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The olfactory adenylyl cyclase type 3 is expressed in male germ cells

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Abstract Elements of the olfactory pathway, such as receptors, receptor-desensitization machinery, and cyclic nucleotide-gated channels, are expressed in male germ cells. Here we report the expression, in rat testis, of both adenylyl cyclase type 3 (AC3) and the olfactory G protein subunit, $G_{\alpha olf}$. Both are expressed in the same sub-population of germ cells, pachytene spermatocytes to spermatids, and in residual bodies. Neither AC3 nor $G_{\alpha olf}$ was found in Sertoli or in peritubular cells, as shown by Western blotting and immunocytochemical analyses. It thus appears that male germ cells contain all the elements of the signaling cascade present in olfactory cells.

Key words: Adenylyl cyclase; Signal transduction; Immunohistochemistry; Germ cell

1. Introduction

It has long been known that cAMP and calcium play a key role in different steps of male germ cell differentiation, in sperm maturation, and in sperm-egg interaction (reviewed in [1]). During the past few years, the presence in male germ cells of various elements of the olfactory pathway has been reported. In the dog and the human germ line, olfactory receptor genes are expressed during the late stages of spermatogenesis, and during the epididymal maturation of the sperm cells; they are present on the tail midpiece of mature sperm cells [2]. This observation has recently been extended to several mammalian species [3] and to other components of the olfactory pathways [4,5]. Signal transduction of certain odorants in olfactory neurons involves the activation of the adenylyl cyclase/cAMP second messenger system, which leads to the opening of cAMP-gated channels, and to an influx of Ca^{2+} causing the opening of Ca^{2+}-activated chloride ions channels (reviewed in [6]). Other odorants stimulate G protein-mediated formation of InsP3 with also a subsequent increase of intracellular Ca^{2+}.

Nine isoforms of adenylyl cyclase (AC) have been isolated in mammals, with a molecular weight of about 120 000 (reviewed in [7–9]). Depending on the AC isoform considered, the AC activity can be modulated by $G_{\alpha s}$, $G_{\alpha i}$, $G_{\beta\gamma}$, Ca^{2+}/calmodulin, or by phosphorylation by either protein kinase C (PKC), cAMP-dependent protein kinase (PKA), or calmodulin-activated kinase II, thus allowing a delicate tuning of the cAMP concentration. The distribution of the isoforms varies with development and is tissue-specific [9]. Adenylyl cyclase type 3 (AC3) was first identified as specific to the olfactory transduction apparatus [10]. Both AC3 and the olfactory $G_{\alpha s}$ subunit ($G_{\alpha olf}$) have been localized in the same receptor cell compartments, the distal segments of the olfactory cilia [11,12]. Here we demonstrate that both AC3 and $G_{\alpha olf}$ are expressed within the testis and that their expression is germ cell-specific and occurs at the same stages of germ cell differentiation. It thus appears that male germ cells contain all the elements of the signaling cascade present in olfactory cells.

2. Materials and methods

2.1. Northern blot analysis

Total RNA were prepared from adult Sprague-Dawley rat testes according to Chomczynski et al. [13]. Poly(A)$^+$ RNAs were isolated using oligo(dT) cellulose and fractionated on 1% agarose/2.2 M formaldehyde gel, transferred onto Hybond N$^+$ membrane (Amersham) and hybridized with the [^{32}P]dCTP-labeled rat cDNA probe for AC3 (amino acids 580–730) previously described [14]. This sequence is specific for AC3 and does not cross-react with any other known adenylyl cyclases. Rat brain poly(A)$^+$ RNAs, prepared under the same conditions, were used as control. Membranes were washed in 0.1×SSC, 0.1% SDS at 65°C for 30 min, and examined by autoradiography. Exposure time: 3 days.

2.2. Cell fractionation and PCR analysis

Sertoli cells were prepared according to Skinner and Fritz [15], pachytene spermatocytes and round spermatids were isolated as described by Pineau et al. [16], and residual bodies were separated according to Meistrich et al. [17]. Total RNA was extracted from the cells and purified by centrifugation through a cushion of CsCl as described by Sambrook et al. [18]. First strands of cDNA were synthesized for 1 h at 37°C from 10 μg of total RNA, with 10 ng/μl random hexanucleotide primers (Pharmacia Biotech, Saclay, France) and 200 U of murine Moloney leukemia virus reverse transcriptase (Gibco), in a reaction buffer (Tris-HCl-$MgCl_2$) containing 0.5 mM of each dNTP (Boehringer Mannheim, Meylan, France), 10 mM dithiothreitol, and 40 U of the RNase inhibitor RNasin (Promega, Charbonnière, France) in a final volume of 20 μl. The reaction volume was then brought up to 50–200 μl. Polymerase chain reaction (PCR) was performed in a Cetus (Perkin-Elmer) apparatus using 1 μl of the cDNA product in 20 μl final volume for 35 cycles (94°C 1 min, 50°C 1 min, 72°C 1 min) and the following oligonucleotides: (sense oligonucleotide): 5′-GGGAGTTCGATGTCGAACCTGGTGATG-3′ (position 1762–1789 of the AC3 rat cDNA sequence [10]); (antisense oligonucleotide): 5′-GTCCCATGTAGTACTGGAGACAGC-TC-3′ (position 2534–2560). 15 μl of the reaction product was loaded on a 1% agarose gel, and transferred to nitrocellulose (Hybond N$^+$, Amersham). An internal AC3-specific oligonucleotide (5′-TGAGCA-CGAACTGAACCAGC-3′) was used to probe the PCR amplification products, after ^{32}P labeling using the T4 polynucleotide kinase.

2.3. Western blotting analysis

Proteins from 3 month old rat testes, homogenized in Laemmli buffer (75 mM Tris-HCl, pH 6.8, 20% glycerol, 15% SDS, 5% 2-mercaptoethanol) [19], were resolved on 7.5% SDS-PAGE, transferred to nitrocellulose membrane (Hybond-C super, Amersham) and probed with either the anti-AC3 (Santa-Cruz Biotechnology) or the anti-$G_{\alpha olf}$

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(Santa-Cruz Biotechnology) antibody. The anti-AC3 antibody is directed against a peptide corresponding to amino acids 1125–1144 (H_2N-PAAFPNGSSVTLPHQVVDNP-COOH) mapping within the highly divergent carboxy-terminus of the AC3 of rat origin. This peptide sequence is not found in any other known adenylyl cyclase. It corresponds to the HAB-1 antibody used by Bakalyar and Reed [10], which specifically labels the epithelial surface of the rat olfactory tissue and makes it possible to detect a single band of 200 kDa in Western blot. No cross-reactivity was observed with adenylyl cyclases 1, 2, 4, 5, 6, and 9. The anti-$G_{\alpha olf}$ antibody was produced against amino acids 87–105 mapping within a highly divergent domain of $G_{\alpha olf}$ of rat origin. Both antibodies were used at the 1/100 dilution. As controls, antibodies preabsorbed with their antigenic peptide, or preimmune serum, were used. Bands were visualized using a horseradish-coupled goat anti-rabbit IgG (Dako) and the ECL system (Amersham Inc.) as mentioned by the manufacturer.

2.4. Immunostaining

Peroxidase histochemistry was performed either on paraffin sections of adult rat testes fixed by perfusion with Bouin's fluid, or on cryosections of adult rat testes rapidly excised, decapsulated and frozen in isopentane. Prior to labeling, the paraffin sections were deparaffinized, and rehydrated through a grade series of ethanol solutions; the cryosections (10 μm) were fixed with acetone and rehydrated with 10 mM phosphate buffer pH 7.4. Labeling was carried out, after a treatment with normal goat serum for 30 min, at room temperature in a moisture chamber, with either the anti-AC3 antibody or the anti-$G_{\alpha olf}$, both diluted at 1/200. Biotinylated goat anti-rabbit IgG was used diluted at 1/200. Immunostaining was performed using the avidin-biotin system (Vector-Biosys) in the conditions described by the manufacturer. No immunoreactivity was observed in the control sections, where the primary antibody was pre-incubated with its antigenic peptide.

3. Results

3.1. AC3 mRNA is expressed in male germ cells

Northern blots performed on poly(A)$^+$ RNAs from adult rat testes showed expression of AC3, although at a low level: a faint band around 4.5 kbp, corresponding to the mRNA observed in rat brain, was detected (Fig. 1A). This result has been confirmed on RNAs isolated from the different testes sub-populations. (i) First, highly enriched populations of pachytene spermatocytes and round spermatids were isolated, and RT-PCR was conducted using pairs of degenerated primers directed against sequences conserved across the different

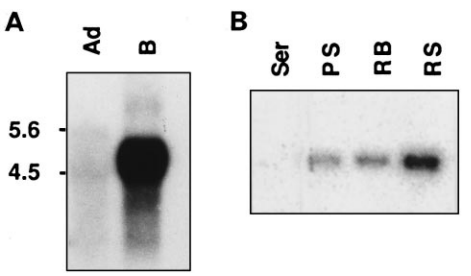

Fig. 1. Testicular expression of AC3 mRNA. A: Northern blot analysis of poly(A)$^+$ RNA from whole testis. Poly(A)$^+$ RNAs (10 μg per lane) isolated from testes (Ad) or brain (B) of adult Sprague-Dawley rats and fractionated on 1% agarose/2.2 M formaldehyde gel were hybridized with a specific rat AC3 probe. Exposure time: 3 days. B: RT-PCR analysis of AC3 expression in sub-populations of testicular cells. Sertoli cells (Ser), pachytene spermatocytes (PS), round spermatids (RS) and residual bodies (RB) were separated as described in Section 2. Complementary DNAs were prepared using random hexanucleotides and 10 μg of total RNAs from the different cell populations. PCR was performed and probed with oligonucleotides specific for rat AC3.

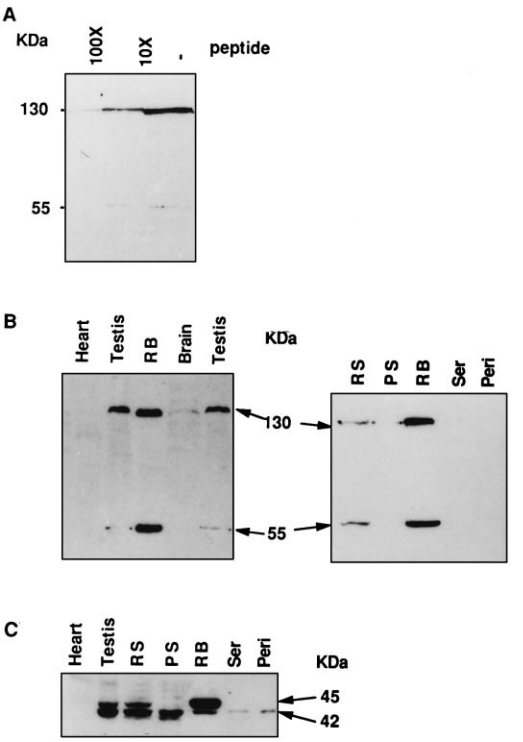

Fig. 2. Western blot analysis of AC3 and $G_{\alpha olf}$ in sub-populations of testicular cells. A: Proteins from 3 month old rats testes were resolved on 7.5% SDS-PAGE and probed with AC3 antibody alone or preincubated with its antigenic peptide. Bands were visualized by a horseradish-coupled goat anti-rabbit IgG and the ECL system (Amersham Inc.). A faint 55 kDa peptide was always detected in rat testis. B and C: Sub-populations of testicular cells were isolated: Sertoli cells (Ser), peritubular cells (Peri), residual bodies (RB), round spermatids (RS) and pachytene spermatocytes (PS). Proteins corresponding to 5×10^5 cells were resolved on SDS-PAGE and revealed using anti-AC3 (B) or anti-G_{olf} (C) antibody.

members of the AC family (in C1a and C2a domains of the catalytic site). Hybridization and sequencing of the PCR products led to the identification of AC3 in both cell populations. Moreover, overlapping PCRs, using specific oligonucleotides directed against the different portions of the rat AC3 sequence, detected the presence of the complete AC3 open reading frame in both types of cells (not shown). (ii) Second, to further establish the expression of AC3 in germ cells, and possibly in other testicular cell types, purified sub-populations of Sertoli cells, pachytene spermatocytes, round spermatids, and residual bodies were isolated, cDNAs were prepared using random hexamers and PCR performed using oligonucleotides located in the highly divergent region between the different ACs (C1b-M2), and specific for AC3. An internal oligonucleotide was used to specifically probe the PCR amplification products. From results shown in Fig. 1B, it is clear that AC3 mRNA is expressed from pachytene spermatocytes to spermatids and is still present in residual bodies. No AC3 mRNA was detected in somatic Sertoli cells.

3.2. AC3 protein is expressed in round spermatids

Since the presence of AC3 transcripts in germ cells does not necessarily imply that the corresponding protein is produced, a specific anti-AC3 antibody was used for immunoblotting experiments. Using a crude total homogenate from adult rat

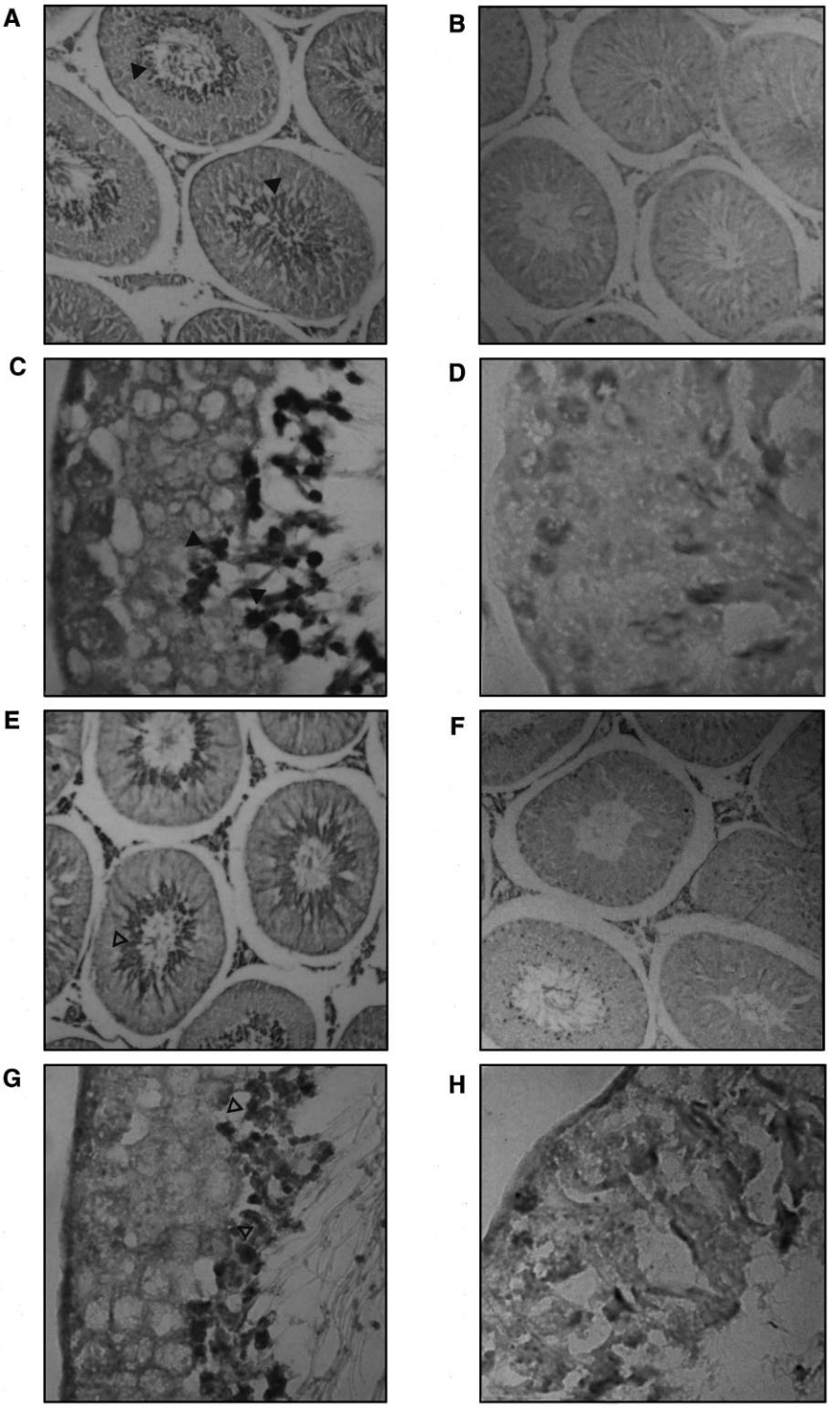

Fig. 3. Immunostaining localization of AC3 and $G_{\alpha olf}$ in adult rat testis sections. Peroxidase localization of AC3 (A–D) or $G_{\alpha olf}$ (E–H). Serial sections of adult rat testes were analyzed with either the specific antibodies (A, C: anti-AC3; E, G: anti-$G_{\alpha olf}$) or the antibody preabsorbed with a 100-fold excess (mol/mol) of its corresponding antigenic peptide (B, D: anti-AC3+AC3 peptide; F, H: anti-$G_{\alpha olf}$ + $G_{\alpha olf}$ peptide). The cells were counterstained with hematoxylin. A, B, E, F: Paraffin-embedded sections, magnification: ×80; C, D, G, H: Cryosections, magnification: ×500. No immunoreactivity was observed in the control sections. Specific AC3 (▲) or $G_{\alpha olf}$ (△) labeling.

testes, we identified two discrete bands of 130 and 55 kDa on Laemmli SDS gel electrophoresis, the most abundant being the high molecular weight entity (Fig. 2A). The apparent molecular weight of 130 kDa is consistent with the calculated molecular size of 129 kDa, and corresponds to a native form of AC3. No AC3 was found in heart. In rat brain, only the 130 kDa form of AC3 was detected (Fig. 2B). The specificity of the reaction was checked by showing that the immunoreactivity was completely abolished by pre-incubation of the antibody with a 100-fold excess (mol/mol) of its anti-

genic peptide to preabsorb the antibody. The analysis of testis sub-populations confirms the PCR results but no AC3 protein was detected in pachytene spermatocytes. Both types of forms of AC3 (130 kDa and 55 kDa) were found in spermatids and, to an even greater extent, in the fraction containing the residual bodies extruded from the mature elongated spermatids. AC3 is absent from the somatic Sertoli and peritubular cells (Fig. 2B).

3.3. $G_{\alpha olf}$ subunit is expressed at the same stages of differentiation

Since both AC3 and olfactory receptors are expressed in male germ cells, the question arises as to whether $G_{\alpha olf}$, which has already been detected in mouse and human testis at levels approaching those found in the olfactory neuroepithelium [20], is also present in the same sub-populations. Analysis of whole testis and series of cell homogenates showed that the anti-$G_{\alpha olf}$ antisera recognized bands of 42 and 45 kDa (Fig. 2C). Such heterogeneity in $G_{\alpha olf}$ proteins has previously been observed [20,21]. In whole testis, as well as in round spermatids, both forms appear expressed at the same level whereas the major forms detected in pachytene spermatocytes and in the residual bodies were the 42 kDa and the 45 kDa, respectively. In rat heart, $G_{\alpha olf}$ immunoreactivity was undetectable.

3.4. Immunohistochemical localization of AC3 and $G_{\alpha olf}$

To determine more precisely the localization of AC3 and $G_{\alpha olf}$ in the adult rat testis, we performed light immunohistochemistry. Serial adjacent testis sections were labeled with either AC3 or $G_{\alpha olf}$ antibody, and revealed with peroxidase reaction. In both cases, most of the specific labeling was restricted to certain categories of cells of the seminiferous tubules and appeared to be strictly intracellular (Fig. 3A,C,E,G). These cells, located next to the lumen of the tubule, correspond to round and elongating spermatids. Moreover, high staining intensity was observed over residual bodies (Fig. 3C,G). Germ cells at earlier stages such as spermatocytes and spermatogonia remained unstained, as did Sertoli cells and the cells of the interstitial tissue. The labeling pattern varied markedly depending upon the stage of the seminiferous epithelium cycle. The specificity of the reaction was assessed by staining adjacent sections with preimmune serum or with preincubated antibodies with their control peptide. Absence of staining was consistently obtained under these conditions (Fig. 3B,D,F,H), as previously observed in the Western blot experiments. Furthermore, a specific anti-AC9 antibody [22], tested under the same conditions, gave no specific signal (not shown). These results are consistent with those obtained from the Western blotting studies.

4. Discussion

In this paper, we demonstrate that both AC3 and the olfactory Gα subunit ($G_{\alpha olf}$) are present in rat male germ cells. AC3 transcripts are present in pachytene spermatocytes and round spermatids while the corresponding protein appears in spermatids. This corresponds to the well known lag between mRNA synthesis and protein expression in germ cells.

By Western blotting, rat testis AC3 appears as two forms of 130 and 55 kDa. In rat brain, the M_r value observed for AC3 was 130 kDa [23]. Bakalyar and Reed [10] demonstrated that treatment of the olfactory ciliary proteins with peptide:*N*-glycosidase F (PNGase-F) altered the mobility of the proteins from an apparent molecular weight of 200 kDa to 130 kDa with the appearance of a small band around 50 kDa. A potential *N*-glycosylation site exists between membrane-spanning regions 9 and 10 of the putative extracellular face of the AC3 molecule. One possibility is that, in round and elongating spermatids, AC3 exists as a non-glycosylated form. This form might be more sensitive to proteolysis, giving rise to the small carboxy-terminal 55 kDa form.

It has long been known that mammalian spermatozoa contain a unique form of adenylyl cyclase, insensitive to G proteins, fluoride and forskolin, and associated with a low molecular weight fraction, ranging from 42 to 69 kDa depending to the authors [24–27]. This low molecular weight form has been described in the cytosol of the early stages of spermatid cells, while it is membrane-bound in mature spermatozoa [28]. Up to now, this specific form of the enzyme has not been purified, and its relationship to any of the nine known AC isoforms is unknown. It is tempting to propose that the soluble AC activity described as present in spermatids [29] is related to AC3. The many proteolytic activities present in male germ cells, such as acrosin [30], could be involved in some specific post-translational modifications of the enzyme. We, indeed, have shown that the adenylyl cyclase activity present as a membrane-bound form in mature ram spermatozoa can be easily solubilized by proteolysis [27]. Recently it has been demonstrated that the enzyme activity can be supported by an artificially shortened molecular species from AC1 and AC2, comprising two non-covalently linked subunits, and even devoid of transmembrane domains [31,32].

Cyclic AMP is known to play an important role in several steps of spermatogenesis; in particular it is required together with Ca^{2+} for sperm motility. It is noteworthy that testis is highly enriched in calmodulin [33] and that in vitro AC3 can be activated by the Ca^{2+}/calmodulin complex [10]. In contrast, in vivo, AC3 has been reported to be inhibited by phosphorylations due to a calmodulin-activated kinase II [34], and to be at the origin of calcium oscillations in some non-excitable cells [35]. However, most of the enzymatic data published so far point to a stimulatory effect of calcium on cyclic AMP production in spermatozoa [36–38].

During the past few years, the presence in male germ cells of various elements of the olfactory pathway has been reported. Olfactory receptor genes were found expressed during the late stages of spermatogenesis, and during the epididymal maturation of the sperm cells [2,3]. Moreover, these olfactory receptors have been colocalized on the midpiece of the rat sperm together with two proteins involved in their desensitization [4] Recently, cyclic nucleotide-gated channels that serve as downstream targets of signaling pathways of vertebrate photoreceptors and olfactory sensory neurons, have also been expressed in bovine sperm [5]. In this context, the presence of AC3 and of $G_{\alpha olf}$ in male germ cells, particularly in the late spermatids, is of special interest since this would demonstrate that those cells use the same transduction pathways as olfactory neurons.

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