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Detection of glycine receptor/Cl^- channel beta subunit transcripts in mouse testis

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Summary

The sperm glycine receptor/Cl^- channel (GlyR) is important to the initiation of the mammalian sperm acrosome reaction by the egg zona pellucida, but its presence in spermatogenic cells has not been demonstrated. Reverse transcriptase–polymerase chain reaction studies confirmed that GlyR beta subunit transcripts similar to those of the neuronal GlyR beta subunit are present in the testis of Swiss Webster mice. *In situ* hybridisation analysis demonstrated that GlyR beta subunit mRNAs were expressed within the germ cells of seminiferous tubules in those mice.

Keywords: Acrosome reaction, *In situ* hybridisation, RT-PCR, Sperm

Introduction

The sperm acrosome reaction (AR) is essential for fertilisation, and the egg zona pellucida (ZP) is generally thought to be an *in vivo* initiator of the AR (Kopf & Gerton, 1991). Our previous pharmacological and immunochemical studies showed that a sperm glycine receptor/Cl^- channel (GlyR) similar to the neuronal GlyR is important for initiation of the porcine sperm AR by the ZP (Melendrez & Meizel, 1995, 1996) and to the initiation of the porcine, human, mouse and hamster AR by glycine (Melendrez & Meizel, 1995; Sato *et al*., 2000*a*; Llanos *et al*., 2001). Indirect immunofluorescence studies detected the GlyR on the periacrosomal plasma membrane of porcine and mouse sperm (Melendrez & Meizel, 1996; Sato *et al*., 2000*a*). Furthermore, our recent studies showed that sperm from mice with mutations in neuronal GlyR alpha and beta subunits (*spastic* and *spasmodic*) (Chai, 1961; Lane *et al*., 1987) were deficient in their ability to undergo the AR initiated *in vitro* by glycine or by solubilised ZP from mouse eggs (Sato *et al*., 2000*b*).

The glycine receptor is a pentameric complex comprised of three alpha subunits and two beta subunits (Becker, 1990; Becker *et al*., 1992). The alpha subunits have binding sites for glycine and strychnine, while the beta subunits are thought to be promoters and stabilisers of glycine receptor assembly (Rajendra *et al*., 1997). The two types of subunits are structurally related, and mutation in either of the GlyR subunits leads to behavioural problems in mice (Chai, 1961; Lane *et al*., 1987). GlyR alpha subunit mRNAs are expressed at high levels only in specific parts of the nervous system (Malosio *et al*., 1990; Matzenbach *et al*., 1994), but beta subunit mRNAs are expressed at high levels throughout the central nervous system (Betz, 1992; Grenningloh *et al*., 1990). While previous studies focused on the mature sperm, there have been no reports dealing with GlyR expression during spermatogenesis. In the present study we investigated the distribution of GlyR beta subunit mRNA in the mouse testis with the reverse transcriptase–polymerase chain reaction (RT-PCR) and by *in situ* hybridisation.

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Materials and methods

Reagents

Chemicals were reagent grade and, unless otherwise indicated, were purchased from Sigma Chemical Co. (St Louis, MO).

RT-PCR

Testis and brain were dissected from 8- to 10-week-old male Swiss Webster mice (Charles River Laboratory, Wilmington, MA). Animal care was in accordance with US Public Health Service guidelines for use of animals, and all procedures were approved in advance by the Institutional Animal Care and Use Committee of the University of California at Davis and National Institute of Environmental Health Science. Total RNA was extracted from the testis and brain using the technique described by Chomczynski & Sacchi (1987). The RNA was treated with DNase I (Gibco BRL, Grand Island, NY) and reverse transcribed into cDNA with the Perkin-Elmer PCR core kit (Perkin-Elmer, Branchburg, NJ), using random primers in the RT reaction. Aliquots of 1 µl of brain-derived cDNA or 10 µl of testis-derived cDNA were used as PCR template with *Taq* DNA polymerase (Gibco BRL). Primers corresponded to mouse brain GlyR beta subunit sequences, i.e. the sense primer S6 (5′-TGGATCCATTCAAGAGAC-3′) and the antisense primer AS1 (5′-TCCGTGACCTACACACC-GAG-3′), as previously reported (Mülhardt *et al*., 1994). One microlitre of brain-derived RT-PCR products and 10 µl of testis-derived products were loaded in each lane of a 1% agarose gel and separated by electrophoresis for ethidium bromide analysis. The absence of genomic DNA was confirmed by running each reaction in parallel with a reaction lacking RT.

Partial cloning and sequencing of the testis GlyR beta subunit

The RT-PCR products amplified from mouse testis (see above) were ligated with the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and the vectors with inserts were transformed into TOP10 competent cells (Invitrogen). The transformation mixture (20–50 µl) was plated on Luria-Bertani (LB) agar plates with kanamycin (50 µg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Gibco BRL) and isopropyl-1-thio-β-D-galactopyranoside (IPTG) (Gibco BRL) and incubated overnight according to the manufacturer's instructions. Twenty colonies from each plate were selected for amplification in LB/kanamycin. Plasmid DNA was extracted from 1.5 ml of overnight growth cultures with the QIA miniprep kit (Qiagen, Chatsworth, CA). Plasmids were subjected to PCR using the GlyR beta-derived primers S6 and AS1. The positive clones were reamplified and sequenced using M13R and M13-21 primers.

In situ hybridisation analysis

The testis and brain from a 10-week-old Swiss Webster mouse were rinsed in diethylpyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS) and fixed overnight with 4% paraformaldehyde in PBS at 4 °C. The fixed tissues were then rinsed in PBS and cryoprotected by immersion for 12 h in 10% sucrose in PBS at 4 °C. Then tissues were immediately embedded in Tissue Freezing Medium (Fisher Scientific, Pittsburg, PA) and frozen in a slurry of methyl butane and dry ice. All tissues were kept at –80 °C until used. Sections were prepared at 5–7 µm, collected on subbed slides (Fisher Scientific), air-dried for 3 h and stored at –20 °C until used. For *in situ* hybridisation, sections were rinsed 3 × 5 min in PBST (PBS with 0.1% Tween), bleached with 0.5% H_2O_2 in PBST and rinsed 3 × 5 min with PBST again. The sections were treated with 1.6 µg/ml proteinase K (Gibco BRL) in PBST for 10 min at room temperature, rinsed with 2 mg/ml glycine in PBS and PBST and post-fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS at room temperature for 10 min. After washing, the sections were prehybridised in the prehybridisation buffer (45% 1 M deionised formamide, 45% 10× SSC (pH 5, adjusted by citric acid), 0.9% SDS, 0.45% tRNA, 0.45% 10 ng/ml heparin) for 2 h at 47 °C. Digoxigenin-labelled cRNA antisense or sense probes for the GlyR beta subunit (see above) synthesised with SP6 or T7 RNA polymerases (Roche, Indianapolis, IN) were added to hybridisation buffer (15 µl prehybridisation buffer and dextran sulphate), covered with an acid-cleaned coverslip, sealed with rubber cement and incubated for 24 h at 47 °C in a humid container. After the hybridisation, the coverslip and rubber cement were removed and the sections washed 3 × 30 min in filtered washing buffer 1 (45% formamide, 23% 10× SSC (pH 5), 0.1% SDS in DEPC water) at 47 °C. Then the sections were washed 3 × 30 min in filtered washing buffer 2 (washing buffer 1 without SDS), and further washed 3 × 5 min in TBST (Tris-buffered saline (TBS) with 0.1% Tween) with 0.05 mg/ml levamisole. After blocking with 10% goat serum in TBST for 2.5 h at room temperature, the sections were incubated at 4 °C overnight with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) diluted 1:500 in TBST with 10% goat serum. Excess antibody was removed with 3 × 1 h washes in TBST with levamisole, and the sections were equilibrated for 3 × 10 min in NTMT buffer (0.1 M NaCl, 0.1 M Tris-HCl (pH 9.5), 50 mM $MgCl_2$, 0.1% Tween-20 and levamisole). Colour development was performed at room temperature for 24 h in NBT (Nitroblue tet-

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razolium chloride) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt; Roche) in NTMT according to the manufacturer's manual. Staining was stopped by a 1 h wash in PBS and fixation with 4% paraformaldehyde in PBS for 1 h. Slides were coverslipped using 50% glycerol/50% PBS and images were recorded using a Nikon Optiphot microscope and an Optronics three-chip colour video camera.

Results and discussion

The RT-PCR analysis of GlyR beta-subunit expression in the brain and testis of the Swiss Webster mouse is summarised in Fig. 1. A product of approximately 720 bp was amplified with GlyR beta-specific primers from both brain-derived total RNA and testis-derived total RNA. This product was not amplified in control reactions lacking reverse transcriptase.

To confirm that the RT-PCR products amplified from testis and brain total RNA were identical and derived from the GlyR beta gene, both were subcloned and sequenced. The first 500 nucleotides downstream from the M13R primer sequence were identical in each. Alignment with BLAST indicated that both products shared 99% nucleotide identity with the corresponding sequence of the mouse brain GlyR beta subunit (Mülhardt *et al*., 1994), and 99% nucleotide identity with the GlyR beta sequence that we previously reported from B6c3Fe-a/aF1 mouse testis and brain (Sato *et al*., 2000*b*). These minor variations in sequence are likely to be the result of gene polymorphisms shown in different mouse strains (e.g. compare Kingsmore *et al*., 1994 with Mülhardt *et al*., 1994).

In situ hybridisation was used to identify the cells containing GlyR beta subunit transcripts in cryostat sections of mature mouse testis (Fig. 2*A*, *C*). GlyR beta subunit mRNA expression was clearest in spermatogonia near the tunica propria at the base of each seminiferous tubule, and was not observed in the interstitial cells or in cells of the tunica propria. In addition to a strong signal in spermatogonia, there were also weaker positive signals in later spermatogenic cells including those nearer the tubule lumen. Sections treated identically except that they were incubated in the sense control probe (Fig. 2*B*, *D*) revealed low levels of background.

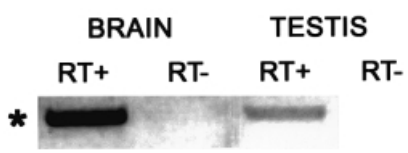

Figure 1 RT-PCR analysis of GlyR beta subunit gene expression in Swiss Webster mouse brain and testis. GlyR beta-specific primers amplify products of the expected size (*) from total RNA isolated from brain and testis if reverse transcriptase is included in the reaction mixture (RT+). No products are detectable in the control reactions lacking reverse transcriptase (RT–), demonstrating that the product is derived from GlyR beta subunit mRNA.

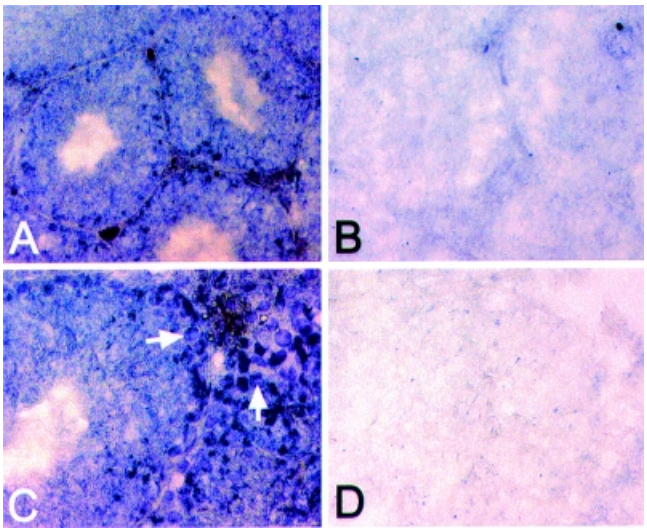

Figure 2 Distribution of GlyR beta subunit mRNA in mouse testis revealed by *in situ* hybridisation. (*A*) A low-magnification overview of the GlyR beta subunit antisense hybridisation signal. (*B*) A sense probe was used as a control with an adjacent section. (*C*) A higher magnification reveals the strongest hybridisation signal in spermatogonia (arrows). (*D*) An adjacent section sense control.

Our *in situ* hybridisation results are the first to demonstrate that the transcripts for any GlyR subunit are present in spermatogenic cells and that they exist in both pre- and post-meiotic cells. Future studies will be required to determine whether an alpha subunit and actual functional GlyRs are present in all the spermatogenic stages that express the beta subunit.

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