Identification and spatial distribution of glycine receptor subunits in human sperm

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Abstract

The human sperm surface glycine receptor (GLR) plays a role in an important fertilization event, the sperm acrosome reaction. Here, by western blot analysis, we report the presence of GLRA1, GLRA2, GLRA3, and GLRB subunits in human sperm. Immunolocalization studies showed that the GLRA1 and GLRA2 subunits are present in the equatorial region, the GLRA3 subunit in the flagellar principal piece, and the GLRB subunit in the acrosomal region of sperm. This first demonstration of isoforms of the sperm GLRA subunit and of a differential spatial distribution of the α and β subunits on the surface of mammalian sperm suggests the possibility that human sperm GLRs have more than one function.

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Introduction

The neuronal glycine receptor/Cl[−] channel (GLR) is a pentameric complex consisting of three α and two β subunits. Four isoforms of the α subunit (α 1, α 2, α 3, and α 4) have been identified and splice variants of the α 1 (α 1 and α 1_{ins}), α 2 (α 2A and α 2B), and α 3 (α 3K and α 3L) have been characterized (reviewed in Lynch 2004, Webb & Lynch 2007). Recently, a splice variant of the β subunit (β Δ7) was cloned from glial cells (Oertel *et al.* 2007).

The acrosome is a secretory granule-like organelle in the anterior two-thirds of the sperm head, and the acrosome reaction (AR) is a modified exocytotic event that is essential for fertilization (Yanagimachi 1994, Meizel 2004). Previous pharmacological and immunochemical studies showed the involvement of a sperm GLR in the porcine, human, mouse, and hamster AR initiated by glycine and the oocyte zona pellucida or its glycoprotein ZP3 (Melendrez & Meizel 1995, Sato *et al.* 2000b, Llanos *et al.* 2001, Bray *et al.* 2002, Meizel & Son 2005). The porcine and mouse sperm studies utilized a mAb (mAb4a) that recognizes all the subunit isoforms, while the human studies used a polyclonal antibody that recognizes both the GLRA1 and GLRA2 subunits but does not distinguish between them (Melendrez & Meizel 1996, Sato *et al.* 2000a, Bray *et al.* 2002). Since the sperm is such a highly differentiated cell composed of a flagellum and head that are each even further compartmentalized (Yanagimachi 1994, Meizel 2004) separate localization of GLR subunits might indicate different functions. In the present study, we utilized

subunit-specific antibodies to identify the GLR subunits present in human sperm and demonstrated their differential cellular localization.

Results

Western blot analysis of Triton-X-100 extracts of uncapacitated human sperm demonstrated the presence of the GLRA1 subunit that ran at an apparent molecular mass of 48.7 ± 0.59 kDa (Fig. 1, lane 1), the GLRA2 subunit at 49.9 ± 1.3 kDa (Fig. 1, lane 2), the GLRA3 subunit at 47.8 ± 2.2 kDa (Fig. 1, lane 3), and the GLRB subunit at 59.6 ± 1.3 kDa (Fig. 1, lane 4). No band was detected when the primary antibody was omitted during western blotting (Fig. 1, lane 5). These molecular masses are in agreement as previously reported for GLRA1 and GLRB subunits of the rat spinal cord GLR (Pfeiffer *et al.* 1982) and as predicted from the amino acid sequence for the GLRA2 and GLRA3 subunits.

Immunolocalization of the subunits in capacitated human sperm with subunit-specific antibodies showed that the GLRA1 and GLRA2 subunits are localized in the equatorial region of the sperm head (Fig. 2a and b respectively), the GLRA3 subunit in the flagellar principal piece (Fig. 2d), and the GLRB subunit in the acrosomal region of the sperm head (Fig. 2e). Immunolocalization using the N-terminal antibody that recognizes both the GLRA1 and GLRA2 subunits also showed their presence in the equatorial region (Fig. 2c). All of the localizations shown were also observed in uncapacitated sperm and were not detected when the primary

Figure 1 Western blot analysis of GLR subunits in human sperm: total lysates of uncapacitated human sperm equivalent to 1×10^6 cells were loaded onto a 10% Tris–glycine gel and blotted onto nitrocellulose membrane as described in Materials and Methods. The blots were developed with subunit specific antibodies: GLRA1 subunit (lane 1), GLRA2 subunit (lane 2), GLRA3 subunit (lane 3), GLRB subunit (lane 4), and only secondary antibody (lane 5). The standards in decreasing molecular weight are: phosphorylase b (97 400), serum albumin (66 200), and ovalbumin (45 000).

antibody was pre-incubated with peptide or when the primary antibody was omitted ($n=3$, data not shown). The dim labeling seen in Fig. 2a is identical to that seen in controls.

Discussion

Our results are the first to demonstrate a detailed characterization (type and localization) of the different isoforms of the GLR subunits in human sperm. Earlier studies of characterization of the GLR in mouse and pig sperm used a MAB (mAb4a) antibody that recognizes all the subunits (Melendrez & Meizel 1996, Sato *et al.* 2000a). In those studies, the GLR was localized on the acrosomal and equatorial regions of sperm. The GLRA1

subunit has been shown to be involved in the zona pellucida-initiated AR using mutant mice lacking the GLRA1 subunit (Meizel & Son 2005). The sperm GLR is apparently present in the plasma membrane of the mammalian sperm head. Antibodies are not known to pass through the plasma membrane of viable sperm; yet, the GLRA1/GLRA2 antibody abolished the rhZP3-initiated AR of human sperm (Bray *et al.* 2002) and immunolocalization of the porcine sperm GLR was identical in both fixed and viable sperm (Melendrez & Meizel 1996).

In this study, both the GLRA1 and GLRA2 subunits were found in the equatorial region of human sperm, presumably in the plasma membrane overlying the equatorial segment, the narrowest part of the acrosome. Neither the GLRA1 nor GLRA2 subunit co-localize with the regulatory GLRB subunit that is present in the main acrosomal region (although we cannot rule out such possible co-localization in the equatorial segment of sperm). Some neuronal GLRs do exist as homomeric GLRA1 subunit receptors and the GLRA1/GLRA2 antibody has been reported to recognize only the homomeric GLRA1 receptor in such neurons (Deleuze *et al.* 2005). Functional homomeric neuronal GLRs consisting of β subunits have not yet been demonstrated (Grenningloh *et al.* 1990). While neuronal GLRs are usually composed of both α and β subunits, the lack of co-expression of the β subunit with α subunit has been reported in several regions of rat brain suggesting a possibility that there could be yet another unidentified α subunit (Malosio *et al.* 1991). The GLRA4 subunit was not tested for its presence in human sperm due to the lack of the availability of a specific antibody. In addition, the human GLRA4 subunit is considered to be a pseudogene (Simon *et al.* 2004). Whether the human sperm head GLRB subunit exists as an unusual GLR homomer or is paired with GLRA1 and/or GLRA2 subunits in the narrow equatorial segment or is paired

Figure 2 Immunolocalization of GLR subunits in human sperm: immunolocalization of GLR subunits was studied in capacitated sperm using (a) GLRA1 subunit, (b) GLRA2 subunit, (c) GLRA1/GLRA2 subunits, (d) GLRA3 subunit, and (e) GLRB subunit primary antibodies as described in Materials and Methods. Arrows indicate localization in the equatorial (eq), acrosomal (a), and principal-piece (p) regions of human sperm. Bar = 5 μ m.

with an as yet undetected α subunit, its spatial separation from the GLRA1 and GLRA2 subunits in the main part of the acrosome and the flagellum suggest the possibility of distinct functions for different human sperm head GLRs. For example, such diverse GLRs might be important in discrete steps during AR initiation.

Earlier studies did not detect any localization of the GLR subunits in the tail region of mice and porcine sperm. Our detection of the GLRA3 subunit in the principal piece region in the human sperm might be due to species and epitope differences. RT-PCR analysis of the GLRA3 subunit in mouse testis and sperm showed the presence of only the low molecular weight α 3K splice variant (P Kumar & S Meizel, unpublished observations). Interestingly, mice lacking the GLRA3 subunit are subfertile (the Jackson Laboratory mice database, www.jaxmice.jax.org/strain/005816.html). The presence of a GLR subunit in the flagellum suggests the possibility of a role for that receptor/channel in sperm motility. While subjective motility assays did not detect motility alterations in human sperm treated with strychnine, a GLR antagonist (Bray *et al.* 2002), such assays do not rule out the possibility that the sperm flagellar GLR might be involved in the hyperactivated form of sperm motility important to fertilization (Yanagimachi 1994).

In conclusion, the spatial distribution of the GLR subunits on the surface of human sperm suggests their involvement in different aspects of sperm function. Further studies will be needed to identify and elucidate such roles.

Materials and Methods

Materials

The following reagents were purchased: Percoll, polyvinyl alcohol from Sigma; Fraction V BSA from Serologicals Corp. (Kankakee, IL, USA); pre-cast SDS-PAGE gels, nitrocellulose membranes and broad-range molecular weight standards from Bio-Rad Laboratories; 15-ml conical polypropylene centrifuge tubes (Grenier Labortechnik) from Applied Scientific (South San Francisco, CA, USA); Donkey anti-goat horseradish peroxidase (HRP) from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA); West Pico chemiluminescence reagents from Pierce (Rockford, IL, USA); and Vectashield from Vector Laboratories (Burlingame, CA, USA). For all experiments, deionized water used was purified to 18 MO cm with a NANO-pure ion-exchange system (Barnstead/Thermolyne, Dubuque, IO, USA). All other reagents were obtained from standard sources and were of the highest purity available.

Methods

Human sperm preparation and capacitation

Protocols for human sperm studies were approved by the Human Subjects committee at the University of California, Davis, CA, USA. Semen samples were obtained by masturbation from a pool of six healthy donors. A population of >95%

motile sperm was obtained by centrifugation of semen samples through a discontinuous Percoll gradient and subsequent washing as described previously (Thomas & Meizel 1988).

In order to undergo the AR, mammalian sperm must undergo cellular changes under defined *in vitro* incubation conditions or *in vivo*, which are collectively called capacitation (Yanagimachi 1994). Here, for immunolocalization studies, sperm were incubated under capacitating conditions (Thomas & Meizel 1988). Washed sperm were diluted to 6×10^6 sperm/ml in a medium containing a balanced salt solution, 26 mg/ml BSA, 25 mM bicarbonate, metabolites (lactate, pyruvate, and glucose), and antibiotics (penicillin and streptomycin), and incubated in 500 μ l aliquots in 15 ml polypropylene centrifuge tubes for 24 h at 37 °C in a 5% CO₂/air atmosphere (Thomas & Meizel 1988). The samples were routinely checked for capacitation by assays of their ability to undergo the progesterone-initiated AR as described earlier (Meizel & Turner 1993).

Antibodies

The antibodies used for western blot analysis and immunolocalizations described in this paper were: goat polyclonal antibodies to human neuronal GLRA1, GLRA2, GLRA3, and GLRB subunits from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); rabbit polyclonal antibody to human neuronal GLRA1/GLRA2 subunits from Sigma; fluorescein isothiocyanate (FITC) goat anti-rabbit IgG from Zymed Laboratories Inc. (San Francisco, CA, USA); and FITC rabbit anti-goat IgG from Jackson ImmunoResearch Laboratories Inc. All western blots and immunolocalizations using these antibodies shown in the present report are representative of three separate experiments.

Western blot analysis of the GLR subunits

Uncapacitated human sperm were prepared by Percoll gradient centrifugation and further washing (Thomas & Meizel 1988), and proteins were extracted using Triton-X-100 in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton-X-100 and a cocktail of protease inhibitors (1 mM EDTA, 20 μ M leupeptin, 1 μ g/ml aprotinin, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1 mM benzamidinium HCl, 1 μ M pepstatin A, and 1 μ M E-64)). The extract was spun at 10 000 *g* for 20 min and the supernatant was run on a 10% SDS-PAGE gel and the transferred to nitrocellulose membrane at 100 mA constant current for 2 h. The membrane was blocked for 2 h with 3% BSA (w/v) in TBST (10 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween 20) and then incubated overnight at 4 °C with a 1:5000 dilution of primary antibody diluted in 1% BSA (w/v) in TBST. The membrane was washed three times with TBST and incubated with a 1:25 000 dilution of donkey anti-goat antibody conjugated to HRP for 1 h at room temperature. The membrane was subsequently washed extensively with TBST and developed by chemiluminescence.

Immunolocalization of GLR subunits

For immunolocalization experiments, uncapacitated or capacitated human sperm (75–85% motile) were fixed with 2% paraformaldehyde for 10 min at room temperature. The cells were then washed three times with PBS (pH 7.4) and plated on

poly-L-lysine coated slides. The cells were allowed to adhere for 15 min and washed twice with PBS and washed four times for 5 min each with PBS. In the case of the $\alpha 1$ antibody, the cells were permeabilized with 0.1% Triton-X-100 for 5 min before washing with PBS. The slides were then blocked with 0.2% gelatin for 2 h and incubated overnight at 4 °C with primary antibody (100 µg/ml antibody). The slides were then washed with PBS and incubated for 1 h with 10 µg/ml of the respective secondary antibodies and were washed as described above. The slide was mounted with Vectashield and sperm was examined using a Nikon E800 epifluorescence microscope at 1000× magnification.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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