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Identification of GABA_BR2 in Rat Testis and Sperm

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Abstract. GABA is capable of mimicking and potentiating the action of progesterone in initiating the acrosome reaction (AR) of mammalian sperm. The GABA-initiated AR is mediated by GABA_AR; whereas GABA_BR1 protein found in rat testis and sperm tends to modify this process. Moreover, the occurrence of GABA_BR2, a subunit essential for the formation of a functionally active GABA_BR, in rat testis and sperm has not been established. In the present study, rat testis and sperm were analyzed for the presence of GABA_BR2 transcript and protein by RT-PCR, Northern blot, Western blot and an indirect immunofluorescence technique. Northern blot shows that the transcript of testis GABA_BR2 is shorter (~3.0 Kb) than that of the brain (~5.6 Kb). The full length testis GABA_BR2 cDNA was prepared by RACE-PCR and found to be shorter by 2.2 Kb in the segment at the extreme terminus of 3'UTR of rat brain GABA_BR2 but, the sequences corresponding to the open reading frame and 5'-UTR of rat testis GABA_BR2 were found to be identical to those of rat brain. GABA_BR2 protein isolated from rat epididymal sperm was slightly larger than those of rat testis and brain. It was principally localized in the acrosome region of the head of rat sperm by an indirect immunofluorescence technique. The present results establish that GABA_BR2 protein is produced in rat testis and sperm and may play a role in GABA triggering of AR.

Key words: GABA_BR2, Testis, Sperm

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γ -Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the vertebrate central nervous system (CNS). Its effects are mediated through two ligand-gated channels (GABA_A and GABA_C receptors), and a metabotropic G protein-coupled receptor, GABA_BR. This species of the receptor acts through G proteins by regulating potassium and calcium channels [1]. The first GABA_B receptor cDNA (termed GABA_BR1) was isolated first from rat brain with a high affinity radio-ligand [2]. After the cloning the GABA_BR1, another subunit of GABA_B

receptor, GABA_BR2, was identified [3–5]. Several lines of evidence demonstrated that the heterodimerization of GABA_BR1 and GABA_BR2 is a requirement for the formation of a functional GABA_B receptor [3–6]. GABA_BR1 and GABA_BR2 form a heterodimer by a linkage involving the interaction at the C-terminal tails [6].

A GABAergic system also occurs in several peripheral tissues [7] e.g. GABA_A receptor is expressed in human sperm [8, 9]. It is interesting to note that rat oviduct contains more than twice the amount of GABA than the brain [10]. Moreover, GABA_A receptor mediates progesterone-induced acrosome reaction (AR), an exocytotic event

essential for successful mammalian fertilization to take place [8, 9]. *In vitro*, GABA can mimic and potentiate the effects of progesterone in inducing AR [9, 11, 12]. These activities show that GABA may participate in the regulation of sperm function, especially the processes of capacitation and AR [13–15].

In our previous studies GABA_AR, GABA_BR1 and GAT1 (GABA transporter 1) have been identified in rat testis and sperm [16–18]. For the first time, we have elucidated the subunit composition of GABA_AR and the splice variants of GABA_BR1 expressed in rat testis and sperm. Furthermore, the role of both receptors in GABA-initiated AR was investigated. The results demonstrate that a low concentration of GABA activates primarily GABA_AR in triggering AR. On the other hand, high concentrations of GABA will tend to activate GABA_BR which will paradoxically suppress GABA_AR activity, thereby inhibiting the neurotransmitter-activated AR [19].

Calver *et al.* [20] found that the expression of GABA_BR1 and GABA_BR2 subunits in the rat CNS differs from that in peripheral tissues. Although GABA_BR1 is found in all rat peripheral tissues including the CNS, GABA_BR2 is restricted to the CNS except for the questionable detection of the transcript in testis based on the results with the PCR technique. Nonetheless, evidence of its production in rat testis has not been validated. It is debatable whether or not the expression of the GABA_BR2 gene is restricted to the CNS. In the present study, compelling evidence is presented showing the production of GABA_BR2 in rat testis and sperm by identifying the respective mRNA and protein. Also the data will show that the transcript of GABA_BR2 of rat testis differs from that of rat brain in that the former isomer lacks the 2.2 Kb at the extreme terminus of the 3'UTR (untranslated region) of the latter. Electrophoretic analysis and immunofluorescence data will show that GABA_BR2 protein in rat epididymal sperm is slightly larger than that of rat testis and brain and that it is localized to the rat sperm head.

Materials and Methods

Materials

The following chemicals and reagents were purchased from Sigma Chemical Co.: Bovine serum

albumin (BSA, Fraction V), EDTA, Tween-20, phenylmethyl sulfonyl fluoride (PMSF), FITC-conjugated goat anti-rabbit IgG secondary antibody, HRP-conjugated goat anti-rabbit IgG secondary antibody, PerfectHyb^{TM} Plus Hybridization Buffers, and rat Multiple Tissue Northern Blots. Trizol and Superscript II Reverse Transcriptase were purchased from GIBCO-BRL; rabbit anti-GABA_BR2 polyclonal antibody [AB5848] from Chemicon. The random prime labeling system was obtained from Amersham Pharmacia Biotech; Smart RACE cDNA Amplification Kit from Clontech; and TaKaRa Ex Taq^{TM} from TaKaRa; and pGEM T-Easy vector from Promega.

Preparation of protein extracts from rat sperm, rat testis and rat brain

Sperm contained in the caudal epididymis of a 3-month old Wistar male rat were released into PBS and prewarmed to 37 C. After washing the sperm with PBS twice, the sperm pellet was suspended in Laemmli buffer and boiled for 5 min. After centrifugation at 12,000 rpm for 5 min, the supernatants were collected as the total protein extract of rat sperm. The rat testis and brain were homogenized in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and 1 mM PMSF. An equal volume of 2 × Laemmli buffer was added to each sample of homogenates and boiled for 5 min. After centrifugation at 12,000 rpm for 5 min, the supernatant was collected and used as the total protein extracts of rat testis and brain.

RNA extraction and RT-PCR

Total RNA from rat testis was extracted with Trizol and isolated according to the procedure outlined by the manufacturer. Reverse transcription was performed according to the manufacturer's instructions (GIBCO-BRL). The first strand of cDNA was generated from 5 µg of total RNA prepared from rat testis with oligo-dT primer and Superscript II Reverse Transcriptase.

The RT-PCR primers were designed according to the cDNA sequence of rat GABA_BR2, namely, the forward primer: ACCATAAGGTTCCAGGGGTC and the reverse primer: AGGCAGAGAGTGATG-GTGCT. PCR was performed first by denaturation at 94 C for 5 min, followed by 35 cycles of denaturation at 94 C for 1 min, annealing at 55 C for 1 min, extension at 72 C for 1 min, and a final extension step at 72 C for 10 min. Amplified PCR

products were electrophoretically fractionated on 1% agarose gels, cloned and sequenced.

Western blot

Protein extracts from rat sperm, rat testis and brain were analyzed by electrophoresis on 10% SDS-PAGE gel, and transferred onto an NC membrane. The membrane was incubated for 2 h with TBS containing 0.1% Tween 20 (TBST) and 5% dehydrated skim milk, followed by 2 h at room temperature in the presence of rabbit anti-GABA_BR2 polyclonal antibody (1:800 dilution). The blots were washed with TBST, for 15 min 3 ×, and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution) at room temperature for 1 h. After washing with TBST for 15 min 4 × the bound anti-GABA_BR2 antibody was visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce).

Indirect immunofluorescence staining

Rat sperm were collected from the caudal epididymis and fixed in 4% paraformaldehyde in PBS for 30 min at 4 C. After three washes with TBS, the cells were incubated with rabbit anti-GABA_BR2 polyclonal antibody in 1% BSA in TBS at 100-dilution overnight at 4 C. After washing with TBS three times, the sperm were incubated with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:400 dilution) for 1 h at room temperature and examined by fluorescence microscopy.

Northern blot

Rat Multiple Tissues Northern Blots were purchased from Sigma. On the membrane, each lane contains approximately 2 µg of poly (A)+ RNA. A probe consisting of 850 bp obtained as a RT-PCR product had been sequenced and found to be homologous to rat brain GABA_BR2. It was radio-labeled by the Random Prime Labeling system (Amersham Pharmacia Biotech). Hybridization was performed by incubating in PerfectHyb^{TM} Hybridization Buffers Plus (Sigma) at 65 C for 3 h. The blots were washed in 2 × SSC, 0.1% SDS at 25 C for 30 min followed by 0.5 × SSC, 0.1% SDS at 50 C for 10 min, and exposed to Kodak BioMax MR film at -80 C.

RACE-PCR

The full-length cDNA of rat testis GABA_BR2 was obtained with a Smart RACE cDNA Amplification

Kit (Clontech). The 5' and 3' RACE-ready cDNA was prepared with mRNA isolated from the testis of a 3-month old rat, according to the procedure supplied by the manufacturer. RACE reactions were performed with 5' or 3' RACE-ready cDNA with the DNA polymerase (TaKaRa Ex Taq^{TM}) suitable for amplification of long regions of DNA with high fidelity. The PCR conditions were set and performed according to the protocols given by Clontech. Four oligonucleotides were used, namely, two for 3' RACE and two for 5' RACE by a nested PCR procedure to obtain high specificity. For 3' RACE, the external primer was GSS1 (5'ACCATAAGGTTCCAGGGGTCCGAGCC 3'), and the internal one was GSS2 (5'TCTGTTTTC-AGGCTGTGGACCCCC3'). For 5' RACE, the external primer was GSA1 (5'CCAAAGGCAGTTG-TGTAGCCACCG 3'), and the internal one was GSA2 (5'GAAGCGAGATCTTCCGAAGCTGCTC3').

The amplification products of 3' and 5' RACE were cloned into pGEM T-Easy vector and sequenced. Ten clones were selected and sequenced both for the 3' and 5' RACE amplification products.

Results and Discussion

GABA_BR functions as a heteromeric assembly of two subunits, namely, GABA_BR1 and GABA_BR2 [3–6]. In our previous study [18], we have identified GABA_BR1 in rat testis and sperm and showed that its splice variants are expressed. But Calver *et al.* [20] concluded that GABA_BR2 is unique and expressed only in CNS tissue. Nonetheless, they did detect GABA_BR2 mRNA in rat testis based on the result of the real-time RT-PCR technique, although the inference is questionable. To confirm that the GABA_BR2 gene is expressed in rat testis and sperm, the presence of GABA_BR2 transcripts and proteins was determined by RT-PCR, Northern blot and Western blot. With GABA_BR2-specific primers in a RT-PCR system, a 850 bp product was obtained (Fig. 1). Sequence analysis showed that it corresponded to GABA_BR2 cDNA at position 1643–2493. (GeneBank, accession No. AJ011318). This finding shows that GABA_BR2 is expressed in rat testis, and supports the claim of Calver *et al.* [20].

With the 850 bp product as probe, GABA_BR2 transcripts were found in brain and testis but not in the other tissues examined (Fig. 2). The size of the

Fig. 1. RT-PCR analysis of GABA_BR2 cDNA prepared from rat testis mRNA. Rat testis total RNA was reverse-transcribed and PCR performed with primers designed according to the cDNA sequence of rat brain GABA_BR2. The cDNA is composed of 850 bp. T: testis. M: Markers (λ /EcoR I+HindIII).

Fig. 2. Northern blot analysis of rat GABA_BR2 mRNA prepared from various rat tissues. The blot was hybridized with rat GABA_BR2 cDNA fragment (the 850 bp product of RT-PCR) radiolabeled by random priming. Lanes: 1, brain; 2, heart; 3, liver; 4, kidney; 5, spleen; 6, testis; 7, lung; 8, thymus; 9, placenta; 10, muscle. Each lane contains approximately 2 μ g of poly (A)+ RNA. Molecular masses (Kb) of RNA standard markers are depicted on the right margin. Note positive staining bands with testis (lane 6) and brain (lane 1).

GABA_BR2 transcript of rat brain was estimated to be 5.6 Kb, which is consistent with the result of Kuner *et al.* [6]. On the other hand, two bands ~3.0 Kb and ~1.8 Kb, were detected in rat testis. The small ~1.8 Kb transcript may not be an effective functional fragment. Although Kuner *et al.* [6] reported that the transcript of GABA_BR2 in rat testis is shorter than the rat brain isomer they were not able to determine the approximate size of the receptor. In addition, the intensity of the blot they had detected was too weak to be considered an unequivocal positive signal. The present study is the first report to show that the size of the transcript of GABA_BR2 of rat testis is approximately 3.0 Kb. It should be noted that there is a significant difference in the amount of GABA_BR2 transcripts in rat brain and testis. The expression of GABA_BR2 is significantly greater in the brain than in the testis.

Since the transcript of GABA_BR2 of rat testis is shorter than that of rat brain, the exact nucleotide sequence of rat testis GABA_BR2 cDNA should be ascertained. Based on the sequence of the RT-PCR product, 5' and 3' primers were designed, synthesized and used to amplify the full-length sequence performed according to the 5' RACE and 3' RACE methods. The full-length rat testis GABA_BR2 sequence is composed of 3263 bp,

generated as 5' RACE and 3' RACE products. The nucleotide sequences of the open reading frame and 5'-UTR of rat testis GABA_BR2 were found to be identical to that of rat brain GABA_BR2. But the 3'-UTR of rat testis GABA_BR2 is 380 bp, which is substantially shorter than the 2576 bp of 3'-UTR of rat brain GABA_BR2 (GeneBank, accession No. AJ011318). Rat testis GABA_BR2 lacks the 2.2 Kb at the extreme terminus of 3'-UTR of rat brain GABA_BR2. The apparent functions of 3'-UTR are to increase mRNA stability, to regulate translational activity and to direct subcellular localization at mRNA [21]. The presence of truncated 3'-UTR in rat testis GABA_BR2 may account for the reduced amount of mRNA transcripts in rat testis relative to that of the brain as based on the Northern blot assay results. The precise role of the truncated 3'-UTR of rat testis GABA_BR2 in regulating its expression needs to be further investigated. The results of RT-PCR and Northern blot assay proved that the GABA_BR2 is produced in rat testis. To show that this gene product is formed, GABA_BR2 protein was determined in rat testis and sperm by Western blot. With a polyclonal antibody to the 20-amino acid peptide at the GABA_BR2 NH₂-terminal, a discrete band of approximately 120 KDa was detected in the extracts of rat testis and brain

Fig. 3. Western blot analysis of GABA_BR2 prepared from rat testis (T), sperm (S), and brain (B). Rabbit anti-GABA_BR2 antibody which recognizes the NH₂-terminal of GABA_BR2 was used. Note that the molecular weight of GABA_BR2 from rat testis and brain is approximately 120 kDa; whereas rat sperm receptor is slightly larger, approximately 125 kDa. The reported Mr of GABA_BR2 is 120 kDa.

proteins (Fig. 3) which corresponded to the reported molecular weight of GABA_BR2 [22]. The molecular size of the band detected in rat sperm protein extracts is slightly greater than those of the rat testis and brain proteins which can be attributed to a greater degree of glycosylation.

By means of the indirect immunofluorescence technique, GABA_BR2 was principally located to the acrosome region of the head of non-capacitated rat sperm (Fig. 4). The sperm tail lacked staining. Most of the fixed sperm with intact plasma membrane exhibited fluorescent staining. Sperm incubated with an equal concentration of nonspecific whole IgG used as the control did not stain (data not shown). The staining pattern on the surface of the sperm head with anti-GABA_BR2 antibody resembled the distribution reported

previously with anti-GABA_BR1 antibody [18], indicating possible congruent localization of both receptors on the head of rat sperm. Lorente *et al.* [23] reported that functional GABA_BR is composed of both GABA_BR1 and GABA_BR2 and is present in rat cardiomyocytes. This finding combined with our present results indicates that the GABA_BR2 subunit occurs in rat testis, sperm and other tissues and is not restricted to the CNS.

Our previous studies, [16–18] show that GABA_AR, GABA_BR1 and GAT1 are components present in rat testis and sperm. Moreover, the functional activity of these receptors was demonstrated by the observation that the rate of AR induced in capacitated rat sperm decreased when pretreated with GABA_BR agonist followed by triggering with a GABA_AR agonist. In conclusion, the action of GABA or progesterone in inducing AR is mediated by GABA_BR [19]. The present study validates the conclusion that the GABA_BR2 gene is expressed in rat testis and sperm at the mRNA and/or protein levels. In addition the detection of GABA_BR1 provides strong molecular evidence of the existence of a functional GABA_BR in rat testis and sperm. The identification of the GABAergic system consisting of GABA_AR, GABA_BR1 and GAT1 in rat testis and sperm will pave the way for a better understanding of the mechanism of AR initiated by GABA as well as revealing a functional role played by GABA in the metabolic pathways of mammalian sperm.

Fig. 4. Indirect immunofluorescence staining of paraformaldehyde-fixed rat sperm with rabbit anti-GABA_BR2 polyclonal antibody. Immunofluorescence (A) and phase-contrast (B) micrographs of mature rat sperm immunostained with rabbit anti-GABA_BR2 polyclonal antibody. Note staining is principally localized to the acrosome region of the rat sperm head.

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