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Title Page

Inhibition of agonist-induced down regulation of the δ opioid receptor with a proteasome inhibitor attenuates opioid tolerance in human embryonic kidney 293 cells

Prem N. Yadav, Kirti Chaturvedi and Richard D. Howells*

Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

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Running Title: Proteasome Inhibition Attenuates Opioid Tolerance

Corresponding address:

Richard D. Howells, PhD

Department of Biochemistry and Molecular Biology

UMDNJ- New Jersey Medical School

185 South Orange Ave, Newark, NJ 07103.

Tel.: 973-972-5652; Fax: 973-972-5594

E-mail: howells@umdnj.edu.

Abbreviations: The abbreviations used are: GPCR, G protein-coupled receptor; DADL, Tyr-D-Ala-Gly-Phe-D-Leu- enkephalin; HEK, human embryonic kidney; ZLLL, *N*-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal; MAP kinase, mitogen-activated protein kinase; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis.

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Abstract:

This study was designed to test the hypothesis that inhibition of agonist-induced δ receptor down regulation would block the development of opioid tolerance in a cell-based model. A HEK293 cell line (DOR) was established that expressed an epitope-tagged δ opioid receptor. Treatment of DOR cells with Tyr-D-Ala-Gly-Phe-D-Leu (DADL) resulted in a time-dependent decrease in the B_{max} of δ opioid receptor binding sites and immunoreactive receptor protein. When cells were co-incubated with the proteasome inhibitor, N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (ZLLL), and DADL, the magnitude of the agonist-induced decrease in B_{max} and immunoreactive receptor protein was reduced compared with DADL treatment alone. Acute treatment of DOR cells with DADL caused a 3-fold increase in the level of phosphorylated mitogen activated protein (MAP) kinase. Prior exposure of DOR cells to DADL completely abrogated the agonist-induced activation of MAP kinase. When DOR cells were co-incubated with DADL and ZLLL, the proteasome inhibitor prevented the loss of agonist activation of MAP kinase. Acute treatment of DOR cell membranes with DADL stimulated [^{35}S]GTPγS binding. When DOR cells were preincubated with DADL, the agonist-induced increase in [^{35}S]GTPγS binding was attenuated. Co-incubation of ZLLL and agonist partially prevented the decreased responsiveness to agonist stimulation. The results of this study demonstrated that inhibition of agonist-induced down regulation with a proteasome inhibitor attenuated opioid tolerance in a cellular model, and suggest that co-administration of a proteasome inhibitor with chronic opioid agonist treatment may be useful for limiting opioid tolerance *in vivo*.

Introduction:

Opioid receptors are members of the G protein-coupled receptor (GPCR) superfamily, and mediate the effects of endogenous opioid peptides in the central, peripheral, and enteric nervous systems. Opioid receptors are also the molecular targets of opioid drugs, such as morphine. Opioids are the most powerful analgesic drugs available currently and are the treatment of choice for the management of moderate to severe pain (McQuay, 1999). Adverse effects, including respiratory depression, nausea, and constipation, impact their use, and protracted opioid therapy leads to drug tolerance and physical dependence. Tolerance is defined as a loss of efficacy following repeated administration, and it is common for patients on long-term opioid therapy to increase their dosage (Sittle et al., 2005). Adaptive changes at the molecular, cellular, synaptic and neural network level are induced by habitual use of opioid analgesics, although much remains to be learned about the mechanisms involved in the adaptive plasticity (Bailey and Connor, 2005). Opioid receptors, like other GPCRs, are subject to agonist-induced desensitization and internalization, involving phosphorylation of receptors by G protein receptor kinases (GRKs) and association with β-arrestins (Lefkowitz and Shenoy, 2005), while chronic exposure leads to receptor down regulation, which involves proteolysis of the receptors. Both desensitization and down regulation are probably involved in opioid tolerance. It has been shown that μ and δ opioid receptors are phosphorylated by GRKs following agonist treatment (Pei et al., 1995), and are sequestered by endocytosis in an arrestin- and dynamin-dependent process via clathrin-coated pits (Chu et al., 1997). Morphine analgesia is enhanced in knockout mice lacking β-arrestin 2, pointing to a role for β-arrestin 2 in desensitization of the μ opioid receptor (Bohn et al., 1999). In mice lacking β-arrestin 2, desensitization of the μ opioid receptor does not occur after chronic morphine treatment unlike in wild-type mice, and the

knockout animals do not develop tolerance to the analgesic effects of morphine (Bohn et al., 2000). Endosome-associated receptors can be resensitized by protein phosphatases and recycled back to the plasma membrane, or degraded within the cell. It has been observed that the δ opioid receptor does not recycle back to the plasma membrane efficiently following internalization (Tsao and von Zastrow, 2000).

The mechanism for GPCR proteolysis has generally been assumed to involve internalization of receptors into endosomes, followed by fusion of endosomes with lysosomes and hydrolysis of the receptor protein by lysosomal proteases. Our laboratory has reported evidence, however, that the ubiquitin/proteasome system plays a role in agonist-induced μ and δ opioid receptor down regulation (Chaturvedi et al., 2001). Proteasome inhibitors blocked DADL-induced down regulation of μ and δ opioid receptors, while inhibitors of calpain, caspases, and lysosomal cathepsins had no effect. Membrane-permeable inhibitors of the proteasome have contributed greatly to our understanding of the involvement of the ubiquitin/proteasome system in protein degradation (Kisselev and Goldberg, 2001). In eukaryotic cells, a wide variety of proteins with roles in cell cycle progression, transcriptional control, signal transduction and metabolic regulation are degraded by the ubiquitin-proteasome system (Hershko et al., 2000), as are damaged and misfolded proteins (Schubert et al., 2000). The presence of ubiquitin-protein conjugates in intracellular deposits in diseased neurons from patients with neurodegenerative disease, along with recent data indicating impairment of the ubiquitin/proteasome system by abnormal protein aggregation, suggests a link between proteasome dysfunction and neuropathogenesis (Betarbet et al., 2006). The 26S proteasome is a 2.4 megadalton complex consisting of a 20S proteolytic core complex and two 19S regulatory complexes, and it is capable of hydrolyzing peptide bonds adjacent to basic, acidic, and hydrophobic amino acids within

substrate proteins. Proteins are targeted to the proteasome by covalent ligation to ubiquitin, a highly conserved 76 amino acid protein. The polyubiquitinated proteins are recognized by the 19S regulatory subunits of the 26S proteasome, the ubiquitin moieties are recycled through the action of ubiquitin hydrolases, and the 20S catalytic core complex degrades the targeted protein substrates.

In an earlier study, we reported that in HEK293 cells, agonist-induced down regulation of the δ opioid receptor was blocked by co-incubation with proteasome inhibitors (Chaturvedi et al., 2001). We then predicted that if receptor down regulation plays a role in opioid tolerance, proteasome inhibitors should reduce the tolerance that develops subsequent to chronic agonist exposure. In this report, [^{35}S]GTPγS binding and phospho-MAP kinase assays were used to assess the effect of prior agonist exposure on δ opioid receptor signal transduction in HEK293 cells, and, if agonist efficacy was attenuated by prior agonist exposure, to determine if co-administration of a proteasome inhibitor would reduce the loss in agonist efficacy.

Methods:

Cell Culture and Transfection

HEK 293 cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin sulfate. HEK 293 cells were transfected using Lipofectamine 2000 (Invitrogen, Gaithersburg, MD), with an expression plasmid encoding the murine δ opioid receptor tagged at the amino terminus with the FLAG epitope (kindly provided by Dr. Mark Van Zastrow). Cells stably expressing the epitope-tagged δ opioid receptor (DOR cells) were selected in media containing 1 mg/ml G418 (Invitrogen).

Membrane Preparation and Radioligand Binding Assays

DOR cells were grown to 60-70% confluence in 100 mm culture dishes. For membrane preparations, the culture medium was aspirated and cells were harvested in 10 ml of 50 mM Tris HCl buffer, pH 7.5, per dish. The cell suspension was homogenized with a Tekmar tissuemizer (Cincinnati, OH), and then centrifuged at 100,000 *g* for 30 min. The membrane pellet was washed twice in Tris buffer and then resuspended by homogenization in 7 ml of 0.32 M sucrose, 50 mM Tris HCl, pH 7.5, per dish, and the membrane preparation was stored at –80°C.

Opioid receptor binding assays were conducted in duplicate with membrane preparations diluted 2-4 fold in 50 mM Tris HCl buffer, pH 7.5. Binding assays were performed at 0°C in a volume of 0.25 ml (containing 20-30 µg of protein/ml) with 0.02 – 6.0 nM (-)-[9-^{3}H] bremazocine ((3-(hydroxycyclopropylmethyl)-1,2,3,4,5,6-hexahydro-6-ethyl-11-dimethyl-2,6-methano-3-benzazocin-8-ol), specific activity 26.6 Ci/mmol, PerkinElmer Life Sciences, Boston, MA), and

non-specific binding was determined in the presence of 10 μM cyclazocine (3-(cyclopropylmethyl)-1,2,3,4,5,6-hexahydro-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol), a benzomorphan similar in structure to bremazocine with high affinity for the δ opioid receptor. Following a 1 h incubation, assays were terminated by filtration through Whatman GF/B filters. Filters were soaked in Ecoscint H liquid scintillation mixture (National Diagnostics, Somerville, NJ) prior to determination of filter bound radioactivity using a Beckman LS 1701 scintillation counter. Receptor binding data were analyzed by nonlinear regression using Prism 3.0 (GraphPad Software, San Diego, CA). Protein concentrations were determined with the BioRad DC protein assay (Hercules, CA), using bovine serum albumin as the standard.

Agonist-Induced Effects on δ Opioid Receptor Binding and Down Regulation

To study the kinetics of agonist-induced receptor desensitization and down-regulation, and to determine the effect of proteasome inhibition on these processes, intact DOR cells in serum-containing media were preincubated for 1 h at 37°C with or without 10 μM ZLLL (N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal, Peptides International, Louisville, KY), then incubation continued in the presence or absence of Tyr-D-Ala-Gly-Phe-D-Leu (DADL, 1 μM) for 2, 6, or 18 h. The level of active opioid receptor binding sites was determined with radioligand binding assays, using membrane preparations as described above. Total cell receptor immunoreactivity was assayed by western blotting. Following incubations as described above, intact DOR cells were washed twice with serum-free DMEM to remove the agonist and proteasome inhibitor, and then challenged with or without 1 μM DADL for an additional 10 min at 37°C. This was the same treatment paradigm used to assess the effect of prior agonist exposure on MAP kinase activation (see below), which is why the additional 10 min incubation

in the absence and presence of DADL was included. Following treatment, control and treated cell extracts were prepared by incubating the cells in culture dishes for 1 h on ice in 0.2 ml of lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace, Maumee, OH), 10% glycerol, a protease inhibitor mixture (containing 4-(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin) and a phosphatase inhibitor cocktail (both from Sigma, St. Louis, MO). The insoluble fraction was pelleted by centrifugation and discarded, and the soluble supernatant was used for further analysis. Protein concentrations in supernatants were determined using the BioRad DC assay with bovine serum albumin as standard. Cell extracts containing approximately 40 μg of protein were mixed with 5X SDS-PAGE gel loading buffer and heated at 40°C for 5 min. Proteins were resolved using 10% or 12% SDS-PAGE and transferred to PVDF membranes (Immobilon P, Millipore, Bedford, MA). Membranes were blocked for 1 h in 3% dried milk, 2% bovine serum albumin, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 10% glycerol and 0.1% Tween 20, followed by overnight incubation at 4°C with mouse anti-FLAG M1 monoclonal antibody (Sigma). Membranes were then washed and incubated with anti-mouse IgG conjugated with alkaline phosphatase (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and developed using CDP-Star western blot chemiluminescence reagent (PerkinElmer Life Sciences). Receptor immunoblots were normalized by stripping the receptor blots and then probing with a mouse monoclonal antibody to α-tubulin (Sigma).

Agonist-induced Phosphorylation of MAP Kinase

MAP kinase assays were conducted as described previously (Chaturvedi et al, 2000). DOR cells were incubated at 37°C in serum-containing culture medium, and were either untreated, or pre-exposed to 1 μM DADL for 2, 6, or 18 h, alone or in combination with the proteasome inhibitor, ZLLL, at 10 μM. Cells that were exposed to the proteasome inhibitor were preincubated with ZLLL for 1 h prior to the addition of agonist. Following incubations, intact DOR cells were washed twice with serum-free DMEM to remove the agonist and proteasome inhibitor, and then challenged with or without 1 μM DADL for an additional 10 min at 37°C. Cells were then extracted in lysis buffer, the insoluble fraction was pelleted by centrifugation and discarded, and the soluble supernatant was assayed for total and phosphorylated MAP kinase by immunoblotting. The protein concentration in the supernatant was determined using the BioRad DC protein assay kit. Equal amounts of protein from each sample were resolved using 12% SDS-PAGE and transferred to PVDF membranes. The PVDF membranes were bathed in blocking buffer, then incubated overnight with mouse monoclonal anti-phospho-MAP kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or a rabbit anti-MAP kinase antibody that recognizes total (phosphorylated and non-phosphorylated) MAP kinase (Upstate Biotechnology, Charlottesville, VA). The immunoblots were then washed and incubated with goat anti-mouse IgG conjugated with alkaline phosphatase or goat anti-rabbit IgG conjugated with alkaline phosphatase (Santa Cruz) for 1 h at room temperature. Following incubation, blots were washed again and developed using CDP Star western blot chemiluminescence reagent and images were captured on Kodak Biomax MR film. Levels of immunoreactivity were quantified using Syngene software (Synoptics Ltd, Cambridge, UK).

Agonist-Induced Stimulation of [^{35}S]GTPγS Binding

As before, DOR cells were incubated at 37°C in serum-containing culture medium, and were either untreated, or pre-exposed to 1 μM DADL for 2, 6, or 18 h, alone or in combination with the proteasome inhibitor, ZLLL, at 10 μM. Cells that were exposed to the proteasome inhibitor were first preincubated with ZLLL for 1 h. Following incubations, intact DOR cells were washed twice with serum-free DMEM to remove the agonist and proteasome inhibitor, then cells were harvested in 10 ml of 50 mM Tris HCl buffer, pH 7.5, per dish. The cell suspension was homogenized with a Tekmar tissuemizer (Cincinnati, OH), and then centrifuged at 100,000 *g* for 30 min. The supernatant was discarded and the membrane pellet was washed twice in Tris buffer, then resuspended by homogenization in 7 ml of 0.32 M sucrose, 50 mM Tris HCl, pH 7.5, per dish, and the membrane preparation was stored at –80°C. DOR cell membrane preparations were thawed on ice and diluted with assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and 0.1% bovine serum albumin), immediately prior to the assay. Cell membranes (containing 5-10 μg of protein) were incubated in assay buffer containing [^{35}S]GTPγS (100,000-150,000 dpm, approximately 80 pM) and GDP (10 μM) with or without DADL (10^{-10} to 10^{-5} M) in a total volume of 1 ml for 90 min at 30°C. Nonspecific binding was defined as [^{35}S]GTPγS binding in the presence of 10 μM unlabeled GTPγS. Nonspecific binding was found to be similar in the presence or absence of 10 μM DADL, and was subtracted from total stimulated and total basal binding. Bound and free [^{35}S]GTPγS was separated by filtration through Whatman GF/B filters under reduced pressure. Filter-bound radioactivity was determined by liquid scintillation counting. Nonlinear regression analysis of the dose response curves was performed using Prism to determine EC_{50} and E_{max} values. Significant differences ($p < 0.05$) in E_{max} values between different treatment groups were determined using ANOVA, Newman-Keuls multiple comparison test.

Results:

Kinetics of the agonist-induced reduction of δ receptor binding sites and down regulation of total cellular δ opioid receptor immunoreactivity and the effect of a proteasome inhibitor on agonist-induced changes

We have established a stable HEK293 cell line that expresses the δ opioid receptor tagged with the 8 amino acid-long FLAG epitope at the amino terminus (DOR cells). The B_{max} of DOR cells was calculated to be 3.2 ± 0.4 pmol/mg of protein, derived from saturation analysis using [^{3}H]bremazocine as the radioligand, and the apparent dissociation constant (K_d) for bremazocine was 2.0 ± 0.2 nM (n=11, see controls in Table 1). Treatment of DOR cells with the peptide agonist, DADL (1 μM), for 2, 6 or 18 h resulted in a time-dependent decrease in the steady-state level of δ opioid receptor binding sites, as measured by determination of the B_{max} following treatment using [^{3}H]bremazocine as the radioligand (Table 1). Specifically, DADL treatment for 2, 6 and 18 h caused a 59%, 79% and 92% decrease, respectively, in the B_{max} ($p<0.05$ for each time point, paired 2-tailed Student's t test compared with controls). Similar decreases in B_{max} were observed using [^{3}H]naltrindole and [^{3}H]diprenorphine as radioligands (data not shown). Apparent K_d values were unchanged following DADL treatment, which provided evidence that the agonist had been effectively washed away prior to the binding assay, since residual DADL would have caused an apparent increase in the K_d. In fact, none of the treatments with agonist, the proteasome inhibitor, or the combination of the two at any time point resulted in a

statistically significant difference in the K_d values listed in Table 1 ($p>0.05$ among groups, ANOVA, Newman-Keuls multiple comparison test). When DOR cells were pre-exposed for 1 h in the presence of the proteasome inhibitor, ZLLL (10 μM) alone, followed by further incubation with the inhibitor for 2, 6, or 18 h, there was no statistically significant change in B_{max}. However, when added to cells together with DADL, the proteasome inhibitor significantly attenuated the agonist-induced decrease in B_{max} following 18 h of DADL treatment (Table 1), but not at 2 or 6 h, changing the 18 h B_{max} ratio from 8 in its absence to 31 in the presence of ZLLL ($p<0.05$, ANOVA, Newman-Keuls multiple comparison test). It is noteworthy (see Discussion) that the B_{max} ratio of 31 was not statistically different from the other B_{max} ratios of 41, 46, 21 and 27 at the other time points in Table 1 ($p>0.05$, ANOVA, Newman-Keuls multiple comparison test).

The time course of agonist-induced changes in total cellular receptor immunoreactivity was also examined using western blot analysis to determine if the agonist induced decrease in receptor binding sites was due down regulation (proteolysis of the receptor). In this series of experiments, we also wanted to assess the effect of prior agonist and proteasome inhibitor exposure on the DADL-induced activation of MAP kinase (see below), so following pretreatment with agonist and/or proteasome inhibitor and washing, an additional 10 min incubation in the absence and presence of DADL was performed prior to making cell extracts for analysis of receptor and MAP kinase immunoreactivity (see Experimental Procedures). To measure total cellular δ receptor immunoreactivity after the various treatments, whole cell lysates were analyzed by immunoblot analysis using the anti-FLAG M1 monoclonal antibody for detection of the δ opioid receptor, and α-tubulin immunoreactivity was used as a control for gel loading and normalization of the receptor protein levels. As expected, acute (10 min) exposure

of DOR cells to DADL did not alter the level of receptor protein (compare lanes 1 and 2, Fig. 1A and 1B), and when cells were pre-exposed to DADL for 2 h, the level of total δ receptor immunoreactive protein also was not changed significantly (compare lane 4 to lanes 1 or 2, Fig. 1A and 1B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). Administration of the proteasome inhibitor, alone or in combination with DADL for 2 h again had no effect on the level of receptor protein (compare lanes 3 and 5 to lanes 1, 2 or 4, Fig. 1A and 1B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). Therefore, it was evident that exposure of DOR cells to DADL for 2 h significantly reduced [^{3}H]bremazocine binding to the receptor (see above) with no effect on total δ receptor protein. The decrease in binding after exposure to DADL for 2 h must be due to an alteration in the receptor ligand binding pocket, since it does not involve a decrease in total receptor protein. More prolonged exposure to DADL for 6 or 18 h decreased total receptor immunoreactivity to 55% and 20% of vehicle-treated controls, respectively, when normalized to the level of α-tubulin (compare lane 4 to lanes 1 or 2 at the 6 and 18 h treatment times, Fig. 1A and 1B, $p<0.05$, ANOVA, Newman-Keuls multiple comparison test). Incubation of DOR cells with the proteasome inhibitor, ZLLL, alone for 2, 6, or 18 h, did not alter the level of total receptor immunoreactivity (compare lane 3 to lanes 1 or 2, Fig. 1A and 1B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). Significantly however, when DOR cells were treated with DADL and ZLLL together for 6 or 18 h, the proteasome inhibitor prevented the DADL-induced down regulation of total immunoreactive δ opioid receptor protein levels (compare lane 5 to lane 4 at the 6 and 18 h treatment times, Fig. 1A and 1B, $p<0.05$, ANOVA, Newman-Keuls multiple comparison test). Indeed, cotreatment of cells with ZLLL and DADL for 6 or 18 h brought the level of receptor immunoreactivity back to the level in samples treated with the proteasome inhibitor alone (compare lane 5 to lane 3, Fig.

1A and 1B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test), and to the receptor levels in controls (compare lane 5 to lanes 1 or 2, Fig. 1A and 1B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test).

Effect of pre-exposure to DADL on agonist-induced activation of MAP kinase and the sensitivity of the DADL-induced changes to co-incubation with a proteasome inhibitor

We examined the effect of DADL pretreatment on agonist-induced phosphorylation of MAP kinase in DOR cells, and determined whether the proteasome inhibitor influenced the effect of prior agonist exposure. It is well documented that activation of MAP kinase occurs via dual phosphorylation at threonine 202 and tyrosine 204 of the kinase, and the activated, phosphorylated form of MAP kinase was detected by immunoblot analysis using a phospho-MAP kinase specific antibody. Total MAP kinase immunoreactivity (detected with a different antibody that recognizes both the phosphorylated and non-phosphorylated forms of the kinase) was used as a control for gel loading and normalization of the phospho-MAP kinase levels. Acute (10 min) treatment of DOR cells with 1 μM DADL caused a 3-fold increase in the level of phospho-MAPK, with no change in total MAPK immunoreactivity (compare lanes 1 and 2, Fig. 2A and 2B, $p<0.01$, ANOVA, Newman-Keuls multiple comparison test). Time course studies (not shown) indicated that activation of MAP kinase was evident at 5 min and maximal after 10 min of agonist exposure, then decayed slowly with time. As a negative control, DADL treatment did not stimulate MAP kinase phosphorylation in non-transfected HEK293 cells in which the δ opioid receptor is not expressed (data not shown). Prior exposure of DOR cells (in which the δ opioid receptor is expressed) to DADL for 2, 6 or 18 h abrogated the agonist-induced activation of MAP kinase (compare lanes 4 and 2 at all treatment times, Fig. 2A and 2B, $p<0.01$, ANOVA, Newman-Keuls multiple comparison test), resulting in levels of phosphorylated MAP kinase that

were not significantly different from basal unstimulated levels (compare lanes l and 4, Fig. 2A and 2B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). Preincubation with ZLLL alone for 2, 6, or 18 h did not alter the acute effect of DADL on MAP kinase activation in cells not previously exposed to agonist (compare lanes 2 and 3 at all treatment times, Fig. 2A and 2B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). When DOR cells were pre-incubated with DADL in the presence of ZLLL for 2 h, there appeared to be a trend that the loss of agonist-dependent activation of MAP kinase was partially inhibited, however, the effect of the proteasome inhibitor did not reach statistical significance relative to DADL pretreatment alone (compare lanes 4 and 5 at the 2 h treatment time, Fig. 2A and 2B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). When the period of co-incubation was extended, statistical significance was reached. The proteasome inhibitor partially prevented the loss of agonist activity to stimulate MAP kinase phosphorylation when DOR cells were co-incubated for 6 h with DADL and ZLLL (compare lane 5 to lane 4 at the 6 h treatment time, Fig 2A and 2B, $p<0.01$, ANOVA, Newman-Keuls multiple comparison test). The magnitude of the MAP kinase phosphorylation, however, was still significantly less than in DOR cells with no prior exposure to DADL (compare lane 5 to lane 2 or 3, Fig. 2A and 2B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test). When DOR cells were co-incubated with DADL and ZLLL for 18 h, the proteasome inhibitor completely prevented the loss of agonist activity to stimulate MAP kinase phosphorylation (compare lane 5 to lane 4 at the 18 h treatment time, Fig 2A and 2B, $p<0.01$, ANOVA, Newman-Keuls multiple comparison test), such that the magnitude of the agonist-induced MAP kinase phosphorylation was not significantly different than in DOR cells with no prior exposure to DADL (compare lane 5 to lane 2 or 3, Fig. 2A and 2B, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test).

Effect of pre-exposure to DADL on agonist-induced stimulation of [^{35}S]GTPγS binding and the sensitivity of the DADL-induced changes to inhibition of the proteasome

Having determined that prior activation of the δ opioid receptor attenuated the agonist stimulation of the effector, MAP kinase, we next sought to study the effect of pre-exposure to agonist on a component of the signaling pathway upstream of an effector, namely G protein activation, and to determine if the agonist-induced changes in G protein activation were also altered by co-treatment with a proteasome inhibitor. As expected, exposure of naive DOR cell membranes to DADL resulted in a significant dose-dependent increase in [^{35}S]GTPγS binding (Fig. 3). The EC_{50} of DADL was 1.2 ± 0.2 μM, and the maximal stimulation with 10 μM DADL was approximately 3.5-fold above basal [^{35}S]GTPγS binding. When DOR cells were preincubated with DADL for 2, 6, or 18 h, analysis of DADL dose-response curves revealed that maximal [^{35}S]GTPγS binding was markedly attenuated following pre-exposure to agonist (Fig. 3). The flatness of the dose-response curve following pre-incubation with DADL precluded accurate determination of the EC_{50} value, however, the EC_{50} values of the control, ZLLL, and ZLLL+DADL samples did not differ significantly from each other at any time point ($p>0.05$, ANOVA, Newman-Keuls multiple comparison test). Following agonist pre-treatment for 2 h, the maximal [^{35}S]GTPγS binding was reduced significantly from 360% to 140% of basal binding (Fig. 3A and 3D, $p<0.001$, ANOVA, Newman-Keuls multiple comparison test, DADL preincubation compared with control). Preincubation of DOR cells with ZLLL alone for 2 h did not alter the agonist stimulation of [^{35}S]GTPγS binding relative to the vehicle-treated control ($p>0.05$). Although a trend was evident, co-administration of the proteasome inhibitor and agonist for 2 h did not significantly alter the reduced maximal [^{35}S]GTPγS binding due to DADL pretreatment alone (Fig. 3A and 3D, $p>0.05$, ANOVA, Newman-Keuls multiple comparison

test). When the agonist pretreatment period was extended to 6 h, the maximal [^{35}S]GTPγS binding in response to agonist was decreased even further from 300% in the vehicle-treated control to 120% of basal [^{35}S]GTPγS binding following pre-exposure to DADL (Fig. 3B and 3D, $p<0.01$, ANOVA, Newman-Keuls multiple comparison test). Preincubation of DOR cells with the proteasome inhibitor alone for 6 h did not alter the agonist stimulation of [^{35}S]GTPγS binding relative to the control ($p>0.05$). Co-administration of ZLLL and DADL for 6 h resulted in a partial prevention of the decreased responsiveness to agonist stimulation, from a maximal [^{35}S]GTPγS binding of 120% of the basal level in the absence of the proteasome inhibitor to 200% in its presence ($p<0.05$, ANOVA, Newman-Keuls multiple comparison test). Pretreatment of DOR cells with DADL for 18 h caused complete inhibition of agonist-stimulated [^{35}S]GTPγS binding, reducing it to basal levels (Fig. 3C and 3D). Pretreatment of DOR cells with the proteasome inhibitor alone for 18 h slightly increased the maximal DADL-induced [^{35}S]GTPγS binding by 30% relative to vehicle-treated controls ($p<0.05$, ANOVA, Newman-Keuls multiple comparison test). Pretreatment of DOR cells with DADL in the presence of the proteasome inhibitor for 18 h partially but significantly inhibited the loss of agonist-stimulated [^{35}S]GTPγS binding from basal levels to a maximal 210% above basal levels (Fig. 3C and 3D, $p<0.05$, ANOVA, Newman-Keuls multiple comparison test).

Discussion:

In this report we have shown that prior agonist exposure abrogates the activation of MAP kinase and the stimulation of [^{35}S]GTPγS binding resulting from subsequent agonist application, and so we have chosen to use these endpoints as a cellular model for δ opioid tolerance. We found that pre-incubation of a proteasome inhibitor together with DADL for 6 or 18 h significantly prevented the loss of MAP kinase activation and stimulation of [^{35}S]GTPγS binding in response to subsequent agonist application.

Receptor desensitization should attenuate agonist activity in the absence of a decrease in total receptor protein, whereas down regulation will decrease the response to agonist as a result of receptor proteolysis which lowers the level of total receptor protein and the B_{max}. Both processes will contribute to the loss of agonist activity. It is generally accepted that desensitization of G protein-coupled receptors is reversed by internalization of the phosphorylated receptor by the endocytic pathway, followed by dephosphorylation of the receptor by endosome-associated phosphatases, allowing recycling of the resensitized receptor to the plasma membrane (Lefkowitz and Shenoy, 2005). In a recent elegant study of the endogenous μ opioid receptor expressed in locus ceruleus neurons, however, it was reported that desensitization and the recovery from desensitization were not dependent on receptor internalization and that the activity of the μ receptor could be modulated at the cell surface (Arttamangkul et al., 2006).

Treatment of DOR cells with DADL for 2, 6, and 18 h caused a progressive time-dependent decrease in the B_{max} of [^{3}H]bremazocine δ receptor binding sites. In contrast, total receptor immunoreactivity did not decrease after a 2 h exposure to agonist (ie., there was no down regulation). While the level of receptor immunoreactive protein did decrease after 6 and 18 h of agonist exposure, the magnitude of the decrease in immunoreactivity was not as great as the

decrease in receptor binding sites as determined by binding assays. We suggest from these results that following agonist exposure for 2 h, a substantial percentage of the receptor pool was in a desensitized state that was incapable of binding bremazocine, a full agonist with high affinity for the δ opioid receptor (Law et al., 1994, Pineyro et al., 2005) This was reflected in the decrease in the apparent B_{max} at this time point. The decrease in bremazocine affinity for the receptor must have been sufficiently large to prevent it from binding at the nanomolar concentrations used in the radioligand binding assay, since the decrease in binding following agonist exposure was due entirely to a 60% decrease in the B_{max}, with no change in the K_d for bremazocine. The immunoblot analysis indicated that the receptor pool that no longer bound bremazocine was still expressed in the cell, since there was no decrease in total cellular receptor immunoreactivity after exposure to DADL for 2 h. We also observed a decrease in B_{max} as measured by [^{3}H]naltrindole and [^{3}H]diprenorphine binding (data not shown). It has also been reported by others that [^{3}H]naltrindole binding is decreased significantly following short-term exposure (<2 h) of NG108-15 cells to the peptide agonist, [D-Ser^2]-Leu-enkephalin-Thr (Breivogel et al., 1997). Our results indicate that the DADL-induced decrease in receptor binding sites was not due to a loss of only high affinity agonist binding, since antagonist binding was also decreased. We suggest that pre-exposure of the receptor to DADL for 2 h caused a significant alteration in the ligand binding pocket in a portion of the receptor population that prevented agonist and antagonist binding. Elucidation of the molecular nature of the alteration will require further study. The proteasome inhibitor, ZLLL, had no effect on the apparent number of bremazocine receptor binding sites after exposure to DADL for 2 h, and it did not alter the level of receptor immunoreactivity. After pre-incubation of DOR cells with DADL for 2 h, the efficacy of DADL to activate MAP kinase and stimulate [^{35}S]GTPγS binding was decreased

significantly. Thus after 2 h of agonist pre-treatment, the fraction (40%) of the receptor population that could bind bremazocine with high affinity was uncoupled from both G protein activation (based on [^{35}S]GTPγS binding) and downstream effector signaling (as measured by assay of phosphorylated MAP kinase). ZLLL did not prevent significantly the decreased response to DADL when the two agents were pre-incubated together for 2 h, although there was a trend in that direction that was not statistically significant. When the proteasome inhibitor was pre-incubated with agonist for 6 h, it significantly blocked the loss in agonist activity for activating MAP kinase and stimulating [^{35}S]GTPγS binding. In concert with the partial but significant protection of signal transduction downstream of the δ opioid receptor, ZLLL prevented the agonist-induced down regulation (proteolysis) of total δ receptor immunoreactive protein, but interestingly, did not significantly increase the number of bremazocine binding sites at 6 h. Following 18 h of co-incubation with agonist, ZLLL completely prevented the loss of DADL activity to activate MAP kinase, partially blocked the loss of agonist-induced stimulation of [^{35}S]GTPγS binding, prevented agonist-induced down regulation of total δ receptor immunoreactive protein, and significantly increased the number of bremazocine binding sites compared with agonist pretreatment alone. From consideration of the 6 h data, it is evident that the proteasome inhibitor was able to prevent the loss of agonist-induced signaling downstream of the receptor in the absence of an increase in receptor binding sites. It was reported recently that chronic morphine treatment decreased the level of Gαi2 and Gαi3 in Chinese hamster ovary cells expressing the μ opioid receptor (Xu et al., 2005), and Gαi2, Gαi3, Gβ1, and Gβ2 in human neuroblastoma SHSY5Y cells endogenously expressing μ and δ opioid receptors (Mouledous et al., 2005). If DADL activation of the δ opioid receptor has a similar effect on these G proteins in DOR-expressing HEK 293 cells, and if the proteasome is involved in the

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agonist-induced decrease in G protein expression, as it is in SHSY5Y cells (Mouledous et al., 2005), the proteasome inhibitor may be able to partially prevent the loss of agonist stimulation of [^{35}S]GTPγS binding and activation of MAP kinase by blocking the agonist-induced degradation of Gαi2 and Gαi3. Other studies have also reported evidence that G proteins are degraded by the proteasome (Obin et al., 1996; Johansson et al., 2005).

We reported previously that proteasome inhibitors were capable of attenuating μ and δ opioid receptor down regulation (Howells et al., 1999; Chaturvedi et al., 2001). All proteasome inhibitors tested, including ZLLL, displayed this activity, whereas inhibitors of calpain, caspase and lysosomal cathepsins were ineffective. ZLLL effectively inhibits the proteasome in cells at concentrations several orders of magnitude lower that are required to inhibit calpain and cathepsins (Tsubuki et al., 1996). The proteasome contains six active sites, two of which are termed "chymotrypsin-like" in that cleavage occurs after hydrophobic residues, two are termed "trypsin-like" that cut after basic residues, and two are termed "caspase-like" since cleavage occurs preferentially after aspartate residues. ZLLL, a peptide aldehyde, inhibits the proteasome by forming a hemiacetal bond with the hydroxyl group of N-terminal threonines at the active sites, and inhibits the chymotrypsin-like >> caspase-like > trypsin-like activities (Kisselev and Goldberg, 2001). Li et al. (2000) reported that proteasomal inhibitors also attenuated down regulation of the κ opioid receptor. In addition, Bouvier and colleagues found that the proteasome, as a component of the endoplasmic reticulum quality control system, was also involved in the turnover of newly synthesized misfolded δ opioid receptors (Petaja-Repo et al., 2001). Reports on the involvement of the ubiquitin/proteasome system in opioid receptor turnover have been extended to other G protein-coupled receptors, including the β2-adrenergic receptor (Shenoy et al., 2001), CXCR4 chemokine receptor (Marchese and Benovic, 2001;

Fernandes et al., 2002), CCR5 chemokine receptor (Fernandes et al., 2002), protease-activated receptor 2 (Jacob et al., 2005) and the neurokinin-1 receptor (Cotrell et al., 2006).

Studies aimed at characterizing the molecular and cellular mechanisms of opioid tolerance have focused mainly on signaling through the μ opioid receptor resulting from activation by μ-selective agonists such as morphine (Bailey and Connor, 2005). There are also numerous reports that the δ opioid receptor is down regulated following agonist treatment of cultured cells (Law et al., 1983; Law et al., 1994; Breivogel et al., 1997; Chaturvedi et al., 2001; Okura et al., 2003) and *in vivo* (Tao et al., 1988; Zhao and Bhargava, 1997). In addition, tolerance develops to the analgesic, convulsive, and locomotor-stimulating action of δ receptor-selective agonists, but not to their antidepressant-like effects (Broom et al., 2002; Zhao and Bhargava, 1997; Tseng et al., 1997; Jutkiewicz et al., 2005).

One might expect that *in vivo* administration of proteasome inhibitors would have pleiotropic side effects, however, the proteasome inhibitor, bortezomib (Velcade, PS-341) has been approved by the FDA for the treatment of multiple myeloma, and is now undergoing clinical trials for many other types of cancer (Joazeiro et al., 2006). It will be a priority now to test whether proteasome inhibitors can inhibit the development of tolerance to the antinociceptive effects of δ opioid receptor selective agonists *in vivo*.

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Footnotes:

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Present address (KC): Department of Animal Sciences, Rutgers-The State University of New Jersey, New Brunswick, NJ 08901.

Legends for Figures:

Figure 1. Immunoblot analysis of the effect of ZLLL on agonist-induced down-regulation of the δ opioid receptor. HEK 293 cells expressing FLAG-tagged δ receptors were pretreated at 37°C for 1 h without (lanes 1, 2 and 4) or with 10 μM ZLLL (lanes 3 and 5), then incubated in the absence (lanes 1, 2 and 3) and presence of 1 μM DADL alone (lane 4) or in combination with ZLLL (lane 5) for 2, 6, or 18 h, as indicated. Culture media was aspirated, cells were washed twice with serum-free media, then challenged for 10 min with 1 μM DADL (lanes 2-5) or not (control, lane 1). Whole cell lysates were prepared, resolved by SDS-PAGE, and immunoblotted with the anti-FLAG M1 monoclonal antibody (panel A). Following development, the blots were stripped and reprobed with α-tubulin antibody for normalization. B. Quantitative analysis of the δ receptor immunoblots, normalized to the level of α-tubulin immunoreactivity, using Syngene software. Each bar represents the mean ± S.E.M. of 3-4 independent experiments. The immunoreactivity in lane 1 was set to 100%, and the immunoreactivity levels in lanes 2-4 are expressed as percentages relative to the controls from lane 1. Since there was a trend for ZLLL treatment to increase receptor immunoreactivity (lane 3), the immunoreactive signals in lane 5 (combined ZLLL and DADL pretreatment) were expressed as a percentage of the immunoreactivity in lane 3 (ZLLL pretreatment alone). DADL pretreatment (lane 4) for 6 and 18 h decreased significantly the level of δ receptor immunoreactivity relative to controls (lanes 1 or 2), and ZLLL pretreatment inhibited significantly the agonist-induced down-regulation of δ receptor immunoreactivity following exposure of cells to DADL for 6 and 18 h (compare lanes 4 and 5). NS, not significant, $p>0.05$, ANOVA, Newman-Keuls multiple comparison test. *, statistically significant, $p<0.05$, ANOVA, Newman-Keuls multiple comparison test.

Figure 2. **Proteasome inhibition blocks the tolerance that develops to the DADL-induced phosphorylation of MAP kinase.** A. DOR cells were treated as described in the legend to Fig. 1. Cell lysates (40 μg protein/lane) were resolved using 10% SDS-PAGE and immunoblotted with a mouse monoclonal anti-phospho-MAP kinase antibody, specific for the phosphorylated, active form of MAP kinase, or a rabbit anti-MAP kinase antibody that detected total MAP kinase levels. B. Quantitative analysis of the immunoblot data displayed in panel A. Each bar represents the mean ± S.E.M. of the phospho-MAP kinase immunoreactivity normalized to total MAP kinase immunoreactivity derived from 3-4 independent experiments. The normalized phospho-MAP kinase immunoreactivity in samples treated for 10 min with DADL (lane 2) was set to 100%, and samples from lanes 1, 3, and 4 were calculated as percentages of those samples. Since ZLLL pretreatment alone (lane 3) tended to increase normalized phospho-MAP kinase immunoreactivity slightly, samples pretreated with the combination of ZLLL and DADL (lane 5) are expressed as a percentage of the immunoreactivity in lane 3. Statistical significance was determined using ANOVA, Newman-Keuls multiple comparison test. NS, not significant, $p>0.05$; *, statistically significant, $p<0.05$; **, statistically significant, $p<0.01$.

Figure 3. Proteasome inhibition blocks the tolerance that develops to the DADL-induced stimulation of [^{35}S]GTPγS binding. DOR cells were preincubated at 37°C for 1 h with or without 10 μM ZLLL followed by incubation with 1 μM DADL, alone or in combination with 10 μM ZLLL for 2, 6, or 18 h. Frozen DOR cell membrane preparations was thawed on ice, and diluted with assay buffer immediately prior to the assay. Membranes (approximately 5-10 μg protein) were incubated in assay buffer containing [^{35}S]GTPγS and GDP with or without DADL (10^{-10} to 10^{-5} M) in a total volume of 1 ml for 90 min at 30 °C. Nonspecific binding was defined as [^{35}S]GTPγS binding in the presence of 10 μM unlabeled GTPγS and was subtracted from total

stimulated and total basal binding. Bound and free [^{35}S]GTPγS was separated by filtration through GF/B filters under reduced pressure. Filter-bound radioactivity was determined by liquid scintillation counting. Nonlinear regression analysis of dose response curves was performed using Prism to determine EC_{50} and E_{max} values. Data are presented as a percent of the basal [^{35}S]GTPγS binding (without DADL stimulation) set to 100%, and represent the mean ± S.E.M. of 3-4 independent experiments measured in duplicate. Panels A, B, and C contain data derived from DOR cells pretreated for 2, 6, or 18 h with DADL, respectively. D. Bar graph depicting the maximal [^{35}S]GTPγS binding following receptor activation with 10 μM DADL derived from panels A, B, and C. Statistical significance was determined using the ANOVA, Newman-Keuls multiple comparison test. NS, not significant, $p>0.05$; *, statistically significant, $p<0.05$; **, statistically significant, $p<0.01$.

Table 1. Kinetics of agonist-induced desensitization and down regulation of the δ opioid receptor and the effect of co-incubation with ZLLL.

DOR cells in serum-containing media were preincubated for 1 h at 37°C with or without 10 μM ZLLL (a proteasome inhibitor), then incubation continued in the presence or absence of 1 μM DADL for 2, 6, or 18 h. Membrane fractions were prepared and saturation analysis was performed using [^{3}H]bremazocine as a radioligand. Saturation curves were analyzed by non-linear regression to calculate the apparent dissociation constant, K_d, and maximum number of receptors, B_{max}. B_{max} ratios represent quotients x 100 of either DADL/control or ZLLL + DADL/ZLLL. Data represent the mean ± S.E.M. of 3-4 experiments conducted in duplicate. *, $p<0.05$ for each time point, paired 2-tailed Student's t test compared with controls; #, $p<0.05$ for 18 h ZLLL+DADL compared with 18 h DADL, paired 2-tailed Student's t test; **, $p<0.05$, ANOVA, Newman-Keuls multiple comparison test compared with 18 h DADL B_{max} ratio.

Time (h)	Treatment	K_d (nM)	B_{max} (pmol/mg)	B_{max} Ratio (%)
2	Control	1.8 ± 0.3	3.9 ± 0.6	
	DADL	2.6 ± 0.2	1.6 ± 0.2*	41
	ZLLL	1.8 ± 0.2	4.8 ± 0.8	
	ZLLL+ DADL	2.3 ± 0.3	2.2 ± 0.5	46
6	Control	1.8 ± 0.3	2.8 ± 0.5	
	DADL	2.2 ± 0.2	0.6 ± 0.1*	21
	ZLLL	1.8 ± 0.4	4.1 ± 1.3	
	ZLLL+ DADL	2.9 ± 0.3	1.1 ± 0.2	27
18	Control	2.5 ± 0.1	3.6 ± 0.2	
	DADL	2.0 ± 0.3	0.3 ± 0.1*	8
	ZLLL	2.3 ± 0.2	3.9 ± 1.5	
	ZLLL+ DADL	2.4 ± 0.4	1.2 ± 0.3#	31**

Figure 1

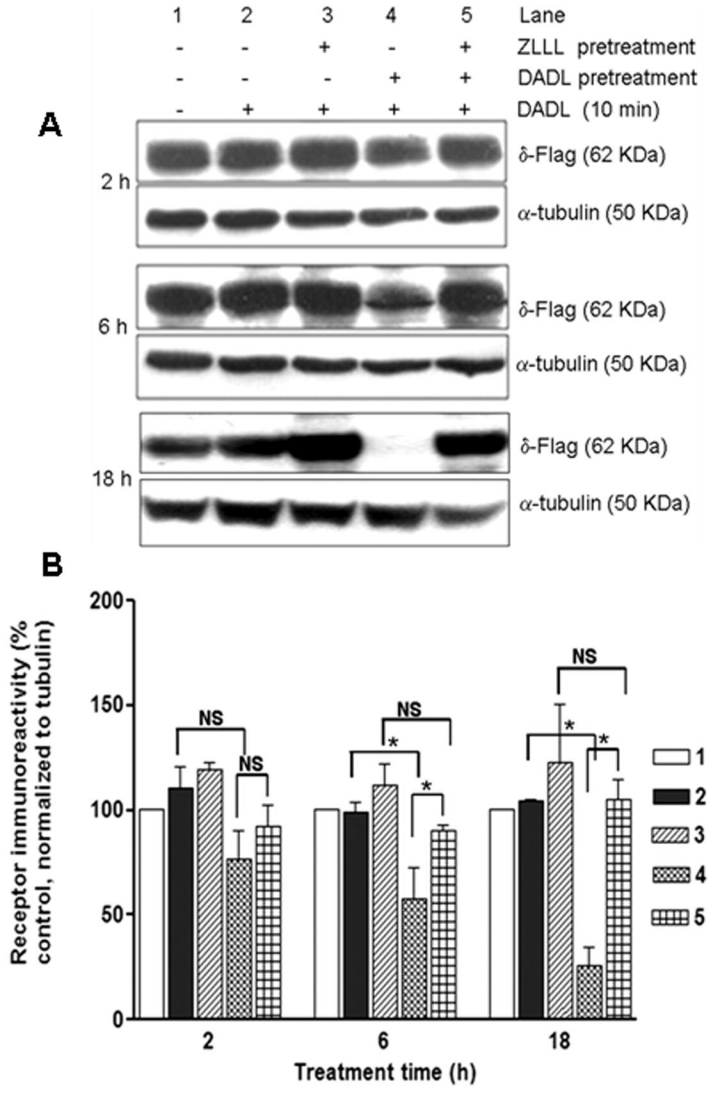

A
1 2 3 4 5 Lane
- - + - + ZLLL pretreatment
- - - + + DADL pretreatment
- + + + + DADL (10 min)
2 h
δ-Flag (62 KDa)
α-tubulin (50 KDa)
6 h
δ-Flag (62 KDa)
α-tubulin (50 KDa)
18 h
δ-Flag (62 KDa)
α-tubulin (50 KDa)
B
Receptor immunoreactivity (% control, normalized to tubulin)
200
150
100
50
0
NS
NS
NS
*
*
NS
*
*
1
2
3
4
5
2
6
18
Treatment time (h)

Figure 2

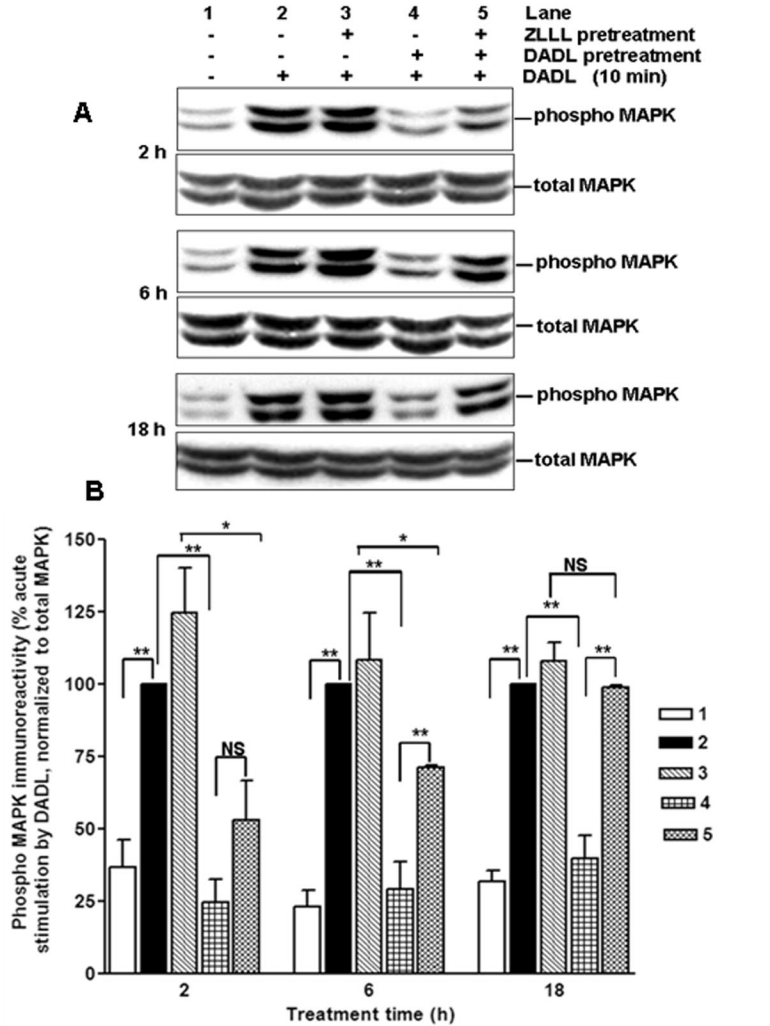
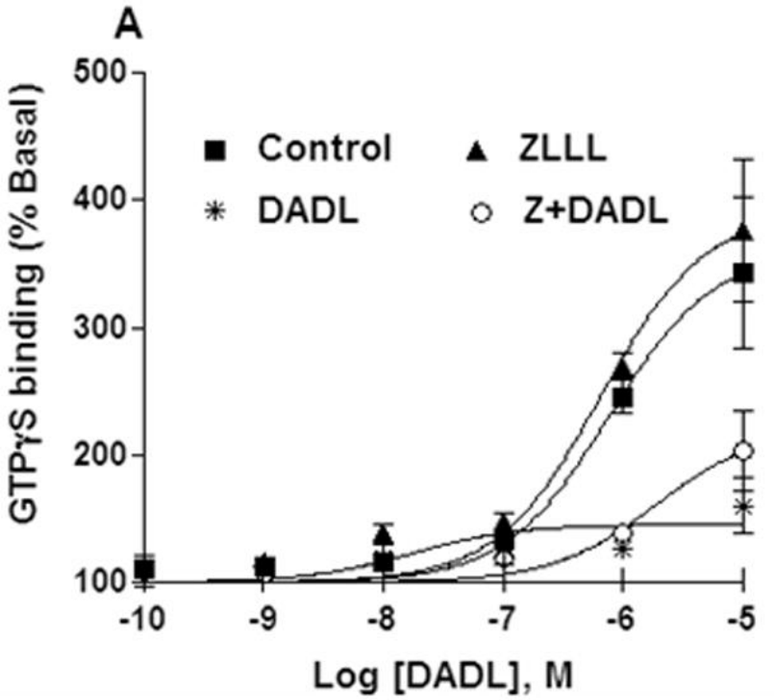
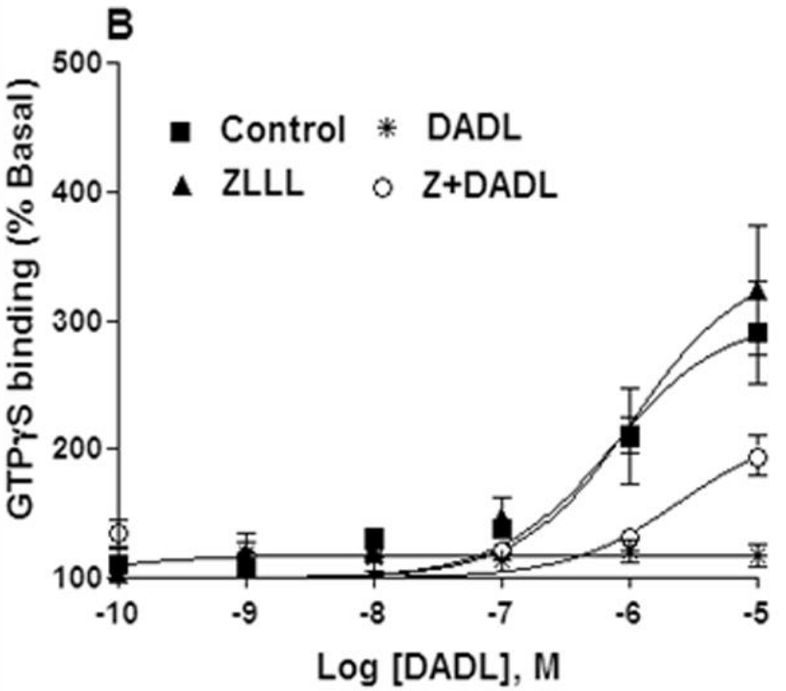
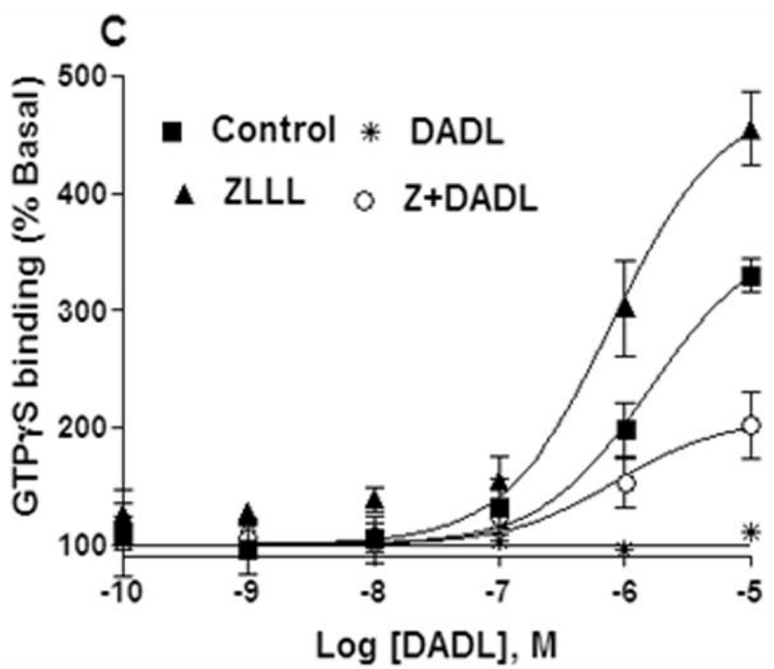
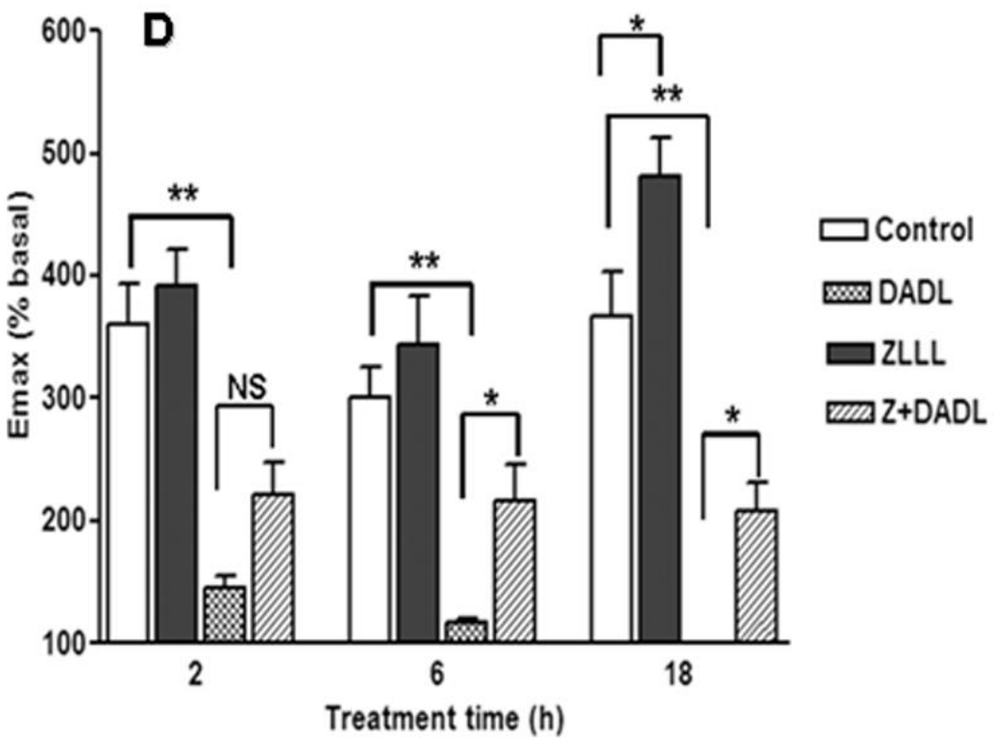

1 2 3 4 5 Lane
- - + - + ZLLL pretreatment
- - - + + DADL pretreatment
- + + + + DADL (10 min)
A
2 h
phospho MAPK
total MAPK
6 h
phospho MAPK
total MAPK
18 h
phospho MAPK
total MAPK
B
Phospho MAPK immunoreactivity (% acute stimulation by DADL, normalized to total MAPK)
150
125
100
75
50
25
0
*
**
NS
2
6
18
Treatment time (h)
1
2
3
4
5

Figure 3


A
GTPγS binding (% Basal)
500
400
300
200
100
Control
ZLLL
DADL
Z+DADL
-10
-9
-8
-7
-6
-5
Log [DADL], M


B
GTPγS binding (% Basal)
500
400
300
200
100
Control
DADL
ZLLL
Z+DADL
-10
-9
-8
-7
-6
-5
Log [DADL], M


C
GTPγS binding (% Basal)
500
400
300
200
100
Control
DADL
ZLLL
Z+DADL
-10
-9
-8
-7
-6
-5
Log [DADL], M


D
Emax (% basal)
600
500
400
300
200
100
**
NS
**
*
*
**
*
2
6
18
Treatment time (h)
Control
DADL
ZLLL
Z+DADL