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Ecto-Cyclic AMP-Receptor in Goat Epididymal Intact Spermatozoa and Its Change in Activity During Forward Motility

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Goat epididymal intact spermatozoa have been shown to possess on the external surface specific receptors that bind with high affinity to exogenous [8-^{3}H]cyclic AMP. The ecto-cyclic AMP-receptor activity was not due to contamination of broken or "leaky" cells, if any. The binding reaction of [^{3}H]cyclic AMP with the receptors was extremely rapid. Uptake of the labeled cyclic AMP to the sperm cytosolic fraction was undetectable. There was little leakage of cyclic AMP-receptors from intact spermatozoa during the binding assays. The binding reaction was proportional to cell concentration, specific and saturable at 250 nM cyclic AMP. The binding of the labelled cyclic nucleotide was nearly completely displaced at saturating concentrations (2.5 μM) of the unlabelled nucleotide. The ecto-receptors showed high specificity for binding to cyclic AMP. The K_d of the binding sites was approximately 1.7×10^{-8} M. The binding interaction was highly sensitive to treatment with proteolytic enzymes: trypsin, chymotrypsin, or pronase (125 μg/ml). Sonication caused a nearly 450% increase of the ecto-receptor activity. The specific activity of the ecto-cyclic AMP-receptor was approximately twofold higher in the vigorously forwardly motile spermatozoa than in the "composite" cells, suggesting that the ecto-receptors may have a role in modulating flagellar motility.

Key words: cell-surface protein, cyclic AMP-receptors, sperm motility, goat spermatozoa

Several lines of studies provided evidence to support the view that protein phosphorylation serves as an important mechanism for the regulation of sperm flagellar motility [1–4]. Intact rat and human spermatozoa have been shown to possess a cyclic AMP-dependent protein kinase (ecto-RC) on their external surface [5–10] and it has been speculated that the ecto-RC may mediate the various stimulatory actions of the extracellular cyclic AMP on spermatozoa [5].

Rosado et al [11] reported the occurrence of cAMP-receptor in the plasma membrane of intact human spermatozoa, although it is not clear whether these

Abbreviations used: cAMP, cyclic adenosine 3′, 5′-monophosphate; cGMP, cyclic guanosine, 3′, 5′-monophosphate; PCMPS, p-chloromercuriphenylsulfonic acid.

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receptors are localised on the external cell surface. Horowitz et al [4] observed that there was little change in the amount of cAMP-receptor sites for interaction with [^{32}P]8-N_3 cAMP or α-chymotrypsin following sonication of intact spermatozoa and it has been concluded that the cells are "leaky" because all the sperm cyclic AMP-binding proteins are readily available to the exogenously added labeled cyclic AMP and α-chymotrypsin. Recently Atherton et al [8] discussed the data of Horowitz et al [4] and disagreed with their observations. They have confirmed intactness of rat spermatozoa and observed that approximately 60% of cyclic AMP-binding proteins of whole spermatozoa require sonication to be readily available to [^{32}P]8-N_3 cAMP or proteolytic enzymes. The data of Atherton et al [8] provided preliminary evidence for the localization of cAMP-receptors on rat sperm external surface. Extensive studies are lacking, however, to rule out the contribution of "leaky" and damaged cells towards the ecto-cAMP-receptor activity.

The present study has therefore examined extensively several parameters including cell "leakiness" while investigating the possible presence of ecto-cyclic AMP binding proteins in goat epididymal spermatozoa. This study demonstrated conclusively the occurrence of an ecto-cyclic AMP binding protein in intact spermatozoa.

MATERIALS AND METHODS

Chemicals

[8-^{3}H]cAMP was a product of Radiochemical Centre (Amersham, England). cAMP, cGMP, ATP (horse muscle), ADP, AMP, Ficoll-400, trypsin (2 × crystallized), α-chymotrypsin (3 × crystallized), pronase (protease, type VI), and trypsin inhibitor were obtained from Sigma Chemical Company (St. Louis, MO).

Isolation of Epididymal Spermatozoa

Highly motile spermatozoa were extracted from the fresh goat cauda-epididymides by the procedure described earlier. Spermatozoa were extracted in medium A—modified Ringer's solution which was free of Ca^{2+} and contained 119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate (pH 6.9), and penicillin, 50 units/ml. After being extracted in medium A spermatozoa (native sperm) were sedimented by gentle centrifugation at 500g for 2 min at room temperature (31°C ± 1°C) and the pellet was washed twice with medium A to remove the contaminating epididymal plasma. Finally the cells were dispersed in medium A (400 × 10^6/ml), and these cell preparations (washed "composite" cells) were used for these studies unless otherwise specified.

Isolation of Vigorously Forwardly Motile Spermatozoa

Spermatozoa that possess a high order of forward progression were separated out from nonmotile and weakly motile cells on the basis of their capacity to migrate upward against the gravity [12]. Freshly extracted native spermatozoa containing 2% Ficoll were layered at the bottom of beakers containing medium A. Forwardly motile cells that moved a vertical distance greater than 8 mm in approximately 15 min at room temperature were collected with a Pasteur pipette. Approximately 100% of these cells showed vigorous forward motility when examined under a phase contrast microscope.

Assay of Sperm Ecto-Cyclic AMP-Receptors

The assay systems contained 25 pmol of [8-^{3}H] cAMP (containing 9–12 × 10^4 cpm), 0.9 μmol of theophylline, 2 μmol of $MgCl_2$, and intact spermatozoa (4 × 10^6) in a total assay volume of 0.2 ml medium A. The incubation was carried out at 37°C for 1 min, and the reaction was arrested with the addition of polyethylenimine (0.1 mg/ml), a polycation that caused instant agglutination of intact cells [13]. Immediately the cell suspensions were filtered through 24-mm Millipore filter discs (0.45 μM) under mild vacuum and washed with 40 ml of medium A. It was found that 40 ml of medium A was adequate to remove all the free radioactive cAMP. The filter discs were then dried and counted for ^{3}H in a liquid scintillation spectrometer in a toluene–0.4% 2,5-diphenyloxazole–0.005%-1,4-bis-(5-phenyloxazole-2-yl) benzene scintillation fluid [14]. Tubes without cells served as blanks. The results were expressed as picomoles of cAMP bound to intact spermatozoa.

RESULTS

Characteristics of Sperm Ecto-cAMP-Receptors

Time course. As shown in Figure 1 [8-^{3}H]cAMP binds rapidly to the receptor sites of intact spermatozoa. The binding reaction was complete in 1 min, and a further increase of the incubation period did not cause any detectable increase in the binding of cAMP to spermatozoa. The rapidity of the reaction strongly suggests that cAMP binds to the receptor sites located on the external surface (ecto-receptors) of spermatozoa.

Uptake of cAMP. As shown in Table I there was little uptake (~0.1%) of the labeled cAMP by the intact cells. The data also show that the binding of [^{3}H]cAMP to the cytosolic receptors was insignificant when the cells were incubated with the labeled cAMP (Table I), although these receptors had the capacity to bind a large amount of cAMP, as evidenced by the association of the [^{3}H]cAMP with the cytosolic proteins when the cell-sonicate was incubated with the labeled nucleotide (data not shown). The data are consistent with the view that there is little penetration of cAMP across the sperm plasma membrane.

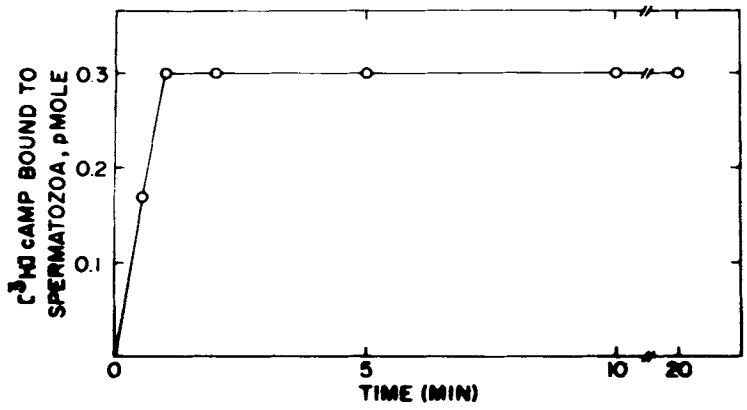

Fig. 1. Time course of the binding of [^{3}H]cAMP to intact spermatozoa under the standard assay conditions.

TABLE I. Determination of the Uptake of [^{3}H]cAMP by Intact Spermatozoa*

Cell system	Distribution of [^{3}H]cAMP	
	cpm	%
Whole spermatozoa	1,886	100
Cytosol	80	4
Cytosolic cAMP-receptors	15	0.8

*Spermatozoa were incubated with [^{3}H]cAMP under the standard assay conditions. One set of tubes was processed for the assay of cell-bound [^{3}H]cAMP by the procedure described above. Spermatozoa from another set of tubes were washed three times by the centrifugation method to remove free radioactive cAMP. Sperm pellets were then dispersed in medium A, sonicated, and centrifuged at 20,000g for 15 min to obtain the cytosolic fraction. An aliquot of the cytosolic fraction was counted for ^{3}H to estimate the total radioactivity in cytosol. Another aliquot of the cytosol was passed through Millipore membrane discs and washed with an excess of medium A to obtain the amount of cytosolic cAMP receptors [18]. A system containing an excess of nonradioactive cAMP (6 μM) served as a blank.

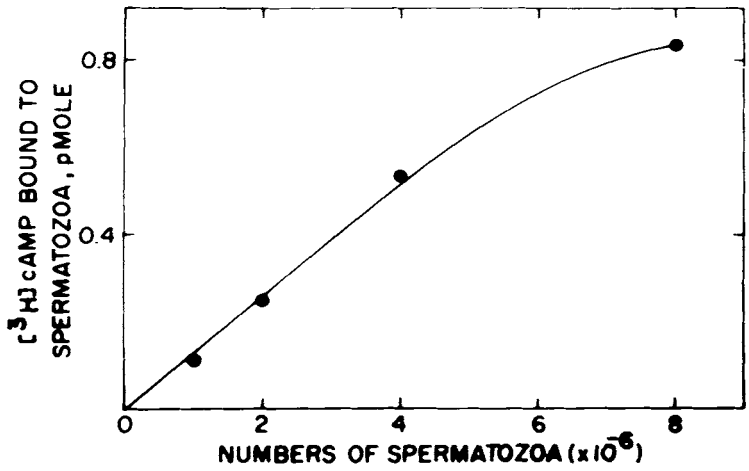

Fig. 2. Effect of varying concentrations of whole spermatozoa on their binding to [^{3}H]cAMP under the standard assay conditions.

Cell concentration. Figure 2 shows the binding of the labeled cAMP as a function of the variation of the sperm concentration. The binding of cAMP to sperm receptors increased linearly with cell concentration up to approximately 4×10^6 cells/assay. Goat cauda-epididymal spermatozoa bind to 0.2 ± 0.1 pmol of cAMP/10^6 cells (data from ten experiments).

[8-^{3}H]cAMP concentration. Figure 3 shows the effect of varying concentrations of the labeled cAMP on its binding to sperm surface receptors. The amount of cAMP bound to the intact cells was nearly maximal at approximately

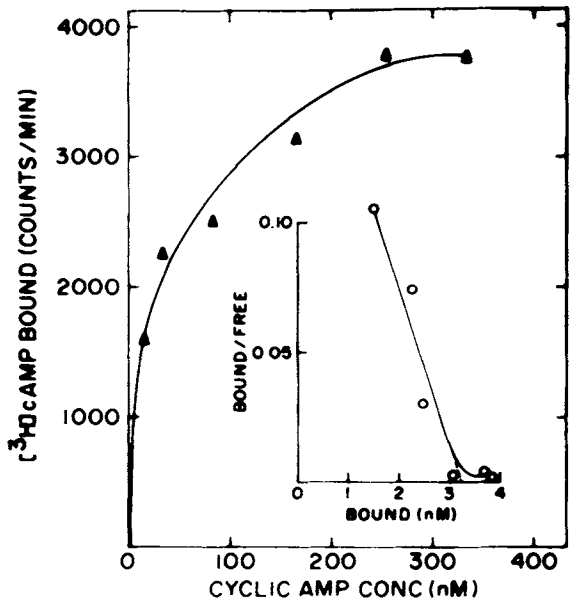

Fig. 3. Effect of varying concentrations of [^{3}H]cAMP on its binding to intact spermatozoa under the standard assay conditions. The insert shows the Scatchard analysis of these data.

250 nM concentration of the cyclic nucleotide. Curvilinearity of the Scatchard plot of these data (Fig. 3) suggests the presence of multiple classes of cAMP-binding proteins having different affinities for binding to cAMP, or there may exist negative cooperative interaction in the binding of cAMP to the sperm surface receptors [13]. cAMP showed high affinity for its interaction with the cell surface since K_d of these sites were approximately 1.7×10^{-8} M.

Chemical nature of the cAMP-receptor interaction. The effect of unlabeled cAMP on the binding of [^{3}H]cAMP to spermatozoa is shown in Figure 4. Unlabeled cAMP competed with [^{3}H]cAMP for the sperm binding sites and therefore caused displacement of the labeled cAMP bound to intact cells. Unlabeled cAMP at 2.5 μM concentration caused nearly complete displacement of the cell-bound [^{3}H]cAMP. The data show that binding of cAMP to intact spermatozoa is specific since there was little nonspecific binding of the labeled cAMP at a saturating concentration of the unlabeled cAMP.

As shown in Table II, unlabeled nucleotides such as cyclic GMP, ATP, ADP, and AMP (0.05 and 0.5 μm) had little effect on the binding of [^{3}H]cAMP to the sperm surface receptors, indicating thereby that these nucleotides compete little with [^{3}H]cAMP for binding to sperm receptors. The data show that sperm receptors have high specificity for binding to cAMP.

Evidence for Ecto-Nature of cAMP-Receptor

Goat cauda-epididymal spermatozoa were highly healthy because they showed a high order of flagellar motility. Previous studies from this laboratory confirmed the intactness of these cells by assaying lactic dehydrogenase as a cytosolic marker enzyme [16,17] and by using [^{203}Hg]-labeled thiol reagent:p-chloromercuriphenylsulfonic acid as a surface probe [13].

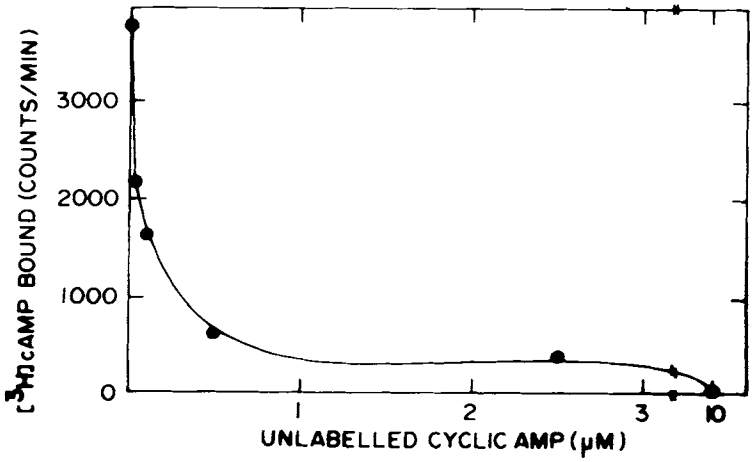

Fig. 4. Effect of varying concentrations of unlabeled cyclic AMP on the binding of [^{3}H]cAMP to intact spermatozoa under the standard assay conditions.

TABLE II. Effect of Various Unlabeled Nucleotides on the Binding of [^{3}H]cAMP to Spermatozoa*

Additions (μM)	[^{3}H]cAMP bound to spermatozoa (cpm)
Control	2,950
cAMP	
0.05	2,550
0.5	590
cGMP	
0.05	2,218
0.5	2,011
ATP	
0.05	2,582
0.5	2,493
ADP	
0.05	2,251
0.5	2,283
AMP	
0.05	2,693
0.5	2,480

*Assays were carried out under the standard conditions, except that the specified additions were made.

Leakage of cAMP-receptors. It is possible that the observed cAMP binding activity may be due to leakage of the receptors from spermatozoa. The data in Table III rule out the possibility of leakage of receptors since there was little cAMP-binding activity in the cell-free filtrates of the cells before and after incubation.

Localization of bound [^{3}H]cAMP. Intact spermatozoa following incubation with [^{3}H]cAMP under the standard assay conditions were filtered through Whatman No. 42 filters, and the clear cell-free filtrates were assayed for the labeled cAMP-receptor complex by passing through the Millipore filters [18]. There was no detectable amount of the labeled complex in the cell-free filtrate (data not shown), indicating that cAMP receptors are associated with intact cells.

TABLE III. Measurement of cAMP-Binding Protein That Had Leaked From the Spermatozoa*

System	cAMP-binding activity (pmol)
Untreated spermatozoa	0.31
Cell-free filtrate (before incubation)	0.01
Cell-free filtrate (after incubation)	0.01

*Spermatozoa were filtered through Whatman No. 42 filter paper before and after incubation at 37°C under the standard assay conditions except that [^{3}H]cAMP was omitted. The resulting cell-free filtrates were assayed for cAMP-binding proteins under the standard assay conditions.

TABLE IV. Effect of Proteolytic Enzymes on the Binding of [^{3}H]cAMP to Spermatozoa*

Proteases (125 μg/ml)	[^{3}H]cAMP bound to spermatozoa (pmol)
Nil	0.140
Trypsin	0.010
Chymotrypsin	0.030
Pronase	0.003

*Intact spermatozoa were preincubated with or without proteolytic enzymes at 37°C for 10 min under the standard assay conditions except that the labeled cAMP has been omitted. Immediately after the preincubation, [^{3}H]cAMP was added to the assays to estimate the activity of sperm ecto-cAMP receptors.

Effect of proteases. Whole spermatozoa were pretreated with various proteases that are believed not to penetrate the plasma membrane of the cells, prior to assay of the ecto-cAMP receptors (Table IV). Treatment of the cells with trypsin, chymotrypsin, or pronase (125 μg/ml) caused marked loss of the sperm cAMP-receptor activity. Addition of trypsin inhibitor (3.75 mg/ml) to the cells pretreated with trypsin had no effect on the rate of binding of the labeled cAMP to the cell-surface receptors. Intactness of spermatozoa preincubated with trypsin was evaluated by assaying lactic dehydrogenase as a cytosolic marker enzyme [16,17]. There was no detectable leakiness in spermatozoa owing to trypsinization (data not shown). These results provided further evidence to support the external surface localization of the cAMP-receptors.

Effect of sonication. Intact spermatozoa were sonicated for varying periods, and it was observed that sonication for 10 sec was adequate for maximal breakage of the cells as assessed by microscopic observation and by assay of lactic dehydrogenase as a marker of the cytosolic enzyme (data not shown). As shown in Figure 5, the cAMP-binding activity of spermatozoa increased markedly with the period of sonication and the activity was maximal after 10 sec of sonication. Further increase of the sonication caused a marked decrease in the cAMP-binding activity. Sonication caused a 3–6-fold increase (4-fold: average of three experiments) of the cAMP-receptor

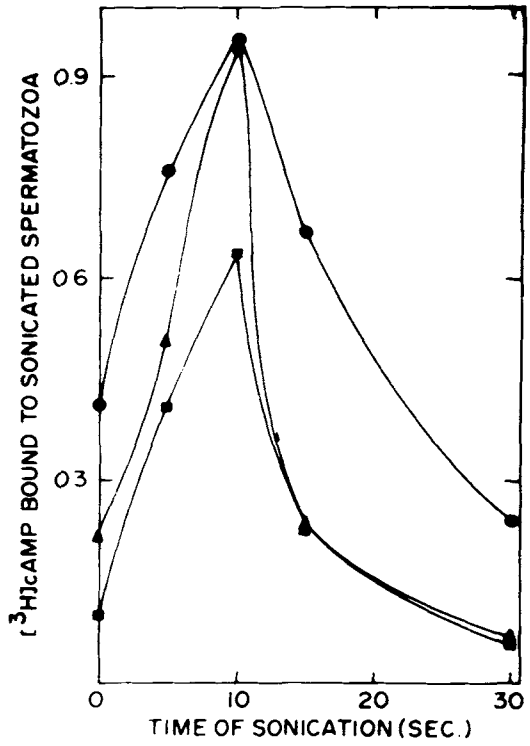

Fig. 5. Effect of varying periods of sonication on the sperm [^{3}H]cAMP binding activity. Two milliliters of washed spermatozoa (400 × 10^6/ml) were sonicated in a plastic test tube using the intermediate probe (length, 127 mm; diameter, 9.5 mm) of a "Braun" sonicator (Labsonic 2000) with a setting of 50 W at "low" position for specified periods (5 sec at a time) at 0–4°C. The cAMP-receptor activity (expressed as pmoles of bound cAMP) of the intact and sonicated cells were measured under the standard assay conditions. Data from three independent experiments have been shown.

activity of the whole spermatozoa. The data show that sperm ecto-cAMP-receptors represent nearly 25% of the total cellular receptor activity.

Figure 6 shows the data on the binding assays as a function of the concentration of the spermatozoa sonicated for 10 sec and 30 sec. The results demonstrate that markedly lower activity of the 30-sec-sonicated cells as compared to the 10-sec-sonicated cells was not an artifact of the cAMP-binding assay. The observed marked sensitivity of the cAMP-receptor activity to excessive sonication was not due to enhanced availability of cyclic phosphodiesterase, since similar results were obtained using a theophylline concentration as high as 13.5 mM. Mixing experiments ruled out the possibility of the appearance of any inhibitor of the binding proteins during sonication (data not shown). It thus appears that excessive sonication (> 10 sec) causes marked inactivation of sperm cAMP-receptors. It is interesting to note that under the same conditions of sonication, sperm cytosolic lactic dehydrogenase did not show any appreciable decrease of the enzymic activity.

Ecto-cAMP-receptors in forwardly motile spermatozoa. In the composite preparation of spermatozoa, less than 2% of cells are found to be damaged. These sperm may be responsible for the binding of cAMP. In that case the exclusively forwardly motile cell preparations are expected to show much less activity for the

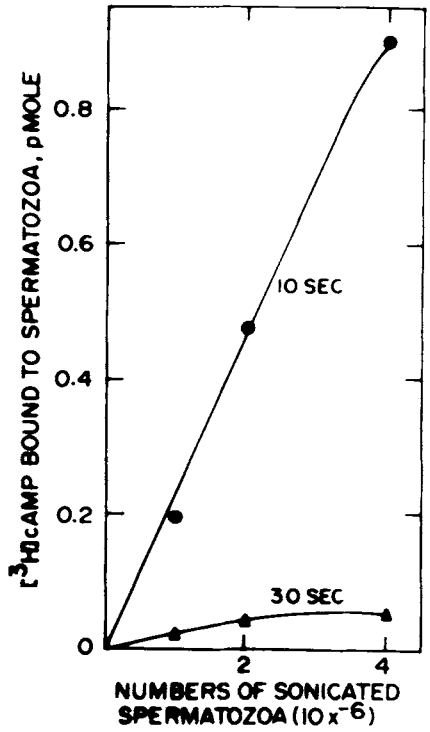

Fig. 6. Effect of varying concentrations of sonicated spermatozoa (●, 10-sec sonicated; ▲, 30-sec sonicated) on [^{3}H]cAMP-binding activity under the standard assay conditions. The conditions of cell sonication have already been described in the legend for Figure 5.

TABLE V. Specific Activity of cAMP-Binding Protein in Intact Forwardly Motile Spermatozoa*

Cell preparation	[^{3}H]cAMP bound to spermatozoa (pmole/10^6 cells): Mean ± SD
Composite	0.054 ± 0.007
Forwardly motile	0.110 ± 0.04
	($P < 0.01$)

*Vigorously forwardly motile spermatozoa were separated from washed composite cells by the procedure described in Materials and Methods. The binding activity was estimated under the standard assay conditions. The data shown are for nine experiments.

binding of cAMP than that of the composite cells. As shown in Table V, the intact forwardly motile cells showed nearly twofold greater specific activity of the cAMP-receptor activity than the composite cells, thereby strengthening the contention that cAMP-receptors are located on the external surface of the sperm plasma membrane.

DISCUSSION

Although several investigators provided evidence for the occurrence of ecto-cyclic AMP-dependent protein kinases on a variety of mammalian cells [19,20]

including spermatozoa [5–10], only a few studies have been devoted to whether cAMP-receptors are present on the external cell surface [4,8]. As already mentioned, these investigations were not sufficiently extensive to eliminate the possibility of the observed ecto-cAMP-receptor activity's being due to the action of intracellular cAMP-receptors and/or to the binding proteins derived from the "leaky" or broken spermatozoa. The present study demonstrated conclusively the occurrence of cAMP-receptors on the goat sperm external surface. Earlier studies from this laboratory confirmed intactness of spermatozoa by using p-chloromercuriphenylsulfonic acid as a surface probe [13] and using lactic dehydrogenase and cyclic AMP-dependent protein kinase as cytosolic enzyme markers [16,17]. Extreme rapidity of the binding of [^{3}H]cAMP to intact spermatozoa, undetectable uptake of the labelled cAMP to the cytosolic fraction of the cells, little leakage of the receptor from the cells, absence of the bound [^{3}H]cAMP in the extracellular medium, sensitivity of the receptors to the proteolytic treatment, and a marked increase of the receptor activity owing to sonication of cells support the "ecto" nature of the cAMP-receptors. The finding that the exclusively forwardly motile spermatozoa possess higher specific activity of the ecto-receptor than the composite cells (Table V) confirmed the contention of the external surface localization of cAMP-receptors. This study has thus eliminated the possibility of artifacts, particularly those resulting from cell "leakiness," as suggested by the data of Horowitz et al [4]. The demonstration of an ecto-cAMP-receptor in spermatozoa has provided further support to the earlier observations of the occurrence of ecto-RC [5–10] since cAMP-receptor protein is a constituent of RC [1].

It is worth noting that the activity of sperm cAMP-receptor is highly sensitive to sonication (Fig. 6), although lactic dehydrogenase activity is relatively resistant to sonication. With the "Braun" sonicator it has been noted that a sonication period greater than 10 sec causes marked inactivation of cAMP-receptor activity. This observation raises a note of caution regarding the actual period of cell sonication to be used for the assay of total activity of biological macromolecules in the sonicate. This is particularly important if one employs sonication as a tool to find out whether a macromolecule is in fact located on the external cell surface [4,8]. Since the degree of sonication differs in the various commercial brands of sonicator, it is important to optimise the instrument setting as well as the period of sonication for each biologically active macromolecule.

Our finding that sonication of intact goat spermatozoa caused a nearly 450% increase in cAMP-binding activity is consistent with that of Atherton et al [8], who observed an increase of 150% following sonication of rat spermatozoa. As mentioned earlier, Horowitz et al [4] did not find an increase of the cAMP-receptor activity after sonicating rat spermatozoa, and it has been concluded that the cells are "leaky." Consequently, the observed ecto-cyclic AMP-dependent protein kinase [5–10] may be an artifact of cell "leakiness" [4]. As discussed above, our data appear to provide an explanation for the controversy in the literature raised by the results of Horowitz et al. The failure of Horowitz et al [4] to demonstrate a marked increase of cAMP-binding activity following cell sonication may perhaps be attributed to excessive sonication (30 sec) that may have caused a profound loss of the cAMP-receptor activity.

Little is known about the chemical nature of cAMP-receptor sites on the sperm surface. It is well known that cAMP-binding proteins R_1 and R_2 play a regulatory role in the cAMP-dependent protein kinases: R_1C and R_2C [1]. It is possible that

sperm ecto-cAMP-receptors may be subunits of the sperm ecto-cAMP-dependent protein kinases [8]. There may also be other types of ecto-receptor sites which may not be subunits of the cAMP-dependent protein kinases [21,22]. Markedly higher specific activity of ecto-cAMP-receptor in forwardly motile spermatozoa (Table V) suggests that the surface receptor may have a role in the regulation of sperm flagellar motility, presumably by modulating the ecto-RC activity. Studies are in progress to characterize the sperm ecto-cAMP-receptor and elucidate its physiological role.

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