in the dramatic increase of photoincorporation in the 49,000 and 40,000 Mr proteins, no such increase occurs in the 55,000 Mr protein. This is a demonstration of qualitative and quantitative differences between the proteins of intact and disrupted cells, and may indicate functional partitioning of the isoenzymes between the internal and external milieu. A further comparison of photoincorporation in the proteolytically derived 40,000 Mr protein between intact and disrupted cells indicates a disproportionate increase in the titer of this protein in disrupted cells. This corresponds to the expected lack of proteolytic activity in intact cells, thereby substantiating their integrity. A second supportive observation is derived from the ability to photolabel and proteolytically digest one set of cAMP receptors on intact cells, while a second set, which can be exposed by cell disruption, is protected from both the analogue and trypsin. This indicates that the cells were either intact during the photolabeling or, if a portion were damaged, the openings in their membranes were large enough to allow regulatory subunits (Mr = 55,000 and 49,000) to escape, or trypsin (Mr = 23,800) to enter. The latter possibilities are refuted by the fact that during the prolonged trypsin treatment, there was no progressive degradation of the bulk of the cellular proteins. These observations, combined with the facts that catalytic activity was observed on intact cells and that the regulatory subunits can be partitioned into the extracellular matrix by gentle washing of photolabeled intact cells, strongly suggest the existence of external enzymes.

A final supportive argument can be inferred from the observation that sperm-free seminal plasma contains a factor which inhibits cAMP-dependent protein kinase activity. The factor is heat-labile and operates in a dose-dependent manner on the catalytic subunit of the enzyme (unpublished data). Gordon [21] speculated that such an inhibitor might have been responsible for the lack of external phosphatase activity in ejaculated sperm, even though it was present on the plasmalemma of epididymal sperm. The existence of this protein-kinase inhibiting factor might logically imply the existence of protein kinases within the domain of the seminal plasma, and the putative external enzyme of the sperm is a good candidate to be the target for the inhibitory action.

The spermatozoa is a highly differentiated cell, virtually devoid of the normal cytoplasmic component as well as transcriptional ability. The cell must carry out those processes necessary for successful fertilization with pre-existing metabolic machinery. Signals, such as cAMP, that trigger these metabolic sequences may originate externally, residing in the seminal plasma or in the female reproductive tract. Examples of other possible external signals are the adenylate cyclase activator FRE [36], Decapacitation Factor [37], Calmodulin [38], and Forward Motility Protein [7]. Such a system of first messenger-type signals to trigger membrane-associated physiological events would be valuable in eliminating the need for a protein-synthesizing system and, by the absence of a signal, might also serve to prevent fertilization in less than optimal conditions.

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