Understanding the molecular and pharmacological basis of selectivity of nicotinic acetylcholine receptor antagonists using reactive methyllycaconitine analogues

N.L. Absalom,[1] G. Quek,[1] J. Ambrus,[2] M.D. McLeod[2] and M. Chebib,[1] [1]Faculty of Pharmacy, University of Sydney, Camperdown, NSW 2006, Australia and [2]Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia.

The nicotinic acetylcholine recepto (nAChR) mediates fast synaptic transmission between neural cells. The nAChR is a pentameric protein that contains a large extracellular domain, four transmembrane domains (M1-M4) where the second M2 lines the channel pore, two short M1-M2 and M2-M3 loops that move to gate the channel and a large intracellular M3-M4 loop. There is a large amount of subunit heterogeneity within the nAChR, which can be formed by specific combinations of α2-10 and β2-4 receptors. The expression patterns of receptor subtypes partly determine the physiological role of each nAChR subtype. Thus, pharmacological agents that can distinguish between receptor subtypes may have greater selectivity for certain physiological process, and may provide superior pharmacological agents. The α7 homomeric is potently and selectivity inhibited by the toxin methyllcaconotine (MLA) from the lakspur plant. Our aim was to identify the site of the receptor that conferred the binding selectivity to MLA on the α7 receptor and compare this to the corresponding residues on the α4β2 receptor. The α7, α4 or β2 cRNA was injected into *Xenopus* oocytes that were removed from frogs anaesthetized with tricaine and ion channel function was measured by the two-electrode voltage clamp technique. For efficient expression of the α7 nAChR, cRNA for the chaperone protein RIC-3 was co-injected. To prevent the large desensitization properties of the α7 nAChR, a mutant L9′T DNA was created by site directed mutagenesis and all further mutations were studied with this background. The L9′T mutation markedly affected acetylcholine activation but not MLA sensitivity. When varying concentrations of ACh were applied to oocytes injected with α7 or α7L9′T after 3 minute incubation with a set concentration of MLA, the maximum response was the same as for the maximum response to ACh alone. This suggests that the ACh is competing for the same binding site with the MLA. Furthermore, the IC50 of MLA is significantly reduced in the α4β2 nAChRs, highlighting the selectivity. When this experiment was performed on oocytes injected with α4β2 nAChRs, the ACh the maximum response with ACh and MLA was significantly lower than ACh alone, indicating that the MLA was also binding at a site different to the ACh-binding site. A previous published crystal structure of the acetylcholine binding protein bound to MLA identified residues that interact directly with the MLA molecule. We focused on two sites where MLA was bound, including the Q79 residue where several antagonists and agonists of the α7 nAChR confer selectivity by interactions with this residue in the extracellular domain. We have mutated this residue to the lysine and threonine residues that are the homologous residues on the α4 and β2 receptors, respectively to create the Q79K L9′T and Q79T L9′T mutant receptors. We have also made the homologous reversal mutations on the α4 and β2 subunits to determine if the MLA inhibition is altered. A second approach was taken by modifying MLA to contain a cysteine-reactive MLA molecule that can tether to introduced cysteines on the target receptor. We applied this molecule to α7 receptors with introduced cysteine residues and identified one residue, S188C L9′T, where the addition of the cysteine reactive MLA causes a permanent reduction in the current elicited by ACh. This indicates a strong association between this residue and the site of the cysteine reactive group in MLA binding. We have made the corresponding mutations in the α4 and β2 subunits to compare the residues that bind to MLA in nAChR subtypes. Here we show that the while the residues that bind to selective antagonists of nAChRs can be predicted with homology models, the mechanism by which these antagonists are selective are best understood by studies using a combination of site-directed mutagenesis and chemical modification.