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Anomalous rectifying properties of 'diazepam-insensitive' $GABA_A$ receptors

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Abstract

Studies using recombinant systems indicate that 'diazepam-insensitive' $GABA_A$ receptors in the central nervous system contain $\alpha 4$ and $\alpha 6$ subunits while 'diazepam-sensitive' $GABA_A$ receptors contain $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$ subunits. Both native and recombinant diazepam-sensitive $GABA_A$ receptors typically exhibit large, outwardly rectifying currents. For example, in patch clamp studies, Human Embryonic Kidney (HEK) 293 cells transfected with cDNAs encoding $\alpha 1\beta 2\gamma 2$ subunits exhibit a rectification ratio ($I_{+60\ mV}/I_{-60\ mV}$) of 1.95 ± 0.21. However, anomalous rectification was observed in recombinant diazepam-insensitive $GABA_A$ receptors composed of either $\alpha 4\beta 2\gamma 2$ (rectification ratio, 0.74 ± 0.09) or $\alpha 6\beta 2\gamma 2$ (rectification ratio, 0.67 ± 0.11) subunits. Based on sequence differences between diazepam-sensitive and -insensitive $GABA_A$ receptor α subunits in the vicinity of the putative channel lining, a point mutation was introduced at His^{273} on the $\alpha 4$ subunit. The rectification ratio in cells expressing a mutated $\alpha 4(Asn^{273})\beta 2\gamma 2$ receptor increased to 1.92 ± 0.17. Moreover, mutation of the homologous residue in the $\alpha 1$ subunit to histidine reduced the rectification ratio of $\alpha 1(His^{274})\beta 2\gamma 2$ to 1.02 ± 0.12. The affinities of benzodiazepine site ligands at diazepam-sensitive and -insensitive $GABA_A$ receptors were unaffected by these mutations. Thus, the electrophysiological properties of diazepam-sensitive and -insensitive $GABA_A$ receptors may be as divergent as their pharmacological characteristics.

Keywords: $GABA_A$ receptor; Rectification; Point mutation; α subunit

1. Introduction

$GABA_A$ receptors are a heterogeneous family of ligand-gated ion channels that may be assembled from at least 16 structurally related subunits (6α, 3β, 3γ, δ, ε and 2ρ) (McKernan and Whiting, 1996; Davies et al., 1997). In the central nervous system, $GABA_A$ receptors are most often assembled as ternary complexes composed of α, β, and γ subunits (Fritschy and Mohler, 1993; De Blas, 1996). While the subunit stoichiometry (i.e., the number of α, β, and γ subunits per receptor) remains controversial (Backus et al., 1993; Chang et al., 1996; Tretter et al., 1997), converging lines of evidence have shown that subunit composition determines the physiological and pharmacological properties of $GABA_A$ receptors (Barnard, 1995; Lüddens et al., 1995).

$GABA_A$ receptors possess multiple, allosterically coupled modulatory sites that are loci for drug action (Skolnick and Paul, 1988; Johnston, 1996). However, from the perspective of both current therapy and future drug development, the group of compounds acting at 'benzodiazepine binding sites' is perhaps the most important. Thus, this structurally diverse group of compounds includes 1,4-benzodiazepines (such as diazepam and flurazepam), imidazopyridines (e.g., zolpidem), cyclopyrrolones (e.g., zopiclone), and β-carbolines (e.g., abecarnil). Studies in recombinant $GABA_A$ receptors have shown that the α subunit is the principal determinant of affinity for this group of compounds (Pritchett and Seeburg, 1990; Lüddens et al., 1990; Hadingham et al., 1993), with the γ subunit playing a smaller, albeit significant role for a subset of these compounds (Lüddens et al., 1994; Benke et al., 1996). While most therapeutically useful benzodiazepine site ligands are high affinity, positive modulators at GABAergic synapses (Haefely and Polc, 1986), Malminiemi and Korpi (1989) demonstrated that high (μM) concentrations of the prototypic 1,4-benzodiazepine

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diazepam are inactive at a subpopulation of $GABA_A$ receptors in primary granule cell neurons. In contrast, the imidazodiazepine Ro 15-4513 retained high affinity for this receptor subpopulation, termed 'diazepam-insensitive' $GABA_A$ receptors. Subsequent cloning studies identified two α subunits ($\alpha 4$ and $\alpha 6$) (Lüddens et al., 1990; Kato, 1990; Wisden et al., 1991) that could reproduce this pharmacological profile when expressed with the appropriate β and γ subunits.

Diazepam-insensitive sites represent only a small fraction of the total $GABA_A$ receptor population. Nonetheless, this group of $GABA_A$ receptors is of considerable interest because several high affinity diazepam-insensitive receptor ligands (e.g., Ro 15-4513 and Ro 19-4603) have been reported to act as amethystic agents (Suzdak et al., 1996; Lister and Durcan, 1989; June et al., 1996). Furthermore, a point mutation in the $\alpha 6$ subunit has been identified in the Alcohol Non-Tolerant (ANT) rat, a strain selected for sensitivity to alcohol (Korpi et al., 1993). These observations prompted efforts to develop benzodiazepine-site ligands that are selective for diazepam-insensitive $GABA_A$ receptors (Ananthan et al., 1993; Wong et al., 1993). While examining the structural requirements for ligand binding to recombinant receptors containing $\alpha 4$ and $\alpha 6$ subunits (Gunnersen et al., 1996), we observed that the large, outwardly rectifying currents that have been characterized in both native and recombinant $GABA_A$ receptors (Verdoorn et al., 1990; Adodra and Hales, 1995) were absent in these diazepam-insensitive isoforms. We now report that a histidine residue located between the second and third transmembrane spanning regions imparts this low rectification to recombinant diazepam-insensitive $GABA_A$ receptors. Mutation of this residue to an asparagine (the amino acid found in the homologous position in $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits) restores the large, outwardly rectifying properties that are observed in diazepam-sensitive $GABA_A$ receptors composed of $\alpha 1\beta 2\gamma 2$ subunits. Moreover, substitution of a histidine residue in the homologous position of the $\alpha 1$ subunit reduces the large, outward rectifying currents characteristic of wild-type receptors composed of $\alpha 1\beta 2\gamma 2$ subunits. These dramatic effects of point mutations on the rectifying properties of $GABA_A$ receptors are independent of the ligand binding properties that have been employed to discriminate between diazepam-sensitive and -insensitive receptor isoforms.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293 cells (American Type Culture Collection, CRL 1573, Rockville, MD) were maintained in Dulbecco's Modified Eagle Medium (MediaTech, Herndon, VA) supplemented with 10% heat inactivated fetal calf serum (Summit Biotechnology, Ft. Collins, CO), 2 mM glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37°C under 5% CO_2. Cells were transfected as previously described (Harris et al., 1995) using a calcium phosphate precipitation procedure (Gorman et al., 1990). Cells were transfected with the cDNAs encoding the specified α, β, and γ subunits at a ratio of 1:1:1 (1 μg per 35 mm dish for electrophysiological studies and 5 μg for a 100 mm dish for radioligand binding studies, respectively). Cells were either harvested for radioligand binding studies or used for electrophysiological recording 48 h after transfection.

2.2. Site-directed mutagenesis of $GABA_A$ receptor subunits

Mutagenesis of the $GABA_A$ receptor $\alpha 1$ and $\alpha 4$ subunits was performed by the PCR-based site-directed mutagenesis method (Ho et al., 1989). Briefly, two overlapping DNA fragments bearing the same mutation were produced in two primary PCR reactions using four primers; the external primers were wild-type and the inner two primers contained the indicated mutation. The upstream external primer and the downstream inner primer were used as one pair and the upstream inner primer and downstream external primer were used as another pair in the primary PCR reactions. Resulting PCR fragments containing an overlap in the region of the desired mutation were gel-purified and small aliquots of purified products were mixed and subjected to five cycles of PCR amplification without primers to allow the two fragments to reanneal and extend. The resulting product was used as a template for the secondary PCR reaction, using only the external two primers. The resulting PCR product was gel-purified, digested with endonucleases *Pme*I and *Eco*NI (for $\alpha 1$ subunit mutagenesis) and recloned into the similarly digested plasmid pRcCMV/$\alpha 1$. Similarly, for $\alpha 4$ subunit mutagenesis, the resulting PCR fragment was digested with endonucleases *Bst*EII and *Eco*RV and ligated into the appropriately digested plasmid pcDNA3/$\alpha 4$.The regions of the resulting constructs generated by PCR were verified by DNA sequencing using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemicals). No additional nucleotide substitutions were found. All PCR reactions were performed with the Pfu DNA polymerase (Stratagene). Primers used in the site-directed mutagenesis were: for $\alpha 1$ subunit mutagenesis: 5′-CAGTGCCAGA*C*ATTCCCTCCCAAAG, 5′-CTTTGGGAGGGAAT*G*TCTGGCACTG, 5′-AAGAGCAGAAGTTGTCTATGAGTGG, 5′-TGGTCGCACTTTTAGCAATAGTGGC, for $\alpha 4$ subunit mutagenesis: 5′-ATCAGTGCTCGG*A*ATTCTTTGCCC, 5′-GGGCAAAGAAT*T*CCGAGCACTGAT, 5′-TGCTGGATGGTTATGACAACAGAC, 5′-CCTCTTGCTGCTGCAGAGATAGTC and for $\alpha 6$ subunit mutagenesis: 5′-GGTAGAGAGT*T*CCGAGCACTGAT. Italicizing indicates mismatched bases as compared to the sequences of the wild-type $\alpha 1$, $\alpha 4$ and $\alpha 6$ subunits, respectively. Mutagenesis of the $\alpha 6$ subunit was per-

formed with the Altered Sites II in vitro Mutagenesis System (Promega) according to the manufacturer's specifications.

2.3. Electrophysiological recording

A standard whole-cell configuration of the patch-clamp technique was used to record Cl^- currents in HEK 293 cells transiently expressing different combinations of $GABA_A$ receptor subunits. Cultures were maintained and transfected as previously described and plated at a density of 20 000 cells/ml on plastic 35 mm dishes. Patch pipettes were prepared from microhematocrit capillary tubes and fire-polished to a tip resistance of 3–5 MΩ when filled with a pipette solution containing (in mM): 143 CsCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). Cells were bathed with an external solution containing (in mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 0.01 mg/ml phenol red (pH 7.3). GABA solutions (10 μM) were rapidly applied and removed by positioning an array of nine glass barrels (406 μm outside diameter) approximately 100 μm away from the target cell as described (Benveniste and Mayer, 1991). GABA was applied for 3 s; intervals of 1 min were allowed between applications, in which cells were continuously perfused with external solution. Whole-cell currents were recorded at different holding potentials with an Axopatch-1C amplifier (Axon Instruments, Burlingame, CA) equipped with a CV-3 headstage. When a gigaseal was formed, capacity transients were canceled. After rupture of the patch, series resistance was compensated (80%) and the pipette potential was set at −60 mV. All experiments were performed at 23°C.

2.4. Radioligand binding

Culture medium was removed and each dish was washed twice with 3-ml ice cold PBS (1.7 mM KH_2PO_4, 5 mM Na_2HPO_4, 150 mM NaCl, pH 7.4). Cells were scraped from the culture dishes, and the cell slurry centrifuged at $1000 \times g$ for 10 min. The cell pellets were resuspended in 50 mM Tris–citrate buffer, pH 7.4, disrupted with a Brinkmann Polytron (setting 7, 10 s) and centrifuged at $20\,000 \times g$ for 20 min. This suspension/centrifugation procedure was repeated a total of 4 times.

Competition studies were performed in a total volume of 250 μl and consisted of 150 μl of tissue suspension (0.1–0.2 mg of protein/assay), 50 μl of [^{3}H]Ro 15-4513 (6 nM), 25 μl drug solution, and 25 μl of 2 M NaCl. Assays (4°C) were terminated after 1 h by rapid filtration (Brandel M-48R, Gaithersburg, MD) through GF/B filters followed by two 5-ml washes with ice-cold 50 mM Tris–citrate buffer. Nonspecific binding was determined by the addition of 10 μM Ro 15-1788 and typically represented 10–15% of the total binding at 6 nM [^{3}H]Ro 15-4513. Radioactivity retained by the filters was measured in an LS 6500 liquid scintillation counter (Beckman Instruments, Palo Alto, CA). Data were analyzed with Inplot4 (Intuitive Software, La Jolla, CA). Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL).

2.5. Materials

[^{3}H]Ro 15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5a][1,4]benzodiazepine-3-carboxylate) [Sp. Act. 30.4 Ci/mmol] was obtained from Dupont/NEN (Boston, MA). Diazepam and compounds with a 'Ro' prefix were donated by Hoffmann-LaRoche (Nutley, NJ). CGS 8216 (2-phenylpyrazolo[4,3-c]quinoline-3(5*H*)-one)

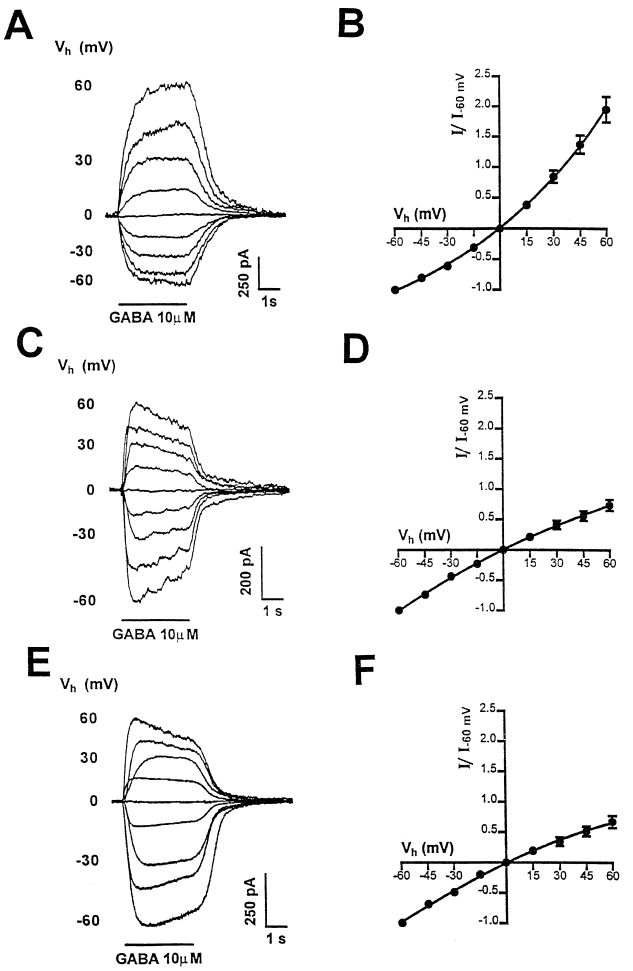

Fig. 1. Voltage-dependence of responses to GABA (10 μM) in HEK 293 cells expressing different combinations of $GABA_A$ receptor subunits. Left panels (A, C, E): Superimposed traces in response to GABA perfusion recorded from cells held at voltages between −60 and +60 mV. Cells were transfected cDNAs encoding $\alpha1\beta2\gamma2$ (A), $\alpha4\beta2\gamma2$ (C) and $\alpha6\beta2\gamma2$ (E) subunits, respectively. Right panels (B, D, F): Current–voltage relationships for responses evoked by GABA recorded from cells transfected with cDNAs encoding $\alpha1\beta2\gamma2$ (B), $\alpha4\beta2\gamma2$ (D) and $\alpha6\beta2\gamma2$ (F) subunits, respectively. Data points represent $X \pm$ S.E.M. of current amplitudes recorded from ≥ 4 cells and normalized to the peak current amplitude at −60 mV of each cell.

Table 1
Rectification ratios ($I_{+60\ mV}/I_{-60\ mV}$) in wild-type and mutant $GABA_A$ receptors

Recombinant $GABA_A$ receptor	Rectification ratio
$\alpha1\beta2\gamma2$	1.95 ± 0.21
$\alpha1(His^{274})\beta2\gamma2$	1.02 ± 0.12[a]
$\alpha4\beta2\gamma2$	0.74 ± 0.09[a]
$\alpha4(Asn^{273})\beta2\gamma2$	1.92 ± 0.17
$\alpha6\beta2\gamma2$	0.67 ± 0.11[a]

HEK 293 cells were transfected with cDNAs encoding wild-type and mutant $GABA_A$ receptor subunits as described in Section 2. Values represent $X \pm$S.E.M. of 4–5 cells.
[a] $P < 0.01$ compared to receptors composed of either $\alpha1\beta2\gamma2$ or $\alpha4(Asn^{273})\beta2\gamma2$ subunits (Newman–Keuls post-hoc test).

was obtained from Ciba-Geigy (Summit, NJ). Other materials were obtained from standard commercial sources.

3. Results

3.1. Current–voltage relationships in wild-type diazepam-sensitive and -insensitive $GABA_A$ receptors

Current–voltage (I–V) relationships were obtained by measuring peak Cl^- current upon fast GABA (10 μM) perfusion during large voltage pulses ranging from -60 mV to $+60$ mV. With symmetrical intracellular and extracellular Cl^- concentrations (146 mM), GABA-induced currents reversed close to the theoretical Cl^- equilibrium potential (0 mV). Consistent with previous studies (Verdoorn et al., 1990), HEK 293 cells transfected with cDNAs encoding $\alpha1\beta2\gamma2$ subunits exhibited large, outwardly rectifying currents (Fig. 1a,b), with a rectification ratio ($I_{+60\ mV}/I_{-60\ mV}$) of 1.95 ± 0.21 (Table 1). On the same cells, the averaged peak current was 483 ± 62 pA at -60 mV. By comparison, anomalous outwardly rectifying currents were observed in cells expressing either $\alpha4\beta2\gamma2$ (Fig. 1c,d) or $\alpha6\beta2\gamma2$ (Fig. 1e,f) $GABA_A$ receptors. Thus, in cells transfected with cDNAs encoding $\alpha4\beta2\gamma2$ subunits, the rectification ratio was 0.74 ± 0.09, with a peak current of 448 ± 57 pA ($n = 5$) at -60 mV. Similarly the rectification ratio in cells transfected with cDNAs encoding $\alpha6\beta2\gamma2$ subunits was 0.67 ± 0.11 with a peak current of 557 ± 67 pA ($n = 4$) at -60 mV.

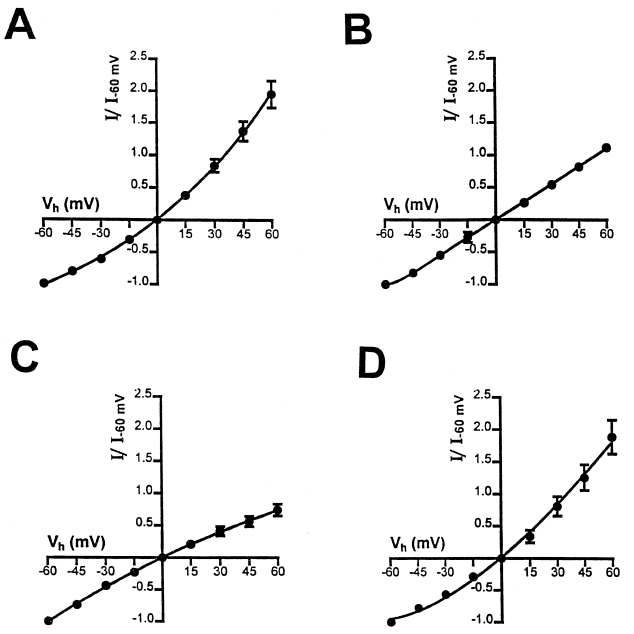

Fig. 3. Current–voltage relationships in wild type and mutated $GABA_A$ receptors. GABA-evoked currents were recorded from HEK 293 cells transfected with cDNAs encoding the specified wild type and mutated subunits. Panel A: Large, outwardly rectifying currents from cells expressing $\alpha1\beta2\gamma2$ $GABA_A$ receptors. Panel B: A dramatic reduction in outward rectifying currents was observed in cells expressing a mutated $\alpha1(His^{274})$ subunit together with $\beta2$ and $\gamma2$ subunits. Panel C: Anomalous outwardly rectifying currents in $GABA_A$ receptors composed of $\alpha4\beta2\gamma2$ subunits. Panel D: Large, outwardly rectifying currents in cells expressing an $\alpha4(Asn^{273})$ mutation together with $\beta2$ and $\gamma2$ subunits. Note that the rectification ratio in receptors composed of $\alpha4(Asn^{273})\beta2\gamma2$ subunits was virtually identical to the value obtained in wild type $\alpha1\beta2\gamma2$ receptors (Table 1). Data points represent the normalized mean current amplitudes ($\pm$S.E.M.) for 4–5 cells. Panels A and C are reproduced from Fig. 1 for clarity.

3.2. Current–voltage relationships in mutated diazepam-sensitive and -insensitive $GABA_A$ receptors

Mutation of $\alpha1(Asn^{274})$ to His^{274} on the putative extracellular loop between TM2–TM3 (Fig. 2) dramatically

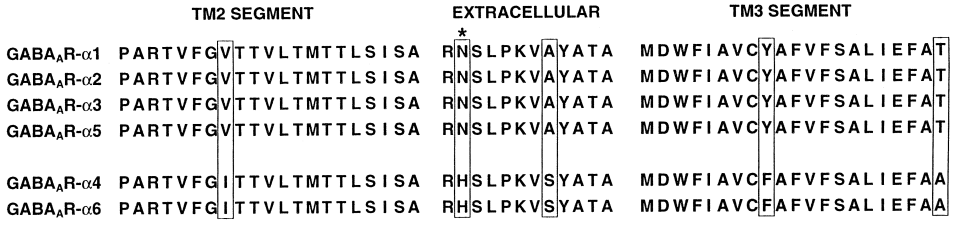

Fig. 2. Amino acid sequences of rat $GABA_A$ receptor α subunits in the vicinity of TM2–TM3. The second transmembrane spanning region (TM2) is thought to line the ion channel (8). The sequences are identical for $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunits ('diazepam-sensitive') and for $\alpha4$ and $\alpha6$ and subunits ('diazepam-insensitive'), respectively. Differences between 'diazepam-sensitive' and 'diazepam-insensitive' subunits are boxed. Asterisk shows location of the point mutations made in the $\alpha1$ and $\alpha4$ subunits. This position (corresponding to His^{273} in $\alpha4$ and His^{273} in $\alpha6$ subunits and Asn^{274} in $\alpha1$ and the homologous position for $\alpha2$, $\alpha3$, and $\alpha5$ subunits) between TM2–TM3, is near the putative channel mouth. Mutations consisted of changing His^{273} in the $\alpha4$ subunit to Asn and the Asn^{274} in the $\alpha1$ subunit to His.

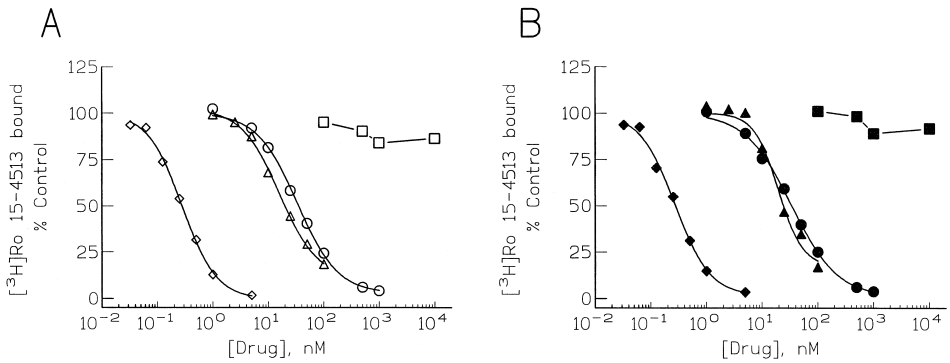

Fig. 4. Ligand affinities at recombinant $\alpha1\beta2\gamma2$ and $\alpha4\beta2\gamma2$ receptors: effect of $\alpha1(His^{274})$ and $\alpha4(Asn^{273})$ mutations. HEK 293 cells were transfected with the appropriate cDNAs for 48 h as described in Section 2. Cells were harvested and membranes incubated with [^{3}H]Ro 15-4513 (6 nM) as described. Values are the average of two experiments and are expressed as the percent of specific [^{3}H]Ro 15-4513 binding obtained in the absence of inhibitors. Panel A, Wild-type receptors: (○), Diazepam; (◇), CGS 8216 in cells expressing $\alpha1\beta2\gamma2$ subunits; (□), Diazepam; (△), CGS 8216 in cells expressing $\alpha4\beta2\gamma2$ subunits. The IC_{50} values for diazepam and CGS 8216 were 33 and 0.28 nM, respectively, in cells expressing $\alpha1\beta2\gamma2$ subunits. The IC_{50} value for CGS 8216 was 16 nM and diazepam did not produce a consistent inhibition at concentrations of up to 10 μM in cells expressing $\alpha4\beta2\gamma2$ subunits. In a typical experiment, specific [^{3}H]Ro 15-4513 binding obtained in the absence of inhibitors was 261 fmol/mg and 276 fmol/mg protein, respectively, in cells expressing $\alpha1\beta2\gamma2$ and $\alpha4\beta2\gamma2$ subunits. Panel B, Mutated receptors: (●), Diazepam; (◆), CGS 8216 in cells expressing $\alpha1(His^{274})\beta2\gamma2$ subunits; (■), Diazepam; (▲), CGS 8216 in cells expressing $\alpha4(Asn^{273})\beta2\gamma2$ subunits. The IC_{50} values for diazepam and CGS 8216 were 33 nM and 0.27 nM, respectively, in cells expressing $\alpha1(His^{274})\beta2\gamma2$ subunits. The IC_{50} value for CGS 8216 was 19 nM and diazepam did not produce a consistent inhibition at concentrations of up to 10 μM in cells expressing $\alpha4(Asn^{273})\beta2\gamma2$ subunits. In a typical experiment, specific [^{3}H]Ro 15-4513 binding obtained in the absence of inhibitors was 313 fmol/mg and 554 fmol/mg protein, respectively, in cells expressing $\alpha1(His^{274})\beta2\gamma2$ and $\alpha4(Asn^{273})\beta2\gamma2$ subunits. In a pilot experiment, IC_{50} values for CGS 8216 and diazepam were similar in cells expressing $\alpha6\beta2\gamma2$ (103 nM and >10 μM, respectively) and $\alpha6(Asn^{273})\beta2\gamma2$ subunits (88 nM and >10 μM, respectively).

reduced the large, outward rectifying currents present in cells expressing $GABA_A$ receptors containing the wild-type $\alpha1$ subunit (Fig. 3a). The rectification ratio in cells transfected with $\alpha1(His^{274})\beta2\gamma2$ was reduced by $\sim$48% to 1.02 ± 0.12, with a peak current of 432 ± 68 pA ($n = 4$) at -60 mV (Fig. 3b). By contrast, mutation of the wild-type $\alpha4$ subunit (Fig. 3c) at His^{273} (the homologous position on the putative extracellular loop between TM2–TM3) to Asn^{308} increased the rectification ratio in receptors composed of $\alpha4(Asn^{273})\beta2\gamma2$ subunits (Fig. 3d) to a value (1.92 ± 0.17; peak current, 517 ± 67 pA at -60 mV) (Table 1) similar to that obtained in receptors composed of $\alpha1\beta2\gamma2$ subunits.

3.3. Ligand binding properties of wild-type and mutated $GABA_A$ receptors

Most benzodiazepine binding site ligands exhibit dramatically lower affinities for diazepam-insensitive compared to diazepam-sensitive $GABA_A$ receptor isoforms (Lüddens et al., 1990; Wong and Skolnick, 1992; Yang et al., 1995). Consistent with previous observations (Wong et al., 1992a), diazepam binds to wild-type $\alpha1\beta2\gamma2$ receptors with high affinity (IC_{50} 33.6 nM) but does not inhibit radioligand binding to $\alpha4\beta2\gamma2$ receptors (Fig. 4a). Similarly, the pyrazoquinolinone CGS 8216 binds to diazepam-sensitive $GABA_A$ receptor isoforms with picomolar affinity (Petrack et al., 1983; Wong et al., 1992b), but is >50-fold less potent at diazepam-insensitive receptor isoforms (Gunnersen et al., 1996; Wong et al., 1992b). In receptors composed of $\alpha1\beta2\gamma2$ and $\alpha4\beta2\gamma2$ subunits, the IC_{50} values of CGS 8216 were 0.28 and 16.2 nM, respectively. The point mutations that dramatically altered the rectification properties of receptors expressing both $\alpha4\beta2\gamma2$ and $\alpha1\beta2\gamma2$ subunits had no effect on the affinities of these ligands (Fig. 4b).

4. Discussion

GABA produces large, outwardly rectifying currents in recombinant $GABA_A$ receptors composed $\alpha1\beta2\gamma2$ subunits (Verdoorn et al., 1990; Fig. 1 a,b) and cultured neurons (Adodra and Hales, 1995) that are likely to contain multiple receptor isoforms. In contrast, recombinant diazepam-insensitive $GABA_A$ receptors composed of either $\alpha4\beta2\gamma2$ (Fig. 1c,d) or $\alpha6\beta2\gamma2$ (Fig. 1e,f) subunits exhibit anomalous rectification characterized by marked reductions in current at positive potentials. This phenomenon results in rectification ratios (a relative measure of the current at depolarizing compared to hyperpolarizing potentials) $\sim$65% lower than in diazepam sensitive receptors composed of $\alpha1\beta2\gamma2$ subunits, a major $GABA_A$ receptor isoform in the mammalian central nervous system (McKernan and Whiting, 1996; Fritschy and Mohler, 1993; De Blas, 1996). These findings demonstrate that the electrophysiological properties of diazepam-sensitive and -insensitive $GABA_A$ receptors may be as divergent as the pharmacological characteristics that were initially em-

ployed to differentiate these isoforms (Lüddens et al., 1995, 1990; Malminiemi and Korpi, 1989).

Charged amino acids located in the vicinity of the putative channel lining have been reported to play a critical role in determining the rectification properties of ligand-gated ion channels (Dingledine et al., 1992; Imoto et al., 1988). Amino acid residues located in and around the putative channel lining (i.e., TM2, the second transmembrane spanning domain [Barnard, 1995]) are highly conserved among rodent $GABA_A$ receptor α subunits (Fig. 2). Thus, these sequences are identical among the α subunits (i.e., $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 5$) that can constitute diazepam-sensitive $GABA_A$ receptors, and between the $\alpha 4$ and $\alpha 6$ subunits that impart insensitivity to diazepam and related 1,4-benzodiazepines (Lüddens et al., 1995). Among the five amino acid residues in the vicinity of TM2 that differ between the groups of α subunits responsible for the characteristic pharmacology of diazepam-sensitive and -insensitive $GABA_A$ receptors, the only charge containing residue is a His (located on the putative extracellular loop between TM2–TM3) on the $\alpha 4$ and $\alpha 6$ subunits (Fig. 2).

In order to test the hypothesis that the His residue located between TM2 and TM3 contributes to the anomalous rectification properties of diazepam-insensitive $GABA_A$ receptors, this amino acid was mutated to an Asn, the residue located in the homologous position in $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits (Fig. 2). When coexpressed together with $\beta 2$ and $\gamma 2$ subunits, the $\alpha 4(Asn^{273})$ mutation exhibited a dramatic increase in rectification (Fig. 3d) compared to receptors containing the wild-type $\alpha 4$ subunit (Fig. 3c). The rectification ratio (1.92 ± 0.17) in receptors composed of $\alpha 4(Asn^{273})\beta 2\gamma 2$ subunits is virtually identical to the value obtained in receptors composed of $\alpha 1\beta 2\gamma 2$ subunits (Table 1). If this His is pivotal to the anomalous rectification observed in recombinant diazepam-insensitive receptor isoforms, then substitution of this moiety on the homologous position should blunt or abolish the large outward rectifying currents observed in wild-type diazepam-sensitive isoforms such as receptors composed of $\alpha 1\beta 2\gamma 2$ subunits. To test this hypothesis, $\alpha 1(Asn^{274})$ was mutated to His^{274}. When expressed with $\beta 2$ and $\gamma 2$ subunits, this mutation resulted in a significant reduction (Fig. 3b) in the large, outward rectifying currents compared to wild-type receptors (Fig. 3a) with a concomitant reduction in the rectification ratio to 1.02 ± 0.12 (Table 1). The rectification ratio in $\alpha 1(His^{274})\beta 2\gamma 2$ receptors is not statistically significantly different than in diazepam-insensitive $GABA_A$ receptor isoforms (Table 1, legend) indicating that Asn^{274} is critical to the large outward rectification observed in wild-type diazepam-sensitive receptors.

The affinities of 1,4-benzodiazepines such as diazepam have been useful in delineating diazepam-sensitive and -insensitive $GABA_A$ receptor isoforms. Studies in recombinant receptors have demonstrated that the potencies of these compounds are primarily determined by a His^{100} (common to $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits) and an Arg^{101} (common to $\alpha 4$ and $\alpha 6$ subunits) residue (Wieland et al., 1992; Kleingoor et al., 1993). However, a subsequent study has shown that three other extracellular residues on the α subunit can also affect the affinities of 1,4-benzodiazepines and other benzodiazepine-site ligands (Wieland and Lüddens, 1994). Based on this report, we determined whether the mutations made in the extracellular region between TM2–TM3 would affect the affinities of diazepam and CGS 8216. The affinities of these compounds in receptors composed of $\alpha 1(His^{274})\beta 2\gamma 2$ and $\alpha 4(Asn^{273})\beta 2\gamma 2$ were unchanged from the values obtained in the corresponding wild-type receptors (Fig. 4 and legend). Thus, despite the dramatic effect of these mutations on the rectification properties of diazepam-sensitive and -insensitive $GABA_A$ receptors, the pharmacological characteristics that are often employed to define these isoforms appear unchanged.

These findings suggest that a histidine residue on the extracellular loop between TM2–TM3 is responsible for the anomalous rectifying properties of recombinant diazepam-insensitive $GABA_A$ receptors. These data are consistent with previous reports demonstrating a pivotal role of charged amino acids in the vicinity of TM2 in the rectifying properties of other ligand-gated ion channels including non-NMDA receptors and nicotinic acetylcholine receptors (Dingledine et al., 1992; Imoto et al., 1988). The functional significance of the very modest outward rectification observed in diazepam-insensitive receptor isoforms is unknown, and $GABA_A$ receptors containing $\alpha 4$ and $\alpha 6$ subunits represent a very minor subpopulation of the total receptor pool (McKernan and Whiting, 1996). Nonetheless, the potential presence of $GABA_A$ receptor subpopulations with electrophysiological properties that are significantly different from those found in the predominant receptor isoform (i.e., $\alpha 1\beta 2\gamma 2$) provides yet another mechanism for modulating the signaling properties of neurons mediating the actions of the principle inhibitory transmitter in the mammalian central nervous system.

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