THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 7, pp. 4354–4363, February 16, 2007

Spontaneous Cross-link of Mutated α1 Subunits during $GABA_A$ Receptor Assembly*[S]

Received for publication, October 13, 2006, and in revised form, November 10, 2006 Published, JBC Papers in Press, December 4, 2006, DOI 10.1074/jbc.M609676200

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γ-Aminobutyric acid, type A ($GABA_A$) receptor α1 subunits containing a cysteine mutation at a position in the channel mouth (H109C) surprisingly formed a spontaneous cross-link with each other in receptors composed of α1H109C, β3, and γ2 subunits. Cross-linking of two α1H109C subunits did not significantly change the affinity of [^{3}H]muscimol or [^{3}H]Ro15-1788 binding in α1H109Cβ3γ2 receptors, but GABA displayed a reduced potency for activating chloride currents. On reduction of the disulfide bond, however, GABA activation as well as diazepam modulation was similar in mutated and wild-type receptors, suggesting that these receptors exhibited the same subunit stoichiometry and arrangement. Disulfide bonds could not be reoxidized by copper phenanthroline after having been reduced in completely assembled receptors, suggesting that cross-linking can only occur at an early stage of assembly. The cross-link of α1H109C subunits and the subsequent transport of the resulting homodimers to the cell surface caused a reduction of the intracellular pool of α1H109C subunits and a reduced formation of completely assembled receptors. The formation of α1H109C homodimers as well as of correctly assembled $GABA_A$ receptors containing cross-linked α1H109C subunits could indicate that homodimerization of α1 subunits via contacts located in the channel mouth might be one starting point of $GABA_A$ receptor assembly. Alternatively the assembly mechanism might have started with the formation of heterodimers followed by a cross-link of mutated α1 subunits at the heterotrimeric stage. The formation of cross-linked α1H109C homodimers would then have occurred independently in a separate pathway.

GABA,[2] quantitatively the most important inhibitory neurotransmitter in the central nervous system, mediates fast synaptic inhibition by opening the chloride ion channel intrinsic to $GABA_A$ receptors (1). $GABA_A$ receptors are the targets of action of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates, steroids, anesthetics, and convulsants. These drugs modulate GABA-induced chloride ion flux by interacting with distinct allosteric binding sites at these receptors (2, 3).

$GABA_A$ receptors are composed of five subunits (4, 5) that can belong to different subunit classes. Recombinant receptor studies (5–8) as well as studies investigating the subunit composition of $GABA_A$ receptors in the brain (9, 10) indicated that the vast majority of $GABA_A$ receptors found are composed of two α, two β, and one γ subunit. Biochemical studies (5, 11, 12) as well as modeling of the $GABA_A$ receptor extracellular domain (13) according to the structure of the acetylcholine-binding protein (14) indicated the absolute arrangement of the subunits in $GABA_A$ receptors (Fig. 1*A*).

To achieve the correct order of subunits around the pore, each subunit must be able to discriminate between different subunits and to interact with its neighbors via specific high affinity contact sites. Several amino acid sequences have been identified that seem to be important for assembly of α, β, and γ subunits of $GABA_A$ receptors (15–23).

In a previous study, we used the comparative models developed by our group (13) for predicting amino acid residues in α1 and γ2 subunits that might form direct contacts with each other (24). These residues were mutated to cysteines, and a possible disulfide bond formation between mutated subunits was investigated. Most of the cysteines introduced into α1 or γ2 subunits did not cause a spontaneous cross-link between subunits, although the respective amino acid residues were found to be important for $GABA_A$ receptor assembly (24). The mutation α1H109C, however, caused a spontaneous cross-link of $GABA_A$ receptor subunits even in receptors composed of α1H109C and wild-type β3 and γ2 subunits and thus in the absence of a mutated γ2 subunit as a possible cross-link partner.[3] In the present study, this surprising observation was investigated in detail. It was demonstrated that the spontaneous disulfide bond formation occurred between two mutated α1 subunits during an early stage of assembly and that functional $GABA_A$ receptors could be formed despite the presence of a disulfide bond between two opposing subunits. Possible mechanisms of this cross-link during $GABA_A$ receptor assembly are discussed.

* This work was supported by Austrian Science Fund Grant P15165. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: GABA, γ-aminobutyric acid; $GABA_A$, γ-aminobutyric acid, type A; HEK, human embryonic kidney; PBS, phosphate-buffered saline; DTT, dithiothreitol; IP, immunoprecipitation; NEM, *N*-ethylmaleimide.

[3] I. Sarto-Jackson, unpublished observations.

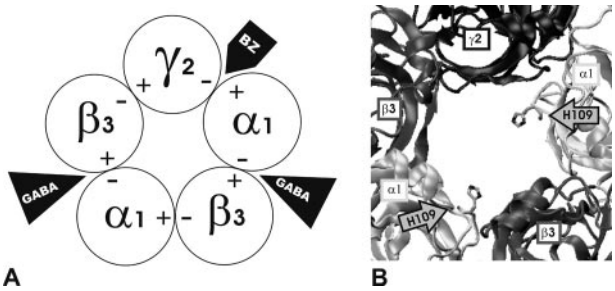

FIGURE 1. **GABA$_A$ receptor structure.** *A*, schematic drawing of the stoichiometry and absolute subunit arrangement of the recombinant $\alpha1\beta3\gamma2$ GABA$_A$ receptor. + and − indicate the principal and complementary side of a subunit, respectively. *BZ* or *GABA* indicate the sites of interaction of benzodiazepines or GABA with GABA$_A$ receptors, respectively. *B*, structure of a model of the GABA$_A$ receptor extracellular domain. View from top of the chloride channel to the $\alpha1$, $\beta3$ and $\gamma2$ subunits, indicating the amino acid residue His-109 investigated in this study. Residue $\alpha1$His-109 is shown in *stick* representations.

EXPERIMENTAL PROCEDURES

Antibodies—The antibodies anti-peptide $\alpha1$-(1–9), anti-peptide $\beta3$-(1–13), anti-peptide $\beta2$-(351–405), and anti-peptide $\gamma2$-(319–366) were generated and affinity-purified as described previously (5, 9, 17).

Generation of cDNA Constructs—For the generation of recombinant receptors, $\alpha1$, $\beta3$, $\beta2$, and $\gamma2$ subunits of GABA$_A$ receptors from rat brain were cloned and subcloned into pCDM8 expression vectors (Invitrogen) as described previously (5). Mutated subunits were constructed by PCR amplification using the wild-type subunit as template. For this, PCR primers were used to construct point mutations within the subunits by the "gene splicing by overlap extension" technique (25). The PCR primers contained XhoI and NsiI, NsiI and EcoRV, or XhoI and XbaI restriction sites, which were used to clone the $\alpha1$, $\beta2$, or $\gamma2$ fragments into pCI vectors, respectively (Promega, Madison, WI). The mutated subunits were confirmed by sequencing.

Culture and Transfection of HEK 293 Cells for Binding Studies and Western Blots—Transformed HEK 293 cells (CRL 1573; American Type Culture Collection, Manassas, VA) were cultured as described previously (5). 3×10^6 cells were transfected with 20 μg of cDNA for a single subunit transfection using the calcium phosphate precipitation method (26). For co-transfection with two different subunits, for each subunit 10 μg of cDNA was used. When cells were co-transfected with three different subunits, 7 μg of cDNA was used per subunit. A total of ~20 μg of cDNA/transfection and a cDNA ratio of 1:1:1 seemed to be optimal for the expression of GABA$_A$ receptors under the conditions used as judged by receptor binding studies in cells transfected with $\alpha1$, $\beta3$, and $\gamma2$ subunits. Changing the subunit ratio by doubling the amount of cDNA for a single subunit at the cost of other subunits did not significantly change the number of [^{3}H]Ro15-1788 binding sites detected.

The cells were then harvested 48 h after transfection. At this time point the number of [^{3}H]Ro15-1788 binding sites formed per milligram of protein was at its maximum for cells transfected with $\alpha1$, $\beta3$, and $\gamma2$ subunits. Results obtained, however, did not change when cells were harvested 34–48 h after transfection. In addition, Western blot analysis revealed that expression levels of wild-type and mutated subunits were comparable at all harvesting times.

Immunoprecipitation of Wild-type and Mutated Subunits—The culture medium was removed from transfected HEK cells, and cells from four culture dishes were extracted with 1 ml of a $C_{12}E_{10}$ extraction buffer (1% polyoxyethylene 10 lauryl ether (Sigma), 0.18% phosphatidylcholine (Sigma), 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, containing one "Mini Complete protease inhibitor mixture" tablet (Roche Diagnostics GmbH)/10 ml of extraction buffer) for 8–12 h at 4 °C. The extract was centrifuged for 40 min at 150,000 × *g* at 4 °C, and the clear supernatant was incubated overnight at 4 °C under gentle shaking with 20 μg of antibodies. After addition of Pansorbin (formalin-fixed *Staphylococcus aureus* cells, purchased from Calbiochem, EMD Bioscience Inc.) and 0.5% nonfat dry milk powder and shaking for an additional 2 h at 4 °C, the precipitate was washed three times with a low salt buffer for immunoprecipitation (IP low buffer) (50 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, and 1 mM EDTA, pH 8.0). The precipitated proteins were dissolved in sample buffer (108 mM Tris sulfate, pH 8.2, 10 mM EDTA, 25% (w/v) glycerol, and 2% SDS with or without 3% dithiothreitol (DTT)).

Immunoprecipitation of Receptors Expressed at the Cell Surface—The culture medium was removed from HEK cells transfected with cDNA (21 μg/3 × 10^6 cells) of GABA$_A$ receptor subunits (cDNA ratio, 1:1:1), and the cells were washed once with PBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, and 4.3 mM Na_2HPO_4, pH 7.3). For experiments with *N*-ethylmaleimide, cells were preincubated with 50 mM NEM (in PBS) for 30 min at room temperature, and then unbound NEM was washed off the culture dishes. Cells with or without NEM treatment were then detached from the culture dishes by incubating with 2.5 ml of 5 mM EDTA in PBS for 5 min at room temperature. The resulting cell suspension was diluted in 6.5 ml of cold Dulbecco's modified Eagle's medium and centrifuged for 5 min at 1000 × *g*.

For Western blot analysis, the cell pellet from two dishes was incubated with 35 μg of $\alpha1$-(1–9) antibodies in 3 ml of the same medium for 30 min at 37 °C. Cells were again pelleted, and free antibodies were removed by washing twice with 10 ml of PBS buffer. Then receptors were extracted with IP low buffer containing 1% Triton for 1 h under gentle shaking. Cell debris were removed by centrifugation (30 min at 150,000 × *g* at 4 °C). After addition of Pansorbin and 0.5% nonfat dry milk powder and shaking for 2 h at 4 °C, the precipitate was centrifuged for 10 min at 10,000 × *g* at 4 °C and dissolved in sample buffer (with or without DTT) and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled antibodies.

To verify that only receptors on the cell surface were labeled by the antibodies, parallel samples were incubated with antibodies directed against the intracellular loop of GABA$_A$ receptor subunits. These antibodies could not precipitate any GABA$_A$ receptor subunits under the conditions used. A possible redistribution of the antibodies during the extraction procedure has been excluded previously (17).

Immunoprecipitation of Subunits after Depletion of Cell Surface Receptors—After immunoprecipitation of receptors expressed at the cell surface, the supernatant of the immuno-

precipitate was again incubated with α1-(1–9) antibodies overnight at 4 °C. Then Pansorbin and 0.5% nonfat dry milk powder were added, and the samples were shaken for another 2 h at 4 °C. The precipitate was centrifuged for 10 min at 10,000 × *g* at 4 °C, dissolved in sample buffer (without DTT), and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies.

SDS-PAGE, Western Blot, and Chemiluminescence Detection—SDS-PAGE was performed according to Neville and Glossmann (27) using gels containing 10% polyacrylamide in a discontinuous system. Proteins separated on the gels were tank-blotted onto prewetted polyvinylidene fluoride membranes. After blocking with 1.5% nonfat dry milk powder in PBS and 0.1% Tween 20 for 1 h at room temperature, the membranes were incubated overnight with digoxigenin-labeled α1-(1–9), β3-(1–13), or γ2-(319–366) antibodies (1 μg/ml) at 4 °C. The membranes were extensively washed and incubated with secondary antibodies (anti-digoxigenin-alkaline phosphatase, F(ab′)$_2$ fragments, Roche Diagnostics GmbH) for 1 h at room temperature. Polyvinylidene fluoride membranes were again washed extensively as described above and then equilibrated in assay buffer (0.1 M diethanolamine and 1 mM $MgCl_2$, pH 10.0) for 10 min, and secondary antibodies were visualized by the reaction of alkaline phosphatase with CDP Star (Applied Biosystems, Bedford, MA). The chemiluminescent signal was quantified by densitometry after exposing the immunoblots to the Fluor-S MultImager (Bio-Rad) and evaluated using the Quantity One quantitation software (Bio-Rad). Under the experimental conditions used, the immunoreactivities were within the linear range, and this permitted a direct comparison of the amount of antigen per gel lane between samples. Data were generated from several different gels and expressed as means ± S.E.

All mutated constructs used in this study could be expressed to a comparable extent after single transfection into HEK cells. After co-transfection of different constructs, however, the stability of mutated subunits that formed non-productive assembly intermediates was reduced presumably by proteolytic degradation (17, 28, 29). Therefore, in all control experiments the extent of expression of mutated constructs was determined in singly transfected HEK cells.

[^{3}H]Ro15-1788 Binding Studies—Frozen membranes from transfected HEK cells were thawed, and cells were homogenized in 50 mM Tris, citrate buffer, pH 7.4, by using an Ultra-Turrax followed by three centrifugation resuspension cycles (200,000 × *g* for 20 min at 4 °C). Cell pellets were resuspended in 50 mM Tris, citrate buffer, pH 7.4, at a protein concentration in the range of 0.1–1 mg/ml as measured with the BCA protein assay kit (Pierce) using bovine serum albumin as standard.

Membranes were then incubated for 90 min at 4 °C in a total of 1 ml of a solution containing 50 mM Tris, citrate buffer, pH 7.4, 150 mM NaCl, and various concentrations (range, 0.1–400 nM) of [^{3}H]Ro15-1788 (74.1 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 100 μM diazepam (Hoffmann-La Roche). Suspensions were filtered through Whatman GF/B filters, and the filters were rinsed twice with 5 ml of ice-cold 50 mM Tris, citrate buffer and then subjected to scintillation counting. Nonspecific binding in the presence of 100 μM diazepam was subtracted from total [^{3}H]Ro15-1788 binding to result in specific binding. Scatchard analysis was performed three times using data from three different transfections and the GraphPad Prism 3.0 program (GraphPad Software, Inc., San Diego, CA).

[^{3}H]Muscimol Binding Studies—Proteins of transfected HEK cells were extracted and immunoprecipitated by the addition of α1-(1–9) and β3-(1–13) antibodies for 2 h at 4 °C, and precipitates were dissolved in 50 mM Tris, citrate buffer, pH 7.4 (+0.1% Triton X-100). These suspensions were incubated for 60 min at 4 °C in a total of 1 ml of a solution containing 50 mM Tris, citrate buffer, pH 7.4 (+0.1% Triton X-100), and various concentrations (range, 0.1–400 nM) of [^{3}H]muscimol (30 Ci/mmol, PerkinElmer Life Sciences) in the absence or presence of 100 μM GABA.

Suspensions were then filtered through Whatman GF/B filters, and the filters were rinsed twice with 3.5 ml of ice-cold 50 mM Tris, citrate buffer and then subjected to scintillation counting. Nonspecific binding in the presence of 100 μM GABA was subtracted from total [^{3}H]muscimol binding to result in specific binding. Scatchard analysis was performed three times using data from three different transfections and the GraphPad Prism 3.0 program (GraphPad Software Inc.).

Electrophysiological Studies Using Transfected HEK Cells—HEK cells for electrophysiological experiments were taken from the batches cultured for binding studies and Western blots and seeded at 1 × 10^5 cells/35-mm cell culture dish. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. The cDNA ratio used was 1:1:1 for α1:β3:γ2 subunits, respectively. pEGFP-N1 (Clontech) was co-transfected to identify transfected cells. Whole-cell recordings were performed at room temperature 1–2 days after transfection. GABA was applied using a DAD-12 superfusion system (Adams and List Associates Ltd., Westbury, NY). Extracellular solution contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.4. The pipette solution contained 140 mM KCl, 11 mM EGTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2. The cells were clamped at −60 mV, and currents were filtered at 1 kHz, recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), and analyzed with Clampfit software (Axon Instruments). DTT was applied immediately prior to experiments by replacing culture media with extracellular solution containing 10 mM DTT for 4 min followed by extensive washing. For experiments with copper phenanthroline, a stock solution of 100 mM $CuSO_4$ was generated. $CuSO_4$ was mixed with *o*-phenanthroline (Sigma) directly before use to a final concentration of 100:400 μM copper:phenanthroline and applied for 10 min after extensive washout of DTT.

Density Gradient Centrifugation—Transfected HEK cells were incubated for 44 h at 37 °C. Cells from four culture dishes were harvested and extracted in 1 ml of $C_{12}E_{10}$ extraction buffer. The extracts were centrifuged for 40 min at 150,000 × *g* at 4 °C, and 200 μl of the extracts was layered onto the top of a density gradient of 5–20% sucrose in $C_{12}E_{10}$ extraction buffer. For the determination of sedimentation coefficients, 2 μg of digoxigenin-labeled catalase (sedimentation coefficient, 11 s),

1.2 μg of digoxigenin-labeled alkaline phosphatase (sedimentation coefficient, 6.1 s), and 1 μg of digoxigenin-labeled carbonic anhydrase (sedimentation coefficient, 3.3 s) were included in the overlays. The gradients were centrifuged at 120,000 $\times$ *g* for 23 h at 4 °C and then fractionated by piercing at the tube bottom (5). Protein in individual fractions was precipitated (30) and dissolved in sample buffer for SDS-PAGE. Proteins were identified by Western blot analysis, and signal intensity per fraction was quantified as described above. In a previous study (5) it has been demonstrated that after co-transfection of HEK cells with α1, β3, and γ2 subunits all three subunits sedimented at a single peak of 8.7 s, representing the pentameric receptor.

Immunofluorescence—HEK cells were fixed with 2% paraformaldehyde in PBS 30–35 h after transfection followed by a 10-min wash in 50 mM NH_4Cl in PBS. Washes between incubation steps were performed in PBS. Primary antibodies were detected with goat anti-rabbit $IgG_{(H+L)}$ Bodipy FL (Molecular Probes, Eugene, OR) in 1% bovine serum albumin in PBS. Labeling was visualized using a Nikon Eclipse TE300 microscope equipped with a high pressure mercury lamp and suitable filter sets. To verify that labeling of cells without permeabilization was restricted to the cell surface, parallel samples were stained with antibodies directed against the intracellular loop of GABA$_A$ receptor subunits. These antibodies detected GABA$_A$ receptor subunits only after permeabilization of transfected cells.

Modeling—All comparative models of the GABA$_A$ receptor extracellular domain were based on the acetylcholine-binding protein structure (Protein Data Bank code 1I9B) (14). Based on seven alignments for the structurally variable regions, a total of 35 models were computed with Modeler version 6 (31). After validation, a total of seven well scoring models (one per alignment) of the GABA$_A$ receptor extracellular domain were used to predict the segments that participate in interface formation. Despite the high uncertainty that is associated with amino acid side chain positions at most interface-forming regions (13, 14), predictions were made with the program WHAT IF (32) on the possible formation of disulfide bridges.

RESULTS

Cells Transfected with α1H109C, β3, and γ2 Subunits Form GABA$_A$ Receptors Containing Cross-linked α1 Subunits—Preliminary experiments performed in the course of a previous study (24) demonstrated that α1 subunits carrying the mutation α1H109C formed a spontaneous cross-link either between themselves or with another protein in HEK cells transfected with α1H109C, β3, and γ2 subunits.[3] To clarify this question, in the present study we first investigated whether the cross-linked α1H109C protein was associated with completely assembled receptors present at the surface of α1H109C-, β3-, and γ2-transfected cells. Intact cells were incubated with α1-(1–9) antibodies, and proteins were then extracted under conditions in which the receptor-antibody complex was not drastically impaired and under which no redistribution of the antibody could be observed (17). Receptors were immunoprecipitated by addition of Pansorbin, dissolved in SDS loading buffer without DTT to allow the detection of subunit dimers formed by disulfide bonds, and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies. Fig. 2*A* shows that cells transfected with wild-type α1, β3, and γ2 subunits under these conditions exhibited a major protein band at about 51 kDa that could be labeled by α1-(1–9) antibodies and thus represented α1 subunits. In contrast, cells transfected with α1H109C and wild-type β3 and γ2 subunits exhibited a major protein band of about 100 kDa that could also be labeled by α1-(1–9) antibodies (Fig. 2*A*). Staining of this 100-kDa protein band was stronger (135 $\pm$ 16%) than that of wild-type α1 subunits and could only be observed in the absence of DTT. In the presence of DTT, a band at around 51 kDa was detected (Fig. 2*D*) indicating that the 100-kDa protein band (Fig. 2*A*) contained α1H109C subunits cross-linked by disulfide bridges. Staining of this protein was still stronger than that of cells transfected with wild-type α1, β3, and γ2 subunits. This indicates that the increase in staining was due to an increased expression of cross-linked α1 subunits at the surface of α1H109C-, β3-, and γ2-transfected cells and not due to a better staining of cross-linked α1 subunits. In addition, this cross-link was not caused or initiated by the α1-(1–9) antibodies used for cell surface precipitation of receptors because it was also observed in Western blots from membranes of appropriately transfected HEK cells that were directly dissolved in SDS sample buffer without incubation with these antibodies (experiments not shown). Finally the cross-link was not induced during homogenization or solubilization because it also could be observed when free cysteines at the cell surface were alkylated with 50 mM NEM before extraction (experiments not shown).

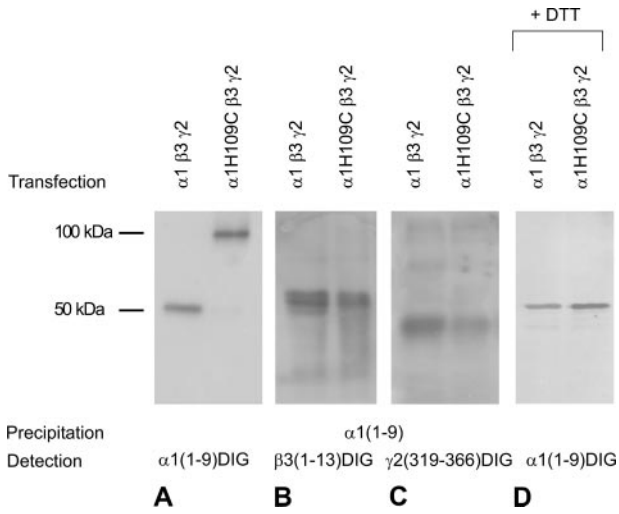

FIGURE 2. **Cell surface expression of GABA$_A$ receptors containing mutated α1 and wild-type β3 and γ2 subunits.** HEK cells were co-transfected with α1, β3, and γ2, or α1H109C, β3, and γ2 subunits. GABA$_A$ receptors expressed at the surface were immunolabeled by an incubation of intact cells with α1-(1–9) antibodies. Receptors were then extracted, precipitated by Pansorbin, and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies (*A*). The blots were then stripped and reanalyzed with digoxigenin-labeled β3-(1–13) antibodies (*B*). Then blots again were stripped and reanalyzed with digoxigenin-labeled γ2-(319–366) antibodies (*C*). Comparable results were obtained when the order of detection of subunits was changed. These experiments were performed six times with comparable results. The quantification of protein bands was performed as described under "Experimental Procedures." The variability ($\pm$S.D. values) of data is given in the text. *D*, the same procedure was performed for surface receptor labeling, extraction, and immunoprecipitation. SDS-PAGE and Western blots were performed under reducing conditions again using digoxigenin-labeled α1-(1–9) antibodies. *DIG*, digoxigenin.

Then the same Western blot (Fig. 2*A*) was stripped and reprobed using β3-(1–13) or γ2-(319–366) antibodies. Fig. 2*B* shows that the β3-(1–13) antibody, in agreement with previous results, could identify a diffuse protein band of about 52–54 kDa (21) in cells transfected with wild-type α1, β3, and γ2 or α1H109C, β3, and γ2 subunits. In cells transfected with α1H109C, β3, and γ2, staining of β3 on average represented only 51 ± 7% of that found in cells transfected with wild-type α1, β3, and γ2 subunits. Stripping and reprobing this Western blot using γ2-(319–366) antibodies indicated a diffuse protein band in the range of 45–49 kDa in cells transfected with wild-type α1, β3, and γ2 or α1H109C, β3, and γ2 subunits that represented the γ2 subunit (Fig. 2*C*). Again in cells transfected with α1H109C, β3, and γ2, the expression of the γ2 subunit was reduced to 44 ± 5% compared with γ2 in cells transfected with wild-type α1, β3, and γ2. The protein smear at around 80–90 kDa in Fig. 2*C* could also be detected in cells transfected with wild-type α1, β3, and γ2 subunits. It presumably resulted from unspecific protein aggregates caused by oxidation of endogenous cysteines in these gels that were run in the absence of DTT to allow the detection of cross-linked proteins.

In contrast to α1-(1–9) antibodies that labeled a 100-kDa protein band in precipitates from α1H109Cβ3γ2-transfected cells, no such protein band could be detected by β3-(1–13) or γ2-(319–366) antibodies indicating that the 100-kDa band did not contain β3 or γ2 subunits. It thus either contained two α1H109C subunits or an α1H109C subunit cross-linked with an unrelated protein. However, density gradient centrifugation indicated that receptors containing cross-linked α1H109C subunits exhibited a maximum sedimentation coefficient similar to completely assembled, wild-type receptors (experiments not shown), excluding the possibility that an unrelated protein or additional α1H109C subunits were cross-linked to the pentameric receptor or that multiple receptors were cross-linked with each other. In addition, residue α1H109C is located at the entrance of the chloride channel formed by GABA$_A$ receptors (Fig. 1*B*). Any cross-link of α1H109C subunits with an unrelated protein would have interfered with the formation of functional GABA$_A$ receptors.

Cross-linked α1H109C Subunits Form Functional GABA$_A$ Receptors with β3 and γ2 Subunits—To investigate the formation of functional GABA$_A$ receptors, whole-cell patch clamp experiments were performed in HEK cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits. GABA enhanced the chloride conductance in both sets of transfected cells (Fig. 3) indicating the formation of functional α1H109Cβ3γ2 receptors. These results argue against a cross-link of α1H109C subunits with an unrelated protein and support a cross-link of two α1H109C subunits within the pentameric receptor. The potency of GABA for stimulating chloride flux, however, was different in α1β3γ2- (EC_{50} = 35 μM, 95% confidence interval = 31–40 μM) and α1H109Cβ3γ2 (EC_{50} = 440 μM, 95% confidence interval = 365–522 μM)-transfected cells (Fig. 3). To investigate whether the 13-fold reduced potency of GABA for activating α1H109Cβ3γ2 receptors was caused by a limited conformational flexibility due to the cross-linked α1H109C subunits, the GABA potency was measured after treatment of transfected cells by 10 mM DTT. Under these conditions the

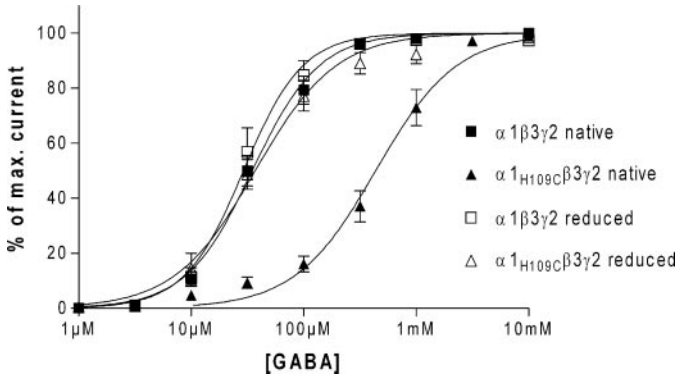

FIGURE 3. **Functional properties of GABA$_A$ receptors containing mutated α1 and wild-type β3 and γ2 subunits.** GABA-elicited whole-cell currents recorded from HEK cells co-transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits in the absence (*native*) or presence (*reduced*) of DTT are shown. Due to variable transfection efficiencies, measured currents varied up to 7-fold from cell to cell even from the same transfected culture dish. Data therefore were normalized to the maximum response to GABA and represent means ± S.E. of six to eight individual dose-response curves obtained from different cells derived from a total of four transfections. Despite the large variability of currents, the average whole-cell currents obtained for wild-type and mutated receptors (under native as well as reduced conditions) were in the same order of magnitude. For technical reasons, GABA concentrations >10 mM could not be applied, and thus maximum currents of the mutated receptors in the absence of DTT treatment could not be determined reliably. The EC_{50} of the mutated receptors under non-reducing conditions is therefore an estimation only. Cells transfected with α1H109C, β3, and γ2 subunits exhibited a Hill coefficient of 1.2 (before and after treatment with DTT) that is comparable to the Hill coefficient of cells transfected with wild-type α1, β3, and γ2 subunits.

EC_{50} for GABA in α1H109Cβ3γ2-transfected cells was shifted to the left resulting in an EC_{50} of about 37 μM (95% confidence interval = 31–45 μM), which was comparable to the EC_{50} of wild-type α1β3γ2 receptors. In control experiments it was demonstrated that treatment of α1β3γ2-transfected cells with DTT prior to the experiment did not change the GABA dose-response curve of wild-type α1β3γ2 receptors (Fig. 3) confirming previously published data (33). However, no spontaneous reoxidation could be observed in α1H109Cβ3γ2-transfected cells after reduction by DTT and subsequent removal of the reducing agent. Attempts to reoxidize the reduced disulfide bonds by the addition of copper phenanthroline were also not successful (experiments not shown) indicating that disulfide bond formation only was possible during assembly and not in completely assembled receptors.

Additional experiments investigated the modulation of wild-type and mutated GABA$_A$ receptors by a benzodiazepine. Diazepam was able to dose dependently modulate GABA receptors formed in α1β3γ2- or α1H109Cβ3γ2-transfected cells with comparable potency but different efficacy. Although diazepam enhanced GABA-induced currents (EC_3) by about 3.5-fold in α1β3γ2-transfected cells, enhancement was only about 2.5-fold in α1H109Cβ3γ2-transfected cells. Upon addition of DTT, diazepam-induced enhancement of GABA currents in α1H109Cβ3γ2-transfected cells was similar to that in α1β3γ2-transfected cells (experiments not shown).

Cells Expressing α1H109C, β3, and γ2 Subunits Exhibit [^{3}H]Ro15-1788 and [^{3}H]Muscimol Binding—In other experiments we studied possible changes in the high affinity [^{3}H]Ro15-1788 or [^{3}H]muscimol binding sites. Scatchard analysis of [^{3}H]Ro15-1788 binding data (Fig. 4*A*) to membranes

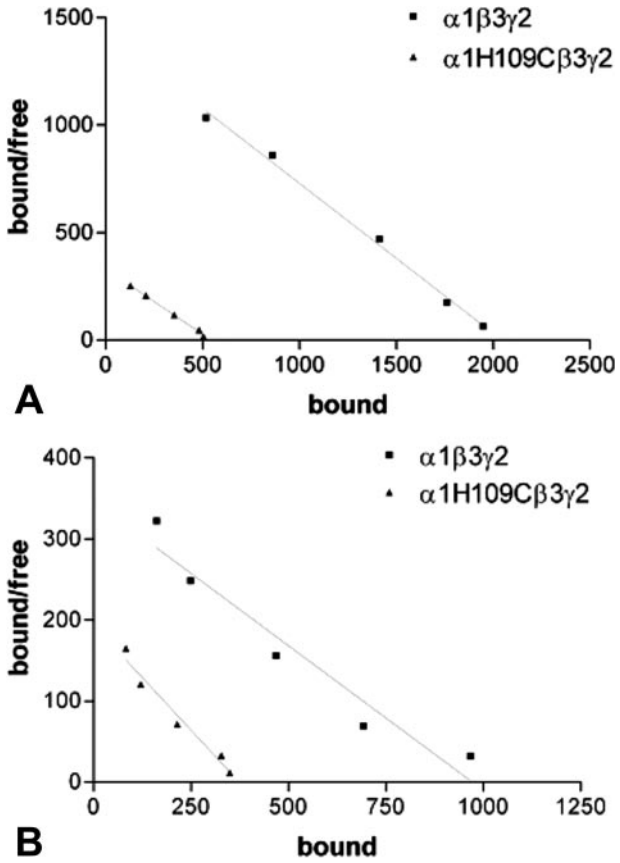

FIGURE 4. **Scatchard analysis of [^{3}H]Ro15-1788 or [^{3}H]muscimol binding studies with GABA$_A$ receptors containing mutated α1 and wild-type β3 and γ2 subunits.** HEK cells were transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits. *A*, membranes were subjected to Scatchard analysis of [^{3}H]Ro15-1788 binding data. *B*, GABA$_A$ receptors were extracted and immunoprecipitated. [^{3}H]Muscimol binding data of the precipitates were subjected to Scatchard analysis. Data shown are from one experiment performed in triplicates in which cells expressing wild-type or mutated GABA$_A$ receptors were transfected and processed in parallel. Experiments were performed a total of three times with comparable results.

from cells transfected with α1H109C, β3, and γ2 subunits indicated a K_D of 1.6 ± 0.2 nM that was comparable with that of wild-type α1β3γ2 receptors (1.4 ± 0.1 nM). This indicated that mutated α1H109C subunits when co-expressed with β3 and γ2 subunits displayed an intact α1(+)/γ2(−) interface forming a benzodiazepine pocket similar to wild-type receptors. Interestingly the B_{max} values of α1H109Cβ3γ2-transfected cells (541 ± 13 fmol/mg) differed significantly from those of α1β3γ2-transfected cells (2028 ± 36 fmol/mg), indicating that the formation of the [^{3}H]Ro15-1788 binding sites in these cells was only 26.7% of that of cells transfected with wild-type subunits. Due to variable transfection efficiencies, B_{max} values varied in different experiments. When data from three different Scatchard analysis experiments were combined, the number of [^{3}H]Ro15-1788 binding sites in the α1H109Cβ3γ2-transfected cells was only 34 ± 5% of that found in cells transfected with α1, β3, and γ2 subunits.

Membrane-bound GABA$_A$ receptors usually exhibit high affinity and intermediate affinity [^{3}H]muscimol binding sites that both are located at the same receptor (2). The exact binding data obtained are variably influenced by endogenous GABA that cannot easily be completely removed. In contrast, [^{3}H]muscimol binding studies performed in extracted and immunoprecipitated receptors are dominated by a single high affinity binding site. Therefore, for measuring [^{3}H]muscimol binding sites, proteins of HEK cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits were extracted and immunoprecipitated by the addition of α1-(1–9) and β3-(1–13) antibodies. [^{3}H]Muscimol binding to precipitated proteins was measured, and Scatchard analysis indicated that the muscimol binding site of mutated (K_D = 2.0 ± 0.5 nM) and wild-type receptors (K_D = 3.3 ± 0.9 nM) exhibited a comparable affinity. B_{max} values of [^{3}H]muscimol binding, however, differed significantly between mutated (382 ± 22 fmol/mg) and wild-type (990 ± 53 fmol/mg) receptors. In three different experiments the amount of [^{3}H]muscimol binding sites found in α1H109Cβ3γ2-transfected cells was reduced to 39 ± 5% of that in α1β3γ2-transfected cells. Due to variable transfection efficiencies as well as the use of different HEK cell pools and different experimental conditions (binding to membranes *versus* extracted and immunoprecipitated receptors), B_{max} values of [^{3}H]muscimol and [^{3}H]Ro15-1788 binding studies cannot be compared.

Assembly Intermediates Can Be Transported to the Cell Surface—As mentioned above, the amount of α1H109C subunits at the surface of cells transfected with α1H109C, β3, and γ2 subunits was increased, whereas the amounts of β3 and γ2 subunits were reduced as compared with cells transfected with α1, β3, and γ2 subunits (Fig. 2). To investigate whether assembly intermediates containing cross-linked α1H109C subunits were transported to the cell surface, HEK cells were transfected with only α1 or α1H109C subunits. Wild-type or mutated α1 subunits possibly expressed at the cell surface were then labeled by α1-(1–9) antibodies and after extraction were precipitated by the addition of Pansorbin. In agreement with previous results (34), α1 subunits when transfected alone into HEK cells could not reach the cell surface. In contrast, however, α1H109C subunits could be detected at the cell surface in a protein band with an apparent molecular mass of 100 kDa (Fig. 5), suggesting that cross-linked α1H109C subunits could reach the cell surface.

This conclusion was confirmed by investigations using immunofluorescence microscopy. On staining with α1-(1–9) antibodies only mutated α1H109C but not wild-type α1 subunits could be detected at the surface of intact HEK cells transfected with either wild-type α1H109C or α1 subunits (experiments not shown).

Transport of Assembly Intermediates to the Cell Surface Resulted in Reduced Amounts of Intracellular α1H109C Subunits—The finding that α1H109C subunits could form cross-linked complexes that could be transported to the cell surface could explain the increased expression of cross-linked α1H109C at the surface of cells transfected with α1H109C, β3, and γ2 subunits (Fig. 2*A*). These results, however, could not explain the reduced expression of β3 or γ2 subunits at the cell surface (Fig. 2, *B* and *C*). To get a more complete picture on what happened in these cells, the experiment of Fig. 2 was repeated, and the distribution of α1H109C subunits was investigated in total cell extracts, in receptors at the cell surface, and in extracts deprived of receptors at the cell surface. For that a

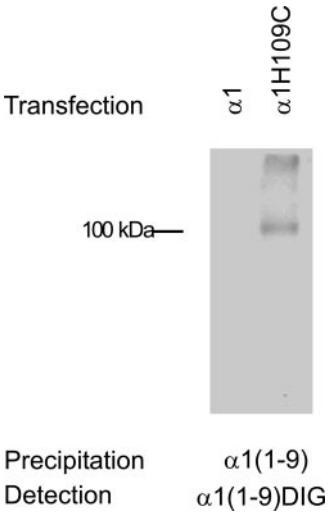

FIGURE 5. **Cell surface expression of assembly intermediates containing mutated α1 subunits.** HEK cells were transfected with α1 or α1H109C subunits. Putative assembly intermediates expressed on the surface were immunolabeled by an incubation of intact cells with α1-(1–9) antibodies. Labeled proteins were then extracted, precipitated by Pansorbin, and subjected to SDS-PAGE and Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies. These experiments were performed twice with comparable results. *DIG*, digoxigenin.

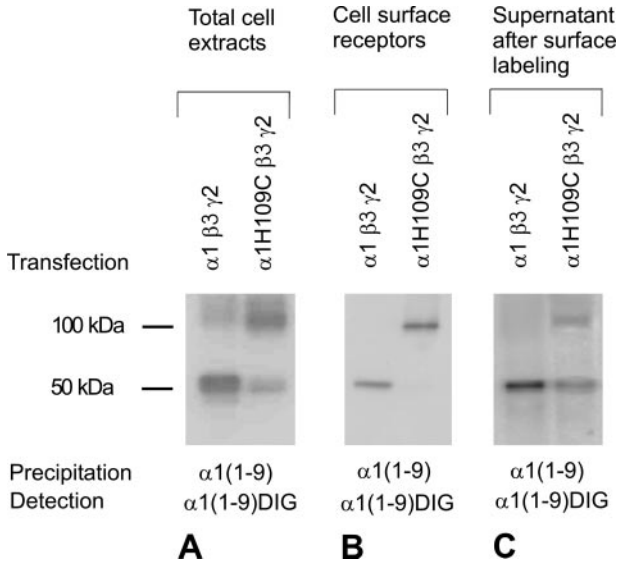

FIGURE 6. **α1 subunit expression in total cell extracts, at the cell surface, and in intracellular compartments of cells transfected with mutated α1 and wild-type β3 and γ2 subunits.** A pool of HEK cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits was generated and divided into two parts. *A*, the total amount of α1 subunits was extracted, and the extracted proteins were immunoprecipitated using α1-(1–9) antibodies and subjected to Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies. *B*, in the second pool of transfected HEK cells, receptors at the cell surface were labeled with α1-(1–9) antibodies, extracted, and precipitated by the addition of Pansorbin. *C*, the supernatant of this precipitate was then again incubated with α1-(1–9) antibodies, thus precipitating receptors or assembly intermediates present in the inside of the cell. These experiments were performed three times with comparable results. The respective variability (±S.D. values) of data is given in the text. *DIG*, digoxigenin.

pool of HEK cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits was generated and divided into two parts. To determine the total amount of α1 subunits, assembly intermediates, or receptors containing α1 subunits, one part was extracted, and the extracted proteins were immunoprecipitated using α1-(1–9) antibodies and subjected to Western blot analysis using digoxigenin-labeled α1-(1–9) antibodies. In the second pool of transfected HEK cells, receptors at the cell surface were labeled with α1-(1–9) antibodies, extracted, and precipitated by the addition of Pansorbin. The supernatant of this precipitate was then again incubated with α1-(1–9) antibodies, thus precipitating receptors or assembly intermediates present in the inside of the cell. As shown in Fig. 6*A*, in total extracts of cells transfected with α1, β3, and γ2 subunits, the majority of α1 subunits migrated as a protein with an apparent molecular mass of 51 kDa as expected. The additional protein smear at around 120–200 kDa presumably resulted from unspecific protein aggregates caused by oxidation of endogenous cysteines in these gels that were run in the absence of DTT to allow the detection of cross-linked proteins. In contrast, in cells transfected with α1H109C, β3, and γ2 subunits, only a minor amount of α1H109C subunits migrated at 51 kDa, whereas the majority of α1H109C subunits migrated at around 100 kDa probably representing the cross-linked α1H109C complex. In addition, as in cells transfected with α1, β3, and γ2 subunits some unspecific protein aggregates could be detected at >100 kDa due to the absence of DTT in these gels. The overall amount of α1H109C subunits (protein bands of 51 and 100–200 kDa) from total extracts of cells transfected with α1H109C, β3, and γ2 subunits was 106 ± 7% of that of α1 subunits in α1β3γ2-transfected cells. This indicated that wild-type and mutated α1 subunits were expressed to the same extent in these cells.

The second pool of cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits was subjected to labeling of cell surface receptors. In agreement with results from Fig. 2*A*, most if not all α1H109C subunits were cross-linked, and the overall expression of α1H109C subunits at the cell surface was increased (Fig. 6*B*). After depletion of cell surface receptors, the same extracts were again incubated with α1-(1–9) antibodies, and the intracellular content of α1 or α1H109C subunits from cells transfected with α1, β3, and γ2 or α1H109C, β3, and γ2 subunits, respectively, was investigated. As shown in Fig. 6*C*, cross-linked as well as non-cross-linked α1H109C subunits could be identified under these conditions, and the amount of α1H109C subunits from cells transfected with α1H109C, β3, and γ2 subunits was drastically reduced (by 67 ± 6%) compared with that of α1 subunits from cells transfected with α1, β3, and γ2 subunits.

Mutations in β2 and γ2 Subunits Homologous to α1H109C Did Not Cause Cross-linking of the Respective Subunits—To investigate whether β2 or γ2 subunits mutated at positions homologous to α1His-109 could also form cross-linked subunit dimers, the respective amino acid residues on the β2 and γ2 subunits were substituted by cysteines giving rise to mutated subunits β2H107C and γ2H122C. However, no cross-link of these mutated subunits could be observed when they were either transfected individually into HEK cells or in combination with the respective wild-type subunits (experiments not shown). In addition, when β2H107C or γ2H122C subunits were co-transfected with α1H109C and wild-type γ2 or α1H109C and wild-type β2 subunits, respectively, only cross-linked α1H109C subunits could be observed (experiments not

shown). For disulfide bond formation it is crucial that the side chains of two residues display a certain distance and angle to each other. These criteria might not have been met by the β2H107C or γ2H122C mutants.

DISCUSSION

The Mutation α1H109C Caused a Spontaneous Disulfide Bond Formation between Two α1 Subunits in α1β3γ2 Receptors—In the present study, it was demonstrated that completely assembled GABA$_A$ receptors composed of the mutated α1H109C and the wild-type β3 and γ2 subunits are formed at the surface of appropriately transfected HEK cells. Interestingly, however, α1H109C subunits that exhibit an apparent molecular mass of 51 kDa migrated as a protein with an apparent molecular mass of about 100 kDa. This 100-kDa protein could be converted to a 51-kDa protein in the presence of DTT, suggesting that α1H109C subunits at the cell surface were cross-linked by disulfide bridges. The cross-linked protein complex, however, did not contain β3 or γ2 subunits indicating that the disulfide bond formation occurred between two α1H109C subunits or between an α1H109C subunit and an unrelated 50-kDa protein. Because residue α1H109C is located within the channel mouth, a cross-link with an unrelated protein would have prevented the formation of functional GABA$_A$ receptors. The demonstration of GABA-gated chloride channels in cells transfected with α1H109C, β3, and γ2 subunits therefore indicated that in these receptors two α1H109C subunits were cross-linked with each other.

Receptors Composed of β3, γ2, and Cross-linked α1H109C Subunits Exhibited the Same Subunit Stoichiometry and Arrangement as Wild-type Receptors—A cross-link of two α1H109C subunits could have changed subunit stoichiometry and arrangement of receptors assembled in α1H109C-, β3-, and γ2-transfected HEK cells. The observation, however, that receptors at the surface of α1H109C-, β3-, and γ2-transfected cells contained only cross-linked but no single α1H109C subunits excluded the formation of receptors containing odd numbers of α subunits. The demonstration of high affinity [^{3}H]muscimol and [^{3}H]Ro15-1788 binding sites formed at the β(+)/α(−) and the α(+)/γ(−) interface, respectively, excluded subunit stoichiometries in which any one of the α1H109C, β3, or γ2 subunits was missing. Finally the observation that GABA$_A$ receptors composed of α1H109C, β3, and γ2 subunits after reduction of their disulfide bond could be activated by GABA with the same potency as wild-type receptors and with a Hill coefficient of 1.2 suggested that the mutated receptor contained two GABA binding sites. Together these results can only be reconciled with GABA$_A$ receptors exhibiting wild-type subunit stoichiometry and arrangement. It thus has to be concluded that the disulfide bond between the two opposing α1H109C subunits bridged the channel mouth of these receptors. The segment around residue α1His-109 protrudes furthest toward the lumen (Fig. 1*B*). In addition, in contrast to the sequence of acetylcholine-binding protein, GABA$_A$ receptor subunits contain an insertion of three amino acid residues in this region that are most likely also located luminally. It is thus possible that the position of α1His-109 might be relatively flexible resulting in a fairly mild distortion of GABA$_A$ receptors containing cross-linked α1 subunits. The low potency of GABA for chloride channel opening before reduction of the disulfide bond then might have been caused by a disulfide bond-induced restriction in the mobility of residue α1H109C or surrounding structures, suggesting that this part of the receptor undergoes a positional or conformational change during GABA-induced opening of the chloride channel.

Cross-linking of α1H109C subunits, however, did not change the affinity of [^{3}H]Ro15-1788 for the benzodiazepine binding site or the potency of diazepam for stimulating GABA-induced chloride flux, indicating that residue α1His-109 is neither involved in direct ligand binding nor important for the benzodiazepine pocket geometry. The efficacy of diazepam for enhancing GABA currents, however, was impaired in receptors containing cross-linked α1His-109 subunits, indicating that the flexibility of residue α1H109C is important also for the allosteric modulation of the receptors by benzodiazepines.

Assembly Intermediates Containing Cross-linked α1H109C Subunits Are Transported to the Cell Surface, Causing a Reduced Formation of Completely Assembled α1H109Cβ3γ2 Receptors—B_{max} values of [^{3}H]muscimol and [^{3}H]Ro15-1788 binding in α1H109Cβ3γ2-transfected cells were only 39 and 34%, respectively, of that in α1β3γ2-transfected cells, suggesting that the mutated receptors represented 34–39% of wild-type receptors. Whereas the amounts of β3 or γ2 subunits identified at the cell surface were consistent with the formation of 34–39% receptors, cross-linked α1H109C subunits present at the cell surface were 135% of wild-type α1 subunits in α1β3γ2-transfected cells. Assuming that 34–39% of α1H109C subunits contributed to α1H109Cβ3γ2 receptors, the surplus of 95–100% α1H109C subunits could only be explained by a transport of cross-linked α1H109C subunits to the cell surface. This was actually demonstrated in cells transfected with only α1H109C subunits. The formation of cross-linked α1H109C subunits, thus, overruled the quality control mechanisms in the endoplasmic reticulum, possibly by covering an endoplasmic reticulum retention signal, thus allowing the exit of incompletely assembled intermediates. This caused an attenuation of α1H109C subunits in the endoplasmic reticulum resulting in an overall reduction in the formation of completely assembled receptors. Wild-type α1 homodimers (as well as higher oligomers) are also formed under these conditions (supplemental Fig. 1). Due to a more labile interaction of two wild-type α1 subunits they might be retained in the endoplasmic reticulum for a longer time period, thus enhancing their chances for interaction with another subunit, leading to completely assembled receptors.

Possible Mechanisms Allowing the Formation of Completely Assembled GABA$_A$ Receptors Containing Cross-linked α1H109C Subunits—The formation of α1H109C dimers as well as of completely assembled receptors containing cross-linked α1H109C subunits most directly can be explained by an interaction of two α1 subunits being at least one starting point of wild-type receptor formation. This possibility is supported by the fact that the α1 subunits are synthesized side by side by different ribosomes on the same mRNA and thus probably have a higher chance to interact with each other than with β3 or γ2 subunits that are synthesized via a different mRNA-ribosome

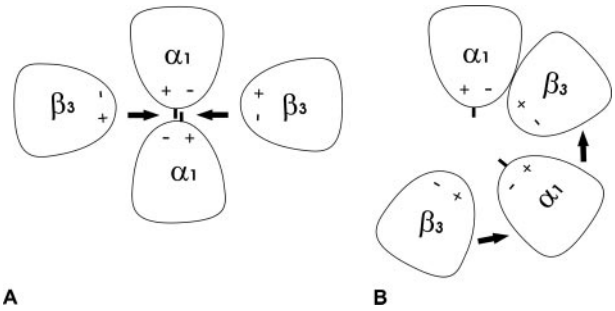

FIGURE 7. **Schematic drawing of possible mechanisms of GABA$_A$ receptor assembly.** The mutated and sterically exposed residues α1H109C are indicated by a *black line* at the luminal side of the subunit. + and − indicate the principal and complementary side of a subunit, respectively. *A*, in one mechanism the two α1H109C subunits interact via their luminal side and offer the possibility that β3 subunits encountering the dimer from the one or the other side could assemble by forming two correct interfaces with two different α1 subunits. *B*, in the second mechanism GABA$_A$ receptor assembly starts with the formation of a heterodimer of α1 and β3 (or γ2) subunits via their respective (+) and (−) sides. As soon as the correct (+)/(−) interface has been established, each additional subunit then has to assemble in the correct order.

complex. In addition, α1H109C homodimer formation and cross-link seemed to occur more rapidly than the assembly between α1H109C and β3 or γ2 subunits as indicated by the large surplus of cross-linked α1H109C dimers over β3 and γ2 subunits as well as over completely assembled receptors at the cell surface. Finally formation of α1 homodimers is also observed in HEK cells transfected with wild-type α1 subunits (supplemental Fig. 1). Because no α1-α1 interface is present in native α1β3γ2 receptors, dimerization of α1 subunits via their respective (+) and (−) sides would lead to a dead end of assembly. This is the reason why the formation of α1-α1 dimers has never been discussed as a possible starting point of assembly. However, interaction of α1 subunits via the side forming the channel mouth (luminal side) offers an alternative possibility avoiding contacts via (+) and (−) sides that could be weakly stabilized, *e.g.* via van der Waals forces to which interaction of the two sterically exposed α1His-109 residues might contribute. Such an assembly intermediate enhances the probability of interaction with a β3 subunit because this subunit can encounter the dimer from the one or the other side (Fig. 7*A*). Formation of two correct α/β interfaces via the (+) and (−) sides of two α1 subunits then probably causes opening of the weak luminal interactions between the two α1 subunits (Fig. 7*A*), thus allowing the fourth and fifth subunits to enter and complete GABA$_A$ receptor assembly. To finally result in receptors with the correct subunit stoichiometry and arrangement, it is mandatory, however, that subunits assembling with the trimer preferentially bind either to the accessible α1(+) side (γ2 subunits) or the α1(−) side (β3 subunits). Overall such a mechanism is consistent with the finding that even in the presence of a covalent cross-link of two α1H109C subunits completely assembled receptors with a wild-type stoichiometry and arrangement are formed presumably before the cross-linked dimers could be transported to the cell surface. Covalent cross-linking with subsequent transport to the cell surface could thus have trapped a labile assembly intermediate that could not have been detected without the use of mutated subunits.

Alternatively the assembly of GABA$_A$ receptors could have started with the formation of a heterodimer either by a direct interaction of α1 and β3 (or γ2) subunits via their respective (+) and (−) sides (Fig. 7*B*) or via an initial contact with a subsequent rearrangement forming the final (+)/(−) interaction. As soon as the correct (+)/(−) interface has been established in the dimer, each additional subunit then has to assemble in the correct order to give rise to a receptor with correct subunit stoichiometry and arrangement. When this assembly mechanism is used, discrimination between subunits occurs already at the stage of the dimer. In this case, cross-linking of the non-neighboring α1H109C subunits could have occurred at the heterotrimeric assembly stage. Here again, α1H109C-α1H109C interaction and subsequent cross-link presumably occurred via luminal contacts before correct formation of all interfaces of the α1-β3-α1 trimer because cross-linking of the two non-neighboring α1H109C subunits after all interfaces have been formed seems to be sterically difficult. An initial luminal contact between subunits might be more efficient in forming the correct interface than a selective contact via the final interfaces because a non-selective initial contact subsequently allows subunits to choose between the available (+) or (−) side of assembly partners. If assembly of GABA$_A$ receptors started with the formation of heterodimers, then the simultaneous formation of a large surplus of cross-linked α1H109C homodimers represented an independent and competitive event that reduced the amounts of α1 subunits available for receptor assembly. Cross-linked homodimers could then have been a dead end of assembly or could have led to completely assembled receptors containing cross-linked α1H109C subunits before the homodimers were transported to the cell surface. The described sequential assembly mechanism (Fig. 7*B*) is similar to that proposed by Green and Claudio (35) for the nicotinic acetylcholine receptor. For the nicotinic acetylcholine receptor an additional assembly mechanism has been discussed in which two subunit dimers could combine with a fifth subunit to form the completely assembled pentameric receptors (36). Further experiments will have to decide between all these possibilities.

Acknowledgments—We thank Susanne Karall, Mirjana Stojanovic, Elisabeth Dögl, and Martina Veit for technical support. We also thank Dr. Noosha Ehya for performing the density gradient experiments shown in supplemental Fig. 1.

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