Effect of a toggle switch mutation in TM6 of the human adenosine A_3 receptor on Gi protein dependent signalling and Gi-independent receptor internalisation

Leigh Stoddart, Steve Briddon, Stephen Hill. *University of Nottingham, Institute of Cell Signalling, Medical School, NG7 2UH, UK*

The highly conserved tryptophan in transmembrane domain 6 of G protein-coupled receptors has been showed to play a central role in forming an active conformation in response to agonist binding. The role of this residue has been studied in a variety of receptors with differing phenotypes. In the adenosine A_3 receptor, mutation of this residue is reported to result in a receptor that is unable to couple to G proteins. We set out to fully characterise the effect of this mutation on the signalling to a variety of G protein-dependent pathways and to G_i-independent internalisation.

The W243F mutation was introduced into a A_3-YFP construct (full length A_3 linked to yellow fluorescent protein) and stably expressed in CHO CRE-SPAP cells. The G_i mediated inhibition of forskolin stimulated CRE gene transcription to a variety of adenosine receptor agonists was monitored. It was found that the maximal response was attenuated in the mutant receptor compared to the wild type receptor to the agonist NECA (% inhibition, A_3 W243F-YFP = 30±4%; A_3-YFP = 79±7%) and this response was completely abolished by pre-treatment with pertussis toxin (PTX). However, the response to highly A_3 selective agonist HEMADO was completely abolished in the mutant cell line. Confocal microscopy confirmed that both A_3-YFP and A_3 W243F-YFP were expressed at the cell surface and treatment with NECA resulted in the accumulation of intracellular granules of fluorescence and loss of receptor from the cell membrane. Quantification of the concentration dependence and time course of the internalisation response was carried out using a MD IX Ultra confocal plate reader, and automated analysis for the appearance of granules was performed on the resulting images. Both A_3-YFP and A_3 W243F-YFP internalised rapidly in response to NECA ($t_{1/2}$ = 7.76±0.37 and 6.34±0.46 min respectively) and the potency of NECA was similar for both receptors. Pre-treatment with PTX had no effect on the time course of internalisation or the potency of NECA in either cell line. There was no change the distribution of A_3 W243F-YFP with HEMADO treatment, whereas A_3-YFP still internalised rapidly ($t_{1/2}$ = 11.8±1.7 min). To confirm that HEMADO still retained the ability to bind to the mutant receptor, A_3 W243F-YFP cells were treated with HEMADO prior to the addition of NECA. HEMADO was able to completely block NECA mediated internalisation of A_3 W243F-YFP, suggesting that it now acted as an antagonist at the mutant receptor. Interestingly, unlike NECA, HEMADO mediated internalisation of A_3-YFP showed some degree of PTX sensitivity.

These data suggest that conformational changes around transmembrane domain 6 play an important role determining the signalling pathway that the adenosine A_3 receptor activates. It also suggests that HEMADO activates the adenosine A_3 receptor in a different manner to NECA.

We thank the MRC for financial support