Expression of metabotropic glutamate receptors in the rat and human testis

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Abstract
The G protein-coupled receptor kinase type 4 mediates the homologous desensitisation of type-1 metabotropic glutamate (mGlu1) receptors and is predominantly expressed in the testis. Hence, we searched for the expression of mGlu1 or other mGlu receptor subtypes in rat and human testes. RT-PCR analysis showed the presence of mGlu1, -4 and -5 (but not -2 or -3) receptor mRNA in the rat testis. The presence of mGlu1 and -5 (but not mGlu2/3) receptor proteins was also demonstrated by Western blot analysis. In the rat testis, both mGlu1a and -5 receptors were highly expressed in cells of the germinal line. It is likely that these receptors are functional, because the agonist, (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid, was able to stimulate inositol phospholipid hydrolysis in slices prepared from rat testes. Immunocytochemical analysis of biopptic samples from human testes showed a high expression of mGlu5 receptors inside the seminiferous tubuli, whereas mGlu1a immunoreactivity was restricted to intertubular spaces. mGlu5 receptors were also present in mature spermatozoa, where they were localised in the mid-piece and tail. This localisation coincided with that of β-arrestin, a protein that is critically involved in the homologous desensitisation and internalisation of G protein-coupled receptors. Taken collectively, these results offer the first evidence for the expression of any glutamate receptor in testes, and suggest that at least mGlu5 receptors are present and functionally active in mature human sperm.

Introduction
Glutamate, the major excitatory neurotransmitter in the central nervous system (CNS), activates both ‘ionotropic’ and ‘metabotropic’ receptors. Ionotropic receptors form membrane ion channels permeable to mono- and divalent cations, whereas metabotropic (mGlu) receptors are coupled to G proteins and regulate the activity of intracellular enzymes, as well as of membrane ion channels (Nakanishi 1992, 1994). mGlu receptors form a family of eight subtypes, subdivided into three groups on the basis of their sequence homology and transduction pathways (Pin & Duvoisin 1995). Group I includes mGlu1 and -5, which are coupled to inositol phospholipid hydrolysis; group II and group III include mGlu2 and -3 and mGlu4, -6, -7 and -8 receptors respectively, which are all negatively coupled to adenylyl cyclase in heterologous expression systems (Pin & Duvoisin 1995). All mGlu receptor subtypes are expressed in the CNS, where they have been implicated in several aspects of physiology and pathology (Nakanishi 1994, Nicoletti et al. 1996, Conn & Pin 1997). However, mGlu receptors are also expressed in peripheral organs, as shown by the presence of mGlu4 receptors in taste buds (Chaudhari et al. 1996), and mGlu5 receptors in rat hepatocytes (Storto et al. 2000), human melanocytes (Frati et al. 2000) and in enteric neurones (Liu & Kirchgessner 2000). Recent evidence indicates that mGlu1 receptors are phosphorylated by the G protein-coupled receptor kinase-type 4 (GRK4) (Sallese et al. 2000), a protein kinase that mediates the homologous
Materials and Methods

Materials

Our studies involving animal experimentation conformed with Guidelines on the Handling and Training of Laboratory Animals, Universities Federation for Animals Welfare at The Old School, Wheathamstead, Herts, UK. Patients were recruited from the University of Catania, Italy and gave their informed consent.

(1S,3R)-1-aminoctylcyclopentane-1,3-dicarboxylic acid (ACPD), 2-methyl-6-(phenethyl)-pyridine (MPEP) and 7-hydroxymimocyclopent[b]chromen-1a-carboxylic acid ethylester (CPCCOEt) were purchased from Tocris Cookson Ltd (Bristol, UK). All other drugs or chemicals were purchased from Sigma (Milan, Italy).

RT-PCR

Total RNA (5 µg) extracted from rat testes or other tissues and 150 ng random hexamers dissolved in 12 µl RNAse-free water were heated to 70 °C for 10 min and then cooled in ice for 1 min. We then added 7 µl Tris–HCl buffer (20 mM, pH 8.4) containing KCl (50 mM), MgCl₂ (2.5 mM), dNTP (500 µM) and dithiothreitol (DTT; 10 mM). After 5 min of incubation at 25 °C, 1 µl Superscript II (200 U) (Gibco-BRL, Life Technologies, Milan, Italy) was added and the incubation was continued for an additional 10 min at 25 °C and then for 50 min at 42 °C. The reaction was terminated by 15 min of incubation at 70 °C. After cooling the samples in ice, 1 µl RNase H (2 U) was added and the samples were incubated at 37 °C for 20 min. PCR was carried out in 50 µl of a Tris–HCl buffer (10 mM, pH 8.4) containing MgCl₂ (1.5 mM), KCl (50 mM), Triton X-100 (0.1%), dTTP, dGTP, dCTP, dATP (200 µM of each), forward and reverse primers (25 pM of each) and 1 µl single-stranded cDNA. Taq polymerase (1 U) was added in hot-start conditions (94 °C for 3–5 min) and 30 cycles were carried out as follows: 94 °C for 1 min, 58 °C for 2 min and 72 °C for 3 min. Amplification products were separated by agarose gel (2%) and visualised with ethidium bromide. Primers and PCR conditions were as follows.

- mGlu1 receptor: accession no. M61099; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 207 bp; forward: 5’-CTGCGACAGATATTTTG-3’ reverse; 5’-GGCCATTCAAGTCCAGAACT-3’
- mGlu2 receptor: accession no. M92075; 1·5 mM MgCl₂; annealing at 60 °C; amplier 677 bp; forward: 5’-GTGACAGCTGCTGCTGAG-3’ reverse; 5’-CTGCGACAGATATTTTG-3’
- mGlu3 receptor: accession no. M92076; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 409 bp; forward: 5’-CCCAGATGAGATGAGTACC-3’ reverse; 5’-TGTCGTGAACAGCTGAGACATA-3’
- mGlu4 receptor: accession no. M5018; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 585 bp; forward: 5’-GGCCATTCAAGTCCAGAACT-3’ reverse; 5’-TGTCGTGAACAGCTGAGACATA-3’
- mGlu5 receptor: accession no. D10891; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 207 bp; forward: 5’-CTGCGACAGATATTTTG-3’ reverse; 5’-GTGACAGCTGCTGCTGAG-3’
- mGlu6 receptor: accession no. M61099; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 677 bp; forward: 5’-GTGACAGCTGCTGCTGAG-3’ reverse; 5’-CTGCGACAGATATTTTG-3’
- mGlu7 receptor: accession no. M92076; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 409 bp; forward: 5’-CCCAGATGAGATGAGTACC-3’ reverse; 5’-TGTCGTGAACAGCTGAGACATA-3’
- mGlu8 receptor: accession no. M92076; 1·5 mM MgCl₂; annealing at 60 °C; amplifier 409 bp; forward: 5’-CCCAGATGAGATGAGTACC-3’ reverse; 5’-TGTCGTGAACAGCTGAGACATA-3’

Immunoprecipitation analysis

Tests removed from male Sprague–Dawley rats (200 ± 20 g body weight; Charles River, Calco, Italy) were homogenised at 4 °C in Tris–HCl buffer (20 mM, pH 7.4) containing 10% sucrose, 1 mM phenylmethyl-sulphonylfluoride, 10 µg/ml leupeptin and 1% aprotinin. Homogenates were centrifuged at 15000 g for 20 min and supernatants were centrifuged twice at 20000 g for 30 min. The resulting pellets were resuspended in ice-cold SDS-lysis buffer containing 50 mM Tris–HCl (pH 7.4), 80 mM β-glycerophosphate, 20 mM EGTA, 150 mM NaCl, 15 mM MgCl₂, 1 mM DTT, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 0·1% Triton-X 100 and 0·2% SDS, and an aliquot was used for protein determination. Five micrograms of immunoprecipitating antibodies directed against mGlu1, -2/3 or -5 receptors (see below) were added to 0·5–1 mg membrane extracts and gently mixed overnight at 4 °C on a rocker shaker. Immunocomplexes were captured by incubation of the extracts for 2 h with protein A-Sepharose (5 mg/sample). Samples were centrifuged at 14 000 g, and washed three times with 1 ml ice-cold phosphate-buffered saline (PBS). After the last centrifugation at 14 000 g, each sample was re-suspended in SDS–bromophenol blue reducing buffer containing 40 mM DTT, to limit the formation of high molecular weight receptor aggregates. After boiling, samples were centrifuged and supernatants were used for Western blot analysis. Western blots were carried out using 8% SDS polyacrylamide gels run on a minigel apparatus (Mini Protein II Cell; Bio-Rad, Milan, Italy); gels were electroblotted on Immobilon PVDF membrane (BioRad) for 1 h using a semi-dry electroblotting system (Trans-blot system SD; Bio-Rad), and filters were blocked overnight in TTBS buffer (100 mM Tris–HCl, 0·9% NaCl and 0·1% Tween 20, pH 7·4) containing 2% non-fat dry milk. Blots were then incubated for 1 h at room temperature with primary polyclonal antibodies (1 µg/ml) which recognise specific carboxy-terminal epitopes of

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mGlu1, mGlu5 (both Upstate Biotecnology, Lake Placid, NY, USA) and mGlu2/3 (Chemicon Int. Inc., Temecula, CA, USA) receptors. Blots were washed three times with TTBS buffer and then incubated for 1 h with secondary antibodies (peroxidase-coupled anti-rabbit; Amersham International plc, Amersham, Bucks, UK) diluted 1:10 000 with TTBS. Immunostaining was revealed by enhanced chemiluminescence (Amersham International plc).

Measurement of inositol phospholipid hydrolysis in slices from rat testes

Agonist-stimulated inositol phospholipid hydrolysis was examined by measuring the accumulation of [3H]inositol monophosphate ([3H]InsP) as described previously (Nicoletti et al. 1986). In brief, 350 × 350 µm slices cut from the rat testis were added to 250 µl Krebs–Henseleit buffer (equilibrated with 95% O2/5% CO2 to pH 7.4) and then incubated for 1 h at 37 °C with 1 µCi/tube myo-d-[3H]inositol (specific activity 16·5 Ci/mmol; NEN Life Science Products Inc., Boston, MA, USA) to label membrane inositol phospholipids. Ten minutes after the addition of 10 mM Li+, the slices were incubated for an additional 40 min in the absence or presence of 1S,3R-ACPD. MPEP or CPCCOEt, when present, were added 2 min prior to 1S,3R-ACPD. The incubation was terminated by the addition of 900 µl methanol:chloroform (1:2). After the addition of 300 µl chloroform and 600 µl water, samples were centrifuged to facilitate phase separation. The [3H]InsP present in the aqueous phase was separated by anion exchange chromatography, as described previously (Nicoletti et al. 1986).

Figure 1 RT-PCR analysis of mGlu1, -2, -3, -4 and -5 receptor mRNA in extracts of rat testes (Te), pancreas (P), thyroid (T) and adrenal glands (A). Extracts from rat cerebellum (CB) or cerebral cortex (CTX) were used as positive controls for mGlu1, -2, -3, -4 and -5 receptors respectively.

Figure 2 Western blot analysis of mGlu1a, -2/3 and -5 receptors in immunoprecipitates from rat cerebellum (CB) or cerebral cortex (CTX) and rat testes (Te). Immunoglobulins used for immunoprecipitation are labelled at 46 kDa. Arrows point to the monomeric (140 kDa) and dimeric forms (above 200 kDa) of mGlu1a, -2/3 and -5 receptors and to the right are the molecular standard sizes.
Male Sprague–Dawley rats were anaesthetised with nembutal (40 mg/kg i.p.) and subjected to intracardiac perfusion with 0·1 M PBS, pH 7·4 and then with 10% formalin. Rat testes or human bioptic samples from the testes of patients with normal or arrested spermatogenesis were dissected, post-fixed in 10% formalin for 24 h at room temperature, dehydrated and paraffin-embedded. Tissue blocks were cut with a microtome (Ernst Leitz GmbH, Vienna, Austria) and 5 µm sections were pretreated with 0·3% H2O2 in ethanol at 4°C for 15 min to inhibit endogenous peroxidase activity and washed with PBS (0·1M, pH7·4) containing 0·05% Tween 20. Sections were incubated with 50% normal goat serum (NGS) for 10 min at 37°C to reduce non-specific immunostaining, and then with mGlu1a (12 µg/ml), mGlu5 (5 µg/ml) or GRK4 (K-20; 1 µg/ml; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) antibodies in 1% NGS at 4°C overnight. Detection was accomplished using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with an anti-rabbit biotin-conjugated IgG (1:100; Calbiochem, La Jolla, CA, USA) in 50% NGS for 30 min at room temperature, and finally incubated with the ABC reagent for 30 min at room temperature. After each incubation step, sections were carefully washed with PBS (0·1 M, pH 7·4). Immunostaining was revealed using 3,5’-diaminobenzidine.
(0.04%) in 0.1 M PBS containing 0.3% H₂O₂, using as counterstain methylene blue or haematoxylin.

**Immunofluorescent analysis of human spermatozoa**

Human spermatozoa from healthy volunteers were washed twice in PBS, smeared on glass slides, air-dried, fixed for 10 min in methanol at −20 °C, extracted for 5 s in acetone at −20 °C, re-dried, washed three times in PBS, and treated with PBS, 5% NGS and 1% BSA for 20 min. Spermatozoa were incubated overnight at 4 °C in the presence of antibodies directed against mGlu1a (5 µg/ml), mGlu5 (5 µg/ml; Upstate Biotechnology), GRK4 or β-arrestin (1 µg/ml; N-19; Santa Cruz Biotechnology Inc.), then washed three times in PBS containing 0.1% BSA, and incubated in fluorescein isothiocyanate goat anti-rabbit antibodies (Calbiochem). Finally, the glass slides were washed three times in PBS, mounted in PBS and glycerol (1:10) containing 5% propylgallate, and observed with a Leitz Aristoplan microscope (E Leitz, Rockleigh, NJ, USA).

**Assessment of human sperm motility**

Sperm motility was assessed in aliquots of seminal fluid from healthy human donors. Ninety microlitres of undiluted semen were added to 10 µl water, MPEP (30 µM) or quisqualate (100 µM), and the percentage of motile sperm was recorded for 60 min. The quality of motility was graded 1 to 3. Spermatozoa with grade 3 motility tend to move rapidly across the field, grade 2 sperm move aimlessly, whereas grade 1 sperm have a beating tail but do not change position.
Results

Expression of mGlu receptor subtypes in rat testes

RT-PCR analysis showed the presence of mGlu1, -4 and -5, but not mGlu2 or -3 mRNA in the rat testes. mGlu1 and -5 receptor mRNA was absent from other endocrine organs including pancreas, thyroid and adrenal glands (Fig. 1). In contrast, mGlu4 receptor mRNA was also detected in the pancreas (Fig. 1). We extended the analysis to mGlu1 and -5 receptor proteins by using polyclonal antibodies specific for either receptor subtype. It is noteworthy that the mGlu1 antibody recognises an epitope that is exclusively present in the major splice variant of mGlu1 receptors (i.e. the mGlu1a receptor) and therefore does not provide any information on the expression of other mGlu1 splice variants (i.e. mGlu1b to -1e receptors). Western blot analysis of immunoprecipitates from rat cerebral cortex or cerebellum (used as reference tissues) showed two major bands corresponding to the monomeric and dimeric form of mGlu1a, -2/3 and -5 receptors (Fig. 2, arrows). mGlu1 and -5 receptors were detected in immunoprecipitates from the rat testis, whereas mGlu2/3 receptors were absent (Fig. 2). Immunohistochemical analysis of the rat testis showed a high mGlu5 immunoreactivity inside the seminiferous tubuli. Cells of the germinal line were intensely labelled beyond the stage of spermatogonia. This distribution pattern was shared by GRK4 immunoreactivity (Fig. 3). mGlu1a immunoreactivity inside the seminiferous tubuli was less intense in germinal cells, but was particularly pronounced in Sertoli cells. An intense mGlu1a immunostaining was noted in the intertubular spaces (Fig. 3).

To assess whether the mGluR1 and -5 receptors expressed in rat testes are functionally coupled to a Gq protein-mediated second messenger response, we measured the stimulation of inositol phospholipid hydrolysis in slices from rat testes challenged with the mGlu receptor agonist, 1S,3R-ACPD. Exposure to 200 µM 1S,3R-ACPD for 60 min induced a significant increase in the accumulation of [3H]InsP. This increase was reduced by about 50% by saturating concentrations of either CPCCOEt (30 µM) or MPEP (30 µM) (Fig. 4), which behave as selective and non-competitive mGlu1 and -5 receptor antagonists respectively (Annoura et al. 1996, Gasparini et al. 1999, Litschig et al. 1999). These results indicate that mGluR1 and -5 receptors are coupled to inositol phospholipid hydrolysis in rat testes.

Expression of mGlu receptors in human testes

To assess whether mGlu receptors are present in human testes, we examined the expression of mGlu1 and -5 receptors by immunocytochemistry in tissue specimens from adult patients who underwent testicular biopsy. Two of these patients (T G and G T of 21 and 28 years of age respectively) had bilateral varicocele. Spermatogenesis was normal in T G and arrested in G T. Another patient (P R, 23 years of age) had left hydrocele with normal spermatogenesis. The last patient (P G) had an intra-abdominal testicular retention that had remained untreated until 37 years of age. This patient was sterile. All patients gave informed consent to the study. Immunohistochemical analysis showed the absence of mGlu1a receptor immunoreactivity inside the seminiferous tubuli and its presence in the intertubular space in bioptic samples from all patients, regardless of the pathological condition (see Fig. 5A and B). In contrast, the seminiferous tubuli of all patients (with normal or abnormal spermatogenesis) were heavily labelled with mGlu5 antibodies. The labelling was apparently present in all cells of the germinal line with the exception of large rounded spermatogonia (see arrows in Fig. 5C and D); Sertoli cells were also immunopositive for mGlu5 receptors (arrowheads in Fig. 5C and D). Thus, while the cellular localisation of the mGlu5 receptor in the human testis was similar to that observed in the rat testis, a difference existed between the expression patterns of mGlu1a receptors in the two species.

Expression of mGlu receptors in human sperm

We performed fluorescent microscopy analysis of human mature spermatozoa to examine the localisation of mGlu1 or -5 receptors in these cells. Immunofluorescent analysis of mGlu1a receptors did not show any positive immunostaining in mature spermatozoa (not shown), consistent with the lack of mGlu1a receptor immunoreactivity in the seminiferous tubuli of human testes. In contrast, mGlu5 receptors were positively labelled in mature sperm, where they were predominantly localised in the mid-piece and tail. mGlu5 receptor immunoreactivity was observed below the acrosomal region in the head of spermatozoa. No labelling of the acrosome region was ever observed (Fig. 6a). The mGlu5 receptor was only partially co-localised with the receptor kinase GRK4, which was expressed in the mid-piece, but was particularly abundant in the acrosome (Fig. 6b). In contrast, a high degree of co-localisation was found between mGlu5 receptors and β-arrestin (Fig. 6c), a protein that is known to interact with the G protein–coupled receptors in the regulation of receptor signalling.

To examine the possibility that activation of mGlu5 receptors could affect sperm motility, we measured the percentage of motile sperms in aliquots of the seminal fluid from healthy human volunteers treated with 100 µM quisqualate (a potent agonist of mGlu1 and -5 receptors) or with 30 µM of the mGlu5 receptor antagonist, MPEP. None of these drugs induced changes in the total percentage of motile spermatozoa, nor in the grading of sperm motility (not shown).
Discussion

We moved from the observation that GRK4 mediates the homologous desensitisation of mGlu1 receptors. GRK4 is co-localised with mGlu1 receptors in cerebellar Purkinje cells, and translocates in response to mGlu1 receptor activation; in addition, GRK4 phosphorylates and desensitises mGlu1 receptors in heterologous expression systems (Sallese et al. 2000). Hence, we decided to examine whether mGlu1 or other mGlu receptor subtypes are present in the testis, where GRK4 shows the highest levels of expression (Sallese et al. 1994). mGlu1 and -5 receptors were both expressed in rat testes with a high degree of specificity because their transcript was absent in other endocrine organs, including the adrenals which can also synthesise steroid hormones. Germinal cells of rat testes were immunopositive not only for mGlu1 and -5 receptors, but also for GRK4, a kinase that regulates receptor signalling. This suggests that mGlu1 and -5 receptors are functional in rat testes. Accordingly, the mGlu1/5 receptor agonist, 1S,3R-ACPD, was able to stimulate inositol phospholipid hydrolysis in slices prepared from rat testes. The use of CPCCOEt and MPEP confirmed that stimulation of inositol phospholipid hydrolysis resulted from the activation of both mGlu1 and -5 receptors. In fact, CPCCOEt and MPEP behave as potent and highly selective non-competitive antagonists of mGlu1 and -5 receptors respectively (Annoura et al. 1996, Gasparini et al. 1999, Litschig et al. 1999). The distribution pattern between rat and human testes was similar for mGlu5 receptors, but divergent with respect to mGlu1a receptors. In human testes, mGlu5 immunolabelling was intense inside the seminiferous tubuli, whereas mGlu1a immunolabelling was detected in Leydig cells of intertubular spaces. The hypothesis that mGlu1a receptors regulate the function of Leydig cells is intriguing. Studies on glutamate receptor activation and testosterone production are still lacking, although d-aspartate (a structural analogue of glutamate) has proved to stimulate testosterone synthesis in isolated rat Leydig cells (Nagata et al. 1999).

The high expression of mGlu5 receptors in the seminiferous tubuli raises the possibility that these receptors regulate the maturation of germinal cells (perhaps after the stage of spermatogonia) or are needed for the physiology of mature spermatozoa. As opposed to mGlu1 receptors, mGlu5 receptors produce oscillatory increases in intracellular Ca\(^{2+}\) when activated in heterologous expression systems (Kawabata et al. 1996). Hence, mGlu5 receptors have the potential to generate intracellular Ca\(^{2+}\) waves in cells of the germinal line, including mature spermatozoa. mGlu5 receptors were predominantly localised in the mid-piece and tail of human sperm, whereas GRK4 was mostly present in the acrosome and mid-piece. However, the localisation of mGlu5 receptors coincided with that of β-arrestin, a functional cofactor of GRKs, which acts as a major determinant in the homologous desensitisation and internalisation of G protein-coupled receptors (Krupnick & Benovic 1998, Lefkowitz 1998, Iacovelli et al. 1999). This particular localisation suggested that mGlu5 receptors could be active in regulating sperm motility. Although mGlu5 receptor agonists or antagonists were unable to induce substantial changes in the percentage of motile sperm, such an action cannot be entirely ruled out because mGlu5 receptors have a significant constitutive activity.

Figure 6 Fluorescent microscopy analysis of (a) mGlu5 receptors, (b) GRK4 and (c) β-arrestin in human spermatozoa.

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and may therefore be active in the absence of any ligand (see Pin & Duvoisin 1995).

In conclusion, these results provide the first evidence that any glutamate receptor is expressed in rat and human testes or human sperm. Understanding the mechanism of activation and the functional role of mGlu receptors might disclose novel aspects in the physiology and pathophysiology of the male reproductive system.

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References


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