Molecular and electrophysiological characterization of a allelic variant of the rat α6 GABA_A receptor subunit

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A 1.45 kb DNA sequence encoding the rat α6 GABA_A receptor subunit (nucleotides 33–1483) was cloned from a Sprague-Dawley rat brain cDNA library by PCR amplification. Dideoxy sequencing of two individual clones revealed that the nucleotide sequence differed at only one basepair (T ~° → G) from that published previously. This difference altered the deduced amino acid sequence, producing a conservative amino acid substitution (His 121 ~ Gln). A Gln residue is present at the same location in the bovine α6 subunit. Restriction endonuclease analysis of the total PCR product demonstrated that this variant of the rat α6 subunit was the only allele found in this particular rat brain library; the original allele was not present. These results were further verified by RNAse protection assays performed with RNA isolated from individual rat cerebella. α6, β1, and γ2S subunits were transiently expressed in L929 cells for electrophysiological analysis. Whole-cell recordings obtained from the cells demonstrated that GABA_A receptor channels with the expected GABA and benzodiazepine pharmacology were produced. Excised outside out single channel recordings from the same cells revealed that GABA elicited brief duration openings to a 33 pS main conductance level and to at least one smaller (approximately 21 pS) subconductance level. Thus this allelic variant of rat α6 subunit could assemble with other subunits to form a functional GABA_A receptor channel with similar properties to the original allelic form.

The GABA_A receptor, the major receptor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), is a member of the ligand-gated ion channel supergene family. Molecular cloning studies from rat, bovine, and human have identified multiple subunit families (α, β, γ, δ, and ρ) and various isoforms within each subunit family have been isolated1. The properties and drug sensitivity of the different subpopulations of GABA_A receptors in vivo are most likely determined by cell-specific expression and assembly of various receptor subunits5.

To date, the best correlation of cell-specific expression of a receptor subunit with its pharmacology has been with the α6 subunit isoform. This subunit was first cloned from rat and bovine brain and subsequently shown to be expressed only in cerebellar granule cells and to possess unique pharmacology at the benzodiazepine-site5,12. Unlike other subunit isoforms which are highly conserved at the amino acid level across various species (> 98% conserved)16, the α6 and also the α4 isoforms only share approximately 90% amino acid identity between the rat and bovine sequences12,23. However, the potential protein kinase phosphorylation sites and transmembrane domains are highly conserved within either the α6 and α4 subunits between the two species. Interestingly, these two isoforms possess similar benzodiazepine pharmacology when co-expressed with rat β2 and γ2 subunits5.

Allelic variants within any species of the GABA_A receptor family have only been described for the human β1 and rat α1 subunit cDNAs6,21. In the human β1 subunit variant, a conservative amino acid change was found at position 10 (Ser10 → Pro). No functional effects, if any, of the amino acid change were demonstrated. However, expression of the rat α1 variant (Phe64 → Leu) with β2 and γ2 subunits produced a 200- or 60-fold decrease in affinity for GABA and GABA-site antagonists, respectively. Similarly, Betz et
tors exist and that they may aker the function of the aquaticus (Taq)
The 3' amplification primer corresponded to nu-
ticles 33–51 (GTC/GGATCC/GTGACCTGGCATTT-
CAGTG, 33-51 (GTC/GGATCC/GTGACCTGGCATTT-
CA). The 5' amplification primer was based upon the
the 50-μM dNTP) into 100 μl of standard buffer (10 mM
cooled on ice prior to initializing the reaction. PCR
weight of α6, β1, and γ2S vector constructs. Positively
and analysis were performed using solutions and
equipment described previously for mouse spinal cord
GABA_A receptor channels13. Amplitude histograms
were constructed using the software IPROC (Axon
Instruments, Burlingame, CA). Only openings with
durations longer than four times the system rise time (rise
time = 130 μs) were analyzed to obtain true amplitude
measurements. Drug solutions were made immediately
prior to use in either water (GABA) or DMSO (di-
azepam (DZ), methyl-4-ethyl-6,7-dimethoxy-β-carbo-
line-3-carboxylate (DMCM)) and diluted into external
recording solution to the indicated final concen-
trations.

In the process of PCR cloning the rat α6 subunit,
we discovered a variant form that possessed a different
amino acid at position 121 (Gln) than that published
previously (His)12. Interestingly, this amino acid substitu-
tion (Gln) was found in the bovine homolog. We thus
undertook this series of studies to determine if the
cloned was a true allelic variant of the rat α6 subunit
and to characterize the whole-cell and single-channel
electrophysiological properties of GABA_A receptor
channels containing this subunit.

The rat α6 subunit cDNA was amplified from a 14
to 16-day-old male/female Sprague–Dawley rat whole
brain cDNA UniZAP library (Stratagene, La Jolla,
CA). The 5' amplification primer was based upon the
published sequence12 and extended from nucleotides
33–51 (GTC/GGATCC/GTGACCTGGCATTT-
CAGTG, GTC/BamHI linker/amplification primer). The 3' amplification primer corresponded to nu-
cleotides 1464–1483 (GTC/GGATCC/CTGTAAGCGAGGAAAATGG, GTC/BamHI/amplification primer). PCR was performed with
Thermus aquaticus (Taq) DNA polymerase (Perkin-Elmer Ce-
tus, Norwalk, CT) in 100 μl of standard buffer (10 mM
Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2, 100 μM dNTP)
containing 1–5 μl of cDNA library and 1 μM primers.
The cDNA library was boiled for 5 min and quickly
cooled on ice prior to initializing the reaction. PCR
products were obtained after 30 cycles of amplification
with protocol A: 60 s, 94°C; 120 s, 55°C; 180 s, 72°C or
alternatively with protocol B: 60 s, 94°C; 120 s, 72°C.
The PCR products were purified after electrophoresis
through a 1% agarose gel using a GeneClean kit
(Bio101, La Jolla, CA) and an aliquot was ligated into
the vector pCR1000 (Invitrogen, San Diego, CA), ac-
cording to the manufacturers’ instructions, to form the
vector pCRα6. Dideoxy sequencing was performed on
individual clones of the vector containing the amplified
fragment using either standard forward or reverse M13
primers or rat α6 specific primers using a Sequenase
2.0 kit with [α-35S]dATP (United States Biochemicals,
Amplification of a rat whole brain cDNA library with primers specific for the α6 subunit produced a 1.45 kb DNA fragment corresponding to the complete open reading frame (data not shown). After subcloning this fragment, an individual bacterial colony harboring the plasmid was picked and grown. Sequence analysis of the purified plasmid revealed a single nucleotide change at position 480 (T→G) that converted the encoded amino acid from His$^{121}$ to Gln (CAT→CAG) (Fig. 1A, arrow). This finding was intriguing since the bovine homolog of the α6 subunit also encoded a Gln at amino acid residue 121$^{12}$. This residue was positioned to the amino terminal side of the third putative N-linked glycosylation site. To verify the authenticity of this change, a second PCR amplification was carried out using the same primers but a slightly different protocol (second protocol listed above). Again, the amplified fragment was subcloned and an individual bacterial colony was grown. Sequencing of purified plasmid DNA again revealed the same single nucleotide change at position 480 (Fig. 1B). No other deviations from the published rat α6 sequence were noted in the amplified DNA (data not shown).

It was possible, but highly unlikely, that two independent clones obtained from the two different PCR reactions possessed the same base pair change due to the result of Taq-induced error at a region of high mutability$. To rule out this possibility, the original samples of amplified DNA prior to subcloning were analyzed by restriction endonuclease digestion. If the nucleotide change at base pair 480 was due to a Taq-induced error, then this should represent some subpopulation of the total amplified DNA, that was just fortuitously cloned. Even if this error occurred during the first round of amplification and only one template copy was present in the reaction, the resulting population of amplified DNA should contain a minimum of 50% wildtype sequences. The wildtype sequence surrounding nucleotide 480 contained a recognition sequence for cleavage by the restriction endonuclease NsiI (5'-ATGCAT-3') and the basepair change removed it (5'-ATGCAG-3'). NsiI digestion of the total amplified DNA from the second PCR reaction produced no fragments (Fig. 2, lane 2), except for the original, uncut DNA (Fig. 2, lane 4). The DNA could be digested however with NcoI to produce the expected fragments of approximately 450 and 950 bp (Fig. 2, lane 3) proving that the DNA was suitable for cleavage. Digestion of the total amplified DNA from the first PCR reaction produced similar results (data not shown). These results suggest that the amplified DNA with the base pair/amino acid change represented a form of the α6 subunit cDNA found in the original library.

To demonstrate that this form of the α6 subunit did not arise from the amplification of a rare, single, mutated cDNA template present in the library, RNase protection assays were performed on total RNA isolated from single cerebella from different rat strains (Fig. 3). The antisense probe (bp 243–656) was designed to span the basepair change (bp 480). The
presence of a G at bp 480 of the mRNA would cause full-length protection of the probe and would produce a band of 413 bp. Alternatively, the presence of the original basepair (T) in the mRNA, would yield two fragments of 176 and 237 bp. Analysis of five different rat strains, including Sprague–Dawley, demonstrated that the probe was fully protected (413 bp). The faint, smaller fragments represent degradation of the probe during the assay. Note that no consistent products were found at the two smaller expected sizes. This result was consistent with the presence of an authentic basepair change at position 480.

Co-expression of this variant of the rat α6 subunit in L929 cells along with the rat β1 and human γ2S subunits produced functional GABA_A receptors (Fig. 4A). Thus this change in amino acid did not effect the assembly of the ion channel. It has been shown by both binding and whole-cell recording that expression of the α6 subunit with β and γ subunits produced receptors which lacked diazepam (DZ) sensitivity and had a much weaker response to the inverse agonist methyl-4-ethyl-6,7-dimethoxy-A-carboline-3-carboxylate (DMCM)^12. The co-application of GABA with DZ to receptors containing the variant α6 subunit similarly demonstrated no enhancement or reduction of current (Fig. 4B). The inverse agonist DMCM when co-applied with GABA produced a slight diminution of current (approximately 10%) (Fig. 4B). Thus the unique pharmacology described for the original form of the α6 subunit was also produced upon expression of this alternate form of the subunit.

Application of GABA to outside-out patches excised from L929 cells expressing the variant α6, rat β1, and human γ2S subunits elicited single-channel currents (Fig. 5A, B). These currents were of brief duration and consisted predominantly of openings to a 33.6 ± 1.1 pS conductance level (2.52 ± 0.08 pA at -75 mV, n = 3 patches) with less frequent openings to at least one smaller subconductance level of approximately 18-23 pS (1.4-1.7 pA) (Fig. 5C). Though no single channel recordings of GABA_A receptors containing the original rat α6 allele have been published, comparison to recordings of receptors composed of α1β2γ2 subunits suggests that these currents were similar in amplitude, but not identical^22. These currents were also larger in amplitude than receptor currents composed of only α and β subunits^12,22.

Various alleles of genes that encode ligand-gated ion channels^10,21, voltage-gated ion channels^19, and anion channels^18 have been reported. In many instances, these allelic variants produce a mutant form of the protein with an altered function and phenotype. It is also possible that “non-mutant” alleles are present which code for conservative^6 or non-conservative amino acid changes.
subunit allelic variant identified by Sigel et al.21. The variant form of the subunit was originally cloned from a neonatal Wistar rat, but it was not found in any other Wistar rat brains. The conservative amino acid change did not drastically effect functional expression of the subunit with rat β1 and human γ2S subunits. Functional receptors were produced which were sensitive to GABA, not sensitive to diazepam, and slightly sensitive to DMCM inhibition (Fig. 3). This was in agreement with a previous study of the pharmacology of α6 subunit containing receptors. Though this previous study co-expressed the α6 subunit with rat β2 and rat γ2 subunits, the homology between the human and rat γ2 subunit isoforms16 and the lack of noticeable effect of switching the β1 and β2 subunits in previous studies of benzodiazepine modulation17 suggested that any effect would be minimal. Single-channel recordings demonstrated that GABA elicited brief openings to one main-and at least one sub-conductance level (Fig. 4) in a manner similar to GABA_A receptors found in vivo14. Thus, this form of the rat α6 subunit had similar basic pharmacological and electrophysiological properties as the original subunit. More importantly, however, was the demonstration that GABA_A receptor channels composed of α6β1γ2S subunits had a main conductance level of 33–34 pS, unlike GABA_A receptor channels composed of α1β1γ2S, α3β1γ2S, and α5β1γ2S subunits, which have main conductance levels of 27–30 pS (T.P. Angelotti, T. Ryan-Jastrow, M.D. Uhler and R.L. Macdonald, manuscript submitted, and unpublished observations). Further studies will be needed to determine if any other receptor properties were modified by this allelic variation of the rat α6 subunit.

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Consistent with this hypothesis could be the rat α1


