Functional asymmetry of the conserved cystine loops in $\alpha\beta\gamma$ GABA_A receptors revealed by the response to GABA activation and drug potentiation

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Abstract

Ligand-gated ion channels respond to specific neurotransmitters by transiently opening an integral membrane ion-selective pore, allowing ions to move down their electrochemical gradient. A distinguishing feature of all members of the ligand-gated ion channel superfamily is the presence of a 13-amino acid disulfide loop (Cys-loop) in the extracellular ligand-binding domain. Structural data derived from the acetylcholine receptor place this loop at the interface between the ligand-binding domain and the transmembrane pore-forming domain where it is ideally located to participate in coupling ligand binding to channel opening. We have introduced specific mutations into a conserved motif at the mid-point of the Cys-loop of the GABA_A receptor subunits $\alpha 1$, $\beta 2$ and $\gamma 2S$ where the sequence reads aromatic, proline, aliphatic (ArProAl motif). Receptors carrying a mutation in the Cys-loop of one of their subunits were expressed in L929 cells and responses to both GABA and drugs were assessed using the whole-cell patch clamp technique. Drug potentiation and direct activation were significantly enhanced by mutations in this Cys-loop but these effects were subunit-dependent. Currents in response to agonists were larger when mutations were carried in the α and β subunits but not in the γ subunit. In contrast, potentiation of current responses by diazepam, etomidate and pentobarbital were all enhanced when mutations were carried in the α and γ subunits, but not the β subunit. Since the disruption of interactions mediated through the ArProAl motif enhances the mutant receptor's response to both agonist and drugs we suggest that this motif in the Cys-loop of the wild-type receptor participates in interactions that create activation barriers to conformational changes during channel gating.

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1. Introduction

γ -Aminobutyric acid type A (GABA_A) receptors are members of the Cys-loop ligand-gated ion channel (LGIC) superfamily that includes the vertebrate nicotinic acetylcholine, 5-HT₃ and glycine receptors (Barnard, Darlison, & Seeburg, 1987; Schofield et al., 1987). Receptors in this family form pentamers of homologous subunits that are arranged around a central ion-conducting pore. It has been proposed, based

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on sequence homology and functional similarity, that all such receptors will share a common mechanism of gating, which is the allosteric coupling of ligand binding to the opening of an ion-selective pore (Ortells & Lunt, 1995). Each subunit has an extracellular, N-terminal, ligand-binding domain incorporating a conserved intramolecular disulfide loop which gives the superfamily its name, followed by four transmembrane (TM) α -helices that form an ion-selective pore embedded in the membrane. A key question regarding the function of LGICs is how the binding signal is transmitted over a distance of about 30 Å to initiate the opening of an ion-selective pore on a time scale of less than a millisecond.

Structural data on LGICs come solely from low resolution (4 Å) electron microscopic images of the nicotinic acetylcholine receptor (nAChR) from the electric ray *Torpedo marmorata* providing a view of the overall organization of the receptor in the closed conformation (Miyazawa, Fujiyoshi, & Unwin, 2003). Additional structural clarity has been provided by atomic resolution structural models of the extracellular ligand-binding domains of LGICs including the nonchannel homologue, the acetylcholine-binding protein (AChBP) of snails (Brejc et al., 2001; Celie et al., 2004) and the binding domain of the $\alpha 1$ subunit of the nAChR in complex with α -bungarotoxin (Dellisanti, Yao, Stroud, Wang, & Chen, 2007). In the receptor structure the conserved Cys-loop of each subunit is positioned at the interface of the ligand-binding domain (LBD) and the pore-forming transmembrane domain (TMD) (Fig. 1A). For this reason it was postulated that the Cys-loop might participate in channel gating (Brejc et al., 2001; Unwin, Miyazawa, Li, & Fujiyoshi, 2002).

The Cys-loop in ligand-gated ion channels exists in a different physiological context compared with the non-channel homo-pentameric AChBP, which is devoid of a hydrophobic transmembrane domain. Furthermore, the functional consequences of ligand binding in the two proteins are entirely distinct. With these differences in mind, we compared the amino acid sequences of the Cys-loop in the acetylcholine binding protein and those of ligand-gated ion channels to see if there were any obvious differences that might suggest an involvement in signal transduction or drug modulation. Although the disulfide loop per se is conserved in both the binding protein and the members of the ligand-gated ion channel family, the chemical nature of this loop is not (Fig. 1B). It is one residue shorter and hydrophilic in the binding protein but hydrophobic and longer in the receptors. Of particular note is the striking conservation in ligand-gated ion channels of a four-residue sequence mid-way

Fig. 1. Schematic diagram of the GABA α receptor β subunit indicating the position and sequence of the conserved Cys-loop. Panel A: (a) The disulfide loop of the β subunit is shown with the residues comprising the 'aromatic, proline, aliphatic' (ArProAl) motif at the tip of the loop indicated. Alongside (b) the position of the Cys-loop relative to the entire subunit is shown where it sits at the boundary between the ligand binding and the pore-forming transmembrane domains. The Cys-loop and the α -helix lining the pore, M2, are indicated. Panel B: Alignment of residues in the Cys-loop of selected members of the ligand-gated ion channel family. Residues mutated in this study in the GABA α subunits are in bold and underlined. Amino acid sequences are of the acetylcholine binding protein, AChBP, from *Lymnaea stagnalis*, human GABA α $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits, the acetylcholine receptor $\alpha 1$ and δ subunits from *Torpedo marmorata*, the human acetylcholine receptor $\alpha 7$ subunit and the human glycine receptor $\alpha 1$ subunit.

through the loop where the sequence reads: aromatic, proline, aliphatic, aspartate at positions 8* through 11* in anionic GABA α and glycine receptors, numbering from the N-terminal cysteine residue, and aromatic, proline, aromatic, aspartate in the cationic acetylcholine and 5-HT $_3$ receptors (Fig. 1B). We postulated that due to its location this motif in the Cys-loop would participate in signal transduction by coupling ligand binding to channel gating in the GABA α receptor. Furthermore, the hydrophobic nature of the motif at the tip of the Cys-loop on the other hand was suggestive that it might

participate in a drug-binding site for similarly lipophilic compounds. In order to test these possibilities we have introduced mutations into the three hydrophobic residues in this region of the Cys-loop of individual GABA_A subunits, $\alpha 1$, $\beta 2$ and $\gamma 2S$ (predicted stoichiometry 2α , 2β , 1γ), to examine the interaction of specific amino acids and the role of individual subunits in GABA-activated and drug-modulated responses. Two types of substitutions were made; first the 8* aromatic, 9* proline and 10* aliphatic residues were individually replaced by alanine, effectively amputating the wild-type side chain. Secondly, ‘swaps’ were performed where the 8* and 10* residues in each subunit were interchanged so as to identify sequential requirements as opposed to general physicochemical constraints. We hereafter refer to this conserved sequence motif as the ArProAl motif (aromatic, proline, aliphatic). The results of site-specific mutation of residues comprising the ArProAl motif of $\alpha\beta\gamma$ GABA_A receptors clearly define distinct roles for the Cys-loops of different subunits in either agonist activation of the GABA_A receptor or drug-mediated potentiation of the GABA response.

2. Materials and methods

2.1. Mutagenesis and expression

The cDNA encoding the human GABA_A $\alpha 1$ or $\beta 2$ subunit was subcloned into pcDNA3.1 (Invitrogen) and the $\gamma 2S$ subcloned into pCMS-EGFP (Clontech, BD Biosciences). Site directed mutagenesis was performed using the QuikChange method (Stratagene, La Jolla, CA). All mutant clones were confirmed by complete sequencing using an ABI 3730 capillary sequencer (ACRF Biomolecular Resource Facility, The John Curtin School of Medical Research, The Australian National University). L929 cells were maintained in standard culture conditions (5% CO₂, 37 °C) and transiently transfected using FUGENE6 (Roche) with plasmids carrying the human GABA_A $\alpha 1$, $\beta 2$ or $\gamma 2S$ plus GFP cDNAs in a 1:1:1 ratio as described previously (Luu, Gage, & Tierney, 2006).

2.2. Drug application and recording

The whole-cell recording technique was used to voltage clamp fluorescent L929 cells and record currents (Hamill, Marty, Neher, Sakmann, & Sigworth, 1981). The external recording medium (bath solution) contained the following in mM: 135 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 glucose and 10 TES (pH 7.4). The intra-pipette solution contained (in mM): 50 NaCl,

80 KCl, 2 CaCl₂, 2 MgCl₂, 5 EGTA and 10 TES (pH 7.3). Electrodes were made using borosilicate glass capillaries (1.5 mm OD $\times$ 0.86 mm ID) fabricated on a pipette puller (Sutter Instruments Co.), and subsequently fire-polished before use. Electrodes had resistances of ~ 7 – 10 M Ω when filled with the internal solution and immersed in bath solution. GABA (Sigma), diazepam (1 μ M for potentiation; diazepam does not directly activate the receptor) (kindly provided by Hoffman-La Roche), pentobarbital (50 μ M for potentiation and 100 μ M for direct activation) (Sigma) and etomidate (1 and 25 μ M for potentiation and 25 μ M for direct activation) (Tocris Bioscience, Missouri, USA) were diluted in bath solution to the final concentration on the day of experiment. Diazepam and etomidate were initially dissolved in DMSO as 1000-fold stock solutions and stored at -20 °C. Etomidate potentiation of the GABA response was tested at two etomidate concentrations after generating an etomidate dose–response curve (data not shown), which was similar to published data (Belelli, Lambert, Peters, Wafford, & Whiting, 1997). The concentrations chosen ensured that the effect would be apparent and reproducible in γ -containing receptors, which are less efficacious, compared to δ -containing GABA_A receptors (Brown, Kerby, Bonnert, Whiting, & Wafford, 2002). The bath had a capacity of 2 ml and flow rate through the bath was approximately 5 ml/min. Drugs were applied via gravity fed flow tubes placed close to the cell. Drug applications were separated by at least 30 s to allow recovery from desensitization. Data from two or more separate transfections were collected for analysis. All recordings were carried out at room temperature (20–22 °C). Currents were monitored with an Axopatch 200A amplifier filtered at 5 kHz (3 dB, 4-pole Bessel; Axon Instruments, CA, USA) and directly recorded on to the computer (Axoscope7; ADC: digidata board 1200; Axon Instruments, CA, USA) sampling at 10 kHz. Voltage offsets between pipette and ground were zeroed once the pipette was immersed in the bath solution. Series resistance compensation was always performed on currents greater than 500 pA.

2.3. Data analysis

The in-house software Channel 2 (written by P.W. Gage and M. Smith) was used for data acquisition and current analysis. Current rise times were measured to ensure consistency with the application speed and to indicate changes in mutant receptor behaviour. Rise times were calculated as the time taken for the current to go from 10% to 90% of the peak current using a saturating GABA concentration (1 mM). As a measure

of desensitization, the current amplitude after a 5 s saturating GABA application (1 mM) was reported as a percentage of the peak whole-cell current. GABA concentration–response curves were generated by applying a range of six GABA concentrations (between 100 nM and 10 mM) to three or more individual voltage clamped whole cells starting at the lowest concentration and working upwards. Data were only included if a 1 μ M response tested at the beginning and end of the GABA concentration series had not reduced by more than 10%. Data were fitted with the equation for a single binding site:

$$I = I_{\max} \frac{[A]^{n_H}}{[A]^{n_H} + EC_{50}^{n_H}}$$

where I is the peak whole-cell current recorded in response to the application of a range of GABA concentrations $[A]$, $I_{\max}$ the maximum peak whole-cell current recorded from that cell, EC_{50} the concentration of GABA that gives a half maximal response and n_H is the Hill co-efficient. Iterative curve fitting was performed using Excel (Microsoft).

The statistical program R (R Development Core Team, 2006) was used to perform one-way ANOVA. Data are expressed as the mean $\pm$ standard error of the mean from n replicates and significant differences relative to the wild-type $\alpha\beta\gamma$ receptor were *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

3. Results

3.1. Mutations in the Cys-loop affect the GABA EC_{50} and Hill co-efficient

All mutations introduced into the Cys-loop significantly reduced the GABA concentration required to elicit a half maximal current response (EC_{50}), with the exception of the (P9*A) mutation in the α and β subunits; these mutant receptors displayed an increased GABA EC_{50} and a response similar to the wild-type receptor, respectively. The shifts in EC_{50} were a relatively modest 2–3-fold change compared to the wild-type $\alpha\beta\gamma$ receptor response of 24.1 μ M (Fig. 2, Table 1). Concomitant with these changes in EC_{50} was a significant decrease in the apparent Hill co-efficient of all mutant receptors. Examples of Cl^- currents activated by a range of GABA concentrations are shown in Fig. 2A for the wild-type $\alpha\beta\gamma$ receptor and those of the Cys-loop mutants $\alpha(P9^*A)\beta\gamma$ and $\alpha\beta(LPY)\gamma$ are shown in Fig. 2B and C, respectively. The change in the apparent Hill co-efficient was greatest for the $\alpha\beta(LPY)\gamma$ mutant receptor and is reflected in the shape of its dose–response curve (Fig. 2D).

Fig. 2. Mutations in the ArProAl motif in the Cys-loop of GABA_A subunits led to ubiquitous changes in GABA sensitivity. Typical recordings of GABA responses from L929 cells transfected with wild-type $\alpha\beta\gamma$ (A), $\alpha(P9^*A)\beta\gamma$ (B) and $\alpha\beta(LPY)\gamma$ (C) receptors are shown. The insert in Panels B and C show, on an expanded time scale, the slow rise time of these mutants relative to the wild-type receptor. The bar above each recording indicates the duration of the GABA application. All recordings were at -60 mV. Panel D: Representative GABA dose–response curves are shown for the wild-type receptor and mutants $\alpha(MPF)\beta\gamma$, $\alpha\beta(LPY)\gamma$ and $\alpha(P9^*A)\beta\gamma$. Data are the mean of pooled values from $n=6$ to 10 cells. Statistical significance was determined using one-way ANOVA with the mutant receptors all significantly different from the wild-type receptor ($p < 0.001$). The mean parameters of best fit are given in Table 1.

3.2. The proline residue in both the α and β subunit Cys-loops is required for rapid gating-dependent conformational changes

Mutation of the 9* proline to alanine in both the α and β subunit Cys-loops significantly reduced the peak whole-cell currents and slowed rise times but no

Table 1

GABA-activated whole-cell current properties of Cys-loop mutant receptors compared to the wild-type $\alpha\beta\gamma$ GABA_A receptor

Receptor	GABA EC ₅₀ (μ M)	n_H	Mean, peak I (pA)	Rise time (ms)	Desensitization (%)	<i>n</i>
$\alpha\beta\gamma$	24.1 $\pm$ 2.3	1.2 $\pm$ 0.1	1086 $\pm$ 165	17.7 $\pm$ 2.4	60 $\pm$ 3	9
α (F8'A) $\beta\gamma$	9.3 $\pm$ 1.2***	0.7 $\pm$ 0.1***	677 $\pm$ 206	30.6 $\pm$ 4.5*	70 $\pm$ 5	5
α (P9'A) $\beta\gamma$	42.4 $\pm$ 4.3***	0.8 $\pm$ 0.1***	141 $\pm$ 26**	65.2 $\pm$ 7.9***	85 $\pm$ 2***	10
α (M10'A) $\beta\gamma$	11.1 $\pm$ 0.7***	0.7 $\pm$ 0.1***	359 $\pm$ 60*	21.4 $\pm$ 6.3	52 $\pm$ 5	4
α (MPF) $\beta\gamma$	12.8 $\pm$ 2.9***	0.7 $\pm$ 0.1***	1317 $\pm$ 568	19.3 $\pm$ 2.2	48 $\pm$ 3*	6
$\alpha\beta$ (Y8'A) γ	11.1 $\pm$ 1.8***	0.6 $\pm$ 0.1***	462 $\pm$ 158	24.2 $\pm$ 2.6	72 $\pm$ 4*	5
$\alpha\beta$ (P9'A) γ	18.5 $\pm$ 2.3	0.6 $\pm$ 0.1***	347 $\pm$ 70*	46.2 $\pm$ 5.1***	77 $\pm$ 2***	7
$\alpha\beta$ (L10'A) γ	9.8 $\pm$ 1.3***	0.8 $\pm$ 0.1***	555 $\pm$ 169	13.4 $\pm$ 2.0	42 $\pm$ 2***	7
$\alpha\beta$ (LPY) γ	10.1 $\pm$ 1.3***	0.4 $\pm$ 0.1***	298 $\pm$ 68**	30.0 $\pm$ 4.3*	87 $\pm$ 1***	9
$\alpha\beta\gamma$ (F8'A)	7.7 $\pm$ 0.4***	1.0 $\pm$ 0.1***	740 $\pm$ 216	24.9 $\pm$ 3.4	44 $\pm$ 4***	9
$\alpha\beta\gamma$ (P9'A)	8.0 $\pm$ 1.7***	0.9 $\pm$ 0.1***	780 $\pm$ 333	14.2 $\pm$ 1.4	62 $\pm$ 2	9
$\alpha\beta\gamma$ (M10'A)	11.4 $\pm$ 0.9***	0.8 $\pm$ 0.1***	658 $\pm$ 148	23.3 $\pm$ 2.6	53 $\pm$ 3***	13
$\alpha\beta\gamma$ (MPF)	7.8 $\pm$ 1.7***	0.7 $\pm$ 0.1***	531 $\pm$ 235	19.3 $\pm$ 1.7	48 $\pm$ 3*	5

Values represent mean $\pm$ S.E.M. from *n* number of experiments. *I*, current; n_H , Hill co-efficient. Significant differences compared to the wild-type $\alpha\beta\gamma$ receptor were determined using one-way ANOVA: *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

such effects were seen for the equivalent mutation in the γ Cys-loop (Fig. 2B, Table 1). Similarly, mutation of the 9* proline residue in the Cys-loops of only the α and β subunits significantly slowed desensitization. Other mutations in these two subunits however displayed opposing affects on desensitization. In contrast, removal of aromatic and aliphatic side chains in the γ subunit Cys-loop produced a consistent trend, increasing the rate of desensitization (Table 1).

3.3. Drug potentiation is affected by mutations in the Cys-loops of the α and γ subunits, but not the β subunit Cys-loop

To determine if the hydrophobic motif at the tip of the Cys-loop either contributes to a drug-binding site or participates in transducing the signal initiated upon drug binding, a range of drugs were tested on the mutant receptors. Specifically the potentiating effects of diazepam, pentobarbital and etomidate were tested.

Strikingly, while mutations in the ArProAl motif of the β subunit Cys-loop had some of the greatest effects on GABA-activated currents, none of the mutations in this subunit significantly altered drug potentiation (Table 2). On the other hand, many of the mutations in the Cys-loop of either the α or γ subunit significantly affected drug potentiation of the GABA response. Typical whole-cell currents in response to potentiation by diazepam, etomidate and pentobarbital on α subunit, Cys-loop mutants are shown in Fig. 3C and E, respectively. In general, amputation of either the 8* aromatic residue or the 10* aliphatic residue in the Cys-loop of the α subunit

increased the potentiation by all three drugs. When the 8* and 10* residues were interchanged in the α (MPF) $\beta\gamma$ mutant receptor the potentiation by all three drugs was enhanced even further with the fold-increases being the greatest for any of the Cys-loop mutant receptors (Table 2).

When the equivalent mutations were introduced into the Cys-loop of the γ subunit, significant differences to wild-type were seen on drug potentiation in a number of mutant receptors whose 8* aromatic or 10* aliphatic residues had been replaced by alanine. The trend once again was toward an increased response to co-activation with drug plus GABA (Table 2). In Fig. 3, Panels F–H show typical currents in response to diazepam, etomidate and pentobarbital potentiation from mutant receptors in which their γ subunit Cys-loop has been mutated. In contrast to the α (MPF) $\beta\gamma$ receptor however, when these 8* and 10* residues were interchanged in the γ subunit, potentiation of the GABA response in the $\alpha\beta\gamma$ (MPF) receptor was not dissimilar to wild-type receptor levels for pentobarbital and etomidate but diazepam potentiation remained significantly higher than wild-type (Table 2).

Significant current enhancement was only evident when the P9*A mutation was carried in the γ subunit (Table 2). The only mutation to significantly impede potentiation was that of the α (P9*A) $\beta\gamma$ mutant receptor but only to etomidate and only at the higher concentration tested (25 μ M). In general however, mutation of the 9* proline residue in the Cys-loop had much less of an effect on drug potentiation than those of the aromatic and aliphatic residues.

Fig. 3. Potentiation of the GABA response by drugs is enhanced by mutations in the ArProAl motif of the GABA_A receptor α and γ subunit Cys-loops. Panels A and B show typical current responses of the wild-type $\alpha\beta\gamma$ GABA_A receptor to potentiation by diazepam (1.8-fold increase of the bracketed EC_{10–20} GABA response) and pentobarbital (3.4-fold), respectively. Mutation of residues in the Cys-loop of the α subunit led to increased potentiation as shown here for diazepam on the $\alpha(F8^*A)\beta\gamma$ receptor (Panel C), etomidate on the $\alpha(MPF)\beta\gamma$ receptor (Panel D) and pentobarbital on the $\alpha(M10^*A)\beta\gamma$ receptor (Panel E). Similar enhancement of drug potentiation was seen in mutant receptors in which the γ subunit Cys-loop was mutated shown here for the $\alpha\beta\gamma(F8^*A)$ receptors response to diazepam (Panel F), the $\alpha\beta\gamma(M10^*A)$ receptors response to pentobarbital (Panel G) and the $\alpha\beta\gamma(M10^*A)$ receptors response to etomidate (Panel H). Drug potentiation of the GABA response was bracketed by applications of GABA

Table 2

Drug potentiation and direct activation of Cys-loop mutant receptors compared to the wild-type $\alpha\beta\gamma$ GABA_A receptor

Receptor	Dz pot'n (1 μ M)	PB pot'n (50 μ M)	Et pot'n		PB direct (100 μ M)	Et direct (25 μ M)
			25 μ M	1 μ M		
$\alpha\beta\gamma$	1.9 $\pm$ 0.1	3.6 $\pm$ 0.3	3.9 $\pm$ 0.3	1.9 $\pm$ 0.2	0.3 $\pm$ 0.04	0.5 $\pm$ 0.05
α (F8'A) $\beta\gamma$	2.8 $\pm$ 0.1***	4.6 $\pm$ 0.3*	4.2 $\pm$ 0.3*	2.2 $\pm$ 0.2*	2.0 $\pm$ 0.1***	0.6 $\pm$ 0.05
α (P9'A) $\beta\gamma$	1.8 $\pm$ 0.1	3.1 $\pm$ 0.1	1.9 $\pm$ 0.2***	1.7 $\pm$ 0.03	1.1 $\pm$ 0.1***	0.6 $\pm$ 0.1
α (M10'A) $\beta\gamma$	2.5 $\pm$ 0.2	6.5 $\pm$ 0.8***	4.7 $\pm$ 0.3	1.5 $\pm$ 0.03*	4.8 $\pm$ 0.3***	1.4 $\pm$ 0.2*
α (MPF) $\beta\gamma$	3.8 $\pm$ 0.5***	5.9 $\pm$ 0.6***	5.6 $\pm$ 0.5*	2.5 $\pm$ 0.1***	5.7 $\pm$ 0.7***	3.2 $\pm$ 0.7***
$\alpha\beta$ (Y8'A) γ	2.1 $\pm$ 0.1	3.6 $\pm$ 0.3	4.2 $\pm$ 0.5	2.0 $\pm$ 0.07	0.6 $\pm$ 0.1	2.4 $\pm$ 0.13***
$\alpha\beta$ (P9'A) γ	2.1 $\pm$ 0.1	3.8 $\pm$ 0.4	4.2 $\pm$ 0.6	2.1 $\pm$ 0.2	2.8 $\pm$ 0.3***	2.1 $\pm$ 0.24***
$\alpha\beta$ (L10'A) γ	2.3 $\pm$ 0.1	3.3 $\pm$ 0.3	3.7 $\pm$ 0.4	2.0 $\pm$ 0.1	1.4 $\pm$ 0.1***	2.0 $\pm$ 0.08***
$\alpha\beta$ (LPY) γ	2.0 $\pm$ 0.2	3.7 $\pm$ 0.4	5.1 $\pm$ 0.4*	2.0 $\pm$ 0.1	2.1 $\pm$ 0.2***	4.2 $\pm$ 0.4***
$\alpha\beta\gamma$ (F8'A)	3.0 $\pm$ 0.2***	4.3 $\pm$ 0.2	3.1 $\pm$ 0.3	1.6 $\pm$ 0.1	0.4 $\pm$ 0.03	0.9 $\pm$ 0.13
$\alpha\beta\gamma$ (P9'A)	2.4 $\pm$ 0.1*	3.7 $\pm$ 0.2	5.3 $\pm$ 0.5*	2.1 $\pm$ 0.1	0.4 $\pm$ 0.04	0.5 $\pm$ 0.06
$\alpha\beta\gamma$ (M10'A)	3.0 $\pm$ 0.1***	5.5 $\pm$ 0.7***	5.5 $\pm$ 0.3**	2.2 $\pm$ 0.03*	0.6 $\pm$ 0.06	0.6 $\pm$ 0.04
$\alpha\beta\gamma$ (MPF)	2.7 $\pm$ 0.2**	3.7 $\pm$ 0.2	4.3 $\pm$ 0.5	1.5 $\pm$ 0.1**	2.9 $\pm$ 0.2***	1.0 $\pm$ 0.04

Drug potentiation is expressed as the fold increase in current relative to a GABA EC_{10–20} response. Whole-cell currents in response to direct drug activation are expressed as a fraction of their EC_{10–20} response to GABA. Values represent mean $\pm$ S.E.M. from $n = 4$ to 16 experiments. Significant differences compared to the wild-type $\alpha\beta\gamma$ receptor were determined using one-way ANOVA: *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. Dz, diazepam; Et, etomidate; PB, pentobarbital; pot'n, potentiation.

3.4. Direct activation of GABA_A receptors by drugs is greatly enhanced when mutations are introduced into the Cys-loop of the α or β subunit

Some drugs such as pentobarbital (Mathers & Barker, 1980; Rho, Donevan, & Rogawski, 1996) and etomidate (Yang & Uchida, 1996) are able to directly activate the GABA_A receptor. Although the actual mechanism through which this occurs is unknown it has been established that the drug-binding site through which channel activation occurs is different to that of the agonist GABA (Amin & Weiss, 1993). Since we saw quite distinct subunit-specific demarcation in the response to drug potentiation for the set of mutants trialed, we tested the ability of these drugs to directly open $\alpha 1\beta 2\gamma 2S$ receptors carrying a mutation in the conserved Cys-loop of one of their subunits.

Direct activation by pentobarbital and etomidate resulted in significantly larger whole-cell currents relative to the wild-type response when any of the mutations in the ArProAl motif were introduced into either the α or β subunit Cys-loop (Table 2). By contrast, the equivalent mutations introduced into the Cys-loop of the γ subunit produced currents similar in amplitude to the wild-type response. Whole-cell currents in response to

direct activation by drugs are expressed as a fraction of their EC_{10–20} response to GABA. Hence, for the wild-type $\alpha\beta\gamma$ receptor 100 μ M pentobarbital generates a current about one-third that of a 1 μ M GABA (EC_{10–20}) response (0.3 $\pm$ 0.04, $n = 10$) (Fig. 4A) and 25 μ M etomidate about half the size (0.5 $\pm$ 0.05, $n = 14$) (Table 2). Cells expressing the α (MPF) $\beta\gamma$ mutant receptor displayed the largest currents upon pentobarbital activation. A typical current response is shown in Fig. 4C for comparison with the wild-type response in Panel A of the same figure. On average the response by this mutant receptor was about 19 times larger than that of the wild-type receptor (Fig. 4E). Similarly, mutations in the Cys-loop of the β subunit lead to significant increases in the amplitude of the current upon activation with either pentobarbital or etomidate (Fig. 4D and E). Mutational analysis of direct drug activation, while showing clear subunit specificity for the α and β subunits, did not show an amino acid preference; all three residues mutated in the ArProAl motif led to enhanced activation although the largest affects of direct drug activation were seen in the mutant receptors whose α or β subunit 8* aromatic and 10* aliphatic residues had been swapped around (Table 2, Fig. 4E).

at an EC_{10–20} concentration (indicated by G above the solid line). Current responses are expressed as the fold increase relative to the bracketed average GABA EC_{10–20} response. Holding potential -60 mV. Averaged data from 4 to 16 cells are displayed in Table 2. Panel I is a summary of the data where the fold increase of each mutant receptor has been normalised to the wild-type receptors response (shown as a line at 1): Dz, diazepam; Pb, pentobarbital; Et, etomidate; sw, swap. Significance was assessed by one-way ANOVA with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Fig. 4. Direct activation by pentobarbital and etomidate is enhanced by mutations in the ArProAl motif of the GABA_A receptor α and β subunit Cys-loops. Panels A and B show typical current responses of the wild-type $\alpha\beta\gamma$ GABA_A receptor to direct activation by 100 μM pentobarbital (0.3 of the GABA EC_{10–20} response) and 25 μM etomidate (0.5 of the GABA EC_{10–20} response), respectively. Mutation of residues in the Cys-loop of the α subunit led to significantly larger currents upon direct drug activation as shown here for the $\alpha(\text{MPF})\beta\gamma$ mutant receptors response to pentobarbital (Panel C). Similar increases in current were seen when the β subunit Cys-loop was mutated as shown here for the $\alpha\beta(\text{LPY})\gamma$ receptors response to etomidate activation (Panel D). Drug activation was bracketed by applications of GABA at an EC_{10–20} concentration (indicated by G above the solid line). Current responses are expressed as the fraction of these GABA EC_{10–20} responses. Holding potential -60 mV. Averaged data from 4 to 16 cells are displayed in Table 2. Panel E is a summary of the data where the average response of mutant receptors has been normalised to that of the wild-type receptor (shown as a line at 1): sw, swap. Significance was assessed by one-way ANOVA with *** $p < 0.001$ and * $p < 0.05$.

4. Discussion

The process of channel gating involves a number of conformational changes that require the co-

ordinated movement of two domains, the extracellular, ligand-binding domain (LBD) and the transmembrane, pore-forming domain (TMD). A number of recent studies have implicated the conserved Cys-loop in the

process of channel gating in LGICs because of its apparent movement subsequent to ligand binding and demonstrated interaction with two other key loops, the transmembrane M2-M3 linker sequence and the extracellular loop 2 (Absalom, Lewis, Kaplan, Pierce, & Schofield, 2003; Kash, Dizon, Trudell, & Harrison, 2004; Kash, Jenkins, Kelley, Trudell, & Harrison, 2003; Schofield, Trudell, & Harrison, 2004; Xiu, Hanek, Wang, Lester, & Dougherty, 2005). However, while its participation has been established, its specific functional role in mediating the series of events associated with gating has not been previously elucidated. Here we have addressed this issue in GABA_A receptors by disrupting the conserved ArProAl motif in the Cys-loop of individual α , β and γ subunits with single-point side-chain amputation mutants and aromatic/aliphatic swap mutants.

All of the mutations introduced in the ArProAl motif of the Cys-loop of GABA_A subunits produced functional, GABA-activated chloride channels. This demonstrates that the integrity of this sequence motif is not essential for normal protein folding and expression. This accords precisely with our initial hypothesis that the ArProAl motif has a functional rather than structural significance, reflected in the conserved sequence differences between the LGIC superfamily and the nonchannel AChBP homolog. Therefore, the ArProAl motif itself does not provide structural features that are required for effective receptor assembly and trafficking. This is contrast to the Cys-loop disulfide bond whose structural integrity is critical for receptor assembly (Blount & Merlie, 1990; Fu & Sine, 1996).

In order to determine if the Cys-loops of the different subunits participate with singular roles in the channel gating process, we examined agonist activation and drug potentiation responses. Activation of the GABA_A receptor by agonist involves the binding of two GABA molecules at the interfaces created between the β and α subunits some 30 Å above the membrane and, hence, 30 Å above the Cys-loop ArProAl motif (Celie et al., 2004; Cromer, Morton, & Parker, 2002). How pentobarbital and etomidate directly activate the GABA_A channel is not known. The results from the systematic mutation of residues in the ArProAl motif of GABA_A subunits reveal that direct activation by either GABA or these drugs is affected principally by the disruption of native interactions in the α and β subunit Cys-loop and not in the γ subunit. Mutation of residues in the ArProAl motif of α and β Cys-loops all produced significantly larger currents in response to direct activation by pentobarbital and etomidate, implying that the interactions of this motif generate resistance to channel opening in

the wild-type receptor by both GABA and other direct activators.

The speed at which these gating-dependent transitions occur is influenced by the ArProAl motif in the Cys-loop. Particularly significant was the slowing of the current rise times and decay times when the proline residue at the tip of the Cys-loop of the α and β subunits was substituted. Mutation of some of the flanking residues in these two subunits also slowed the rising phase of the current but less so. In contrast, none of the mutations in the γ subunit had any affect upon this process. These results suggest that in the wild-type receptor the rigidity imposed by the proline at the tip of the Cys-loop is important for orchestrating a rapid response upon ligand binding through the interactions of both the α and β subunit Cys-loops. The recent atomic structure of the nAChR α 1 subunit LBD co-crystallized with α -bungarotoxin revealed an intimate interaction in the Cys-loop between the α 1 motif residues F8*, P9* and F10* in the absence of a transmembrane domain. The 9* proline had adopted the relatively less common cis-conformation due to stabilisation by phenylalanine at position *8. The presence of this constrained conformation, unique to the proline residue in the ArProAl motif of the principle agonist binding subunit of the nAChR, suggests why its substitution has profound functional consequences in the GABA binding subunits α and β . Interestingly, previous studies using unnatural amino acid substitutions have suggested that cis-trans isomerization of a conserved proline residue in the M2-M3 loop (Pro272) of the 5-HT_{3A} receptor is important in channel gating (Lummiss et al., 2005). It may turn out that such a transition also underlies the Cys-loop gating conformational changes although further experimentation is required to determine the influence of transmembrane domain residues in stabilising the Cys-loop structure.

The one agonist-mediated gating process affected by mutation in the γ subunit Cys-loop was desensitization where the loss of native aromatic and aliphatic interactions in the ArProAl motif all accelerated the process. Previous studies have implicated the involvement of the γ subunit in desensitization (Verdoorn, Draguhn, Ymer, Seeburg, & Sakmann, 1990). One possible interpretation of our results is that in the wild-type receptor the hydrophobic interactions of the γ subunit ArProAl motif act to resist desensitization, opposing the action of the proline-constrained α and β subunit Cys-loops whose function it is to facilitate a rapid conformational change, thereby modulating the rate of desensitization and subsequent pore closure in the continued presence of GABA. Hence, the different subunits in the GABA_A receptor

confer different response capabilities to the receptor during GABA activation.

Taken together the results from direct activation of mutant GABA_A receptors either by GABA or drugs reveal that the Cys-loops of the α and β subunits are the principal participants in the channel activation process and, by contrast, the native interactions of the ArProAl motif of the Cys-loop of the γ subunit contribute little to the coupling of the binding of direct activators to channel opening events. These ligand-activation data clearly support asymmetric transmission of the binding signal in the extracellular domain. The observation that both GABA activation and pentobarbital- and etomidate activation were affected by mutations in the same two subunits suggests that the Cys-loop plays the same role in receptor activation, irrespective of the ligand. The finding that only the Cys-loops of the principal binding subunits participate in mediating resistance during agonist activation is consistent with observations of a natural mutant of the nAChR where effects on channel gating were only observed when the Cys-loop V5*L mutation was carried in the principle binding subunits α or δ (Shen, Ohno, Sine, & Engel, 2005).

It has been proposed, based on a chemical interpretation of the main functional regions of the nAChR, that the Cys-loop may function as a pivot that stabilises conformations during gating (Unwin, 2005; Unwin et al., 2002). The functional data presented here derived from mutated GABA_A receptors reveal that the structural constraints imposed by the proline 9* residue at the tip of the Cys-loop of the α and β subunits (but not the γ subunit) are consistent with the Cys-loop acting as a pivot during gating-dependant conformational changes.

Potentiation of the GABA response by diazepam, pentobarbital and etomidate revealed that the conserved Cys-loops in the different subunits are not participating equivalently in the gating of the heteromeric $\alpha\beta\gamma$ GABA_A receptor. Drugs were effectively more potent in the presence of GABA when mutations were introduced into the Cys-loop of the α or γ subunit. By contrast, equivalent mutations in the β subunit Cys-loop had no effect on potentiation, despite using drugs that are predicted to bind to different domains (LBD vs. TMD) and have different predicted subunit specificities (e.g., references Belelli et al., 1997; Brown et al., 2002; Dalziel, Cox, Gage, & Birnir, 1999; Hill-Venning, Belelli, Peters, & Lambert, 1997; Li et al., 2006; Mihic, Whiting, Klein, Wafford, & Harris, 1994; Sigel, Schaerer, Buhr, & Baur, 1998). Mutation of the aromatic and aliphatic residues in the α and γ Cys-loops all increased the potency of the drugs implying that the functional role of the Cys-loops

in these subunits in the native receptor is also to impede drug-potentiated channel gating.

The binding site for diazepam is proposed to be at an extracellular site, at the unique interface created by the single γ subunit and one of the two α subunits (Sigel & Baur, 1997). The results from this mutational study of residues in the Cys-loop ArProAl motif are also consistent with the involvement of these same two subunits in diazepam potentiation. Thus, the potentiation of the GABA response by diazepam may be thought of as resulting from the recruitment of the γ subunit into the channel opening process, thereby creating a functional pseudo-symmetry in the LBD of the pentamer. The similarity in the results obtained using different drugs to potentiate the GABA response suggests that in the wild-type receptor all three drugs act via a common mechanism, i.e., viz principally involving the α and γ Cys-loops. Our findings thus suggest a novel target for the pharmacological modulation of this class of receptors. Any drug designed to interfere with the interactions of the Cys-loop of particular GABA_A subunits would be expected to enhance inhibitory activity.

In conclusion, we propose that the strikingly different sequence at the tip of the Cys-loop (ArProAl motif) in LGICs compared to the hydrophilic sequence of the nonchannel AChBP (TES) reflects the divergence of function between these proteins. Our mutational analysis has identified specific roles for the conserved ArProAl motif in the Cys-loop of the GABA_A receptor subunits, in which disruption of just a single amino acid side-chain interaction reduces both the EC₅₀ (with two exceptions) and Hill co-efficient and, additionally, increases the potency of different classes of drugs. We propose that the native interactions of these ArProAl motifs in the Cys-loops participate in communication between the LBD and the TMD and, furthermore, act in a general sense as an impedance to channel gating in GABA_A receptors. The subunit-specific mutations reveal that the α and β subunit Cys-loops mediate rapid conformational changes upon agonist activation that require the 9* proline residue but that the γ subunit Cys-loop does not participate in this process because of its insensitivity to the replacement of its 9* proline. Potentiation by drugs (and presumably endogenous potentiators) however, recruits the γ subunit into the activation process thereby channelling additional ligand-binding energy into driving the gating-dependant conformational changes and rendering the activation process pseudo-symmetrical in the ligand-binding domain.

Given the similarity of sequence and the likelihood of common molecular mechanisms among all members of the Cys-loop LGIC family we predict that the conserved

loop will act equivalently in resisting gating-dependant conformational changes, but that different subunits may participate asymmetrically in agonist activation and drug modulation as shown here for the $\alpha\beta\gamma$ GABA_A receptor.

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