INTERNALIZATION AND SRC ACTIVITY REGULATE THE TIME COURSE OF ERK ACTIVATION BY DELTA OPIOID RECEPTOR LIGANDS.

by

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Running title: Internalization and Src regulate kinetics of ERK stimulation

Abbreviations: BSA: Bovine serum albumine; DMEM: Dulbecco’s Modified Eagle Medium; DMSO: Dymethyl sulfoxide; δOR: delta opioid receptor; DPDPE: D-Pen 2,5 – Enkephalin; ERK: extracellular signal-regulated kinase; GPCRs: G protein couple receptors; GRKs: G protein couple receptor kinase; IBMX: 3-Isobutyl-1-methylxanthine; MAPK: mitogen-activated protein kinase; PP2: 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4,d]pyrimidine; PTX: Pertussis Toxin; TICP: Tyr-Ticpsi [CH2-NH]Cha-Phe-OH

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ABSTRACT

The present study showed that delta opioid receptor (δOR) ligands TICP and ICI174864 behaved as inverse agonists in the cyclase pathway but induced agonist responses in the ERK cascade. Unlike ligands that behaved as agonists in both pathways, and whose stimulation of ERK was marked but transient (10min), ERK activation by ICI174864 and TICP was moderate and sustained, lasting for more than one hr in the case of TICP. Biochemical experiments showed that duration of ERK activation by agonists and “dual efficacy ligands” was inversely correlated with their ability to trigger receptor phosphorylation and degradation. Thus, while TICP stabilized δORs in a conformation that did not incorporate $^{32}$P, was not a substrate for tyrosine kinase Src and was not down-regulated following prolonged exposure to the drug, the conformation stabilized by DPDPE incorporated $^{32}$P, was phosphorylated by Src and suffered degradation within the first two hrs of treatment. Inhibition of endocytosis by sucrose prolonged ERK activation by DPDPE increasing the decay half-life of the response to values that resembled those of “dual efficacy ligands” (from 2min decay t$_{1/2}$ increased to 12min). Src inhibitor PP2 also prolonged ERK stimulation by DPDPE. It did so by maintaining a sustained activation of the kinase at ≈20% of maximum following an initial rapid reduction in the response. These results show that specific kinetics of ERK activation by agonists and “dual efficacy” ligands are determined, at least in part, by the differential ability of the two types of drugs to trigger mechanisms regulating δOR responsiveness.
INTRODUCTION

Occupation of GPCRs by agonist ligands has two distinct consequences, the generation of an intracellular signal and the concomitant activation of a series of regulatory mechanisms that modulate receptor responsiveness over time. The chain of regulatory events triggered by agonist occupation of the receptor has been extensively characterized and has led to an established model of desensitization in which phosphorylation of the receptor by GRKs is the first step in the process (1,2). Phosphorylation then promotes the recruitment of β-arrestin (3,4), which is responsible for uncoupling the receptor from the G-protein (5) and for its targeting to clathrin-coated pits. From there receptors will be removed from the cell surface via dynamin-dependent endocytosis (6). Once inside the cell the receptor is either degraded or is quickly redirected to the cell membrane (7) for a new signalling cycle.

Despite the overwhelming evidence supporting this tightly knit model of activation and desensitization, there are also increasing number of observations indicating that activation and regulatory phenomena can be dissociated. For example, antagonist ligands for cholecystokinin (8) and endothelin receptors (9) selectively induce internalization without causing neither receptor activation nor phosphorylation. Agonists for parathyroid hormone type 1 receptor stabilize an active state that promotes signalling but does not recruit β-arrestin or induce internalization (10). In contrast, certain beta2-adrenergic receptors (β2ARs) ligands that preclude G-protein activation are still able to recruit β-arrestin to the receptor (11).
Ligands that stabilize GPCRs in a conformation that prevents activation of
the G-protein are classified as inverse agonists, and are commonly thought to
induce an inactive conformation of the receptor (12,13). More recently, some of
these drugs have been described as “proteans” or “dual efficacy ligands”,
referring to their ability to display both agonist and inverse agonist behaviour
(11,14-16). For example, we have recently shown that ICI118551 and
propranolol, two ligands of the β_{2}AR display dual efficacy since they behave as
inverse agonists in the cyclase pathway but produce agonist responses in the
ERK cascade (11).

The observation that some “inverse agonists” may produce agonist
responses indicates that the conformation they stabilize is not inactive, but rather
a signalling state that is distinct from the one stabilized by classical agonists. If
receptor states stabilized by agonists and dual efficacy ligands are distinct, then
one would expect that the responses that they elicit also be regulated in a distinct
manner. The present study focused on this question, assessing whether
agonistic responses generated by “dual efficacy ligands” for the δOR were
regulated as agonist responses induced by it’s classical agonists. Results show
that ERK activation by dual efficacy ligands like TICP and ICI174864 was
considerably longer, though more modest than the response induced by agonists
such as SNC-80 and DPDPE. Differences in time course were associated with
the distinct ability of dual efficacy ligands to stabilize δORs in an ERK stimulating
conformation that eluded regulatory steps typically triggered by highly efficacious
agonists.
EXPERIMENTAL PROCEDURES

Reagents. Buffer chemicals, protease inhibitors, DPDPE, morphine, naloxone, forskolin, IBMX, PTX, sucrose, anti-FLAG M2 affinity resin, and FLAG peptide were purchased from Sigma. \[^{35}\text{S}]\text{GTP}^\gamma\text{S}, \[^{3}\text{H}]\text{adenosine and }[^{32}\text{P}]\text{orthophosphoric acid were from Perkin-Elmer. ICI174864 and SNC-80 were obtained from Tocris Cookson, TIPP and TICP}_\Psi\text{ were synthesized as previously described (17). 4-amino-5-}(4\text{-chlorophenyl})\text{-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was from calbiochem. G418, DMEM, fetal bovine serum, fungizone, glutamine, penicillin, streptomycin were purchased from Wisent.}

DNA Constructs. The human \(\delta\text{OR cDNA was subcloned into the pcDNA3 expression vector (Invitrogen) as previously described (18), and was tagged at the C-terminal end using CLONTECH site-directed mutagenesis kit to remove the stop codon and introduce the sequence coding for the FLAG epitope (DYKDDDDD). The construction was confirmed by restriction enzyme mapping and DNA sequencing, and its signalling properties shown to be identical to those of the wild type \(\delta\text{OR (19,20). A truncated mutant of the murine }\delta\text{OR (}\delta\text{OR344T), was kindly provided by Dr. M. von Zastrow (UCSF). Wild-type and inhibitory mutant forms of c-Src (K295R/Y527F) were a gift from Dr Bouvier's laboratoty (Université de Montréal).}

Cell Culture and Transfection. HEK 293s cells were transfected using the calcium-phosphate precipitation method and clones stably expressing full length or truncated receptors were selected using 400 \(\mu\text{g/ml of G-418. Cell lines stably expressing full length }\delta\text{ORs and wild type c-Src were similarly selected, following lipofectamine transfection (Invitrogen). The dominant inhibitory form of c-Src}
(K295R/Y527F) was transiently transfected (0.25-3 µg DNA) onto cell lines expressing the full length δOR using polyethylenimine as previously described (21). Cells were grown and maintained in complete DMEM containing 10% (v/v) fetal bovine serum, 1000 units/ml penicillin, 1 mg/ml streptomycin, and 1.5 µg/ml fungizone) in a humidified atmosphere of 5% CO₂ at 37°C.

**Phosphorylation and immunoprecipitation of flag-tagged receptors.** For [³²P] incorporation assays, cells were incubated for 2 h in phosphate-free DMEM, after which [³²P]orthophosphoric acid was added at a final concentration of 1 mCi/ml and incubation allowed to proceed for an additional hr. At this time, DPDPE (1µM), TICP (1µM) or vehicle (0.01% DMSO) were added to the incubation medium for 30 min. Cells were then recovered, membranes prepared as indicated below and finally suspended in solubilization buffer (0.5% n-DDM (w/v), 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 140 mM NaCl, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 2µg/ml aprotinin, 0.5 mM PMSF, and 2 mM 1,10-phenantroline). Following agitation at 4°C for 60 min, the solubilized fraction was centrifuged at 12 000 g for 60 min and the receptor immunoprecipitated from the supernatant fraction using anti-FLAG M2 antibody resin. 40 µl of antibody-coupled resin equilibrated in solubilization buffer and supplemented with 0.1% BSA (w/v) were used to purify the receptor overnight at 4°C under gentle agitation. The next morning the resin was pelleted, washed twice with 500 µl of solubilization buffer and four times with 500 µl of modified solubilization buffer (containing 0.1% instead of 0.5% n-DDM (w/v). The receptor was then eluted by incubating the resin for 10 min at 4°C with 100 µl of modified solubilization buffer containing 175 µg of FLAG peptide/ml. This elution was
repeated three times, the eluates combined and concentrated by membrane filtration over Microcon-30 concentrators (Millipore). SDS sample buffer was then added, and samples used for SDS-PAGE. Similar immunoprecipitation procedure was used to assess TYR-phosphorylation of δORs.

**SDS-PAGE and Western Blotting.** SDS-PAGE was performed as described by Laemmli using a 4% stacking gel and 10% separating gel. Proteins resolved in SDS-PAGE were then transferred (50 mA, 16 h, Bio-Rad Mini-Trans Blot apparatus) from the gels onto nitrocellulose (Amersham Pharmacia Biotech;). In the case of [32P] incorporation studies, membranes were first exposed for autoradiography (BIOMAX films; KODAK). When assessing TYR-phosphorylation of δORs, membranes were probed overnight at 4°C with monoclonal antibodies raised against phosphorylated TYR (1:500; PY99, Santa Biotechnologies, CA). In both cases antisera directed against the FLAG M2 antibody (1:1000; Sigma) was used in order to detect the total amount of receptor protein present in each sample. Horseradish peroxidase-conjugated antimouse secondary antibodies (1:4000, Sigma) and enhanced chemiluminescence detection reagents (NEN Life Science Products) were used to reveal the blotted proteins, and relative intensities of the labeled bands were analyzed by densitometric scanning using MCID (Imaging Research Inc). Receptor phosphorylation was expressed as the ratio between phosphorylation and FLAG signals in order to normalize to the amount of receptor protein present in each sample.

For detection of ERK1/2 activation, cells were grown in six well plates and serum starved overnight. The day of the experiment they were cultured for 2 hr in serum-free medium and then exposed to different ligands. Following treatment,
cells were washed with ice-cold phosphate buffered saline and whole cell extracts prepared by lysis in SDS sample buffer. Samples were sonicated and then boiled for 5 min before loading for SDS-PAGE. Phospho-ERK1/2 detection was done by probing membranes with antiphospho-ERK1/2 antibody (1:1000; Santa Cruz, Biotechnology). Total ERK protein was determined after stripping by using 1:20,000 dilution anti-ERK1/2 antibody (Santa Cruz, Biotechnology). Secondary antimouse (1:5000; Sigma) and antirabbit (1:40000; Amersham) horseradish-conjugated antibodies were used to visualize proteins by chemiluminescence. ERK1/2-phosphorylation was normalized according to protein contents by expressing results as the ratio between pERK1/2 and total ERK1/2.

To assess Src activation cells were grown in 100mm petri dishes and prepared for the experiment as described for ERK1/2. Following treatment with different ligands cells were washed, harvested, and solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% triton X100, 0.25% deoxycholate acid, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor, 10 µg/ml benzamidine, 1 µg/ml aprotinin, 1 mM Na3VO4) at 4°C for 90 min. After centrifugation of non-solubilized debris at 12 000 g for 20 min samples were concentrated, suspended in SDS sample buffer and separated in SDS-PAGE. Anti-phospho-Src (Y416) monoclonal antibody (Upstate Biotechnology, NY) at a dilution of 1:1000 was used in order to determine the presence of activated Src and total amount of protein loaded was detected by probing with antibody anti Src (1:1000; Upstate Biotechnology).

cAMP accumulation assays. Cells were labeled overnight (16 hours) with 1 µCi/ml of [3H]adenine in complete DMEM medium. The day of the experiment
radioactive medium was replaced with fresh DMEM, cells mechanically detached, thoroughly washed (3 times) with PBS (4°C) and viability assessed using trypan blue (mortality was never higher than 5%). 5 x 10^5 cells were then incubated for 20 min at 37°C in 300 µl assay mixture containing PBS, 25 µM forskolin, 2.5 µM IBMX, and different drugs at the indicated concentrations. At the end of the incubation period, the assay was terminated by adding 600 µl ice cold solution containing 5% trichloroacetic acid, 5mM ATP and 5mM cAMP. [^3H]ATP and [^3H]cAMP were separated by sequential chromatography on Dowex exchange resin and aluminum oxide columns. Results were expressed as the ratio of [^3H]cAMP/[^3H]ATP + [^3H]cAMP.

[^35S]GTPγS binding assays were carried out on whole cell membrane preparations as previously described (20), cells were suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 2 mM EDTA, 5 µg/ml leupeptin, 5 µg/ml soybean trypsin inhibitor, and 10 µg/ml benzamidine) and homogenized with a Polytron homogenizer (Ultra-Turrax T-25, Janke and Kunkel) using three bursts of 5 s at maximum setting. Homogenates were centrifuged at 700 x g for 5 min, and the supernatant was further centrifuged at 27,000 x g for 20 min. Pellets were washed twice in lysis buffer and were immediately resuspended in [^35S]GTPγS assay buffer (50 mM Hepes; 200 mM NaCl; 1 mM EDTA; 5 mM MgCl₂; 1 mM DTT; 0.5% BSA and 3 µM GDP; pH 7.4) to yield 10 µg protein/tube. [^35S]GTPγS was used at 50nM and non-specific binding was determined in the presence of 100 µM GTP. The test compound SNC-80 was introduced at a final concentration of 100 nM and incubation was allowed to proceed for one hour at RT. The reaction was terminated by rapid filtration onto Whatman GF/C glass
filters pre-soaked in water. Filters were washed twice with ice-cold wash buffer (pH 7) containing 50 mM Tris, 5 mM MgCl₂ and 50 mM NaCl, and the radioactivity retained was determined by liquid scintillation.

Data analysis. Statistical analysis and curve fitting were done using GraphPad Prism 2.01 (San Diego, California)
RESULTS

Comparison of the effects of δOR ligands in cAMP and ERK signalling cascades. It has been previously shown that certain ligands for β2ARs display dual efficacy, inducing inverse agonist responses in the cAMP signalling pathway, but producing agonist effects in the ERK cascade (11). To determine whether this type of dual behaviour was specific to Gs-coupled receptors or could be extended to receptors coupled to Gi/o proteins, different ligands for the δOR were compared in adenylyl cyclase and ERK signalling pathways. In the cAMP pathway ligands produced effects that spanned the complete spectrum of efficacy ranging from agonism to inverse agonism. At maximally effective concentrations (1µM) SNC-80 and DPDPE were highly efficacious agonists, morphine, TIPP and naloxone were partial agonists while ICI174864 and TICP displayed typical inverse agonist responses. Figure 1A shows these different ligands ranked according to magnitude and vectorial aspects of their efficacies (SNC-80 ≥ DPDPE >MOR ≥ TIPP ≥ Nx >ICI174864 > TICP). In contrast with the diversity of responses observed in cAMP accumulation assays all drugs tested in the ERK cascade behaved as agonists, except for naloxone that was neutral. Indeed, ERK phosphorylation was induced not only by drugs that behaved as agonists in the cyclase cascade but also by TICP and ICI174864, which had produced inverse agonist responses when tested in this pathway. Moreover, when ranked according to the magnitude of their effect on ERK phosphorylation, TICP, the most efficacious inverse agonist in the cyclase pathway was now more
effective than partial agonists TIPP and morphine in activating ERK (SNC-80 > DPDPE > TICP ≥ TIPP ≥ ICI174864 ≥ MOR > Nx).

Dual efficacy ligands for the $\beta_{2AR}$ produce ERK activation via $\beta$-arrestin recruitment and independently of G protein activity (11). In order to determine whether this was also the case for Gi/o-coupled receptors, cells were treated overnight with PTX, and ERK activity assessed the following day. Without modifying basal activity of the kinase (pERK/totalERK ratio in controls: 0.6 ± 0.1; following PTX: 0.6 ± 0.1), PTX abolished ERK stimulation by classical agonist DPDPE and by dual efficacy ligand TICP (figure 1C). These results indicate not only that ERK stimulation by DPDPE and TICP requires Gi/o protein activity but also that simple inactivation of spontaneous Gi/o signalling cannot account for ERK stimulation. Neither TICP, ICI174864 nor classical agonists were able to evoke ERK activation in non-transfected cells (not shown), confirming that ligand-induced stimulation of ERK signalling was specifically mediated by the $\delta$OR.

**$\delta$OR ligands differ in their kinetics of ERK activation.** To determine whether the time course of ERK activation by classical agonists differed from that of dual efficacy ligands cells were exposed to a maximally effective concentration (1 $\mu$M) of each drug, and ERK phosphorylation measured following increasing periods of time. Two main types of kinetic profiles could be recognized. One was characteristic of highly efficacious ligands like SNC-80 and DPDPE which produced quick and pronounced ERK activation that peaked within 5 min (figure 2A), decaying right away with a calculated half-life ($t_{1/2}$) of approximately 2 min (figure 2B and 2C). The other type of response, induced by partial agonists and
dual efficacy ligands was less pronounced but more sustained, decaying with a 
t_{1/2} that ranged between 11 and 14 min (figure 2B and 2C). Among ligands 
inducing sustained responses, the effect of TICP could be distinguished from the 
rest of the drugs in the same category, because its effect was more pronounced 
and particularly more sustained (p<0.001 two way ANOVA; figure 2A and 2B).

**The time course of ERK activation by highly efficacious agonists and dual efficacy ligands is correlated with desensitization parameters.** One of the primary checkpoints that controls drug effects over time 
is the receptor itself. In particular, δOR signalling efficacy is regulated by 
phosphorylation of C-terminal Ser/Thr residues (22,23). In order to determine 
whether differences in the time course of ERK activation could be related to the 
distinct ability of different ligands to trigger phosphorylation of δORs cells were 
exposed for 30 min to DPDPE or TICP (1μM) in the presence of 
[^32P]orthophosphoric acid. Receptors were immunopurified, resolved on SDS-
PAGE and transferred onto nitrocellulose membranes that were first exposed for 
autoradiography and then used for western blot analysis using an anti-FLAG M2 
antibody. Immunoblots revealed two broad bands at ≅55 and ≅46 kDa, 
corresponding to mature and immature monomeric forms of the receptor, 
respectively (24). Autoradiograms showed that 30 minutes incubation with 
DPDPE increased ^32P incorporation by the ≅55 kDa species, but this effect was 
absent for TICP. Thus, at a time when the ERK response for the agonist was no 
longer present δORs were heavily phosphorylated. In contrast, ERK activation by 
the dual efficacy ligand was still at its maximum and no phosphorylation of the 
receptor could be detected.
Phosphorylation is an initial step in the process of desensitization, but if exposure to an agonist is allowed to proceed long enough δORs will start to be targeted for degradation (7). Hence, to confirm whether the different time course of ERK activation by DPDPE and TICP also correlated with later events in the process of desensitization cells were treated for 2 hrs either with the agonist or the dual efficacy drug. Following treatment the total amount of δOR protein present in membrane preparations was assessed by immunoblot (figure 3B). While incubation with TICP caused no detectable change in the mature receptor species (∼55 KDa) there was a decrease of the corresponding immunoreactive band following treatment with DPDPE. These results confirm that differences in the time course of ERK activation by DPDPE and TICP is inversely correlated with the ability of each ligand to trigger different events within the process of desensitization.

If indeed differences in time course of ERK activation by agonists and dual efficacy ligands were due to their distinct ability to trigger regulatory mechanisms of receptor responsiveness, interfering with these mechanisms should transform ERK activation by the agonist, into the more prolonged type of response observed for the dual efficacy ligand. To test this assumption the time course of ERK activation by DPDPE was assessed in presence of sucrose, which is an inhibitor of clathrin-mediated endocytosis. Though sucrose did not turn ERK activation into a stable response, it prolonged the effect of DPDPE by increasing the decay t_{1/2} of activation from 2 to 12 min (p<0.001 for interaction; two way ANOVA; figure 4), a value that falls within the 11-14 min range observed for dual efficacy ligands. Another means to modify mechanisms regulating δOR
responsiveness is to mutate amino acids that are implicated in the process. For δORs, Ser/Thr residues located in the C-terminal domain of the receptor are the principal target for G-protein-coupled receptor kinases and their phosphorylation is an essential step in the desensitization of full-length δORs (22,23,25). To explore the contribution of these residues to the kinetics of ERK activation by DPDPE experiments were repeated using a receptor truncated at its C-terminus (δOR344T). This approach also yielded results in which the DPDPE response decayed more slowly than in the full-length receptor (t_{1/2} of 6 min; two way ANOVA; p< 0.01 for interaction; figure 4). However, the effect of truncation was far less noticeable than that observed with sucrose on the full length δOR. Moreover, the addition of sucrose further prolonged the decay t_{1/2} for DPDPE in truncated receptors (t_{1/2} of 38 min; two way ANOVA; p<0.02 for interaction; figure 4).

**Agonists but not dual efficacy drugs induce TYR-phosphorylation of δORs.** The fact that the time course of ERK activation by DPDPE was only modestly prolonged by removal of the C-terminus suggests that there could be a complementary mechanism capable of regulating ERK activation by agonists in the absence of C-terminal Ser/Thr residues. In this sense mutation of a TYR residue located proximal to Ser/Thr aminoacids of the C-terminus has been shown to attenuate agonist-induced internalization and down-regulation of the receptor (26). Thus, it was deemed of interest to determine whether δORs could be differentially phosphorylated at their tyrosine residues following exposure to agonists and dual efficacy ligands. To do so, cells expressing full-length receptors were incubated for 30 min either with DPDPE or TICP, receptors
immunopurified and separated by SDS-PAGE. Immunoblots with antibodies that recognize phosphorylated TYR residues showed that TICP and DPDPE distinctively modified phosphor-TYR contents of δORs (figure 5A). While 30 min incubation with TICP (1 μM) produced no significant change, DPDPE induced an increase in immunoreactivity for phospho-TYR in the band corresponding to the mature receptor (figure 5A). Furthermore, the introduction of Src inhibitor PP2 (20 μM) prior to exposure to DPDPE prevented the increase in phospho-TYR content (figure 5B), indicating that a non-receptor TYR-kinase of the Src family was involved in agonist-induced TYR-phosphorylation of δORs. In order to determine whether failure of TICP to induce TYR-phosphorylation of the receptor was due to its incapacity to stimulate Src, cells overexpressing the kinase were exposed either to DPDPE or TICP and changes in Src activity assessed by immunoblot. Figure 5C shows that both, the agonist and the dual efficacy ligand increased immunoreactivity for the active form of Src, confirming that the observed difference in tyrosine phosphorylation of δORs was not related to diminished capacity of TICP to stimulate Src.

The goal of the next series of experiments was to assess whether Src could differentially regulate ERK responses generated by DPDPE or TICP. To accomplish this, cells were pretreated with increasing concentrations of Src inhibitor PP2 and ERK stimulation was assessed following exposure to each of the two δOR ligands. It was found that concentrations of 20-40 μM PP2 had opposite effects on ERK activation by the dual efficacy ligand and the agonist. While the effect of TICP was blocked (figure 6A) that of DPDPE was enhanced, and it was only at a concentration of 80 μM that PP2 interfered with ERK
activation by DPDPE (figure 6B). The inhibitory effect of low, specific, concentrations of PP2 on the response to TICP is compatible with the notion that this drug induces ERK activation in Src-dependent manner. On the other hand, the higher non-specific concentrations needed to block the effect of DPDPE do not allow to conclusively implicate Src as an intermediate in agonist-induced ERK stimulation. So as to overcome this problem of specificity, ERK activation by DPDPE was re-assessed transfecting cells with increasing concentrations of a kinase-impaired Src mutant (K296R/Y528F). This procedure generated a similar biphasic pattern as described for PP2, with low levels of the mutant (0.25-0.5 µg DNA) enhancing DPDPE responses and higher levels (3µg DNA) inhibiting ERK activation by the agonist. The observed inhibition of DPDPE responses by high levels of inactive Src confirms the idea that activity of this kinase is necessary for ERK activation by agonists and is consistent with previous reports showing that pharmacological inhibition of Src interfered with agonist-induced ERK activation (27).

On the other hand, the fact that low levels of mutant Src or modest concentrations of PP2 enhanced ERK activation by DPDPE suggest that Src could also be involved in the negative regulation of agonist signalling. This possibility was assessed by pretreating cells with DPDPE (1 µM; 30 min) in the presence or absence of PP2, and by then assessing the ability of SNC-80 to induce GTPγ35S binding. By itself, PP2 (20 µM; 1hr) enhanced the ability of SNC-80 (100 nM) to promote GTPγ35S binding (Controls: 169 ± 17 fmole/mg; PP2: 209 ± 17 fmole/mg; p < 0.05; figure 7A), but this effect was accompanied by a marked increase in basal nucleotide binding (Controls: 86 ± 9 fmole/mg; PP2: 128 ± 17
f mole/mg; \( p < 0.05 \), figure 7A). Therefore, to avoid any possible confounding, subsequent comparisons of the ability of SNC-80 to promote \( \text{GTP}^3_{\gamma} \) binding were expressed as percentual changes with respect to the corresponding non-stimulated condition under study. As shown in figure 7B, the efficacy of SNC-80 to induce nucleotide binding was greatly reduced following pre-treatment with DPDPE. However, if PP2 was introduced into the incubation medium before DPDPE, the ability of SNC-80 to induce \( \text{GTP}^3_{\gamma} \) binding was not significantly modified, confirming that inhibition of Src activity had a protective effect against agonist-induced desensitization.

Finally, to specifically examine whether Src may have contributed to the distinct kinetic profile of ERK activation by DPDPE and TICP, time course for DPDPE was assessed in presence of 20 \( \mu \text{M} \) PP2. In the absence of Src inhibition, ERK phosphorylation by DPDPE had completely disappeared within the first 30 min of incubation. In contrast, in the presence of PP2, ERK activity at 30 min was still 18 ± 5\% of the maximal response (which corresponds approximately to a 95\% increase above ERK activity in non-stimulated cells; \( p<0.01 \); two way ANOVA; figure 7C). Moreover, PP2 had a stabilizing effect on ERK activation by DPDPE since following 1 hr incubation with the agonist phosphorylation of the kinase was not significantly changed from the value observed 30 min before (16 ± 5\% of maximal).
DISCUSSION

The present study provides new insight into the regulation of agonistic responses induced by "double efficacy ligands". In particular, results presented indicate that when occupied by this type of dual ligands δORs elude phosphorylation and induce prolonged activation of the ERK cascade. These properties contrast with those of highly efficacious agonists, whose activation of the ERK cascade is transient and correlated with heavy phosphorylation and degradation of the receptor.

ERK was activated not only by drugs like SNC-80, DPDPE, TIPP and morphine which also produced agonistic effects in the cyclase pathway, but also by ligands like TICP and ICI174864 that stimulated ERK activity despite displaying inverse agonist behaviour in cAMP accumulation assays (figures 1A and 1B). Inverse agonists are commonly thought to produce their actions via an inactive receptor conformation that precludes Gprotein signalling (12,13). However, it is improbable that activation of the ERK cascade by TICP or ICI174864 could be due to inhibition of spontaneous Gi/o signalling since the inactivation of Gi/o proteins by PTX failed to produce any effect on ERK activity (figure 1C). Moreover, the fact that PTX abolished ERK activation by TICP indicates that similar to agonists, ERK activation by dual efficacy δOR ligands requires the activation of a Gi/o protein. This property is in marked contrast with dual efficacy ligands previously described for the β_{2AR} whose activation of the ERK cascade was not dependent on Gproteins (11).
The fact that TICP could stimulate Gi/o proteins to activate ERK signalling and simultaneously block Gi/o activity regulating the cyclase pathway may be explained by the fact that δORs are promiscuous receptors, capable of interacting with more than one Gi/o protein subtype (28,29). Hence, it is possible for ligands like TICP or ICI174864 to simultaneously display opposite agonist and inverse agonist behaviours in ERK and cAMP cascades by respectively stabilizing a conformation of the receptor that activates one Gi/o subtype but inactivates another. In contrast, classical agonists would stabilize a conformation that activates Gi/o proteins responsible for the regulation of both pathways.

Consistent with the idea that classical agonists and dual efficacy ligands may stabilize δORs in different conformations is the observation that the rank order of efficacy with which TICP, TIPP and morphine modified cyclase signalling was reversed with respect to their efficacies to promote ERK activation (figures 1A and 1B). In fact, reversal in rank order of efficacy (or potency) for ligands that regulate more than one signalling cascade via the same receptor is considered as one of the most compelling proofs in favour of a model of ligand-specific active receptor states (30,31). In particular, the observed swap in position between TICP on the one hand and TIPP and morphine on the other make it possible to conclude that the conformation via which the dual efficacy ligand stimulated ERK signalling is different from the one(s) involved in ERK stimulation by the two partial agonists.

The observations that receptors stabilized by DPDPE but not those occupied by TICP incorporated [$^{32}$P] (figure 3A) and were a target for the tyrosine
kinase Src (figure 5A and figure 5B) further supports the idea that δORs may exist in ligand-specific conformations, and points to the fact that these different receptor states have distinct desensitization properties. The response observed for DPDPE is in keeping with previous reports showing that highly efficacious agonists promote δOR phosphorylation by GRKs and tyrosine kinases (23,25,27,32,33). On the other hand, TICP resembles morphine in its ability to induce a receptor conformation that is poorly phosphorylated by receptor kinases (32). Though the failure of morphine to trigger phosphorylation of different opioid receptors has been frequently attributed to its stabilization of a receptor conformation that differs from the one stabilized by more efficacious agonists (34), there is some controversy as to whether lack of receptor phosphorylation is not just the reflection of the low efficacy of the drug (35,36). Given the fact that DPDPE and TICP induced similar levels of Src activity (figure 5C) differential tyrosine phosphorylation of δORs by the two ligands (figure 5A) cannot be attributed to drug efficacy, further pointing to the existence of distinct conformations that are differentially recognized as Src substrates. At the same time, similar magnitude of Src activation by TICP and DPDPE poses the question as to why do they differ in their ability to stimulate the MAPK (figure 1B). A possible explanation to this observation would be that DPDPE activates ERK via more than one pathway while TICP would only depend on Src.

Phosphorylation of Ser/Thr residues in the C-tail of δORs is a major regulatory event that triggers the internalization (22,33) and desensitization (22,23) of the full length receptor. Hence, it was reasoned that if the differential
phosphorylation of these residues by TICP and DPDPE contributed to their distinct kinetics of ERK activation, removal of the C-terminus or interference with the process of internalization of the full-length receptor should convey ERK activation by DPDPE some of the characteristics of the TICP response. Both of these interventions resulted in prolonged ERK stimulation by DPDPE, but only inhibition of internalization by sucrose prolonged the decay $t_{1/2}$ of ERK activity to values within the range observed for dual efficacy ligands. Furthermore, the discrete increase in decay $t_{1/2}$ associated with the effect of DPDPE in the truncated δOR could be further prolonged by sucrose, indicating that the time course of ERK responses generated by the truncated mutant was still dependent on internalization. The latter observation, that is consistent with previous studies showing that in HEK293 cells this truncated mutant internalizes as the wild-type (37), stresses the determinant role played by receptor sequestration in the kinetics of ERK stimulation by agonists. On the other hand, the prolonged decay $t_{1/2}$ associated with the stimulation of ERK by dual efficacy ligands (figure 2B) is consistent with previous results showing that 30 min exposure to ICI174864 did not change the total amount of δORs present at the cell membrane (20).

Though inhibition of internalization slowed down the decay of ERK activity induced by the agonist, the response never attained the characteristic sustained profile observed for ERK activation by TICP (figure 2A). This incapacity to recreate the complete “phenotype” of TICP stimulation, together with the fact that DPDPE produced a very transient activation of ERK in the truncated mutant
suggested that mechanisms different from phosphorylation of C-terminus, could also contribute to the distinct kinetics of ERK stimulation by DPDPE and TICP.

Since only DPDPE stabilized δORs in a conformation that was recognized by Src, and given that tyrosine residues have been implicated in the regulation of δOR signalling (26,27), one possibility that was assessed was whether Src could distinctively regulate ERK activation by agonists and dual efficacy ligands. Low concentrations of Src inhibitor PP2 (20-40 μM) blocked ERK activation by TICP but enhanced the response to DPDPE (figure 6A and 6B) revealing that indeed, Src had a distinct effect on ERK responses elicited by the two types of drugs. The ability of low concentrations of PP2 (figure 6B) or very discrete amounts of inactive Src (figure 6C) to increase the magnitude of ERK activation by DPDPE may be interpreted as an indication that Src negatively controls δOR responsiveness to agonists, an assumption that was confirmed in GTPγS binding assays, where PP2 was found to protect against agonist-induced desensitization. On the other hand, the fact that ERK activation by TICP was blocked at all levels of Src inhibition (figure 6A) not only indicates that this non-receptor tyrosine kinase is an intermediate in ERK stimulation by the dual efficacy ligand, but also argues against a regulatory role of Src in the effects of these drugs. The observation that DPDE-dependent activation of ERK was blocked by high concentrations of PP2 (figure 6B) or by the transfection of high quantities of inactive Src indicate that agonists too rely on Src for the stimulation of the MAPK. However, this effect is not apparent at the same level of Src
inhibition at which the effect of TICP is blocked, due to the additional regulatory
effect of Src on agonist responses.

The interpretation that Src may have contributed to determine the
transient kinetics of ERK activation by DPDPE is supported by the fact that PP2
had a stabilizing effect on ERK stimulation by the agonist. Indeed, though PP2
had no significant effect on the decay $t_{1/2}$ it was shown to prolong DPDPE
responses by preventing complete fading of ERK activity after the first 10 min of
stimulation (figure 7C). The mechanism whereby Src inhibition had this stabilizing
effect on DPDPE responses is not clear. Though Src has been implicated in the
regulation of receptor endocytosis including $\delta$ORs (27,38,39), the fact that the
effect of PP2 had no resemblance to that of sucrose indicates that inhibition of
sequestration is probably not the main mechanism involved. On the other hand,
results from GTP$_{\gamma}^{35}$S binding assays showing that PP2 enhanced agonist-
induced nucleotide binding as well as basal GTP$_{\gamma}^{35}$S binding activity (figure 7A)
suggest that Src may also influence $\delta$OR signalling by reducing $\delta$OR-Gprotein
coupling. It is possible then, that the stabilizing effect of PP2 on DPDPE-
dependent activity could be linked to a better coupling between agonist stabilized
receptors and the corresponding Gprotein.

In conclusion, this study showed that the agonistic responses of dual
efficacy ligands for $\delta$ORs were more sustained and decayed much slower than
those of classical agonists. These kinetics were associated with the distinct
ability of dual efficacy ligands to stabilize $\delta$ORs in an active conformation that
does not trigger the same regulatory mechanisms as classical, highly efficacious agonists.

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LEGEND TO THE FIGURES

Figure 1: Comparison of the efficacy of δOR ligands in adenylyl cyclase and ERK cascades. (A), HEK293s cells stably expressing full length δORs (1-1.5 pmole/mg protein) were treated with saturating concentrations (1 µM) of the indicated ligands and cAMP accumulation assays performed in presence of 25 µM forskolin as detailed in Experimental Procedures. Statistical significance of drug effects on cAMP production was established by comparing the amount of cAMP counts obtained in the presence of each ligand to cAMP counts produced in the control situation (all drugs differed from control as determined using one way ANOVA and Dunnett’s post hoc test; p<0.05; not shown). Drug effects as they appear in the figure are expressed as % change with respect to total amount of cAMP produced in absence of ligand (% change in cAMP accumulation = ([cAMP_ligand - cAMP_no_ligand] / cAMP_no_ligand) × 100) and correspond to mean ± SEM of at least 9 experiments carried out in triplicates. Differences among drug effects were established by comparing % changes induced by different ligands using one way ANOVA and Tukey’s post-hoc test. (B) HEK293s cells stably expressing full length δORs were serum starved (16 hrs) prior to exposure to saturating concentrations (1 µM) of the indicated ligands for 5 min following which ERK signalling was assessed by immunoblot. Band immunoreactivity was quantified using MCID to measure optical density and ERK1/2 phosphorylation was normalized to the amount of protein loaded per lane, by expressing the data as a ratio of phosphoERK1/2 over total ERK1/2 optic density. The statistical significance of drug effects was established by comparing ratio obtained in the presence of each ligand to the ratio obtained in
the basal condition (all drugs except naloxone differed from basal using one way ANOVA and post hoc Dunnett’s test; p<0.05; not shown). Drug effects as they appear in the figure were expressed as % of the basal ratio (% of basal = \(\frac{[pERK/totalERK_{ligand}]/pERK/totalERK_{no\ ligand}}{x100}\)), and represent mean ± SEM of at least 7 experiments. Statistical differences that appear in the figure were established using one way ANOVA followed by Tukey’s post-hoc test. Immunoblots above the histogram bars correspond to representative examples of results obtained for each of the indicated drugs. (C) Effect of PTX (100 ng/ml for 16 hs) on DPDPE or TICP-induced ERK phosphorylation. Cells were serum-starved and exposed or not to PTX prior to treatment with either DPDPE or TICP (1 µM; 5 min). Results, expressed as in B, correspond to mean ± SEM of 4 independent experiments. Statistical difference between drug effects obtained in presence and absence of PTX was determined using Student’s t test and appear in the figure.

**Figure 2: δOR ligands differ in their kinetics of ERK activation.**

HEK293s cells stably expressing full length δORs were serum starved (16 hs) prior to exposure to saturating concentrations (1 µM) of different ligands for the indicated times, and phosphoERK1/2 immunoreactivity measured as before. (A) Time course of ERK activation by different ligands. Results represent mean ± SEM of at least 3 experiments and are expressed as % of basal as in 1B. (B) Decay of ERK responses represented in A. Results are expressed as % of maximal response observed with each drug. Curves were fit to one phase exponential decay using GraphPad Prism 2.01 and compared by to way ANOVA to generate statistics discussed in the text. Calculated t\(_{1/2}\) values appear in the
figure. (C) Representative immunoblots of phosphorylated ERK1/2 bands obtained with indicated ligands at different time points

**Figure 3:** DPDPE and TICP differ in their ability to induce receptor phosphorylation and down-regulation. (A) Effect of DPDPE and TICP on the phosphorylation of full-length δORs. HEK293s cells stably expressing δORs (1 – 1.5 pm/mg protein) were metabolically labelled with $^{32}$P and exposed for 30 min (1 μM) to either of the drugs. The receptor was purified by immunoprecipitation using anti-FLAG M2 antibody resin and subjected to electrophoresis on 10% SDS-PAGE. Representative autoradiograms and corresponding western blots for different conditions are shown on the right panel. Band immunoreactivity was quantified using MCID, and receptor phosphorylation was normalized to the amount of protein loaded in each lane by expressing data as the phospho/protein ratio calculated from densitometric analysis of the autoradiogram and its corresponding western blot. Results represent mean ± SEM of at least 5 experiments and are expressed as % of the phospho/protein ratio obtained in the basal condition (% of basal = \{[phospho-protein ratio\_ligand]/[phospho-protein ratio\_basal]\}x100). Statistical significance of differences between drugs was established using Student’s t test, and the result of the analysis appears in the figure. (B) Effects of DPDPE and TICP on receptor protein contents. Immunoblots of flag-tagged δORS were performed on crude membrane preparations derived from cells that had been treated or not for 2 hrs with 1 μM of the indicated drug. Identical amounts of membrane proteins were loaded for each condition (100 μg/well) and the total amount of receptor protein was estimated by densitometric analysis of the mature monomeric band. Results represent mean ± SEM of 4 experiments
and are expressed as % of densitometric values obtained in basal conditions (% of basal = [(densitometric values ligand)/(densitometric values basal)]x100). Statistical significance of differences between drugs was established using Student’s t test, and the result of the analysis appears in the figure.

**Figure 4:** ERK activation by DPDPE may be modified to yield a prolonged decay $t_{1/2}$ typical of TICP. HEK293 cells stably expressing wild type or truncated δORs (0.5 - 1 pmole/mg protein) were serum starved (16 hrs) and the day of the experiment were incubated or not with 0.4M sucrose for 4 hrs, followed by exposure to saturating concentrations (1 µM) of DPDPE for the indicated times. PhosphoERK1/2 immunoreactivity was measured as described in previous figures. Results are expressed as % of maximal response and correspond to mean ± SEM of at least 7 experiments. Curves were fit to one phase exponential decay using GraphPad Prism 2.01 and calculated $t_{1/2}$ values appear in the figure to the right of representative immunoblots obtained for each condition. Statistical comparison among curves was done using two way ANOVA and p values are discussed in the text.

**Figure 5:** Tyrosine kinase Src phosphorylates δORs occupied by DPDPE but not by TICP. (A) HEK293s cells stably expressing flag-tagged δORs were exposed for 30 min to either of the drugs, and the receptor purified as described before. Tyrosine phosphorylation was assessed by immunoblot by calculating the ratio between phospho-TYR immunoreactivity of the mature species (shown on the right panel) and FLAG-immunoreactivity of the same band (not shown). Results represent mean ± SEM of at least 5 experiments and are expressed as % of the ratio between phosphoTYR immunoreactivity and FLAG.
immunoreactivity obtained in basals. Statistical significance of differences between the two drugs was established using Student’s $t$ test, and the result of the analysis appears in the figure. (B) 20 $\mu$M PP2 or vehicle (DMSO 0.01%) were introduced to cell cultures 1 hr before the experiment followed by stimulation by DPDPE (1 $\mu$M; 30 min) and assessment of pTYR immunoreactivity in purified receptors. Results are expressed as % of the of phosphoTYR/FLAG ratio obtained in basals of the corresponding control or PP2 condition, and represent mean $\pm$ SEM of at least 5 experiments. Student’s $t$ test was used to compare phosphorylation induced in presence and absence of the Src blocker. The result of the analysis appears in the figure. (C) Effects of DPDPE and TICP on Src activity. Cells were serum starved overnight (16 hs) and then exposed to the indicated drug (1 $\mu$M) for 5 min. Src activation was assessed as described in Experimental Procedures by measuring phospho-TYR$^{416}$ immunoreactivity. Src phosphorylation was normalized according to the amount of protein present in each sample by expressing the data as a ratio of phosphoTYR$^{416}$ over total Src immunoreactivity. Results represent mean $\pm$ SEM of seven experiments, and are expressed as pSrc/totalSrc ratio. Statistical significance of drug effects was determined using one way ANOVA followed by Dunnett’s post hoc test to compare drugs to basal. ** $p < 0.01$.

**Figure 6:** Src inhibitor PP2 produced opposite effects on ERK activation by DPDPE and TICP. HEK293s cells stably expressing full-length $\delta$ORs (0.5-1 pm/mg protein) were serum starved overnight (16 hrs) and the day of the experiment exposed to indicated concentration of PP2 or vehicle (DMSO, 0.01%) 1 hr before addition of TICP (A) or DPDPE (B) (1 $\mu$M; 5 min).
PhosphoERK1/2 immunoreactivity was measured as described before and expressed as % change of phosphoERK/ERK total ratio obtained in the absence of δOR ligand. Results represent mean ± SEM of at least 4 experiments. Statistical significance of the effect of PP2 on DPDPE and TICP responses was established using one way ANOVA followed by Dunnett’s post hoc test to compare ERK activation in the absence of PP2 to activation induced in presence of increasing concentrations of the Src inhibitor. * p < 0.05. (C) HEK293s cells stably expressing full length δORs were transiently transfected with the indicated concentrations of DNA encoding for kinase-impaired Src mutant (K296R/Y528F) Results and statistical analysis as in (B). On top of representative immunoblots for ERK activity are the blots corresponding to the total amount of Src immunoreactivity present in cell lysates following transfection with the indicated quantities of Src K296R/Y528F.

**Figure 7:** PP2 protects against desensitization by DPDPE and modifies the time course of ERK activation by this agonist. (A). Cells were exposed to PP2 (20 µM) or vehicle (DMSO, 0.01%) for 1 hr following which membranes were prepared, and used to assess GTPγ[35S] binding. Results are expressed as fmole of GTPγ[35S] bound/mg of membrane protein and represent the mean ± SEM of 4 independent experiments. Statistical differences between different conditions were established using two way ANOVA * p < 0.05; ** p < 0.01. (B) Cells were treated or not with PP2 as in (A) before adding DPDPE (1 µM) for additional 30 min. Membranes where then prepared and SNC-80 effects (100 nM) assessed in naïve membranes and in membranes obtained from desensitized cells. Results are expressed as % change with respect to basal
values obtained in each condition (% change with respect to basal = \{[\text{GTP}\gamma^{35}\text{S}] \text{bound}_{\text{ligand}} - \text{GTP}\gamma^{35}\text{S}] \text{bound}_{\text{no ligand}}]/\text{GTP}\gamma^{35}\text{S}] \text{bound}_{\text{no ligand}}\times100\) and represent the mean ± SEM of 3 independent experiments. Differences in SNC-80 actions among the different conditions were established using two way ANOVA and results appear in the figure. (C) HEK293 cells stably expressing wild type δORs were serum starved (16 hrs), and the day of the experiment exposed to PP2 (20 µM) or vehicle (DMSO, 0.01%) 1 hr before addition of DPDPE (1 µM) for the indicated times. ERK phosphorylation was measured as described previously and results are expressed as % of maximal response. They correspond to the mean ± SEM of at least 4 experiments. Curves were fit to one phase exponential decay using GraphPad Prism 2.01. Calculated $t_{1/2}$ values appear in the figure to the right of representative immunoblots obtained for each condition. Statistical comparison among curves was done using two way ANOVA and p values are discussed in the text.
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Figure 1
Figure 2

A. ERK phosphorylation (% of basal)

B. ERK phosphorylation (% of maximal response)

C. Time (min)

- ▼ - SNC
- ■ - DPDPE
- □ - TICP
- □ - IC17
- ■ - MOR
- □ - TIPP
- □ - ICI17
- □ - MOR

Figure 2
Figure 3

A. 

\[ \delta P \text{ incorporation by } \delta \text{ORs (% basal)} \]

| Basal | TICP | DPDPE |
|-------|------|-------|
| 0     | 100  | 300   |

\[ p < 0.05 \]

B. 

\[ \delta \text{OR protein levels (% basal)} \]

| Basal | TICP | DPDPE |
|-------|------|-------|
| 100   | 100  | 75    |

\[ p < 0.05 \]
Figure 4

WT δOR

Control

Sucrose

basal

5 min

10 min

30 min

1 hr

2 hr

t_{1/2}=2 min

t_{1/2}=12 min

δOR344T

Control

Sucrose

basal

5 min

10 min

30 min

1 hr

2 hr

t_{1/2}=6 min

t_{1/2}=38 min

ERK phosphorylation (% of maximal response)

Time (min)

0

20

40

60

80

100

120

0

20

40

60

80

100

120

Figure 4

WT δOR

δOR344T
A. pTYR contents (% of basal)

- Basal
- TICP
- DPDPE

p<0.01

Mature δOR (≈ 55 KD)

B. pTYR contents (% of basal)

- CTL
- PP2 20 µM

p<0.01

Mature δOR (≈ 55 KD)

C. Src activity (pERK/totalERK ratio)

- Basal
- TICP
- DPDPE

**

Figure 5
A. TICP

ERK phosphorylation (% of basal)

| Treatment | 0 | 50 | 100 | 150 |
|-----------|---|----|-----|-----|
| PP2 20 µM | - | +  | +   | -   |
| PP2 40 µM | - | +  | +   | -   |

B. DPDPE

ERK phosphorylation (% of basal)

| Treatment | 0 | 100 | 200 | 300 |
|-----------|---|-----|-----|-----|
| PP2 20 µM | - | +   | +   | -   |
| PP2 40 µM | - | +   | +   | -   |
| PP2 80 µM | - | +   | +   | -   |

C. DPDPE

ERK phosphorylation (% of basal)

| Treatment | 0 | 100 | 200 | 300 |
|-----------|---|-----|-----|-----|
| Src (K296R/Y528F) 0.5 µg | - | +   | +   | -   |
| Src (K296R/Y528F) 3 µg | - | +   | +   | -   |

Figure 6
Figure 7

A. 

![Graph showing GTPγS binding comparison between CTL and PP2 20 µM treated groups.](image)

B. 

![Graph showing % change in GTPγS binding between CTL and PP2 20 µM treated groups.](image)

C. 

![Graph showing ERK phosphorylation over time.](image)
Internalization and SRC activity regulate the time course of ERK activation by delta opioid receptor ligands
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