Biochemical and Biophysical Research Communications **283,** 243–247 (2001)
doi:10.1006/bbrc.2001.4732, available online at http://www.idealibrary.com on IDEAL®

Identification of $GABA_B$ Receptor in Rat Testis and Sperm

Xiao Bing He,* Jing Hua Hu,* Qi Wu,* Yuan Chang Yan,*[,1] and S. S. Koide†
Institute of Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China; and †Center for Biomedical Research, Population Council, 1230 York Avenue, New York, New York 10021

Received March 10, 2001

γ-Aminobutyric acid (GABA) can mimic and potentiate the action of progesterone in initiating the acrosome reaction (AR) of mammalian sperm, indicating that sperm contain receptors for GABA. This contention was validated by identifying the receptor (R) subtype, $GABA_AR$, in mammalian sperm. In the present study a second subtype, $GABA_BR$, was identified in rat testis and sperm. Total RNAs of rat testis and sperm were prepared and used as template to synthesize the respective cDNAs by the RT-PCR method. Two splice variants of the cDNA coding $GABA_BR1$ ($GABA_BR1a$ and $GABA_BR1c$) and $GABA_BR2$ were identified. Extracts of rat testis, spermatogenic cells and sperm contained two proteins with estimated molecular sizes of 130 and 100 kDa, corresponding to $GABA_BR1a$ and $GABA_BR1c/1b$, respectively, determined by Western blot using polyclonal anti-$GABA_BR1$ antibody. By an indirect immunofluorescence technique, $GABA_BR1$ was located on the head of rat sperm. The present finding is the first direct demonstration that mammalian sperm contain $GABA_BR$. © 2001 Academic Press

Key Words: **GABA receptors; spermatogenic cells.**

γ-Aminobutyric acid (GABA) is the most prevalent neurotransmitter with inhibitory activity in the mammalian central nervous system (CNS). Its effects are mediated through two ligand-gated pathways, namely, the neurotransmitting receptors, $GABA_AR$ and GABAcR, and a metabotropic G-protein-coupled receptor, $GABA_BR$. The latter receptor interacts with G proteins and appears to regulate potassium and calcium channels (1). The cDNA coding the $GABA_B$ receptor ($GABA_BR1$) was initially isolated from rat brain with the use of a high-affinity radioligand (2). Two N-terminal variants of $GABA_BR1$, $GABA_BR1a$ and $GABA_BR1b$, were identified. Subsequently, two additional novel splice variants, $GABA_BR1c$ and $GABA_BR1d$ (3), and a second subtype, $GABA_BR2$, were identified (4–6). Moreover, it was demonstrated that heterodimerization of $GABA_BR1$ and $GABA_BR2$ was a prerequisite for the formation of an active functional $GABA_BR$ (4–7). When coexpressed, the $GABA_BR1$ and $GABA_BR2$ form a heterodimeric complex by the interaction of their C-terminal segments (7). In addition to CNS, GABAergic system exists in various somatic tissues (8). For example, human sperm possesses $GABA_A$ receptor (9, 10) while the rat oviduct contains twice the amount of GABA as found in rat brain (11). $GABA_AR$ has been shown to mediate the progesterone-induced AR (9, 10, 12), an exocytotic event essential for fertilization to be successful. Furthermore, specific binding sites for GABA occur in sperm of several species (13, 14) that allow this transmitter to trigger AR, thereby mimicking progesterone action (10, 12, 15). These findings suggest that GABA may regulate sperm functions, such as capacitation and AR via its interaction with the receptors (15–17). $GABA_BR$ has also been implicated as an inducer of AR (18). A study with ram spermatozoa, however, demonstrated that baclofen (a specific agonist of $GABA_BR$) did not promote capacitation or trigger AR (16). To date, no experimental evidence of $GABA_BR$ in mammalian sperm has been reported.

In the present study, splice variants of $GABA_B$ receptors were identified in rat testis, sperm and validated by sequence analysis.

MATERIALS AND METHODS

Materials. The following chemicals and reagents were purchased from Sigma Chemical Co.: bovine serum albumin (BSA), EDTA, 2-mercaptoethanol, NP-40, Tween 20, PMSF, Triton X-100, FITC-conjugated rabbit anti-goat IgG antibody and HRP-conjugated rabbit anti-goat IgG antibody. Trizol was purchased from GIBCO and goat anti-$GABA_BR1$ antibody from Santa Cruz. This antibody recognized the epitope mapped at the carboxyl terminus of $GABA_BR1$. Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Pharmacia Biotech.

Preparation of protein extracts from rat sperm, spermatogenic cell and testis. Sperm from the caudal epididymis were collected and dispersed in PBS at 37°C. Motile sperm were selected by the swim-up method, collected and washed twice in PBS containing 1 mM PMSF. Germ cells were isolated from rat testis using a mechanical method as described by Aravindan *et al.* (19). Specimens of testis, spermatogenic cells and sperm were homogenized in a lysis buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 1% NP-40, 0.1% SDS, 2 mM EDTA, 1 mM PMSF, 1% Triton X-100), vortexed at 4°C for 1 h,

[1] To whom correspondence should be addressed. Fax: 86-21-6433-1090. E-mail: ycyan@sunm.shcnc.ac.cn.

0006-291X/01 $35.00

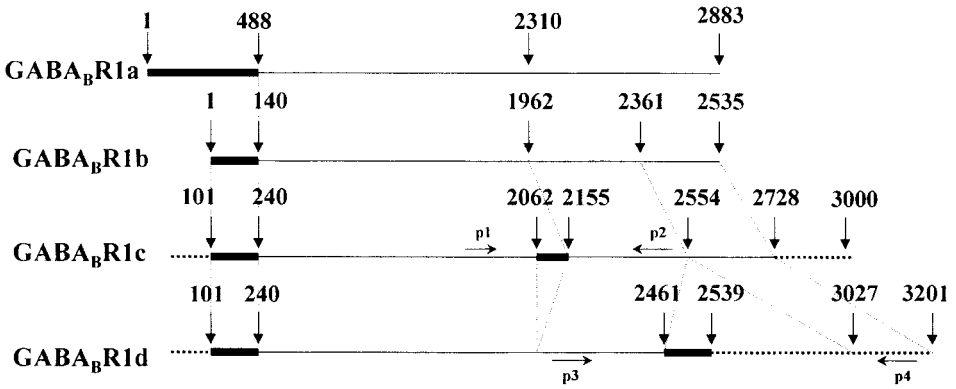

FIG. 1. Alignment of cDNA sequences of rat GABA$_B$R1a, 1b, 1c, and 1d. The N-terminus of GABA$_B$R1b differs from that of GABA$_B$R1a by the replacement of a 1- to 140-nucleotide segment by a 488-nucleotide section. GABA$_B$R1c contains a 93-bp insert (2062–2155) and GABA$_B$R1d contains a 78-bp insert (2461–2539). The dashed line represents untranslatable sequences. Note that the nucleotide segment, designated as p4 (3027–3201) of GABA$_B$R1d, is identical to the nucleotide segment 2554–2728 of GABA$_B$R1c. pl and p4 represent the forward- and reverse-directed primer GBRlab/c, respectively, at nucleotide positions 1747–1766 and 2216–2197 of GABA$_B$R1c cDNA. p3 and p4 represent the forward- and reverse-directed primer GBRlab/d, respectively, at nucleotide positions 2253–2272 and 3027–3201 of GABA$_B$R1d cDNA.

sonicated and centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was collected and considered as protein extract of the respective tissues.

RNA extraction and RT-PCR. Total RNA from rat testis was extracted using Trizol and isolated according to the procedure supplied by the manufacturer. Reverse transcription was carried out according to the manufacturer's instructions (GIBCO-BRL). The first strand of the cDNA was generated from 5 μg of total RNA prepared from rat testis using oligo-dT primer and superscript II reverse transcriptase (GIBCO-BRL).

Nucleotide primers were prepared based on the sequences of four GABA$_B$R1 splice variants and GABA$_B$R2. Three pairs of primers of GABA$_B$R1 (GBR1ab/c, GBR1ab/d and GBR1a) and one of GABA$_B$R2 (GBR2) were synthesized according to Isomoto *et al.* (3). Primer GBR1ab/c was used to discriminate GABA$_B$R1a, GABA$_B$R1b and GABA$_B$R1c; primer GBR1ab/d to discriminate GABA$_B$R1a, GABA$_B$R1b and GABA$_B$R1d. Primer GBR1a was specific for GABA$_B$R1a, and primer GBR2 was specific for GABA$_B$R2. The sequences of these oligonucleotide primers are as follows: Primer GBR1ab/c, 5′-AGTGGAGGAAGACCCTAGAG-3′ (forward) and 5′-ATCATGGTCACAGGAGCAGT-3′ (reverse). Primer GBR1ab/d, 5′-TGAAACGCAGGACACCATGA-3′ (forward) and 5′-TCACTTGTAAAGCAAATGTACT-3′ (reverse). Primer GBR1a, 5′-CCATCAACTTCCTGCCTGTG-3′ (forward), and 5′-TTGCTGTCGTGGTGGATAAG-3′ (reverse). Primer GBR2, 5′-ACCATAAGGTTCCAGGGGTC-3′ (forward) and 5′-AGGCAGAGAGTGATGGTGCT-3′ (reverse).

The PCR was performed initially by denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, extension at 72°C for 1 min (for GABA$_B$R2, duration of 90 s), and a final extension step at 72°C for 10 min. Amplified DNA fragments were electrophoretically fractionated on 1% agarose gels, cloned and sequenced to validate the structure of GABA$_B$R1 variants and GABA$_B$R2.

Western blot. Samples of 50 μg of proteins extracted from rat testis, germ cell and sperm were analyzed by electrophoresis on 10% SDS–PAGE gel, and transferred onto a PVDF membrane. The membrane was incubated for 2 h with PBS containing 0.1% Tween 20 (PBST) and 5% dehydrated skim milk to block nonspecific binding. The membrane was then incubated with anti-GABA$_B$R1 antibodies (1:1000 dilution) in PBST for 18 h at 4°C. The blots were washed with PBST three times, and incubated with HRP-conjugated anti-goat IgG as the secondary antibody (1:10000 dilution) at room temperature for 1 h. After washing with PBST three times, the bound anti-GABA$_B$R1 antibody was detected by visualization using ECL Western blot analysis system (Amersham).

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 PBS, the cells were incubated with anti-GABA$_B$R1 antibody in 1% BSA in PBS at a 100-fold dilution overnight at 4°C. After washing with PBS three times, the spermatozoa were incubated with FITC-conjugated anti-goat IgG as the secondary antibody (diluted 1:400) for 1 h at room temperature and examined by fluorescence microscopy.

RESULTS AND DISCUSSION

In previous studies, GABA$_A$R and GABA transporter (GAT-1) were identified in rat testis and sperm (20, 21). To show that GABA$_B$R is also produced in these tissues, total RNA was prepared from rat testis and analyzed by RT-PCR and proteins from testis and sperm were analyzed by Western blot. Sequence determination of the cDNAs synthesized from testis RNAs show the presence of four variants of GABA$_B$R1 (GABA$_B$R1a, GABA$_B$R1b, GABA$_B$R1c, and GABA$_B$R1d) (Fig. 1) (2, 3). GABA$_B$R1b differs from GABA$_B$R1a in that the N-terminal consisted of a 47-amino-acid residue segment (1–140) in place of a 163-residue segment (1–488). The N-terminus of GABA$_B$R1c (101–240) was identical to that of GABA$_B$R1b with the addition of a 93-bp insert (2062–2155) that coded a 31-amino-acid segment in the fifth transmembrane region of GABA$_B$R1b. GABA$_B$R1d also contained an N-terminus identical to that of GABA$_B$R1b with the addition of an insert of 78 bp (2461–2539) that coded a divergent polypeptide sequence (25 residues) in the carboxyl terminus (3). In addition to GABA$_B$R1, a second subtype, designated as GABA$_B$R2, was identified. This variant encoded a protein consisting of 940 amino acid residues. The

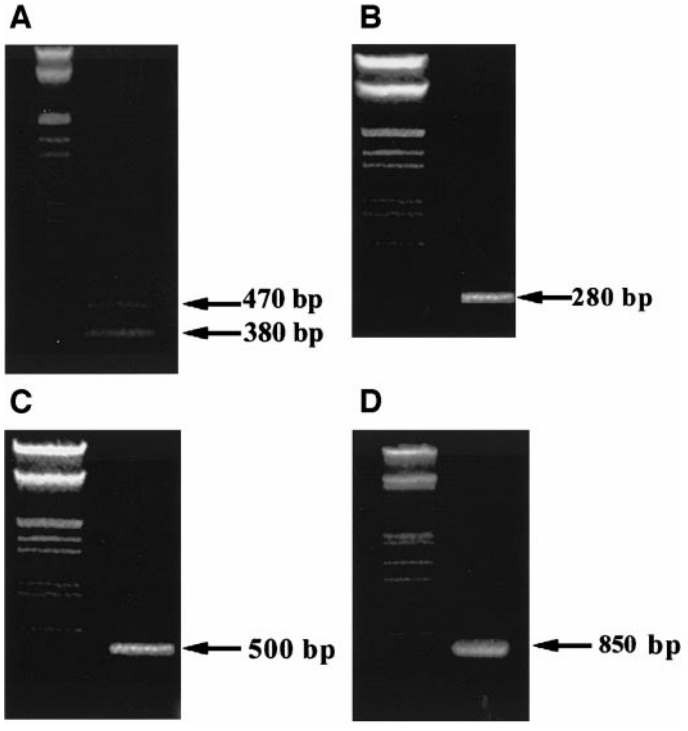

FIG. 2. Electrophoresis analysis of $GABA_BR$ cDNAs prepared from rat testis RNAs by RT-PCR using various primers. Left lane, molecular size markers (lambda/*Eco*RI + *Hin*dIII); right lane, the amplified RT-PCR products. (A) Use of primer GBRlab/c yielded two bands: a 380 bp ($GABA_BR1a$, lb, or 1d) and a 470 bp ($GABA_BR1a$, lb, or lc). (C) Use of GBR_B R1a-specific primer GBR1a yielded a single band of 500 bp. (D) Use of $GABA_BR2$-specific primer GBR2 yielded a single band of 850 bp.

amino acid sequences of $GABA_BR2$ and $GABA_BR1$ are closely related, exhibiting 35% identity overall and 41% identity within the predicted transmembrane domains. Coexpression of $GABA_BR1$ and $GABA_BR2$ appears to be a prerequisite for the production and transport of $GABA_BR1$ to the plasma membrane and for the development of the high affinity GABA binding capacity and for the G-protein activation potential (4–7).

Expression of $GABA_BR1a$, $GABA_BR1c$ and $GABA_BR2$ in rat testis, sperm and spermatogenic cells. With the use of primer GAR1ab/c, two PCR products with estimated sizes of 470 and 380 bp were obtained (Fig. 2A). The 470-bp product was sequenced and found to be identical to rat $GABA_BR1c$ cDNA at position 1848–2316. Sequence analysis of the 380-bp product showed that it is identical to a segment within $GABA_BR1a$ cDNA at position 2096–2471, to $GABA_BR1b$ cDNA at position 1748–2123 and to $GABA_BR1d$ cDNA at position 1848–2223. We were unable to identity the variant from which the 380-bp product was derived. The possible sources are: $GABA_BR1a$, $GABA_BR1b$ or $GABA_BR1d$. Nonetheless, this finding shows clearly that $GABA_BR1c$ and other variants occur in rat testis, substantiating the results of Isomoto *et al.* (3).

Using primer GBRab/d, a single 280-bp product was obtained (Fig. 2B). The amplified product was sequenced and found to be identical to a segment in $GABA_BR1a$ cDNA at position 2601–2883, to $GABA_BR1b$ cDNA at position 2253–2535 and to $GABA_BR1c$ cDNA at position 2446–2728. Since primer GBRlab/d also matched a segment of $GABA_BR1d$, the failure to detect an 870-bp product which is the estimated size of $GABA_BR1d$, indicated that either this variant was not expressed in rat testis or was expressed at a level undetectable by RT-PCR.

Using primer GBR1a, specific for $GABA_BR1a$, a cDNA consisting of 500 bp was obtained (Fig. 2C). Sequence analysis confirmed that it is identical to the segment at position 94–594 of $GABA_BR1a$ cDNA. With $GABA_BR2$-specific primer GBR2, a cDNA composed of 850 bp was obtained (Fig. 2D). Sequence analysis showed that it corresponded to a segment of $GABA_BR2$ cDNA at position 1643–2493. These finding indicated that the genes coding $GABA_BR1a$ and $GABA_BR2$ are expressed in rat testis.

Because of the similarity in the structure of $GABA_BR1b$ to $GABA_BR1a$ and $GABA_BR1c$, the existence of $GABA_BR1b$ in rat testis cannot be proven definitively with the RT-PCR method using the primers GBRab/c or GBRlab/d. Although four splice variants of $GABA_BR1$ occur in rat brain (2, 3), only the two variants, la and lc, were found in the rat testis. In conclusion, rat testis contains $GABA_BR1a$, $GABA_BR1c$, and $GABA_BR2$, but lacks $GABA_BR1d$. The occurrence of $GABA_BR1b$ has not been established.

Two proteins with estimated sizes of 130 and 100 kDa were identified in the extracts of rat testis, germ cells and sperm by Western blot, using polyclonal anti-$GABA_BR1$ antibody (Fig. 3). The 130-kDa protein corresponds to $GABA_BR1a$ as reported by Kaupmann *et al.* (2). The 100-kDa protein, however, may be $GABA_BR1c$ or $GABA_BR1b$ (3). The molecular sizes of these two variants differ by less than 3 kDa, although they possess identical carboxyl terminals (3). Since the polyclonal anti-$GABA_BR1$ antibody used recognizes the 20-peptide segment at the carboxyl terminus of $GABA_BR1$, the issue of whether the 100-kDa protein is

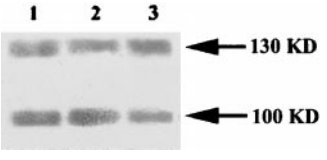

FIG. 3. Western blot analysis of proteins prepared from rat testis, germ cells, and sperm. Stained with anti-$GABA_BR1$ antibody. Lane 1, rat testis proteins; lane 2, rat sperm proteins; lane 3, rat germ cell proteins. The 130 kDa band corresponds to $GABA_BR1a$ and the 100-kDa band to $GABA_BR1c/ld$.

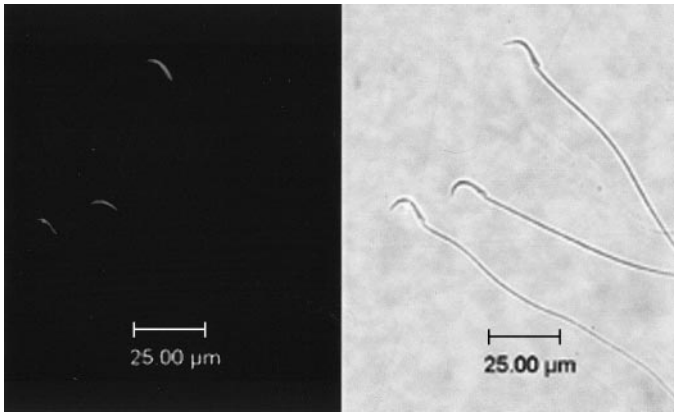

FIG. 4. Indirect immunofluorescence staining of rat sperm with anti-$GABA_BR1$ antibody. Note localization of staining to sperm head.

$GABA_BR1b$ or $GABA_BR1c$ has not been resolved. Based on the results with of RT-PCR, however, the 100-kDa protein is probably $GABA_BR1c$.

The study of Billinton *et al.* (22) on the localization of $GABA_BR1$ variants in the cells of the cerebellum demonstrated that transcripts of $GABA_BR1a$ occurred predominantly in the granule cell layer; whereas that for $GABA_BR1b$ occurred principally in Purkinje cells, suggesting that these variants are associated with the pre- and postsynaptic elements, respectively. Thus the expression of the different splice variants of $GABA_BR1$ may vary in germ cells with the progress of differentiation.

Localization of $GABA_BR1$ on the rat sperm. Using an immunofluorescence technique, $GABA_BR1$ was located on the head of rat sperm (Fig. 4). Almost all of the fixed sperm with intact plasma membrane exhibited fluorescence. The location of $GABA_BR1$ on rat sperm differed from that of GAT1. The latter component was localized to the tail and the entire head except for the equatorial sector (21). Thus the immunolocalization together with the identification of the cDNA coding for $GABA_BR$ provide direct evidence of its existence in rat sperm.

The interaction of $GABA_BR$ with Gi proteins will have an inhibitory influence on adenylyl cyclase activity and on voltage-dependent calcium channels; whereas potassium channels will be activated. Also presynaptic $GABA_BR$ has been shown to be coupled to N-, P-, and T-types of calcium channels (1). Since voltage-dependent calcium channels in mammalian sperm need to be activated before AR can occur (23–27), $GABA_BR$ may play a functional role in the fertilizing potential of sperms and be involved in the induction of AR in human sperm by GABA. The two potential roles of $GABA_BR$, i.e., one of mediating AR and the other of inhibiting voltage-dependent calcium channel, appear to be paradoxical. By elucidating the molecular mechanisms involved in the action of GABA on voltage-dependent calcium channels, adenylyl cyclase activity and AR of mammalian sperm, the functional role of $GABA_BR$ can be clarified.

ACKNOWLEDGMENTS

The present project was supported by grants of the National "973" Project and Laboratory of Molecular Cell Biology, Shanghai Institute of Cell Biology, China.

REFERENCES

1. Bettler, B., Kaupmann, K., and Bowery, N. (1998) $GABA_B$ receptors: Drugs meet clones. *Curr. Opin. Neurobiol.* **8,** 345–50.
2. Kaupmann, K., Huggel. K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., Bettler, B. (1997) Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. *Nature* **386,** 239–246.
3. Isomoto, S., Kaibara, M., Sakurai-Yamashita, Y., Nagayama, Y., Uezono, Y., Yano, K., and Taniyama, K. (1998) Cloning and tissue distribution of novel splice variants of the rat $GABA_B$ receptor. *Biochem. Biophys. Res. Commun.* **253,** 10–15.
4. Jones, K. A., Borowsky, B., Tamm, J. A., Craig, D. A., Durkin, M. M., Dai, M., Yao, W-J., Johns, M., Gunwaldsen, C., Huang, L-Y., Tang, C., Shen, Q., Salon, J. A., Morse, K., Laz, T., Smith, K. E., Nagarathnam, D., Noble, S. A., Brancheck, T. A., and Gerald, C. (1998) $GABA_B$ receptors junction as a heteromeric assembly of the subunits $GABA_BR1$ and $GaBA_BR2$. *Nature* **396,** 674–679.
5. Kaupmann, K., Malitscheck, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., and Bettler, B. (1998) GABA(B) receptor subtypes assemble into functional heteromeric complexes. *Nature* **396,** 683–687.
6. White, J. H., Wise, A., Main, M. J., Green, A., Fraser, N. J., Disney, G. H., Barnes, A. A., Emson, P., Foord, S. M., and Marshall, F. H. (1998) Heterodimerization is required for the formation of a functional GABA(B) receptor. *Nature* **396,** 679–682.
7. Kuner, R., Kohr, G., Grunewald, S., Eisenhardt, G., Bach, A., and Kornau, H. C. (1999) Role of heteromer formation in $GABA_B$ receptor function. *Science* **283,** 74–77.
8. Erdö, S., and Wolff, J. R. (1990) γ-aminobutyric acid outside the mammalian brain. *J. Neurochem.* **54,** 363–72.
9. Wistrom C. A., and Meizel, S. (1993) Evidence suggesting involvement of a unique human sperm steroid receptor/C1-channel complex in the progesterone-initiated acrosome reaction. *Dev. Biol.* **159,** 679–690.
10. Shi, Q. X., and Roldan, E. R. (1995) Evidence that GABAA-like receptor is involved in progesterone-induced acrosome reaction exocytosis in mouse spermatozoa. *Biol. Reprod.* **52,** 373–381.
11. Erdö, S. L., Rosdy, B., and Szporny, L. (1982) Higher GABA concentrations in fallopian tube than in brain of rat. *J. Neurochem.* **38,** 1174–1176.
12. Roldan, E. R., Murase, T., and Shi, Q. X. (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. *Science* **266,** 1578–1586.
13. Aanesen, A., Fried, G., Andersson, E., and Gottlieb, C. (1995) Evidence for γ-aminobutyric acid specific binding sites on human spermatozoa. *Hum. Reprod.* **10,** 1885–1890.
14. Erdö, S. L., and Wekerle, L. (1990) $GABA_A$ binding sites on membranes of spermatozoa. *Life Sci.* **47,** 1147–1151.
15. Shi, Q. X., Yuan, Y. Y., and Roldan, E. R. (1997) γ-Aminobutyric

acid (GABA) induces the acrosome reaction in human spermatozoa. *Mol. Hum. Reprod.* **3,** 677–683.

16. de las Heras, M. A., Valcarcel, A., and Perez, L. J. (1997) *In vitro* capacitating effect of gamma-aminobutyric acid in ram spermatozoa. *Biol. Reprod.* **56,** 964–968.
17. Calogero, A. E., Hall, J., Fishel, S., Green, S., Hunter, A., and D'Agata, R. (1996) Effects of gamma-aminobutyric acid on human sperm motility and hyperactivation. *Mol. Hum. Reprod.* **2,** 733–738.
18. Calogero, A. E., Burrello, N., Ferrara, E., Hall, J., Fishel, S., and D'Agata, R. (1999) Gamma-aminobutyric acid (GABA) A and B receptors mediated the stimulatory effects of GABA on the human sperm acrosome reaction: Interaction with progesterone. *Fertil. Steril.* **71,** 930–936.
19. Aravindan, G. R., Mruk, D., Lee, W. M., and Cheng, C. Y. (1997) Identification, isolation, and characterization of a 41-kilodalton protein from rat germ cell-conditioned medium exhibiting concentration-dependent dual biological activities. *Endocrinology.* **138,** 3259–3268.
20. Yang, S. H., Yan, Y. C., Wu, W. L., Hu, J. H., and Chen, X. Z. (1998) Preliminary study on γ-aminobutyric acid ($GABA_A$) like receptor in rat testis. *Reprod. Contracep.* **9,** 27–33.
21. Hu, J. H., He, X. B., and Yan, Y. C. (2000) Identification of γ-aminobutyric acid transporter (GATI) on the rat sperm. *Cell Res.* **10,** 51–58.
22. Billinton, A., Upton, N., and Bowery, N. G. (1999) GABA(B) receptor isoforms GBR1a and GBR1b appear to be associated with pre-and post-synaptic elements, respectively, in rat and human cerebellum. *Br. J. Pharmacol.* **126,** 1387–1392.
23. Babcock, D. F., and Pfeiffer, D. R. (1987) Independent elevation of cytosolic [Ca^{2+}] and pH of mammalian sperm by voltage-dependent and pH-sensitive mechanisms. *J. Biol. Chem.* **262,** 15041–15047.
24. Arnoult, C., Zeng, Y., and Florman, H. M. (1996) ZP3-dependent activation of sperm cation channels regulates acrosomal secretion during mammalian fertilization. *J. Cell Biol.* **134,** 637–645.
25. Florman, H. M., Corron, M. E., Kim, T. D., and Babcock, D. F. (1992) Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. *Dev. Biol.* **152,** 304–314.
26. O'toole, C. M. B., Roldan, E. R., and Fraser, L. R. (1996) Role for Ca^{2+} channels in the signal transduction pathway leading to acrosomal exocytosis in human spermatozoa. *Mol. Reprod. Dev.* **45,** 204–211.
27. Garcia, M. A., and Meizel, S. (1999) Progesterone-mediated calcium influx and acrosome reaction of human spermatozoa: Pharmacological investigation of T-type calcium channels. *Biol. Reprod.* **60,** 102–109.