Identification of intracellular and extracellular domains mediating signal transduction in the inhibitory glycine receptor chloride channel

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Fast synaptic neurotransmission is mediated by transmitter-activated conformational changes in ligand-gated ion channel receptors, culminating in opening of the integral ion channel pore. Human hereditary hyperekplexia, or startle disease, is caused by mutations in both the intracellular or extracellular loops flanking the pore-lining M2 domain of the glycine receptor $\alpha 1$ subunit. These flanking domains are designated the M1–M2 loop and the M2–M3 loop respectively. We show that four startle disease mutations and six additional alanine substitution mutations distributed throughout both loops result in uncoupling of the ligand binding sites from the channel activation gate. We therefore conclude that the M1–M2 and M2–M3 loops act in parallel to activate the channel. Their locations strongly suggest that they act as hinges governing allosteric control of the M2 domain. As the members of the ligand-gated ion channel superfamily share a common structure, this signal transduction model may apply to all members of this superfamily.

Keywords: β -alanine/glycine/ligand-gated ion channel superfamily/signal transduction/taurine

Introduction

Fast synaptic neurotransmission in the mammalian central nervous system is mediated by members of the ligand-gated ion channel (LGIC) superfamily, which includes receptors for acetylcholine, GABA_A, serotonin and glycine (Grenningloh *et al.*, 1987; Schofield *et al.*, 1987; Maricq *et al.*, 1991). These channels, which are located in the post-synaptic membrane, are opened by pre-synaptically released neurotransmitter and permit an ionic flux which changes the membrane potential. Receptors of this family consist of five similar membrane-spanning subunits arranged in a ring to form a central ion-conducting pore. The subunits of the different receptor types each comprise a large N-terminal extracellular domain, which contains the ligand binding sites and four putative membrane spanning domains (M1–M4), the second of which forms the channel-lining α -helix (reviewed in Devillers-Thiery *et al.*, 1993; Karlin and Akabas, 1995). Since the ligand binding domain is distant from the M2 region (Valenzuela *et al.*, 1994; Unwin, 1995), channel activation requires

long-range allosteric interactions. Although the functional molecular architecture of the ligand binding region and the ion channel pore has been intensively investigated, little is known about the transduction mechanism linking these domains.

Analysis of mutations underlying the human hereditary disorder hyperekplexia (or startle disease) initially identified one residue involved in gating of the glycine receptor (GlyR) chloride channel. Two separate point mutations at the same base pair in the gene encoding the human GlyR $\alpha 1$ subunit resulted in the arginine at position 271 (R271) being substituted by either a leucine (R271L) or a glutamine (R271Q) (Shiang *et al.*, 1993). R271 lies at the external vestibule of the ion channel pore and forms part of the extracellular loop linking the second and third membrane-spanning domains (the M2–M3 loop). The physiological effect of these mutations is to reduce the glycine efficacy and single channel conductance of recombinant homomeric and heteromeric GlyRs (Langosch *et al.*, 1994; Rajendra *et al.*, 1994, 1995a). In addition, both mutations converted β -alanine and taurine from full agonists into competitive antagonists without dramatically affecting their binding affinities (Laube *et al.*, 1995; Rajendra *et al.*, 1995a). As these results are consistent with an uncoupling of the agonist binding process from the channel activation gate, it was concluded that R271 formed a crucial component of the allosteric signal transduction pathway (Langosch *et al.*, 1994; Rajendra *et al.*, 1994, 1995a; Lynch *et al.*, 1995).

Additional startle disease mutations have recently been identified in the M2–M3 loop (Y279C, Shiang *et al.*, 1995; K276E, Elmslie *et al.*, 1996) and in the intracellular M1–M2 loop (I244N, Rees *et al.*, 1995). We show here that the Y279C and K276E mutations, like the R271L and R271Q mutations, convert taurine and β -alanine into competitive antagonists with little disruption to their binding affinities. The I244N mutation also results in a dramatic reduction in taurine efficacy. These results demonstrate that the startle disease mutations may act by disrupting the allosteric transduction (or gating) process between ligand binding and channel activation and raise the possibility that the M1–M2 loop and the M2–M3 loop act as the structural components mediating this process.

To investigate this possibility, we constructed sequential alanine substitution mutants of all residues in the M1–M2 intracellular loop and the M2–M3 extracellular loop and for each mutant GlyR measured the binding affinities of taurine and β -alanine and their agonist efficacies relative to glycine. Since numerous mutations in both domains did indeed reduce or eliminate the taurine and β -alanine efficacies with minimal change to their binding affinities, we conclude that both domains act in parallel to activate the channel. The locations of these domains suggest they act as hinges to allow the M2 domain to move with

Fig. 1. Examples of agonist-activated currents (A–D) and averaged dose–response curves (E) in cells expressing GlyRs incorporating naturally occurring human startle disease mutations. (A) Currents activated by 100 mM glycine, β -alanine and taurine for the I244N, K276E and Y279C mutant GlyRs. (B) Complete inhibition by taurine of currents activated by a half-maximal concentration (2 mM) of glycine for the L276E mutant GlyR. (C) Partial inhibition by taurine of currents activated by a half-maximal concentration (500 μ M) of glycine for the I244N mutant GlyR. (D) Examples of glycine dose–response curves for I244N, K276E and Y279C mutant GlyRs. Numbers above each trace indicate glycine concentration in mM. (E) Averaged dose–response curves for currents activated by glycine (upper panel), β -alanine (centre) and taurine (lower panel) for wild-type GlyRs (●) and for GlyRs expressing human startle disease mutations where β -alanine and taurine have a reduced efficacy (open symbols) or no efficacy (shaded symbols). In this and all subsequent figures, the glycine dose–response curves are normalized to 1 and the β -alanine and taurine dose–response curves are normalized to the maximal glycine-activated current for each mutant GlyR. The data for the R271L and R271Q mutant GlyRs in (E) and Figure 4 are reproduced from Rajendra *et al.* (1995a).

respect to the rest of the protein. Additionally, we show that two intracellular mutations (W243A and I244A) also dramatically increase the desensitization rate, suggesting they form a link between the mechanisms mediating channel activation and desensitization.

Results

Human startle disease mutations act by disrupting transduction

All experiments described in this report were performed on human $\alpha 1$ homomeric GlyRs recombinantly expressed in mammalian HEK 293 cells. Taurine and β -alanine act as full agonists of human $\alpha 1$ GlyRs when expressed in this system (Pribilla *et al.*, 1992; Rajendra *et al.*, 1995a,b), although they act as partial agonists of the same GlyRs when expressed in *Xenopus* oocytes (Schmieden *et al.*, 1992; Laube *et al.*, 1995).

We sought to investigate the mechanism by which the following recently identified startle disease mutations

disrupt the function of the human $\alpha 1$ GlyR homomer: I244N (Rees *et al.*, 1994), K276E (Elmslie *et al.*, 1996) and Y279C (Shiang *et al.*, 1995). For each mutant GlyR, we examined the agonist efficacies of taurine and β -alanine relative to glycine, the concentration of each agonist required for half-maximal current activation (EC_{50}) and, in mutant GlyRs where β -alanine and taurine exhibited partial or no agonist efficacy, the concentration required for half-maximal inhibition of glycine-gated currents (IC_{50}). Sample electrophysiological responses from cells expressing these mutant GlyRs are shown in Figure 1A–D, averaged dose–response curves are shown in Figure 1E and the results of all electrophysiological measurements are summarized in Table I. The binding properties of the competitive antagonist [3 H]strychnine were also examined with the dissociation constant (K_d), number of expressed receptors (B_{max}) and inhibition constants (K_i) for glycine, β -alanine and taurine displacement of bound [3 H]strychnine being determined for wild-type and mutant GlyRs. Averaged results from all binding experiments are summar-

Table I. Functional properties of mutant GlyRs as determined by electrophysiological recording

GlyR	Phenotype	Glycine activation		β -alanine activation		Taurine activation		β -alanine inhibition		Taurine inhibition	
		EC ₅₀ (μ M)	$I_{\max}$ (nA)	EC ₅₀ (μ M)	Max $I_{\beta\text{-ala}}/I_{\text{gly}}$	EC ₅₀ (μ M)	Max $I_{\text{tau}}/I_{\text{gly}}$	IC ₅₀ (μ M)	Min $I_{\beta\text{-ala}}/I_{\text{gly}}$	IC ₅₀ (μ M)	Min $I_{\text{tau}}/I_{\text{gly}}$
Wild-type	wild-type	18 $\pm$ 2	10.0 $\pm$ 2.5	52 $\pm$ 4	0.99 $\pm$ 0.01	153 $\pm$ 43	1.07 $\pm$ 0.06	^a	1 $\pm$ 0	^a	1 $\pm$ 0
Disease											
I244N	partial	274 $\pm$ 44	0.41 $\pm$ 0.09	396, 502	0.85 $\pm$ 0.03	2210 $\pm$ 460	0.50 $\pm$ 0.04	^a	1 $\pm$ 0	13500 $\pm$ 620	0.56 $\pm$ 0.04
R271L	complete	6700 $\pm$ 1500	5.7 $\pm$ 2.8	^a	0 $\pm$ 0	^a	0 $\pm$ 0	^b	0 $\pm$ 0	^b	0 $\pm$ 0
R271Q	complete	12000 $\pm$ 600	1.8 $\pm$ 0.6	^a	0 $\pm$ 0	^a	0 $\pm$ 0	^b	0 $\pm$ 0	^b	0 $\pm$ 0
K276E	complete	1820 $\pm$ 68	1.1 $\pm$ 0.7	^a	0 $\pm$ 0	^a	0 $\pm$ 0	367 $\pm$ 21	0 $\pm$ 0	276 $\pm$ 52	0 $\pm$ 0
Y279C	complete	1310 $\pm$ 380	0.19 $\pm$ 0.03	^a	0 $\pm$ 0	^a	0 $\pm$ 0	^a	0 $\pm$ 0	^a	0 $\pm$ 0
M1–M2 loop											
W243A	partial	114 $\pm$ 9	1.47 $\pm$ 0.40	2100 $\pm$ 570	0.82 $\pm$ 0.05	2940 $\pm$ 490	0.46 $\pm$ 0.03	^a	^a	^b	0.56
I244A	partial	719 $\pm$ 141	1.23 $\pm$ 0.45	3010 $\pm$ 360	0.54 $\pm$ 0.03	^a	0.20 $\pm$ 0.04	^b	^b	1066 $\pm$ 72	0.32 $\pm$ 0.10
N245A	wild-type	20, 17	3.7 $\pm$ 1.1	69 $\pm$ 9	0.96 $\pm$ 0.02	131 $\pm$ 26	0.97 $\pm$ 0.05	^a	^a	^a	^a
M246A	partial	125 $\pm$ 15	2.34 $\pm$ 0.41	880 $\pm$ 230	0.92 $\pm$ 0.03	810 $\pm$ 90	0.69 $\pm$ 0.03	^a	^a	^b	0.71, 0.73
D247A	wild-type	19 $\pm$ 4	2.21 $\pm$ 0.41	60 $\pm$ 17	0.98 $\pm$ 0.01	146 $\pm$ 24	0.99 $\pm$ 0.02	^a	^a	^a	^a
P250A	wild-type	27 $\pm$ 5	2.89 $\pm$ 0.56	37, 42	1.02 $\pm$ 0.04	83, 89	0.98 $\pm$ 0	^a	^a	^a	^a
M2–M3 loop											
S270A	wild-type	22 $\pm$ 3	2.99 $\pm$ 0.82	42 $\pm$ 4	0.99 $\pm$ 0.03	153 $\pm$ 42	0.95 $\pm$ 0.06	^a	^a	^a	^a
R271A	complete	3170 $\pm$ 500	9.5 $\pm$ 1.1	^a	0.03 $\pm$ 0.01	^a	0.01 $\pm$ 0	401 $\pm$ 57	0.02 $\pm$ 0	983 $\pm$ 330	0 $\pm$ 0
S273A	wild-type	27 $\pm$ 4	4.43 $\pm$ 0.63	51 $\pm$ 4	1.01 $\pm$ 0.05	221 $\pm$ 11	0.99 $\pm$ 0.03	^a	^a	^a	^a
L274A	partial	465 $\pm$ 100	3.41 $\pm$ 0.51	2100 $\pm$ 450	0.75 $\pm$ 0.04	12500 $\pm$ 3600	0.39 $\pm$ 0.05	708 $\pm$ 226	0.69 $\pm$ 0.06	1140 $\pm$ 470	0.24 $\pm$.07
P275A	wild-type	27 $\pm$ 4	2.47 $\pm$ 0.62	50 $\pm$ 8	1.00 $\pm$ 0.01	66 $\pm$ 9	0.96 $\pm$ 0.02	^a	^a	^a	^a
K276A	complete	1020 $\pm$ 180	3.06 $\pm$ 0.80	^a	0.08 $\pm$ 0.03	^a	0.03 $\pm$ 0.01	338, 456	0.25, 0.19	562 $\pm$ 113	0.10 $\pm$.03
V277A	complete	1920 $\pm$ 450	2.83 $\pm$ 0.51	^a	0 $\pm$ 0	^a	0 $\pm$ 0	712 $\pm$ 149	0 $\pm$ 0	323 $\pm$ 14	0 $\pm$ 0
S278A	partial	104 $\pm$ 15	3.63 $\pm$ 0.32	1240 $\pm$ 380	0.84 $\pm$ 0.03	2830 $\pm$ 910	0.49 $\pm$ 0.05	^a	0.90 $\pm$.02	8800 $\pm$ 1800	0.49 $\pm$ 0.08
Y279A	complete	1400, 1930	0.035, 0.04	^a	0, 0	^a	0, 0	^a	0, 0	^a	0, 0
V280A	wild-type	21 $\pm$ 4	1.45 $\pm$ 0.34	53 $\pm$ 9	0.96 $\pm$ 0.04	130 $\pm$ 25	0.89 $\pm$ 0.07	^a	^a	^a	^a
K281A	partial	40 $\pm$ 4	3.89 $\pm$ 0.85	177 $\pm$ 6	0.97 $\pm$ 0.02	442 $\pm$ 205	0.80 $\pm$ 0.02	^a	^a	^a	0.73, 0.75
I283A	wild-type	24 $\pm$ 8	2.88 $\pm$ 0.47	48 $\pm$ 8	1.01 $\pm$ 0.01	98 $\pm$ 23	0.95 $\pm$ 0.02	^a	^a	^a	^a
R271											
R271N	partial	480 $\pm$ 130	6.5 $\pm$ 0.8	810 $\pm$ 100	0.46 $\pm$ 0.09	1480 $\pm$ 380	0.17 $\pm$ 0.03	^b	0.40 $\pm$ 0.04	^b	0.14 $\pm$ 0.03
R271K	partial	840 $\pm$ 150	10.2 $\pm$ 1.1	760 $\pm$ 70	0.36 $\pm$ 0.07	2740 $\pm$ 730	0.08 $\pm$ 0.03	^b	^b	^b	^b
R271H	partial	340 $\pm$ 70	8.9 $\pm$ 1.3	1200 $\pm$ 220	0.29 $\pm$ 0.06	2600, 5800	0.07 $\pm$ 0.02	^b	^b	^b	^b

Values are given as mean $\pm$ SE in cases where $n \geq 3$. In cases where $n < 3$, individual values are shown. Min $I_{\beta\text{-ala}}/I_{\text{gly}}$ and Min $I_{\text{tau}}/I_{\text{gly}}$ represent the mean proportional glycine-gated currents remaining in the presence of 100 mM β -alanine or taurine respectively. Hill coefficients are not displayed as there was no significant difference in Hill coefficient values between mutant and wild-type GlyRs. In both Tables I and II data for R271L and R271Q mutant GlyRs only are reproduced from Rajendra *et al.* (1995a).

^aParameters that could not be quantified as currents were either non-existent or too small to measure.

^bNot determined.

ized in Table II. Hill coefficients were determined for all electrophysiological and binding experiments. However, since the Hill coefficients for all mutant GlyRs were not significantly different from wild-type GlyR values, they have been omitted from Tables I and II for reasons of brevity.

In the K276E and Y279C mutant GlyRs, concentrations of up to 100 mM of either taurine or β -alanine failed to activate detectable currents, although glycine-gated currents were readily observed (Figure 1A and Table I). Indeed, both mutations converted taurine and β -alanine into full antagonists of currents activated by half-saturating concentrations of glycine. An example of taurine inhibition of currents activated by 2 mM glycine in the K276E mutant GlyR is shown in Figure 1B. Averaged IC₅₀ values for taurine and β -alanine inhibition of glycine-gated currents in the K276E mutant GlyR are shown in Table I. We also confirmed that 100 mM taurine or β -alanine completely inhibited glycine-gated currents in the Y279C mutant GlyR ($n = 3$ for each), but as glycine-gated currents were particularly small in this mutant GlyR (Table I), the IC₅₀ values could not be measured precisely.

The K276E and Y279C mutations also resulted in large (73- to 100-fold) increases in the glycine EC₅₀ with respect to the wild-type value (Figure 1D and E and Table I). However, for both mutant GlyRs, the strychnine K_d and $B_{\max}$ values were not significantly different to wild-type values (Table II), suggesting that the mutations did not induce non-specific alterations in the structure of the N-terminal domain or reduce the efficiency of GlyR expression. The ability of bound glycine to displace [³H]strychnine was, however, significantly diminished in both K276E and Y279C mutant GlyRs, although the changes in taurine and β -alanine K_i values were less dramatic (Table II). All of these measured properties indicate that the K276E and Y279C mutant GlyRs exhibit a similar phenotype to that of the R271L and R271Q mutant GlyRs. It may therefore be concluded that each of these startle disease mutations acts in a similar manner to disrupt the ability of the receptor to convert agonist binding into channel activation. For brevity, this set of properties is designated the ‘complete’ disruption phenotype, as the transduction mechanism for taurine and β -alanine is completely disrupted. As each of the mutated

Table II. Binding properties of mutant GlyRs

GlyR	Phenotype	^{[3]H} strychnine binding			Glycine displacement			β-alanine displacement			Taurine displacement		
		K _d (nM)	B _{max} (×10 ⁶)	n	K _i (μM)	Fold increase	n	K _i (μM)	Fold increase	n	K _i (μM)	Fold increase	n
Filtration													
Wild-type	wild-type	8.4 ± 0.4	1.32 ± 0.19	5	79 ± 111	1	9	115 ± 16	1	5	177 ± 27	1	4
R271Q	complete	9.0 ± 0.9	1.65 ± 0.26	4	9600 ± 1900	121	6	1357 ± 100	12	3	818 ± 70	5	3
R271L	complete	14.4 ± 2.2	3.91 ± 0.58	3	4840 ± 730	61	6	1547 ± 260	13	3	1190 ± 140	7	3
R271N	partial	9.9 ± 0.6	2.10 ± 0.21	3	890 ± 160	11	3	580 ± 41	5	3	536 ± 96	3	3
R271A	complete	8.7 ± 1.2	1.83 ± 0.98	3	4000 ± 800	51	4	1390 ± 130	12	4	1210 ± 80	7	4
R271K	partial	10.6 ± 0.4	2.18 ± 0.26	4	162 ± 23	2	5	126 ± 27	1	4	246 ± 62	1.4	5
R271H	partial	9.8 ± 1.0	1.24 ± 0.32	4	1258 ± 140	16	4	884 ± 109	8	3	913 ± 94	5	4
Y279C	complete	9.9 ± 1.8	1.18 ± 0.25	3	213 ± 12	3	3	76 ± 6	0.7	3	100 ± 4	0.6	3
Centrifugation													
Wild-type	wild-type	18.2 ± 1.9	1.18 ± 0.11	3	33 ± 3	1	3	64 ± 11	1	3	145 ± 21	1	3
W243A	partial	23.1 ± 11.3	0.14 ± 0.06	3	42 ± 8	1.3	3	^a			^a		
I244A	partial	18.7 ± 0.7	0.50 ± 0.26	3	23 ± 4	0.7	4	29 ± 7	0.5	3	92 ± 39	0.6	3
I244N	partial	binding not detected											
N245A	wild-type	22.9 ± 3.7	1.71 ± 0.19	3	53 ± 23	1.6	3	^b			^b		
M246A	partial	18.4 ± 1.0	0.95 ± 0.10	3	50 ± 18	1.5	3	72 ± 9	1.1	2	228 ± 32	1.6	2
D247A	wild-type	25.0 ± 3.0	0.77 ± 0.06	3	56 ± 5	1.7	3	^b			^b		
P250A	wild-type	20.7 ± 1.7	1.48 ± 0.57	3	24 ± 2	0.7	3	^b			^b		
S270A	wild-type	25.5 ± 3.6	0.91 ± 0.32	3	44 ± 7	1.3	3	^b			^b		
S273A	wild-type	23.1 ± 2.1	1.48 ± 0.24	3	47 ± 7	1.4	3	^b			^b		
L274A	partial	18.8 ± 1.8	1.34 ± 0.51	3	263 ± 87	8	4	208 ± 111	3	3	384 ± 87	2.6	4
P275A	wild-type	34.5 ± 4.8	0.78 ± 0.28	3	39 ± 4	1.2	3	^b			^b		
K276A	complete	16.7 ± 2.0	0.49 ± 0.15	4	503 ± 82	15	6	276 ± 22	4	3	421 ± 18	2.9	3
K276E	complete	18.6 ± 1.5	1.13 ± 0.44	3	405 ± 58	12	4	200 ± 10	3	3	365 ± 19	2.5	3
V277A	complete	14.9 ± 1.5	0.85 ± 0.29	3	251 ± 33	8	4	132 ± 26	2	4	264 ± 76	1.8	4
S278A	partial	23.2 ± 3.4	1.35 ± 0.40	3	232 ± 30	7	3	146 ± 15	2	3	294 ± 35	2	3
Y279A	complete	17.3 ± 2.6	0.71 ± 0.10	3	200 ± 45	6	4	63.9 ± 7.2	1	3	72 ± 8	0.5	3
V280A	wild-type	21.9 ± 2.9	1.04 ± 0.29	3	18 ± 2	0.5	3	^b			^b		
K281A	partial	21.1 ± 3.1	1.31 ± 0.10	3	182 ± 31	5.5	4	276 ± 40	4	3	412 ± 35	2.8	3
I283A	wild-type	32.5 ± 6.2	0.77 ± 0.26	2	35 ± 6	1	3	^b			^b		

Results are separated into those obtained using filtration assays and centrifugation assays. Although there was a consistent ~2-fold difference in the K_d values between the two assays, fold increases in all K_i values are directly comparable.

^aNot determined.

^b K_i values for β -alanine and taurine were not measured in mutant GlyRs which expressed a wild-type phenotype.

residues (R271, K276 and Y279) are distributed throughout the M2–M3 loop, they raise the possibility that this entire loop may form an important structural component of the allosteric signal transduction mechanism.

In the I244N mutant GlyR, taurine was converted into a partial agonist, although β -alanine remained virtually a full agonist (Figure 1A and E and Table I). Taurine was also converted into a partial antagonist of glycine-gated currents (Figure 1C) and, as expected, β -alanine did not display detectable antagonism ($n = 3$; data not shown). In the I244N mutant GlyR, the average EC_{50} values of glycine, β -alanine and taurine were increased by a factor of 10–15 over their respective wild-type values (Figure 1E and Table I). We were unable to detect significant [³H]strychnine binding in this mutant GlyR, although in electrophysiological experiments we confirmed that 1 μ M strychnine did completely inhibit currents activated by a half-saturating concentration (200 μ M) of glycine ($n = 2$; data not shown). Since cells expressing recombinant GlyRs containing the I244N mutation were characterized by extremely small peak currents (I_{max} , see Table I), it would appear that this mutation greatly impaired the efficiency of GlyR expression while leaving the strychnine binding site essentially intact. This interpretation is supported by the observation that when I244 was mutated to the more conservative alanine, strychnine binding was

regained but B_{max} was significantly reduced (42%) relative to the wild-type value (Table II).

Since the β -alanine and taurine efficacies and glycine EC_{50} values of the I244N mutant GlyR lie between those of the wild-type GlyR and the K276E and Y279C mutated GlyRs, the I244N mutation may be considered to partially disrupt the agonist transduction mechanism and is consequently designated as having a ‘partial’ disruption phenotype. These properties imply that the M1–M2 loop, like the M2–M3 loop, is important for transducing agonist binding into channel activation.

Residues in the M1–M2 and M2–M3 loops control agonist efficacy

To investigate further the possibility that the M1–M2 and M2–M3 loops may act in parallel to mediate activation of the channel, we sought to examine the contribution of all residues in both loops to this process. As the various startle disease mutations are not conservative, they may have imposed additional functions on mutated residues that were not characteristic of the wild-type GlyR. A more conservative method of assessing the role of each residue is to investigate the effect of alanine substitution, since alanine causes minimal disruption to α -helical and β -sheet structures and is unlikely to introduce additional functions. Accordingly, we analysed consecutive alanine substitution

Fig. 2. Examples of agonist-activated currents (A–D) and averaged dose–response curves (E) for cells expressing either wild-type GlyRs or GlyRs incorporating alanine substitutions in the M2–M3 loop. (A) Currents activated by 100 mM glycine, β-alanine and taurine for the wild-type GlyR and L274A and V277A mutant GlyRs. (B) Partial inhibition by taurine of currents activated by a half-maximal concentration (500 μM) of glycine for the L274A mutant GlyR. (C) Complete inhibition by taurine of currents activated by a half-maximal concentration (2 mM) of glycine for the L277A mutant GlyR. (D) Examples of glycine dose–response curves for wild-type GlyR and L274A and V277A mutant GlyRs. Numbers above indicate glycine concentrations in mM. (E) Averaged dose–response curves for currents activated by glycine (upper panel), β-alanine (centre) and taurine (lower panel) for wild-type GlyRs (●) and selected mutant GlyRs where β-alanine and taurine have a reduced efficacy (open symbols) or no efficacy (shaded symbols).

mutants of all non-alanine residues in the M1–M2 intracellular loop from W243 to R252 and in the M2–M3 extracellular region from S270 to I283. Each mutant GlyR was investigated using similar electrophysiological and radioligand binding procedures as previously described. The averaged results of all electrophysiological measurements are summarized in Table I and averaged results from all radioligand binding studies are summarized in Table II. With the exception of R252A, all mutations resulted in expressed GlyRs which displayed glycine-gated currents and [³H]strychnine binding. Cells transfected with cDNA encoding the R252A mutant GlyR displayed no glycine-gated currents ($n = 25$ cells) and no significant [³H]strychnine binding ($n = 3$), suggesting that the R252A mutation precluded expression of functional GlyRs. This is consistent with the previous observation that other mutations of this residue (R252Q and R252E) prevented GlyR protein from being inserted into the cell membrane (Langosch *et al.*, 1993). As discussed above, the I244N, I244A and W243A mutant GlyRs were also characterized by reduced $B_{\max}$ values, suggesting that these residues are also important for receptor expression.

Several GlyRs mutated in the M1–M2 loop (N245A,

D247A and P250A) and in the M2–M3 loop (S270A, S273A, P275A, V280A and I283A) exhibited phenotypes that were indistinguishable from wild-type GlyRs in both their electrophysiological properties and their ligand binding affinities (Tables I and II). We conclude that such mutated residues do not form part of the agonist transduction pathway. However, four other mutations in the M2–M3 loop (R271A, K276A, V277A and Y279A) essentially abolished the agonist efficacies of β-alanine and taurine (Table I). For the V277A mutant GlyR, examples of currents activated by 100 mM concentrations of glycine, β-alanine and taurine are shown in Figure 2A. In this mutant GlyR, taurine and β-alanine were converted into full antagonists (Table I). Examples of taurine inhibition of currents activated by a half-saturating (2 mM) concentration of glycine in this mutant GlyR are shown in Figure 2C. Other characteristics of the V277A mutant GlyR include a large (107-fold) increase in the glycine EC_{50} with respect to the wild-type value (Table I and Figure 2D and E), an 8-fold increase in the glycine K_i and smaller (2-fold) increases in the K_i values for β-alanine and taurine (Table II). Very similar electrophysiological and pharmacological properties were shared by the R271A,

Fig. 3. Examples of agonist-activated currents (A–C) and averaged dose–response curves (D) for GlyRs incorporating alanine substitution mutations in the M1–M2 loop. (A) Currents activated by 100 mM glycine, β -alanine and taurine for I244A and D247A mutant GlyRs. (B) Partial inhibition by taurine of currents activated by a half-maximal concentration (1 mM) of glycine for the I244A mutant GlyR. Due to the rapid desensitization, glycine was co-applied with the indicated concentration of taurine. (C) Onset and recovery of desensitization for the I244A mutant GlyR. A half-maximal concentration (1 mM) of glycine was applied for 2 s in order to desensitize >90% of channels, then re-applied for 5 s commencing at the times indicated by the dots. (D) Examples of glycine dose–response curves for D247A and I244A mutant GlyRs. Numbers above indicate glycine concentration in mM. (E) Averaged dose–response curves for wild-type GlyRs (●) and selected mutant GlyRs (open symbols) activated by glycine (upper panel), β -alanine (centre) and taurine (lower panel).

K276A and Y279A mutant GlyRs (Tables I and II). As these mutant GlyRs clearly exhibit the ‘complete’ disruption phenotype, we conclude that V277 in addition to R271, K276 and Y279 comprise crucial elements of the allosteric transduction mechanism.

Three additional mutations within the M2–M3 loop resulted in the conversion of β -alanine and taurine from full agonists into partial agonists. The mutations which conferred this characteristic were L274A, S278A and K281A (Table I). An example of the reduced agonist efficacy of taurine and β -alanine in the L274A mutant GlyR is shown in Figure 2A. As expected for competitive partial agonists, taurine and β -alanine also displayed properties of partial antagonists (Table I). An example of partial inhibition by taurine of glycine-activated currents in the L274A mutant GlyR is displayed in Figure 2B. The L274A, S278A and K281A mutant GlyRs were also characterized by less pronounced (2- and 47-fold) increases in their respective glycine EC_{50} values relative to the wild-type value (see Table I and Figure 2D and E). Taurine and β -alanine EC_{50} values were also dramatically increased over their respective wild-type values (Figure 2E and

Table I). The binding affinities for glycine, β -alanine and taurine in these mutant GlyRs were intermediate between those of wild-type GlyRs and mutant GlyRs displaying the complete disruption phenotype (Table II). The L274A, S278A and K281A mutant GlyRs may therefore be classified as having a ‘partial’ disruption phenotype.

Three mutations in the M1–M2 loop (W243A, I244A and M246A) also reduced the agonist efficacies of β -alanine and taurine. Sample recordings from cells expressing GlyRs mutated in the M1–M2 loop are displayed in Figure 3. Figure 3A shows examples of saturating agonist responses for the I244A and D247A mutant GlyRs. The latter mutant exhibited a wild-type phenotype (Table I) and is included for comparison. An example of partial antagonism by taurine of glycine-gated currents in the I244A mutant GlyR is shown in Figure 3B. The same mutation also resulted in a dramatically reduced sensitivity (40-fold) to activation by glycine (Figure 3D and E). Averaged dose–response curves for all M1–M2 loop mutations which resulted in conversion of taurine and β -alanine into partial agonists are shown in Figure 2E. In terms of their electrophysiological properties, the W243A,

I244A and M246A mutant GlyRs exhibit the 'partial' disruption phenotype and therefore also form crucial elements of the allosteric signal transduction mechanism. However, apart from their reduced $B_{\max}$ values, the radioligand binding properties of these intracellular mutant GlyRs are indistinguishable from the wild-type GlyR in their K_d values for strychnine or in their K_i values for glycine, β -alanine and taurine (Table II).

Increased desensitization rate in W243A and I244A mutant GlyRs

An unexpected finding was that the W243A and I244A mutations also conferred a dramatically increased desensitization rate. Following a 2 s application of a saturating concentration (1 mM) of glycine, wild-type GlyR currents declined to an average of $89 \pm 3\%$ ($\pm$ SEM, $n = 14$) of their peak values. In contrast, after a 2 s application of 100 mM glycine, currents in W243A and I244A mutant GlyRs had decreased to $11 \pm 5\%$ and $10 \pm 2\%$ ($n = 11$ for both) respectively of their peak values. This difference is highly significant ($P < 0.0001$, unpaired t -test) for both mutants with respect to the wild-type value. The I244N mutant GlyR also displayed a significantly more rapid rate of desensitization than the wild-type GlyR (mean $77 \pm 3\%$, $n = 10$, $P < 0.025$), but the desensitization rates of all other mutant GlyRs were not significantly different to the wild-type GlyR desensitization rate. Examples of strongly desensitizing responses for the I244A mutant GlyR can be seen in Figure 3. As displayed in Figure 3C, recovery from desensitization was also rapid, with a half-recovery time of ~ 2 s ($n = 2$) for the I244A mutant GlyR.

Progressive disruption to transduction caused by mutations at R271

To determine whether modifications to the side chain group on a single residue may result in a continuum of phenotypes from partial to complete disruption, we examined a series of mutations to R271. This residue was investigated as it has already been characterized via three mutations to differing side chain groups (R271L, R271Q and R271A). In an attempt to characterize the side chain characteristics required for minimal disruption to the transduction process, we constructed and examined the following mutations: R271K, R271H and R271N. The conservative R271K substitution retains the positive charge and large side chain. The R271H substitution conserves the positive charge but not the large side chain. On the other hand, the R271N substitution is uncharged and polar and, like the structurally similar R271Q substitution, may be expected to result in a complete disruption phenotype. Averaged dose-response curves for glycine, β -alanine and taurine are shown for all R271 mutant GlyRs in Figure 4. Surprisingly, these experiments revealed that the R271K, R271H and R271N mutant GlyRs each exhibited the partial disruption phenotype, in contrast to those of the R271L, R271Q and R271A mutant GlyRs, which each exhibited the complete disruption phenotype. As expected for mutant GlyRs with the partial disruption phenotype, the radioligand binding properties of the R271K, R271H and R271N mutant GlyRs were also intermediate between those of the wild-type GlyR and the R271L, R271Q and R271A mutant GlyRs (Table II). Hence, mutations to the

Fig. 4. Averaged dose-response curves for GlyRs incorporating various mutations to R271. Averaged dose-response curves are shown for currents activated by glycine (upper panel), β -alanine (centre) and taurine (lower panel) for wild-type GlyRs (●) and mutant GlyRs where β -alanine and taurine have a reduced efficacy (open symbols) or no efficacy (shaded symbols).

same residue can result in a continuum of phenotypes from partially disrupted to completely disrupted. Although we were unable to distinguish an obvious correlation between the structural and chemical properties of the side chain and the resulting phenotype, these results demonstrate that residues identified by partial disruption phenotypes contribute to the transduction process.

Discussion

The M1-M2 and M2-M3 loops form allosteric signal transduction domains

Alanine substitution mutations to a total of four out of 11 possible residues in the M2-M3 loop resulted in the complete disruption phenotype. This phenotype is characterized by a complete loss of agonist efficacy for β -alanine and taurine, dramatic increases (>55 -fold) in the glycine EC_{50} , the conversion of β -alanine and taurine into complete antagonists of glycine-gated currents and large increases in the K_i values for glycine relative to those for β -alanine and taurine. In addition, mutations to a further six out of a possible 14 residues throughout the M1-M2 and M2-M3 loops resulted in a partial disruption phenotype. This phenotype is characterized by the conversion of taurine and β -alanine from full agonists into partial

Fig. 5. A model for signal transduction for members of the ligand-gated ion channel superfamily. (A) The first three membrane-spanning domains of the human GlyR α1 subunit are illustrated showing a summary of results obtained in this study. Residues attached by stems represent the startle disease mutations examined in this study. Lightly shaded residues indicate those startle disease mutations or alanine substitution mutations which resulted in β-alanine and taurine being converted into partial agonists. Darkly shaded residues indicate those mutations which resulted in a complete loss of efficacy for these ligands. Thus, the lightly and darkly shaded residues represent 'partial' disruption and 'complete' disruption phenotypes respectively. Residues outlined in bold indicate those where mutations induced dramatically increased rates of desensitization. (B) Alignment of the M2–M3 signal transduction domains from various members of the ligand-gated ion channel superfamily. Circles indicate residues that convert agonists into antagonists, squares indicate residues that redistribute single channel conductances and diamonds indicate residues that alter allosteric enhancement or Hill coefficients. See text for details.

agonists with partial antagonist efficacy, less dramatic increases (2- to 47-fold) in the glycine EC_{50} and relatively smaller increases in the K_i values for glycine. Figure 5A illustrates the locations of the mutations which cause both the complete disruption phenotype (darkly shaded residues) and the partial disruption phenotype (lightly shaded residues). In addition, mutated residues which cause an increased desensitization rate are outlined in bold.

Since glycine, β-alanine and taurine share overlapping binding sites (Schmieden *et al.*, 1992, 1993), any mutation which converts β-alanine and taurine from agonists into antagonists without changing their binding affinities may act by simply disconnecting their binding sites from the channel activation gate (Rajendra *et al.*, 1995a). An alternative proposal is that the R271L and R271Q mutations selectively disrupt a common agonist recognition subsite and thereby unmask antagonist subsites for β-alanine and taurine (Laube *et al.*, 1995; Schmieden and Betz, 1995). It is difficult to differentiate between these models by analysing mutations of a single residue, as there is no functional difference between an uncoupled

competitive agonist site and an antagonist site. However, as the antagonist subsite model (Laube *et al.*, 1995; Schmieden and Betz, 1995) would require a highly specific lesion to disrupt the agonist recognition site selectively in a common ligand binding pocket, it is clearly incompatible with the involvement of numerous intracellular and extracellular residues, as observed in the present study (Figure 5A). Hence, our results strongly support the idea that mutations which convert taurine and β-alanine from agonists into antagonists do indeed act by disrupting the allosteric transduction mechanism. This interpretation is also supported by a theoretical analysis using an allosteric receptor model with multiple discrete conformational states (Galzi *et al.*, 1996). Since numerous residues in both loops are capable of disrupting the allosteric transduction process, we propose that the M1–M2 and M2–M3 loops act in parallel to transduce the ligand binding signal into channel activation in GlyR. Furthermore, since all members of the ligand-gated ion channel superfamily share considerable sequence and structural homology (Devillers-Thiery *et al.*, 1993; Karlin and Akabas, 1995),

this model is likely to apply to all members of this superfamily.

A variety of experimental evidence from various members of the ligand-gated ion channel superfamily supports the involvement of residues in the M2–M3 loop in mediating allosteric transformations. In the GlyR $\alpha 1$ subunit, we have demonstrated that the R271L and R271Q mutations convert the competitive antagonist picrotoxin into an allosterically acting potentiator, suggesting a convergence of allosteric pathways (Lynch *et al.*, 1995). Furthermore, in R271L and R271Q mutant GlyRs, single channel conductance states are redistributed towards lower unitary levels (Langosch *et al.*, 1994; Rajendra *et al.*, 1995a) without radically changing the anion–cation selectivity ratio or the current–voltage relationship (Rajendra *et al.*, 1994). These mutations may therefore prevent allosteric transitions to one or more conducting conformations. A similar interpretation may also apply to the E297S mutation in the GlyR β subunit (Bormann *et al.*, 1993) and to the K→M substitution at position +21 of the *Xenopus* nicotinic acetylcholine receptor (nAChR) γ subunit (Murray *et al.*, 1995), which are both homologous to A273 of the GlyR $\alpha 1$ subunit and both also result in significant changes to unitary channel conductances. In both the $\alpha 7$ and $\beta 4$ nAChRs, the aspartic acid residues at the positions corresponding to Y279 and V280 respectively in the GlyR $\alpha 1$ subunit were recently shown to disrupt the coupling between agonist binding and channel activation (Campos-Caro *et al.*, 1996). In the GABA_A receptor (GABA_AR) $\rho 1$ subunit, the R316A mutation (homologous to position K276 in the GlyR $\alpha 1$ subunit) increased the Hill coefficient for GABA activation, suggesting a modification to the allosteric coupling mechanism (Kusana *et al.*, 1994). Collectively, these results, which are summarized in Figure 5B, support the notion of a common signal transduction mechanism applying to all ligand-gated ion channel receptors.

Based on comparison of electron diffraction images of nAChR in the open and closed states, Unwin (1995) proposed a structural model for the activation of members of this receptor superfamily. The model postulates that in the closed state the M2 domains are kinked inwards to constrict the channel pore and upon activation by acetylcholine are twisted sideways to create an open passage. This rotation is clearly a crucial step in the transduction process and implies that the intracellular and extracellular loops flanking the M2 domain act as hinges to permit this rotation. Consistent with this model, the simplest explanation of our results is that the M1–M2 and the M2–M3 loops act as the hinges and that mutations that disrupt transduction act by preventing this rotation from taking place. However, since other structural rearrangements are also associated with channel activation (Karlin and Akabas, 1995; Unwin, 1995), further signal transduction domains may also exist.

The M2–M3 loop may interact with the glycine binding site

Mutant GlyRs expressing the complete disruption phenotype are also characterized by dramatic increases in the glycine K_i values (Table II). An increase in K_i is frequently taken as evidence that the mutated residue forms part of a binding domain. However, due to the principle of

reciprocity between transduction and binding processes, binding experiments alone cannot unequivocally define whether a residue comprises a binding site or a transduction site (Colquhoun and Farrant, 1993). We have demonstrated that the M1–M2 loop mutations which result in partial disruption phenotypes have K_i values for glycine, β -alanine and taurine which are not significantly different to wild-type values (Table II). However, M2–M3 loop mutations with similar partial disruption phenotypes generally have dramatically increased glycine K_i values (Table II), suggesting that the M2–M3 loop interacts more directly with the glycine binding site. Furthermore, the relatively larger increases in glycine K_i values, compared with those for taurine and β -alanine, imply that the glycine binding site is more directly linked to the M2–M3 loop than are the binding sites for taurine and β -alanine. In this respect, we propose that R271 forms the point of closest functional contact between the M2–M3 loop and the glycine binding site, as mutations to this residue are characterized by much larger increases in glycine K_i values than are observed at any other residue (Table II).

Desensitization

Mutations to two residues (W243A and I244A) in the intracellular M1–M2 loop not only partially disrupt the transduction process but also result in a greatly increased rate of desensitization onset and recovery (Figure 3). If such a property is conferred by a simple side chain substitution, then a similar property could also be imposed by allosteric interactions converging on this residue. A major determinant of desensitization in the related nAChRs and GABA_ARs is the phosphorylation state (Raymond *et al.*, 1993; Levitan, 1994). Like these receptors, the GlyR $\alpha 1$ subunit contains functional phosphorylation sites in the intracellular M3–M4 loop (Vaello *et al.*, 1994), but it is as yet unclear as to whether phosphorylation modulates the desensitization rate of this receptor. It is, however, tempting to speculate that phosphorylation sites, or other intracellular modulatory sites, may control the desensitization rate by interacting with the channel gating mechanism via residues in the M1–M2 loop.

A common human startle disease phenotype

Inherited startle syndromes in humans and a variety of animal species are characterized by a reduced glycinergic tone (Rajendra and Schofield, 1995). Molecular analysis of human startle disease pedigrees has so far identified a total of six mutations, each of which maps to the GlyR $\alpha 1$ subunit (R271L and R271Q, Shiang *et al.* 1993; I244N, Rees *et al.*, 1994; Y279C, Shiang *et al.*, 1995; K276E, Elmslie *et al.*, 1996; Q266H, Milani *et al.*, 1996). The disease phenotype conferred by the R271L and R271Q mutations was characterized by both a reduced glycine efficacy and a reduced single channel conductance (Langosch *et al.*, 1994; Rajendra *et al.*, 1994, 1995a). In the present study, we investigated the I244N, Y279C and K276E startle mutations and found in each case that the glycine sensitivity was profoundly decreased (Table I). In addition, the K276E and Y279C mutations resulted in dramatically diminished $I_{\max}$ values (11 and 2% respectively of the wild-type value) without corresponding decrements in the [³H]strychnine $B_{\max}$ values (Table II), strongly suggesting that these mutations also lowered either the

single channel conductance or the channel open probability, or both. However, in the I244N mutant GlyR, the sharp reduction in $I_{\max}$ was accompanied by a complete loss of [^3H]strychnine binding. Since strychnine-inhibitable glycine-gated currents were readily observed in these mutant GlyRs, the most likely explanation for the lack of strychnine binding was a drastically decreased efficiency of expression of functional GlyRs. It remains to be determined whether reduced GlyR expression also occurs *in vivo*.

In summary, decreased receptor glycine efficacies caused by disruptions to the allosteric transduction mechanism can account for the phenotypes conferred by all human startle disease mutations studied so far. Decreased chloride conductances and receptor expression efficiencies may also contribute to the phenotype caused by some mutations.

Conclusion

The major finding of this study is that numerous residues in both the M1–M2 and M2–M3 loops are involved in the transduction process between ligand binding and channel activation. This conclusion is supported by a variety of experimental evidence from other members of the LGIC superfamily, reviewed above, as well as the structural model of Unwin (1995). The Unwin model postulates that in the closed state the M2 domains are kinked inwards to constrict the channel pore and upon activation are twisted sideways to create an open passage. Consistent with this model, the simplest explanation of our results is that the M1–M2 and M2–M3 loops act as the hinges to facilitate this rotation and that mutations which disrupt signal transduction act by preventing this rotation from taking place. Whereas agonists interact with the external M2–M3 loop to activate the channel, other intracellular sites may interact with the M1–M2 loop to modulate channel desensitization. Given their highly conserved structures, it is likely that all ligand-gated ion channel receptors share this mechanism for channel activation. A second major finding is that human startle disease mutations reduce or eliminate the agonist efficacies for taurine and β -alanine without dramatically affecting their ligand binding affinities. This indicates that these mutations act in a common manner to disrupt the allosteric transduction process linking agonist binding to channel activation.

Materials and methods

Mutagenesis and expression of human GlyR $\alpha 1$ subunit cDNA

All procedures have recently been described in detail (Rajendra *et al.*, 1995a). Briefly, mutations were constructed to human GlyR $\alpha 1$ cDNA in the pCIS2 expression vector using oligonucleotide-directed PCR and confirmed by sequencing the cDNA clones. Mutant plasmid constructs were transiently transfected into the human embryonic kidney 293 cell line using the modified calcium phosphate precipitation method of Chen and Okayama (1987). Patch-clamp studies and [^3H]strychnine binding assays were conducted at least 48 h after transfection.

Electrophysiology

Glycine-gated currents were measured using whole-cell recording (Hamill *et al.*, 1981) at a holding potential of -60 mV. Cells were continually superfused with a bathing solution containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 10 mM

glucose, pH 7.4 with NaOH. Patch pipettes had tip resistances of 1–4 M Ω when filled with the standard intracellular solution containing 145 mM CsCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM HEPES, 10 mM EGTA, pH 7.4 with CsOH. The empirical Hill equation, fitted by a non-linear least squares algorithm, was used to calculate the apparent 50% effective concentrations for activation (EC_{50}) and for inhibition (IC_{50}) under conditions as described in Results. Hill coefficients were also calculated but were excluded from Tables I and II for brevity. There was, however, no consistent trend between the respective Hill coefficients and any other parameter measured in this study.

[^3H]Strychnine binding assays

Transfected cells or crude membrane preparations were incubated with [^3H]strychnine (1–50 nM, 23 Ci/mM; New England Nuclear) with and without 10 μM cold strychnine to determine non-specific binding or with 20 nM [^3H]strychnine and 0–10 $^{-1}$ M glycine, β -alanine or taurine to generate agonist competition curves. After incubation to equilibrium at 4°C for 60 min, cells were collected by rapid filtration onto Whatman GF/B filter paper and crude membranes were collected by centrifugation at 13 000 r.p.m. for 5 min. The amount of radioactive strychnine remaining bound to either filters or to resuspended pellets was determined. The K_d and $B_{\max}$ for the [^3H]strychnine saturation isotherms and the IC_{50} and K_i values for glycine, β -alanine and taurine displacement of bound [^3H]strychnine were estimated using the Inplot program (Graphpad Software, San Diego, CA).

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