Messenger Ribonucleic Acids Encoding a Serotonin Receptor and a Novel Gene Are Induced in Sertoli Cells by a Secreted Factor(s) from Male Rat Meiotic Germ Cells*

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ABSTRACT

Using Sertoli cell-germ cell cocultures and messenger RNA (mRNA) differential display, we have identified a complementary DNA of 355 nucleotides that is up-regulated in Sertoli cells by pachytene spermatocytes. The mRNA differential display pattern was confirmed by Northern blotting. Sequence analysis revealed a homology of 91% (nt) and 86% (aa) to a serotonin receptor. The mRNA encoding the serotonin receptor was detected in Sertoli cells after 18 h of coculture. Its induction did not require cell contact, as germ cell-conditioned medium also induced the mRNA. The germ cell factor(s) inducing the serotonin receptor mRNA is more than 10 kDa, survives freezing and thawing, and is heat sensitive. A high dose of serotonin (10 μM) or the serotonin receptor agonists (-)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl and quipazine induce the serotonin receptor mRNA in Sertoli cells after 24 h. The antagonists, ketanserin and spiperone, inhibit the serotonin-mediated mRNA induction but fail to inhibit the germ cell-mediated induction, suggesting that the germ cell factor(s) up-regulates the serotonin receptor by a distinct pathway. A second clone of 380 nucleotides, induced in Sertoli cells by pachytene spermatocytes or germ cell-conditioned medium, did not show homology to database sequences. The germ cell factor(s) inducing the second clone is larger than 10 kDa, but is inactivated by freezing/thawing and boiling. The induction of a serotonin receptor mRNA and a second novel mRNA in Sertoli cells by pachytene spermatocytes demonstrates that meiotic germ cells induce mRNA encoding an important receptor in Sertoli cells. (Endocrinology 140: 5754–5760, 1999)

C OIN CIDENT with the mitotic, meiotic, and postmeiotic phases of spermatogenesis, a wide variety of genes are developmentally regulated. Many of the genes are expressed exclusively in either somatic cells or germ cells of the testis, whereas others are expressed in both cell types (1, 2). Within the seminiferous tubules, cell-cell interactions between the somatic Sertoli cells and germ cells regulate germ cell development (3, 4). In close association with the differentiating germ cells, Sertoli cells secrete many factors presumably needed for germ cell development, including transferrin, androgen-binding protein, testin, and plasminogen activator, and metabolites such as lactate and pyruvate (3, 4). In turn, germ cell factors affect Sertoli cell function, enhancing the secretion of regulatory molecules such as inhibin, transferrin, and androgen-binding protein (5–8). As germ cells differentiate, their cellular interactions vary, with the meiotic pachytene spermatocytes forming specialized junctions with Sertoli cells, whereas the postmeiotic round spermatids form weaker associations with Sertoli cells (9). These differences in germ cell-Sertoli cell interactions can occur in culture. More preproenkephalin, glutamyl transpeptidase, transferrin, and ceruloplasmin are expressed in cocultures with pachytene spermatocytes than in Sertoli cells cocultured with round spermatids (10–12). In contrast, spermatid-conditioned medium increases inhibin messenger RNA (mRNA) levels more than conditioned medium from pachytene spermatocytes (13). Based on the differential induction of proteins in Sertoli cells by pachytene spermatocytes or round spermatids, each germ cell type appears to differentially control Sertoli cell function according to its specific needs (3, 4).

To identify genes that are up- or down-regulated by Sertoli cell-germ cell interactions, we cocultured male germ cells with Sertoli cells and used mRNA differential display to detect specific mRNA inductions (14). Recently, we demonstrated that soluble factors secreted by Sertoli cells induce a von Ebner’s-like protein and the Huntington disease protein in rat male germ cells (15). Here we demonstrate that a soluble factor(s) secreted by meiotic pachytene spermatocytes, but not by postmeiotic round spermatids, induces a serotonin receptor mRNA and a second novel mRNA in rat Sertoli cells in culture and in testes.

Materials and Methods

Cell isolation and culture

Sertoli cells were isolated from the testes of 20-day-old Sprague Dawley rats (Charles River Laboratories, Inc., Kingston, MA) by sequential enzymatic digestion as previously described (14–16). Germ cells were isolated from the testes of 60-day-old Sprague Dawley rats and cultured as previously described (14–16).
Cocultures of Sertoli cells and germ cells

Sertoli cells (2 x 10^6) from 20-day-old rats were cocultured with pachytene spermatocytes (8 x 10^6) or round spermatids (8 x 10^6) for 24 h. Germ cells were cultured in medium supplemented with 2 mM sodium pyruvate and 6 mM D,L-lactate at a density of 8 x 10^6/ml. After 24 h, the cells were scraped from the plates, and RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA). To determine the cellular sites of mRNA induction, Sertoli cells were cocultured with germ cells for 2–24 h. At the termination of each culture, the germ cells were separated from Sertoli cells by aggressive washing. Total RNA was purified from the cocultures and from separated Sertoli cells and germ cells. Throughout the culture period, trypan blue exclusion revealed greater than 97% cellular viability.

To delineate whether the mRNA inductions required physical contact between cells or whether secreted factors induced the mRNAs, germ cells and Sertoli cells were cultured separately for 24 h, and conditioned media were collected. The media collected from Sertoli cells were added to germ cells, and the media from germ cells were added to Sertoli cells, which were cultured for 24 h and collected for RNA extraction. To determine whether cellular contact was needed for mRNA induction, crude germ cell membrane fraction was prepared using the procedure of Charreau et al. (17). In brief, germ cells were homogenized in PBS and centrifuged at 1000 x g for 30 min at 4°C. The supernatants were further centrifuged at 100,000 x g for 1 h at 4°C. The pellets were resuspended in DMEM/Ham’s F-12 medium, and 500 μg membranes were cultured with 2 x 10^6 Sertoli cells for 24 h.

To characterize the factor(s) inducing mRNAs in coculture, the conditioned media described above were boiled for 10 min, frozen and thawed five times, or passed through Ultrafree-15 columns (Sigma Chemical Co., St. Louis, MO) containing Biomax membranes with molecular mass limits of 10 kDa.

To examine the effects of serotonin, serotonin agonist, and serotonin antagonist on the induction of the serotonin receptor mRNA, Sertoli cells were cultured alone with 2.5–10.0 μM serotonin, 10 μM serotonin agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-amino propane HCl or quipazine, or 10 μM serotonin antagonist ketanserin or spiperone in the presence of 10 μM serotonin or with germ cells and 10 μM of the receptor antagonists, ketanserin or spiperone. After 24 h, RNA was extracted.

Differential display RT-PCR and isolation of clones

mRNA differential display was performed on total RNA from Sertoli cells, from cocultures of Sertoli cells with pachytene spermatocytes or round spermatids, from pachytene spermatocytes, or from round spermatids.

Amplicons showing reproducibly unique expression patterns were cut from the dried gels and reamplified by PCR using the same set of primers. After gel purification the amplified PCR fragments were subcloned into a TA cloning vector (Invitrogen, San Diego, CA). Subcloned fragments were sequenced and used as probes for Northern analysis (14–16).

Results

RNA isolated from rat Sertoli cells cocultured with rat pachytene spermatocytes or round spermatids for 24 h was analyzed by differential display using T11 GT and OPA-12 (TCCGGCATGAG) as primers (Fig. 1). Two amplicons of 335 and 380 nucleotides were detected in cocultures of Sertoli cells and pachytene spermatocytes (Fig. 1, lane SC+P), but not in the Sertoli cell-round spermatid cocultures (Fig. 1, lane SC+RS) or in cultures of individual cell types (Fig. 1, lanes SC, P, and RS).

The mRNA of a serotonin receptor is up-regulated in Sertoli cell-pachytene spermatocyte cocultures

The complementary DNA (cDNA) of 355 nucleotides (clone 2 in Fig. 1), up-regulated in cocultures of Sertoli cells with pachytene spermatocytes, showed 91% similarity at the nucleotide level (from nucleotides 1862–2216) and a coding region amino acid similarity of 86% (from amino acids 399–450) to a Rattus norvegicus serotonin receptor 5HT-2 (18) (Fig. 2A). The serotonin receptor expresses two transcripts of 7 and 0.8 kb in Sertoli cell-pachytene spermatocyte cocultures or in testis, which were not detectable in RNA from cocultures of Sertoli cells and round spermatids, Sertoli cells, pachytene spermatocytes, round spermatids, lung, or brain (Fig. 3A). As serotonin receptors are known to be present in brain (19, 20), the blot was exposed for 18 h (Fig. 4A) and also for 3 days (Fig. 4B). After the longer exposure, 7- and 0.8-kb transcripts were detected in brain (Fig. 4B), but not in RNAs from Sertoli cells, pachytene spermatocytes, or round spermatids (data not shown).

Serotonin receptor transcripts are induced in Sertoli cells by a germ cell factor(s) after 18 h of coculture

To define the time and cellular site of induction of the serotonin receptor mRNA, Sertoli cells were cocultured with germ cells for 2–24 h. At the termination of culture, germ cells were separated from Sertoli cells, and RNAs were prepared from each cell type. The 7- and 0.8-kb transcripts were first detected in Sertoli cell and pachytene spermatocyte cocultures and in separated Sertoli cells from these cocultures after 18 h of culture (Fig. 5A). No induction was seen in Sertoli cells cocultured with round spermatids or when Sertoli cells were cultured alone.

To determine whether cell contact is needed or the serotonin receptor-like mRNAs are induced by secreted factors, germ cell-conditioned medium or a germ cell membrane fraction were added to Sertoli cells. The 7- and 0.8-kb transcripts were induced in Sertoli cells by conditioned medium, but not by a crude germ cell membrane fraction. The mRNAs were not induced in germ cells (Fig. 6A). Preliminary fractionation studies indicated that the germ cell factor(s) is larger than 10 kDa and survives freezing and thawing, but is inactivated by boiling (Fig. 7A).
Effects of serotonin, serotonin agonists, and antagonists on induction of serotonin receptor mRNA

To determine whether the germ cell factor(s) that induces the serotonin receptor mRNA in Sertoli cells is serotonin, Sertoli cells were cultured with serotonin, serotonin agonists, or serotonin antagonists for 24 h, and RNA was isolated for Northern analysis. Sertoli cells that received increasing amounts of serotonin (Fig. 8, lanes C–E) or serotonin agonists (Fig. 8, lanes F and G) expressed serotonin receptor mRNA. The antagonists, ketanserin and spiperone, inhibited the serotonin-mediated increase in serotonin receptor mRNA (Fig. 8, lanes H and I). However, these antagonists failed to inhibit serotonin receptor mRNA induction by germ cells (Fig. 8, lanes J and K).

A novel gene (clone 1) is also up-regulated in Sertoli cell-pachytene spermatocyte cocultures

A cDNA of 380 nucleotides (clone 1, Fig. 1) was also induced in Sertoli cell-germ cell cocultures. Clone 1 showed no homology to any known sequence in the GenBank database.
base (Fig. 2B). It hybridized to a 2.8-kb transcript from Sertoli cell-pachytene spermatocyte cocultures and from testis, but was not induced in Sertoli cells by round spermatids and did not hybridize with RNA from brain or lung (Fig. 3B).

**Clone 1 is induced in Sertoli cells by a germ cell factor(s) after 8 h of coculture**

When pachytene spermatocytes were cocultured with Sertoli cells for 8 h or more and then separated, the clone 1 transcript was detected in RNA isolated from Sertoli cells, but not from pachytene spermatocytes (Fig. 5B). The 2.8-kb transcript was induced in Sertoli cells by pachytene spermatocytes and germ cell-conditioned media, but not by germ cell membranes (Fig. 6B). The inducing factor appeared to be larger than 10 kDa and was inactivated by freezing/thawing and boiling (Fig. 7B).

**Discussion**

Germ cell development is not an autonomous process, but is subject to regulation by endocrine and paracrine cues transmitted through Sertoli cells (3). Experimentation over the last decade has revealed that Sertoli cells make and secrete a large number of proteins (4). Here we report that pachytene spermatocytes in culture can induce Sertoli cells to up-regulate two genes: a serotonin receptor and a novel gene not in the database. Serotonin, a biogenic amine, is a major neurotransmitter in the brain and functions in a number of tissues, including chromaffin cells of the gastrointestinal mucosa, pineal gland, pancreas, lung, thymus, thyroid, and testis (19, 21). In the central nervous system, serotonin acts as neurotransmitter with an involvement in gonadotropin regulation (22). Peripherally, serotonin affects steroidogenesis in ovary, testis, and adrenal (23, 24). Serotonin and monoamine oxidase, the enzyme that metabolizes serotonin, are present in the testis (21), and studies have indicated that serotonin may cause significant decreases in testosterone production and testis weight. At high doses, serotonin causes disruption of the seminiferous epithelium ranging in severity from an increased degeneration of spermatogenic cells to complete loss of the germinal epithelium. In addition, serotonin induces vasoconstriction in the main testicular artery, causing testicular ischemia and atrophy (25). Serotonin exerts its effect by binding to cell surface receptors. A large number of serotonin receptor subtypes, representing 13 distinct gene products and 7 families, have been reported (26).

The induction of novel serotonin receptor mRNA in Sertoli cells indicates that meiotic male germ cells can induce an additional neurotransmitter receptor in Sertoli cells. Neurotransmitters stimulate the aromatization of testosterone (27, 28), and receptors for neurotransmitters, such as the adrenergic β1 and β2 receptors, have been identified in Sertoli cells (29). Many adrenergic and cholinergic receptors are similar to serotonin receptors (30). In the testis, serotonin is secreted by Leydig cells (31), and a high concentration of serotonin has been reported in the interstitial fluid (32). High and low
affinity binding sites for serotonin on Leydig cell membranes have been reported (31). Taken together, these data suggest that Sertoli cells are in contact with significant quantities of natural ligand, thereby implicating serotonin as a potential regulator of Sertoli cell activity. Our data support such a role for serotonin. We propose that pachytene spermatocytes, through a soluble factor(s), induce serotonin receptor mRNAs in Sertoli cells. Serotonin secreted by Leydig cells then can bind to Sertoli cells, leading to the synthesis of specific proteins needed by germ cells.

The Sertoli cell serotonin receptor could also be involved in nitric oxide (NO) production in Sertoli cells. NO production in Sertoli cells has been proposed to be a regulatory agent in the local control of spermatogenesis (33). In the rat brain and in carcinoid tumor cells, serotonin receptors are coupled to NO activation (34, 35). Based on the induction of the serotonin receptor gene in Sertoli cells by pachytene spermatocytes, we suggest that pachytene spermatocytes could modulate Sertoli cell NO production through this novel serotonin receptor.

Our ability to detect the serotonin receptor mRNA in rat brain only after long overexposures supports the previous report of a low level of the serotonin receptor (5HT2) mRNA in brain (36). The reduced hybridization signal also could indicate that the testis contains distinct isoforms of serotonin receptor that differ in sequence from the brain serotonin receptors. The 7- and 0.8-kb transcripts detected by our cDNA differ from the 6-kb mRNA found in myometrial smooth muscle cells (37) or the 2.3-kb transcript reported in human tissues (20).

The ability of high concentrations of serotonin to up-regulate this serotonin receptor mRNA is surprising, but not unexpected. We also find that selective serotonin receptor agonists can mimic the inductive effects of serotonin on serotonin receptor mRNA. This effect of serotonin on serotonin receptor mRNA synthesis is completely abolished by selective serotonin receptor antagonists. However, the expression induced by germ cells is not inhibited by antagonist, suggesting that the germ cell factor(s) mediates the effect through a pathway distinct from the serotonin pathway.

**Fig. 5.** Time-dependent expression and cellular localization of the serotonin receptor cDNA and clone 1. Total RNAs (10 μg) were isolated from 2-, 4-, 8-, 18-, and 24-h cultures of Sertoli cells with pachytene spermatocytes (SC+P), Sertoli cells cocultured with either pachytene spermatocytes or round spermatids and then separated (SC), pachytene spermatocytes that had been cocultured with Sertoli cells and then separated (P), Sertoli cells cocultured with round spermatids and then separated (SC+RS), and round spermatids that had been cocultured with Sertoli cells and then separated (RS). The three lanes underlined with C represent Sertoli cells (SC), pachytene spermatocytes (P), or round spermatids (RS) cultured alone for 24 h as controls. The blot was hybridized with cDNAs encoding the serotonin receptor (A) and clone 1 (B). The blot was rehybridized with an actin-coding region probe.
Moreover, we believe that the inducing germ cell factor(s) is not serotonin, because our inducing factor has a molecular mass in excess of 10 kDa. However, we cannot rule out that serotonin might be bound to a larger molecule.

Clone 1 represents a second gene up-regulated in Sertoli cells by pachytene spermatocytes, but not by round spermatids. A search of databases does not detect any similar sequence, suggesting that this cDNA encodes a novel gene. When germ cells and Sertoli cells are dissociated after 8 h in coculture, the transcript was only detected in Sertoli cells. The germ cell factor(s) inducing clone 1 is greater than 10 kDa and does not survive freezing/thawing or boiling, suggesting that it is a protein. As the factor that induces the serotonin receptor mRNA survives freezing/thawing, it is likely that different factors induce clone 1 and the serotonin receptor.

In summary, our results show induction of mRNAs encoding a serotonin receptor and a novel gene in Sertoli cells by soluble factors from pachytene spermatocytes. We believe that the germ cell factor(s) inducing the serotonin receptor mRNA mediates its effect by a mechanism distinct from serotonin. As the serotonin receptor mRNA and clone 1 are not induced when Sertoli cells are cocultured with round spermatids or the kidney cell line NRK-5ZE (data not shown), we propose that these inductions represent specific meiotic germ cell regulation of Sertoli cell function.
FIG. 8. Northern blot analysis demonstrating the effects of serotonin, serotonin agonist, and serotonin antagonist on serotonin receptor expression. Total RNAs (10 μg) were extracted from Sertoli cells cocultured with germ cells (A), Sertoli cells cultured alone (B), Sertoli cells cultured with 2.5 μM serotonin (C), Sertoli cells cultured with 5.0 μM serotonin (D), Sertoli cells cultured with 10.0 μM serotonin (E), Sertoli cells cultured with 10 μM of the receptor agonist quipazine (G), Sertoli cells cultured with 10 μM of the serotonin receptor antagonist ketanserin (H), Sertoli cells cultured with 10 μM of the serotonin receptor antagonist spiperone (I), Sertoli cells cultured with 10 μM of the serotonin receptor antagonist ketanserin and germ cells (J), Sertoli cells cultured with 10 μM of the receptor antagonist spiperone (K). RNAs were electrophoresed and hybridized with cDNA encoding the serotonin5-HT2 receptor. The blots were rehybridized with an actin-coding region cDNA.

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