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Phosphorylation of Juxtamembrane C-terminal of CB1 Cannabinoid Receptor in NT18TG2 Cells

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Activation of CB1 cannabinoid receptor primarily stimulates the G protein family of Gi/o to produce its effects. The aim of the present study is to investigate the effect of phosphorylation of the juxtamembrane C-terminal domain of the CB1 cannabinoid receptor on G protein activation. To do so, we used phosphopeptides that mimic this domain to investigate activation of individual Gi proteins in N18TG2 neuroblastoma cell membranes using an antibody-capture [35S]-GTPγS scintillation proximity assay (SPA). An N18TG2 membrane fraction was prepared by homogenization and sedimentation at 40,000 X g, and the protein concentration was determined by the BCA method with bovine serum albumin as standard. C-terminal juxtamembrane peptides were incubated with N18TG2 membranes in a reaction mix including [^{35}S]-GTPγS, NaCl, dithiothreitol and GDP in 96-well plates for 1 h at 30 °C. The reaction was terminated by detergent-solubilization of the membrane proteins, followed by incubation with specific primary and secondary antibody associated with beads covalently bonded to the scintillation fluor. The non-specific binding was measured in the presence of 10 μM unlabelled GTPγS. Data are presented as the mean ± standard error from three or more experiments. Agonist-stimulated values were transformed to "percent over basal" [% = (stimulated-basal)/(basal)*100] then were analyzed by One-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post hoc test. We demonstrated that In N18TG2 membranes, the unphosphorylated peptide (Arg401 to Glt420) was able to stimulate [35S]-GTPγS binding in a dose-dependent fashion (30-300μM). When applied at 100μM the unphosphorylated peptide was able to stimulate [35S]- GTPγS binding mediated by Gi1: Emax = 18%, Gi2: Emax = 34%, Gi3: Emax = 25%, Go: Emax =18% over basal, respectively. We investigated the effect of phosphorylation of the three Ser residues (Ser402, Ser411, and Ser415) (100μM) and found that phosphorylation of Ser402 was able to stimulate [35S]-GTPγS binding mediated by Gi3: Emax = 33%, Go: Emax = 27% over basal. Phosphorylation of Ser415, was able to stimulate [35S]-GTPγS binding mediated by Gi3: Emax = 38%, Go: Emax =29% over basal. Phosphorylation of Ser411 was able to stimulate [35S]-GTPγS binding mediated by Gi3: Emax = 31%, Go: Emax =33.6% over basal. The order of potency for Go activation was pSer411>pSer415>pSer402>unphosphorylated and for Gi3 activation was pSer415>pSer411>pSer402>unphosphorylated. In conclusion, these data show that the 8th helix C-terminal peptides were able to stimulate G protein activation in N18TG2 cell membranes, and the activation could be augmented by phosphorylation of Ser residues.

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