Phospho-proteomic analysis of the M_1 muscarinic reveals phosphorylation *in vivo* and identifies the action of the allosteric modulator BQCA.

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G-protein coupled receptors are hyperphosphorylated in a process that controls receptor coupling to down-stream signalling pathways. The pattern of receptor phosphorylation has been proposed to generate a "phosphorylation barcode" that can be varied to direct physiologically relevant receptor signalling. Here we employ a proteomic approach using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) to identify 12 phosphorylation sites on the M_1-receptor. Eleven of these are in the 3rd intracellular loop and 1 site in the C-terminal tail. We have used this data to generate phosphorylation specific antibodies to target four of the sites, serine 228, serine 273 and serine 322 all in the intracellular loop and serine 451 in the C-terminal tail. These antibodies have been used in western blotting experiments to probe the phosphorylation status of these sites in mouse M_1-muscarinic receptors expressed in CHO cells. Anti phosphoserine 228 and 273 antibodies reported low basal and a strong agonist regulated increase in phosphorylation 3.26 +/- 0.03 n=4 and 3.86 +/- 0.24 n=4 fold increase over basal respectively in response to 5 minute stimulation with methacholine (100μM). In contrast to this, anti phosphoserine 322 and 451 reported higher basal and reduced agonist regulation 1.0 +/- 0.07 n=4 and 1.57 +/- 0.06 n= 4 fold increase over basal respectively. M_1 muscarinic receptors expressed in the hippocampus and cerebral cortex of adult C57/B6 mice were examined by immunoprecipitation followed by western blotting to determine if the phosphorylation profile of the M_1-muscarinic receptor is dependent on the brain area and cell type in which the receptor is expressed. These experiments revealed that the M_1 muscarinic receptor is highly phosphorylated on serine 228 and phosphorylated to a lesser extent on serine 451. There was no observable difference in the pattern of phosphorylation of M_1 muscarinic receptors in the hippocampus compared to the cortex.

We have extended these studies by using one of these antibodies, anti phospho-serine 228, to determine changes in the M_1-muscarinic receptor phosphorylation following treatment with the M_1-receptor specific ligand 1-(4-Methoxybenzyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid (BQCA), a positive allosteric modulator of the M_1-receptor which has been shown to reverse scopolamine induces deficit in memory and learning in mice (Ma, *et al*.,2009). We examine the phosphorylation status of M_1-muscarinic receptors on serine 228 in the hippocampus of wild type C57/B6 mice by western blotting 30 minutes after IP injection (15mg/kg) of BQCA and show that injection of BQCA induces a 1.4 +/- 0.04 n=2 fold increase in phosphorylation of serine 228, this is consistent with BQCA acting at hippocampal M_1-receptors and correlates well with its ability to mediate changes in hippocampal based learning and memory.