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Characterization of the Expression and Regulation of Genes Necessary for *myo*-Inositol Biosynthesis and Transport in the Seminiferous Epithelium[1]

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ABSTRACT

In many mammals, the concentration of *myo*-inositol in the fluid of the seminiferous tubules is dramatically higher than levels found in serum. Two enzymes involved in *myo*-inositol synthesis: *myo*-inositol-1-phosphate synthase (ISYNA1) and *myo*-inositol monophosphatase-1 (IMPA1), are known to have high activity in the testes. ISYNA1 is an isomerase that catalyzes the conversion of glucose-6-phoshate to *myo*-inositol-1-phosphate. IMPA1 then hydrolyzes the phosphate group to produce *myo*-inositol. Although no physiological role for the high concentration of *myo*-inositol has yet to be elucidated, it has been suggested that it could be involved in osmoregulation. Previous research on these enzymes in the testis has focused on enzyme activity. The objective of this study was to evaluate the expression of these genes and the *myo*-inositol transporter, *Slc5a3,* within the testis. Using Northern blot analyses, we found that all three genes, *Impa1, Isyna1,* and *Slc5a3* are expressed in Sertoli cells. *Isyna1* is highly expressed in two types of germ cells, pachytene spermatocytes and round spermatids. IMPA1 was expressed in round spermatids. *Slc5a3* expression is upregulated when Sertoli cells are treated with 0.1 mM dibutyryl cAMP. When Sertoli cells were cultured in a hypertonic medium, there was an increase in the expression of *Isyna1* and *Slc5a3.* We postulate that this upregulation is a result of the capability of the Sertoli cell to sense and then react to a change in osmolarity by increasing the transport and production of the osmolyte *myo*-inositol.

cyclic adenosine monophosphate, follicle-stimulating hormone, Sertoli cells, testis

INTRODUCTION

Spermatogenesis is the process by which spermatozoa are produced from a nondifferentiated germ cell population in the testis. The multicellular epithelium of the seminiferous tubule is made up of two cell types, the germ cells and the somatic Sertoli cells. The Sertoli cells create two compartments within the seminiferous epithelium, designated the basal and adluminal compartments. The barrier between these compartments is created by the formation of specialized tight junctions between adjacent Sertoli cells. The presence of this barrier creates a microenvironment where the concentration of ions and other molecules can be very different than serum levels or levels in other organs in the body [1–4]. One compound that has an unusually high concentration in the seminiferous tubule fluid is the polyol *myo*-inositol [5, 6].

Although the concentration of *myo*-inositol can vary in the seminiferous tubule fluid between species [2, 6], every mammalian species tested contains millimolar levels, but the exact function of the high concentration of *myo*-inositol within the testis is unknown [7]. Although *myo*-inositol is an important precursor for the phosphatidyl-inositol signaling pathway, the high concentration of *myo*-inositol within the fluid suggests that it may have another function within the seminiferous epithelium. *Myo*-inositol is a nonperturbing solute that does not affect enzyme function [8, 9] and can be broken down to D-glucuronic acid by *myo*-inositol oxygenase [8]. This breakdown of *myo*-inositol does not occur within the testis and is most likely only functional within the kidney [10]. It is known that *myo*-inositol is a compatible osmolyte, and it is postulated that the molecule can aid in maintaining an ideal osmotic state when a cell is placed in a hypertonic environment [8, 9]. In the kidney, *myo*-inositol is known to be a very important osmolyte. As urine is concentrated, cells in the thick ascending loop of Henle lose water to the hypertonic surroundings and concentrate ions [8]. *Myo*-inositol enters the cell along with sodium to offset the change in osmolarity generated by the loss of water molecules [8].

Myo-inositol is made de novo in the testis by two enzymes, *myo*-inositol-1-phosphate synthase (ISYNA1, EC 5.5.1.4) and *myo*-inositol monophosphatase-1 (IMPA1, EC 3.1.3.25). Glucose-6-phosphate is converted to *myo*-inositol-1-phosphate by the isomerase ISYNA1 [11–14]. Inositol-1-phosphate is then converted to *myo*-inositol by the phosphatase IMPA1 [12, 15]. It has been well documented that both of these enzymes have high activity in the testis [12, 14, 16, 17]. The testis is one of the only organs in the body that can produce *myo*-inositol de novo from glucose-6-phosphate [18] and it appears all of the *myo*-inositol within the testis is produced this way. Studies show that, when rats were injected with radiolabeled *myo*-inositol, no radioactivity could be found within the testis [19]; this suggests that the testicular *myo*-inositol was produced within the testis, and there is no mechanism for serum *myo*-inositol to pass through the tight junctions and into the seminiferous tubule fluid.

Myo-inositol is transported into a cell by a sodium/*myo*-inositol cotransport protein, SLC5A3, a member of the solute carrier (SLC) superfamily of sugar transport proteins [20]. In the mouse, this protein is composed of 718 amino acids and is proposed to have 14 transmembrane domains. Mice that have a targeted deletion of the *Slc5a3* gene have a severe shortage of *myo*-inositol throughout their bodies and die shortly after birth due to hypoventilation [21]. It

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has been demonstrated that in cells, such as Madin-Darby kidney cells [22], primary peritoneal mesothelial cells [23], and primary cultured astrocytes [24], *Slc5a3* expression is sensitive to changes in osmolarity or when the cells are exposed to a hypertonic environment. The mechanism of how the cell recognizes the change in osmolarity and the subsequent upregulation of the *myo*-inositol transport protein are not well understood.

Most studies that examine *myo*-inositol in the testis have focused on the enzyme activity of ISYNA1 and IMPA1 [12, 16, 17] or the concentration of the molecule itself [1, 2, 4, 6]. Our focus here was to investigate where these genes are expressed in the testis. We also wanted to determine if and where the gene important for transport of *myo*-inositol, *Slc5a3*, was expressed in the testis. Robinson and Fritz [12] reported that there was an increase in *myo*-inositol production in rat Sertoli cells treated with dibutyryl cAMP. We hypothesized that Sertoli cells expressed *Impa1*, *Isyna1*, or *Slc5a3*, and the expression of these genes was regulated by FSH and dibutyryl cAMP (dbcAMP). Furthermore, we hypothesized a hypertonic environment would affect the expression pattern of the genes important for *myo*-inositol biosynthesis and the uptake of *myo*-inositol by Sertoli cells.

MATERIALS AND METHODS

Animals and Cell Culture

Mice (B6/129 strain) were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility and fed standard mouse lab chow and water ad libitum. Mice were humanely killed before culture of Sertoli cells or extraction of tissues. All animal protocols were approved by the Washington State University Animal Care and Use Committee and were in accordance with National Research Council standards established by the *Guide for Care and Use of Laboratory Animals.*

Sertoli cells were isolated from 16- to 20-day-old mice as previously described [25] with some modifications, which are noted here. Cells were plated into 100-mm culture dishes that were treated with 1 mg/ml gelatin (Sigma, St. Louis, MO). The medium used was a 1:1 mixture of DME and F-12 (Gibco BRL, Invitrogen Corporation, Carlsbad, CA). Cells were allowed to attach to the culture plates for 2 days before use. Fresh medium was used in all treatments and the purity of the Sertoli cells was ≥95%. The osmolarity of the medium was made hypertonic by increasing the sodium chloride concentration with an additional 100 mM to a final concentration of 237 mM. In some experiments, cells were treated with FSH (provided by Dr. AF Parlow; National Hormone and Peptide Program, Torrance, CA) at a concentration of 25 ng/ml (1.5 IU) or dibutyryl cAMP (Sigma) at 0.1 mM.

Primitive type A spermatogonia and type B spermatogonia were isolated from Day 8 postpartum mice, whereas pachytene spermatocytes and round spermatids were isolated from Day 30 mice and Day 60 postpartum mice, respectively. Enriched testicular cells were isolated by gravity sedimentation [26]. The purity of recovered spermatogonia was ≥85% by morphological criteria and the purity of pachytene spermatocytes and round spermatids was ≥95%.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

One microgram of DNase-treated total RNA isolated from testis was reverse transcribed into cDNA using Oligo$(dT)_{12–18}$ at 45°C for 60 min in a solution with SuperScript II reverse transcriptase (Invitrogen Corporation) and RNasin (Promega, Madison, WI). Primers used to amplify *Impa1*, *Isyna1*, and *Slc5A3* were as follows: IMPA1 forward primer, 5′-GGGTGTTCCAGATGGATGGT-3′; IMPA1 reverse primer, 5′-CGGCAGCTTTAAGCAGAACT-3′; ISYNA1 forward primer, 5′-GCTCCAAGGAGGTG ACAAAG-3′; ISYNA1 reverse primer, 5′-AGCAGCACTAGGTCCAG CAT-3′; SLC5A3 forward primer, 5′-AGGCCTTGTTGCAGTGATCT-3′; and SLC5A3 reverse primer, 5′-CTGCTTCCACACACTTGCAT-3′. The PCR was performed using the following conditions: 30 cycles at 95°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec, and a final extension step of 72°C for 10 min. The reverse RT-PCR products were purified and cloned into pGEM-T easy vectors (Promega) and sequenced to verify that the PCR product was correct.

Northern Blots

Total RNA was isolated from cultured Sertoli cells or from specific tissues by using TRIzol according to the manufacturer's instructions (Invitrogen). RNA concentration and purity was determined spectrophotometrically. Ten micrograms of total RNA was resolved on a 1.2% denaturing agarose gel, transferred onto a Hybond-N nylon membrane (Amersham Pharmacia, Piscataway, NJ), and cross-linked to the membrane by ultraviolet (UV) irradiation (UV Stratagene 1800; Stratagene Inc., La Jolla, CA). The poly-A enriched RNA was prepared using a standard oligo-(deoxythymidine) affinity chromatography method (Sigma). To examine poly-A RNA, 3 μg of enriched poly-A RNA were loaded per lane. Membranes were prehybridized for 1 h at 65°C in Church and Gilbert solution (0.5 M sodium phosphate, 7% SDS, 1 mM EDTA, 1.0% BSA). The cDNA probes were radiolabeled with 50 μCi of [α-^{32}P] deoxyadenosine triphosphate at 37°C for 1 h using a random primer labeling kit (Invitrogen). The reaction was terminated by adding 50 μl of 0.5 M EDTA. Free nucleotides were removed from the reaction with a Sephadex G50 spin column. Blots were hybridized with the labeled probe overnight at 65°C in Church and Gilbert solution. After hybridization, blots were washed one time in 2× saline-sodium citrate (SSC), 0.1% SDS at 26°C for 30 min, two times in 0.2× SSC, 0.1%SDS at 65°C for 30 min, and then exposed to a phosphorimaging screen (Molecular Dynamics, Sunnyvale, CA) overnight. Membranes were stripped with 1% SDS and each blot was hybridized with a probe to ribosomal S2 to ensure equal loading of RNA [27]. Levels of mRNA for each specific probe are expressed as a ratio of (specific probe): ribosomal protein S2 using ImageQuant analysis software version 1.1 (Molecular Dynamics). All experiments were performed on at least three different Sertoli cell preparations.

^{3}H-*myo*-Inositol Uptake

Sertoli cells were isolated as described above and plated in six-well culture dishes. To each well was added 1.5 μCi of ^{3}H-*myo*-inositol at the beginning of the experiment. Cells were treated with vehicle (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $NAHPO_4 \cdot 7H_2O$), sodium chloride (final concentration 237 mM), or dibutyryl cAMP (0.1 mM; Sigma).

After treatment, each well was rinsed three times with PBS and cells were lysed with 0.5 N NaOH and then neutralized with 1 N HCl. An aliquot of the cell extract was analyzed in a scintillation counter and another aliquot was used in a Lowry protein assay to determine protein concentration. To control for differences in cell density between the six wells of the plate, results are shown as the cpm divided by the amount of total protein present in each well. All samples were processed in duplicate in at least three separate experiments from three Sertoli cell preparations.

Data Analysis

All results are expressed as means ± SEM. Statistical comparisons were performed with a paired Student *t*-test.

RESULTS

Northern Analysis

To localize where the genes *Isyna1*, *Impa1*, and *Slc5a3* were expressed in the mouse, Northern blots were used with either total RNA or poly-A enriched RNA isolated from various tissues. *Isyna1* is expressed at high levels in the testis, specifically in Sertoli cells, and at low levels in the brain, epididymis, kidney, lung, and ovary (Fig. 1A). Expression of *Impa1* is ubiquitous, with no apparent enrichment of the message in any specific tissues (Fig. 1B). *Slc5a3* expression is considerably lower than the other two genes that were being investigated. To see a clear pattern of *Slc5a3* expression, we used a poly-A-enriched Northern blot (3 μg). Expression of the *Slc5a3* gene is apparent in most tissues that were investigated, including Sertoli cells (Fig. 1C). All organ Northern blots were performed three times with different RNA samples.

To investigate further into the expression of *Isyna1*, *Impa1*, and *Slc5a3* in the testis, Northern blots with total RNA from specific, enriched germ cell populations were probed. *Isyna1* was highly expressed in pachytene sper-

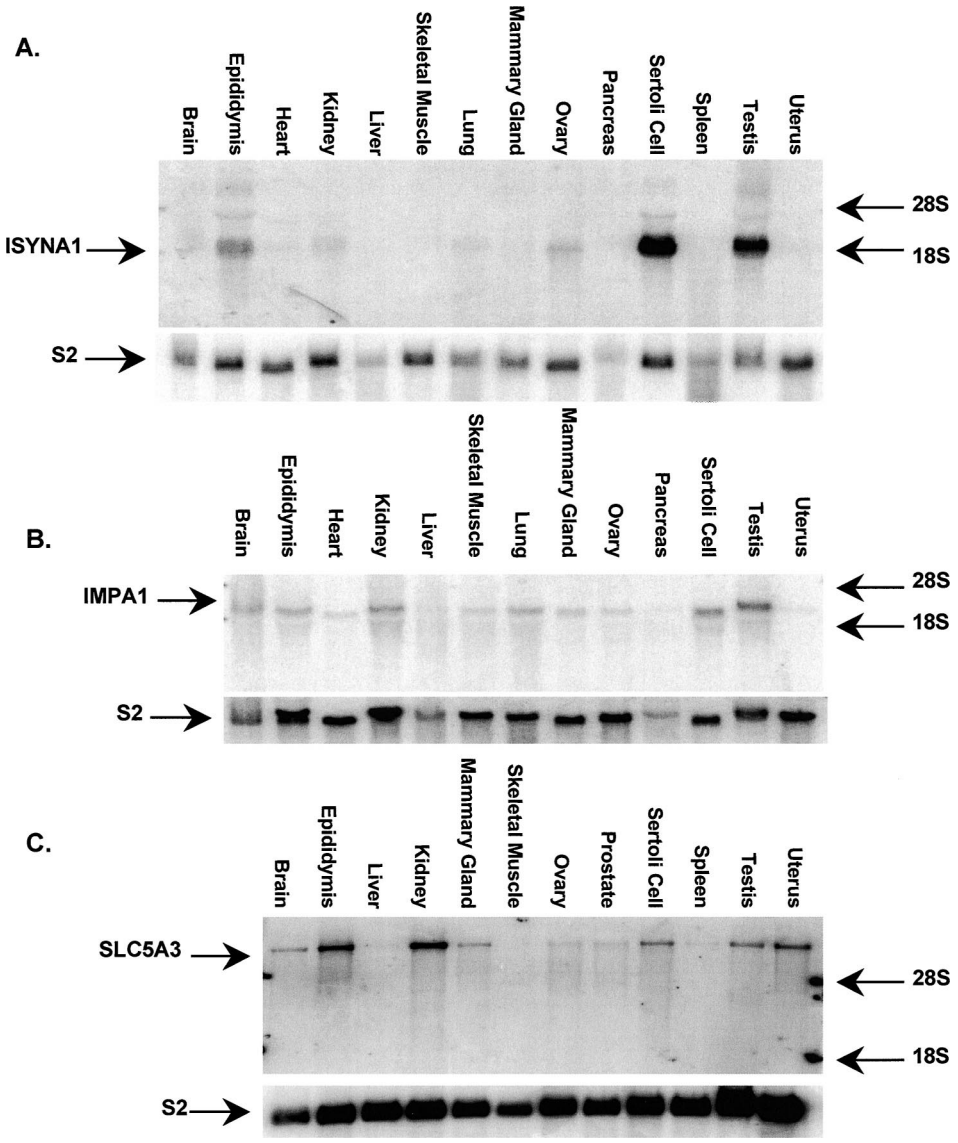

FIG. 1. Organ Northern blot analyses of ISYNA1 (**A**), IMPA1 (**B**), and SLC5A3 (**C**) mRNA expression in various tissues in the mouse. For (**A**) and (**B**), 10 μg of total RNA was purified and membranes were probed with either ISYNA1 (**A**) or IMPA1 (**B**). In (**A**), there is a band around 2.2 kilobase (kb) that corresponds to the ISYNA1 message. In (**B**) is a band around 2.1 kb that corresponds to the IMPA1 message. **C**) A poly-A RNA-enriched blot where 3 μg of mRNA was isolated and probed for SLC5A3. A band can be seen around 7.5 kb that corresponds to the SLC5A3 message. All blots were then stripped and probed with ribosomal protein S2 to control for loading.

matocytes and round spermatids (Fig. 2), while *Impa1* expression was found in significant amounts only in round spermatids (Fig. 2). *Slc5a3* was not expressed in any type of germ cell examined (data not shown). Northern blot analysis on germ cell RNA was performed two times with different RNA samples.

Effects of a Hypertonic Environment in Sertoli Cells

Sertoli cells were cultured and then subjected to hypertonic conditions by adding an additional amount of sodium chloride for a final treatment of 237 mM. Total RNA was extracted from these cells after exposure to hypertonic conditions for 0, 4, 8, and 24 h and was used for Northern blot analysis. Expression of *Slc5a3* was elevated significantly at 4, 8, and 24 h (Fig. 3, A and B) of hypertonic exposure. Peak expression of *Slc5a3* was at 4 h of exposure with a 12.4 ± 4.8-fold (n = 3, where n is equal to the amount of different Sertoli cell preparations) increase when compared with controls (Fig. 3B). The expression of *Isyna1* also increased when Sertoli cells were subjected to hypertonic conditions at 4, 8, and 24 h (Fig. 3A). The peak expression of *Isyna1* occurred at 24-h exposure with a 5.9 ± 1.2-fold (n = 3) increase of expression as compared with controls (Fig. 3B). The hypertonic conditions increased *Impa1* expression 1.8 ± 0.2-fold (n = 5) at the 24-h time point (Fig. 3, A and B).

Effects of Follicle Stimulating Hormone and Dibutyryl cAMP on *Slc5a3* Expression

Cultured mouse Sertoli cells were treated with either 0.1 mM dibutyryl cAMP or 25 ng/ml of ovine FSH for 4, 8, or 24 h. A representative Northern blot is shown in Figure 4A. With both treatments, a peak in *Slc5a3* expression was evident at 4 h. There was an increase (a mean of 2.6- ± 1-fold, [n = 3]) in *Slc5a3* expression when Sertoli cells were treated with FSH at 4 h (Fig. 4B), and peak expression of *Slc5a3* by treatment with dibutyryl cAMP increased approximately 4.4 ± 1.5-fold (n = 3) at 4 h (Fig. 4B). Longer treatments with either dbcAMP or FSH had a slight induction of expression, although not as significant as the 4-h treatment (Fig. 4, A and B).

Myo-Inositol Uptake in Mouse Sertoli Cells

Hypertonic exposure or dbcAMP treatment of Sertoli cells resulted in an upregulation in expression of the *myo*-

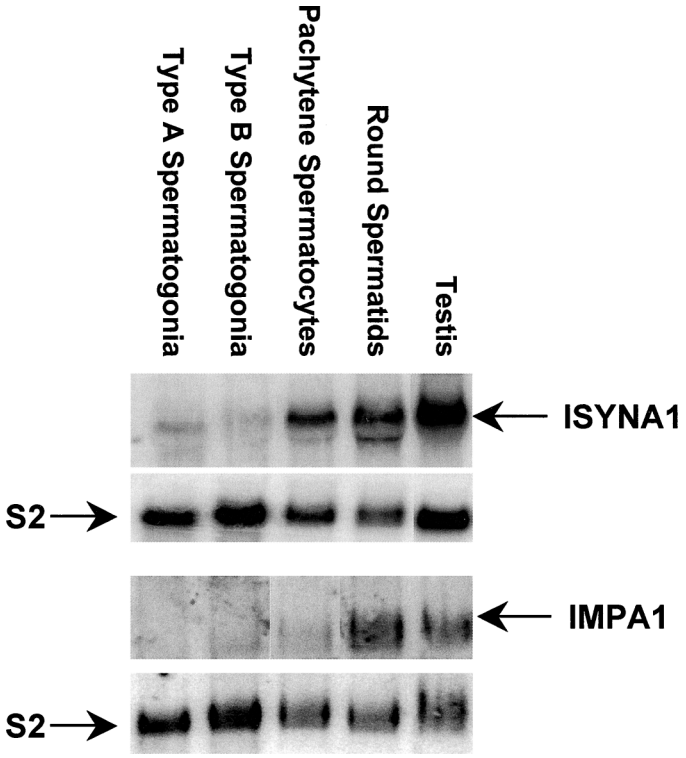

FIG. 2. Northern blot analyses of ISYNA1 and IMPA1 mRNA expression in enriched germ cells. Each lane contains 10 μg of total RNA and the membranes were probed with either ISYNA1 or IMPA1. Ribosomal protein S2 was used for the loading control.

inositol transporter gene, *Slc5a3* (Figs. 3 and 4). To determine if Sertoli cells would transport *myo*-inositol under these conditions, experiments were performed using ^{3}H-*myo*-inositol during the treatments. Sertoli cells that were in a hypertonic medium took up significantly more ^{3}H-*myo*-inositol at 12, 16, 20, and 24 h than cells that were in an isotonic medium (Fig. 5). Last, expression of *Slc5a3* is induced by dbcAMP (Fig. 4), and treatment of Sertoli cells with dbcAMP increased the amount of ^{3}H-*myo*-inositol uptake when compared with untreated cells at 12, 16, 20, and 24 h (Fig. 6).

DISCUSSION

Previous studies have established that there is a high level of *myo*-inositol in the seminiferous tubule fluid of many mammalian species [2, 6] and that the concentration is significantly higher in tubules than in serum and other organs [19]. All of the earlier studies concentrated on enzyme activity of ISYNA1 and IMPA1 within the testis or reported on the levels of *myo*-inositol in the testicular fluid, but as of yet, the purpose for such high levels of enzyme activity and concentration of *myo*-inositol has yet to be elucidated. In addition, no molecular data have been reported to identify the specific cells in the testis that produce IMPA1 and ISYNA1 or how the two genes are regulated. This is the first study to localize the expression of genes involved in *myo*-inositol biosynthesis. Using Northern blot analyses, we report that these two genes are expressed in Sertoli cells and specific germ cells. This study also investigated a very important missing part of the *myo*-inositol story within the testis, i.e., the localization of the Na^{+}/*myo*-

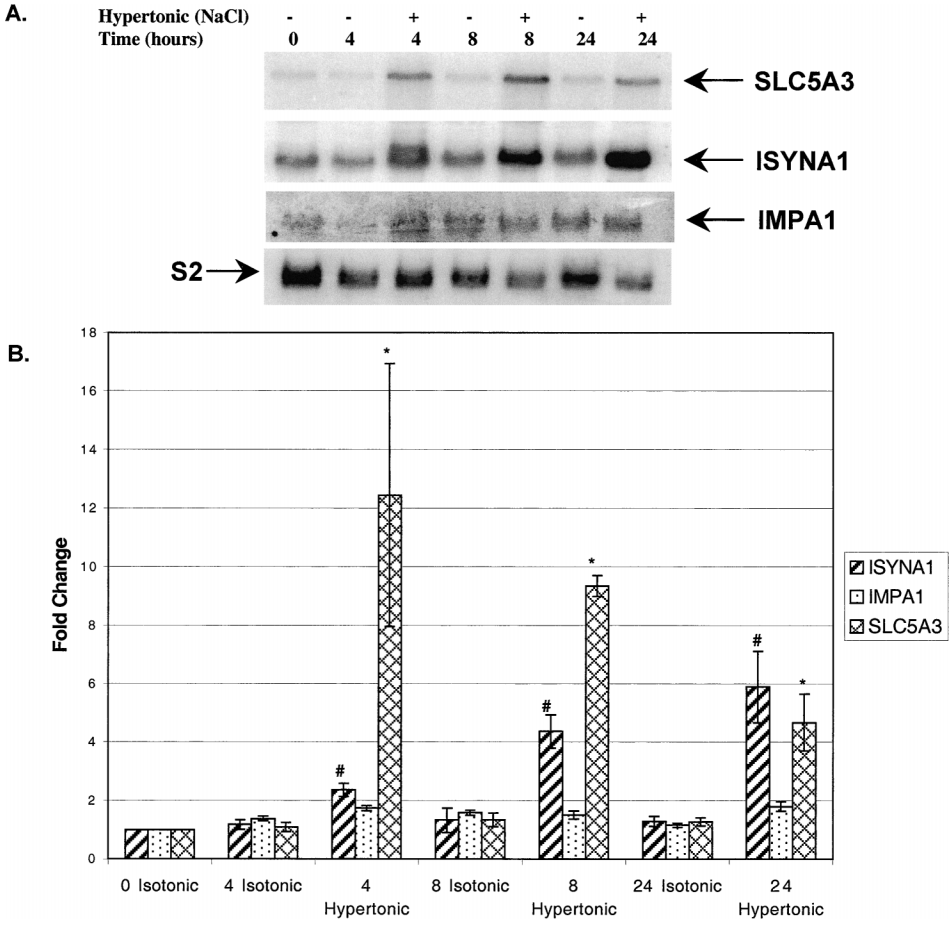

FIG. 3. Northern blot analyses of SLC5A3 and ISYNA1 mRNA expression isolated from hypertonic- and isotonic-treated Sertoli cells. **A**) Total RNA from cultured Sertoli cells was isolated at each time point and 10 μg loaded per lane. Membranes were probed with either SLC5A3 or ISYNA1 and ribosomal protein S2 was used as a loading control. **B**) Graphic representation of ISYNA1 and SCL5A3 normalized to ribosomal S2. Significant increases in ISYNA1 (#) and SLC5A3 (*) expression in hypertonic Sertoli cells versus isotonic Sertoli cells ($P \leq 0.05$).

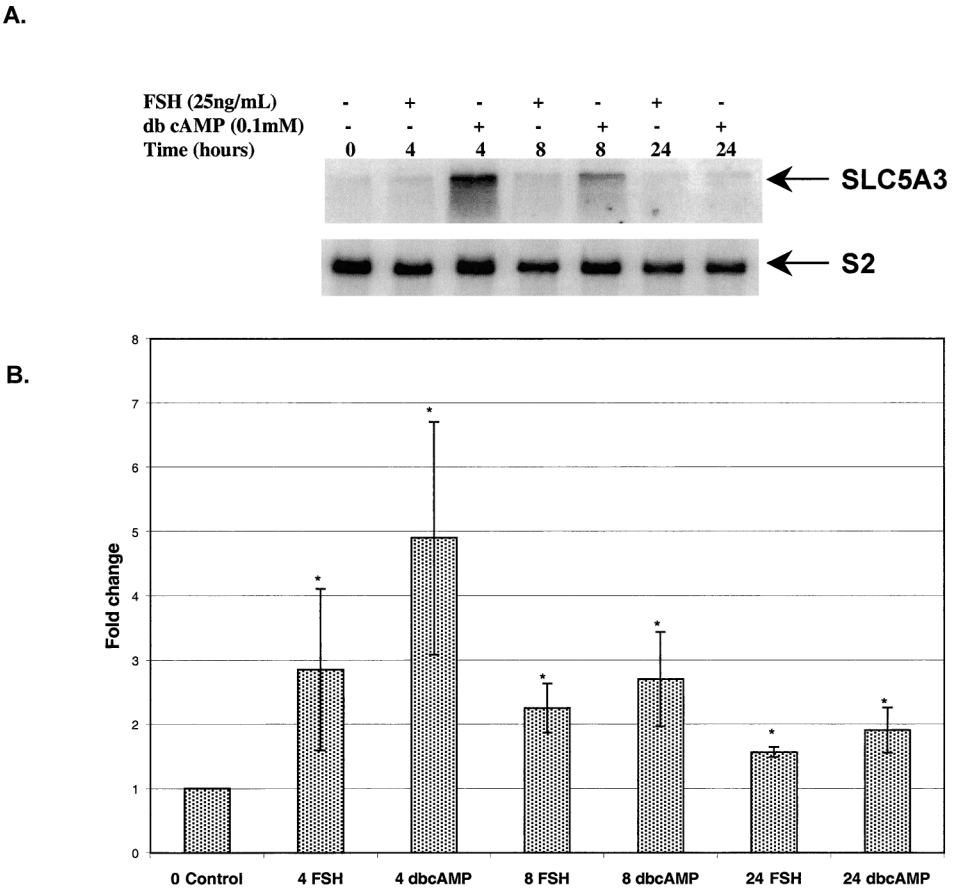

FIG. 4. Northern blot analyses of SLC5A3 mRNA expression from isolated Sertoli cells treated with FSH or dbcAMP. Controls for each time point are not shown because basal expression levels of SLC5A3 do not change (see Fig. 3). **A**) Total RNA from cultured Sertoli cells was isolated at each time point and 10 μg loaded per lane. Membranes were probed with SLC5A3 and ribosomal protein S2 was used as a loading control. **B**) Graphic representation of SLC5A3 normalized to ribosomal S2. Significant increase in SLC5A3 (*) expression in treated Sertoli cells when compared with control Sertoli cells at time zero ($P \leq 0.05$).

inositol transport gene, *Slc5a3*, to Sertoli cells and the epididymis.

ISYNA1 is an enzyme that isomerizes glucose-6-phosphate to inositol-1-phosphate. This enzyme is highly expressed in the testis as compared with other tissues and can be localized to Sertoli cells, pachytene spermatocytes, and round spermatids. All these cells are in contact with the luminal compartment, where a unique microenvironment exists, especially when compared with the basal compartment of the seminiferous epithelium. It is in this unique microenvironment where the *myo*-inositol concentration is high. Because *myo*-inositol is known to be important for osmoregulation in the kidney, we hypothesize that it may be performing the same function in the lumen as well. For example, the concentration of potassium is much higher in the luminal fluid as compared with the serum [1, 3, 28]. Because Sertoli cells, pachytene spermatocytes, and round spermatids are all expressing *Isyna1* at an elevated level, it

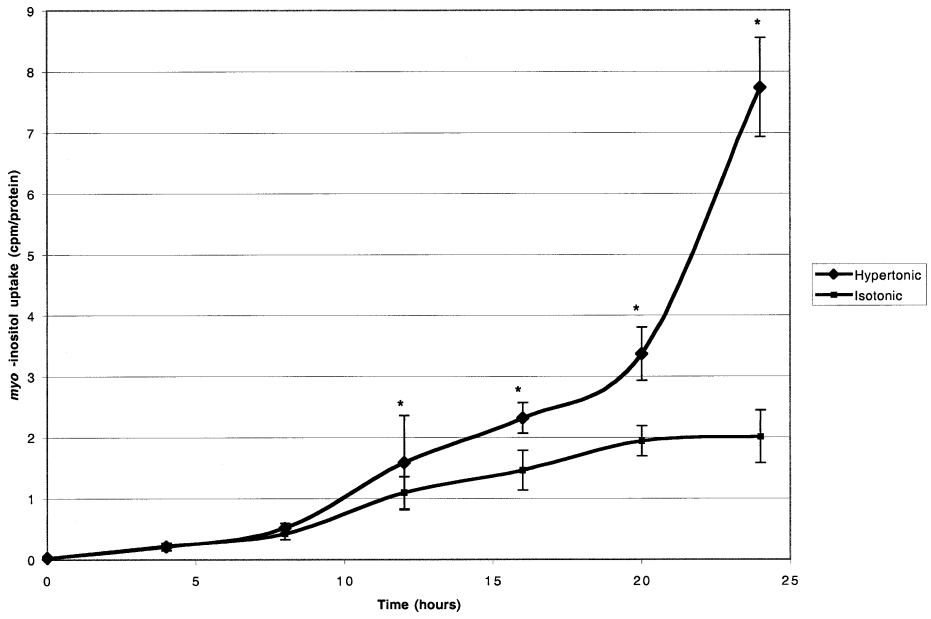

FIG. 5. *Myo*-inositol uptake by primary Sertoli cell cultures exposed to isotonic and hypertonic medium. Cultures were incubated either isotonic (normal medium) or hypertonic (additional 100 mM NaCl added) and containing 1.5 μCi of ^{3}H-*myo*-inositol. Cells were lysed at each time point and used for scintillation counting. Normalization was performed by determining the amount of protein that was present in each well. Each point is the mean ± SEM of at least three separate experiments performed in duplicate. *, Indicates a significant difference between the hypertonic and isotonic cells ($P \leq 0.05$).

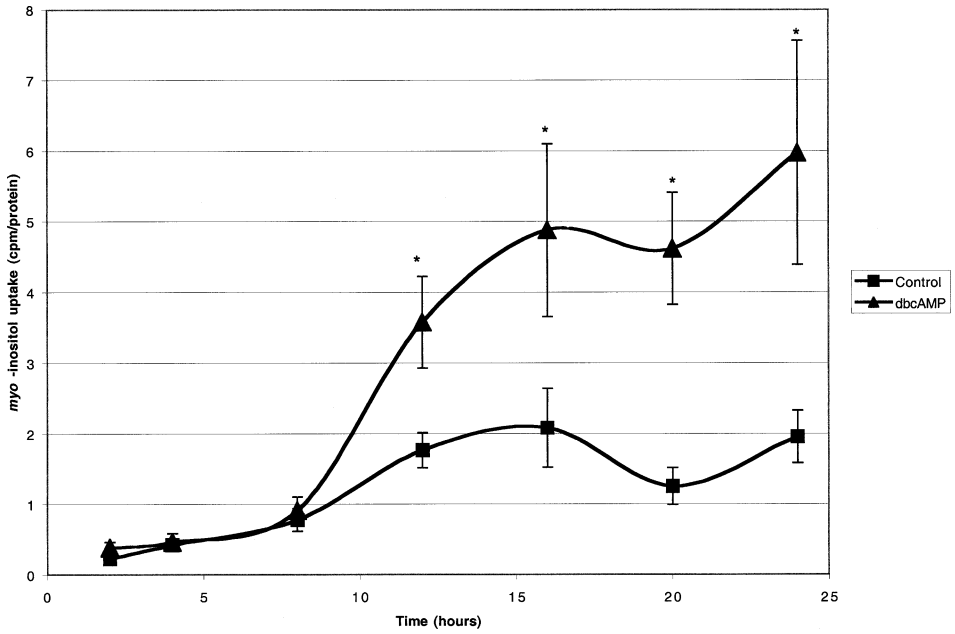

FIG. 6. *Myo*-inositol uptake by primary Sertoli cell cultures treated with 0.1 mM dbcAMP. Cultures were treated with either vehicle or 0.1 mM dbcAMP as well as 1.5 μCi of ^{3}H-*myo*-inositol. Cells were lysed at each time point and used for scintillation counting. Normalization was performed by determining the amount of protein that was present in each well. Each point is the mean ± SEM of at least three separate experiments performed in duplicate. *, Indicates a significant difference between the hypertonic and isotonic cells ($P \leq 0.05$).

can be assumed that all three of these cell types are producing *myo*-inositol and contributing to the high concentration in the luminal fluid. In contrast, type A and type B spermatogonium are not expressing *Isyna1*, and therefore are not synthesizing significant levels of *myo*-inositol de novo from glucose. This observation makes biological sense because the type A and type B cells are in the basal compartment of the seminiferous tubule and are not in contact with the high *myo*-inositol environment of the lumen. *Isyna1* expression is also induced when Sertoli cells are cultured in a hypertonic environment. The data indicates that Sertoli cells can sense a change in the osmolarity and thereby induce *Isyna1* to help offset that change in osmolarity.

IMPA1 will dephosphorylate *myo*-inositol-1-phosphate to *myo*-inositol and is ubiquitously expressed in all organs [15]. It is one of the enzymes that is crucial for *myo*-inositol recycling, which is essential for the phosphatidyl inositol signaling pathways [7, 29]. It was previously reported that rat germ cells lack IMPA1 activity [12]. In the mouse, we found that the message for *Impa1* is present in round spermatids (Fig. 2B). The expression level of *Impa1* is slightly induced when Sertoli cells were grown under hypertonic conditions. This slight upregulation of *Impa1* is probably coupled to the upregulation of *Isyna1* because ISYNA1 is producing more *myo*-inositol-1-phosphate, which is the substrate for IMPA1. The delayed induction of *Impa1* is logical because there is more substrate to act on after *Isyna1* is induced.

We also report the localization of the *Slc5a3* transcript to Sertoli cells. *Slc5a3* expression was upregulated when Sertoli cells were cultured in hypertonic medium made by the addition of sodium chloride. The Na^+/*myo*-inositol transport protein SLC5A3 is very important for regulating osmolarity in the thick ascending loop of Henle in the kidney [30]. SLC5A3 is also hypothesized to be important for osmoregulation in nerve tissue [24]. We hypothesized that SLC5A3 may be important for osmoregulation within the seminiferous epithelium just as it is in the kidney. It has been reported that, when a number of different cell types and cell lines were placed in a hypertonic state, they would take up *myo*-inositol when compared with cells in an isotonic medium [23, 24, 31]. To investigate if Sertoli cells were actually taking up *myo*-inositol, we used ^{3}H-*myo*-inositol. Sertoli cells in hypertonic medium took up significantly more ^{3}H-*myo*-inositol as compared with when they were placed in isotonic medium. This supports the hypothesis that Sertoli cells are taking up *myo*-inositol from the medium to offset the change in osmolarity.

Regulation of the *Slc5a3* gene is not well understood. In cultured astrocytes treated with dbcAMP, there is a downregulation of *Slc5a3* expression [32], and it was suggested by the authors that dbcAMP is important for maturation of astrocytes and that the biochemical stage is important for osmoregulation in astrocytes [32]. Primary mouse Sertoli cells were treated with either FSH or dbcAMP, and a significant increase in transcription of the *Slc5a3* gene was observed at all time points. The increase in transcription was higher in dbcAMP-treated cells than in FSH-treated cells. When cultured primary Sertoli cells were incubated with ^{3}H-*myo*-inositol and 0.1 mM dbcAMP, an increase in ^{3}H-*myo*-inositol uptake was observed versus the control cells. These results were opposite of the results in another report, which found cultured astrocytes treated with dbcAMP had a reduced amount of *Slc5a3* expression and ^{3}H-*myo*-inositol uptake [32]. The reason for the differences in the results is probably due to the different specialized functions of Sertoli cells and astrocytes.

The transcriptional control of *Slc5a3* is very interesting because both FSH and hypertonicity induce its expression. Hypertonicity induces *Isyna1* as well, but FSH does not (results not shown). It could be possible that FSH is regulating transcription of *Slc5a3* early in development during the establishment of the tight junctions and the seminiferous tubular microenvironment. After development and the onset of spermatogenesis, the degree of hypertonicity within the fluid could regulate both genes. The transcription factor TonEBP/OREBP is induced by hypertonic situations and can bind ORE/TonE response elements [33]. *Slc5a3* is known to contain these response elements [34], and a partial promoter sequence of *Isyna1* contained in GenBank indicates that the *Isyna1* promoter has at least one of these sites as well. Because both of these promoters contain the

hypertonic response elements, it strongly suggests they are both very important for osmoregulation.

Although the hypothesis that *myo*-inositol is important for osmoregulation within the microenvironment of the seminiferous tubule has been suggested before [35], to our knowledge, no experiments have been performed to substantiate the hypothesis. The upregulation of *Slc5a3* and *Isyna1* in Sertoli cells exposed to hypertonic conditions supports the osmoregulation hypothesis. We believe functional studies to silence *Isyna1* or *Slc5a3* would result in a state of azoospermia.

Inhibition of the enzymes IMPA1 and/or ISYNA1 could result in decreased spermatogenesis. Lithium chloride is known to be an inhibitor of the IMPA1 enzyme and, subsequently, decreases the amount of free *myo*-inositol available. Lithium chloride is used to treat patients with bipolar disorder. When rats were treated with lithium chloride for 21 days, a decrease in type A spermatogonia, pachytene spermatocytes, and round spermatids was observed [36, 37]. Valproate is a drug that is used to treat both seizure and bipolar disorders [29]. It has been hypothesized to be an inhibitor of one of the enzymes necessary for creating *myo*-inositol [29]. Originally, it was speculated that valproate was an inhibitor of IMPA1 similar to lithium, but further experiments revealed that this was not the case and, in actuality, valproate may inhibit ISYNA1 [38]. While mammalian studies have not directly assessed this possibility, studies in *Saccharomyces cerevisiae* indicate that valproate is inhibiting ISYNA1 [38, 39]. It should be noted that ISYNA1 and INO1 (the yeast homologue of *Isyna1*) are greater than 50% identical at the amino acid level. Rats that were administered high doses of valproate had severe germ cell loss and a 50% decrease in testicular weight [40]. While the authors of this study did not speculate as to why this occurred, we believe that it was due to the loss of de novo synthesis of *myo*-inositol within the seminiferous tubule. Without a high amount of *myo*-inositol, the optimal microenvironment could not be established for efficient spermatogenesis to occur. No other abnormal effects were reported in other organs of the treated rats, and this is most likely because *Isyna1* is highly expressed in the testis when compared with other tissues.

In conclusion, these studies have identified that the *Slc5a3 myo*-inositol transporter is located in the testis and specifically in the Sertoli cells. *Isyna1* and *Impa1* are expressed in meiotic germ cells as well as Sertoli cells. The data presented here support the hypothesis that the high concentrations of *myo*-inositol are important for osmoregulation in the seminiferous epithelium.

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