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Calcium-Modulated Rod Outer Segment Membrane Guanylate Cyclase Type 1 Transduction Machinery in the Testes

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Abstract

The importance of the second messengers, Ca^{2+} and cyclic GMP, for the process of fertilization is well established; the mechanisms for their intracellular regulations in the testes are, however, poorly understood. This study documents the biochemical, molecular, and functional identity of a Ca^{2+}-modulated membrane guanylate cyclase transduction machinery in bovine testes. The machinery is both inhibited and stimulated by free Ca^{2+} levels. The Ca^{2+}-sensor component of the inhibitory mode of the machinery is GCAP1 (guanylate cyclase activating protein type 1) and for the stimulatory mode is S100B. The transduction component is a Ca^{2+}-driven rod outer segment membrane guanylate cyclase type 1, ROS-GC1. The cyclase is predominantly expressed in spermatogenic cells. GCAP1 expression is restricted to a small population of spermatogonia, whereas S100B is present in the majority of spermatocytes and spermatids. The expression of GCAP1 and S100B in spermatocytes and spermatids is mutually exclusive.

Key words: ROS-GC1, GCAP1, S100B, signal transduction

Intracellular second messenger cyclic guanosine monophosphate (cGMP) is involved in the regulation of a broad spectrum of physiological processes ranging from peripheral to the central nervous system. In testes, it has been implicated to affect motility in spermatozoa, development of testicular germ cells, relaxation of peritubular lamina propria cells, testosterone synthesis in Leydig cells, dilatation of testicular blood vessels, and fertilization (Ong et al, 1975; Rossi et al, 1985; Mukhopadhyay et al, 1986; Pandey et al, 1986; Armstrong et al, 1994; Sanchez et al, 1996; Wiesner et al, 1998; Middendorff et al, 2000; Wayman et al, 2005). Guanylate cyclase-activating substances, which are secreted by several tissues in the genital tract, affect sperm motility, capacitation, and acrosomal reactivity; stimulation of sperm metabolism; and promotion of sperm ability to approach the oocyte, interact with it, and finally, to fertilize it (Garbers, 1989; Shapiro et al, 1990; Armstrong et al, 1994; reviewed in: Revelli et al, 2002; Kawase et al, 2004).

In the process of fertilization Ca^{2+} plays a key role. The mechanisms controlling Ca^{2+} entry into the sperm, Ca^{2+} conductance, and ultimately the spermatozoa movement toward the oocyte, remain unknown but are thought to involve various types of Ca^{2+} channels (reviewed in: Benoff, 1998; Publicover and Barratt, 1999; Son et al, 2000; Jagannathan et al, 2002a,b; Castellano et al, 2003; Chiarella et al, 2004; Stamboulian et al, 2004; Trevino et al, 2004). The acrosomal activity of human spermatozoa is thought to be triggered by Ca^{2+} influx mainly through α1H T-type Ca^{2+} channels, expressed exclusively in spermatogenic cells (Son et al, 2000; Jagannathan et al, 2002a,b). A novel Ca^{2+} channel, CatSper, present in the sperm has been cloned (Nikpoor et al, 2004). This gene encodes a unique and testes-specific Ca^{2+} channel. A significant reduction in the level of CatSper gene expression has been observed among patients with lowered sperm motility (Nikpoor et al, 2004). Also a cyclic nucleotide-gated channel has been identified in the mammalian sperm (Weyand et al, 1994; Wiesner et al, 1998). The homooligomeric α subunit cloned from bovine testes is roughly 200-fold more sensitive to cyclic GMP then to cyclic AMP, suggesting that the channel is specific and is a part of the cyclic GMP signaling pathway (Wiesner et al, 1998).

Cyclic GMP is synthesized by a group of enzymes termed guanylate cyclases. Depending on their cellular distribution, these enzymes have been classified into 2 families, membrane-bound and soluble. The soluble form is heterodimeric in structure and is stimulated by nitric oxide and carbon dioxide (Murad, 1994; Middendorff et al, 1997; Pyriochou and Papapetropoulos, 2005). The membrane-bound form is a single transmembrane-spanning protein; it is monomeric, yet requires homodimerization for its activity (Chinkers and Wilson, 1992; Vaandrager et al, 1994; Wilson and Chinkers, 1995; Labrecque et al, 1999; Yu et al, 1999). On a biochemical basis, the membrane guanylate cyclase family is divided into 2 subfamilies, surface receptors and rod outer segment guanylate cyclase (ROS-GC). A central feature of the surface receptor subfamily is that all its members are receptors for peptide hormones or bacterial toxins (Fulle and Garbers, 1994; Leitman et al, 1994; Amin et al, 1996; Sharma and Duda, 1997; reviewed in: Sharma, 2002; Kuhn, 2003). These receptors, by binding their ligands, transduce the signal into production of the second messenger cyclic GMP.

The Ca^{2+}-modulated membrane guanylate cyclase subfamily has been named ROS-GC. ROS-GC subfamily is designed to transduce Ca^{2+} signals (reviewed in: Koch et al, 2002; Sharma, 2002; Sharma and Duda, 2006). ROS-GC machinery is a 2-component transduction system, the enzyme ROS-GC and the Ca^{2+} sensor. It is expressed specifically in the sensory and intermediary neurons of the retina (Hayashi and Yamazaki, 1991; Dizhoor et al, 1994; Goraczniak et al, 1994; Lowe et al, 1995; Goraczniak et al, 1997), pinealocytes (Venkataraman et al, 2000), the olfactory bulb (Duda et al, 2001a,b), and the anterior portion of the gustatory epithelium (Duda and Sharma, 2004). There are 3 members of this subfamily, ROS-GC1, ROS-GC2, and ONE-GC, expressed specifically in the olfactory neuroepithelium (Duda et al, 2001a,b). These cyclases, in contrast to the peptide hormone receptor guanylate

cyclases, receive signals indirectly through Ca^{2+} sensor proteins, which belong to 2 classes, guanylyl cyclase activating proteins (GCAPs) and calcium dependent-GCAPs (CD-GCAPs). GCAPs receive the signals, undergo conformational change and inhibit ROS-GC. CD-GCAPs receive the signals, also undergo conformational change, and stimulate ROS-GC (reviewed in Koch et al, 2002; Sharma, 2002). GCAPs and CD-GCAPs sense Ca^{2+} signals through their EF-hand domains. There are 3 known functional GCAPs, 1, 2, and 3, and 2 CD-GCAPs, S100B and neurocalcin δ (Palczewski et al, 1994; reviewed in Haeseleer et al, 1999; Sharma, 2002; Sharma and Duda, 2006).

The presence and functional activity of the soluble and particulate (ANF-RGC and CNP-RGC) guanylate cyclase has been shown in testes (Marala and Sharma, 1988; Middendorff et al, 1996; Middendorff et al, 1997; Middendorff et al, 2000; Muller et al, 2004). In the sperm of marine invertebrates, the presence of chemotactic peptide receptors guanylate cyclases has also been shown (Ramarao and Garbers, 1985; Matsumoto et al, 2003), but there is no evidence of their presence in the mammalian sperm. Until now, the presence of Ca^{2+}-modulated ROS-GC transduction system has been thought to be the exclusive domain of sensory or sensory-linked neuronal cells. This study documents the biochemical, molecular, and functional identity of a Ca^{2+}-modulated membrane guanylate cyclase transduction machinery in bovine testes. The machinery is both inhibited and stimulated by free Ca^{2+} levels. The Ca^{2+}-sensor component of the inhibitory mode of the machinery is GCAP1 and for the stimulatory mode is S100B. The transduction component is the guanylate cyclase ROS-GC1.

Materials and Methods

- Top
- Abstract
- Materials and Methods
- Results and Discussion
- References

Reagents

GCAP1 and GCAP2 were cloned, expressed, and purified as described previously (Venkataraman et al, 2000). S100B used was obtained commercially (Sigma Chemical Co, St Louis, Mo) or recombinant, prepared in our laboratory as described previously (Duda and Sharma, 2004).

Antibodies

Characterization of highly specific antibodies raised against GCAP1, GCAP2, S100B, and ROS-GC1 has been described previously (Duda et al, 1998; Venkataraman et al, 2000; Duda et al, 2001a,b; Duda and Sharma, 2004). The antibodies were enriched by precipitating the immunoglobulin fraction using ammonium sulphate. ELISA and Western blotting were used to test the specificity and to determine the titer of purified antibodies.

Reverse Transcription Polymerase Chain Reaction

Bovine testes were purchased from a local slaughterhouse. The tunica albuginea was removed, and total RNA was isolated from the fraction containing seminiferous tubules and interstitial tissue using TriPure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols. The cDNA library was constructed using Advantage RT for PCR kit (BD-Biosciences, San Jose, Calif) and used for the amplification of the 548-bp fragment of ROS-GC1, 373 bp of ROS-GC2, 627 bp of GCAP1, 601 bp of GCAP2, and 279 bp of S100B coding region. The amplified fragments were purified on agarose gel and sequenced to confirm their identities. Additionally, as a control, the 238-bp specific fragment of a housekeeping gene, large ribosomal subunit (L30), was amplified from the cDNA library.

Preparation of the Membrane Fraction of Testes

Membrane fraction of bovine testes was isolated according to the protocol described previously

(Marala et al, 1991). The tissue fragments devoid of tunica albuginea, including seminiferous tubules and interstitial tissue, were homogenized in a buffer containing 250 mmol sucrose, 10 mmol Tris-HCl (pH 7.4), and 1 mmol phenylmethylsulfonyl fluoride and centrifuged at 150 *g* for 10 minutes to remove cell debris and nuclei. The post-mitochondrial supernatant (after centrifugation at 400 x *g* and 10 000 x *g*) was centrifuged at 40 000 x *g*. The pellet, designated as the membrane fraction, was suspended in the homogenization buffer and stored at −150 °C until use.

Guanylate Cyclase Activity

The membrane fraction (~0.1 µg protein/sample) was assayed for guanylate cyclase activity as described previously (Paul et al, 1987; Duda et al, 1998; Venkataraman et al, 2000; Duda and Sharma 2004). Briefly, membranes were preincubated on an ice-bath with or without regulatory proteins in the assay system containing 10 mmol theophylline, 15 mmol phosphocreatine, 20 µg creatine kinase, and 50 mmol Tris-HCl, pH 7.5, adjusted to appropriate free Ca^{2+} concentrations with precalibrated Ca^{2+}/EGTA solutions (Molecular Probes, Eugene, Ore). The total assay volume was 25 µL. The reaction was initiated by the addition of the substrate solution [4 mmol $MgCl_2$ and 1 mmol GTP (final concentrations)] and maintained by incubation at 37 °C for 10 minutes. The reaction was terminated by the addition of 225 µL of 50 mmol sodium acetate buffer, pH 6.2, followed by heating in a boiling water bath for 3 minutes. The amount of cyclic GMP formed was determined by radioimmunoassay (Nambi et al, 1982). The RIA system detects 2 fmol of cyclic GMP/sample.

Western Blotting

The procedure was carried out according to the previously published protocols (Venkataraman et al, 2000; Duda and Sharma, 2004). Briefly, after boiling in gel-loading buffer (62.5 mmol Tris-HCl [pH 7.5], 2% SDS, 5% glycerol, 1 mmol ß-mercaptoethanol, and 0.005% bromophenol blue) ~150 µg of membrane protein was subjected to SDS-PAGE in a buffer containing 0.025 mmol Tris-HCl (pH 8.3), 0.192 mol glycine, and 0.1% SDS. The resolved proteins were transferred to nitrocellulose membranes, and the blot was incubated in Tris-buffered saline containing 0.05% Tween 20 (TBS-T), 5% powdered nonfat Carnation milk (blocking buffer) overnight at 4 °C. The anti-ROS-GC1, GCAP1, GCAP2, or S100B rabbit polyclonal antibodies were added individually at dilution 1:1500, 1:1000, 1:1000, and 1:800, respectively. After 1 hour incubation the blot was rinsed with TBS-T and the incubation was continued with the secondary antibodies conjugated to horseradish peroxidase (1:10 000 dilution) in the blocking buffer for another hour. Finally the blot was treated with SuperSignal blaze chemiluminescent substrate (Pierce Biotechnology, Rockford, Ill) for 5 minutes according to the manufacturer's protocol. The immunoreactive band was detected by exposing the blot to Kodak X-ray film for 15 seconds. Images of the membranes with the immunoreactive bands were acquired by scanning and processed using Photoshop 6.0 software.

Immunohistochemistry

Paraffin sections of bovine testes fixed in 4% paraformaldehyde were used for immunohistochemical detecting of ROS-GC1, GCAP1, and S100B. Antigens were retrieved by microwave activation in citrate buffer (10 mmol, pH 6.0). After being blocked in PBS containing 3% bovine serum albumin and 0.1% Tween 20, sections were incubated with primary antibodies against ROS-GC1 diluted 1:200, GCAP1 diluted 1:100, and S100B diluted 1:50 in the same solution for 60 minutes at 37 °C in a humidified chamber and washed for 60 minutes in PBS containing 0.1% Tween 20. AP-conjugated anti-rabbit IgG, diluted 1:200 (Sigma-Aldrich, Saint Louis, Mo) and NBT/BCIP as the substrate were used for detection. Incubation and washing conditions were as described for primary antibodies. Control included detection reactions carried out under identical conditions, except that the primary antibodies were replaced by nonimmune serum.

Results and Discussion

- Top
- Abstract
- Materials and Methods
- Results and Discussion
- References

With the background information that both cyclic GMP and Ca^{2+} play an important role in the spermatogenesis and fertilization processes, the present study was aimed at determining if the activities of these 2 messengers are interlocked in the testes. Through molecular, biochemical, and functional approaches, this investigation shows that they are indeed linked and provides a mechanism for the interlocking process.

Testes Contain a Functional Ca^{2+}-Dependent Membrane Guanylate Cyclase

Membrane fraction of bovine testes was isolated and tested for guanylate cyclase activity. It was 3.5 pmol of cyclic GMP/mg protein/min. This is a cumulative value representing all putative guanylate cyclases present in the fraction. To assess whether the activity is Ca^{2+}-modulated, the membrane fraction was exposed to increasing concentrations of free Ca^{2+} and the guanylate cyclase activity was measured. The results (Figure 1A) show that the fraction contained a guanylate cyclase, which was either stimulated or inhibited depending on the free Ca^{2+} concentration. A range of Ca^{2+} concentration from 10 nmol to 0.8 µmol caused a dose-dependent decrease, and the higher range caused stimulation of the membrane guanylate cyclase. The inhibitory IC_{50} value for Ca^{2+} was ~300 nmol, and the stimulatory EC_{50} value was ~5 µmol (Figure 1A). These results show that the testes contain membrane guanylate cyclase, which is both inhibited and stimulated by Ca^{2+} signals.

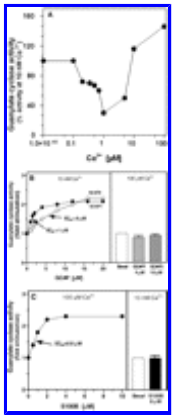

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Figure 1. Ca^{2+} regulation of guanylate cyclase activity in bovine testes. Membrane fraction of bovine testes was prepared and assayed for guanylate cyclase activity, as described in "Materials and Methods." **(A)** Response to Ca^{2+}. Membranes were incubated in the presence of indicated concentration of Ca^{2+}. **(B)** Response to GCAP1 and GCAP2. Membranes were incubated with increasing concentrations of GCAP1 or GCAP2 in the presence of 10 nmol free Ca^{2+} and 4 µmol GCAP1 or 10 µmol GCAP2 in the presence of 100 µmol free Ca^{2+}. **(C)** Response to S100B. Membranes were incubated with indicated concentrations of S100B in the presence of 100 µmol Ca^{2+} and 8 µmol S100B in the presence of 10 nmol Ca^{2+}. The molecular mass of 24 000 daltons was used to calculate the concentration of GCAP1 and GCAP2; 20 kd for S100B. Each experiment was done in triplicate and repeated 2 times. The results shown are from 1 representative experiment. Error bars are within the size of the symbols.

Testes Express a Guanylate Cyclase With the Biochemical Attributes of ROS-GC1

The 3 known Ca^{2+}-dependent membrane guanylate cyclase forms, ROS-GC1, ROS-GC2, and ONE-GC, can be differentiated biochemically (Koch, 1991; Goraczniak et al, 1998; Duda et al, 2001a,b). The ROS-GCs are stimulated in low nanomolar Ca^{2+} concentrations and inhibited in the semimicro- to micromolar Ca^{2+} concentrations by the GCAPs: ROS-GC1 by GCAP1 and GCAP2, and ROS-GC2 only by GCAP2 (reviewed in Palczewski et al, 1994; Haeseleer et al, 1999; Koch et al, 2002; Duda et al, 2005; Duda and Sharma, 2006). In the semimicro- to micro-molar Ca^{2+} concentrations, both ROS-GCs are stimulated by the CD-GCAP, S100B and ROS-GC1 also by neurocalcin δ (reviewed in Koch et al, 2002; Sharma and Duda, 2006). ONE-GC is stimulated only by Ca^{2+}-bound neurocalcin δ (Duda et al, 2001a,b; Sharma and Duda, 2006).

To determine which 1 (or more) of these cyclases is expressed in the testes, the membrane fraction of bovine testes was exposed to GCAP1, GCAP2, or S100B in the presence of 10 nmol and 100 µmol Ca^{2+}. The stimulation by GCAP3 was not tested, since its expression was not detected in bovine tissue (Haeseleer et al, 1999). In 10 nmol Ca^{2+} both GCAPs stimulated the cyclase activity in a dose-dependent manner, with an EC_{50} of ~1 µmol for GCAP1 and of ~6 µmol for GCAP2 (Figure 1B). The maximal stimulation of the cyclase was ~2-fold above the basal value (Figure 1B). There was no stimulation by either GCAP in 100 µmol Ca^{2+} (Figure 1B). The Ca^{2+}-bound S100B also stimulated the cyclase activity in a dose-dependent fashion (Figure 1C). The half-maximal stimulation was achieved at 0.8 µmol S100B, and the maximal stimulation was ~2.5-fold. Ca^{2+}-free S100B did not stimulate the cyclase activity (Figure 1C). All these stimulatory profiles are in accord with those established earlier for the reconstituted systems consisting exclusively of recombinant ROS-GC1 and individual Ca^{2+} sensor proteins, GCAP1, GCAP2, or S100B (Duda et al, 1996a,b; Duda et al, 1998; Goraczniak et al, 1998; Krishnan et al, 1998). The ability of the cyclase to respond to both GCAPs (ROS-GC2 does not respond to GCAP1) and S100B indicates that the membrane fraction of bovine testes contains a guanylate cyclase that functionally mimics ROS-GC1 (Goraczniak et al, 1998; Heaseleer et al, 1999). It is noted that the ability of exogenous GCAPs or S100B (Figure 1B and C) to stimulate ROS-GC1 in the testes membrane fraction beyond the point achieved through only the removal or addition of Ca^{2+} (Figure 1A) indicates that during the membrane preparation some Ca^{2+} sensor proteins were lost; hence, their levels were lower than that necessary for maximal activation of the cyclase.

The Transcript of ROS-GC1 Is Present in the Testes

To verify at molecular level the presence of ROS-GCs in bovine testes, total RNA was isolated from the tissue, reverse transcribed, and a 548-bp fragment corresponding to ROS-GC1 nucleotides 2042–2589 (GenBank accession number P55203) and a 373-bp fragment matching to ROS-GC2 nucleotides 3276–3649 (GenBank accession number U95958) were amplified. Amplification of a 238-bp fragment of the 30 kd ribosomal subunit (L30) served as a control. In the cases of ROS-GC1 and L30, the amplification yielded single bands of the predicted size (Figure 2A, lane 3, ROS-GC1; lane 4, L30). Sequencing of the amplified ROS-GC1 fragment confirmed its identity with bovine ROS-G1. The amplification of the ROS-GC2 fragment yielded no results (Figure 2B, lane 2); however, when cDNA from bovine retina was used as a control, the amplification yielded a single band of the predicted size of 373 bp (Figure 2B, lane 3). It is thus concluded that the ROS-GC1 but not ROS-GC2 transcript is present in the bovine testes.

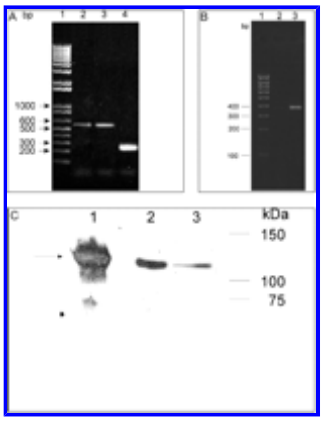

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Figure 2. Expression of ROS-GC1 in bovine testes. **(A)** Detection of the ROS-GC1 transcript. RNA was isolated from the bovine testes and reverse transcribed. From the resulting cDNA a 548-bp fragment of ROS-GC1 (lane 3) and a 238-bp fragment of L30 (lane 4) were amplified. The predicted size fragment of ROS-GC1 corresponded to PCR product obtained from retinal cDNA (lane 2). **(B)** Detection of the ROS-GC2 transcript. ROS-GC2 was not detected in bovine testes (lane 2). When cDNA from bovine retina was used as a control, the amplification yielded a single band of the predicted size, 373 bp (lane 3). Molecular size markers are given in lane 1. **(C)** Detection of the ROS-GC1 protein. Membranes of COS cells expressing ROS-GC1 (100 µg of total proteins; lane 1), retinal membranes (150 µgof total proteins; lane 2) and the membranes of bovine testes (150 µgof proteins; lane 3) were separated on a SDS-6% PAGE, transferred to nitrocellulose membrane, and probed with anti ROS-GC1 antibody, as described in "Materials and Methods." The immunoreactive bands were visualized by enhanced chemiluminescence (ECL). The ROS-GC1 immunoreactive band of the testes is indicated by an arrow. The positions of the molecular size markers are given

alongside.

Biochemical Identification of ROS-GC1 in the Membranes of Bovine Testes

To determine the direct biochemical presence of ROS-GC1 in testes, Western blot analysis was carried out. The particulate fraction was electrophoresed, transferred onto nitrocellulose membrane, and probed with specific antibodies against ROS-GC1. Specificity of the antibody has been described previously (Venkataraman et al, 2000; Duda and Sharma, 2004). Membranes of COS cells expressing ROS-GC1 as well as membranes isolated from bovine retina were processed in parallel as a positive control. A single immunoreactive band of apparent mobility of ~116 kd was observed in control membranes (Figure 2C, lane 1 and 2). A band of identical mobility was detected in the membrane preparation from bovine testes (Figure 2C, lane 3). Identical results were obtained when antibodies against the catalytic domain of membrane guanylate cyclase corresponding to ROS-GC1 amino acid residues M872-S1016 (Fik-Rymarkiewicz et al, 2006) were used in Western blots (data not shown). Thus through 3 independent criteria, functional, molecular, and biochemical, it is concluded that the sole ROS-GC expressed in the membrane fraction of bovine testes is ROS-GC1.

Expression of GCAP1 and S100B in Bovine Testes

To determine whether GCAP1 and/or GCAP2 are responsible for the cyclase stimulation in the absence of Ca^{2+} (Figure 1A; 10 nmol Ca^{2+}) and if S100B is responsible for the Ca^{2+}-dependent stimulation (Figure 1A; 0.8–100 µmol Ca^{2+}), their presence was investigated at both the RNA and protein levels. Using the specific primers designed based on the known sequences of S100B (GenBank accession number DQ195377), GCAP1 (GenBank accession number X95352), and GCAP2 (GenBank accession number U32856), RT-PCR was performed. A 279-bp fragment corresponding to the total coding region of S100B and a 627-bp fragment corresponding to the total coding region of GCAP1 were amplified (Figure 3A, lanes 2 and 3). Sequencing of the amplified fragments gave exact match to bovine GCAP1 and S100B cDNAs. There was, however, no amplification of a GCAP2 fragment (Figure 3A, lane 4). A single band of the predicted for GCAP2 size, 601 bp, was obtained in parallel control reaction with cDNA from bovine retina (Figure 3A, lane 5).

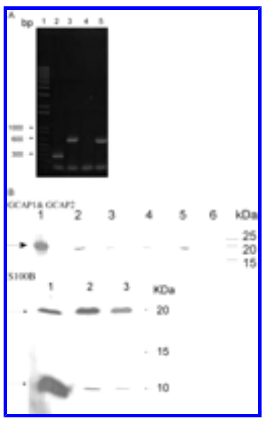

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Figure 3. Expression of ROS-GC1 modulators in the bovine testes. **(A)** Detection of GCAP1, GCAP2, and S100B transcripts. From a cDNA library constructed from bovine testes, a 279-bp coding region of S100B (lane 2) and a 627-bp fragment of GCAP1 (lane 3) were amplified. There was no amplification of GCAP2 fragment (lane 4). A single band of 601 bp characteristic for GCAP2 was obtained when cDNA from bovine retina was used as a control (lane 5). The products were resolved on agarose gel and visualized with ethidium bromide under UV light. Molecular size markers are in lane 1. **(B)** Detection of GCAP1, GCAP2, and S100B proteins. Membrane fraction from bovine testes was isolated and subjected to Western blot analyses with specific antibodies against GCAP1 (panel "GCAP1&GCAP2," lane 3), GCAP2 (panel "GCAP1&GCAP2," lane 6), and S100B (panel "S100B," lane 3), as described in "Materials and Methods." Recombinant GCAP1 (1 µg) and GCAP2 (0.5 µg) (panel "GCAP1&GCAP2," lanes 1 and 4, respectively) as well as commercial S100B (0.3 µg) (panel "S100B," lane 1) were used as controls. Additionally, retinal membranes were probed with GCAP1, GCAP2, and S100B antibodies as a positive control of the study (panel "GCAP1&GCAP2," lane 2 for GCAP1 and 5 for GCAP2; panel "S100B," lane 2). The band corresponding to GCAP1 as well as monomeric and dimeric forms of S100B are indicated by arrows. Molecular size markers are given alongside.

At the protein level, the identities of GCAPs and S100B in the membranes of bovine testes were established by Western blot analysis using specific antibodies against these proteins (Figure 3B). Anti-GCAP1 antibody detected a single band of apparent mobility of ~20 kd, identical to that of purified recombinant GCAP1 and retinal membranes (Figure 3B, panel "GCAP1&GCAP2," lanes 1, 2, and 3 for recombinant, retinal, and testes, respectively). In agreement with the cDNA amplification results, in testes membrane fraction there was no immunoreactivity with anti-GCAP2 antibody (Figure 3B, panel "GCAP1& GCAP2," lane 6). Anti-S100B antibody showed the presence of 2 immunoreactive bands (Figure 3B, panel "S100B," lane 3). Bands of identical mobility were observed for recombinant S100B and membranes isolated from retina (Figure 3B, panel "S100B," lanes 1 and 2). The mobility of these bands, ~10 and 20 kd, corresponds to monomeric and dimeric forms of S100B. It is therefore concluded that GCAP1, but not GCAP2, and S100B are expressed in bovine testes.

ROS-GC1 Is Expressed in Germinal Cells of Bovine Testes

To determine the localization of the components of the ROS-GC1 transduction system, immunohistochemical analyses were carried out with specific antibodies against ROS-GC1, GCAP1, and S100B. In all specimens, the labeling was randomly distributed in seminiferous tubules. The results are presented in Figure 4. Staining for ROS-GC1 was observed predominantly in spermatogenic cells, especially in primary spermatocytes and spermatids. However, single positively stained spermatogonia were observed as well (Figure 4A). The increase in staining was detected across the cells in different stages of the spermatogenesis process. The highest accumulation visible in long spermatids appears to correlate with the stage of the seminiferous cycle. The majority of spermatocytes and spermatids were stained for S100B (Figure 4B). A different pattern of immunostaining was observed with anti-GCAP1 antibody. Only a small population of spermatogonia was stained for GCAP1 (Figure 4C). It can be suggested that the expression of GCAP1 in these cells can be associated with their biological activity. No labeling was observed in the controls, where the primary antibodies were omitted (Figure 4D).

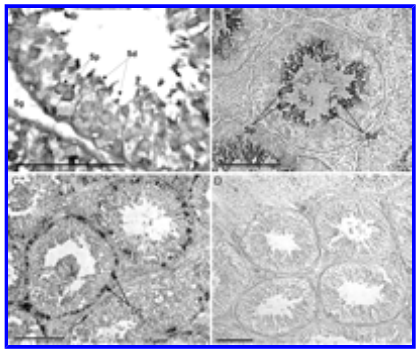

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Figure 4. Immunolocalization of ROS-GC1 in the bovine testes. **(A)** Immunohistochemistry was performed using specific antibodies against ROS-GC1 diluted 1:100 on paraffin section of bovine testes. The ROS-GC1 was localized in spermatogenic cells. The positive staining was observed in spermatogonia (Sg), primary spermatocytes (Sp), and spermatids (Sd), as indicated by the arrows. Bar = 100 µm. **(B)** When the section were subjected to analysis with anti-S100B antibodies, the majority of positively stained spermatocytes and spermatids were observed. **(C)** Incubation of paraffin section of bovine testes with antibodies against GCAP1 resulted in staining of only a small population of spermatogonia. **(D)** Control. The procedure was exactly the same as for detection of ROS-GC1, except that nonimmune serum was used instead of ROS-GC1 antibodies. Lack of staining is characteristic for all cells of spermatogenic cycle.

These results suggest that the ROS-GC1/GCAP1 and ROS-GC1/S100B transduction systems are mutually exclusive and are functional in different phases of the spermatogenic cycle.

The Ca^{2+} Modulated GCAP1/S100B - ROS-GC1 Signal Transduction Model

The present study, through functional, molecular, and biochemical approaches, shows the presence of ROS-GC1 in the testes. The enzyme coexists with its 2 Ca^{2+}-dependent modulators, GCAP1 and S100B. In

response to localized changes in free calcium concentration, these proteins bind to the specific domains, residing in the intracellular region of the cyclase, and inhibit or stimulate ROS-GC1. In this manner Ca^{2+} pulses precisely regulate the levels of cyclic GMP generated in 1 (or more) of the testes cells. Given the presence of a cyclic GMP-gated channel in these cells, the possibility exists that the levels control the hyper- or depolarized state of those cells. This prediction opens up a new venue of research. And the finding demonstrates that the presence of the ROS-GC transduction machinery is not unique to the neurosensory and neurosensory-linked systems; it also exists in the endocrinal system of the testes.

Footnotes

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References

- Top
- Abstract
- Materials and Methods
- Results and Discussion
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