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CB1 cannabinoid receptor-phosphorylated fourth intracellular loop structure-function relationships

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Abstract

A peptide comprising the juxtamembrane C-terminal intracellular loop 4 (IL4) of the CB₁ cannabinoid receptor possesses three Serine residues (Ser402, Ser411 and Ser415). Here we report the effect of Ser phosphorylation on the CB₁ IL4 peptide conformation and cellular signaling functions using nuclear magnetic resonance spectroscopy, circular dichroism, G protein activation and cAMP production. Circular dichroism studies indicated that phosphorylation at various Ser residues induced helical structure in different environments. NMR data indicates that helical content varies in the order of IL4pSer411 > IL4pSer415 > IL4 > IL4pSer402. The efficacy of phosphorylated IL4 peptides in activating Go and Gi3 ([³⁵S]GTPγS binding) and inhibiting cAMP accumulation in N18TG2 cells were correlated with helicity changes. Treatment of cells with bradykinin, which activates PKC, augmented CB₁-mediated inhibition of cAMP accumulation, and this was reversed by a PKC inhibitor, suggesting that phosphorylation of serine might be a physiologically relevant modification in vivo. We conclude that phosphorylation-dependent alterations of helicity of CB₁ IL4 peptides can increase efficacy of G protein signaling.

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Author Contributions

ACH, SMC designed the study, interpreted data, wrote and revised the manuscript. IRC and SMC designed, performed and analyzed the experiments shown in Figure 1. ADG, LWP, JB and SMC designed, performed and analyzed the experiments shown in Figures 2 and 3 and table 2. ADG and SMC designed, performed and analyzed the experiments shown in Figures 4 and 8. KE and ACH designed, performed and analyzed the experiments shown in Figures 5, 6 and 7 and table 1. All authors reviewed the results, wrote section of the manuscript, and approved the final version of the manuscript.

Conflict of interest

The authors have no Conflicts of Interest to disclose.

Keywords

Bradykinin; endocannabinoid; G protein-coupled receptors (GPCR); peptidic; phosphorylation; protein kinase C (PKC)

Introduction

The CB₁ cannabinoid receptor (CB₁R) is the most prominent G protein-coupled receptor (GPCR), in the CNS and has been characterized for its coupling to the Gi/o family to reduce cAMP levels in a pertussis toxin-sensitive manner (1,2), regulate K⁺ and Ca²⁺ channels, and stimulate the mitogen-activated protein kinase (MAPK) pathway (3). Using peptides representing the N-terminal side of the 3rd and 4th intracellular loop (IL4) and site-directed antibodies, the ability of the CB₁R intracellular loop regions to interact with G proteins was investigated (4). The ability of these peptide fragments to reduce cAMP levels suggests that these peptides represent the receptor domains that interact with and contribute to the activation of Gi/o. Thus, these peptides are among the first GPCR-derived peptides shown to produce functional cellular signaling.

The CB₁R IL4 domain (amino acids 401–417 in rodent) is critical for Gi/o protein coupling (5). The synthetic peptide (CB₁R IL4) was able to reduce cAMP levels in a CB₁R-independent manner. In the absence of CB₁R expression in Chinese hamster ovary cells, the CB₁R IL4 peptide was still functional. These data are in line with direct activation of G_i by the CB₁R IL4 peptide, although alternative sites of interaction are possible. Studies by Mukhopadhyay and colleagues on the CB₁R IL4 peptide and its analogs were focused on helicity and charge within the peptide (5).

Circular dichroism spectropolarimetry (CD) showed that in an aqueous environment the CB₁R IL4 peptide exists in a random coil conformation (5). Although significant α -helix formation was not inducible in an hydrophobic environment, sodium dodecylsulfate (SDS) micelles induced significant helical structure as characterized by CD as well as NMR analyses (5,6). Less active peptide analogs that were truncated or had the charged groups blocked, exhibited helical conformation in a hydrophobic environment (5). These peptide analogs of the CB₁R IL4 retained the full efficacy but reduced apparent affinity to activate Gi/o.

The presence of one Lys and three non-consecutive Arg residues within the CB₁R IL4 sequence suggests a cationic patch in the helical structure. In many GPCRs, a BBXB or BBXXB motif has been proposed to exist on the receptor domains that bind to G proteins (7,8). It was hypothesized that an α -helical structure of the peptide could be stabilized by the negatively charged region of the G protein. Phosphorylation and dephosphorylation reactions are important regulatory mechanisms in receptor signaling. The CB₁R IL4 peptide possesses three Ser residues (Ser402, Ser411 and Ser415) which could be potential sites for phosphorylation-dephosphorylation modifications (Table I). If one or more of these residues is phosphorylated, the charge distribution along this region is expected to be modified. In the present study, we examined the effect of phosphorylation on the CB₁R IL4 peptide conformation using CD and NMR spectroscopy, and demonstrated that structural

modifications were related to cellular signaling functions mediating Gi/o activation and cAMP production.

Experimental Section

Chemicals

NMR reagents were obtained from Aldrich chemical company (Milwaukee, Wisconsin, USA). Deuterated SDS was obtained from Cambridge Isotope Laboratories (Massachusetts, USA). [^{35}S]GTP γ S, [^3H]cAMP, and antibody capture scintillation proximity assay (SPA) polyvinyl toluene beads were obtained from Perkin-Elmer (MA, USA). GDP, forskolin, bradykinin, calf thymus histone, protein kinase A (PKA) from bovine heart, polyethelene glycol 8000 and fatty acid free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanandamide, 3-isobutyl-1-methylxanthine, rolipram and bisindolylmaleimide were from Cayman Chemical (Ann Arbor, MI, USA). CP55940 was provided as solutions in ethanol by the Drug Supply Program of the National Institute on Drug Abuse (NIDA, Rockville, MD, USA) and WIN55212-2 was from Cayman Chemical (Ann Arbor, MI, USA). Gi3 and Go antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Peptide synthesis

CB₁R IL4 phosphopeptides (IL4pSer402, IL4pSer415, IL4pSer411; Table 1), were manufactured using an ABI431 synthesizer, purified over a Microsorb C-18 reverse-phase HPLC column, and the structures of the peptides were verified by GC/MS (Midwest Biomolecules, Inc., St. Louis, MO or Pepton Inc. South Korea). The peptides purity by HPLC analysis was >98% (MW=2131.28).

Circular Dichroism Spectropolarimetry

The CD spectra were measured on a Jasco J-720 spectropolarimeter. The equipment was standardized as described in (5). Phosphopeptide samples for CD measurements were 75 μM in solvents designed to mimic various cellular or biomembrane compartments (5).

NMR Spectroscopy and Structure Calculations

NMR experiments were carried out at 5 mM phosphopeptide analogs in 90% H₂O, 10% D₂O, pH 4.5. Perdeuterated SDS solution (300 mM), which was well above critical micelle concentration, was added to the same peptide samples, making the molar ratio of peptide to detergent as 1:60. The experiments in SDS were performed as in (6). 1D spectra were recorded from 300 °K to 320 °K at 5 °K intervals. The phase sensitive homonuclear 2D double-quantum filtered correlation spectroscopy (DQF-COSY), nuclear Overhauser effect spectroscopy (NOESY) and total correlation spectroscopy (TOCSY) experiments were performed using the time-proportional phase incrementation method (9–13). NOESY experiments with mixing times at 100, 200, 300 and 340 ms were used for sequential assignments. The ^1H – ^1H NOE build-up curve was optimal at 340 ms mixing time and was used for structural analysis. These experiments, were performed as described in (14) and NMR spectra were recorded as in (15).

3D structure calculation was performed as described in (15,16). NOESY peak volumes on the 340 ms NOESY spectra were converted into distance constraints, and were designated as strong, medium and weak (1.8–2.7 Å, 1.8–3.5 Å and 1.8–5 Å, respectively). A total of 114, 112 and 114 NOEs were used as input for CYANA for IL4pSer402, IL4pSer415, and IL4pSer411, respectively. Inputs for CYANA were: for IL4pSer402 peptide, a total of 180 angle and NOE distance constraints (68 intra-residue, 41 i, i+1; 13 i, i+2 and 7 i, i+3); for IL4pSer415 peptide, a total of 183 angle and NOE distance constraints (88 intra-residue, 75 i, i+1; 15 i, i+2 and 5 i, i+3); for IL4pSer411 peptide, a total of 183 angle and NOE distance constraints (88 intra-residue, 75 i, i+1; 15 i, i+2 and 5 i, i+3). Of 50 generated structures, 20 were selected based upon low target function values ($\approx 1\text{\AA}$). Restrained energy minimization was performed, and further refinement of the structure was done using $^3J_{\text{HNH}\alpha}$ -coupling constants obtained from high-resolution 1D spectra (pre-saturation) and converted to dihedral angles (17,18).

Cell culture and membrane preparation, [^{35}S]GTP γ S Binding and Adenylyl Cyclase

N18TG2 neuroblastoma cells were cultured, membranes prepared, and [^{35}S]GTP γ S binding assays were performed as described in (19,20). The assay for cAMP accumulation was performed as described in (19,21).

Data Analysis

Data are presented as mean values $\pm$ standard error of the mean (SEM) of at least three independent experiments. cAMP accumulation is represented as a percentage of that stimulated by forskolin or secretin alone. Data were analyzed using GraphPad/Prism.

Results

CD spectroscopy in different membrane mimetic environments

The CD spectra for IL4pSer402, IL4pSer415, and IL4pSer411 were acquired in Phosphate buffer, a mix of Na₂HPO₄ and KH₂PO₄ (pH 7.0) at various concentrations of SDS, methanol and TFE (titration data not shown). The CD spectra of all CB₁R IL4 peptides exhibited an intense negative minimum around 198 nm in phosphate buffer (Fig.1), suggestive of a random coil configuration (23). The spectra indicate maximum negative ellipticity at 222 nm in SDS (Fig. 1), suggestive of α -helix structure (24–26). The appearance of an isodichroic point at 203 nm confirms development of α -helical structure in SDS (Fig.1A). Our results show that IL4pSer402 is unstructured in methanol (Fig.1A). α -Helical conformation was observed in SDS and 90% TFE.

CD spectra recorded for IL4pSer415 in phosphate buffer, TFE and methanol (Fig. 1B) indicated its existence as a random coil. At 90% TFE, a slight shift toward helicity was initiated. The spectra indicate induction of α -helix in SDS. Methanol as high as 50% failed to induce structure, contrary to the results observed for IL4pSer402. This indicates that a hydrophobic environment does not induce α helical structure.

CD spectra for IL4pSer411 exhibited an intense negative band at 198 nm in phosphate buffer, methanol, and TFE (Fig. 1C), indicative of random coil structure. Spectra in SDS at

very low concentrations showed characteristics of α -helical structure, and an isodichroic point was detected at 203 nm indicating stabilization of α -helix. SDS was observed to induce more structure than TFE.

NMR assignments and chemical shift analyses

The proton spectra of the three IL4 phosphopeptides in SDS was assigned from the two-dimensional TOCSY and NOESY spectra as previously detailed (15,22,23). Complete proton resonance assignments for IL4pSer402, IL4pSer415, and IL4pSer411 peptides in SDS are summarized in supplementary information tables (Table S1 and Table S2).

The CB₁ IL4 peptide shows upfield chemical shifts in SDS micelles for residues Leu5 to Met12 (Fig. 2A), indicating helix in the middle region of the peptide (24,25). However, both N- and C-termini show extended conformation from residues Arg1 to Asp4 and Phe13 to Glu17 (6). As is seen from the chemical shift index for IL4pSer402 (Fig. 2B), the helix is broken at His7 in the middle of the peptide. A downfield chemical shift index indicates extended structure for both N- and C-termini. Fig. 2C shows the chemical shift index for IL4pSer415, in which residues Asp4 to Met12 show upfield chemical shifts indicating a stretch of helical structure from Asp4 to Met12. Fig. 2D shows the chemical shift index for IL4pSer411 indicating an upfield chemical shift from Arg1 to Met12 and thus a longer helical stretch. As compared to the CB₁R IL4 peptide, IL4pSer411 shows a longer helical stretch from Arg1 to Met12 (N-terminus to middle region). The C-terminus does not show any defined conformation, as also seen in the case of IL4pSer415. The percentage of helical content was semi-quantitatively determined (6,26) to be 43% for CB₁R IL4 from residues L5 through M12. Helical content was 33% for IL4pSer402 A8 through M12, 74% for IL4pSer415 D4 through M12, and 81% for IL4pSer411 D4 through M12.

Phosphopeptide Secondary Structure determined from NOE connectivity

Weak $d_{\alpha N(i,i+2)}$ NOEs in addition to the standard $d_{\alpha N(i,i+3)}$ NOEs indicate the presence of a 3_{10} -helical structure in the IL4 peptide(6). The relative intensities of d_{NN} NOEs being stronger than $d_{\alpha N}$ NOEs for IL4pSer402 (A8–M12) (Fig. 3A) are consistent with helical conformation (23). All these NOEs indicate that in the SDS environment, IL4pSer402 is significantly structured but not completely helical between residues K3 and F13. In IL4pSer415, a helical conformation is indicated by stronger d_{NN} NOEs, and the presence of 3_{10} -helix from residue D4-M12 is supported by both NOE connectivities of medium range (3 $d_{\alpha\beta(i,i+3)}$, 4 $d_{\alpha N(i,i+3)}$, 6 $d_{NN(i,i+2)}$ and 9 $d_{\alpha N(i,i+2)}$) as well as by a negative chemical shift index for these residues.

IL4pSer411 shows greater helical conformation as compared to IL4pSer415 and IL4pSer402. The presence of medium range NOE connectivities (6 $d_{\alpha\beta(i,i+3)}$, 5 $d_{\alpha N(i,i+3)}$, 6 $d_{NN(i,i+2)}$ and 7 $d_{\alpha N(i,i+2)}$) supported by $d_{\alpha N(i,i+4)}$ connectivity between L5-F9 indicates predominant α -helix along with some degree of conformational averaging around the helical core.

Additional criteria for helicity are found as $^3J_{HNH\alpha}$ coupling constants less than 6 Hz (27,28). IL4pSer402 shows $^3J_{HNH\alpha}$ values around 4–6 Hz for residues A8-M12, supported by few medium range NOEs, indicating a non-random coil conformation. IL4pSer415 D4-M12 coupling constants are around 4–6 Hz, suggestive of helical structure. In the case of

IL4pSer411, the central core L5-M12 shows coupling constants around 4–6 Hz, indicating core helix as supported by various medium and long range NOEs. The terminal residues show $^3J_{\text{HNH}\alpha} > 6$ Hz, suggestive of fraying at the termini.

Generation of three dimensional structure

The 340 ms NOESY spectra were used to generate distance restraints. A total of 114, 114 and 119 NOEs along with 16 dihedrals were derived, respectively, for IL4pSer402, IL4pSer415 and IL4pSer411. The number of sequential, medium range, long range and intra-residue contacts for the peptides are summarized in Table II. Three-dimensional structure was then calculated by simulated annealing in torsion angle space and restrained molecular dynamics (CYANA) (6,16). Twenty calculated structures having the lowest RMSD values were selected: 0.5 ± 0.11 Å, 0.30 ± 0.10 Å and 0.26 ± 0.08 Å, respectively, for IL4pSer402, IL4pSer415 and IL4pSer411. The phosphopeptide backbone atoms for the superposition are shown in Fig. 4, and the structural statistics for the ensemble are described in Table II. The Ramachandran plot (PROCHECK) (29,30) indicated that the backbone dihedral angles reside within favored regions for all 20 refined structures. These structures along with NMR data are deposited in PDB (IDs: 2mz2, 2mz3, 2mza) and BMRB (Accession codes: 25470, 25471, 25478) databases.

CB₁R IL4 phosphopeptides evoke Gi/o protein activation and inhibit cAMP accumulation in N18TG2 Cells

To study the effect of CB₁R peptide phosphorylation on Gα_o and Gα_{i3} activation, phosphopeptides (20 to 100 μM) were incubated with N18TG2 membranes, and G protein activation was quantitated using a GTPγS binding immune-targeted scintillation proximity assay (SPA) (Fig.5). At 100 μM IL4pSer402, stimulated [³⁵S]GTPγS binding to Gα_o and to Gα_{i3} with similar efficacy to IL4. IL4pSer415 and IL4pSer411 were more efficacious than IL4 in stimulating [³⁵S] GTPγS binding to Gα_o and to Gα_{i3}. Interestingly, when compared to the maximal stimulation by full agonist WIN55212–2 (1 μM), activation of the two G proteins by phosphopeptides appeared to exhibit a quite different profile. The phosphopeptides activated Gα_o to an equal or greater extent compared with WIN55212–2 (Fig. 5A–5C). In particular, IL4pSer411 stimulated 3-fold greater than WIN55212–2 or the unphosphorylated IL4 peptide. In contrast, neither IL4 peptide nor the phosphopeptides activated Gα_{i3} to greater than 50% of the activation by WIN55212–2 (Fig. 5D–5F). IL4pSer411 appeared to have reached its maximal activation at a lower concentration (50 μM) than the other phosphopeptides (100 μM).

Fig. 6 demonstrates the ability of the phosphopeptides to transduce the Gα_{i/o} signal to the effector adenylyl cyclase, which is AC6 in N18TG2 cells (31). IL4 peptide inhibited cAMP accumulation in a dose-dependent fashion to the same maximal extent as the full agonist CP55940 acting at the CB₁R. Phosphopeptide analogs also inhibited cAMP accumulation in a dose-dependent fashion but more efficaciously than IL4. At 100 μM, IL4pSer415 and IL4pSer411 inhibited cAMP accumulation to a greater extent than IL4 or IL4pSer402. Mastoparan activates Gi/o proteins after passing across the cell membrane as an amphipathic α-helix (32–34). As proof of principle, mastoparan evoked a dose-dependent inhibition of cAMP accumulation that was identical to that of IL4pSer415.

PKC stimulation augments cannabinoid inhibitory effect on cAMP accumulation

Using NetPhos3 (35), IL4 Ser411 was expected to have a high possibility of being a substrate for PKC. The IL4pSer411 peptide appeared to exhibit a somewhat greater activation of Gi/o proteins, and was one of the two more effective peptides in the IL4 series to inhibit cAMP production. If phosphorylation at the Ser411 site can augment CB₁R activity *in vivo*, then one would expect that physiological stimulation of PKC would result in an augmented inhibition of adenylyl cyclase. To investigate whether a Gq-coupled GPCR that acts via PKC can modulate the cannabinoid effect on adenylyl cyclase in neuronal cells, we tested the response to bradykinin. The B2 bradykinin receptor is known to stimulate Ca²⁺ mobilization in the N18TG2 cells (36–38). We incubated cells with CP55940 (30–300 nM) with and without bradykinin. Bradykinin was able to augment the maximal response of CP55940 to inhibit either forskolin- or secretin-stimulated adenylyl cyclase in N18TG2 cells (Fig. 7). This effect of bradykinin was reversed by the PKC inhibitor, bisindolylmaleimide 1 (100 nM) Fig.7C.

Discussion

The CB₁R structure has been extensively studied to determine the critical sites for receptor recognition by agonists (39,40), determination of cannabimimetic activity (41), or interaction with G proteins leading to signal transduction (42). The C-terminal of the CB₁R juxtamembrane region has been suggested as a critical area for G protein activation, and studies using the strategy of co-immunoprecipitation indicated that unique domains of the CB₁R direct activation of each G protein subtype (4,43,44). We have recently demonstrated that phosphorylation of serine or threonine residues in the central and distal C-terminus of the CB₁R can alter the association with β -arrestin or cannabinoid receptor interacting protein 1 a (CRIP1a) (45). The current study highlights the impact of phosphorylation of proximal juxtamembrane C-terminus residues on helicity and signaling strength to the Gi3 and Go proteins.

Makriyannis laboratory studies on helix 8 peptide structure in dodecylphospho-choline (DPC) and SDS micelles revealed significant α -helical structure with similar cross-peaks in the NOESY spectra of the peptide in both micelles (46). As was found in our studies, under aqueous conditions, helix 8 exhibited a random coil conformation without secondary structural features (46). Using a combined high resolution NMR and computer modeling approach (47), Xie and Chen also reported that the C-terminal juxtamembrane domains of both CB₁R and CB₂R acquire helical conformations in DPC micelles.

In the present study, CD spectro-polarimetry was used to characterize the impact of phosphorylation on the conformation of the three phosphorylated analogs of IL4 (IL4pSer402, IL4pSer411, IL4pSer415). CD results indicated that a hydrophobic environment of TFE and methanol failed to induce helical conformation in IL4. Interestingly, induction of helical conformation of IL4pSer402 occurs at concentrations as low as 40% TFE and in 50% methanol. Although all phosphorylated IL4 peptides lacked pronounced secondary structure in aqueous environment, addition of low concentration of SDS induced helical structure (5,6,46). Thus, it is proposed that the structural effects of phosphorylation depend upon local context (48).

The peptides carry a net positive charge characterized by Lys 403, Arg406 and Arg410 under the conditions of these experiments and therefore are attracted to the negatively charged SDS micelles by electrostatic interactions. As represented in the helical wheel representation His407, Lys403, Arg410, and Arg406 form an electrostatically distinct cluster towards N-terminus (Figure 9). In addition, the C-terminal hydrophobic part of the peptide forms stable adducts in which the aromatic side chains of Phe 409 and Phe 413 residues penetrate into the interior of micelles. Introduction of a phosphate group on the Ser402 residue is possibly disturbing hydrophobic interactions of Phe residues as seen from helical wheel. Ser415 and Ser411 are located away from the electrostatic cluster of N-terminus.

We propose that the helicity of the cluster formed by Lys 403, His 407, Arg 406 and Arg 410 is most likely to modulate the binding and selectivity for the G protein. The phosphorylation of the CB₁R IL4 peptides might facilitate stability of peptide-protein associations that mimic functional protein-protein allosteric interactions. NMR data from our phosphopeptides in membrane-mimetic SDS show the unique structural impact of phosphorylation of serine at different positions (Fig.8). Calculations based on chemical shift index (Wishart Plot) for the structured regions of the phosphorylated analogs suggest that % helicity is in the order of IL4pSer411 > IL4pSer415 > IL4 > IL4pSer402. Calculation of the distance between the C α carbons of Arg401 and Glu417 for the average of representative NMR conformers shows that length varies in the order of unphosphorylated IL4 (26Å) > IL4pSer411 (23.68Å) ~ IL4pSer402 (23.61Å) > IL4pSer415 (21.52Å).

We proposed that the effect of charge distribution towards the C-terminus (IL4pSer415), middle (IL4pSer411) and N-terminus (IL4pSer402) would alter biological activity. The G protein activation and cAMP studies reported here showed improvement in both G protein activation and cAMP inhibition with specific middle and C-terminus serine phosphorylation. The enhancement may be explained by the change in helicity associated with the introduction of negative charge, which we propose disrupts the hydrophobic residues in IL4pSer402. In contrast, in IL4pSer411 and IL4pSer4015, the negative charge did not affect the hydrophobic residues which were reported to be critical for strong helical structure (49).

Biologically functional cell-penetrating peptides, termed pepducins, can act as GPCR activity modulators based on the peptide structure (first defined in reports describing palmitoylated peptides) (50–54). Pepducins have been used in models of cardiovascular diseases, inflammatory diseases, as well as cancer and angiogenesis (55). Studies using the peptides from CB₁R (4,5,43) showed that the IL4 peptide can activate G proteins autonomously as a pepducin.

In the present study, [³⁵S]GTP γ S binding to G α i3 and G α o was not different between IL4 and IL4pSer402, the peptide with the least % of helicity. IL4pSer415 stimulated [³⁵S]GTP γ S binding to both G α o and G α i3 with higher efficacy than IL4. IL4pSer411, the peptide with greatest % of helicity, stimulated [³⁵S]GTP γ S binding to G α o (as well as binding to G α i3) with significantly greater efficacy than did IL4.

The G α o protein activation induced by the control unphosphorylated peptide 401 was as effective as when the entire CB₁ receptor-G α o- $\beta\gamma$ complex was stimulated by a maximally-

effective concentration of the full agonist WIN55212–2. However, the Gi3 protein activation induced by the control unphosphorylated peptide 401 was 2.5 to 3-fold lower than when the CB₁ receptor-Gαi3-βγ complex was stimulated by WIN55212–2. We can interpret this to mean that the conformational change promoting release of GDP from Gi3 is more efficiently performed by the agonist-stimulated CB₁ receptor complex than by the peptide alone. In contrast, for Go, the stimulation of GDP release occurred equally well by both the agonist-driven CB₁ receptor as for the peptide alone.

Both IL4pSer411 and IL4pSer415 (at 100 μM) were able to inhibit cAMP accumulation in neuronal cells more efficaciously than IL4 and IL4pSer402, the least two peptides in the helicity order. To our knowledge, this is the first report of improved functionality of CB₁R IL4 pepducins with specific residues phosphorylated. Other pepducins require a lipid moiety to assist their movement and attachment to the plasma membrane cytosolic surface near the target receptor. The CB₁R IL4 pepducins were able to cross the cell membrane and evoke intracellular actions similar to the amphipathic α-helix mastoparan (56). We speculate that phosphorylated pepducins may have an application in treating CB₁R-deficient pathological conditions.

Regarding physiological relevance of phosphorylation in the 8th helix, we report that activation of PKC, via the B₂ bradykinin Gq-coupled receptor, is able to augment the CB₁R-mediated inhibition of adenylyl cyclase. This can be reversed by a PKC inhibitor, highlighting the crosstalk between G protein pathways. This finding supports the suggestion that PKC can promote phosphorylation to modify 8th helix structure. The altered conformation could modulate function, consistent with other studies showing that PKC phosphorylation can mediate regulation of GPCR signaling (37). Of note, an earlier study from the Mackie laboratory indicated that PKC can phosphorylate Ser317 in the third intracellular loop, which negatively regulates CB₁R signaling to ion channels (57).

Conclusion

Ser phosphorylation of the CB₁R IL4 peptide induces changes in peptide helicity with the order of IL4pSer411 > IL4pSer415 > IL4 > IL4pSer402. This change in helicity correlates with improved cellular signaling in both G protein activation (mainly Gα_o) and also inhibition of cAMP accumulation in neuronal cells. This change may be physiologically relevant under conditions of agonist-stimulated, Gq-coupled, PKC-mediated phosphorylation at Ser411 of CB₁R. These studies also demonstrate the application of phosphopeptides as pepducins in pharmacotherapeutic stimulation of G protein signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(DQF-COSY)	2D double-quantum filtered correlation spectroscopy
(DMEM)	Dulbecco's Modified Eagle's Medium
(DTT)	dithiothreitol
(CD)	Circular dichroism
(SDS)	sodium dodecyl sulfate
(GPCR)	G protein coupled receptor
(IL4)	intracellular loop 4
(MAPK)	mitogen-activated protein kinase
(NOESY)	nuclear Overhauser effect spectroscopy
(TOCSY)	total correlation spectroscopy
(TFE)	trifluoroethanol
(FAF-BSA)	fatty acid free bovine serum albumin
(SPA)	antibody capture Scintillation Proximity Assay
(DPC)	dodecylphosphocholine

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Figure 1.

CD spectroscopic analysis of (A) IL4pSer402, (B) IL4pSer415, and (C) IL4pSer411. Peptides were suspended in Sodium-Potassium phosphate buffer alone, or including the indicated concentrations of SDS, TFE, or methanol, and data were acquired as described in the experimental section. Non-phosphorylated peptide data were published (5).

Figure 2. Secondary CH_α proton chemical shifts for (A) Control peptide IL4, (B) IL4pSer402, (C) IL4pSer415, or (D) IL4pSer411 in SDS micelles. Data were generated and calculated according to random coil chemical shifts from Wüthrich (28).

Figure 3.

Summary of the NOE connectivities that define the secondary structure of (A) IL4pSer402, (B) IL4pSer415, or (C) IL4pSer411. NMR studies were performed on peptides in the presence of SDS micelles, as described in the Experimental Section.

Figure 4.

Stereo views of IL4pSer402, IL4pSer415 and IL4pSer411. Data are shown for the 20 final conformers determined for phosphopeptides in SDS micelles. The superposition of backbone atoms calculated using CYANA is shown.

Figure 5.

Effect of the CB₁R phosphorylated peptides, IL4pSer402, IL4pSer415 and IL4pSer411 on [35S]GTPγS binding to G_αo and G_β3 in membranes from N18TG2 cells. Membranes (5μg protein) were incubated with [35S]GTPγS plus either WIN55212-2 (1μM), control peptide (IL4 100μM) or phosphorylated peptides at the indicated concentrations (μM) and were then subjected to SPA using anti-Gαo (A, n=3; B, n=4; C, n=3) or anti-Gβ3 antibodies (D, n=3; E, n=5; and F, n=3). Specific GTPγS-binding (CPM) was determined by subtracting non-specific activity. For presentation, data have been normalized to the mean stimulation by WIN55212-2 as 100%. The % over stimulation by WIN55212-2 for G_αo and G_β3 are 19±2.8 and 62.7 ±6.6 respectively. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post hoc test. At reported concentrations, all peptides and WIN55212-2 were significantly different from basal. For Gαo IL4pSer411 was significantly different compared to WIN55212-2 and IL4. (***)P<0.0001. For Gβ3

IL4pSer411 and IL4pSer411 were significantly different compared to WIN55212–2 but not IL4. (* $P < 0.05$). WIN55212–2 was significantly different compared to IL4 in Gi3 (** $P < 0.005$).

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Figure 6.

Phosphopeptides inhibit adenylyl cyclase in a concentration-dependent manner. N18TG2 cells were pre-incubated in PBS-HEPES-BSA plus PDE inhibitors and 100 nM(A) or the indicated concentrations (B) of CB₁RIL4, n=7; IL4pSer402,n=3; IL4pSer411, n=3; IL4pSer415, n=3;mastoparan, n=3; or CP55940 (300 nM), n=7. After 15 min, cells were incubated for an additional 4 min with or without forskolin(1μM), and cAMP accumulation was determined. 100% represents the amount of cAMP produced by forskolin-stimulated cells. Data are presented as the mean ±SEM. Statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post hoc test. Significant difference was found between CP55940 and CP55940 plus SR141716 (**P<0.005). No differences were observed between 100 nM peptides in the absence or presence of SR141716.

Figure7.

Bradykinin-stimulated PKC augments CP55940-mediated inhibition of both forskolin- and secretin-stimulated adenylyl cyclase and this is blocked by a PKC inhibitor. N18TG2 cells were preincubated in PBS-HEPES-BSA plus phosphodiesterase inhibitors and the indicated concentrations of CP55940, bradykinin (100 nM) or PKC inhibitor bisindolylmaleimide I (BIM I) (100 nM). After 15 min, cells were incubated for an additional 4 min with 1 μ M forskolin (A) or 30nM secretin (B). cAMP levels were then determined. 100% represents the amount of cAMP produced by forskolin- (A,C) or secretin- (B) stimulated cells. Data are presented as the mean $\pm$ SEM, $n=3-7$. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison post hoc test. All were significantly different from forskolin or secretin alone. A significant difference was found comparing the effect of CP55940 with and without BK on CP55940-inhibited cAMP values at (* $P<0.05$, ** $P<0.005$).

Figure 8.

Effect of charge distribution towards C-terminus (IL4pSer415), middle (IL4pSer411) and N-terminus (IL4pSer402). The figure was prepared using Delphi charge spectrum which uses red for negative charge, blue for positive charge, and white for neutral, assuming phosphoserine to be negatively charged at physiological pH.

Figure 9.

The helical wheel representation of phosphorylated peptides. His 407, Lys 403, Arg 410, Arg 406 form electrostatically distinct cluster towards N-terminus.

TABLE 1. The CB₁R IL4 phosphopeptides: Sequence and Probability of phosphorylation

Peptide	Sequence	Probability of phosphorylation by kinases
CB ₁ IL4 (amino acids 401–417)	RSKDLRHAFRSMFPSSE	
IL4pSer402	RpSKDLRHAFRSMFPSSE	PKA 59%
IL4pSer415	RSKDLRHAFRSMFPpSSE	CKII 50%
IL4pSer411	RSKDLRHAFRpSMFPSSE	PKC 66%

Prediction results were obtained from NetPhos 3.1 Server <http://www.cbs.dtu.dk/services/NetPhos-3.1/>

Table 2.

Structural statistics for the ensemble of 20 structures in SDS micelles

	NOEs	
IL4pSer402	Intra residue	68
	Sequential	41
	Medium Range	20
	Long Range	-
	Average backbone RMSD to mean	0.5±0.11 Å
	Average heavy atom RMSD to mean	1.45±0.20 Å
IL4pSer415	Intra residue	88
	Sequential	44
	Medium Range	22
	Long Range	-
	Average backbone RMSD to mean	0.30±0.10 Å
	Average heavy atom RMSD to mean	1.26±0.16 Å
IL4pSer411	Intra residue	18
	Sequential	42
	Medium Range	24
	Long Range	1
	Average backbone RMSD to mean	0.26±0.08 Å
	Average heavy atom RMSD to mean	1.21±0.21 Å