Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 607-613

PARTICULATE AND SOLUBLE ADENYLATE CYCLASE ACTIVITIES OF MOUSE MALE GERM CELLS

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Received July 7,1980

SUMMARY

Germ cells from the mouse testis possess both a particulate and a soluble form of adenylate cyclase (EC 4.6.1.1). Germ cell adenylate cyclase activity is Mn^{++} dependent and is not stimulable with either NaF or 5'guanylylimidodiphosphate. Both particulate and soluble adenylate cyclase specific activities increase as germ cells progress through their differentiative stages, but epididymal spermatozoa seem to lack a significant amount of soluble activity. Somatic cells of the seminiferous tubule possess only a membrane bound activity, which is Mg^{++} and Mn^{++} dependent, NaF and 5'guanylylimidodiphosphate stimulable. It is suggested that germ cell adenylate cyclases represent incomplete forms of the enzyme, devoid of regulative subunits.

INTRODUCTION

Considerable interest exists in the study of the cyclic nucleotide system of male germ cells as this could represent a clue to the understanding of the regulation of germ cell differentiation. In fact, while spermatogenesis appears to be a hormone controlled process, long lasting efforts by many research groups failed to demonstrate any specific interaction of circulating hormones with male germ cells (1). The possibility exists, however, that local events can regulate germ cell differentiation within the seminiferous tubule (1).

Using isolated male mouse germ cells Conti et al. have shown the presence of two isoenzymes of cAMP dependent protein kinase, whose ratio drastically changes as spermatogenesis proceeds (2). Adenylate cyclase of rat seminiferous tubule has been studied by Braun et al., who reported the presence of a Mn^{++} dependent, soluble form of AC, attributed to spermatids, and of a particulate AC activity attributed to the somatic components of the seminiferous tubule (3,4). Further characterization of soluble AC was performed by Neer (5) who demonstrated that it is a true soluble protein with a MW of 56,000 Dalton.

Abbreviations: AC, adenylate cyclase; cAMP, adenosine 3':5'cyclic monophosphate; GppNHp, 5'guanylylimidodiphosphate; BSA, bovine serum albumin; Hepes, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

This work was initiated under the stimulating leadership of Professor Valerio Monesi, who died prematurely on December 29, 1979.
His collaborators deeply feel the loss of his guidance and friendship.

0006-291X/80/220607-07$01.00/0
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In this paper we further evaluate the properties of germ cell AC using mouse testis germ cells separated into homogeneous populations corresponding to definite stages of differentiation.

MATERIALS AND METHODS

Seminiferous tubules were prepared as described (2) from 3 months old Swiss CD1 mice, and dissociated by incubation with collagenase, 1 mg/ml in Minimum Essential Medium (Eagle) containing 0.5 mg/ml BSA, for 45-60 min at 30°C. The resulting suspension was separated into homogeneous cell populations by unit gravity sedimentation in albumin gradients (2,6). Spermatogonia were separated with the same procedure from 17-18 days old unirradiated mice. Spermatozoa were obtained from the cauda epididymis according to the method of Majumder (7). Controls performed incubating spermatozoa in collagenase and, subsequently, in BSA in conditions similar to those employed for preparation and separation of tubular cells indicated that no difference in AC activity existed between the two preparations.

Cells were homogenized in 25 mM Hepes pH 7.4, 1 mM EDTA (buffer A) after addition of 2 mM PMSF, using a Dounce homogenizer, and centrifuged at 20,000 x g for 10 min. Pellets were washed and recentrifuged twice, resuspended in buffer A and used as the particulate fraction. The 20,000 x g supernatants were further centrifuged at 100,000 x g for 1 hr, and the resulting supernatants were used as the soluble fraction.

AC activity was measured by the method of Salomon (8). Incubation mixtures contained, in a final volume of 100 μl, 25 mM Hepes pH 7.4, 20 mM phosphocreatine, 1 mM cAMP, 1 mM ATP, 0.5 mg/ml BSA, 200 U/ml creatine kinase, 1 μCi of $[^{32}P]$ ATP, 25 to 50 μg of particulate fraction protein or 10 to 25 μg of soluble fraction protein, and other additions as indicated. Final EDTA concentration in the assay was 0.25 mM. In these conditions AC activity was linear for at least 15 min at 34°C. The reaction product was identified as authentic $[^{32}P]$ cAMP by thin layer chromatography (9). Experimental values are indicated as mean $\pm$ standard deviation of triplicate samples. AC interassay variation was lower than 15%.

Protein was measured by a modification of the standard Lowry procedure (10), using BSA as a standard.

Labelled compounds were from NEN; collagenase and GppNHp were from Boheringer;cAMP, ATP, phosphocreatine,creatine kinase, Hepes and PMSF were from Sigma; BSA was from Miles and MEM from Gibco.

RESULTS AND DISCUSSION

Adenylate cyclase activity of germ cells at various stages of differentiation was measured in different conditions as shown in Fig.1. The specific activity of both the particulate and the soluble enzyme is barely detectable when Mg^{++} is the only cation present in the assay, and no relevant NaF stimulation can be observed. On the contrary, when the particulate AC is measured in the presence of Mn^{++}, a significant activity is evident, which increases as germ cells differentiate from spermatogonia to spermatids and to spermatozoa. The increase observed for the specific activity of the soluble enzyme parallels that of the particulate AC only to the stage of elongating spermatids, while it drops in spermatozoa. It is also evident that, with the exception of spermatozoa, the specific activity of the soluble enzyme is consistently higher than that of the particulate AC.

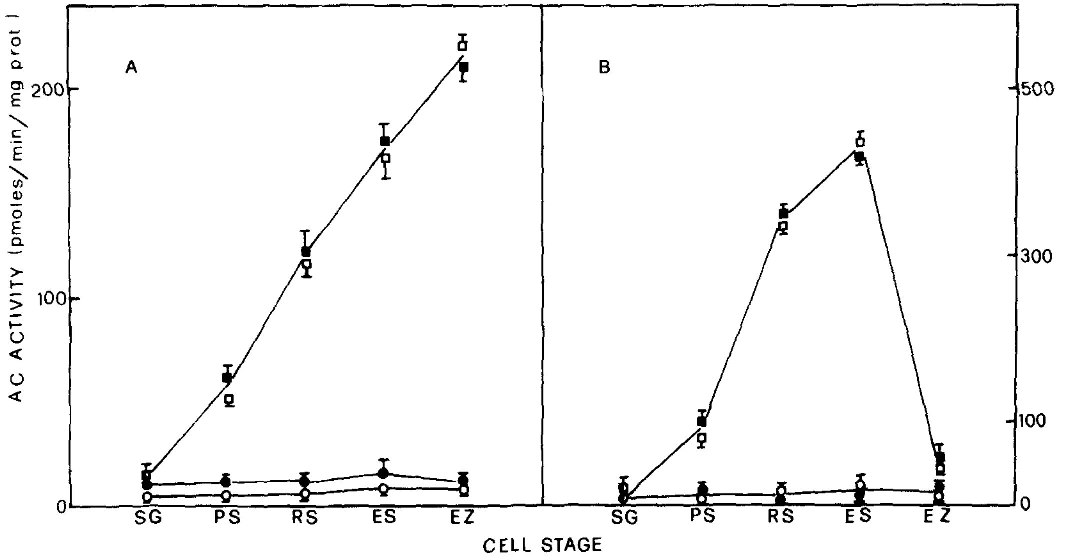

FIGURE 1. Adenylate cyclase activity of the particulate (A) and soluble (B) fraction of germ cells at various stages of differentiation. Cells were obtained as described under "Materials and Methods". Cell stages examined were: spermatogonia, SG; pachytene spermatocytes, PS; round spermatids, RS; elongating spermatids, ES; epididymal spermatozoa, EZ. AC incubation mixtures contained 20 mM $MgCl_2$ (○——○), 20 mM $MgCl_2$ and 10 mM NaF (●——●), 20 mM $MnCl_2$ (□——□), or 20 mM $MnCl_2$ and 10 mM NaF (■——■). Identical values were obtained when cells were homogenized in buffer A containing 13.5 or 27% (w/v) sucrose. Some solubilization of AC activity was observed when spermatozoa were homogenized in buffer A containing 27% sucrose.

It was of interest to verify whether the Mn^{++} dependent activities could actually be attributed to the germ cells or rather to some contaminants of the germ cell preparation. For this purpose a group of animals was irradiated with X rays (300 Rad, total body) in order to gradually deplete tubules of germ cells. This low dose irradiation causes the death of cycling spermatogonia but does not affect other germ cell stages, which proceed along spermatogenesis or somatic cells. Depletion of the more differentiated germ cell stages from the seminiferous tubule follows, according to spermatogenesis timing (11, 12). Irradiated animals and control animals of the same age were sacrificed at weekly intervals after irradiation, and particulate and soluble AC activity of the seminiferous tubules was measured (Figure 2). The loss of both particulate and soluble total protein in irradiated animals is evident from Figure 2A, and it is a rough estimate of ongoing germ cell depletion. Morphological examination of the seminiferous tubules confirmed germ cell depletion in irradiated animals. Repopulation rate was such that leptotene spermatocytes were observed in some of the seminiferous tubule cross sections by the end of the fourth week after X rays. As an effect of germ cell depletion, both soluble and particulate AC

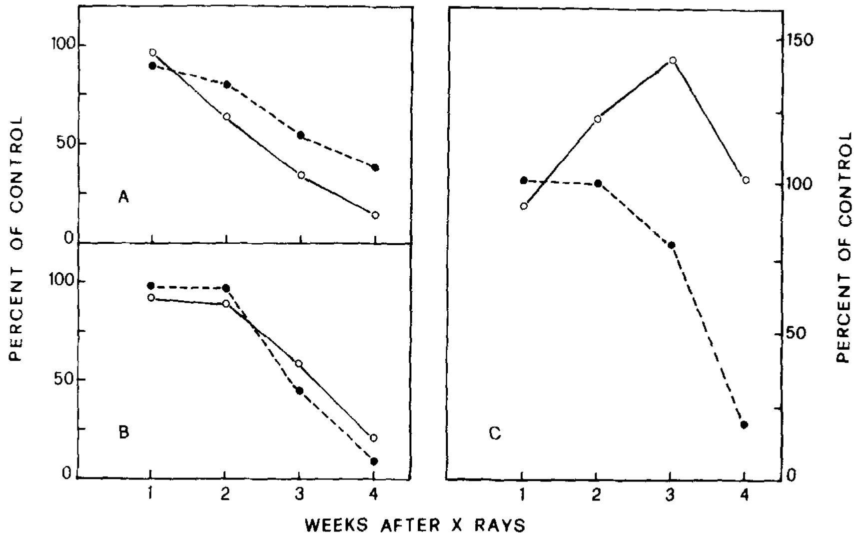

FIGURE 2. Time course of the effect of X irradiation on seminiferous tubule protein and AC activity. Seminiferous tubules were prepared from control and X irradiated animals sacrificed at weekly intervals after irradiation. Tubules were homogenized in buffer A, particulate and soluble fractions were prepared and assayed for protein and AC activity as described under "Materials and Methods". Panel A, soluble and particulate protein per testis. Panel B, soluble and particulate AC activity per testis. Panel C, soluble and particulate AC activity per mg of protein. Solid symbols: soluble fraction. Empty symbols: particulate fraction. AC assays were run in the presence of 20 mM $MnCl_2$ and 10 mM NaF.

activities per testis decrease (Figure 2B), after a lag corresponding to the period in which only meiotic germ cells, low in AC, are lost from the seminiferous tubules. Attribution of soluble AC to the germ cells is indicated by the decrease in the specific activity of the soluble enzyme as germ cell depletion occurs (Figure 2C). The decrease in specific activity is the expression of the disappearance of those cell stages which possess such soluble activity, and the specificactivity minimum is reached when those cells with a high specific activity of soluble AC (e.g. spermatids) disappear from the tubule. The different behaviour of the specific activity of the particulate enzyme, which does not fall below the control value (Figure 2C) is not in contrast with the results shown in Figure 1A if one assumes that (a), the particulate enzyme is present not only in germ cells but also in somatic cells, which remain in the seminiferous tubule after X irradiation (1), and that (b), the specific activity of the particulate AC in somatic cells is equal or higher than that of the germ cells which are disappearing from the tubule.

To further verify the presence of a particulate AC activity also in the germ cells, and to differentiate particulate AC activity of germ cells from that of somatic cells, the AC activities of a purified germ cell preparation were compared with those of total

Table 1. Adenylate cyclase activity of different cell populations of the seminiferous tubule (pmole/mg protein/min ± s.d.)

	Mg	Mg + NaF	Mg + NaF / Mg	Mn	Mn + NaF	Mn + NaF / Mn	Mn / Mg
PARTICULATE							
Germ cells	4.8 ± 0.4	12.1 ± 0.1	2.5	57.2 ± 3.8	62.3 ± 8.7	1.1	12.0
Control tubules	7.3 ± 0.7	41.6 ± 2.9	5.7	59.6 ± 3.2	96.1 ± 5.4	1.6	8.2
X irradiated tubules	9.6 ± 0.9	81.3 ± 5.6	8.5	21.6 ± 0.6	89.3 ± 2.7	4.1	2.2
SOLUBLE							
Germ cells	0.6 ± 0.1	0.6 ± 0.1	1.0	144.7 ± 3.1	145.7 ± 4.7	1.0	241.2
Control tubules	0.7 ± 0.2	1.4 ± 0.1	2.0	104.0 ± 8.9	99.8 ± 2.8	1.0	148.6
X irradiated tubules	1.2 ± 0.3	2.4 ± 0.9	2.0	7.4 ± 1.2	7.3 ± 0.4	1.0	5.9

Germ cells were prepared by dissociation of the seminiferous tubules and separation on albumin density gradients as described under "Materials and Methods". Fractions corresponding to middle-late pachytene spermatocytes and round spermatids were pooled for these experiments. Tubules from control animals and from animals which had been X irradiated four weeks prior to the experiments were dissociated with collagenase as described and the totalcell suspensions were kept in 1% BSA while germ cells were being separated into homogeneous populations. Particulate and soluble fractions were prepared as described. AC assay incubation mixtures contained either 20 mM $MgCl_2$ or 20 mM $MnCl_2$, in the presence or absence of 10 mM NaF.

Table 2. Effect of GppNHp on adenylate cyclase activity of different cell populations of the seminiferous tubule (pmole/mg prot/min ± s.d.)

	basal	GppNHp	GppNHp / basal
PARTICULATE			
Germ cells	27.7 ± 1.4	31.2 ± 2.0	1.1
Control tubules	29.5 ± 1.9	60.7 ± 4.0	2.1
X irradiated tubules	19.2 ± 1.3	109.6 ± 1.6	5.7
SOLUBLE			
Germ cells	79.9 ± 0.6	82.3 ± 2.5	1.0
Control tubules	65.7 ± 2.2	64.3 ± 2.4	1.0
X irradiated tubules	4.7 ± 0.4	4.0 ± 0.7	0.9

Preparation of cells and subcellular fractionation were as described for Table 1. AC was measured in the presence of 10 mM $MgCl_2$ plus 10 mM $MnCl_2$ ± 0.125 mM GppNHp.

seminiferous tubule cells and with those of total X irradiated germ cell depleted seminiferous tubule cells (Tables 1 and 2). The results shown in Table 1 indicate that (a), germ cells possess a particulate activity of the same order of magnitude of somatic cell particulate activity; (b) while germ cell particulate activity is Mn^{++} dependent and scarcely stimulable by NaF, somatic cell particulate activity is Mn^{++} and Mg^{++} dependent and can be stimulated with NaF; (c), where NaF stimulable activity is present, stimulation occurs in the presence of both Mg^{++} and Mn^{++}, although higher stimulation is observed with the former ion; (d), soluble AC activity is present only in germ cells and (e), it is Mn^{++} dependent and insensitive to fluoride stimulation. Also GppNHp, as it occurs for fluoride, is unable to stimulate AC activity of germ cells, while its effect is evident on the particulate fraction of somatic cell enriched seminiferous tubules(Table 2).

It appears, therefore, that at least three different AC activities are present in the seminiferous tubule of the mouse. A particulate, responsive activity of the somatic cells (e.g. Sertoli and/or peritubular myoid cells (1,3)), and a particulate plus a soluble activity in the germ cells. Both forms of germ cell AC are Mn^{++} dependent and unresponsive to NaF and GppNHp. The finding of a germ cell particulate AC with the described properties is in good agreement with the observations that spermatozoa possess a membrane bound AC, Mn^{++} dependent (3,14) and practically unresponsive to cholera toxin and GppNHp (15). Germ cell soluble AC, on the other hand, represents an exception to the general concept of AC as a membrane bound enzyme. However, it does not appear that germ cell soluble AC represents an artefact due to extraction of membrane bound enzyme, as no

difference are observed in the levels of soluble AC when cells or tissue are homogenized in hypotonic or hypertonic buffer (up to 27% sucrose), in low or high salt (up to 0.4 M KCl) (data not shown). Furthermore, soluble AC does not appear to be a degradation product of the particulate enzyme, as shown by Neer (5).

Our data indicate that both particulate and soluble germ cells adenylate cyclases share some properties, such as Mn^{++} dependence and unresponsiveness to NaF and GppNHp. These properties correspond to those of the C component, the catalytic subunit of AC (16). On the other hand, testicular soluble AC, unlike authentic C component, does not reconstitute Mg^{++} dependent, NaF and GppNHp regulated AC activity when combined with plasma membrane extracts containing the regulatory subunit of AC (16). Further work is necessary to verify whether particulate and soluble AC represent modified C components and to elucidate the possible physiological role of the peculiar adenylate cyclases of germ cells.

ACKNOWLEDGEMENT: work supported by Consiglio Nazionale delle Ricerche, grant N° 79.01166. 85 and by Ford Foundation grant N° 790-0659.
The authors are indebted to Mr. R. Pezzotti and Mrs. A.M. Fonzi Zani for their skillfull assistance, and to Prof. M.Molinaro for critically reading the manuscript.

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