The \( \beta \) Subunit Determines the Ion Selectivity of the GABA\(_A\) Receptor

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The \( \gamma \)-aminobutyric acid, type A (GABA\(_A\)) receptor is a chloride-conducting receptor composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits assembled in a pentameric structure forming a central pore. Each subunit has a large extracellular agonist binding domain and four transmembrane domains (M1–M4), with the second transmembrane (M2) domain lining the pore. Mutation of five amino acids in the M1–M2 loop of the \( \beta \) subunit to the corresponding amino acids of the \( \alpha_2 \) nicotinic acetylcholine subunit rendered the GABA\(_A\) receptor cation-selective upon coexpression with wild type \( \alpha_2 \) and \( \gamma_2 \) subunits. Similar mutations in the \( \alpha_2 \) or \( \gamma_2 \) subunits did not lead to such a change in ion selectivity. This suggests a unique role for the \( \beta \) subunit in determining the ion selectivity of the GABA\(_A\) receptor. The pharmacology of the mutated GABA\(_A\) receptor is similar to that of the wild type receptor, with respect to muscimol binding, Zn\(^{2+}\) and bicuculline sensitivity, flumazenil binding, and potentiation of GABA-evoked currents by diazepam. There was, however, an increase in GABA sensitivity (EC\(_{50} = 1.3 \mu M\)) compared with the wild type receptor (EC\(_{50} = 6.4 \mu M\)) and a loss of desensitization to GABA of the mutant receptor.

The \( \gamma \)-aminobutyric acid receptor (GABA\(_A\)R)\(^1\) is a member of the superfamily of ligand-gated ion channels (LGICs), which also includes the nicotinic acetylcholine receptors (nAChR), the glycine receptors (GlyR), and a subtype of the 5-hydroxytryptamine receptors (5-HT\(_{3}\)R) (1–3). The LGICs are involved in mediating fast neurotransmission in the central nervous system but play different roles, which is reflected in their different ion selectivities. GABA\(_A\)Rs and GlyRs are anion-selective whereas the nAChRs and 5-HT\(_{3}\)Rs are cation-selective (3–6). All LGICs are integral membrane proteins, which are formed by assembly of five homologous subunits around a central ion channel (7–10). Each subunit has a large extracellular N-terminal domain and a C-terminal domain containing four transmembrane segments, designated M1–M4, connected by relatively short loops. The extracellular N-terminal domains are believed to form the agonist binding sites, whereas the transmembrane domains form the channel; with the five M2 domains being the primary lining of the ion-conducting pore of the receptor (11, 12).

The structure of the ion-conducting pore of the LGICs have been described thoroughly using the substituted-cysteine-accessibility method (12–15). All the data support a hypothesis of a predominant \( \alpha \)-helical structure of the M2 domains with a slight kink in the center. The pore was found to be widest at the extracellular end narrowing toward the cytoplasmic side (12–16). The selectivity filter and gate was found to be at the intracellular end of the M2 domains and to include a part of the M1–M2 loop (16, 17). Differences between the individual LGICs were observed, but in general both anions and cations can enter the extracellular vestibules, charge selection occurs at a more intracellular position than 13', and the gate is constituted by amino acids in positions –3 to 2' (see Fig. 1).

Besides the structural homology, the LGICs also show considerable sequence homology, particularly in the pore forming domains. This suggests a common quaternary structure of the channels, irrespective of the charge of the permeating ion, with charge selection being accommodated through minor alterations of the overall design of the channel.

The first example of conversion of the ion selectivity filter of a member of the LGICs was provided by Galzi et al. (18). By conducting a series of mutations of the homomeric nAChR \( \alpha_7\), it was demonstrated that mutating three residues was sufficient to alter the ion selectivity from cationic to anionic: A proline insertion at position –2', a mutation of the adjacent glutamate (E-1'A) near the intracellular border of the M2 domain, and a mutation in the central part of the M2 domain (V13'T) (see Fig. 1). Moreover, the ion selectivity of the homomeric 5-HT\(_{3}\)R and that of the homomeric GlyR \( \alpha_4\) have been changed by the same or inverse set of mutations, respectively (19, 20), thereby proving that charge selection is indeed a function of minor alterations in the M1–M2 loop and in the M2 domains of all LGICs.

This evidence has all been established using members of the LGICs that are capable of assembling into homomeric receptors. However, the majority of the LGICs are known to function as heteromeric complexes consisting of up to four different subunits. The predominant GABA\(_A\)R subtypes in the central nervous system are believed to consist of two \( \alpha \) subunits, two \( \beta \) subunits, and one \( \gamma \) subunit (8, 9, 21). When expressed \textit{in vitro}, receptors composed of only \( \alpha \) and \( \beta \) subunits are believed to consist of two \( \alpha \) and three \( \beta \) subunits (9, 22). Thus, even though the underlying principle and actual location of the ion selectivity filter have been unequivocally resolved, the question still remains as to how this knowledge extends to the heteromeric receptors. The present study provides the first example of

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\(^\dagger\) The abbreviations used are: GABA\(_A\)R, \( \gamma \)-aminobutyric acid receptor; M2, second transmembrane domain; TBPS, tert-butylcyclophosphorothionate; NMDG, N-methyl-D-glucosamine; GlyR, glycine receptor; nAChR, nicotinic acetylcholine receptor; 5-HT\(_{3}\)R, subtype of the 5-hydroxytryptamine receptors; wt, wild type; LGIC, ligand-gated ion channel; CHO, Chinese hamster ovary cells.
conversion of the ion selectivity of a heteromeric receptor, namely the GABA\textsubscript{A}R\textsubscript{a2b2\alpha 2}.
As for other LGICs, the selectivity filter was shown to be located at the intracellular border of the M2 domain. However, the different subunits were shown to have different roles in defining the ion selectivity. A number of data sets ascribed a unique role of the \beta\textsubscript{2} subunit in the determination of the ion selectivity. This raises important questions as to the function of the individual subunits in a pentameric receptor.

**EXPERIMENTAL PROCEDURES**

**Cloning of GABA\textsubscript{A}R Subunits**

The GABA\textsubscript{A}R receptor subunits \alpha\textsubscript{2}, \beta\textsubscript{2}, and \gamma\textsubscript{2L} were cloned from human hippocampus poly(A\textsuperscript{+}) mRNA (Clontech) using reverse transcription-PCR. First-strand cDNA was obtained using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Amersham Biosciences). Full-length cDNA sequences were obtained by PCR reactions using the Expand DNA polymerase (Roche Molecular Biochemicals) and the specific primer sets (MWG Biotech) listed below. The cDNAs were ligated using the rapid ligation kit (Roche Molecular Biochemicals). The cDNAs were uracilated by means of the Expand DNA polymerase. The elongation conditions were as follows: 25 °C for 1 min, and ending with an incubation at 72 °C for 10 min.

**Expression of Mutated Receptors**

All constructs were prepared in CHO-K1 cells (ATCC no. CCL61). CHO-K1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10 mM HEPES and 2 mM glutamine supplemented with 10% fetal bovine serum and 2 mM l-proline (Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air and passaged twice a week. CHO-K1 cells were co-transfected with the plasmids described above and a plasmid encoding enhanced green fluorescent protein using the LipofectAMINE Plus kit (Invitrogen) according to manufacturer's protocol. Binding experiments and electrophysiological measurements were performed 24–48 h after transfection.

**Binding Assays**

Membranes were prepared from CHO-K1 cells expressing recombinant GABA\textsubscript{A}R subunits. The cells were washed in phosphate-buffered saline (Invitrogen), trypsinized, washed twice in Tris-citrate buffer (50 mM, pH 7.1), and centrifuged for 10 min at 5000 × g.

[H\textsubscript{3}]Muscimol Binding—Membranes were resuspended in membrane wash buffer (20 mM KH\textsubscript{2}PO\textsubscript{4}/K\textsubscript{2}HPO\textsubscript{4}, pH 7.5, 50 mM KCl, 0.025% bovine serum and 2 mM l-proline (Invitrogen). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO\textsubscript{2} and 95% air and passaged twice a week. CHO-K1 cells were co-transfected with the plasmids described above and a plasmid encoding enhanced green fluorescent protein using the LipofectAMINE Plus kit (Invitrogen) according to manufacturer’s protocol. Binding experiments and electrophysiological measurements were performed 24–48 h after transfection.

[H\textsubscript{3}]Plumazenil Binding—Membranes were resuspended in Tris-citrate buffer (50 mM, pH 7.1) and centrifuged for 10 min at 22,000 × g at 4 °C. The pellet was resuspended in Tris-citrate buffer to a protein concentration of 100–200 μg/ml. Binding was performed with 0.5, 1, 2, or 3 μM [H\textsubscript{3}]Plumazenil (8 Ci/mmol, PerkinElmer Life Sciences) in triplicate in a final volume of 550 μl containing 50–100 μg of protein, and nonspecific binding was determined in the presence of 1 μM GABA (Sigma). Samples were incubated at 4 °C for 30 min, and labeled membranes were harvested on a Brandel cell harvester using GF/C filters (Whatman). The filters were washed with 3 × 4 ml binding buffer, and the amount of radioactivity was determined by liquid scintillation counting.

[\textsuperscript{35}S]TPBS Binding—Membranes were resuspended in Tris-citrate buffer and centrifuged for 10 min at 22,000 × g at 4 °C. The pellet was resuspended in 20 mM KH\textsubscript{2}PO\textsubscript{4} buffer (pH 7.4) containing 200 mM KCl
to a protein concentration of 200–400 μg/ml. Binding was performed in triplicate with 5 nM [3H]TBPS (89Ci/mmol, PerkinElmer Life Sciences) and 0.6 μM GABA in a final volume of 500 μl containing 100–200 μg of protein, and nonspecific binding was determined in the presence of 200 μM picrotoxin (Sigma). Samples were incubated at 20–22 °C for 150 min, and labeled membranes were harvested using rapid filtration over GF/C filters. The filters were washed with 2 × 5-ml 20 mM KH2PO4 buffer (pH 7.4) containing 200 mM KCl, and the amount of radioactivity was determined by liquid scintillation counting.

Data Analysis

Kd and Bmax values were calculated from a one-site binding equation using GraphPad Prism software.

**Electrophysiology**

All experiments were performed under voltage clamp using conventional whole-cell patch clamp methods (24) at 20–22 °C. The EPC-9 amplifier (HEKA Electronics) was controlled by a Macintosh G3 computer via an ITC-16 interface. The experimental conditions were defined by the Pulse-software accompanying the amplifier, and data were sampled at 1 kHz and low pass-filtered at 333 Hz.

Pipettes were pulled from borosilicate glass (Modulohm) using a horizontal electrode puller (Zeitz Instrumente). The pipette was filled with an intracellular solution containing 120 mM KCl, 2 mM MgCl2, 10 mM EGTA, and 10 mM HEPES adjusted to pH 7.2. The pipette electrode was a chlorided silver wire, and the reference electrode was a silver chloride pellet electrode (In Vivo Metric) fixed to the experimental chamber. The electrodes were zeroed with the open pipette in the bath just prior to sealing, and the pipette resistances were 1.6–2.6 MΩ.

Coverslips with cells were transferred to a 15-ml experimental chamber mounted on the stage of an inverted microscope (Olympus). Transfected cells were identified by the emission of green fluorescence when exposed to UV-light. After gigaseal formation, the whole-cell configuration was attained by suction.

Cells were continuously superfused at a rate of 2.5 ml/min with an extracellular solution (Na-R) containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 10 mM HEPES adjusted to pH 7.4. Chloride permeability was addressed using an extracellular solution (Gluconate-R) with a low chloride concentration containing 5 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 135 mM sodium gluconate, and 10 mM HEPES adjusted to pH 7.4. When Gluconate-R was used, the electrodes were zeroed in this solution. Cation permeability was addressed using an extracellular solution (NMDG-R) with a low sodium concentration containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 126 mM NMDG, and 10 mM HEPES adjusted to pH 7.4 with HCl. Liquid junction potentials were estimated using the Henderson liquid junction potential equation.

The cells were voltage-clamped at −60 mV and the holding current was monitored for 30 s at the start of each experiment to ensure a stable baseline. The I-V experiments were performed by holding the cells at potentials of −60, −50, −40, −30, −20, −10, 0, 10, 20, 30, 40, 50, and 60 mV and recording the currents activated by 3 or 30 μM GABA at each membrane potential. GABA-containing solutions were delivered to the chamber through a custom-made gravity-driven U-tube, the tip of which was placed ~50 μm from the cell. In general, GABA was applied for 0.5–1 s every 30–40 s. Currents were measured at the peak of the response, and I-V plots were fitted to a polynomial function using GraphPad Prism software. Reversal potentials (Vrev) were estimated from I-V plots covering two to three membrane potentials on each side of the reversal potentials for each cell. I-V curves and reversal potentials were corrected for the estimated liquid junction potentials.

When evaluating the effect of modulators, cells were preincubinated with the modulator for at least 10 s before GABA was applied. The effects of the modulators Zn2+ (ZnCl2, Sigma), bicuculline (Sigma), and diazepam (Roche Molecular Biochemicals) were quantified against the GABA response evoked prior to addition of the modulator. Results are presented as mean ± S.E.M., and comparisons were made using a two-tailed t test.

**RESULTS**

Wild type and mutated GABA_A receptors were characterized with respect to ligand binding properties and by electrophysiological experiments. I-V curves were recorded in different extracellular solutions and the reversal potentials (Vrev) were estimated from the curves. Anion permeability was assessed by measuring the change in reversal potential when switching from a high (Na-R) to a low (Gluconate-R) extracellular chloride concentration, and cation permeability was assessed by measuring the change in reversal potential when switching from a high (Na-R) to a low (NMDG-R) extracellular sodium concentration. Data from the electrophysiological measurements for the wild type (wt) GABA_A receptors (Table I). As expected for chloride-selective channels, the reversal potentials were sensitive to changes in the concentration of extracellular anions. Replacement of 90% of the extracellular chloride ions by the less permeable gluconate ion resulted in a shift in reversal potentials in the range of 39–43 mV. The permeability of the gluconate ion has been shown to be ~10% of the chloride permeability in GABA_ARs (4); using this value, the expected shift in reversal potential is +43 mV according to the Nernst equation. Reduction of the extracellular sodium concentration by replacement of 90% of the extracellular sodium by the less permeable NMDG ion did not change the reversal potentials significantly, indicating that wt GABA_ARs are cation-impermeable.

**Binding of the GABA agonist [3H]muscimol and of the GABA_A pore blocker [3S]TBPS** were observed for both receptors (Table II). Binding of the benzodiazepine binding site antagonist [3H]flumazenil was observed for the α2β2γ2 receptor but not for the α2β3 receptor, which is consistent with the location of the benzodiazepine binding site at the interface between the α and γ subunits (25).
A Mutated Heteromeric GABA<sub>A</sub> Receptor with Cation Selectivity

fig. 1. Sequence alignments of anion- or cation-conducting LGICs. Amino acid sequences of the pore lining region (M2) of selected subunits from the anion-conducting receptors GlyR (α<sub>1</sub>) and GABA<sub>A</sub>R (α<sub>2</sub>, β<sub>1</sub>, and γ<sub>2</sub>) and selected subunits from the cation conducting receptors nAChR (α<sub>7</sub>, β<sub>2</sub>, and γ<sub>2</sub>) and 5-HT<sub>3</sub>R (3A) numbered according to Miller (28). The intra- and extracellular borders of the M2 domain are outlined, and the amino acids that are shaded above the amino acid sequences mark the amino acids that are exposed to the channel lumen of the GABA<sub>A</sub>R α<sub>2</sub> subunit (12), and the open arrow indicates that the selectivity filter is located at a more intracellular position than 6'. The stars below the amino acid sequence show the amino acids that are exposed to the channel lumen of the nAChR α<sub>7</sub> subunit (14), and the filled arrow indicates that the selectivity filter is located at a more intracellular position than 13'. The region of the nAChR α<sub>7</sub> subunit inserted into the GABA<sub>A</sub>R subunits (named α<sub>7M2α<sub>2</sub></sub>, β<sub>2M2α<sub>2</sub></sub>, and γ<sub>2M2α<sub>2</sub></sub>) is indicated by a box (dotted line). The amino acids used to design GABA<sub>A</sub>Rs with homologous M2 domains are also shown as a box (hatched line) as are the amino acids mutated at the intracellular border (solid line).

Table III

| α<sub>2</sub> subunit | β<sub>3</sub> subunit | γ<sub>2</sub> subunit | Whole-cell currents at -60 mV evoked by 30 μM GABA | V<sub>rev</sub> | K<sub>d</sub> |
|---------------------|---------------------|---------------------|-----------------------------------------------|-----------------|----------|
| Na-R | Gluconate-R | n | [3H]Flumazenil | n |
| α<sub>2</sub>M2γ<sub>2</sub> | β<sub>3</sub> | γ<sub>2</sub> | 516 ± 433 | -0.1 ± 0.7 | 40.1 ± 0.02 | 3 | 1.7 ± 0.7 | 3 |
| α<sub>2</sub> | β<sub>3</sub>/M2γ<sub>2</sub> | γ<sub>2</sub> | No current | 5 | | 2.9 ± 0.4 | 3 |
| α<sub>2</sub>M2γ<sub>2</sub> | β<sub>3</sub>/M2β<sub>3</sub> | γ<sub>2</sub> | 425 ± 264 | -2.9 ± 0.7 | 35.5 ± 0.2 | 3 | 4.4 ± 0.1 | 3 |
| α<sub>2</sub> | β<sub>3</sub>| γ<sub>2M2β</sub> | 403 ± 91 | -1.6 ± 0.4 | 36.0 ± 0.1 | 3 | 2.0 ± 0.7 | 3 |
| α<sub>2</sub>M2β<sub>3</sub> | β<sub>3</sub>| γ<sub>2M2β<sub>3</sub></sub> | 45 ± 11 | -4.6 ± 0.03 | 35.5 ± 0.6 | 3 | 3.1 ± 0.3 | 3 |
| α<sub>2</sub> | β<sub>3</sub>/M2α<sub>2</sub> | γ<sub>2M2α<sub>2</sub></sub> | No current | 5 | | 3.6 ± 0.6 | 3 |
| α<sub>2</sub> | β<sub>3</sub>| γ<sub>2M2α<sub>2</sub></sub> | 304 ± 178 | -1.3 ± 0.1 | 36.3 ± 0.5 | 3 | 2.2 ± 0.3 | 3 |
| α<sub>2</sub>M2γ<sub>2</sub> | β<sub>3</sub> | γ<sub>2</sub> | No current | 5 | | 1.7 ± 0.2 | 3 |
| α<sub>2</sub>M2γ<sub>2</sub> | β<sub>3</sub>| γ<sub>2M2γ<sub>2</sub></sub> | No current | 5 | | ND* | |
| α<sub>2</sub> | β<sub>3</sub>| γ<sub>2M2γ<sub>2</sub></sub> | 613 ± 213 | -3.0 ± 2.6 | 41.7 ± 4.0 | 3 | ND | |

* ND, not determined.

Chimeras between GABA<sub>A</sub>R Subunits—By aligning the M2 domains of several members of the LGIC family, we observed (Fig. 1), that the GABA<sub>A</sub>R M2 domains are not identical for the subunits α<sub>2</sub>, β<sub>3</sub>, or γ<sub>2</sub>. To investigate whether a GABA<sub>A</sub>R consisting of subunits with identical M2 domains would be functional, a set of “internal” chimeric GABA<sub>A</sub>R subunits was designed. The chimeras were expressed in combination with either wt or chimeric GABA<sub>A</sub>R subunits.

As shown in Table III, substituting the M2 domains of the α<sub>2</sub> or γ<sub>2</sub> subunits with that of either the α<sub>2</sub>, β<sub>3</sub>, or γ<sub>2</sub> subunits did not affect the receptors with respect to GABA-evoked whole-cell currents and reversal potentials in either Na-R or Gluconate-R. In contrast, substituting the M2 domain of the β<sub>3</sub> subunit with that of the α<sub>2</sub> or γ<sub>2</sub> subunits invariably led to non-functional receptors. Interestingly, when the M2 domains of both the α<sub>2</sub> and γ<sub>2</sub> subunits were substituted with that of the β<sub>3</sub> subunit, a GABA-evoked current could be measured, but it was reduced by an order of magnitude with respect to amplitude. Interchanging the M2 domains did not affect binding affinity of the benzodiazepine antagonist [3H]Flumazenil for any of the chimeras (Table III). These data show that it is possible to make functional receptors with identical M2 domains.

Chimeras of GABA<sub>A</sub>R Subunits and the nAChR α<sub>7</sub> Subunit—In a first attempt to change the ion selectivity of the heteromeric GABA<sub>A</sub>Rs, chimeric GABA<sub>A</sub>R/nAChR α<sub>7</sub> subunits were generated. The design of chimeras containing the M2 domain of the nAChR subunit α<sub>7</sub> (α<sub>7M2α<sub>2</sub></sub>, β<sub>2M2α<sub>2</sub></sub>, and γ<sub>2M2α<sub>2</sub></sub>) outlined in Fig. 1. The chimeras were expressed in combination with either wt or chimeric GABA<sub>A</sub>R subunits.

As seen in Table III, no GABA-evoked current (30 μM GABA) could be detected when the chimeric subunits α<sub>2</sub>M2α<sub>2</sub> or β<sub>2</sub>M2α<sub>2</sub> were expressed. The only receptor responding to GABA was that of wt α<sub>2</sub> and β<sub>3</sub> subunits co-expressed with the chimeric γ<sub>2M2α<sub>2</sub></sub> subunit. However, because co-expression of the subunits α<sub>2</sub> and β<sub>3</sub> can form functional receptors without the γ<sub>2</sub> subunit, it cannot be ruled out that this functionality can be attributed to wt α<sub>2</sub>β<sub>3</sub> receptors. Moreover, the shift in reversal potential from high to low extracellular chloride concentration for this complex was indistinguishable from that of the wt receptor (Table I), suggesting chloride selectivity. Because the chimeras containing the nAChR α<sub>7</sub> M2 domain were all non-
TABLE IV  
Characterization of GABA$\alpha$Rs mutated in the M1-M2 loop  
Reversal potentials estimated from I-V curves (30 $\mu$m GABA) recorded in Na-R and NMDG-R to identify cation conductance.

| $\alpha_2$ subunit | $\beta_2$ subunit | $\gamma_2$ subunit | Whole-cell currents at $-60$ mV evoked by 30 $\mu$m GABA | $V_{rev}$ Na-R | $V_{rev}$ NMDG-R | $n$
|---|---|---|---|---|---|---|
| $\alpha_2$ | $\beta_2$ | $\gamma_2$ | 4.1 ± 1.3 | $-5.9 \pm 1.1$ | $-6.2 \pm 2.9$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | 12.5 ± 3.7 | $-0.8 \pm 0.4$ | $-16.8 \pm 0.7$ | 4 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | 74.6 ± 167 | $-0.2 \pm 0.7$ | $-1.2 \pm 0.5$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | No current | $-2.0 \pm 1.9$ | $-5.8 \pm 1.9$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | 15.0 ± 5.2 | $-4.4 \pm 1.0$ | $-4.4 \pm 1.6$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | 8.0 ± 3.8 | $-7.4 \pm 0.6$ | $-38.7 \pm 1.9$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | No current | $3.4 \pm 1.4$ | $-5.9 \pm 2.0$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | 21.2 ± 11.6 | $-8.5 \pm 1.5$ | $-42.4 \pm 4.3$ | 3 |
| $\alpha_2$ | $\beta_2$, G-E | $\gamma_2$ | No current | $11.6 \pm 0.5$ | $-7.5 \pm 0.7$ | 3 |

TABLE V  
Characterization of the significance of the amino acids in the SG-EK motif  
Reversal potentials estimated from I-V curves (30 $\mu$m GABA) recorded in Na-R and NMDG-R to identify changes in cation conductance.

| $\alpha_2$ subunit | $\beta_2$ subunit | Whole-cell currents at $-60$ mV evoked by 30 $\mu$m GABA | $V_{rev}$ Na-R | $V_{rev}$ NMDG-R | $\Delta V_{rev}$ Na-R to NMDG-R | $n$
|---|---|---|---|---|---|---|
| $\alpha_2$ | $\beta_2$, SG-EK | 24.5 ± 3.0 | $-7.6 \pm 0.7$ | $-36.2 \pm 2.0$ | $-28.6 \pm 0.6$ | 6 |
| $\alpha_2$ | $\beta_2$, SG-EK | 90.7 ± 13.7 | $-3.7 \pm 0.8$ | $-30.0 \pm 0.6$ | $-26.3 \pm 1.2$ | 3 |
| $\alpha_2$ | $\beta_2$, G-EK | 9.3 ± 2.7 | $-6.0 \pm 0.2$ | $-25.8 \pm 2.1$ | $-19.6 \pm 2.0$ | 3 |
| $\alpha_2$ | $\beta_2$, G-EK | 32.0 ± 3.5 | $-5.3 \pm 0.6$ | $-24.4 \pm 1.1$ | $-19.1 \pm 0.9$ | 3 |
| $\alpha_2$ | $\beta_2$, G-EK | 8.0 ± 3.7 | $-4.1 \pm 0.3$ | $-11.6 \pm 0.5$ | $-7.5 \pm 0.7$ | 3 |

As seen in Table IV, GABA-evoked whole-cell current amplitudes from GABA$\alpha$Rs containing $\alpha_2$DSG-EK or $\beta_2$SG-EK subunits were reduced by a factor of 10–50 and, as for the first set of mutations, no whole-cell current could be measured when both the $\alpha_2$ and the $\beta_2$ subunits were mutated. Expression of the $\alpha_2$DSG-EK subunit did not change the reversal potentials; on the other hand, expression of the $\beta_2$SG-EK subunit changed the reversal potential by $-31.3 \pm 1.3$ mV for the $\alpha_2$$\beta_2$SG-EK$\gamma_2$ receptor and by $-33.9 \pm 1.8$ mV for the $\alpha_2$$\beta_2$SG-EK$\gamma_3$SG-EK receptor. This indicates that GABA$\alpha$Rs containing the $\beta_2$SG-EK subunit are cation-permeable. Again, it was not possible to detect any differences between receptors containing the $\gamma_2$SG-EK or the wt $\gamma_2$ subunit, with respect to either current amplitude or reversal potentials. For reasons of simplicity, the $\gamma_2$ subunit was therefore omitted in a number of the following evaluations.

**Significance of Serine, Glycine, and Lysine in the SG-EK Motif**—As seen in Table IV the $\beta_2$-E subunit gave rise to a small shift in reversal potential when expressed with wt $\alpha_2$ and $\gamma_2$ subunits, whereas the $\beta_2$SG-EK gave rise to a larger shift. To investigate in more detail the role of the amino acids —4’ serine, —3’ glycine, and 0’ lysine in the determination of the ion selectivity, a set of $\beta_2$ mutations were designed and co-expressed with wild-type GABA$\alpha_2$ subunit. As seen in Table V, all the mutated $\beta_2$ subunits resulted in receptors, which displayed a negative shift in reversal potentials ($\Delta V_{rev}$) measured in NMDG-R compared with Na-R. It is also evident that the mutated receptors fall into three categories with respect to shifts in reversal potentials. The shift in reversal potentials for receptors containing $\beta_2$SG-EK is significantly different from that of receptors containing $\beta_2$G-EK ($p = 0.02$), which again is significantly different from that of receptors containing $\beta_2$E ($p = 0.04$). However, whether the 0’ position holds a lysine or arginine makes no difference, because the $\alpha_2$$\beta_2$SG-EK and the $\alpha_2$$\beta_2$SG-EK receptors show identical shifts in reversal potentials.

functional or chloride conducting, two other sets of mutated constructs were designed in which smaller portions of the GABA$\alpha$-M2 domains were mutated to the corresponding nACH$\alpha$ residues.

Chimeras of GABA$\alpha$ Subunits and the nACH$\alpha_2$ M1–M2 Loop—The first set of mutations was constructed on the basis of the work presented in Refs. 18–20. The —2’ proline (—2’ alanine) for the GABA$\alpha$ subunit $\beta_2$ was deleted, and the —1’ alanine was mutated to a glutamate for the three GABA$\alpha$ subunits as shown in Fig. 1 (designated $\alpha_2$E, $\beta_2$E, and $\gamma_2$E). Because the T13 V mutation in the $\beta_2$ subunit has previously been shown to abolish [3H]muscimol binding and activation by GABA (26), this mutation was not included. Again, the chimeras were expressed in combination with either wt or chimeric GABA$\alpha$ subunits.

As seen in Table IV, GABA-evoked whole-cell currents from GABA$\alpha$Rs containing either the $\alpha_2$E or the $\beta_2$E subunit were reduced by two orders of magnitude, relative to the wt GABA$\alpha$Rs, whereas no whole-cell currents could be detected from receptors containing both of these mutated subunits. To facilitate detection of cation permeability, reversal potentials were measured in Na-R and NMDG-R rather than in Glucinate-R. Expression of the $\alpha_2$E subunit did not lead to any changes in the reversal potentials of the receptor. However, a small change in the reversal potential measured in NMDG-R of $-13$ mV was observed for receptors containing the $\beta_2$E subunit. Receptors containing the $\gamma_2$E subunit were indistinguishable from counterparts containing the wt $\gamma_2$ subunit with respect to both amplitude and reversal potentials. In the second set of mutations, a block of six amino acids at the M2 border (—5’—0’) of the GABA$\alpha$ subunits was replaced by the five amino acids (DSGKE) of the corresponding region of the nACH$\alpha_2$ as shown in Fig. 1 (designated $\alpha_2$DSG-EK, $\beta_2$DSG-EK, and $\gamma_2$DSG-EK, because the aspartate is conserved for the $\beta_3$ and $\gamma_2$ subunits).
**Fig. 2.** Current-voltage relationships for the GABA<sub>AR</sub> α<sub>2</sub>β<sub>3</sub> and α<sub>2</sub>β<sub>3</sub>SG-EK. GABA-activated (30 μM) whole-cell currents recorded in different extracellular solutions; Na-R, 140 mM Na<sup>+</sup> and 150 mM Cl<sup>-</sup>; NMDG-R, 14 mM Na<sup>+</sup> and 150 mM Cl<sup>-</sup>; and Gluconate-R, 140 mM Na<sup>+</sup> and 15 mM Cl<sup>-</sup>. Representative traces are shown at the right, and the peak responses in picoamps are plotted against the membrane potential in millivolts corrected for liquid junction potentials to generate I-V plots. The solid curves represent fits of the data points to a polynomial function, as described under “Experimental Procedures.” Reversal potentials (V<sub>rev</sub>) and shift in reversal potentials compared with Na-R (ΔV<sub>rev</sub>) are indicated (n = 3). A positive shift in reversal potential is observed for the α<sub>2</sub>β<sub>3</sub> receptor when extracellular chloride is reduced from 150 to 15 mM, whereas this shift is not observed for the α<sub>2</sub>β<sub>3</sub>SG-EK receptor. Contrary, a negative shift in reversal potential is observed for the α<sub>2</sub>β<sub>3</sub>SG-EK receptor when extracellular sodium is reduced from 140 to 14 mM, whereas no shift is observed for the α<sub>2</sub>β<sub>3</sub> receptor.
Fig. 3. Muscimol binding and GABA concentration-response for the GABA<sub>A</sub>Rs α₂β₃ and α₂β₃SG-EK. Saturation curves from [³H]muscimol binding studies and K<sub>d</sub> values (n = 3) of the GABA<sub>A</sub>Rs α₂β₃ and α₂β₃SG-EK. Representative examples from whole-cell currents evoked by different concentrations of GABA (0.3–30 μM) for the GABA<sub>A</sub>R α₂β₃, SG-EK, and concentration-response relationships for α₂β₃ (•) and α₂β₃SG-EK (□) normalized to the response evoked by 30 μM GABA for each cell. EC<sub>50</sub> values and n<sub>H</sub> are indicated as an inset.

This demonstrates that substitution of four amino acids (ASAA) with three (SG-E) in the β₃ subunit resulted in conversion to cationic selectivity. No [³⁵S]TBPS binding was observed for any of the mutated receptor combinations, indicating substantial structural changes of the pore domain associated with just a few amino acid mutations (data not shown).

Characterization of the α₂β₃SG-EK Receptor—To further address the ion selectivity of the GABA<sub>A</sub>R α₂β₃SG-EK compared with the wt α₂β₃ receptor, full I-V curves from −60 to 40 mV were recorded. The I-V curves for the wt α₂β₃ receptor shown in Fig. 2 exhibits a shift in reversal potentials of ∼39 mV for Gluconeate-R compared with Na-R and a minor shift of ∼3 mV for NMDG-R compared with Na-R. This demonstrates anion but no significant cation permeability. Furthermore, outward rectification was observed for the α₂β₃ receptor in all extracellular solutions, although it was less pronounced in NMDG-R.

In contrast, the I-V curves for the α₂β₃SG-EK receptor revealed a shift in reversal potential of ∼29 mV for NMDG-R compared with Na-R, whereas a shift of ∼5 mV was seen for Gluconeate-R compared with Na-R. This demonstrates that the α₂β₃SG-EK receptor is a cation-selective channel almost insensitive to changes in extracellular chloride. Besides the change in ion selectivity, the outward rectification observed for the wt α₂β₃ receptor was lost for the cation-selective α₂β₃SG-EK receptor.

As seen in Fig. 3, binding affinity of [³H]muscimol was not significantly different for the α₂β₃SG-EK receptor (K<sub>d</sub> 58 ± 8.0 nM, n = 3) compared with the wt α₂β₃ receptor (K<sub>d</sub> 45 ± 0.7 nM, n = 3) (p = 0.23). Moreover, the B<sub>max</sub> value for the α₂β₃SG-EK (0.20 ± 0.02 pmol/mg, n = 3) compared with that of the α₂β₃ receptor (0.44 ± 0.03 pmol/mg, n = 3) indicate uniform expression levels. As seen in Fig. 3, the GABA concentration-response curves for the two receptors were only slightly different, with an EC<sub>50</sub> of 1.3 μM (n<sub>H</sub> of 0.9) for the α₂β₃SG-EK receptor and an EC<sub>50</sub> of 6.4 μM (n<sub>H</sub> of 1.3) for the wt α₂β₃ receptor.

Zn<sup>2+</sup> (3 μM) was found to block GABA responses evoked by 10 μM GABA by 63 ± 6% for the mutated and by 95 ± 1% for the wt receptor. The GABA<sub>A</sub>R antagonist bicuculline (10 μM) was found to inhibit responses evoked by 30 μM GABA by 38 ± 1% for the mutant α₂β₃SG-EK receptor and by 59 ± 7% for the wt α₂β₃ receptor; representative traces are shown in Fig. 4. These observations indicate similar pharmacology. Besides the obvious difference in current amplitudes, it is notable that desensitization is reduced for the mutated receptor.

Although binding of the GABA<sub>A</sub>R channel blocker [³⁵S]TBPS could be observed for the wt α₂β₃ receptor in the range of 0.15–0.25 pmol/mg of protein at 5 nM [³⁵S]TBPS, no binding could be demonstrated for the α₂β₃SG-EK receptor, indicating a fundamental functional change in pore structure of the α₂β₃SG-EK receptor.

Assembly of the γ₂ Subunits into the α₂β₃SG-EK Receptor—To investigate whether the γ₂ subunit integrates functionally in the α₂β₃SG-EK receptor, binding studies using the benzodiazepine antagonist [³H]flumazenil were performed. As seen in Fig. 5A, the GABA<sub>A</sub>R α₂β₃SG-EKY₂ binds [³H]flumazenil with a K<sub>d</sub> of 1.8 ± 0.6 nM, which is not significantly different from the wt α₂β₃γ₂ receptor (2.4 ± 0.7 nM). In contrast, no binding of [³H]flumazenil could be detected for cells transfected with the GABA<sub>A</sub>R subunits α₂ and γ₂ only (up to 6 nM) indicating assembly of the γ₂ subunit with the α₂ and β₃SG-EK subunits. To investigate whether the modulatory effect of classic benzodiazepines was retained for a receptor containing the β₃SG-EK subunit, the ability of diazepam (1 μM) to potentiate responses evoked by 1 or 3 μM GABA was investigated, and representative traces are shown in Fig. 5B.
As seen from the traces, diazepam was capable of potentiating the response evoked by 1 μM GABA for both the wt α2β2γ2 receptor and the α2β2γ-EKγ2 receptor, although the potentiation appeared reduced for the β2γ-EK containing receptor. As seen from the bar graph in Fig. 5B, diazepam potentiation was more pronounced at 1 μM GABA than at 3 μM GABA, consistent with a benzodiazepine agonist induced leftward shift of the concentration-response curve of GABA. In line with this, the reduced potentiation seen for α2β2γ-EKγ2 receptor may in part be due to a slightly lower EC50 value for GABA. Collectively, these experiments strongly suggest that the γ2 subunit can be incorporated into the complex and that the α2β2γ-EKγ2 receptor has retained its ability to be modulated by benzodiazepines.

DISCUSSION

The majority of GABA4Rs are believed to be expressed as heteromeric complexes of two α, two β, and one γ subunit (8, 9, 21). An alignment of the subunit members α1–6, β1–3, and γ2–3 shows that, with a few exceptions, the M2 domains as well as the important loops between M1 and M2 are identical within each subclass (data not shown). To simplify the work on locating/changing the ion selectivity filter, it was first investigated whether a GABA4R in which the M1–2 loop and M2 domain were identical in all subunits could be functional. The results were somewhat surprising with a number of chimeric receptors being indistinguishable from wt counterparts, some quite different, and others again non-functional. As a general feature it was not possible to distinguish receptors containing only a chimeric α2 or a chimeric γ2 subunit from those with wt subunits. By contrast, a receptor giving reduced current amplitudes in response to GABA was obtained when chimeric αβ2M2β3 and γ2M2β3 subunits were co-expressed with a wt β3 subunit. Non-functional receptors were obtained whenever the β3 subunit was the chimera (Table III). These data hint at a unique function of the β3 subunit in pore formation but also imply the importance of features absent in the β3 M1–2 loop or M2 domain but found in both the α2 and γ2 counterparts.

The fact that it was possible to express a GABA4R in which all subunits had identical M2 regions, albeit with low current amplitudes, prompted us to make chimeric receptors with the nAChR α7 M1–M2 loop and M2 domain. No GABA-evoked currents could be recorded from receptors containing either the α7M2α7 or the β3M2α7 chimeras. Receptors with only a chimeric γM2α7 subunit were indistinguishable from the wt αβ2E receptor, questioning the incorporation of the γ2M2α7 subunit into the receptor (Table III).

Because the chimeras with the nAChR α7 M1–M2 loop and M2 domain resulted in non-functional receptors, it was decided to make chimeric receptors with smaller changes. The first attempt was to make the reverse set of mutations presented in Ref. 18 (P-2Δ (A-2Δ for the β3 subunit) and A-1′E) but without the T13′V mutation, because other work had indicated the importance of this threonine for wt GABA4R function (26). Receptors containing either the α2′E or β3′E mutation suffered a current amplitude loss of almost two orders of magnitude compared with the wt receptors. Moreover, no whole-cell currents could be recorded when the α2′E and β3′E subunits were co-expressed. No discernible effects of the γ2′E versus the γ2

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Fig. 5. Assembly of the α2 and β2γ-EK subunits with the γ2 subunits. A, saturation curve from a [3H]flumazenil binding experiment for the α2β2γ-EKγ2 receptor with the Kd value (n = 3). B, the ability of 1 μM diazepam to potentiate the whole-cell current evoked by 1 μM GABA is presented as traces for the α2β2γ2 and the α2β2γ-EKγ2 receptors. The bar graph shows the potentiation of the GABA response by 1 μM diazepam at 1 and 3 μM GABA for the GABA4Rs α2β2γ2 (■) and α2β2γ-EKγ2 (○).
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subunit were noticeable. Interestingly, a small shift in reversal potential, indicative of a change in ion selectivity, could be observed in receptors containing the β<sub>3</sub>-E subunit but not in receptors with the α<sub>2</sub>-E subunit.

When looking at an alignment of cation-selective receptors (Fig. 1) it is clear that the M1–M2 loop has a conserved motif in the region shown to influence selectivity: DSG-EK. In some receptors aspartate, serine, and lysine are replaced by glutamate, cysteine, and arginine, respectively, but these are the most conservative substitutions possible. It was therefore decided to make chimeras of GABA<sub>AR</sub> subunits with this motif in the second attempt. The results were actually similar to the results for the P-2Δ (A-2Δ), A-1/Δ mutations with respect to functionality of the receptors and current amplitudes. Most importantly, however, α<sub>2</sub>β<sub>2</sub>SG-EKγ<sub>2</sub> and α<sub>2</sub>β<sub>2</sub>SG-EKγ<sub>2</sub>-SG-EK receptors displayed large shifts in reversal potentials of approximately ~30 mV when extracellular sodium was replaced with NMDG, demonstrating cation selectivity. Apart from a further loss in current amplitudes, no discernable effect of the γ<sub>2</sub>-EK version was observed. Furthermore, the shifts in reversal potentials were independent of the γ<sub>2</sub> subunit presence.

Additional characterization showed that the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor was by far more sensitive to changes in the extracellular sodium concentration, relative to changes in the chloride concentration, which demonstrates predominant cation conductance. Evaluation of the contribution of the individual amino acids in the SG-EK motif to the change of ion selectivity showed that only the 0<sup>1</sup> lysine does not contribute significantly to cation selectivity. [3H]Muscimol binding experiments revealed that the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor has the same K<sub>d</sub> and same B<sub>max</sub> as wt α<sub>2</sub>β<sub>2</sub> receptors. From GABA concentration-response curves, the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor is seen to be slightly more sensitive to GABA but with a similar Hill slope, and a pharmacological characterization showed that Zn<sup>2+</sup> and bicuculline still block the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor. Although no evident effects of the γ<sub>2</sub> subunit were observed when studying reversal potentials, the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor still binds [3H]flumazenil, and very importantly GABA-evoked currents can be potentiated by diazepam. These data demonstrate that the γ<sub>2</sub> subunit is able to integrate in the receptor and form a functional benzodiazepine site.

Besides the fundamental change in ion selectivity the α<sub>2</sub>β<sub>2</sub>-SG-EK channel shows distinct changes in gating behavior, similar to the “gain-of-function” characteristics of mutated nAChR α<sub>2</sub> receptors (18, 27). Thus, the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor displayed an increased GABA sensitivity and a loss of desensitization. This stands in contrast to the GlyR-STM mutation (19), where it was speculated that the T13<sup>V</sup> mutation could be responsible for the loss-of-function characteristics. Interestingly, these features were attributed to the V13<sup>T</sup> mutation (27), which fit well with the present data. As observed for the mutated nAChR α<sub>2</sub> (27), a 10-fold loss of current amplitudes was observed for the α<sub>2</sub>β<sub>2</sub>-SG-EK receptor, whereas binding data indicate that the total number of receptors is similar. Possible explanations for this could be either a decrease in transport to the surface of the cells, a decrease in unitary conductance, or altered gating efficiency.

Corringer et al. (27) proposed the term “exclusive” for the P-2<sup>Δ</sup> insertion (P236<sup>Δ</sup>), based on the findings that a proline in the selectivity filter is incompatible with a cation channel for the homomeric nAChR α<sub>2</sub> and necessary for a conversion to an anion channel. The data presented here indicate that other features in the heteromeric GABA<sub>AR</sub> substitute for this “exclusivity” making the role of the proline less obvious. The wt β<sub>3</sub> subunit has an alanine instead of a proline in position –2′ for which reason a wt α<sub>2</sub>β<sub>2</sub>γ<sub>2</sub> receptor only holds a total of three proline residues. However, the data for the “internal” chimeras demonstrate that it is possible to make functional anion permeable heteromeric GABA<sub>AR</sub> subunits lacking the proline in all subunits (α<sub>2</sub>β<sub>2</sub>M2γ<sub>2</sub>). Furthermore, the data for α<sub>2</sub>β<sub>2</sub>-SG-EKγ<sub>2</sub> receptors show that it is possible to make heteromeric cation-permeable GABA<sub>AR</sub> channels containing a total of three prolines in the α<sub>2</sub> and γ<sub>2</sub> subunits.

Corringer et al. (27) showed that the exact nature of the amino acid side chains in the 234–238 (~5′ to 0′) loop in the anionic version of the nAChR α<sub>2</sub>, besides the proline insertion, is less important. Interestingly, the present study demonstrates that the identity of the side chains in this same region is highly important for the mutated β<sub>3</sub> subunit generating a cationic GABA<sub>AR</sub>. This seems to support the hypothesis that the M1–M2 loop has differential contribution in ion selectivity in anion and cation channels (27).

Because all previous reports on the determinants of ion selectivity of LGICs have been concerned exclusively with homomeric ion channels, it has not yet been possible to address the issue of the symmetry of the selectivity filter. If the selectivity filter were assumed to be symmetrical, all five subunits in a receptor would contribute equally to ion selectivity. It would then be expected that all subunits would have to be mutated for full conversion of charge selectivity, and if only some subunits were mutated, the phenotype of the resulting receptors in terms of ion selectivity would be independent of which subunits were mutated. It is evident from the present data that the results are essentially the opposite, i.e. mutations in all subunits abolish function and α<sub>2</sub>DSG-EKβ<sub>3</sub> conducts anions, whereas α<sub>2</sub>β<sub>2</sub>-SG-EK conducts cations. One explanation for these findings could be that not all subunits contribute equally or at all to the ion selectivity filter; indeed, the present data strongly suggest that the β<sub>3</sub> subunit has a dominant role in determining the ion selectivity of the heteromeric GABA<sub>AR</sub>. The present data demonstrate that integration of the γ<sub>2</sub> subunit into the α<sub>2</sub>β<sub>2</sub>-SG-EKγ<sub>2</sub> receptor has no effect on the shift in reversal potentials, which means that only two β<sub>3</sub>-SG-EK subunits are sufficient for conversion of the ion selectivity. It seems possible that, although all five subunits are involved in the overall design of the pore, only the two β<sub>3</sub> subunits are responsible for ion selectivity. Future studies will show whether other features such as Ca<sup>2+</sup> permeability and unitary conductance are also determined by the β<sub>3</sub> subunit exclusively or by all subunits in unison.

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