Activation of brain adenylate cyclase by a factor derived from bovine sperm

Roger A. Johnson+, Joseph A. Awad, Karl H. Jakobs* and Günter Schultz*

*Pharmakologisches Institut der Universität Heidelberg, D-6900 Heidelberg, FRG and +Department of Physiology, School of Medicine, Vanderbilt University, Nashville, TN 37232, USA

Received 5 November 1982

Adenylate cyclase *Sperm membrane Activating factor* *Solubilized brain adenylate cyclase Forskolin*

1. INTRODUCTION

Mammalian spermatozoa contain a membrane-bound adenylate cyclase which is not sensitive to fluoride, guanine nucleotides, forskolin, or cholera toxin, and which exhibits a striking dependency on Mn^{2+} relative to Mg^{2+} for activity [1,2]. Available data suggest that the sperm enzyme lacks a functional guanine nucleotide regulatory component, but may be reconstituted from human erythrocytes [3]. The lack of responsiveness of the sperm adenylate cyclase and the possibility that regulation could be imparted to it, suggested to us that the sperm enzyme may be a useful test system for identifying other components of the adenylate cyclase and for characterizing the interactions of the enzyme's various subunits. In studies with brain adenylate cyclase analogous to those reported with erythrocytes [3], we found unexpectedly large increases in cyclase activity when preparations from sperm and brain were combined. As we report here, rather than being due to a contribution of a component from brain to the sperm, the superadditive activity was found to be due to a factor in sperm which activated the brain adenylate cyclase.

2. MATERIALS AND METHODS

2.1. *Enzyme preparation*

Bovine testes were obtained from a local slaughterhouse. Sperms were removed by rinsing the epididymal ducts. After several washes in isotonic buffer, sperms were frozen in small aliquots in liquid nitrogen. Frozen sperm ($\sim 10^8$ cells/ml) were thawed with the addition of 1 or 2 volumes of 10 mM triethanolamine–HCl (pH 7.4) and were homogenized with a motor-driven glass-Teflon homogenizer. The particulate material was then collected by centrifugation at 30000 × *g* for 10 min. The particles were resuspended in 10 mM triethanolamine–HCl (pH 7.4) and were then used for assay of adenylate cyclase.

Detergent-dispersed adenylate cyclase from rat brain was prepared as in [4] with the modification that the initial centrifugation speeds were increased from 3000 to 17000 × *g*. The Lubrol-PX dispersed enzyme was chromatographed on DEAE-Sephadex (A-25) as in [5], with the following modification. Proteins were first eluted with 600 mM NaCl containing 1 mM ethyleneglycol-bis-(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA) (instead of only 300 mM NaCl). The column was then washed with 600 mM NaCl to wash out the EGTA. The enzyme was then eluted with a solution of 0.25% Lubrol-PX, containing neither salt nor EGTA.

2.2 *Adenylate cyclase assay*

Adenylate cyclase activity was determined and the [^{32}P]cAMP was purified as in [6,7]. Comparable results to those shown were obtained in at

Published by Elsevier Biomedical Press
00145793/83/0000—0000/$3.00

least two separate experiments in each case. Standard deviations of triplicate determinations were less than 5% of the means.

2.3. *Materials*

[α-^{32}P]ATP was prepared enzymatically [6]. Calmodulin (CaM) was isolated from pig testes by an ammonium sulfate precipitation procedure developed by Keravis and Wells (personal communication) and was purified to near homogeneity by chromatography on 2-trifluoro-10-(3-aminopropyl)phenothiazine-Sepharose CL 4B, essentially as in [8]. The phenothiazine analog was generously provided by Dr J.N. Wells, Vanderbilt University. Forskolin was a generous gift of Dr H. Metzger, Hoechst AG (Frankfurt).

3. RESULTS

3.1. *Sperm and brain adenylate cyclase*

In agreement with the observations of other workers, we found the adenylate cyclase of bovine sperm particles to be considerably more active with Mn^{2+} than with Mg^{2+}. The Mn^{2+}:Mg^{2+} activity ratio ranged from about 30 to 100. Moreover, the activity of this enzyme was unaffected by guanyl 5'yl-(β,γ-imino)diphosphate (GPP(NH)P), fluoride, or prostaglandin E_1. The Mn^{2+}-requiring characteristic of the sperm enzyme and the lack of stimulation by GPP(NH)P or NaF suggest at least a functional deficiency in the guanine nucleotide regulatory component (N_s). This characteristic of the enzyme from ram sperm was taken advantage of in [3] as a source of cyclase with which the regulatory component (N_s) of erythrocytes could

Table 2

Lack of effect of EGTA on activation of the brain adenylate cyclase by the sperm factor

Enzyme	Adenylate cyclase activity[a]	
	Control	Calmodulin (25 μg/ml)
	pmol cAMP (25 min.tube)$^{-1}$	
Bovine sperm	0.1	0.1
+ EGTA (500 μM)	0.1	0.1
Brain	32.8	140
+ EGTA (500 μM)	35.6	33.5
Sperm + brain[b]	64.8	127
+ EGTA (500 μM)	72.7	79.5

[a] Activities were determined with a medium containing 50 μM ATP, 3.7 mM $MgCl_2$, 50 μM $CaCl_2$, 1 mg bovine serum albumin/ml, and 10 μM forskolin, without or with 500 μM EGTA as indicated. Reactions were for 25 min at 30°C

[b] Bovine sperm particles (from 10^7 cells) were mixed with solubilized brain adenylate cyclase (240 μg protein). These mixtures were allowed to sit on ice 15–20 min prior to assay

Table 1

Effects of forskolin and GPP(NH)P on combinations of brain and sperm adenylate cyclases

Enzyme source	Adenylate cyclase activity[a]			
	Basal	Forskolin (10 μM)	GPP(NH)P (100 μM)	GPP(NH)P + Forskolin
	pmol cAMP (30 min.tube)$^{-1}$			
Sperm (8.4 μg/tube)	0.3	0.4	0.1	0.2
Brain (40 μg/tube)	77	255	151	476
Sperm + brain	365	676	603	1000

[a] Sperm particulate and solubilized brain adenylate cyclases were preincubated for 5 min at 30°C with a cyclase reaction mixture which was complete except for [α-^{32}P]ATP. The determination of enzyme activity was initiated by the addition of labelled ATP. Reactions were for 30 min at 30°C

be combined. Since the apparent interaction of the cyclase constituents in [3] occurred with mixtures of particles, it was of interest to us to conduct similar experiments with detergent-solubilized cyclase components from other tissues.

Following chromatography on DEAE-Sephadex, detergent-solubilized adenylate cyclase from rat brain is sensitive to stimulation by forskolin, GPP(NH)P (table 1), and to calmodulin (table 2, see below). The combination of forskolin and GPP(NH)P resulted in activity greater than with either agent alone (table 1). The forskolin (10 μM) concentration used in these experiments resulted in about half-maximal stimulation of the brain enzyme. Maximal stimulation could be achieved with about 1 mM forskolin, when correction is made for the inhibitory effect of solvent ethanol (not shown). By comparison, forskolin was without effect on either the particulate or Lubrol-PX solubilized sperm adenylate cyclase at concentrations up to 1 mM.

3.2. *Combinations of sperm and brain cyclase preparations*

When sperm particles were mixed with the solubilized brain enzyme considerably greater than

Table 3

Combination of soluble brain adenylate cyclase with heat- or *N*-ethylmaleimide-inactivated adenylate cyclase from sperm

Enzyme source	Adenylate cyclase activity		
	Control (Mn^{2+})	Control (Mg^{2+})	Forskolin (100 μM)
Experiment I	pmol cAMP (25 min.tube)$^{-1}$		
Sperm	26.0	3.0	1.9
Brain - control	–	22.9	94.2
- heat-treated	–	0.5	0.9
Sperm + brain - control	–	48.9	142.6
Sperm + brain - heat tx'd	–	0.4	2.0
Experiment II	pmol cAMP (10 min.tube)$^{-1}$		
Brain	–	2.73	25.5
Sperm - control	25.8	0.01	–
- heat-treated	0.08	0.01	–
- NEM-treated	3.14	0.01	–
Brain + sperm - control	–	6.15	60.4
Brain + sperm - heat tx'd	–	6.66	46.1
Brain + sperm - NEM tx'd	–	4.77	56.7

For expt I heat treatment of the solubilized brain enzyme was for 5 min at 47°C. Cyclase assays were with either 10 mM $MgCl_2$ or 10 mM $MgCl_2$ + 10 mM $MnCl_2$ as indicated. Forskolin-stimulated activity was measured with Mg^{2+}. Reactions were for 25 min at 32°C

For expt II sperm particles were heat-treated for 20 min at 58°C or were incubated for 10 min at 30°C with 3 mM *N*-ethylmaleimide (NEM). The treatment with NEM was stopped by the addition on ice of a 17-fold molar excess of dithiothreitol. Cyclase assays were with either 5 mM $MgCl_2$ or 5 mM $MgCl_2$ + 10 mM $MnCl_2$ as indicated. Forskolin-stimulated activity was measured with Mg^{2+}. Brain (10.5 μg/tube) and sperm (22 μg/tube) preparations were preincubated 15 min at 30°C with a reaction mixture, complete except for [α-^{32}P]ATP. Cyclase assays were initiated by the addition of labelled ATP and were for 10 min at 30°C

additive activity was observed (table 1). This superadditivity was evident for both basal and stimulated forms of the brain enzyme and was also seen when a washed particulate preparation from brain was used instead of the solubilized preparation shown here. The superadditivity evident from table 1 was not due simply to the addition of calmodulin from the sperm preparation, since similar results were also obtained in the presence of 500 μM EGTA (table 2). The EGTA was sufficient to prevent activation of the brain enzyme by exogenously added calmodulin, whether the brain cyclase was assayed alone or in combination with the sperm particles.

The data shown in table 3 suggest that the superadditivity was due to an effect of the sperm particles to alter the activity of the brain enzyme. When the brain adenylate cyclase was inactivated (table 3, expt.I), no significant superadditivity could be measured. When the sperm adenylate cyclase was inactivated (expt.II), by treatment with either heat or *N*-ethylmaleimide, the combination of sperm particles and the brain enzyme resulted in the same activity as that seen with the untreated (control) sperm particles. Heating of the sperm particles at 95° for 5 min completely prevented their activating the brain enzyme (not shown). Thus, the sperm adenylate cyclase was not activated by something in the brain preparation, but rather, a relatively heat-stable component of the sperm particles caused a marked activation of the solubilized cyclase from brain.

The superadditive activities of the brain and sperm preparations shown in tables 1–3 were determined with Mg^{2+}/MgATP as substrates. When Mn^{2+} was added to the reactions activity of the sperm cyclase was increased 30-fold and activity of the brain cyclase 10-fold. Although diminished due to the differing effects of Mn^{2+} to increase activities of the two cyclases, superadditivity of the brain and sperm cyclase activities was still evident (table 4).

The concentration-dependency of the effect of the sperm particles on the brain adenylate cyclase is illustrated in fig.1. Increasing amounts of sperm particles resulted in increasing adenylate cyclase activities of the solubilized brain enzyme. Since GPP(NH)P and forskolin had no effect on the sperm cyclase but stimulated the brain enzyme 3- to 9-fold, the increase was most readily seen in the presence of these agents.

The stimulatory effect of the sperm particles on the solubilized brain adenylate cyclase was rapid in onset but exhibited a short lag phase (fig.2). Following the lag phase the formation of cAMP was linear with respect to time for at least 25 min.

Table 4

Comparison of combinations of brain and sperm adenylate cyclase preparations in the presence of manganese or magnesium

Enzyme	Adenylate cyclase activity	
	Mg^{2+}	Mn^{2+}
	pmol cAMP (25 min.tube)$^{-1}$	
Sperm	9.8	324
Brain	61.5	685
Sperm + brain	136	1369

Bovine sperm particles were mixed with brain adenylate cyclase and the mixture was allowed to sit on ice 15–20 min prior to assay. Cyclase activities were determined with a medium containing 300 μM ATP with either 3 mM $MgCl_2$ or 3 mM $MgCl_2$ + 10 mM $MnCl_2$ as indicated

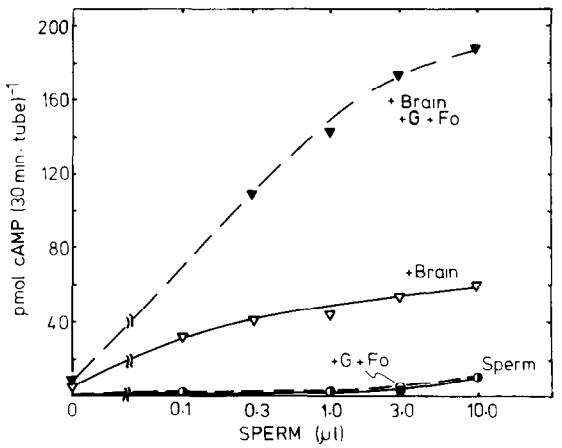

Fig.1. Concentration-dependent activation of solubilized brain adenylate cyclase by bovine sperm particles. Adenylate cyclase activities were assayed as described in section 2 with 2.2 μg brain protein/tube and 1.2 μg sperm particulate protein/μl at the indicated volumes added. Indicated additions were 100 μM GPP(NH)P (G) or 10 μM forskolin (Fo).

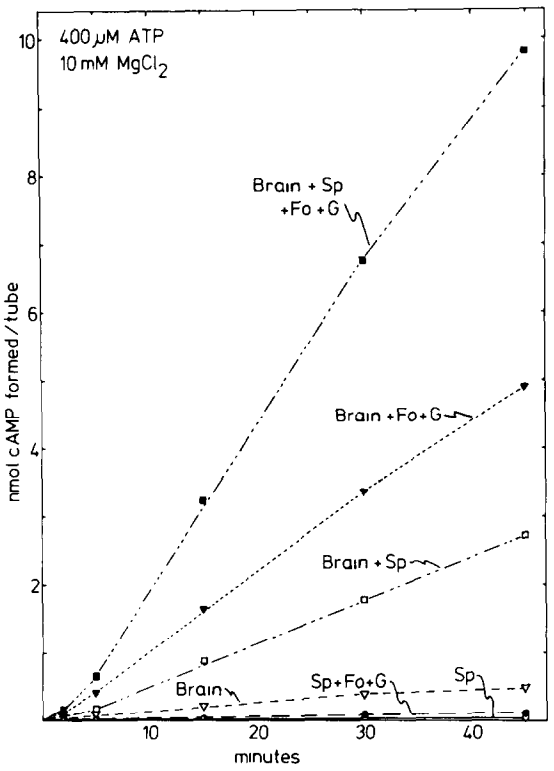

Fig.2. Time course of cAMP formation with combined sperm particulate and solubilized brain adenylate cyclases. Adenylate cyclase activity was determined as in section 2 with 400 μM ATP and 10 mM $MgCl_2$. Solubilized brain protein was 72 μg/tube (100 μl) and particulate sperm protein was 28.6 μg/tube. Indicated additions were 100 μM GPP(NH)P (G) or 10 μM forskolin (Fo).

It is worth noting that this linearity of cAMP formation was observed at considerably greater amounts of sperm particles (28.6 μg/tube) and the brain enzyme (72 μg/tube) than those used in the experiment shown in fig.1 (2.2 μg brain protein/tube and maximally 12 μg sperm protein/tube). That is, the saturation suggested in fig.1 was not due to a decrease in activity of the brain enzyme due to increased ATP hydrolysis at the higher protein concentrations.

4. DISCUSSION

The data presented here indicate the presence in bovine sperm particles of a component which activates the solubilized adenylate cyclase of rat brain. The observation is interesting in light of the magnitude of the activation and the small amount of sperm protein required. The effect is of further interest in view of a number of reconstitution studies involving sperm. Due to their reported lack of a guanine nucleotide regulatory component (N_s) and the lack of effect of forskolin on the sperm cyclase, sperms have been considered to be useful for the identification and quantification of components of the cyclase system mediating the disparate effects of guanine nucleotides, hormones, and forskolin. The present data, though, suggest that in reconstitution experiments sperm particles may cause changes in measured adenylate cyclase activity, which, although suggestive of component reconstitution to sperm, may in fact be due to donation from sperm of completely unrelated factor(s). The results presented here would be consistent with the factor either enhancing activation of the receiving cyclase (e.g., stimulation of brain N_s) or with blocking an inhibitory process (e.g., inhibition of N_i). An understanding of the component present in sperm particles and its mechanism of activation of adenylate cyclases should aid our understanding of adenylate cyclase regulation in general, and be of use in cyclase reconstitution studies in particular.

ACKNOWLEDGEMENTS

We are indebted to Ms Christina Stannek and Ms Gabriele Gabel for their excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft and grant AM 18185 from the United States National Institutes of Health. R.A.J. is a recipient of an Alexander von Humboldt Foundation fellowship on sabbatical leave to Heidelberg University.

REFERENCES

[1] Garbers, D.L. and Kopf, G.S. (1980) Adv. Cyclic Nucleotide Res. 13, 251–306.
[2] Johnson, R.A., Awad, J.A., Steinmeyer, C. and Schultz, G. (1982) Naunyn-Schmiedeberg's Arch. Pharmacol. 319, R28.
[3] Stengel, D. and Hanoune, J. (1981) J. Biol. Chem. 256, 5394–5398.
[4] Johnson, R.A. and Sutherland, E.W. (1974) in: Methods in Enzymology (Hardman, J.G. and O'Malley, B.W. eds) vol.38, pp.135–143, Academic Press, New York.

[5] Johnson, R.A., Garbers, D.L. and Pilkis, S.J. (1976) J. Supramolecular Structure 4, 205–220.
[6] Walseth, T.F. and Johnson, R.A. (1979) Biochim. Biophys. Acta 562, 11–31.
[7] Jakobs, K.H., Saur, W. and Schultz, G. (1976) J. Cyclic Nucleotide Res. 2, 381–392.
[8] Jamieson, G.A. Jr. and Vanaman, T.C. (1979) Biochem. Biophys. Res. Commun. 90, 1048–1056.