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Molecular determinants of desensitization and assembly of the chimeric $GABA_A$ receptor subunits ($\alpha1/\gamma2$) and ($\gamma2/\alpha1$) in combinations with $\beta2$ and $\gamma2$

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Abstract

Two γ-aminobutyric $acid_A$ ($GABA_A$) receptor chimeras were designed in order to elucidate the structural requirements for $GABA_A$ receptor desensitization and assembly. The ($\alpha1/\gamma2$) and ($\gamma2/\alpha1$) chimeric subunits representing the extracellular N-terminal domain of α1 or γ2 and the remainder of the γ2 or α1 subunits, respectively, were expressed with β2 and β2γ2 in *Spodoptera frugiperda* (Sf-9) cells using the baculovirus expression system. The ($\alpha1/\gamma2$)β2 and ($\alpha1/\gamma2$)β2γ2 but not the ($\gamma2/\alpha1$)β2 and ($\gamma2/\alpha1$)β2γ2 subunit combinations formed functional receptor complexes as shown by whole-cell patch–clamp recordings and [^{3}H]muscimol and [^{3}H]flunitrazepam binding. Moreover, the surface immunofluorescence staining of Sf-9 cells expressing the ($\alpha1/\gamma2$)-containing receptors was pronounced, as opposed to the staining of the ($\gamma2/\alpha1$)-containing receptors, which was only slightly higher than background. To explain this, the ($\alpha1/\gamma2$) and ($\gamma2/\alpha1$) chimeras may act like α1 and γ2 subunits, respectively, indicating that the extracellular N-terminal segment is important for assembly. However, the ($\alpha1/\gamma2$) chimeric subunit had characteristics different from the α1 subunit, since the ($\alpha1/\gamma2$) chimera gave rise to no desensitization after GABA stimulation in whole-cell patch–clamp recordings, which was independent of whether the chimera was expressed in combination with β2 or β2γ2. Surprisingly, the ($\alpha1/\gamma2$)($\gamma2/\alpha1$)β2 subunit combination did desensitize, indicating that the C-terminal segment of the α1 subunit may be important for desensitization. Moreover, desensitization was observed for the ($\alpha1/\gamma2$)β2γ2 receptor with respect to the direct activation by pentobarbital. This suggests differences in the mechanism of channel activation for pentobarbital and GABA.

Keywords: Chimeric $GABA_A$ receptor; Desensitization; N-terminal domain; SF-9 cells

The γ-aminobutyric acid (GABA)-activated chloride channel, a member of the ligand-gated ion channel family, is an oligomeric receptor complex composed of five subunits. The topology of each subunit is a large extracellular N-terminal domain with tentative glycosylation sites, four transmembrane segments (M1–M4), a relatively large intracellular loop between M3 and M4 containing possible phosphorylation sites and a small extracellular C-terminus (Smith and Olsen, 1995). Molecular cloning studies have revealed 19 different mammalian $GABA_A$ receptor subunits, which are divided into eight distinct classes, some containing several isoforms (α1–6, β1–3, γ1–3, δ, ε, π, θ, ρ1–3, Macdonald and Olsen, 1994; Stephenson, 1995; Ogurusu and Shingai, 1996; Davies et al., 1997; Hedblom and Kirkness, 1997; Bonnert et al., 1999). The most abundant $GABA_A$ receptor subunits are α1, β2, and γ2 and it is assumed that these three subunits form part of the most common $GABA_A$ receptors (Laurie et al., 1992a,b; Wisden et al., 1992; Fritschy and Möhler, 1995). Moreover, it is believed from studies on αβγ recombinant receptors that only a single species of receptor is assembled, even though αβ receptor complexes readily are formed in the absence of the γ subunits (Angelotti and

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Macdonald, 1993; Hartnett et al., 1996). $GABA_A$ receptors composed of α1, β2 or β3, and γ2 subunits are assumed to be assembled into $(\alpha1)_2(\beta2/3)_2\gamma2$ receptors (Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999). Moreover, Im et al. (1995a) have proposed the stoichiometry of the α6β2γ2 $GABA_A$ receptor subtype to be α6-β2-α6-β2-γ2 by using an α6-β2 tandem.

The extracellular N-terminal domain has been shown to be involved in agonist/antagonist binding as well as in forming sites for modulators such as benzodiazepines (Smith and Olsen, 1995). This region has also been shown to be important for channel assembly. The assembly of the $GABA_A$ receptor is thought to take place in the endoplasmic reticulum (ER) followed by transport of the receptor pentamer to the cytoplasmic cell membrane (Hurtley and Helenius, 1989). This assembly process involves highly specific folding of the subunits and the formation of recognition sites for other subunits to guide the stepwise assembly of the pentameric receptor complex. The best characterized ion channels, in terms of structure, are the muscle-type nicotinic acetylcholine receptors (nAChR), which provide detailed information about specific amino acid segments in the extracellular N-terminal domain involved in the assembly process (Kreienkamp et al., 1995). These residues are in the region preceding the conserved disulfide loop. This also applies for the glycine receptor (Kuhse et al., 1993). Additionally, it has been proposed that post-translational modifications such as disulfide bond formation, N-linked glycosylation, phosphorylation and fatty acid acylation may be important for folding that allows the association of unassembled subunits (Merlie and Smith, 1986; Green and Wanamaker, 1997). For the $GABA_A$ receptor it has been shown that two invariant tryptophans (W69 and W94) in the extracellular N-terminal domain of the α1 subunit are critical for assembly of pentamers (Srinivasan et al., 1999). Moreover, Taylor et al. (2000) revealed that residues 58–67 of the α1 subunit mediated oligomerization of the α1 and β3 subunits. Furthermore, Klausberger et al. (2000) demonstrated that amino acid sequences γ2-(91–104) and γ2-(83–90) formed the sites mediating assembly with α1 and β3 subunits, respectively. Likewise, site-directed mutagenesis of N-linked glycosylation sites has demonstrated profound effects on the processing of the α1 $GABA_A$ receptor subunit (Buller et al., 1994), and it has been shown that an intact cysteine loop is necessary for complete assembly (Amin et al., 1993).

With respect to desensitization, several reports have shown the M2 domain to be important (Im et al., 1995b; Birnir et al., 1997a,b; Tierney et al., 1998). All tested $GABA_A$ receptors have the ability to desensitize to some extent. However, the ρ-containing receptors as well as subunit combinations including the δ subunit exhibit very low desensitization (Saxena and Macdonald, 1994; Chang and Weiss, 1999). The effect of desensitization is to decrease the single-channel open probability and has the important characteristic of decreasing the effect of the transmitter (Frosch et al., 1992). However, after a brief agonist exposure, as at a GABAergic synapse, when channels leave the desensitized state, they may reopen. In this way desensitization can prolong a synaptic response (Jones and Westbrook, 1996).

The present study was undertaken to investigate the importance of the extracellular α1-N-terminal domain for $GABA_A$ receptor desensitization and assembly. The (α1/γ2) and (γ2/α1) chimeric subunits representing the extracellular N-terminal region of α1 or γ2 and the remainder of the γ2 or α1 subunits, respectively, were expressed with β2 and β2γ2. It was shown that the extracellular α1-N-terminal domain is important for assembly of the α1β2 and α1β2γ2 receptor complexes. Moreover, a pharmacological characterization of the (α1/γ2)-containing receptors showed an overall resemblance to similar α1-containing receptors. However, one striking difference was observed, since the (α1/γ2) chimera in combination with β2 and γ2 subunits did not give rise to desensitization in whole-cell patch–clamp recordings.

1. Experimental procedures

1.1. Insect cell culture and infection

Sf-9 cells were maintained in serum-free medium (supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin) as shaking cultures (140 rpm) at 27°C. At the time of infection the concentration of Sf-9 cells was 2×10^6 cells per ml and a multiplicity of infection (MOI) of five was used for each type of baculovirus added unless otherwise specified. Virus titer was determined with a plaque assay following the protocol from GibcoBRL™ Instruction Manual 'Guide to baculovirus expression vector systems (BEVS) and insect cell culture techniques'.

1.2. Cloning of chimeric receptor subunits

Rat α1 and γ2 cDNAs were cloned into the pBluescript SK vector at the EcoRI restriction site and into the pSP72 vector at the HindIII (5′) and XbaI (3′) restriction sites, respectively. The γ2 cDNA was further subcloned into the pBluescript KS vector at the XhoI (5′) and EcoRI (3′) sites. Polymerase chain reaction (PCR) based mutagenesis was performed to introduce a unique NsiI site in γ2, which was in the vicinity of the 11th amino acid in the beginning of the first transmembrane domain. At the amino acid level this resulted in an exchange of a threonine to an isoleucine, however for the purpose of creating chimeras of α1 and γ2

subunits this change is trivial, since α1 already contains an isoleucine at that position. Furthermore, the α1 subunit already contains a unique site for NsiI in the homologous position. The following mutagenic oligonucleotides were designed for the PCR reaction 5′-TACATTCCATGCATACTCAT-3′ and 5′-CAATGAGTATGCATGGAATGT-3′ and used in conjunction with the T7 and SP6 primers of pSP72, respectively. A DNA fragment of 217 bp containing the newly introduced NsiI cutting site was obtained from the γ2 PCR product using BbsI and StyI digestion. This cassette was then subcloned back into wild type γ2 in pBluescript vector and subsequently sequenced (Sequenase 2.0 kit, Amersham, Denmark). Chimeric subunits ((α1/γ2) and (γ2/α1)) representing the extracellular N-terminal of α1 or γ2 and the remainder of the subunit γ2 or α1, respectively, were generated by digesting α1 pBluescript with NsiI, SmaI (5′) and EcoRI (3′) and by digesting γ2 pBluescript with NsiI, PvuII (5′) and XbaI (3′) and isolating and religating of the desired fragments into pVL1393 vectors (BaculoGold™ Transfection Kit) cut with XbaI together with SmaI and EcoRI together with PvuII. Chimeras were confirmed by restriction enzyme mapping. Incorporation of the chimera cDNA into the Baculovirus genome was performed with the BaculoGold™ transfection kit from PharMingen (San Diego, CA).

1.3. Sucrose density gradient centrifugation

All tissue preparations were performed as described before (Elster et al., 2000). Concentrated solubilized protein solution was layered (200 μl) on top of a 3–30% sucrose gradient made of 1 × solubilization buffer containing 0.1% (v/v) Triton X-100. Sedimentation coefficients (*S*) were determined by adding 200 μl of cytochrome *C* (1 mg, 1.9*S*), bovine serum albumin (2 mg, 4.3*S*), alcohol dehydrogenase (2 mg, 7.4*S*) and catalase (4 mg, 11.4*S*) to different gradients followed by detection of these standards in the gradients with Pierce Micro BCA™ protein assay. The total volume of the gradients was 11.2 ml. Gradients were centrifuged in a Beckman SW40 rotor for 26 h, 4°C, at 34 500 rpm with acceleration and deceleration programming. Fractions of 225 μl were collected from the top downwards.

1.4. Filtration binding assays

Sf-9 cell membranes were prepared for binding assay as described previously (Elster et al., 2000). Measurement of 25 nM [^{3}H]muscimol and 10 nM [^{3}H]flunitrazepam binding was performed in triplicate using 200 μl of membrane homogenate with 25 μl of radioactive ligand in the presence of 25 μl modulator, competing drug, or assay buffer in a final volume of 250 μl. For saturation binding, membranes were incubated with 3–300 nM [^{3}H]muscimol (SA = 5 Ci/mmol) or 0.1–10 nM [^{3}H]flunitrazepam (SA = 82 Ci/mmol). The protein concentration in the assay was between 0.46 and 1.24 mg/ml for saturation binding experiments and 0.37–0.78 mg/ml for drug modulation experiments. Samples were incubated at 4°C for 30 min for [^{3}H]muscimol and 1 h for [^{3}H]flunitrazepam. Labeled membranes were harvested on a Brandel cell harvester using glass microfiber filters (GF/B). The filters were washed with 3 × 4 ml of cold assay buffer. The amount of radioactivity on the filter discs was determined by conventional liquid scintillation counting (Packard, 1900 TR), at a counting efficiency of ∼40%. Specific binding was obtained by subtracting from the total bound radioactivity the amount that was not displaced by saturating concentrations of competing drugs (i.e. non-specific binding). GABA (100 μM) and flurazepam (10 μM) were used as competing drugs for [^{3}H]muscimol and [^{3}H]flunitrazepam binding, respectively. Binding to fractions from sucrose gradients was performed as described previously (Elster et al., 2000).

1.5. Immunofluorescence

Sf-9 cells were seeded in plastic chamber slides (4-well) at a concentration of 0.1×10^6 cells per well, followed by infection with virus, each applied at a MOI of 10, for ∼40 h. The cells were washed in 0.1 M sodium phosphate buffer, pH 7.2 (3 × 2 min), and subsequently fixed in 4% *para*-formaldehyde in 0.1 M sodium phosphate buffer, pH 7.2, for 2 h at 4°C. The cells were washed in phosphate buffer saline (PBS) buffer, pH 7.4 [2.7 mM KCl; 137 mM NaCl; 1.5 mM KH_2PO_4; 6.5 mM Na_2HPO_4] (3 × 2 min), before being treated with 3% (w/v) bovine serum albumin (BSA) in PBS for 30 min at room temperature. The cells were incubated in a moist chamber for 20 h at 4°C with a polyclonal antibody directed against the γ2 $GABA_A$ receptor subunit (residues # 1–29, Somogyi et al., 1996). After wash in PBS (3 × 2 min), the cells were incubated for 1 h at room temperature in TRITC-conjugated swine anti-rabbit immunoglobulins, at a dilution of 1/20, supplemented with 0.25% BSA. The cells were then washed three times in PBS buffer before being examined using a fluorescence light microscope (Leica DMIRB) at a magnification of 200 × .

1.6. Electrophysiological recordings

For whole-cell patch–clamp recordings two million cells were plated out in 35 mm Petri dishes and cells were infected for 28–34 h. The culture dish was placed on the stage of a Zeiss Axiovert 10 (Zeiss, Germany) inverted phase-contrast microscope, where the individual cells were viewed at 200 × . The recording chamber contained 2–3 ml artificial balanced salt solution

(ABSS) (10 mM HEPES, pH 7.35; 162.5 mM NaCl; 3.5 mM KCl; 1.25 mM Na_2HPO_4; 2 mM $MgSO_4$; 2 mM $CaCl_2$ and 10 mM glucose) that was renewed by constant perfusion at 0.5 ml/min at room temperature. Standard patch–clamp techniques (Hamill et al., 1981) were used to record from cells in the whole-cell configuration using an EPC-9 amplifier (HEKA, Germany). The patch electrodes were manufactured from 1.5 mm o.d. glass (World Precision Instruments, USA) and filled with electrode buffer (10 mM HEPES, pH 7.3; 160 mM KCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 10 mM EGTA and 2 mM MgATP). Electrodes had resistances of 2–5 MΩ. Series resistance (5–17 MΩ) was 65% compensated. A holding potential of −40 mV was used unless specified. With 6 mM GABA in the present application system, 10–90% rise-times of as low as 70 ms were achieved, but events on a faster time scale may not be resolved. The signals were stored on computer and also recorded on videotape using a VR-10B digital data recorder (Instrutech, USA). Results were analyzed using Pulse (HEKA, Germany) and Igor Pro (Wavemetrics, USA) software.

The drugs used were premixed at the required concentrations in ABSS buffer and were applied using a multi-barreled perfusion pipette as previously described (Elster et al., 1998). The time constant for solution exchange was approximately 50 ms. Agonists (GABA and pentobarbital) were added for 5 s every minute. When pentobarbital (50 μM) was used as a modulator, it was applied for 15 s immediately before GABA and also concurrent with GABA. Midazolam was only applied for 15 s immediately before GABA application. Responses were quantified by measuring the peak current during application of agonist and also the current remaining after 5 s of application. Prior to each modulation experiment a constant response level was established for GABA. The modulated responses were followed by a series of GABA applications, which were continued until a stable level was reached (1–2 min for pentobarbital, 2–4 min for midazolam). Results were only used if this level was within ±15% of the original GABA response level. For concentration–response experiments the experimental data were fitted to an equation of the form $I = I_{max}/[1 + (EC_{50}/A)^n]$ in which I is the peak current; A, the agonist concentration; EC_{50}, the concentration giving half-maximum current; and n is the Hill coefficient.

1.7. Materials

Sf-9 cells and SF-900 II serum-free insect medium were obtained from Life Technologies (Denmark). Plastic tissue culture flasks were purchased from Corning (Corning, NY) and NUNC A/S (Denmark). [methylene-^{3}H]muscimol (20 Ci/mmol), and [methyl-^{3}H]flunitrazepam (82 Ci/mmol) were purchased from Dupont-New England Nuclear (Boston, MA). GF/B filters were from Whatman (Maidstone, UK). Sample concentrators, Centripep 30, were purchased from Micron (Beverly Hills, CA). Ultra-clear centrifuge tubes were purchased from Beckman (Palo Alto, CA). Protein Micro BCA kit was purchased from Pierce (Rockford, IL). Restriction enzymes and other molecular biological enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were obtained from Pharmacia Biotech (Sweden). Other drugs and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or similar commercial suppliers.

2. Results

2.1. Importance of the extracellular α1-N-terminal domain for assembly

The (α1/γ2) and (γ2/α1) chimeric subunits were designed (see details in method section) in order to elucidate the structural requirements for $GABA_A$ receptor assembly. The subunits were spliced together, at a position 11 amino acids into the first transmembrane domain. The Sf-9 cell expression system used in the present study was chosen because it is known to express high quantities of proteins and can readily be used for both binding and electrophysiological studies. Several combinations of α1, β2, γ2, (α1/γ2) and (γ2/α1) subunits, including homomeric expression, were tested in [^{3}H]muscimol and [^{3}H]flunitrazepam binding assays (Fig. 1). As expected, both the α1β2 and α1β2γ2 subunit combinations had binding sites for [^{3}H]muscimol and the latter combination for [^{3}H]flunitrazepam as well. All other combinations of the α1, β2 and γ2 subunits did not produce notable binding activity. Specific binding was detected for the (α1/γ2) chimera but not the (γ2/α1) chimera in receptor combinations with β2 or β2γ2 (Fig. 1). Whole-cell patch–clamp recordings on these two chimeras in combinations with β2 and γ2 confirmed this by showing that (α1/γ2)-containing receptors were capable of sustaining GABA-mediated chloride currents (see below). Moreover, the current elicited by the (α1/γ2)-containing receptors could be blocked by bicuculline (data not shown). Surface expression of the $GABA_A$ receptors was shown by immunofluorescence studies using an antibody directed against the extracellular N-terminal domain of the γ2 subunit. The (γ2/α1)β2 receptor construct was incorporated into the cell plasma membrane, albeit at a significantly reduced level compared with the (α1/γ2)β2γ2 subunit composition (Fig. 2.). To explain the above observations, the (α1/γ2) and (γ2/α1) chimeras may act like α1 and γ2 subunits, respectively, indicating that the extracellular N-terminal segment is important for assembly.

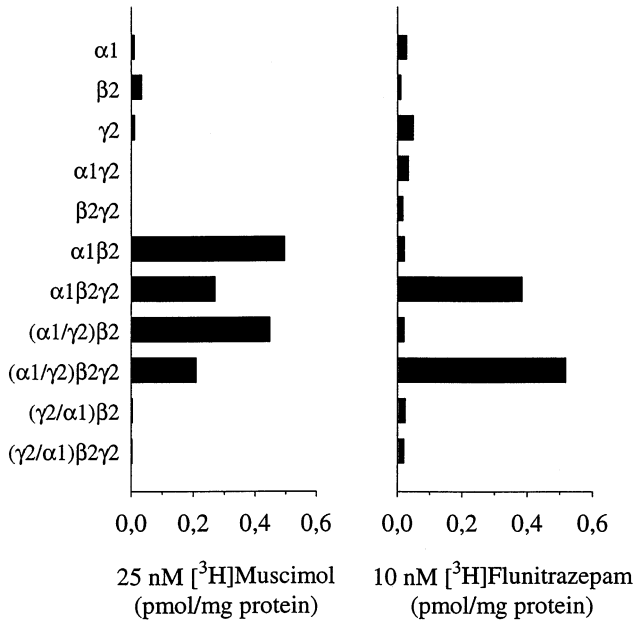

Fig. 1. Sf-9 cells infected for approximately 60 h using different subunit combinations were prepared for 25 nM [^{3}H]muscimol and 10 nM [^{3}H]flunitrazepam binding experiments. Non-specific binding was measured in the presence of 100 μM GABA and 10 μM flurazepam, respectively.

To verify the ability of these chimeras to form pentamers, sucrose density gradient centrifugation was performed on receptor protein solubilized in Triton X-100 in order to separate assembly intermediates from the pentameric complex (Fig. 3A and B). Sf-9 cells were infected with recombinant baculoviruses to produce (α1/γ2)β2, (α1/γ2)β2γ2 and (γ2/α1)α1β2 $GABA_A$ receptors and sucrose gradient fractions were tested for [^{3}H]muscimol and [^{3}H]flunitrazepam binding activity. The pentameric receptor complex has previously been shown to have a sedimentation coefficient of about 7.2 under the applied experimental conditions (Elster et al., 2000). Binding at positions corresponding to the pentameric complex (fraction 22) was detected for all subunit combinations with sedimentation values of 7.2 (Fig. 3A and B).

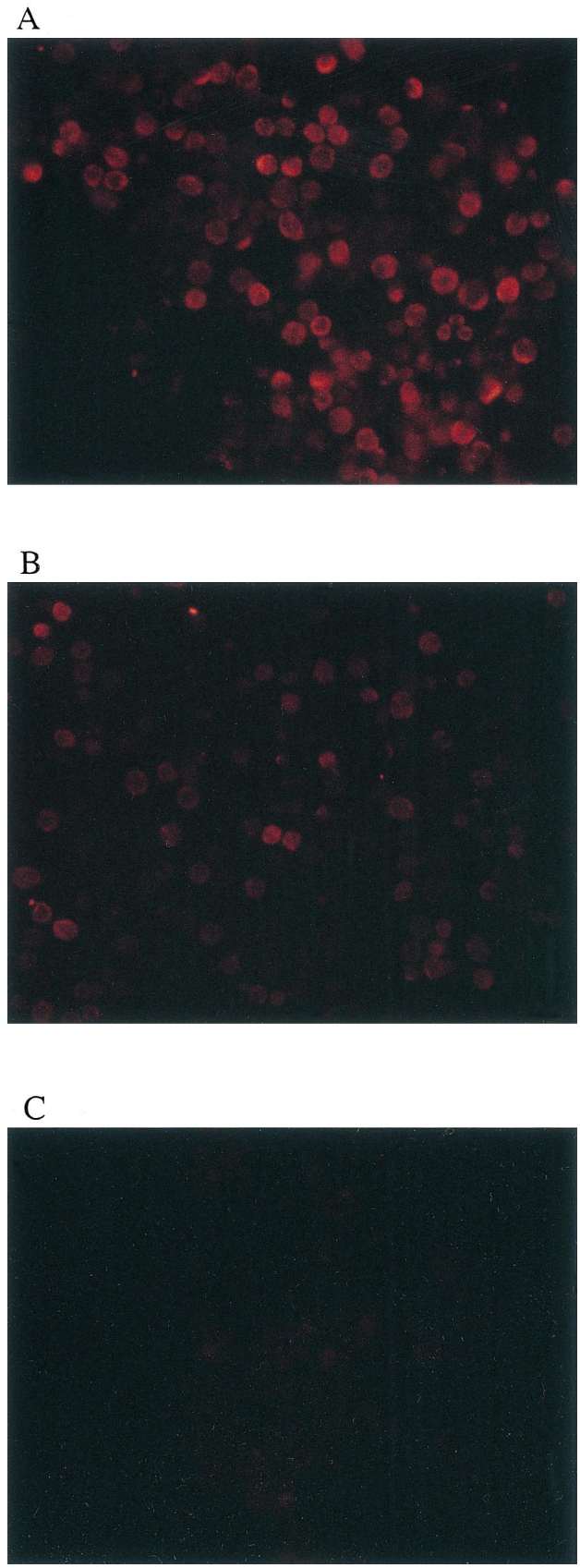

Fig. 2. Surface expression of (α1/γ2)β2γ2 (A), (γ2/α1)β2 (B), and α1β2 (C) subunit combinations was detected by immunofluorescence using an antibody directed against the extracellular N-terminal region of the γ2 subunit. Sf-9 cells were infected for ~40 h. The cells were fixed in 4% *para*-formaldehyde and TRITC-conjugated swine anti-rabbit immunoglobulins were used to visualize the $GABA_A$ receptors on the surface of the cells (light microscope (Leica DMIRB; magnification of 200× (8–40 nm))). The experiments were performed three times with comparable results.

Fig. 2.

2.2. Pharmacological characterization of $GABA_A$ receptors including the ($\alpha1/\gamma2$) chimera

Saturation binding curves from membrane preparations of Sf-9 cells infected with α1β2, α1β2γ2, (α1/γ2)β2 and (α1/γ2)β2γ2 subunit combinations were performed (Table 1). The chimera-containing receptor ((α1/γ2)β2γ2) was shown to have the same affinity for [^{3}H]flunitrazepam as the control (α1β2γ2, Table 1). For [^{3}H]muscimol binding, a slightly higher affinity was observed for the chimera-containing receptor complexes ((α1/γ2)β2, K_d, 32 ± 0.5 nM ($\pm$S.E.M.); (α1/γ2)β2γ2, K_d, 27 ± 0.4 nM) compared with controls (α1β2, K_d, 58 ± 2.2 nM; α1β2γ2, K_d, 71 ± 5.1 nM). Moreover, Scatchard analysis indicated the presence of a single population of binding sites for all subunit combinations. The modulatory effect of pentobarbital was altered in the (α1/γ2) chimera-containing receptor (Fig. 4). The percentage potentiation of [^{3}H]flunitrazepam binding by pentobarbital was significantly lower for the (α1/γ2)β2γ2 complex ($\sim$10%) compared with the α1β2γ2 combination ($\sim$35%). In contrast to this, modulation of [^{3}H]flunitrazepam binding by GABA was not significantly different for the two receptor constructs (Fig. 4).

In whole-cell patch–clamp recordings, GABA concentration–response curves from membrane preparations of Sf-9 cells infected with the α1β2, α1β2γ2, (α1/γ2)β2 and (α1/γ2)β2γ2 subunit combinations showed no significant differences in EC_{50} values between the α1β2 and (α1/γ2)β2 subunit combinations and the α1β2γ2 and (α1/γ2)β2γ2 receptors (Table 2). Likewise, the Hill coefficients were found to be the same for the α1β2; (α1/γ2)β2 and α1β2γ2; (α1/γ2)β2γ2 receptor pairs (Table 2). Moreover, the reversal potentials for the α1β2γ2, (α1/γ2)β2 and (α1/γ2)β2γ2 subunit combinations were not significantly different from each other and they were close to the Nernst potential of -3 mV for Cl^- with the solutions used (Fig. 5). However, in cells infected with α1β2γ2 receptors a prominent fade of the GABA-stimulated current could be observed while in the (α1/γ2)β2γ2-expressing cells only a small fade was observed, suggesting the absence of desensitization (Fig. 6A). Surprisingly, desensitization was seen for the direct response of pentobarbital (Fig. 6A and B). This suggests differences in the mechanism of channel activation for pentobarbital and GABA. In contrast to the direct effect of pentobarbital, no desensitization of the midazolam- and pentobarbital-enhanced GABA responses was seen (Fig. 6C and D). Moreover, in agreement with the binding studies, the direct activation of the channel by 500 μM pentobarbital was significantly different for the (α1/γ2)β2γ2 subunit combination compared with control (α1β2γ2), since pentobarbital gave rise to a peak current of $\sim$173% of the maximum GABA-induced peak current

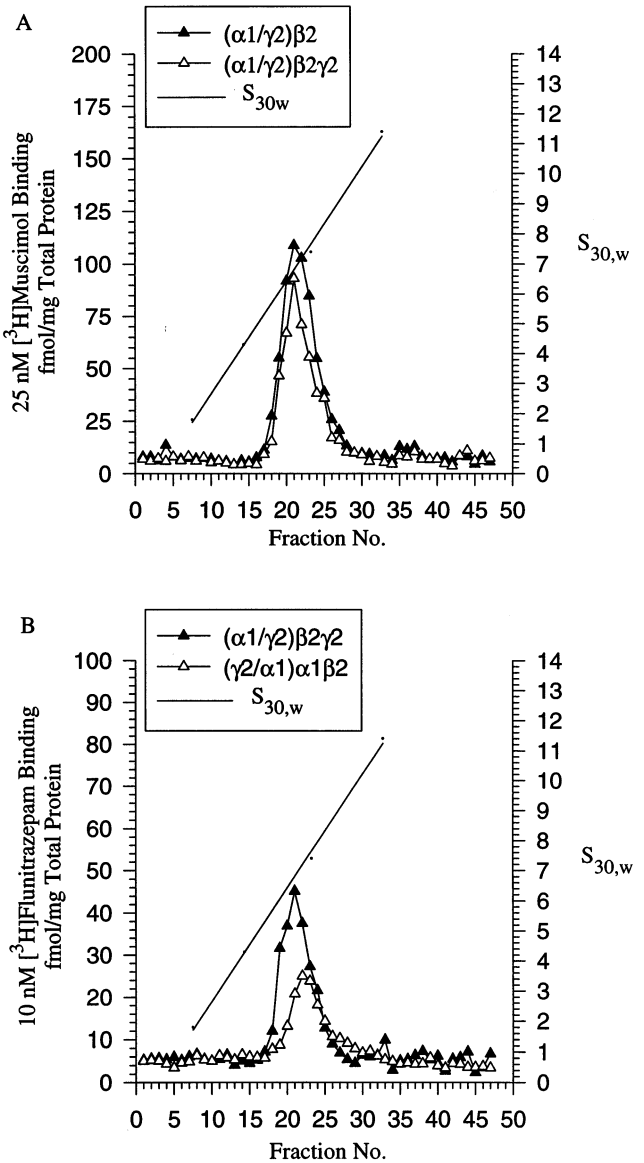

Fig. 3. Sucrose density gradient centrifugation of (α1/γ2)β2, (α1/γ2)β2γ2 and (γ2/α1)α1β2 $GABA_A$ receptor proteins. Sf-9 cells were infected for approximately 60 h. $GABA_A$ receptors were extracted with 1% Triton X-100 and solubilized protein was size-fractionated on 3–30% sucrose gradients. [^{3}H]muscimol (25 nM) (A) and 10 nM [^{3}H]flunitrazepam (B) binding was performed on 225 μl gradient fractions, which were fractionated from the top of the gradients. The column was calibrated using cytochrome *C* (*S*, 1.9), BSA (*S*, 4.3), alcohol dehydrogenase (*S*, 7.4) and catalase (*S*, 11.4). *S*, sedimentation coefficient; 30, 30% sucrose; w, weight. The experiments were performed three times with comparable results.

for the (α1/γ2)β2γ2 complex compared with only $\sim$66% for the α1β2γ2 combination (Fig. 6B). To facilitate comparison of the maxima for pentobarbital and GABA, a saturating concentration of pentobarbital on the α1β2γ2 combination was used (Carlson et al., 2000), and since a relatively larger signal was acquired for the direct response of pentobarbital compared with GABA the comparison stands even stronger. Contrary to this,

Table 1
[^{3}H]muscimol and [^{3}H]flunitrazepam saturation binding curve data from membrane preparations of Sf-9 cells infected with different combinations of subunits[a]

Subunit combinations	[^{3}H]muscimol		[^{3}H]flunitrazepam	
	K_d (nM ± S.E.M.)	B_{max} (pmol/mg pro. ± S.E.M.)	K_d (nM ± S.E.M.)	B_{max} (pmol/mg pro. ± S.E.M.)
α1β2	58 ± 2.15	0.63 ± 0.016	ND	ND
(α1/γ2) β2	32 ± 0.52	0.75 ± 0.006	ND	ND
α1β2γ2	71 ± 5.10	0.42 ± 0.023	2.1 ± 0.65	0.31 ± 0.09
(α1/γ2) β2γ2	27 ± 0.40	0.65 ± 0.056	2.0 ± 0.47	0.75 ± 0.17

[a] ND, not determined. Concentrations used for [^{3}H]muscimol and [^{3}H]flunitrazepam binding were between 3–300 and 0.1–10 nM, respectively. Non-specific binding was measured in the presence of 100 μM GABA and 10 μM flurazepam, respectively. Each value is the mean ± S.E.M. of three experiments each performed in triplicate.

the pentobarbital enhancement of the GABA current was not significantly different for the subunit constructs, eventhough a ~393% enhancement was seen for the (α1/γ2)β2γ2 complex compared with only ~240% for the α1β2γ2 combination (Fig. 6C). Again, in accordance with the binding studies, no difference was observed for the benzodiazepin (midazolam)-enhanced GABA current comparing the two subunit constructs (Fig. 6D).

2.3. Desensitization properties of the (α1/γ2) chimera in combination with β2 or β2γ2 subunits

In order to test whether the small fade seen for the (α1/γ2)-containing receptors was due to desensitization or change of the chloride gradient across the cell membrane, in some cells the holding potential was stepped between −40 and +40 mV while applying GABA or pentobarbital (Fig. 7). Since the equilibrium potential for Cl^- is close to zero, this prevents any net transport of Cl^- during the response. Any remaining fade will presumably be due to desensitization. No desensitization was seen at all for the (α1/γ2)β2γ2 subunit combination (Fig. 7). The same was observed for the (α1/γ2)β2 subunit combination (data not shown). Interestingly, and contrary to this, desensitization was observed for the (α1/γ2)(γ2/α1)β2 subunit combination (Fig. 7). This indicates that the α1-C-terminal fragment may be important for desensitization. Furthermore, an increase in the current after termination of pentobarbital application was seen in three out of seven cells expressing the (α1/γ2)β2γ2 combination (Fig. 7) and in two out of six cells expressing the α1β2γ2 combination (data not shown).

3. Discussion

3.1. Importance of the extracellular N-terminal domain in assembly of GABAA receptors

Specific binding and whole-cell membrane currents were only detected for the (α1/γ2) chimera and not the (γ2/α1) chimera in receptor combinations with β2 or β2γ2. Moreover, surface immunostaining of Sf-9 cells expressing the (α1/γ2)-containing receptors was pronounced, as opposed to the staining of the (γ2/α1)-containing receptors, which was only slightly higher than background. To explain this, the (α1/γ2) and (γ2/α1) chimeras may act like α1 and γ2 subunits, respectively. The (α1/γ2)-containing receptors will resemble the α1-containing receptors (α1β2 and α1β2γ2) in the same way as the (γ2/α1)-containing receptors resemble the γ2-containing complexes (β2γ2). This is also in agreement with the fact that the β2γ2 subunit combination does not form functional receptors (Hartnett et al., 1996; Nabekura et al., 1998). Moreover,

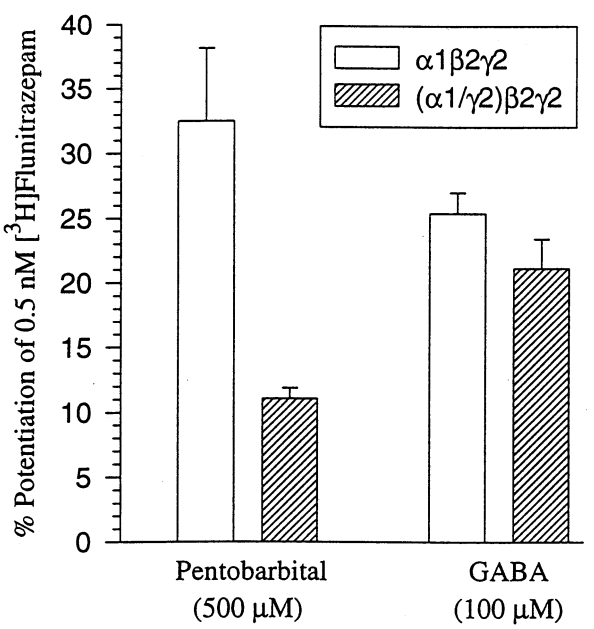

Fig. 4. Pentobarbital (500 μM) and GABA-enhancement (100 μM) of 0.5 nM [^{3}H]flunitrazepam binding to membranes prepared from Sf-9 cells infected for approximately 60 h with α1β2γ2 or (α1/γ2)β2γ2 subunit combinations. For pentobarbital enhancement, the two means are significantly different from each other; $P = 0.0094$, Student's t-test. Non-specific binding was measured in the presence of 10 μM flurazepam. Each value is the mean ± S.E.M. of four experiments performed in triplicate.

Table 2
Concentration–response curves from membrane preparations of Sf-9 cells infected with different combinations of subunits[a]

	GABA concentration–response curves peak currents	
	EC_{50} (μM ± S.E.M.)	Hill coefficient
α1β2	12.2 ± 1.32	0.78 ± 0.06
(α1/γ2)β2	23.9 ± 7.08	0.82 ± 0.15
α1β2γ2	140 ± 19	1.19 ± 0.16
(α1/γ2)β2γ2	139 ± 10	1.53 ± 0.13

[a] Membrane currents were obtained with whole cell patch–clamp recordings. The membrane potential was held at −40 mV. The peak currents were recorded after GABA application. For each cell, the currents were normalized with respect to the peak current induced by 6 mM GABA. There was no significant differences in EC_{50} values between the α1β2 and (α1/γ2)β2 subunit combinations and the α1β2γ2 and (α1/γ2)β2γ2 receptors ($P = 0.97$ and $P = 1.00$, respectively, Analysis of variance followed by multiple comparison procedures, Tukey test). Each value is the mean ± S.E.M. from seven to eight experiments.

Tierney et al. (1996) have shown surface staining of non-permeabilized Sf-9 cells infected exclusively with α1 or β1 subunits, but as expected at a significantly reduced level compared with the α1β2 subunit combination. However, the lack of surface expression of the (γ2/α1)-containing receptors could have been due to a

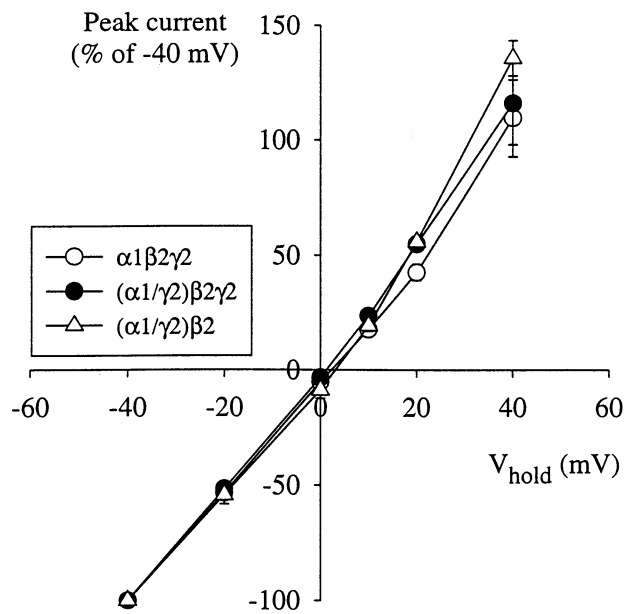

Fig. 5. Current voltage relationships for maximum GABA induced peak currents in α1β2γ2, (α1/γ2)β2 and (α1/γ2)β2γ2 receptors. The reversal potentials for the three combinations were not significantly different (α1β2γ2, 2.3 ± 0.8 mV, $n = 5$; (α1/γ2)β2γ2, 1.3 ± 0.6 mV, $n = 3$; and (α1/γ2)β2, 3.0 ± 1.2 mV, $n = 4$; analysis of variance, $P = 0.537$) and they were close to the Nernst potential of −3 mV for Cl^- with the solutions used. The current voltage relationships show outward rectification, which is a characteristic of $GABA_A$ receptor mediated Cl^- currents.

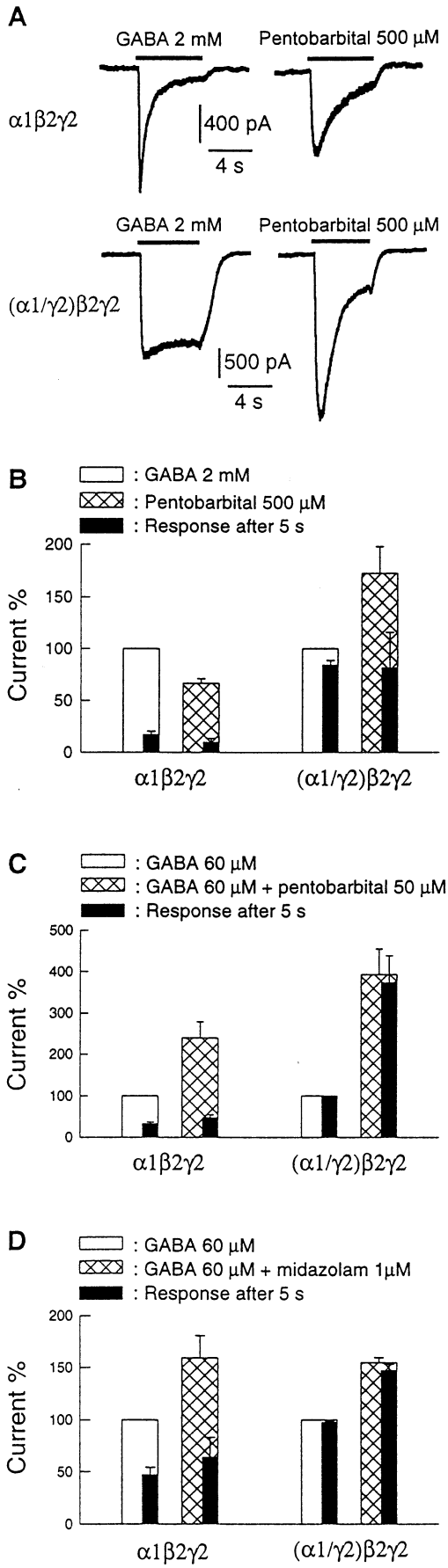

Fig. 6. (*Continued*)

defect in recombinant protein synthesis. The presence of (γ2/α1) protein was confirmed by the detection of [^{3}H]flunitrazepam binding to sucrose fractions corresponding to (γ2/α1)α1β2 pentamers, since detection of [^{3}H]flunitrazepam binding requires the (γ2/α1) subunit to be part of the receptor complex. The GABA (muscimol) and benzodiazepine binding sites have been speculated to reside in the interface between the extracellular N-terminal of α1 and β2, and α1 and γ2 subunits, respectively (Smith and Olsen, 1995). This leads to the conclusion that the extracellular α1-N-terminal segment may be important for assembly and that the extracellular N-terminal fragment is enough for correct folding of the protein to form binding sites for muscimol and flunitrazepam as well as functional receptors shown by electrophysiological recordings.

The similarity between the (α1/γ2) and α1 subunit-containing receptors suggests that the (α1/γ2) chimera has not introduced any gross changes in the receptor structure. Similar GABA EC_{50} values in whole-cell patch–clamp measurements and K_d values for [^{3}H]flunitrazepam and [^{3}H]muscimol binding were seen. Additionally, no differences were seen for the pentobarbital- and midazolam-induced potentiation of GABA currents and the GABA-mediated enhancement of [^{3}H]flunitrazepam binding. Moreover, the reversal potentials for the tested subunit combinations were not significantly different from each other. The K_d values for [^{3}H]muscimol binding to α1β2 (58 nM) and α1β2γ2 (71 nM) $GABA_A$ receptors were slightly higher than previously reported. In Sf-9 cells, K_d values between 14.5–29.0 nM for the α1β2(or β1) and 10.2–17.9 nM for the α1γ2β2(or β1) subunit combinations have been observed (Pregenzer et al., 1993; Hartnett et al., 1996; Tierney et al., 1998). However, a K_d of ~2 nM for [^{3}H]flunitrazepam binding to the α1β2γ2 subunit combination resembled that previously reported (Pregenzer et al., 1993). Also, it may seem peculiar that the K_d values for the chimera-containing receptors were lower than for the wild type receptors, since desensitization is proposed to increase affinity of the ligand (Lohse, 1993). However, for Sf-9 cells expressing the α1β2 or α1β2γ2 subunit combination [^{3}H]muscimol binding to intermediates has been detected, which makes the interpretation more complex, as other factors than desensitization appear to be involved (Elster et al., 2000). The EC_{50} value (140 μM) for the GABA-induced currents

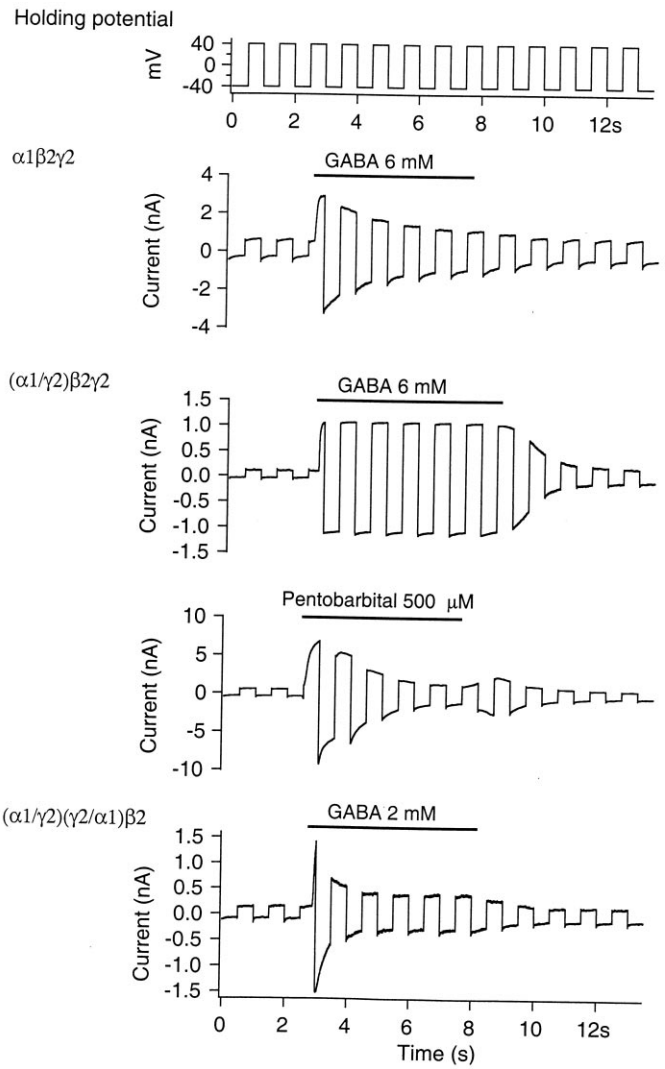

Fig. 7. Examples of whole-cell patch–clamp recordings from Sf-9 cells infected with α1β2γ2, (α1/γ2)β2γ2 or (α1/γ2)(γ2/α1)β2 subunit combinations. Cells were infected for approximately 30 h. The holding potential was stepped between −40 and +40 mV in order to avoid fading due to net Cl^- flux out of the cell while applying 2 or 6 mM GABA, both of which give rise to maximum responses, or while applying 500 μM pentobarbital. When using this voltage protocol, any remaining fade is presumably due to desensitization. These experiments were performed four to seven times with similar results.

Fig. 6. Whole-cell patch–clamp recordings from Sf-9 cells infected with the α1β2γ2 or (α1/γ2)β2γ2 subunit combinations. Cells were infected for approximately 30 h. (A) Examples of GABA and pentobarbital induced currents at a holding potential of −40 mV. (B) For the wild-type receptor (α1β2γ2) the peak current induced by 500 μM pentobarbital (hatched bars) was significantly smaller than a maximum GABA induced peak current (open bars, $P < 0.001$, $n = 6$, Student's t-test), while for the chimeric (α1/γ2)β2γ2 receptor the pentobarbital induced peak current was significantly larger ($P = 0.014$, $n = 7$). The corresponding currents remaining after 5 s of drug application are shown with solid bars. Prominent fade was seen for pentobarbital in both combinations, while with GABA this was only the case for the wild-type receptor. (C) Pentobarbital at a lower concentration (50 μM) enhanced currents induced by GABA at approximately EC_{20} in both subunit combinations (α1β2γ2, $P = 0.0057$, $n = 6$; (α1/γ2)β2γ2, $P < 0.001$, $n = 9$, Student's t-test). The enhanced peak currents were not significantly different from each other ($P = 0.087$). (D) Midazolam enhanced currents induced by GABA at approximately EC_{20} in the two subunit combinations (α1β2γ2, $P = 0.011$, $n = 13$; (α1/γ2)β2γ2, $P < 0.001$, $n = 10$, Student's t-test) to a similar extent. For the (α1/γ2)β2γ2 combination, only a small fade during the GABA application was seen, even for the pentobarbital (C) and midazolam (D) enhanced currents (α1β2γ2, $n = 13$; (α1/γ2)β2γ2, $n = 10$).

was likewise somewhat higher than that reported earlier for the $\alpha1\beta2\gamma2$ receptor. EC_{50} values of 31 and 47 μM have been measured in Sf-9 cells for the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ subunit combinations, respectively (Nabekura et al., 1998). The reason for these discrepancies is not clear, but differences in pH or salt concentrations in the applied buffers or in the infection time before execution of experiments could play a role. Additionally, because of desensitization the EC_{50} will depend on the speed of application.

3.2. Lack of desensitization for the ($\alpha1/\gamma2$) chimeric subunit in combination with $\beta2$ or $\beta2\gamma2$

Desensitization of currents elicited by high agonist concentrations is a prominent feature of the $GABA_A$ receptor, whereas the homomeric ρ-containing $GABA_A$ receptors exhibit very low desensitization (Chang and Weiss, 1999). Looking at specific $GABA_A$ receptors, those consisting of the $\alpha1\beta3$ or the $\alpha1\beta3\gamma2$ subunit combinations desensitize with similar mean rates and to a similar extent, whereas desensitization of the $\alpha1\beta3\delta$ combination is slower and less extensive, but still significant (Haas and Macdonald, 1999). Moreover, in one investigation comparing the $\alpha1\beta2\gamma2$ with the $\alpha6\beta2\gamma2$ combination, the latter showed no desensitization during a 200 ms application of 1 mM GABA (Tia et al., 1996). However, other investigators have found desensitization for the $\alpha6\beta3\gamma2$ combination in response to high concentrations of GABA applied for 5–15 s (Saxena and Macdonald, 1994; Fisher et al., 1997). In this investigation, we found that while the $(\alpha1/\gamma2)\beta2$ and $(\alpha1/\gamma2)\beta2\gamma2$ receptors were similar to their α1-containing counterparts with respect to EC_{50} values for GABA and sensitivity to modulation by midazolam and pentobarbital, a conspicuous difference was the amount of desensitization accompanying channel activation. The receptors containing the chimeric construct exhibited a total lack of desensitization even in response to 6 mM GABA. This demonstrates that the $(\alpha1/\gamma2)$-containing receptors are biophysically distinct from all other $GABA_A$ receptors that have been characterized.

The M2 domain has repeatedly been shown to be important for $GABA_A$ receptor desensitization (Im et al., 1995b; Birnir et al., 1997a,b; Tierney et al., 1998). For the $(\alpha1/\gamma2)\beta2$ and $(\alpha1/\gamma2)\beta2\gamma2$ chimeric receptors, the channel pore, which is believed to be formed by the M2 domains of the five subunits (Imoto et al., 1986), will only be formed by amino acid residues from β2 and γ2 subunits. These $(\alpha1/\gamma2)$-containing receptors did not desensitize. However, the ability to desensitize could be restored by expressing the $(\alpha1/\gamma2)$ chimera together with its 'inverted' construct $(\gamma2/\alpha1)$ and the β2 subunit. Taken together, these results suggest that the C-terminal part of the α1 subunit is required for desensitization of GABA-gated currents to occur. In transfected human embryonic kidney (HEK) cells, a high level of expression of $\alpha1\gamma2$, $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ $GABA_A$ receptors has been reported (Verdoorn et al., 1990). It was shown that the $\alpha1\beta2$ and $\alpha1\beta2\gamma2$ $GABA_A$ receptors had faster desensitization and channel kinetics than the $\alpha1\gamma2$ $GABA_A$ receptor. These authors concluded that β2-containing $GABA_A$ receptors desensitize faster and that this subunit may control channel gating properties. Moreover, Birnir et al. (1997a) have shown the β1 subunit in $\alpha1\beta1$-expressing Sf-9 cells to dominate conformational changes activated by GABA. They performed site-directed mutagenesis at the M2 region of the α1 and β1 subunits and it was shown that β1 affects the rate of desensitization. Obviously, desensitization is likely caused by a complex interplay between subunits. However, the present study clearly shows that the shift to a desensitized state is related to the interaction of agonist with its binding site and not just the passage of currents, since differences in the desensitization pattern were seen for the $(\alpha1/\gamma2)\beta2\gamma2$ subunit combination when activated by pentobarbital and GABA. The receptors containing the chimeric construct exhibited a total lack of desensitization in response to GABA contrary to a pronounced desensitization in response to pentobarbital. This elucidates the importance of α and β subunits in desensitization, since the binding site for GABA, as mentioned before, has been suggested to be located in the interface between α1 and β2 (Smith and Olsen, 1995).

For the wild type $\alpha1\beta2\gamma2$ receptor, peak currents gated by 500 μM pentobarbital were significantly smaller than the maximum GABA (2 mM) gated peak currents, while for the $(\alpha1/\gamma2)\beta2\gamma2$ receptor they were significantly larger. This difference in the pharmacological effects of pentobarbital on $(\alpha1/\gamma2)$- and α1-containing receptors may not be surprising, since barbiturates are believed to have their binding site near the channel lumen, which as mentioned above is involved in desensitization (Im et al., 1995b; Birnir et al., 1997a,b; Tierney et al., 1998). For comparison, Birnir et al. (1997a) have shown that mutation of a threonine residue to glutamine in the M2 region of the β1 subunit abolished potentiation by pentobarbital in Sf-9 cells expressing $\alpha1\beta1$ receptors. Moreover, Tierney et al. (1996) have also reported the abolishment of pentobarbital enhancement in $\alpha1\beta1$ receptors after mutation of leucine to threonine in the M2 domain of the α1 subunit.

Desensitization of $GABA_A$ receptors has also been reported to be influenced by several other factors including various allosteric ligands (Frosch et al., 1992) as well as phosphorylation of the receptors by different protein kinases (Moss et al., 1992). The influence of phosphorylation on the desensitization of the $(\alpha1/\gamma2)$-containing receptor is of great interest. Moreover, the $(\alpha1/\gamma2)$ chimera will allow more detailed studies of $GABA_A$ receptor activation and deactivation kinetics,

which so far only have been possible for the very slowly desensitizing ρ-containing $GABA_A$ receptors (Chang and Weiss, 1999). Additionally, since one cannot exclude that desensitization might occur within the 70 ms time limit of the application system used it is possible that extremely fast desensitization may have been missed. For that reason it would be interesting to test the chimera in a system equipped with an even faster application system. Finally, in a similar experimental paradigm as used in the present study, a new set of chimeras, with varying lengths of the α1 and γ2 subunits, or point mutations, may provide more detailed information about the structural requirements for assembly and desensitization. Such studies are currently underway in our laboratory.

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