Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 636-642

Function of Calmodulin in Mammalian Sperm: Presence of a Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase Associated with Demembranated Rat Caudal Epididymal Sperm

Wilma M. Wasco and George A. Orr

Department of Molecular Pharmacology
Albert Einstein College of Medicine
Bronx, New York 10461

Received December 12, 1983

A calmodulin dependent cyclic nucleotide phosphodiesterase is associated with the head and tailpieces of demembranated rat caudal epididymal sperm. The phosphodiesterase was stimulated two-fold in the presence of Ca^{2+}, while the simultaneous addition of Ca^{2+} and calmodulin resulted in a four-fold increase in activity. Ca^{2+} stimulation was abolished if demembranated sperm were extracted with EGTA and was recovered upon the addition of exogenous calmodulin. Micromolar levels of Ca^{2+} were required for full stimulation. Trifluoperazine inhibited the Ca^{2+} stimulated enzyme in a dose dependent manner (ID_{50} = 50 μM) but had no effect on the basal phosphodiesterase activity.

INTRODUCTION: Calcium is involved in the regulation of both the motility and the acrosomal reaction of mammalian sperm (1). Since it is generally recognized that Ca^{2+} plays a central role in the regulation of cyclic nucleotide metabolism (2), a correlation between Ca^{2+} and cAMP levels in sperm has been sought. Although sperm contain high concentrations of calmodulin, a cellular mediator of many Ca^{2+} dependent processes, they are reported to lack calmodulin-dependent cyclic nucleotide phosphodiesterases (1,3,4,5). In light of the fact that calmodulin-dependent phosphodiesterases can lose Ca^{2+} sensitivity after mild proteolysis, and since sperm are rich in proteases, we have reinvestigated the possible presence of any calmodulin-sensitive phosphodiesterases in sperm. This report demonstrates that if precautions are taken to limit proteolysis during sperm isolation and fractionation, it is possible to demonstrate the association of a calmodulin-dependent phosphodiesterase with demembranated sperm heads and tails. The cytosolic and membrane bound enzymes were non-responsive to Ca^{2+}, EGTA or exogenous calmodulin.

MATERIALS. Tritiated cyclic nucleotides were purchased from Amersham. Protease inhibitors and nucleotides were purchased from Sigma and PEI-cellu-

0006-291X/84 $1.50

lose TLC plates were obtained from Baker. Calmodulin was isolated from bovine testes by the method of Dedman et al. (6). All other chemicals were of reagent grade.

METHODS. Caudal epididymal sperm from male Sprague-Dawley rats (> 400 g) were fractionated into cytosolic, detergent soluble and detergent resistant fractions as previously described (7). Separation of heads and tails was carried out according to the method of Calvin (8). cAMP dependent phosphodiesterase activity was m asured by the method of Rangel-Aldao et al. (9), the only modifications being the change of the assay temperature from 30°C to 37°C and the addition of calmodulin (10 ug/assay), Ca^{2+} or EGTA where appropriate. Free Ca^{2+} concentration was determined by the method of Pershadsingh and McDonald (10).

RESULTS: Rat caudal epididymal sperm were fractionated into cytosolic, detergent soluble and detergent resistant material and assayed for cyclic nucleotide phosphodiesterase activity in the presence and absence of Ca^{2+}/calmodulin. As can be seen from Table 1, at 1 uM [^{3}H]cAMP, the addition of Ca^{2+} and calmodulin to the detergent resistant fraction caused a 3-fold increase in activity over the basal activity as measured in the presence of 200 µM EGTA. The activity of the fully stimulated enzyme and the fold stimulation varied from sample to sample and with sample age, but were generally in the range of 0.8 to 1.4 pmol [^{3}H]cAMP hydrolyzed/min/10^6 sperm and 2-4-fold, respectively, at 1 µM [^{3}H]cAMP. No stimulation of either the cytosolic or detergent soluble fraction activity was observed. If sperm fractionation was performed in the absence of protease inhibitors, then enzymatic activity and fold stimulation were consistently lower than those observed in the presence of protease inhibitors (data not shown).

TABLE I. Subcellular Distribution of Cyclic Nucleotide Phosphodiesterases in Rat Caudal Epididymal Sperm.

Sample	Phosphodiesterase Activity[a] (pmol/min/10^6 cells)		Fold Stimulation
	200 µM EGTA	200 uM Ca^{2+}/10 ug CaM	
cytosolic	0.33	0.33	-
membrane bound	0.36	0.37	-
detergent resistant	0.33	0.99	3.0

[a] Assayed in the presence of 1 µM [^{3}H]cAMP.

TABLE II. Effect of Ca^{2+} and Calmodulin on Cyclic Nucleotide Phosphodiesterases Associated with Demembranated Rat Caudal Epididymal Sperm.

Additions	Phosphodiesterase Activity (pmol/min/10^6 cells)	
	1 μM [^{3}H]cAMP	1 μM [^{3}H]cGMP
200 μM EGTA	0.20	0.80
200 μM EGTA, 250 μM Ca^{2+}	0.56	1.70
200 μM EGTA, 250 μM Ca^{2+}, 10 μg CaM	0.83	6.00

The detergent resistant sperm structures were assayed in the presence of 1 μM [^{3}H]cAMP or [^{3}H]cGMP and the effects of added EGTA, Ca^{2+} and calmodulin determined (Table II). With both substrates, the stimulation was lower when Ca^{2+} alone was added rather than the Ca^{2+}/calmodulin combination. This would suggest that a portion of the enzyme was depleted of endogenous calmodulin. At this substrate concentration, the fully stimulated enzyme was more active against cGMP than cAMP.

Removal and Reassociation of Calmodulin - If detergent resistant structures were subjected to several cycles of EGTA (1 mM) washing and centrifugation, it was found that greater than 90% of the Ca^{2+}-dependent stimulation was lost after the first EGTA extraction. No phosphodiesterase activity, either Ca^{2+} independent or dependent, was found in the supernatants from the EGTA washings. Ca^{2+} stimulation was regained by the addition of exogenous calmodulin to the EGTA treated sperm structures (Table III).

Effect of Ca^{2+} on Cyclic Nucleotide Phosphodiesterase - We determined the effects of Ca^{2+} on the detergent resistant sperm phosphodiesterase by using an EGTA buffering system to control the free Ca^{2+} ion concentration. In an effort to bind calmodulin to all available sites, we added exogenous calmodulin to all assay tubes. The concentration dependence for the activation of the enzyme by Ca^{2+} is reported in Figure 1. As can be seen, the enzyme is fully activated when the free Ca^{2+} concentration is in the low micromolar range; half maximal stimulation occurred at 0.7 μM. This value is in agreement with

TABLE III. Effect of EGTA Extraction on Cyclic Nucleotide Phosphodiesterases Associated with Demembranated Rat Caudal Epididymal Sperm.

Treatment	Phosphodiesterase Activity[a] (pmol/min/10^6 cells)
None[b]	0.83
1st EGTA extraction: pellet[c]	0.22
2nd EGTA extraction: pellet[c]	0.21
3rd EGTA extraction: pellet[c]	0.17
3rd EGTA extraction: pellet + Ca^{2+}/CaM[b]	0.73

[a] Assayed in the presence of 1 μM [^{3}H]cAMP.

[b] Assayed in the presence of 200 μM EGTA, 250 μM Ca^{2+} and 10 ug CaM.

[c] Assayed in the presence of 200 μM EGTA and 250 μM Ca^{2+}.

published reports for other calmodulin-dependent phosphodiesterases (6,11). Ca^{2+} had no effect on the basal phosphodiesterase activity (demembranated and EGTA extracted sperm) over the same concentration range.

Inhibition by Trifluoperazine - Trifluoperazine, a known antagonist of calmodulin-dependent processes, inhibited the Ca^{2+} stimulated enzyme in a concentration dependent manner but had no effect on the basal activity, i.e. EGTA treated demembranated sperm (Fig. 2). The ID_{50} was approximately 50 μM.

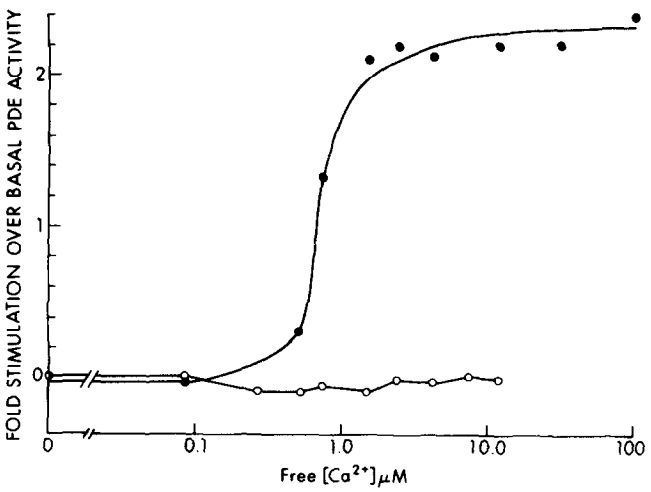

Figure 1. Calcium dependency for calmodulin activation of cyclic nucleotide phosphodiesterase of demembranated rat sperm. EGTA concentration in the assay was 200 μM and total Ca^{2+} concentration ranged from 0 to 300 uM. (●), Demembranated sperm in the presence of 10 μg calmodulin; (o) Demembranated and EGTA-extracted sperm.

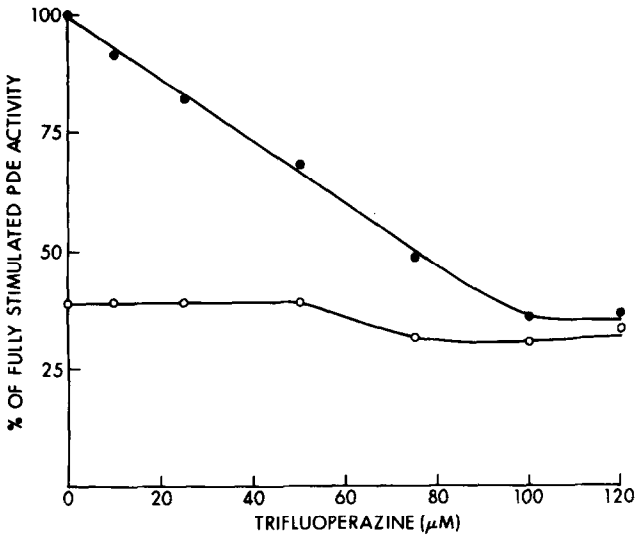

Figure 2. Effect of Trifluoperazine on Cyclic Nucleotide Phosphodiesterase of Demembranated Rat Sperm. Phosphodiesterase activity was measured in the presence of increasing concentrations of trifluoperazine and either 200 uM EGTA (o) or 250 μM Ca^{2+} and 200 μM EGTA (●).

Location of Calmodulin-Dependent Phosphodiesterase on Detergent Treated Sperm Structures - Separation of sperm heads and tails was carried out by brief sonication followed by discontinuous sucrose gradient centrifugation. When individual components were assayed in the presence of 1 μM [^{3}H]cAMP, approximately 60% of the calmodulin-dependent activity was located on the tail. This value should not be considered as the absolute percentage of calmodulin-dependent phosphodiesterases on sperm heads and tails, since we do not know whether the two activities possess similar kinetic parameters.

DISCUSSION: Several investigators have studied the cyclic nucleotide phosphodiesterases present in sperm and in all cases, the enzyme was observed to be unresponsive to either Ca^{2+} or calmodulin (1,3-5). The results of this communication refute these earlier reports and conclusively demonstrate the presence of a calmodulin-dependent phosphodiesterase hydrolyzing both cAMP and cGMP, associated with the head and tailpieces of demembranated rat sperm. The following findings helped to establish the presence of a calmodulin-dependent activity: 1) The addition of Ca^{2+} and calmodulin to demembranated sperm resulted in a 2-4-fold stimulation of the phosphodiesterase towards 1 uM cAMP;

2) Extraction of demembranated sperm with EGTA removed all Ca^{2+} stimulation; Ca^{2+}-dependency was recovered upon addition of exogenous calmodulin; 3) Micromolar levels of Ca^{2+} were required for full activation; 4) Trifluoperazine inhibited the Ca^{2+} stimulated enzyme but had no effect on the basal phosphodiesterase activity.

Both cyclic nucleotides and Ca^{2+} appear to play an intimate role in regulating flagellar motion (1). It has been demonstrated that cAMP stimulates motility when added directly to demembranated sperm in the presence of ATP (12,13). In contrast to the positive effects of cAMP, micromolar levels of Ca^{2+} have been shown to inhibit motion in both demembranated mammalian and sea urchin sperm (12). It has been suggested that the regulation of flagellar motion by cAMP and Ca^{2+} probably involves phosphorylation and dephosphorylation of specific axonemal proteins (13). It was observed that whereas cAMP stimulated the phosphorylation of specific proteins, Ca^{2+} was inhibitory. Our recent finding that the majority of cAMP-dependent protein kinases are firmly associated with the tail lends further support for the idea that a phosphorylation type mechanism may be involved in the cAMP-dependent regulation of flagellar motion (7). The results of this communication, demonstrating that a significant proportion of the calmodulin-dependent phosphodiesterases in sperm are also associated with sperm tails, provides one possible mechanism whereby Ca^{2+} could negate the positive effects of cAMP on sperm motion. An increase in Ca^{2+} concentration in the vicinity of the tail would decrease cAMP concentration by activation of the phosphodiesterase. This would result in a decrease in protein phosphorylation due to the reassociation of the catalytic and regulatory subunits of the tail cAMP-dependent protein kinase. This is probably not the sole mechanism whereby Ca^{2+} could influence sperm motility since Tash and Means observed that the phosphorylation state of certain proteins phosphorylated via cAMP independent pathways were also reduced by Ca^{2+} (13). Further work is necessary to establish whether the structure and/or activity of other flagellar proteins can be regulated by Ca^{2+} in a calmodulin-dependent or independent manner.

REFERENCES

1. Garbers, D.L., and Kopf, G.S. (1980) Adv. Cyc. Nucl. Res. **13**, 251-306.
2. Rasmussen, H., and Goodman, D.B.P. (1977) Physiol. Rev. **57**, 421-509.
3. Wells, J.N., and Garbers, D.L. (1976) Biol. Reprod. **15**, 46-53.
4. Tash, J.S. (1976) J. Reprod. Fert. **47**, 63-72.
5. Bhatnagar, S.K., and Anand, S.R. (1982) Biochem. Biophys. Acta **716**, 133-139.
6. Dedman, J.R., Potter, J.D., Jackson, R.L., Johnson, J.D., and Means, A.R. (1977) J. Biol. Chem. **252**, 8415-8422.
7. Horowitz, J.A., Toeg, H. and Orr, G.A. (1984) J. Biol. Chem., in press.
8. Calvin, H.I. (1976) Meth. Cell Biol. **13**, 85-104.
9. Rangel-Aldao, R., Schwartz, D., and Rubin, C.S. (1978) Anal. Biochem. **87**, 367-375.
10. Pershadsingh, H.A., and McDonald, J.M. (1980) J. Biol. Chem. **255**, 4087-4093.
11. Piascik, M.T., Wisler, P.L., Johnson, C.L., and Potter, J.D. (1980) J. Biol. Chem. **255**, 4176-4181.
12. Lindemann, C.B. (1978) Cell **13**, 9-18.
13. Tash, J.S., and Means, A.R. (1982) Biol. Reprod. **26**, 745-763.