Delta Opioid Receptors Stimulate Akt-Dependent Phosphorylation of c-jun in T-cells

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Abbreviations: DOR, delta opioid receptor; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; JNK, c-jun NH₂-terminal kinase; AI, Akt inhibitor 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; ai, Akt inhibitor C₃₁H₂₇In₄S; DADLE, [D-Ala₂-D-Leu₅]-enkephalin;
SP600125, JNK inhibitor (Anthra[1,9-\(cd\)]pyrazol-6(2\(H\))-one 1,9-pyrazoloanthrone); L, LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one].
Abstract

Activation of naïve T-cells markedly up-regulates the expression of delta opioid receptors (DORs). These receptors are bound by DOR peptides released by T-cells, modulating T-cell functions such as interleukin-2 production, cellular proliferation and chemotaxis. Previous studies have shown that DOR agonists (e.g., [D-Ala²-D-Leu⁵]-enkephalin; DADLE) modulate T-cell antigen receptor signaling through mitogen-activated protein kinases (MAPKs; i.e., ERKs 1,2), and that DORs directly induce phosphorylation of activating transcription factor-2 (implicated in cytokine gene transcription) and its association with the MAPK, c-jun NH2-terminal kinase (JNK). Such observations suggest that DORs may induce the phosphorylation of c-jun. These experiments were performed to test this hypothesis and determine the potential roles of phosphoinositide 3-kinase (PI3K) and Akt (protein kinase B). DADLE (10⁻¹⁰–10⁻⁶M) dose-dependently induced c-jun phosphorylation. This was blocked by pertussis toxin and the DOR-specific antagonist, naltindole. Fluorescence flow cytometry showed that DADLE significantly stimulated c-jun phosphorylation by T-cells. DADLE stimulated phosphorylation of membrane-associated Akt; wortmannin and LY294002, specific inhibitors of PI3K, abolished the DADLE-induced phosphorylation of c-jun. Finally, inhibitors of Akt and JNK blocked DADLE-induced phosphorylation of c-jun. Thus, activated DORs directly stimulate c-jun phosphorylation through a PI3K-dependent pathway in T-cells, apparently involving Akt. This implies that DORs activate JNK through a novel pathway dependent on PI3K and Akt, thereby regulating the function of AP-1 transcription complexes containing c-jun and other transcription partners.
Introduction

The enhanced expression of delta opioid receptors (DORs) prepares activated mature peripheral T-cells for the immunomodulatory effects of exogenous and endogenous opioids. A relatively small fraction of naïve quiescent peripheral T-cells express DORs (Miller, 1996; Sharp et al., 2001; Sharp et al., 2005). However, activation through the T-cell receptor (TCR) markedly up-regulates both the fraction of T-cells that express DORs and the number of DORs present on each T-cell. These findings are based on studies showing that DORs were induced in vivo by antigenic stimulation with staphylococcal enterotoxin B (SEB) and in vitro by mitogen treatment or crosslinking the TCR (Miller, 1996; Shahabi et al. 2000; Sharp et al., 2001).

DOR agonists exert diverse immunomodulatory effects on T-cell responses including T-cell proliferation, cytokine production, chemotaxis, and thymic T-cell selection (Shahabi and Sharp, 1995; Rogers et al., 2000; McCarthy et al., 2004). Recent studies indicate that DOR agonists affect intracellular signaling pathways in T-cells involving mitogen activated protein kinases (MAPK). For example, DADLE ([D-Ala₂-D-Leu₅]-enkephalin), a selective DOR agonist, rapidly and dose-dependently attenuated the anti-CD3-ε-induced phosphorylation of ERKs 1, 2 (Shahabi et al., 2000). In contrast, DADLE alone was sufficient to stimulate the hyperphosphorylation of ATF-2 (activating transcription factor-2), which is involved in cytokine gene transcription (Shahabi et al., 2003). DADLE also induced the association of the MAPK, c-jun NH₂-terminal kinase (JNK), with hyperphosphorylated ATF-2. Apart from immunomodulatory interactions between DORs and certain TCR-dependent signaling cascades, the effects of DADLE alone on...
JNK suggest that activation of DOR may be sufficient to signal through certain intracellular pathways. Thus, we hypothesized that DADLE might stimulate c-jun phosphorylation in T-cells.

JNK is known to phosphorylate transcription factors and an array of other molecules known to regulate cell viability, cellular stress responses, apoptosis, and proliferation (for review, Manning and Davis, 2003). c-Jun is one of multiple transcriptional proteins (e.g., ATF-2 and Nuclear Factor of Activated T-cell or NFAT) implicated in JNK signaling within T-cells. In addition to c-jun, JNK phosphorylates other transcription proteins that form activator protein-1 (AP-1) dimers (Hibi et al., 1993; Kallunki et al., 1996). Phosphorylation of the c-jun NH2-terminal activation domain enhances the activity of AP-1, promoting the transcription of T-cell cytokine genes such as interleukin-2 (IL-2), granulocyte-macrophage colony stimulating factor (GM-CSF; Thomas et al., 1997), gamma-interferon (IFN-gamma; Ye et al., 1996), and tumor necrosis factor-alpha (TNF-alpha; Becker et al., 1999).

Recent studies of opioid receptor signaling in transfected COS-7, SH-SY5Y, and NG108-15 cell lines showed that opioid receptors activate JNK, as indicated by the detection of phosphorylated JNK and the transient phosphorylation of c-jun, using in vitro kinase assays (Kam et al., 2003; Kam et al., 2004). Activation of JNK by the µ opioid receptor was dependent on phosphoinositide 3-kinase (PI3K), whereas DOR and κ opioid receptor signaling were not. In addition, Akt, a serine-threonine kinase that is one of the key downstream targets of PI3K (Cantrell, 2001), was not implicated in JNK phosphorylation.
by opioid receptors in these neuronal cell lines. However, both PI3K and Akt have been shown to facilitate agonist-induced mu opioid receptor desensitization in sensory neurons (Tan et al., 2003). Thus, we determined whether DADLE induced the PI3K and Akt-dependent phosphorylation of c-jun in splenic T-cells. We report herein that DADLE-induced JNK-dependent c-jun phosphorylation in normal T-cells, requiring both PI3K and Akt.

**Methods and Materials**

RPMI-1640 (Roswell Park Memorial Institute), fetal bovine serum (FBS), penicillin-streptomycin-glutamine, [D-Ala2-D-Leu5]-enkephalin (DADLE), sodium orthovanadate, protease and phosphatase inhibitors (Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails 1 and 2) were from Sigma Chemical (St. Louis, MO). Antibodies specific for total and phosphorylated c-jun (anti-p-serine 63 c-jun) and Akt (anti-p-Ser 473 Akt) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other antibodies and the BCA (bicinchoninic acid) Protein Assay Kit were purchased from Pierce (Rockford, IL). Akt inhibitor (AI; [1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate]) (Hu et al. 2000), a second Akt inhibitor, C₃₁H₂₃In₄S (ai; ChemBridge Corp. identification #5233705) (Kau et al., 2003), a JNK inhibitor (SP600125; Anthra[1,9-cd]pyrazol-6(2H)-one 1,9-pyrazoloanthrone) (Bennett et al., 2001), wortmannin and LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] were obtained from CalBiochem, San Diego, CA)
Animals and cell culture:

Balb/c mice 5-7 weeks old were obtained from NCI (Bethesda, MD) and maintained for one week in specific pathogen-free facilities (12 h light/12 h dark) at a constant temperature (20°C) with ad libitum access to food and water. They were sacrificed by cervical dislocation under isofluorane anesthesia. All procedures were conducted in accordance with NIH guidelines and approved by the Animal Care Committee of the University of Tennessee. Spleens were dispersed through a wire mesh screen, erythrocytes were eliminated with lysing buffer, and then cells were spun, washed, and layered on Ficoll-Hypaque, followed by centrifugation at 400xg for 10 min. The interface layer was recovered, washed, and cultured at 2x10^6 cells/ml in RPMI-1640 with penicillin-streptomycin-glutamine, 5% (FBS), and 5x10^{-5}M β-mercaptoethanol. Pooled splenocytes were cultured for 48 h in flasks coated with anti-CD3-ε (80 ng/cm^2), rested for 24 hours in the absence of anti-CD3-ε, and then starved for 4 h in the absence of serum. Thereafter, cells were divided into separate aliquots equivalent to the number of samples necessary for all treatment groups. Each of these samples was treated with the specific reagents identified in the figures and Results section. Each study was based on 4-6 total samples per treatment group.

Membrane preparation:

Cells were washed briefly with PBS, lysed using 500 ml of RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM sodium orthovanadate, and a mixture of protease and phosphatase inhibitors), and then sonicated 3 times (2-3 sec each) on ice. Lysates were centrifuged at 120,000xg for 60 min at 4°C. Pellets were
resuspended in RIPA buffer containing 0.1% Triton X-100, sonicated briefly, and protein concentrations were determined using the BCA Protein Assay Kit. Membranes were boiled for 5 min and 5-10 mg protein were loaded on 11% SDS polyacrylamide gels (1.5 mm depth).

**Western Blotting:**

SDS polyacrylamide gels were transferred to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) over 16 hours at 24°C. Membranes were briefly washed with Tris-buffered saline (TBS; 20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20, 0.05% NP-40, pH 7.7), and then blocked in TBS containing 5% nonfat dry milk for 2 h. They were incubated at room temperature with primary antibody for 90 min. Membranes were washed extensively, incubated with a peroxidase tagged secondary antibody (Pierce) for 1 h at room temperature, and chemiluminescence was detected (Super Signal ULTRA; Pierce).

**Immunofluorescence Flow Cytometry:**

Splenocytes were cultured for 48 h in tissue culture flasks coated with purified anti-mouse-CD3-ε (80 ng/cm²; Pharmingen, San Diego, CA). Thereafter, cells were rested 24 h, starved 15 h in the absence of serum, and then incubated for 15 min with PBS, DADLE (10⁻⁶ M) or phorbol 12-myristate13-acetate (PMA 50ng/ml; Sigma) plus A23187 (1µM; Calbiochem, La Jolla, CA). Cells were centrifuged, incubated with 1ml FCM fixation buffer (Santa Cruz, Santa Cruz, CA) for 20min at 4°C, washed twice with PBS, and incubated with 1ml FCM permeabilization buffer (Santa Cruz) for 15 min at 4°C.
Cells were then washed (2x) with FCM wash buffer (Santa Cruz) and stained for 60 min at room temperature with PE-labeled anti-p-c-jun (Santa Cruz) and FITC-labeled anti-CD90 (Thy-1; Pharmingen). Cytofluorometric analyses (10^4 cells per run) were performed using an Epics XL flow cytometer (Beckman-Coulter, Miami, FL) equipped with an argon laser, and filtered for excitation at 488 nm and emission at 525 to 695 nm. Mouse isotypes (i.e., IgG1-PE and IgG1,κ-FITC) were used for background subtraction.

Statistical Analyses: one-way analysis of variance (ANOVA) was performed and post-hoc testing was done using Bonferroni test. Differences were considered significant at p<0.05. Values are expressed as mean ± sem.
Results

The initial set of experiments sought to determine whether DADLE, a specific DOR agonist, induced phosphorylation of c-jun in T-cells through DORs coupled to Gi proteins. In order to obtain data from normal lymphocytes, primary murine splenocyte cultures were utilized to minimize variation in responses. Since these cells transfected poorly, pharmacological rather than molecular approaches were taken in these studies. Figure 1 demonstrates that DADLE induced phosphorylation of c-jun in murine splenocyte cultures that had been stimulated with anti-CD3-ε for 48 h, rested for 24 hours and then starved for 4 h in the absence of serum prior to adding a specific delta opioid receptor (DOR) agonist. Phosphorylation of c-jun by DADLE was concentration dependent \([10^{-12}-10^{-6}\text{M (D12-D6)}; \text{n}=3\text{ experiments; } F=5.08, p=0.002]\); phospho-c-jun \((p-c\text{-j}un)\) increased approximately 5-fold over basal levels after treatment with 1 \(\mu\text{M}\) DADLE. Phosphorylated c-jun also was detected by immunofluorescence flow cytometry, as shown in figure 2. Using the same protocol for cell culture and treatment with DADLE, splenocytes were labeled with anti-Thy-1 to identify T-cells and with anti-phospho-c-jun after permeabilization. The percentage of double positive cells (T-cell containing phospho-c-jun) significantly increased from 101±3.9% (mean±sem) in controls to 153.6±6.1 in cells treated with 1 \(\mu\text{M DADLE (D6)}\) and 241.1±15.7 in response to PMA \((\text{n}=9; F=49.9, p<0.001; p=0.005\text{ for control vs. DADLE})\). In addition, after stimulation with DADLE, 95% of the cells positive for phospho-c-jun were T-cells.
Naltrindole, a specific DOR antagonist, abolished the induction of phospho-c-jun by DADLE (fig. 3), whereas naltrindole alone had no effect. Since neuronal DORs are known to signal through Gi-proteins, which are inhibited by pertussis toxin (PTX), the effect of this agent on DADLE-induced phosphorylation of c-jun was studied. Figure 4 demonstrates that pertussis toxin (100 ng/ml) also abolished the phosphorylation of c-jun by DADLE. Thus, DADLE dose-dependently induced the phosphorylation of c-jun in T-cells through DORs coupled to pertussis toxin-sensitive G-proteins.

The compound SP600125 (S) is a potent, specific inhibitor of the c-jun NH$_2$-terminal kinases (JNK), which mediate phosphorylation of c-jun (Hibi et al., 1993; Bennett et al., 2001). Thus, this agent was used to demonstrate that DADLE-induced c-jun phosphorylation is dependent on JNK. Figure 5 shows that S alone had a small effect on the level of phospho-c-jun, and it largely prevented DADLE-induced phosphorylation. Densitometric analysis confirmed this, demonstrating that DADLE (D6) significantly increased phospho-c-jun and S + D6 was significantly less ($F = 6.67$, $p = 0.001$; D6 vs. control, $p < 0.001$; D6 vs. S10 + D6, $p = 0.001$; D6 vs. S30 + D6, $p = 0.01$).

Phosphoinositide 3-kinase (PI3K), a lipid kinase that generates phosphatidylinositol (PtdIns)-3,4,5-P$_3$ (PIP$_3$), and Akt (protein kinase B), one of its key downstream effectors, have been directly implicated in signaling through G-protein coupled receptors in certain cell types (Hawes et al., 1996; Shah et al., 2005). Moreover, activation of JNK by the µ opioid receptor required PI3K (Kam et al., 2003; Kam et al., 2004). Therefore, we evaluated the potential role of PI3K and Akt in phosphorylation of c-jun by DADLE. In
the first experiments, the ability of DADLE to activate PI3K was evaluated indirectly by assessing the phosphorylation of membrane Akt, which initially docks to PIP3 at the inner leaflet of the plasma membrane and subsequently becomes phosphorylated by phosphoinositide-dependent kinase 1 (PDK1; Vanhaesebroeck and Alessi, 2000). Figure 6 shows that DADLE significantly induced phosphorylation of Akt (p-Akt), increasing p-Akt by 300-400% of baseline for at least 30 min (F=3.6, p=0.013; p=0.03, 0.006 and 0.001 for upper band of control vs. 10, 20 and 30 min, respectively). Experiments shown in figure 7 demonstrate that wortmannin (100 nM; WT), a specific inhibitor of PI3K (Ui et al., 1995; Carpenter and Cantley, 1996), abolished DADLE-induced phosphorylation of c-jun without affecting basal levels of c-jun phosphorylation. Densitometric analysis confirmed this (lower panel), showing that the phosphorylation of c-jun by DADLE (D6) in the presence of wortmannin (WT/D6) was significantly less than D6 alone [F=25.69, p<0.0001; D6 vs. control, p<0.0001; WT + D6 vs. D6, p=0.0001]. Figure 8 demonstrates that LY294002 (10-20 µM; L), a specific inhibitor of PI3K, also reduced c-jun phosphorylation by DADLE (D6). Densitometric analysis, (lower panel) showed that phosphorylation of c-jun by D6 in the presence of LY294002 (L10/D6 or L20/D6) was significantly less (**) than D6 alone [F=10.22, p<0.0001; D6 vs. control, p<0.0001 (*); L (10 or 20) + D6 vs. D6, p<0.0001]. Thus, experiments with wortmannin and LY294002 demonstrate that DADLE-induced c-jun phosphorylation depends on PI3K.

The potential role of Akt in c-jun phosphorylation was studied further, using two different inhibitors. The first, 1L-6-Hydroxymethyl-chiro-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate] (abbreviated as AI; Hu et al., 2000), a phosphatidylinositol ether
analog (EC\textsubscript{50} = 5.0 \mu M vs. 83 \mu M against Akt compared to PI3K, respectively), dose-dependently blocked the phosphorylation of c-jun by DADLE (D6) (figure 9). This occurred within a concentration range with high Akt specificity. Indeed, c-jun phosphorylation was reduced by more than 50\% at 10 \mu M AI, and 20 \mu M AI caused no greater inhibition (F=5.65, p=0.002; D6 vs. control, p=0.0003; AI10 + D6 vs. D6, p=0.038). The second Akt inhibitor, C\textsubscript{31}H\textsubscript{27}In\textsubscript{4}S (ChemBridge Corp. identification #5233705; Kau et al., 2003; abbreviated as ai;), is a benzimidazole compound that blocks the phosphorylation and activation of Akt, apparently without affecting PI3K. The representative experiment in figure 10 (upper panel) showed that ai dose-dependently reduced the phosphorylation of c-jun by DADLE; both 5 and 10 \mu M ai abolished the response. Densitometric analysis (lower panel) demonstrated that ai 5 or 10 \mu M significantly inhibited D6-induced phosphorylation of c-jun [F=9.48, p<0.0001; D6 vs. control, p<0.0001; ai5 + D6 vs. D6, p<0.0001; ai10 + D6 vs. D6, p<0.0001]. Thus, Akt appears to be required for c-jun phosphorylation by DADLE.

**Discussion**

These investigations demonstrate that DORs stimulate phosphorylation of c-jun in splenic T-cells, and that this depends, as expected, on JNK. Although a prior report showed that DORs can enhance JNK activity in a neuronal cell line, this was independent of PI3K (Kam et al., 2003). In contrast, we observed that PI3K was required for DADLE-induced c-jun phosphorylation in T-cells. As such, Akt, a proxy for PI3K activity, was phosphorylated by DADLE, and both wortmannin and LY294002 blocked c-jun
phosphorylation. We also found that two inhibitors of Akt, which are chemically diverse in structure, blocked DOR-dependent c-jun phosphorylation. One of these, a first generation compound, inhibited c-jun phosphorylation at concentrations that would affect Akt, but not PI3K. The other antagonist, a second generation compound, is known to inhibit phosphorylation of Akt, without interacting with PI3K (Kau et al., 2003); however, its precise mechanism of action is unknown. And, in general, the specificity of Akt inhibitors has not been studied definitively. This second generation inhibitor was effective at lower concentrations than the first generation compound. Thus, in addition to PI3K, Akt appears to be required for DOR activation of JNK and c-jun.

Although opioid receptors activate JNK and c-jun, the potential role of Akt in signaling from 7-transmembrane G-protein coupled receptors to JNK and c-jun is largely undefined. In sensory neurons chronically exposed to the specific MOR agonist, [d-Ala$^2$,N-MePhe$^4$,Gly-$\text{ol}^5$]-enkephalin (DAMGO), desensitization of $\mu$ opioid receptor (MOR)-dependent modulation of voltage gated calcium channels was shown to require persistent activation of both Akt and PI3K (Tan et al., 2003). This, however, appears to be independent of JNK. In contrast, both MOR and DOR have been reported to stimulate JNK phosphorylation, although only MOR required PI3K, and this did not involve Akt (Kam et al., 2003; Kam et al., 2004). Other studies indicate that Akt can regulate protein kinases upstream of JNK, inhibiting the phosphorylation of JNK and protecting against JNK-dependent apoptosis in susceptible cells (reviewed by Brazil et al., 2004). Since there are a variety of kinases capable of activating JNK (reviewed by Kyriakis and Avruch, 2001), the specific cellular context, including factors such as cell type and
compartmentalization of signaling molecules responsive to specific extracellular stimuli, may determine how Akt regulates JNK activity.

The JNKs are one branch of the mitogen-activated protein kinase (MAPK) family, which are activated by upstream kinases termed MAPK kinases (MAPKK; i.e., MKK4). MAPKKs are themselves activated by serine/threonine phosphorylation mediated by MAPKK kinases (MAPKKK). Three JNK isoenzymes have been identified: JNK-1, JNK-2 and JNK-3. These are specifically phosphorylated by MKK4 and MKK7, although more than 10 MAPKKKs activate the JNK pathway (reviewed by Manning and Davis, 2003). Thus, a wide variety of stimuli, most commonly related to environmental stressors, converge on the activation of JNKs. Within the T-cell, JNK is involved in thymocyte apoptosis and T-cell proliferation and differentiation. Specific roles of isoenzymes of JNK in T-cell differentiation were demonstrated by the following: jnk1-/- mice exhibited enhanced T helper cell type 2 (Th2) differentiation and nuclear accumulation of the transcription factor termed Nuclear Factor of Activated T cells (NFATc; Dong et al., 1998); JNK-2 was necessary for Th1 differentiation (Yang et al., 1998). In addition, JNK-2 may be involved in proliferation and differentiation of peripheral T-cells (Dong et al., 1998; Sabapathy et al., 1999). Thus, both JNK-1 and –2 appear to be involved in the T helper cell type-specific differentiation.

Recent investigations indicate that N-terminal phosphorylation of c-jun by JNK is not required for T-cell proliferation and differentiation (Behrens et al., 2001). However, JNK-2 is required in these processes, implicating another nuclear effector(s). JNK-2 has
been shown to regulate the DNA binding and transactivational properties of the NFAT transcriptional complex upon stimulation of peripheral T-cells (Behrens et al., 2001). Recent studies have demonstrated that JNK activates NFATc2-dependent transcription, and this is associated with phosphorylation of multiple residues within the NFATc2 regulatory domain (Ortega-Perez et al., 2005). Mutations of T116 and s170 indicate that these residues are critical for NFATc2 transactivation by JNK in Jurkat T-cells. In contrast to previous studies indicating that JNKs exclusively inhibited NFAT (i.e., NFATc1 and NFATc3), Ortega-Perez et al. have clearly demonstrated positive subtype-specific regulation of NFATc2.

The Akt-dependent inhibition of kinases upstream of JNK, which attenuates JNK activity, may be an incomplete picture of Akt interaction with JNK. Analogous to the foregoing observations, one may postulate that Akt phosphorylates and activates a subset of JNK, defined by compartmentalization and/or different isoenzyme subtype. The data in the current study of DADLE-induced c-jun phosphorylation is potentially consistent with such a model of selective JNK activation by Akt. In summary, these experiments demonstrate that DORs stimulate c-jun phosphorylation through JNK, which is dependent on the upstream activity of PI3K and most likely Akt.
References


Footnotes

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Legends for Figures

Figure 1. Effect of DADLE on c-jun phosphorylation in murine splenocytes. Splenocytes were stimulated with anti-CD3-ε for 48 h, rested for 24 h and starved for 4 h. Thereafter, splenocytes (approximately 85% T-cells) were treated with either vehicle (C) or indicated concentrations of DADLE (D) for 15 min [10^{-12}-10^{-6}M, (D12-D6)]. Cells were harvested and lysed, and then phospho-serine-63 c-jun (p-c-jun) was detected by western immunoblotting (upper panel). Blots were re-probed with an antibody that recognized total c-jun (middle panel). Results of densitometric measurements are shown in the lower panel; values are mean ± sem. Phosphorylation of c-jun by DADLE was concentration dependent (n=6 total samples per treatment, obtained from 3 immunoblots; F=5.08, p=0.002 by ANOVA); *, p<0.05 for D vs. control (CONT).

Figure 2. Detection of DADLE-induced phosphorylation of c-jun in splenic T-cells by immunofluorescence flow cytometry. Splenocytes were stimulated with anti-CD3-ε for 48 h and rested for 24 h. After starvation for 15 hours, cells were incubated with either vehicle, DADLE 10^{-6} M (D6) or PMA (50ng/ml) plus A23187 (1µM). Fixed, permeablized cells were incubated with phycoerythrin-labeled anti-phospho-c-jun (p-c-jun) and FITC-labeled anti-CD90 (Thy-1). The percentage of double-positive cells [(+)Thy-1 and (+)p-c-jun] was determined by fluorescence flow cytometry (values are mean ± sem). Both D6 and PMA increased the percentage of T-cells positive for p-c-jun (n = 9 total samples per treatment, obtained from 3 experiments; F=49.9, p<0.001 by
ANOVA); *, p=0.005 compared to control.

Figure 3. Effect of naltrindole (NTI), a DOR-specific antagonist, on DADLE-induced c-jun phosphorylation. Splenocytes were cultured as described in figure 1. After starvation, cells were pretreated with 10^{-8} or 10^{-7} M NTI (NTI8, NTI7) for 15 min and then challenged with 1\mu M DADLE (D6) or vehicle (C) for 15 min. Phospho-c-jun (p-c-jun) was detected by western immunoblot, using anti-phospho-serine-63 c-jun. A representative immunoblot (n=4-6 total samples per treatment, obtained from 2 blots) demonstrates that NTI abolished D6-induced c-jun phosphorylation.

Figure 4. Effect of pertussis toxin (PTX) on DADLE-induced c-jun phosphorylation. After starvation, splenocytes were pretreated with PTX (100 ng/ml) or vehicle (C) overnight and then stimulated with vehicle (C) or 1\mu M DADLE (D6) for 15 min. Additionally, lane 13 (PA) shows the extract of cells treated as positive controls with PMA (50ng/ml) plus A23187 (1\mu M) for 15 min. Anti-phospho-serine-63 c-jun was used to detect p-c-jun by western immunoblot. A representative immunoblot (n=6 total samples per treatment, obtained from 2 blots) demonstrates that PTX abolished D6-induced c-jun phosphorylation.

Figure 5. Effect of the c-jun NH\textsubscript{2}-terminal kinase (JNK) inhibitor, SP600125 (S), on
DADLE-induced phosphorylation of c-jun. After starvation, splenocytes were pretreated with 10 or 30 µM S for 30 min and then challenged with 1µM DADLE (D6) for 15 min. Phosphorylation of c-jun was determined in cell lysates by western immunoblotting (upper panel). A representative immunoblot (n=4-6 total samples per treatment with D6 ± S, obtained from 2 blots) shows that S inhibited most of the phosphorylation of c-jun stimulated by DADLE. Densitometric analysis (lower panel) demonstrated that D6 significantly increased phospho-c-jun (*) and S + D6 was significantly less (**) (F = 6.67, p = 0.001; D6 vs. control, p < 0.001; D6 vs. S10 + D6, p = 0.001; D6 vs. S30 + D6, p = 0.01). Values are mean ± sem.

Figure 6. Time course of DADLE-induced phosphorylation of Akt in splenocytes. After starvation, splenocytes were treated with vehicle (CONT) for 10 min or DADLE 10^{-6} M (D6) for 5, 10, 20 or 30 min. Thereafter, cells were harvested and membrane was obtained, as described in Methods. Phospho-Akt (p-Akt) was determined in membrane lysate by western immunoblotting with anti-phospho-Ser 473 Akt (upper panel). The blots were re-probed with an Akt antibody against total Akt (middle panel). The lower panel shows that DADLE increased p-Akt, which remained significantly elevated for at least 30 min (n=10 total samples per time interval, obtained from 5 immunoblots; F=3.6, p=0.013); *, p=0.03, 0.006 and 0.001 for upper band of control vs. 10, 20 and 30 min, respectively. Values are mean ± sem.
Figure 7. Effects of wortmannin, a PI3K antagonist, on DADLE-induced c-jun phosphorylation. Splenocytes, prepared as described in figure 1, were pretreated with wortmannin (100 nM; WT) for 60 min prior to treatment with DADLE $10^{-6}$ M (D6) or vehicle (C) for 15 min. Phosphorylation of c-jun was determined in cell lysates by western immunoblotting (upper panel). A representative immunoblot (n=7 total samples per treatment, obtained from 2 blots) demonstrates that WT abolished D6-induced c-jun phosphorylation. Densitometric analysis confirmed this (lower panel), showing that the phosphorylation of c-jun by D6 in the presence of wortmannin (WT/D6) was significantly less (**) than D6 alone [$F=25.69, p<0.0001$; D6 vs. control, $p<0.0001$ (*); WT + D6 vs. D6, $p=0.0001$]. Values are mean ± sem.

Figure 8. Effects of LY294002, a PI3K antagonist, on DADLE-induced c-jun phosphorylation. Splenocytes, prepared as described in figure 1, were pretreated with LY294002 10 or 20 µM (L10 or L20) for 60 min prior to treatment with DADLE $10^{-6}$ M (D6) or vehicle (C) for 15 min. Phosphorylation of c-jun was determined in cell lysates by western immunoblotting (upper panel). A representative immunoblot (n=5-6 total samples per treatment, obtained from 2 blots) demonstrates that LY294002 abolished D6-induced c-jun phosphorylation. Densitometric analysis confirmed this (lower panel), showing that the phosphorylation of c-jun by D6 in the presence of LY294002 (L10/D6 or L20/D6) was significantly less (**) than D6 alone [$F=10.22, p<0.0001$; D6 vs. control, $p<0.0001$ (*); L (10 or 20) + D6 vs. D6, $p<0.0001$]. Values are mean ± sem.
Figure 9. Effects of an Akt inhibitor (AI) on DADLE-induced phosphorylation of c-jun. Splenocytes were pretreated with 10 or 20 µM Akt inhibitor (AI; 1L-6-Hydroxymethyl-chiro-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) for 2 h prior to stimulation with DADLE $10^{-6}$ M (D6) or vehicle (C) for 15 min. Phosphorylation of c-jun was determined in cell lysates by western immunoblotting (upper panel). Blots were re-probed with a c-jun antibody against total c-jun (middle panel). A representative immunoblot (n=6 total samples per treatment, obtained from 2 blots) demonstrates that AI dose-dependently inhibited D6-induced c-jun phosphorylation. Densitometric analysis confirmed this (lower panel), showing that AI 10 or 20 µM significantly reduced (** D6-induced phosphorylation of c-jun [F=5.65, p=0.002; D6 vs. control, p=0.0003 (*) ; AI10 + D6 vs. D6, p=0.038; AI20 + D6 vs. D6, p=0.008]. Values are mean ± sem.

Figure 10. Effects of a second Akt inhibitor (ai; C$_{31}$H$_{27}$In$_{4}$S) on DADLE-induced phosphorylation of c-jun. Splenocytes were pretreated with 1, 5 or 10 µM of ai (ChemBridge Corp. identification #5233705) for 2 h prior to stimulation with DADLE $10^{-6}$ M (D6) or vehicle (C) for 15 min. Phosphorylation of c-jun was determined in cell lysates by western immunoblotting (upper panel). A representative immunoblot (n=4-6 total samples per treatment, obtained from 2 blots) demonstrates that ai dose-dependently inhibited D6-induced c-jun phosphorylation. Densitometric analysis (lower panel) showed that ai 5 or 10 µM significantly inhibited (** D6-induced phosphorylation of c-jun [F=9.48, p<0.0001; D6 vs. control, p<0.0001 (*) ; ai5 + D6 vs. D6, p<0.0001; ai10 + D6 vs. D6, p<0.0001]. Values are mean ± sem.
Figure 1
Figure 2
Figure 3
Figure 4

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Figure 7
Figure 8
Figure 9

A graph showing the percentage of control for different conditions:

- C
- D6
- AI20/D6
- AI10/D6
- AI20/C
- AI10/C

The graph compares p-c-jun and total c-jun across these conditions.