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# Importance of Arg-219 for correct biogenesis of α1 homooligomeric glycine receptors

Dieter Langosch*, Astrid Herbold, Volker Schmieden, Joachim Borman, Joachim Kirsch

*Max-Planck-Institut für Hirnforschung, Deutschordenstraße 46, 60528 Frankfurt, Germany*

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The inhibitory glycine receptor is characterized by a pentameric arrangement of subunits with four predicted transmembrane segments (M1–M4) each. Here, we have mutagenized arginine residues located at both termini of the α1 subunit segment, M2, which lines the receptor's anion channel. No glycine-gated channel formation could be detected in the plasma membrane of expressing cells for any of the mutants. In addition, mutating the arginine at the cytoplasmic terminus of M2 (R219) generated proteins which were only core-glycosylated, retained within intracellular compartments, and aggregated to high molecular weight complexes. Thus, residue R219, which corresponds to an arginine/lysine conserved in other ligand-gated ion channel polypeptides, is essential for correct biogenesis of the receptor.

Glycine receptor; Receptor biogenesis; Transmembrane segment

## 1. INTRODUCTION

Ligand-gated ion channel proteins are formed by a pseudo-symmetric pentameric arrangement of membrane-spanning subunits [1,2] which are made up of a large glycosylated extracellular domain followed by four predicted transmembrane segments (M1–M4) [2,3]. Of particular interest is segment M2 which has been shown to line nicotinic acetylcholine receptor (nAChR) [6,7] and glycine receptor (GlyR) [4,5] channels, and the N- and C-terminal borders of which face the cytoplasmic and extracellular membrane faces, respectively [7]. Due to the co-translational nature of polypeptide insertion into the endoplasmic reticulum (ER) membrane during biosynthesis, the final transmembrane topology of receptor subunits is determined by the orientation of the first hydrophobic segment interacting with the lipid bilayer, i.e. their cleaved N-terminal signal peptide [8]. Generally, a conserved basic charge motif is believed to retain a signal peptide's N-terminus at the cytoplasmic membrane face and thus determines the transmembrane topology of the whole polypeptide [9–11]. For internal transmembrane segments, a preferential occurrence of positively charged residues within cytoplasmic vs. extracellular flanking regions was also found [12]. Their functional role, however, is not clear [10].

In line with the latter observations, a survey of 7 GlyR, 21 $GABA_AR$, and 44 nAChR subunit sequences revealed that an arginine or lysine residue is conserved at the N-terminal, i.e. cytoplasmic terminus of the channel-forming M2, but not of the other transmembrane segments (our unpublished results). Upon mutating this residue within the GlyR α1 subunit, no glycine-gated channel function could be detected in the plasma membrane of cells expressing the mutants. This was paralleled by their retention and aggregation in intracellular compartments. These results suggest a requirement of this arginine for the correct topogenesis of receptor subunits.

*Corresponding author. Fax: (49) (69) 9676 9441.

## 2. EXPERIMENTAL

### 2.1. *Mutagenesis*

Oligonucleotide-directed mutagenesis of GlyR α1 subunit cDNA was performed as published previously [13]. Oligonucleotides used were: R219Q, 5′GCTGCACCTGCTCAAGTGGGCCTAGGC3′; R219E, 5′GCTGCACCTGCTGAAGTGGGCCTAGGC3′; R238Q, 5′GCTGCACCTGCTCAAGTGGGCCTAGGC3′; and R238E, 5′GCTGCACCTGCTGAAGTGGGCCTAGGC3′. For oocyte injection, cRNA was transcribed from the plasmids linearized with *Eco*RV (Stratagene mCAP kit). For subcloning into the eukaryotic expression vector, pCIS2 [14], the 0.7 kB *Apa*I fragment of pCIS2 harbouring the wild-type GlyR α1 sequence was replaced by the mutated *Apa*I fragments. The sequences of the subcloned fragments were verified by dideoxysequencing.

### 2.2. *Heterologous expression in HEK-293 cells*

HEK-293 cells (ATCC CRL 1573) were transfected by calcium phosphate precipitation [15] using ca. 2 μg of plasmid DNA per $10^6$ cells. Where indicated, tunicamycin (Sigma) dissolved in ethanol was added at 1 μg/ml immediately after transfection and again after 15 h. Cells were harvested 40 h after transfection. For immunocytochemistry, transfected cells were grown on fibronectin-coated glass coverslips (Menzel #1, Germany, 14 mm diam.) and processed for immunofluorescent detection of the recombinant proteins with mAb4a as described [16].

### 2.3. *Electrophysiology*

*Xenopus laevis* oocytes injected with cRNA or transfected HEK-293 cells were analyzed for glycine-gated whole-cell currents as described [5,17].

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2.4. *Biochemical procedures*

Preparation of membranes and solubilisation was done according to [4]. Combined P1/P2 pellets were collected from the homogenized cells by 15 min centrifugation at 10,000 × *g*, P1 pellets by 3 min centrifugation at 1,000 × *g*, and P2 pellets by 15 min centrifugation of the S1 supernatant at 10,000 × *g*. Membranes were solubilized with Triton X-100 as described [18].

For endoglycosidase H digestion, the solubilized P1/P2 pellets (100 μg total protein) were precipitated with methanol and the precipitates dissolved in 0.5% (w/v) SDS, 1% (v/v) β-mercaptoethanol for 15 min at 95°C. 0.1 vol. of 0.5 M sodium citrate, pH 5.5, and 2,500 U of endoglycosidase H (Biolabs) were added and the digestion performed for 2 h at 37°C. The reactions were stopped by the addition of SDS sample buffer.

Detergent extracts of P2 pellets were fractionated by sedimentation on 5–20% sucrose gradients underlayered with a 60% sucrose cushion [1,18] in a SW41 rotor at 41,000 rpm for 24 h. Fractions of 1 ml each were analysed for GlyR α subunit immunoreactivity by a dot-blot immuno-assay using mAb4a [19]. The position of the following marker proteins run in parallel was determined by standard enzymatic assays: β-galactosidase, 15.92 S; catalase, 11.3 S; lactate dehydrogenase, 6.95 S; malate dehydrogenase, 4.32 S; cytochrome *c*, 1.8 S.

Western-blotting with GlyR mAb4a was performed as described [1].

2.5. *Confocal microscopy*

HEK-293 cells expressing the GlyR were immunolabelled as described in section 2.2. and analyzed with a Sarastro 2000 confocal microscope (Molecular Dynamics). Optical sections of 0.1 μm pixel size were scanned. A Gaussian filter with 3 × 3 × 3 kernel size was applied to the primary data. After background subtraction, the signals were amplified by a factor of 1.5 for better visualization of membrane labelling.

## 3. RESULTS

To examine the functional role of arginine residues bordering the M2 segment of GlyR α1 subunits, we mutated these residues and compared channel function, state of glycosylation, subcellular localization, and oligomeric state of the mutants with wild-type (wt) receptor.

### 3.1. *Channel function*

Expression of wt α1 subunit in *Xenopus* oocytes or HEK-293 cells generates functional GlyRs [5,17]. To functionally assay α1 mutants generated by exchanges at the cytoplasmic (R219) or extracellular (R238) termini of the M2 segment, cRNAs of the α1 subunit single point mutants (R219Q, R219E, R238Q, R238E) or double-mutants (R219Q/R238Q, R219E/R238Q, R219Q/R238E, R219E/R238E) were injected into oocytes. Alternatively, cDNAs of mutants R219Q, R219E, R238Q, or R238E subcloned into a eukaryotic expression vector were transiently transfected into HEK-293 cells. Whole-cell recordings under voltage-clamp conditions revealed that none of the mutants responded to glycine application (not shown). As control oocytes or cells expressing wt α1 subunit showed large glycine-gated currents (not shown), we conclude that all mutations rendered the α1 subunit incapable of forming functional GlyR channels in the plasma membrane.

### 3.2. *Glycosylation state*

Membrane preparations of HEK-293 cells expressing either wt α1 subunit or mutants R219Q, R219E, R238Q, or R238E were analysed by Western blotting. Fig. 1A shows that R238 mutants (lanes 4,5) were expressed with efficiencies and subunit molecular weights comparable to those of the wt α1 subunit (lane 1). In contrast, less intense staining, a smaller apparent molecular weight, and a micro-heterogenous appearance were observed for the R219 mutants (lanes 2,3). To test whether these altered properties reflect incomplete processing of the attached sugar moieties, wt α1 GlyR and R219 mutants were solubilized from transfected cell membranes and compared in their sensitivity to endoglycosidase H. The electrophoretic migration behaviour of the vast majority of the wt α1 subunit protein was unaltered upon glycosidase digestion (Fig. 1B, lanes 1,2), as expected for a fully processed glycoprotein residing in the plasma membrane. In contrast, protein bands corresponding to the R219 mutants were shifted to significantly lower apparent molecular weights (lanes 3–6), corresponding to that of unglycosylated wt α1 subunit obtained by expression in the presence of tunicamycin (lane 7). Tunicamycin treatment of cells expressing the R219 mutants resulted in the same lower

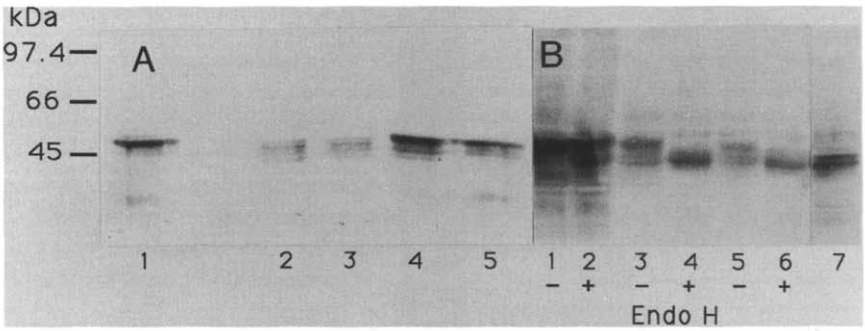


Fig. 1. Heterologous expression of GlyR mutants in HEK-293 cells as analysed by Western blot analysis. (A) Apparent molecular weights. Note that the dominant band representing mature polypeptides with wt α1 subunit (lane 1) or mutants R238Q (lane 4) and R238E (lane 5) is lacking with mutants R219Q (lane 2) and R219E (lane 3). (B) Endoglycosidase H sensitivity. Whereas the major fraction of wt α1 subunit (lanes 1,2) was resistant to digestion, mutants R219Q (lanes 3,4) and R219E (lanes 5,6) were quantitatively digested to the apparent molecular weight of the wt α1 subunit expressed in the presence of tunicamycin (lane 7).

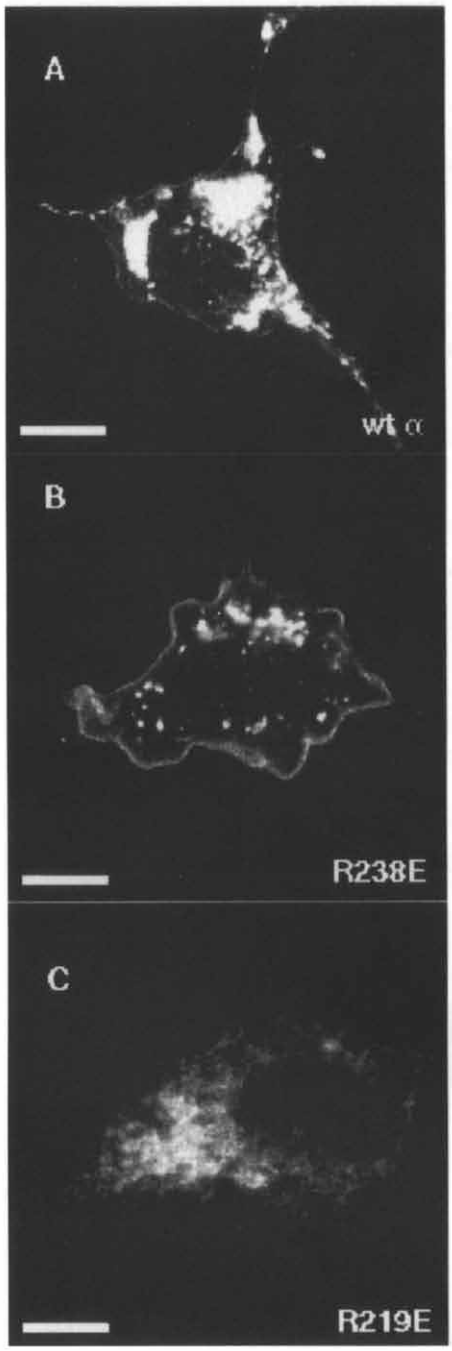


Fig. 2. Immunocytochemical detection of GlyR in transfected HEK-293 cells. The single optical sections show that wt α1 subunit (A) and mutants R238E (B) or R238Q (not shown) were found both in intracellular vesicles and the plasma membrane. No membrane staining was detected in cells expressing mutants R219E (C) and R219Q (not shown). These latter mutants displayed a reticular staining pattern reminiscent of ER labelling. Bars = 10 μm.

apparent molecular weight, corroborating that their altered electrophoretic migration behavior was due to smaller carbohydrate chains (not shown). Thus, the R219 mutants appeared to be only core-glycosylated, i.e. presumably retained within the ER of the cell.

### 3.3. *Subcellular localization*

Confocal microscopy of transfected HEK-293 cells revealed that wt α1 GlyR and the R238 mutants were primarily localized to intracellular vesicles (Fig. 2 and not shown). These vesicles most likely corresponded to Golgi and/or post-Golgi compartments. Residence of receptor protein within the cell's plasma membrane could also be demonstrated by significant mAb4a immunoreactivity continuously lining the cell's circumference (Fig. 2A and B). By contrast, in cells transfected with the R219 mutants, only intracellular labelling was observed (Fig. 2C and not shown). These cells displayed a fine reticular immunostaining pattern reminiscent of ER labelling. No membrane labelling was detected.

### 3.4. *Oligomeric state*

Solubilized membranes from transfected HEK-293 cells were fractionated by sucrose gradient centrifugation, and the fractions analyzed immunochemically for GlyR distribution. The sedimentation profiles given in Fig. 3 reveal that wt α1 receptors and the R238 mutants migrated at 7.9 S, a sedimentation value comparable to that of native GlyR (7.8 S) [1]. In contrast, only a minority of the R219 mutants migrated at this position. These antigens preferentially sedimented to the bottom fractions of the gradients, indicating high molecular weight aggregate formation.

## 4. DISCUSSION

Our results demonstrate that mutating arginine residues bordering transmembrane segment M2 of the GlyR α1 subunit profoundly affects the function, structure, and subcellular localization of recombinant receptors. Mutating R219, cytoplasmic to M2, generated proteins which were sensitive to endoglycosidase H, i.e. only core-glycosylated [20]. Cells expressing the R219 mutants lacked GlyR-specific immunoreactivity in the plasma membrane but exhibited an intracellular staining pattern reminiscent of reticular ER staining. Together, we interprete these data to mean that these mutants are retained within the ER. Further, they exhibited a principal ability for correct oligomerisation but strongly tended to high molecular weight aggregate formation; the latter property might reflect complexation with the molecular chaperone, BiP, as demonstrated for other oligomeric membrane proteins under conditions impeding subunit maturation [21–23]. Upon mutating R238, extracellular to M2, subcellular localization and sedimentation behaviour were indistinguishable from wt GlyR. Therefore, these latter mutants appear to oligomerize correctly and are routed to the plasma membrane. With both types of mutants, the glycine-gated currents seen with heterologously expressed wt α1 subunit [5,17] were completely abolished. For R219 mutants, this finding is explained by their absence from the plasma membrane. Mutating residue R238, on the other

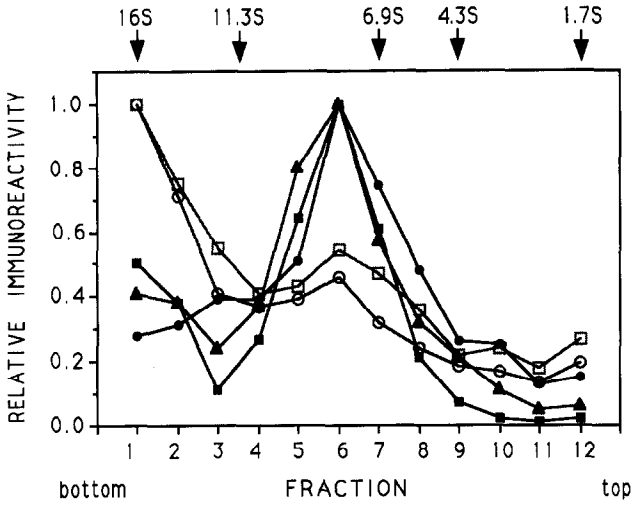


Fig. 3. Hydrodynamic properties. On 5–20% sucrose gradients, the mutant R238Q (■) and R238E (▲) proteins co-migrated with the wt α1 receptor (●) whereas the majority of mutants R219Q (○) and R219E (□) sedimented to the bottom of the gradients. The positions of marker proteins run in a parallel gradient are indicated by arrows.

hand, appears to interfere directly with GlyR channel function.

What could be the reason for the retention of R219 mutants in intracellular compartments? For other membrane proteins, specific transmembrane segments have been shown to direct their sorting to Golgi [24] or nuclear [25] compartments. It is conceivable that the M2 segment of ligand-gated ion channel proteins subserves such a sorting function to the plasma membrane, which is disrupted by altering its primary structure. This possibility is considered unlikely, however, as the M2 segment is the primary constituent of the GlyR's central ion channel [4,5] and as such should be inaccessible to recognition by an external 'sorting receptor'. Alternatively, 'helical hairpin' formation between adjacent transmembrane segments may be required for insertion into the ER membrane [26]. Assuming that salt-bridge formation may be essential to stabilize such a hairpin structure, we inspected homologs of the GlyR α1 sequence for conserved negatively charged residues possibly interacting with a positive charge at the N-terminus of segment M2. A negative residue was found at the C-terminus of segment M3 (GlyR α1 E300) in all ligand-gated anion channel subunits (28 GlyR and $GABA_AR$ sequences) and close to the N-terminus of segment M4 (GlyR α1 D488) in all ligand-gated anion/cation channel subunits (72 GlyR, $GABA_AR$, and nAChR sequences) examined. Ion bonding of these residues with the positive cytoplasmic end of M2 might thus stabilize interaction between M2 and M3 or M2 and M4, respectively. To invert this hypothesized ion pair, we constructed the double-mutants GlyR α1 R219E/E300R and R219E/D488R. However, high-molecular weight aggregate formation and retention in intracellular compartments of these double-mutants were indistinguishable from the behaviour of mutant R219E (unpublished results). Thus, we consider the above hypothesis unlikely to account for our observations. On the other hand, a recent model study revealed that the efficiency of weakly hydrophobic sequences as stop–transfer signals is dramatically enhanced in the presence of positively, but not of negatively, charged amino acids at their cytoplasmic termini [27,28]. Intriguingly, the M2 segments are the least hydrophobic transmembrane domains of ligand-gated ion channel proteins due to the accumulation of hydroxylated side-chains facilitating ion flux [29]. We may speculate, therefore, that GlyR α1 R219 is required for the efficient stop of segment M2 transfer through the lipid bilayer during polypeptide synthesis. This would be consistent with the stringent conservation of arginine/lysine at this position throughout all GlyR, $GABA_AR$, and nAChR subunit sequences we have examined. Interestingly, a conservation of this type is not observed within the subunits of AMPA, kainate, or NMDA subtypes of the ionotropic glutamate receptor [30,31]; this deviation from the ligand-gated ion channel proteins mentioned above may be related to a different transmembrane topology of glutamate receptor subunits [32].

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