Regulation of the M_1 RASSL by Phosphorylation

Sajjad A. Riaz[1], Steven J. Charlton[2], Andrew B. Tobin[1]. [1]*University of Leicester, Lancaster Road, Leicester LE1 9HN, UK,* [2]*Novartis Institutes for Biomedical Research, Wimblehurst Road, Horsham RH12 5AB, UK*

G-protein coupled receptors or seven-transmembrane receptors have been shown to undergo phosphorylation in response to agonist occupation that mediates downstream signalling in the cell. To investigate these receptors, *in vivo*, our laboratory is currently employing a chemical genetics approach based on a mutant receptor called a RASSL (receptors activated solely by synthetic ligands). RASSL receptors are engineered so that the receptor no longer responds to its endogenous ligand(s) but instead can be activated by a previously inert pharmacological ligand. A RASSL based on the acetylcholine M_1-muscarinic receptor has been developed which contains modifications in the third- (T106C) and fifth- (A196G) trans-membrane domains. The M1-RASSL receptor mutant has been shown to be unresponsive to acetylcholine (Ach) but responds to the chemical ligand, clozapine-N-oxide (CNO). This was determined in assays that measure the accumulation of inositol phosphate in the presence of a lithium block of inositol 1-phosphatase. Basal levels of phosphate as measured by the Inositol Phosphate accumulation assay (IP_x) = 6067.664 ± 1414.394 cpm/mg. Stimulation with 10mM CNO resulted in levels to increase to 49456.65 ± 12166.24 cpm/mg. Initial studies from other laboratories have shown that the M1-RASSL receptor couples normally to phosphoinositide hydrolysis and ERK1/2 phosphorylation (Roth et al*.,* 2007). However, the phosphorylation profile of this receptor mutant has up until now not been described. Here we show using Western blotting with phospho-specific antibodies raised against residues which have been shown to be phosphorylated, that the M_1-RASSL receptor is phosphorylated in response to CNO in a similar manner to the wild type M_1-receptor following stimulation with Ach. In the wild-type receptor phosphorylation at residue 228 was seen to increase by approximately 7-fold in response to Ach but no response was seen to CNO. In contrast, the M1-RASSL receptor did not respond to Ach but CNO stimulation resulted in an approximate 7-fold increase in receptor phosphorylation at residue 228. We also show that the M1-RASSL receptor when stimulated with CNO behaves in a similar manner when the wild-type M_1-muscarinic receptor is stimulated with Ach with regards to its coupling to phosphoinositide hydrolysis. We anticipate these results to be the starting point for more sophisticated studies using the M_1-RASSL receptor, for example, studying-protein mediated signalling and phosphorylation/arrestin dependent signalling *in vivo* and *in vitro.*