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A Role for the 2′ Residue in the Second Transmembrane Helix of the $GABA_A$ Receptor γ2S Subunit in Channel Conductance and Gating

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Abstract. $GABA_A$ receptors composed of α, β and γ subunits display a significantly higher single-channel conductance than receptors comprised of only α and β subunits. The pore of $GABA_A$ receptors is lined by the second transmembrane region from each of its five subunits and includes conserved threonines at the 6′, 10′ and 13′ positions. At the 2′ position, however, a polar residue is present in the γ subunit but not the α or β subunits. As residues at the 2′, 6′ and 10′ positions are exposed in the open channel and as such polar channel-lining residues may interact with permeant ions by substituting for water interactions, we compared both the single-channel conductance and the kinetic properties of wild-type α1β1 and α1β1γ2S receptors with two mutant receptors, αβγ(S2′A) and αβγ(S2′V). We found that the single-channel conductance of both mutant αβγ receptors was significantly decreased with respect to wild-type αβγ, with the presence of the larger valine side chain having the greatest effect. However, the conductance of the mutant αβγ receptors remained larger than wild-type αβ channels. This reduction in the conductance of mutant αβγ receptors was observed at depolarized potentials only (E_{Cl} = −1.8 mV), which revealed an asymmetry in the ion conduction pathway mediated by the γ2′ residue. The substitutions at the γ2′ serine residue also altered the gating properties of the channel in addition to the effects on the conductance with the open probability of the mutant channels being decreased while the mean open time increased. The data presented in this study show that residues at the 2′ position in M2 of the γ subunit affects both single-channel conductance and receptor kinetics.

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Key words: GABA — Single-channel — Conductance — Gating — Kinetics — Gamma subunit

Introduction

Physiological ion channels must conduct ions at a rapid rate whilst selecting between ions of different charges and sizes. There is good evidence that in many cases these properties derive from the functional groups lining the narrowest region of the channel that interact with permeant ions, for example, backbone carbonyl groups in the P loop of the KcsA channel (Doyle et al., 1998). The Cys-loop superfamily of ligand-gated ion channels, which includes nicotinic acetylcholine (nAChR), serotonin type 3 ($5HT_3R$), γ-aminobutyric acid type A and C ($GABA_{A,C}R$) and glycine (GlyR) receptors, exhibit charge and size selectivity that is suggestive of interactions between the channel and the permeant ion. There is now an abundance of experimental evidence indicating that the second transmembrane domains, M2, predominantly form the walls of the ion channel. Biochemical data from the nAChR first implicated M2 as lining the pore of this pentameric receptor. Such studies include the photoaffinity labelling of residues in the M2 region with the open-channel blockers chlorpromazine and QX-222 (Giraudat et al., 1986; Hucho et al., 1986; Giraudat et al., 1987; Leonard et al., 1988; Charnet et al., 1990), the generation of chimaeric mouse and calf δ subunits that demonstrated conductance was influenced by a region including the M2 domain (Imoto et al., 1986), and, subsequently, site-specific mutagenesis of the rings of charged amino acids at either end of the M2 region, which altered the rate of ion transport (Imoto et al., 1988). More recently, mutational studies that

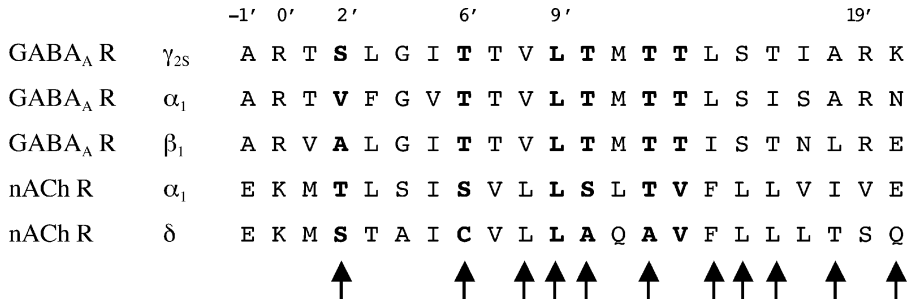

Fig. 1. Alignment of the pore-lining amino acids from the M2 transmembrane region of selected ligand-gated ion channels. The residues are numbered according to Miller (1989). Amino acids in the $GABA_A$ subunits in bold indicate those we have mutated and shown to affect channel properties (Tierney et al., 1996, 1998; Dalziel et al., 1999a, 1999b, 2000) and the equivalent residues in the other subunits are also in bold. Arrows indicate those residues in the $GABA_A$ β1 subunit exposed in the pore in the presence of saturating agonist concentrations (Goren et al., 2004). $GABA_A$ subunits are the human isoforms and the nAChR α1 and δ subunits are from *T.* marmorata (Tierney et al., 2004).

involved switches of ion selectivity in numerous Cys-loop ligand-gated ion channels have led to the proposal that the narrowest region of the pore lies at the cytoplasmic end of the M2 helices, between the −3′ and 2′ positions (Galzi et al., 1992; Keramidas et al., 2000; Gunthorpe & Lummis, 2001; Jensen et al., 2002; Carland et al., 2004). Finally, recent structural data from the nAChR confirm the position of the M2 helices, which comprise the lining of the central pore and indicate that the volume bounded by the M2 helices tapers towards the cytoplasmic side of the membrane (Miyazawa, Fujiyoshi & Unwin, 2003).

Hydroxylated side-chains on the channel-facing surface of M2, specifically at the 2′ and 6′ positions (*see* Fig. 1), have been proposed to form the ion permeation pathway in ligand-gated ion channels (Unwin, 1989; Betz, 1990; Sansom, 1992). Substitution of hydroxyl groups, particularly at the 2′ position, does reduce the conductance of the cation-selective nAChR (Imoto et al., 1988; Imoto et al., 1991; Villarroel et al., 1991; Cohen et al., 1992a; Cohen et al., 1992b; Villarroel et al., 1992; Villarroel & Sakmann, 1992; Wilson & Karlin, 1998). A reduction in single-channel conductance was also observed when all three hydroxylated side-chains in the mouse nAChR were substituted at the 6′ position, but only at depolarized potentials (Leonard et al., 1988).

Polar residues in M2, homologous to those lining the nAChR channel, also line the channel of the anion-selective $GABA_A$ receptor. Mutagenesis studies using the SCAM approach (substituted cysteine accessibility method) have provided information regarding the relative exposure of the $GABA_A$ α and β subunit's pore-lining residues to the aqueous environment as well as inferences regarding the narrowing of the pore towards its intracellular end (Xu & Akabas, 1993, 1996; Goren, Reeves & Akabas, 2004). The M2 region provides rings of hydroxylated residues at the 6′, 10′ and 13′ positions in $GABA_A$ receptors, although only γ and δ subunits have hydroxylated residues at 2′ (Fig. 1). We have shown previously that polar residues are not required at the 10′ position for appropriate ion channel function (Tierney et al., 1998) and that in the β1 subunit the 13′ polarity appears to be important for gating rather than ion permeation (Dalziel et al., 1999a). At the 6′ position, however, polar side chains are required for ligand-gated ion channel function (Tierney et al., 1998). Polar residues are clearly not essential at the 2′ position for ion permeation, as they are not present in αβ receptors, which are perfectly functional as chloride-selective ion channels. However, by analogy to the role of the 2′ residues in the Na^+ channel of the nAChR, the polar 2′ residue in the γ subunit could contribute to the properties of Cl^- conductance in the $GABA_A$ αβγ receptor.

We mutated the γ2S serine 2′ residue in $GABA_A$ α1β1γ2S receptors to either its α (2′V) or β (2′A) subunit counterpart and recorded GABA-activated single-channel currents from transiently transfected L929 cells in outside-out patches. Comparison of the single-channel conductance and kinetic properties displayed by both wild-type αβ and αβγ combinations with mutant receptors (αβγ(S2′V) and αβγ(S2′A)) revealed that the conductance of both the mutant receptors was decreased compared to the wild-type αβγ receptors. This reduction in the conductance, however, was observed at depolarized potentials only (E_{Cl} = −1.8 mV).

Materials and Methods

Plasmid Construction

The $GABA_A$ α1–pcDNA3.1$^+$ plasmid was constructed by subcloning the human $GABA_A$ α1 cDNA as a 1.4 kB fragment into the pcDNA3.1$^+$ vector (Stratagene) digested with *Bgl2* and *Eco*R1 restriction enzymes. Similarly, human β1-pcDNA3.1$^+$ was constructed by excising the β1 1.4 kB cDNA fragment with the restriction enzyme *Bam*HI from the previously prepared αβpAcUW31 plasmid and ligating the fragment into pcDNA3.1$^+$ cut with the same restriction enzyme. A *Bam*HI/*Eco*RI 1.4 kB cDNA fragment of the γ2S was subcloned from BP8 (B.C. Cromer,

Ph.D. Thesis, ANU) into pcDNA3.1$^+$ digested with the same restriction enzyme, creating the γ2S-pcDNA3.1$^+$ construct. The same procedure was used to generate the mutant γ(S2′A)-pcDNA3.1$^+$ plasmid, subcloning the γ(S2′A) 1.4 kB *Bam*HI/*Eco*RI fragment from BP8γ(S2′A) (B.C. Cromer, Ph.D. Thesis, ANU) into pcDNA3.1$^+$. The 2′ serine to valine substitution in the γ2S subunit was generated using the oligonucleotide, 5′-CCA GCC AGA ACA G**TC C**TA GGT ATC ACC AC-3′ and the γ(S2′A)-pcDNA3.1$^+$ as template, following the directions of the U.S.E. Mutagenesis Kit (Amersham Pharmacia Biotech). The mismatched base pairs are indicated in bold. The authenticity of all the plasmids was verified by DNA sequencing. Large quantities (0.5–1.5 mg) of highly purified plasmids were made using the CsCl-density gradient centrifugation procedure (Sambrook et al., 1989).

Cell Culture and DNA Transfection

Mouse L929 fibroblasts (American Type Culture Collection, Rockford, MD) were grown in minimum essential medium (MEM), containing 200 IU/mL penicillin and 200 μg/mL streptomycin, supplemented with 10% heat-inactivated fetal bovine serum (Trace biosciences) and incubated at 37°C in 5% CO_2, 95% air. For transfections, cells were passaged with phosphate-buffered saline containing 0.025% trypsin and plated on to coverslips at a density of 1×10^5 cells in each well, in a 6-well plate. Cells were transfected the next day using a lipid-mediated reagent (Lipofectin, Sigma) with various combinations of $GABA_A$ subunit cDNAs. For identification of successfully transfected cells, the plasmid encoding enhanced-green fluorescent protein (EGFP-NI, Clontech) was used in every transfection. Various combinations of human α1, β1, γ2S, γ(S2′A), γ(S2′V) and EGFP plasmids were combined and added to the cells in ratios of 1:1:1 (α:β:GFP) or 1:1:1:1 (α:β:γ:EGFP) using 5 μg of each cDNA. Lipofectin (20 μL) and the mixture of cDNAs were then incubated in 100 μL of Opti-MEM (GibcoBRL, Life Technologies) in separate polystyrene tubes for 30 min before combining together for 15 min. Opti-MEM 1 mL, was further added to the final mixture, forming the final transfection solution. L929 cells were washed with 2 mL Opti-MEM prior to incubation with the transfection solution for 3–5 h at 37°C. After the incubation the transfection solution was replaced with normal medium. Only cells that had bright green fluorescence (viewed under UV excitation 450–495 nm) were used for electrophysiological recordings between 24–72 h later.

Recording Solutions and Techniques

Standard whole-cell and outside-out patching techniques were used to voltage clamp cells and record currents (Hamill et al., 1981). Prior to recording, cells were washed with external recording medium (bath solution) containing the following in mM: 135 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose and 10 TES (pH 7.4). The intra-pipette solution contained (in mM): 50 NaCl, 80 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 EGTA and 10 TES (pH 7.3). This combination of bath and intra-pipette solution produced a chloride equilibrium potential (E_{Cl}) of approximately −1.8 mV, whereas the equilibrium potentials for sodium and potassium were at +19 and −70 mV, respectively. Patch-pipettes were made using borosilicate glass capillaries (1.5 mm OD × 0.86 mm ID) fabricated on a pipette puller (Sutter Instruments), and subsequently fire-polished and coated with Sylgard (Dow Corning) before use. Patch-pipette resistances ranged from 10–20 MΩ for both whole-cell and single-channel recordings when filled with the internal solution and immersed in bath solution. GABA (Sigma), diazepam (kindly provided by Hoffman-La Roche) and zinc chloride (Sigma) were diluted in bath solution to the final concentration on the day of experiment. The flow rate of the bath solution was approximately 5 mL/min. Drugs were applied via gravity-fed flow tubes and placed close to the cell or directly in front of the patch by lateral movement of the flow tube using a hydraulic micromanipulator. The external diameter of a single flow tube was approximately 300 μm. For whole-cell recordings, drug applications were separated by at least 30 s to allow recovery from desensitization. Data from two or more separate transfections were pooled for analysis. All recordings were carried out at room temperature (20–22°C). Currents were monitored with an Axopatch 200A amplifier filtered at 5 kHz (3 dB, 4-pole Bessel; Axon Instruments, CA) and directly recorded on to the computer (Axoscope7; ADC: digidata board 1200; Axon Instruments) sampling at 10 kHz. Voltage offsets between pipette and ground were zeroed once the pipette was immersed in the bath solution. We did not correct for the small liquid junction potential between the pipette and bath solution. Series resistance compensation was not always performed, because we were looking for qualitative rather than quantitative differences.

Analysis of Currents

All currents were analyzed using software developed in-house, Channel 2 (written by M. Smith & P. Gage). For single-channel analysis the recordings were first filtered at 2 kHz using Channel 2. Segments of single-channel data were used for kinetic analysis only if superimposed openings of 2 independent channels were rare. The baseline (closed state) was determined by visual inspection of the whole file and adjusted to zero and a current-amplitude probability histogram of the data points was constructed, normally with a bin width of 0.1 pA, using the program Channel 2. To determine open probability (P_o) the total open time was divided by the total time (at least 30 s). Single-channel current amplitudes were measured directly and were only accepted as valid events if their open duration was at least 0.3 ms (i.e., 3 times the sampling rate). Amplitude histograms were then constructed in which the bin widths were 0.05 pA and were fitted to the sum of Gaussian components using least-squares minimization. The number of Gaussian components required to fit the histogram was determined by the criteria set out by Horn (1987).

Open times and closed times were measured from the time in which a single-channel opening crossed the set "open" and "closed" threshold. These thresholds were set just above the baseline noise. Bursts of openings were defined as openings or groups of openings separated by relatively long closed periods (t_c). The minimum closed period (t_c) was set at the shortest closed time constant for each experiment. The values of t_c ranged between 1.26 and 2.12 ms for single channels recorded from αβ receptors (mean 1.53 ± 0.14 ms) and between 0.84 and 1.67 ms for αβγ receptors (mean 1.14 ± 0.15 ms). Open, closed and burst time histograms were plotted using logarithmic binning, with the logarithmic time on the x-axis and a square-root ordinate transformation, applied according to Sigworth and Sine, (Sigworth & Sine, 1987). The number of exponential functions required to fit the distribution was increased until additional components did not significantly improve the fit as determined by the criteria set out in Horn (1987).

Results

Incorporation of a Mutant γ Subunit into Functional $GABA_A$ Receptors

The potentiation of the GABA response by diazepam is strictly dependent upon the presence of the γ

subunit in $GABA_A$ receptors (Horne et al., 1993) and zinc inhibition of the response to GABA is effective only on αβ receptors (Draguhn et al., 1990). These properties were used to confirm the composition of the wild-type and mutant receptors expressed in L929 cells.

Diazepam does not affect the response to GABA of wild-type αβ receptors (Fig. 2*A*) but potentiates the GABA response in the wild-type αβγ receptor (Fig. 2*B*). Diazepam invariably increased the whole-cell response to GABA in the mutant αβγ receptors. On average, diazepam (1 μM) increased the current in response to GABA (1 μM) in the αβγ(S2′A) and αβγ(S2′V) mutants by 4.1 ± 0.5 fold (mean ± standard error of the mean, SEM, $n = 7$) and 3.8 ± 0.6 fold ($n = 7$), respectively (Fig. 2*C*, *D*). This potentiation by diazepam was similar to the wild-type αβγ receptor response measured under the same conditions (3.6 ± 0.2 fold, $n = 13$; Fig. 2*B*). Zinc (10 μM) did not alter the magnitude of the GABA-activated currents in these mutants (Fig. 2*C*, *D*), providing further evidence that the receptors were composed of the α, β and γ subunits (Draguhn et al., 1990). These tests also showed that the presence of an alanine or valine residue at the 2′ position of the γ subunit did not prevent its assembly into normal functional αβγ receptors.

L929 cells co-transfected with a plasmid carrying either the γ(S2′A) or the γ(S2′V) mutation together with the wild-type α and β cDNAs displayed whole-cell currents significantly larger than those recorded from cells expressing αβ receptors alone but similar or smaller in amplitude than those expressing wild-type αβγ receptors (Fig. 2). On average, the peak whole-cell current response of the αβγ(S2′V) receptor to GABA was smaller (471 ± 70 pA, $n = 54$, −40 mV) than those recorded from cells that expressed the wild-type αβγ receptor (653 ± 77, $n = 47$, −40 mV, $P < 0.5$) and this effect was observed at both hyperpolarized and depolarized potentials (Fig. 2, Table 1). In contrast, no significant change in the peak amplitude of the whole-cell response to GABA was observed when alanine was substituted for serine at the γ2′ position (684 ± 62 pA, ($n = 70$), −40 mV). The difference in the whole-cell data measurements for the αβ and αβγ combinations is consistent with previous reports (Angelotti & Macdonald, 1993) and the peak current measurements of the mutants serve here only to indicate that the γ2′ mutation has not disrupted receptor assembly.

Single-Channel Conductance Properties

Outside-out membrane patches were pulled from L929 cells after transfection with either αβγ(S2′A) or αβγ(S2′V) subunit cDNAs. Single-channel currents were recorded at two holding potentials, +40 mV and −40 mV, and compared with currents recorded under identical conditions from cells expressing either

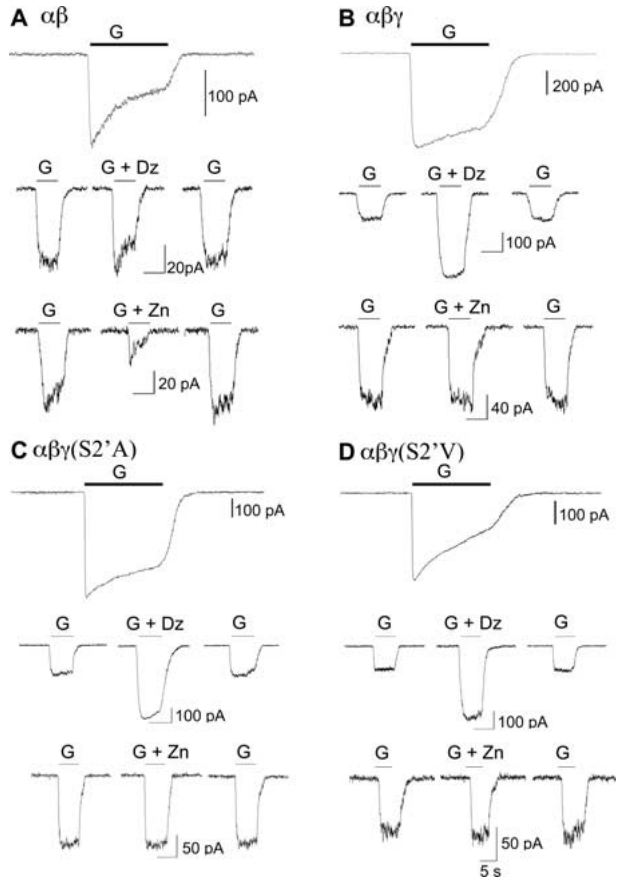

Fig. 2. $GABA_A$ receptor subunit combinations distinguished by their whole-cell current responses to GABA plus diazepam and GABA plus zinc. GABA-activated whole-cell currents from cells expressing αβ, αβγ, αβγ(S2′A) or αβγ(S2′V) receptors are shown at the top of each panel from *A* to *D*, respectively. Subsequent panels show each receptor combination tested with GABA (*G*, 1 μM plus diazepam (*Dz*, 1 μM) and GABA (*G*, 1 μM) plus zinc (10 μM). GABA (1 μM) is applied to the cell before and after each GABA plus drug combination to ensure no run down in the response. All recordings were at −40 mV. The time scale in all panels is 5 seconds.

Table 1. Summary of whole-cell currents activated by 1 mM GABA at ±40 mV

Membrane Potential	$GABA_AR$	*I* (pA)	*n*
−40 mV	αβ	223 ± 60	29
	αβγ	653 ± 77	47
	αβγ(S2′A)	684 ± 62	70
	αβγ(S2′V)	471 ± 70	54
+40 mV	αβ	216 ± 50	4
	αβγ	731 ± 116	6
	αβγ(S2′A)	751 ± 115	10
	αβγ(S2′V)	523 ± 101	8

Mean current ± standard error of the mean from *n* experiments.

the wild-type αβ or αβγ subunit combinations. GABA (1 μM), applied through the bath, activated channels in outside-out patches containing either

wild-type αβ or αβγ receptors. Their conductance and kinetic properties were clearly different. Channels formed by αβγ $GABA_A$ receptors had a higher conductance, longer openings and more prolonged bursts of activity than αβ receptors (Fig. 3*A*, *C*). Average characteristics are listed in Table 2 and are consistent with previous reports of the single-channel properties of recombinant $GABA_A$ αβ and αβγ receptors (Angelotti & Macdonald, 1993). The amplitudes of single-channel openings at a holding potential of +40 mV or −40 mV were measured by constructing all-point amplitude histograms (Fig. 3*B*, *D*). Both wild-type subunit combinations opened to both a main conductance level and at least one subconductance level; at +40 mV these conductance levels were 30 and 22 pS, respectively, for αβγ receptors compared with 15 and 11 pS, respectively, for αβ receptors. Hence, wild-type receptors that do not contain the γ subunit have significantly lower conductances. The conductance values were similar when measured at either +40 or −40 mV for both αβ and αβγ receptor combinations, indicating a lack of rectification (Table 2).

Typical recordings of single-channel activity from $GABA_A$ receptors containing αβ together with either an (S2′A) or (S2′V) mutation in the M2 region of the γ subunit are shown in Fig. 3*E* and *G*, respectively. As in wild-type receptors at a holding potential of +40 mV, the mutant αβγ(S2′A) receptors exhibited channels with both a main and a subconductance level (mean values: main level = 24.7 ± 0.3 pS, subconductance level = 19.2 ± 0.3 pS, n = 21; Table 2), which were approximately 17% lower and significantly different from αβγ wild-type receptors (P < 0.01). Both wild-type combinations and the αβγ(S2′A) mutant channels opened more frequently to their main conductance level (~75%) than their corresponding subconductance level (~25%). When a valine was substituted for the serine residue at the 2′ position in M2 of the γ subunit, the conductance of these receptors was altered more dramatically. In cells expressing $GABA_A$ αβγ(S2′V) receptors only a single conductance state was observed at 21.2 ± 0.4 pS (n = 9; +40 mV). As this conductance level of the mutant is very similar to the subconductance level of wild-type αβγ receptors, it is possible that this reduced conductance represents a shift in the equilibrium between conductance states rather than a reduction in conductance of the main state, i.e., loss of the main conductance state.

Interestingly, significant differences in single-channel conductance were observed between wild-type and both mutant receptors recorded at +40 mV, but not at −40 mV (~20 and ~30 pS). In fact, the amplitudes of single-channel openings measured from wild-type and mutant receptors were indistinguishable at −40 mV (Table 2) and, furthermore, the αβγ(S2′V) receptors opened to both a main level and a subconductance level, showing a slightly higher probability for the subconductance state compared with wild-type αβγ receptors.

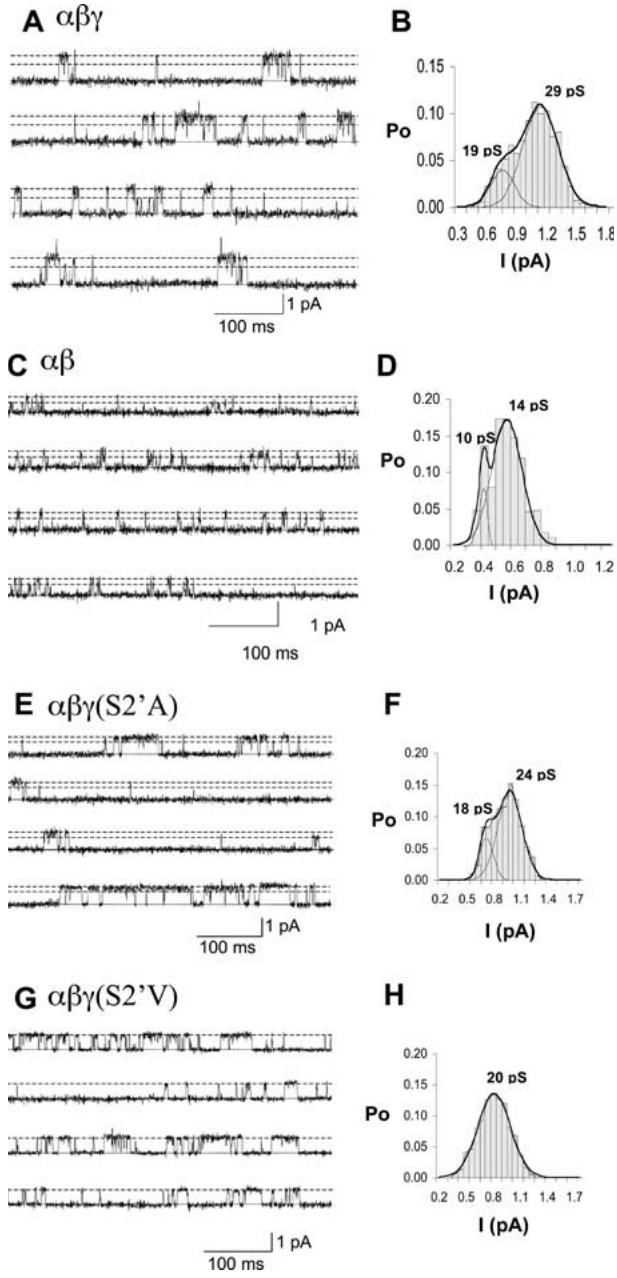

Fig. 3. A comparison of single-channel conductance states from wild-type αβ and αβγ receptors with those from mutant αβγ(S2′A) and αβγ(S2′V) receptors. Typical outside-out traces recorded at +40 mV of single-channel openings activated by 1 μM GABA of αβγ, αβ, αβγ(S2′A) and αβγ(S2′V) receptors are depicted in panels *A*, *C*, *E* and *G*, respectively. Single-channel amplitudes were measured at this potential and an amplitude histogram was constructed for each receptor type. The histograms in panels *B*, *D*, *F* and *H* correspond to experiments recording αβγ, αβ, αβγ(S2′A) and αβγ(S2′V) receptors, respectively. Single channels from αβγ, αβ and αβγ(S2′A) receptors opened to 2 conductance states. This is reflected in the 2 peaks in the corresponding histograms. Only 1 conductance state could be clearly resolved for αβγ(S2′V) receptors.

Table 2. Single-channel properties of GABA-activated channels from wild-type αβ and αβγ receptors compared with mutant αβγ(S2′A) and αβγ(S2′V) receptors at both +40 and −40 mV

Potential	$GABA_AR$	Conductance (pS)		Mean Open Probability (P_o)	Mean open time (ms)	Mean burst time (ms)	*n*
		Main	Sub				
+40 mV	αβ	14.8 ± 0.4* (76 ± 6)	11.4 ± 0.4* (24 ± 6)	0.08 ± 0.01	0.89 ± 0.1*	2.23 ± 0.15	10
	αβγ	29.7 ± 0.8 (73 ± 3)	21.9 ± 0.5 (27 ± 3)	0.08 ± 0.02	1.61 ± 0.14	3.50 ± 0.21	14
	αβγ(S2′A)	24.7 ± 0.3* (77 ± 3)	19.2 ± 0.3* (23 ± 3)	0.05 ± 0.01	2.11 ± 0.13*	4.78 ± 0.32*	21
	αβγ(S2′V)	21.2 ± 0.4* (100)		0.03 ± 0.01*	2.24 ± 0.17*	4.74 ± 0.42*	9
−40 mV	αβ	15.5 ± 0.3* (80 ± 5)	11.6 ± 0.2* (20 ± 5)	0.04 ± 0.01	0.83 ± 0.08*	2.32 ± 0.25*	8
	αβγ	29.6 ± 0.5 (75 ± 5)	20.6 ± 0.4 (25 ± 5)	0.06 ± 0.01	1.57 ± 0.09	3.20 ± 0.27	13
	αβγ(S2′A)	29.7 ± 0.6 (74 ± 3)	21.9 ± 0.5 (26 ± 3)	0.03 ± 0.01*	2.32 ± 0.21*	5.50 ± 0.75*	21
	αβγ(S2′V)	28.2 ± 0.9 (63 ± 3)	19.8 ± 0.6 (36 ± 3)	0.02 ± 0.01*	2.14 ± 0.16*	4.37 ± 0.31*	9

Mean ± SEM from *n* experiments. Relative frequency (%) of each conductance is given in parentheses; * denotes a significant difference ($P < 0.05$) between equivalent values of wild-type αβγ receptors and αβ, αβγ (S2′A) or αβγ (S2′V)

To examine the voltage dependence of conductance further, we recorded complete current-voltage relationships. The chord conductance (the conductance as measured from the slope of the line) of the main conductance level for wild-type and mutant receptors was derived from single-channel current-voltage relationships. Both wild-type αβγ and αβ channels had current amplitudes that varied linearly with potential and reversed at approximately the chloride equilibrium ($E_{Cl} = -1.8$ mV) (Fig. 4*A–D*). In the experiment shown, the calculated chord conductances were 28.8 pS ($n = 3$–13) and 14.4 pS ($n = 3$–13) for the αβγ and αβ receptors, respectively. By contrast, co-expression of wild-type α and β subunits together with either the γ(S2′A) or γ(S′2V) mutant subunit gave rectifying channels. Figure 4 shows a typical experiment in which GABA-activated single channels were recorded at potentials ranging from +80 to −80 mV (Fig. 4*E–H*). The current-voltage relationship of receptors carrying a mutation at the 2′ position in γM2 deviates from linearity and now shows inward rectification.

Single-Channel Kinetics

To help interpret the conductance data of the mutant channels the open- and closed-time behavior of their single channels was analyzed at the same membrane potentials, ±40 mV. The mean open probability of αβ and αβγ receptors was similar at +40 mV ($P_o = 0.08$) but both wild-type subunit combinations opened less frequently at −40 mV (αβγ $P_o = 0.06$ and αβ 0.04) (Table 2 and also Fig. 4). Removing the polar group at the 2′ position in M2 of the γ subunit greatly reduced the open probability of both the mutant receptors. Compared to wild-type αβγ receptors, the mean open probability of each of the mutant receptors was significantly reduced at both membrane potentials, with the valine substitution causing a slightly greater reduction than the alanine substitution (e.g., $P_o = 0.02 \pm 0.01$ αβγ(S2′V) versus 0.03 ± 0.01 αβγ(S2′A) compared to 0.06 αβγ, −40 mV) (Table 2). This reduction in open probability of the mutant receptors was unlikely to be due to changes in EC50 (concentration of GABA required to elicit a half maximal response) since the mutations did not appear to alter GABA efficacy. In our expression system, wild-type αβγ receptors have an EC50 of 22 ± 2.1 μM ($n = 6$) (*data not shown*). Under identical recording conditions the αβγ(S2′A) mutant had a similar EC50 value, 25 ± 3 μM ($n = 3$) (*data not shown*) and although a complete EC50 curve was not determined for the S2′V mutant, we reason that its EC50 value is similar, as there did not appear to be a great difference in the macroscopic behavior of the two mutant receptors in response to either GABA or potentiation by diazepam (Fig. 2*C* and *D*).

To investigate the basis for the changes in P_o observed in the mutant receptors, the open- and closed-time properties of the mutant channels were compared with those of wild-type αβ and αβγ receptors. In view of the voltage dependency observed in the single-channel conductance measurements from mutant αβγ(S2′A) and αβγ(S2′V) receptors (Table 2), kinetic properties were analyzed at both +40 mV and −40 mV.

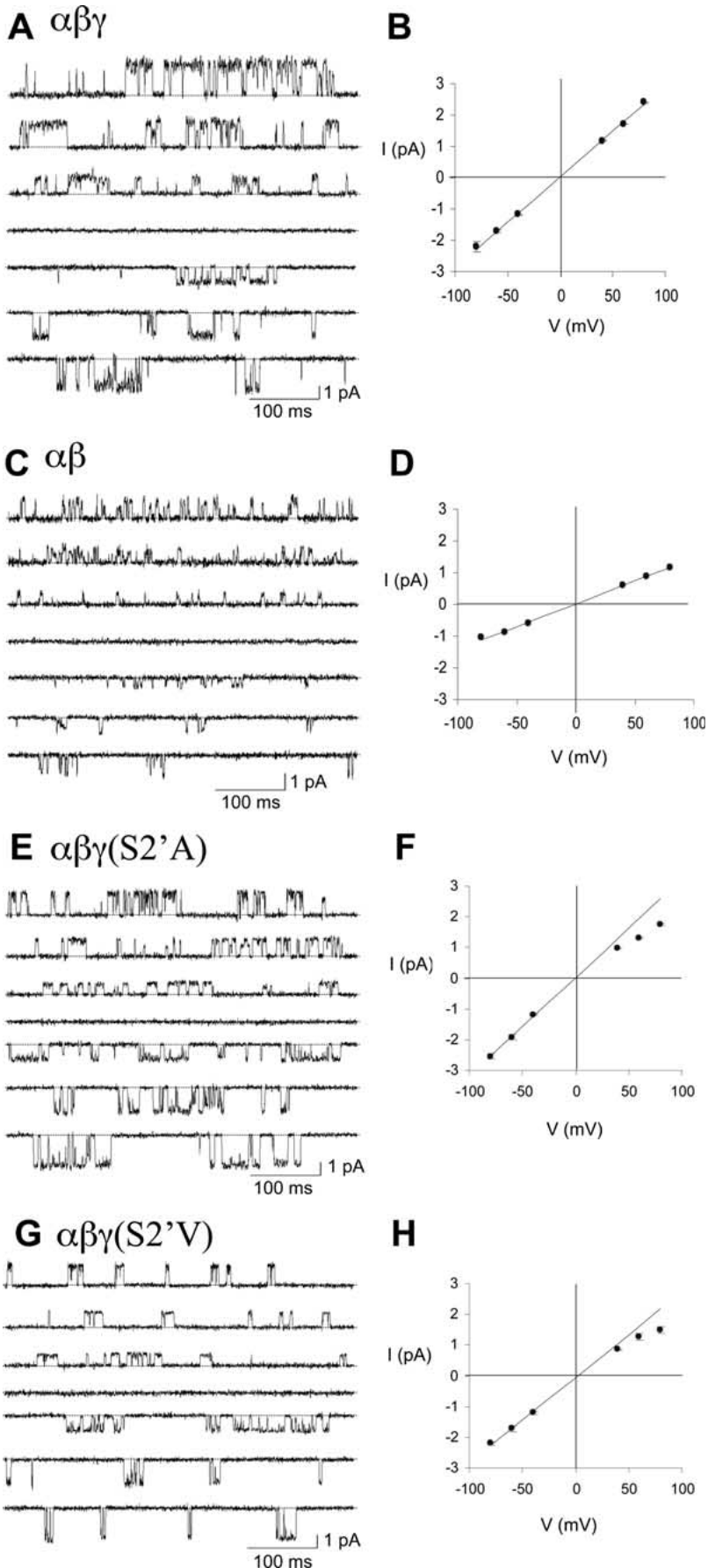

Fig. 4. Single-channel current-voltage (I/V) relationship of wild-type αβγ and αβ receptors compared with mutant αβγ(S2′A) and αβγ(S2′V) receptors. In panels *A*, *C*, *E* and *G* single-channel traces were recorded at different membrane potentials (traces from top to bottom were; +80, to −80 mV in −20 mV steps) from αβγ, αβ, αβγ(S2′A) and αβγ(S2′V) receptors, respectively. The single-channel current amplitude of the main conductance at each membrane potential was then plotted against the voltage. These are shown in panels *B*, *D*, *F*, and *H* for αβγ, αβ, αβγ(S2′A) and αβγ(S2′V) receptors, respectively. The I/V plot for both wild-type receptor combinations was fitted best with a straight line. In contrast, the single-channel amplitudes recorded at positive membrane potentials from both mutant receptors deviated away from the linear relationship and show inward rectification.

Table 3. Time constants of GABA-activated single channels composed of wild-type αβ and αβγ receptors compared with mutant αβγ(S2′A) and αβγ(S2′V) receptors at +40 mV.

	Open times				Closed times			
	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)
τ_1	0.49 ± 0.06*	0.23 ± 0.03	0.66 ± 0.09*	0.39 ± 0.05*	1.53 ± 0.14	1.14 ± 0.15	1.04 ± 0.08	1.19 ± 0.10
	(73 ± 4*)	(50 ± 4)	(33 ± 2*)	(44 ± 7)	(50 ± 4)	(44 ± 5)	(34 ± 5)	(41 ± 9)
τ_2	1.63 ± 0.22	1.21 ± 0.15	2.40 ± 0.18*	1.64 ± 0.09*	15.2 ± 1.84	4.45 ± 0.79	8.33 ± 1.19*	11.6 ± 1.39*
	(27 ± 4*)	(34 ± 6)	(50 ± 4)	(43 ± 6)	(36 ± 3)	(25 ± 6)	(32 ± 3)	(19 ± 4)
τ_3		3.33 ± 0.12	5.24 ± 0.25*	4.66 ± 0.14*	123 ± 16.5	32.6 ± 2.4	46.3 ± 8.0	41.8 ± 8.0
		(16 ± 3)	(17 ± 4)	(13 ± 4)	(14 ± 2)	(16 ± 4)	(23 ± 4)	(23 ± 8)
τ_4						153 ± 12	378 ± 21*	217 ± 31
						(15 ± 6)	(11 ± 1)	(18 ± 4)
n	10	5	11	5				

	Burst duration			
	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)
τ_1	0.40 ± 0.06	0.29 ± 0.06	0.57 ± 0.09*	0.59 ± 0.10*
	(55 ± 5)	(46 ± 6)	(19 ± 3*)	(43 ± 6)
τ_2	3.69 ± 0.32	2.17 ± 0.39	4.16 ± 0.35*	3.87 ± 0.55*
	(45 ± 5)	(26 ± 4)	(47 ± 7*)	(37 ± 4)
τ_3		7.88 ± 0.59	10.0 ± 0.60*	11.5 ± 0.46*
		(28 ± 5)	(34 ± 8)	(19 ± 5)

Mean ± standard error of the mean from *n* experiments. Relative frequency (%) of each conductance is given in parentheses; * denotes a significant difference ($P < 0.05$) between equivalent values of wild-type αβγ receptors and αβ, αβγ(S2′A) or αβγ(S2′V)

The gating properties of wild-type $GABA_A$ αβ and αβγ receptor combinations are obviously different (Figs. 3 and 4). The presence of the γ subunit results in about a 40% increase in the mean open time (e.g., 0.89 ± 0.07 ms ($n = 10$) for αβ, 1.61 ± 0.07 ($n = 14$) for αβγ, +40 mV) and about a 40% increase in mean burst times (2.23 ± 0.15 versus 3.5 ± 0.21 at +40 mV) (Table 2). The values at −40 mV were similar to those given for +40 mV (Table 2). The substitution of a serine for alanine residue at the 2′ position in γM2 significantly increased both the mean open times (2.11 ± 0.13 ms at +40 mV, 2.32 ± 0.21 ms at −40 mV) and mean burst times (4.78 ± 0.32 at +40 mV, 5.5 ± 0.75 ms at −40 mV) compared with the αβγ receptor ($P < 0.05$). The result of introducing a valine residue had similar consequences with mean open and burst times also increasing (Table 2). This trend towards longer open times and increased burst lengths in the mutant receptors was observed at both +40 and −40 mV.

These 'mean' measurements were analyzed further in terms of channel open- and closed-time frequency distributions. The open-duration frequency distributions of both the αβγ(S2′A) and αβγ(S2′V) receptors were best fitted with the sum of three exponentials, as were wild-type αβγ receptors, suggesting the presence of at least three open states. By contrast, αβ receptors produced open distributions best fitted with the sum of two exponential functions. Hence an additional longer open-time constant is conferred by the presence of the γ subunit (Table 3).

Substitution to remove the polar moiety at the 2′ position in M2 of the γ subunit resulted in each of the open-time constants becoming significantly longer ($P < 0.05$) with some open-time constants increasing as much as two-fold compared to wild-type αβγ receptors (e.g., τ2: αβγ 1.21 ± 0.15 ms, αβγ(S2′A) 2.4 ± 0.18 ms, αβγ(S2′V) 1.64 ± 0.09 ms, +40 mV). This increase in the open duration of the mutant channels was observed at both +40 mV and −40 mV. Overall, the relative probability of each open-time constant did not vary greatly between the wild-type αβγ and mutant receptors (Tables 3 and 4).

In general, the closed time constants of receptors containing either the γ(S2′A) or γ(S2′V) mutation tended towards longer durations compared to wild-type αβγ receptors. The closed-time distributions of both mutant and wild-type receptors were best fitted with four time constants, suggesting the presence of at least four closed states. Primarily, the longest (τ4) and intermediate (τ2) closed-time constants of the mutants doubled in length (Table 4). This trend was observed at both +40 mV and −40 mV. In most cases, the probability distributions of closed-time constants were not significantly altered in the mutant receptors compared to the wild-type αβγ receptor. The exception being the probability distributions of the longer closed-time constants displayed by the αβγ(S2′V) mutant at −40 mV, which were higher than the equivalent wild-type αβγ values.

The burst time displayed by both the γ(S2′A)- and γ(S2′V)-containing mutant receptors was significantly

Table 4. Time constants of GABA-activated single channels composed of wild-type αβ and αβγ receptors compared with mutant αβγ(S2′A) and αβγ(S2′V) receptors at −40 mV.

	Open times				Closed times			
	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)
τ_1	0.43 ± 0.06*	0.28 ± 0.05	0.70 ± 0.15*	0.51 ± 0.07*	1.84 ± 0.20	1.20 ± 0.20	1.03 ± 0.12	1.18 ± 0.16
	(80 ± 5*)	(43 ± 4)	(35 ± 4)	(49 ± 5)	(51 ± 4)	(38 ± 3)	(35 ± 5)	(24 ± 5*)
τ_2	2.12 ± 0.41	1.39 ± 0.21	2.55 ± 0.27*	2.20 ± 0.15*	15.0 ± 1.95	8.87 ± 1.59	12.4 ± 1.26	15.0 ± 1.55*
	(20 ± 5*)	(42 ± 5)	(49 ± 4)	(43 ± 6)	(38 ± 3)	(31 ± 6)	(28 ± 3)	(16 ± 3*)
τ_3		3.88 ± 0.28	5.64 ± 0.40*	6.00 ± 0.74*	128 ± 15.8	76.0 ± 10.5	67.0 ± 14.1	80.8 ± 8.4
		(15 ± 4)	(16 ± 3)	(8 ± 2)	(11 ± 2)	(15 ± 4)	(19 ± 3)	(30 ± 5*)
τ_4						306 ± 36	429 ± 28*	434 ± 41*
						(15 ± 3)	(17 ± 1)	(31 ± 5*)
n	6	7	11	6				

	Burst duration			
	αβ	αβγ	αβγ(S2′A)	αβγ(S2′V)
τ_1	0.46 ± 0.12	0.24 ± 0.01	0.64 ± 0.14*	0.42 ± 0.04*
	(57 ± 8)	(37 ± 6)	(24 ± 8)	(32 ± 7)
τ_2	4.14 ± 0.34	1.84 ± 0.48	3.56 ± 0.19*	4.01 ± 0.47*
	(43 ± 8)	(32 ± 4)	(51 ± 8)	(44 ± 7)
τ_3		6.72 ± 0.09	9.23 ± 0.9*	8.55 ± 0.57*
		(31 ± 7)	(25 ± 9)	(24 ± 9

Mean ± standard error of the mean from *n* experiments. Relative frequency (%) of each conductance is given in parentheses; * denotes a significant difference ($P < 0.05$) between equivalent values of wild-type αβγ receptors and αβ, αβγ(S2′A) or αβγ(S2′V).

longer than in wild-type αβγ receptors, to a degree reflecting the increased open times of the mutant channels (Tables 3 and 4). For example, at −40 mV, αβγ receptors had burst times of 1.84 and 6.72 ms compared to 3.56 and 9.23 ms for the αβγ(S2′A) receptor and 4.01 and 8.55 ms for the αβγ(S2′V) receptor. This trend of longer burst times was also seen at +40 mV. Overall, the burst-time distributions of the mutant channels were not too dissimilar from wild-type αβγ receptors (Tables 3 and 4).

Discussion

The data presented in this study show that the polar 2′ residue in M2 of the γ subunit contributes to the conductance of the $GABA_A$ αβγ receptor. To examine whether the polar 2′ residue in M2 of the γ subunit contributes to the conductance properties of the $GABA_A$ αβγ receptor, a comparison of the macroscopic current and single-channel properties of wild-type and γ2′ mutant receptors was undertaken. Diazepam-sensitivity and zinc-insensitivity of whole-cell currents demonstrated that the mutant γ subunits were incorporated into functional αβγ receptors. Furthermore, the similarity between wild-type and mutant receptor responses to drugs and the agonist GABA suggested that no gross structural perturbation had arisen from mutation at the 2′ position in M2 of the γ subunit.

The single-channel data were much more informative for investigating the influence of the γ subunit and it's 2′ residue. The single-channel conductance was reduced at positive potentials by the mutations at the 2′ position in M2, indicating that the polar nature of the γS2′ residue does assist ion flow and contributes to the increased conductance of $GABA_A$ receptors when the γ subunit is present. In wild-type receptors the absence of the γ subunit in αβ receptors reduces the single-channel conductance by 50% with respect to αβγ receptors. The decrease in single-channel conductance for the αβγ(S2′A) and the αβγ(S2′V) mutant receptors was 18% and 29%, respectively, with respect to wild-type αβγ receptors (Fig. 3, Table 2). Hence a single point mutation in only one of the five subunits forming a receptor reduced the conductance of the channel disproportionately, by almost 30%. Other features of the γ subunit other than the M2 2′ residue, however, must also contribute to the increased conductance of wild-type αβγ receptors. Interestingly, however, this reduction in the rate of ion flow through the mutant channels was seen only for inward currents (outside-out patch, +40 mV, $E_{Cl} = -1.8$ mV) (Fig. 4, Table 2). These data are complex by virtue of the presence of a main and subconductance state. For the γ(S2′A) mutation, both of these conductance levels decreased by a similar amount and the relative frequency of the two states was unaffected by the mutation, providing convincing evidence that the mutation reduces conductance through each state without affecting the states themselves. For the γ(S2′V) mutation, however, only one conductance state was apparent and we have interpreted this as

being the main state, with a conductance decreased by 29%. In support of this interpretation, the open-time kinetics of the mutant receptor are similar to the main conductance state of the wild-type αβγ receptor, with the mutant channel displaying much longer openings than the brief spikes of the subconductance state (Figs. 3 and 4). However, the conductance of the solitary state of the αβγ(S2′V) mutant is similar to the wild-type αβγ subconductance state and, although unlikely, we cannot rule out the possibility that the effect of the mutation is both to shift the equilibrium 100% to the subconductance state and, concomitantly, increase the open time of this state.

The removal of the solitary polar moiety at the 2′ position in the γ subunit of αβγ receptors not only decreased the conductance of the channel but it also altered the gating of the receptor. The changes were not subtle. For example, the open probability (P_o) of the channel was reduced by around 50% but when the channel did open it stayed open longer and continued to flicker quickly between open and closed states (i.e., burst) for longer time periods. Open probability (P_o) is a measure of the frequency of channel openings, the open times of the channel and the number (N) of receptors in the membrane patch. Mutations that affect receptor targeting to the plasma membrane would affect N. As the peak whole-cell currents were not grossly different in the wild-type and mutant channels, nor was the fold potentiation by diazepam using approximately an EC20 concentration for GABA, we infer that receptor density in the plasma membrane was not greatly affected. Therefore, the major cause of the reduction in open probability most likely reflects a decrease in the frequency of receptor openings in the 2′ mutant channels. We interpret this to suggest that the removal of the polar residue at the 2′ position made opening the channel more difficult.

For $GABA_A$ receptors lacking the γ2′ polar group, inward currents through the channel were selectively reduced. The change in single-channel properties may be interpreted in a straightforward fashion as an increased energy barrier for ion flux within the pore and near its intracellular end (*see* Fig. 1). The rectification implies that the role of the γS2′ residue in conduction is either voltage-dependent or dependent on the direction of ion flow. Measurements of single-channel conductance under a chloride gradient rather than an applied electric field could be used to distinguish between these possibilities. Directional dependence of ion flow may be explained in terms of rate theory and in a way that fits with our current view of the pore lining. For example, consider if the ring of polar 6′ residues in the $GABA_A$ receptor is a Cl^- ion-binding site with lower energy than the intracellular bulk solution and the largely non-polar 2′ position represents a barrier to ion permeation between the 6′ position and the intracellular solution. In this scenario, because the 2′ barrier is effectively higher for an inward moving than for an outward moving Cl^- ion, increasing the barrier by removing the polar side-chain at the γS2′ will cause a greater reduction of inward ion flow than outward ion flow. Alternatively, based on the concepts of Dang and McCleskey (1998), the 2′ position of the γS2′ could be viewed as a weak ion-binding site that provides a small well or step between a strong ion-binding site at 6′ and a more intracellular barrier. Removal of this step with either γ(S2′A) or γ(S2′V) substitutions would reduce inward Cl^- ion flow but not affect outward flow. Both of these models could explain in theory the available data but there are many other possible explanations that cannot be ruled out.

Mutation of polar residues at the 2′ position in M2 of the homologous Na^+ channel of the nAChR also resulted in a decrease in channel conductance (Imoto et al., 1988, 1991; Villarroel et al., 1991; Cohen et al., 1992a,b; Villarroel et al., 1992; Villarroel & Sakmann, 1992; Wilson & Karlin, 1998). Conductance was seen to decrease both with reduced polarity and increased hydrophobic bulk of the side chain at the 2′ position (Imoto et al., 1991). Similarly, we observe decreased conductance in the $GABA_A$ receptor mutants as the hydrophobic bulk of the side chain increases. Unlike the Na^+ channel of the nAChR (Charnet et al., 1990; Cohen et al., 1992b), however, we found that removal of all polar residues at the 2′ position in the $GABA_A$ receptor does not abolish activity. Together, these results suggest that hydroxylated residue(s) at the corresponding position of $GABA_A$ and nACh receptors are able to act as surrogate water molecules in the hydration shell of either a Cl^- ion or a Na^+ ion but the densely charged Na^+ ion's greater requirement for solvating hydroxyl groups requires a higher number of participating side chains in the nAChR.

In the study described here, the whole-cell recordings conceal the effects of the mutations and only by analyzing the single-channel properties of the mutant receptors are the details revealed. The reduced conductance of channels at positive potentials, decreased open probability and longer closed times are counterbalanced by the increase in the mean open and burst times, which together may account for the similar-sized or slightly reduced whole-cell currents of the αβγ(S2′A) and αβγ(S2′V) receptors, respectively, compared with those of the wild-type αβγ receptor. There was no significant difference in the kinetic properties of the two mutant receptors although the values of the S2′V mutant tended to be more exaggerated. The only kinetic data published on nAChR M2 2′ mutants has been mean open times, which were seen to increase with the removal of polar residue(s) (Leonard et al., 1988; Charnet et al., 1990), in accord with our results in the mutant 2′ $GABA_A$ receptors. The detailed single-channel kinetic analyses performed here provide a further insight into

channel transitions, revealing that the removal of the single polar moiety at the γ2′ position of αβγ $GABA_A$ receptors may help to keep the channel open but it actually impedes channel transitions into the open state, as evidenced by the mutant channels' reduced open probability and increased closed times.

The results presented here bring to light a number of interesting observations. Firstly they highlight the potentially complex consequences of a single point mutation in the pore region of the $GABA_A$ receptor. Indeed, the single polar residue at the 2′ position in the γ subunit of $GABA_A$ αβγ receptors finely balances the conductance and kinetic behavior of the channel. Our results suggest that in the wild-type αβγ $GABA_A$ receptor, at the depolarized potentials (relative to the resting membrane potential) where this receptor is normally active, this single serine residue helps to impart the highest conductance while keeping the channel in the open state for the shortest time. There now exists a significant pool of data showing that conductance through a single ligand-gated ion channel is influenced by a number of distinct regions on the receptor. In addition to our point mutation in the $GABA_A$ γM2 region, we have previously described effects of the $GABA_A$ receptor clustering protein GABARAP on increasing channel conductance in recombinant αβγ $GABA_A$ receptors (Everitt et al., 2004) and Kelley et al. have described the inhibitory effects of arginine residues, located in the M3-M4 cytoplasmic loop region of the $5HT_3$ α subunit, on single-channel conductance (Kelley et al., 2003). Together these studies reveal a communication between spatially distinct regions of the polypeptide chain in co-ordinating and controlling ion flow through the channel.

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