Crosslinking of α1β1 $GABA_A$ receptor subunits *via* cysteines introduced into the transmembrane domain

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$GABA_A$ receptors belong to the ligand gated ion channel superfamily. Activation of these channels is thought to involve movement of the pore lining M2 domain. A model for α1β1 $GABA_A$ receptor activation has been proposed on the basis of disulphide bond trapping experiments at an M2 residue (T6′) located near the activation gate (Horenstein *et al.*, 2001). This residue was mutated in both subunits to produce αT6′C and βT6′C subunits. Western analysis of $GABA_A$ receptors expressed in HEK293 cells plus electrophysiology of the same receptors in *Xenopus* oocytes suggested that disulphide bonds between adjacent β subunits lock the channels in the open state. The authors conclude that activation is mediated by an asymmetric rotation of adjacent β subunits. Data from our laboratory presented previously (Shan *et al.*, 2002) and here question this model.

To facilitate Western analysis, all αT6′C subunits were tagged with a FLAG epitope and all βT6′C subunits were tagged with a myc epitope. HEK293 cells were transfected with different combinations of wild type α, wild type β, αT6′C and βT6′C subunits and investigated by whole-cell electrophysiological recording. The oxidising agent, copper phenanthroline (Cu:phen), was used to promote formation of disulphide bonds and the reducing agent, dithiothreitol (DTT), was used to break them. Crude membrane preparations of the same transfected subunit combinations were used for Western analyses. Surface expression of functional receptors was confirmed by immunocytochemistry.

Disulphide bond formation between βT6′C subunits was observed in the closed state. In cells expressing channels containing βT6′C subunits, GABA-gated currents decreased irreversibly upon Cu:phen treatment. As this effect was reversed only upon DTT application, we conclude that the disulphide bonds lock the channel closed. These bonds also formed spontaneously at a much slower rate. Western analysis provided direct evidence that formation of disulphide bonds in the closed state occurs between β subunits. The inclusion of DTT at several stages of channel protein preparation was sufficient to reduce the inter-subunit disulphides. Similarly, when Cu:phen was applied in combination with GABA, cells expressing channels containing βT6′C subunits again drastically reduced GABA current magnitude. Since the channels could only be reopened upon a subsequent application of DTT, we conclude that disulphide bonds lock the channel in a closed conformation. Since channels containing βT6′C subunits desensitize rapidly relative to wild type channels, the conclusion that the βT6′C subunits are crosslinked in the open state (simply because GABA is present) should be treated with caution. Due to the very short lifetime of the open state, we suggest that disulphide bond formation occurs predominantly in the desensitized state. Western analysis again provided direct evidence that formation of disulphide bonds in the desensitized state occurs between β subunits. No inter-subunit disulphides were observed with channels containing only αT6′C subunits and β wild type subunits. Indeed, αT6′C subunits were observed to form DTT-sensitive intramolecular disulphides. However, a weak band corresponding to a small population of mixed disulphides was observed with channels containing both αT6′C subunits and βT6′C subunits.

Because we find no evidence for a state-dependent disulphide trapping of T6′C residues, our results are inconsistent with the model for LGIC activation proposed by Horenstein *et al.* (2001).

Horenstein, J., Wagner, D.A., Czajkowski, C. & Akabas, M.H. (2001) *Nature Neuroscience*, **4**, 477-485.
Shan, Q., Haddrill, J.L. & Lynch, J.W. (2002) *Journal of Biological Chemistry*, 277, 44845-44853.