Coincident Regulation of PLCβ Signaling by Gq-Coupled and μOpioid Receptors Opposes Opioid-Mediated Antinociception

Gissell A. Sanchez, Emily M. Jutkiewicz, Susan Ingram and Alan V. Smrcka

Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109 (G.A.S., E.M.J., A.V.S.); Department of Neurological Surgery, Oregon Health Sciences University, Portland, OR 97239 (S.I.)
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Corresponding authors:

Alan Smrcka: Department of Pharmacology, University of Michigan Medical School, 1150 W. Medical Center Dr. Ann Arbor, MI 48109, Phone: 734-615-4945
Email: avsmrcka@umich.edu

Susan Ingram: Current Address: Department of Anesthesiology, University of Colorado Anschutz Medical Campus, 12801 E. 17th Ave., Aurora, CO 80045, Phone: 303-724-6628
Email: Susan.ingram@cuanschutz.edu

Emily Jutkiewicz: Department of Pharmacology, University of Michigan Medical School, 1150 W. Medical Center Dr. Ann Arbor, MI 48109: 734-764-8612
Email: ejutkiew@umich.edu

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Non standard abbreviations: MOR, μOpioid Receptor; PLC, phospholipase C; DAG, diacylglycerol; PAG, periaqueductal gray; PKC, protein kinase C; PTX, pertussis toxin; eIPSCs, evoked GABAergic inhibitory postsynaptic currents; myrGαq-CT, myristoylated peptide from the C terminus of Gαq; i.c.v., intracerebroventricular
Abstract

Pain management is an important problem worldwide. The current frontline approach for pain-management is the use of opioid analgesics. The primary analgesic target of opioids is the μ-opioid receptor (MOR). Deletion of phospholipase Cβ3 (PLCβ3), or selective inhibition of Gβγ regulation of PLCβ3, enhances the potency of the antinociceptive effects of morphine suggesting a novel strategy for achieving opioid sparing effects. Here we investigated a potential mechanism for regulation of PLC signaling downstream of MOR in HEK293 cells and found that MOR alone could not stimulate PLC, but rather required a coincident signal from a Gq-coupled receptor. Knockout of PLCβ3, or pharmacological inhibition of its upstream regulators, Gβγ or Gq, ex vivo in periaqueductal gray (PAG) slices increased the potency of the selective MOR agonist DAMGO in inhibiting presynaptic GABA release. Finally, inhibition of Gq-GPCR coupling in mice enhanced the antinociceptive effects of morphine. These data support a model where Gq and Gβγ-dependent signaling cooperatively regulate PLC activation to decrease MOR-dependent antinociceptive potency. Ultimately this could lead to identification of new non-MOR targets that would allow for lower dose utilization of opioid analgesics.

Significance Statement: Previous work demonstrated that deletion of PLCβ3 in mice potentiates MOR-dependent antinociception. How PLCβ3 is regulated downstream of MOR had not been clearly defined. We show that PLC dependent DAG generation is cooperatively regulated by MOR-Gβγ and Gq-coupled receptor signaling through PLCβ3, and that blockade of either Gq-signaling or Gβγ signaling enhances the potency of opioids in ex vivo brain slices and in vivo. These results reveal potential novel strategies for improving opioid analgesic potency and safety.
Introduction

Pain management is an important problem worldwide. The current frontline approach for clinical pain-management is the use of opioid analgesics. While these compounds are highly effective, they come with substantial drawbacks. Prolonged use results in the development of tolerance and physical dependence, which severely limits their use in treatment of chronic pain. Hence, μ-opioid receptor (MOR) agonists produce reinforcing effects and, thus, have abuse liability. Severe respiratory depression as a result of opioid overdose is the major cause of opioid related deaths.

The primary analgesic target of opioids is MOR. MORs are G protein-coupled receptors (GPCRs) that are expressed in both pre- and postsynaptic locations throughout the nervous system and can activate many different signaling pathways. As GPCRs, MORs activate G proteins and are desensitized and/or internalized through recruitment of β-arrestins (Bohn et al., 2000; Kliewer et al., 2020; Shenoy and Lefkowitz, 2011). One approach to improving opioid analgesics has been to find strategies that improve potency and efficacy of opioid agonists while limiting MOR desensitization and internalization (Kandasamy et al., 2021; Schmid et al., 2017). Work by our laboratory has identified phospholipase C signaling as a process that limits the antinociceptive effects of MOR agonists (Xie et al., 1999) and that pharmacological attenuation or blockade of activation of this pathway enhances the potency of opioid analgesics in mice (Bianchi et al., 2009; Bonacci et al., 2006; Campbell and Smrcka, 2018; Hoot et al., 2013; Mathews et al., 2008; Smrcka et al., 2008).
Phospholipase C is the upstream enzyme responsible for PKC activation, and this pathway can be activated by GPCRs. PKC has been implicated in adaptations involved in morphine tolerance through alterations in MOR signaling (Bailey et al., 2009a; Bailey et al., 2009b; Gabra et al., 2008; Ingram and Traynor, 2009; Seksiri et al., 2015), but the mechanisms for upstream regulation of PKC activation in the opioid system have not been examined. Our laboratory has been interested in understanding the mechanisms for activation of phospholipase Cβ by opioid receptors. Since PLCβ3 is activated by Gβγ subunits released from Gi-coupled receptors (Li et al., 2000; Smrcka, 2008; Smrcka and Sternweis, 1993) we hypothesized that PLCβ3 would be activated downstream of MOR via a Gβγ-dependent signal transduction pathway. Indeed, inhibition of Gβγ signaling with M119 or gallein enhanced the antinociceptive effects of morphine in wild type (wt) mice but not in PLCβ3/− mice (Bonacci et al., 2006).

Here we further explored potential mechanisms for MOR-dependent PLC activation and the relevance of these mechanisms to presynaptic opioid-dependent inhibition of neurotransmitter (GABA) release, and to antinociception in mice. In vitro, MOR activation alone did not stimulate PLC signaling, but rather, required coincident activation of a Gq coupled receptor, consistent with the previously described property of PLCβ3 as a coincidence detector for Gαq and Gi signaling (Philip et al., 2010). In PAG brain slices, inhibition of either Gβγ or Gαq signaling through PLCβ3 enhanced opioid-dependent inhibition of neurotransmitter (GABA) release. Finally, blockade of either Gαq or Gβγ in mice enhanced morphine-dependent antinociception in mice. These data show that MOR signaling is inhibited in presynaptic
terminals through a PLCβ3-dependent mechanism that utilizes coincident inputs from Gq-coupled receptors and MOR to modulate antinociception.
Materials and Methods

Reagents

Gallein (Tocris, Minneapolis MN), myrGq-CT inhibitor and scrambled peptide (GenScript USA Inc., Piscataway NJ), YM-254890 (MedChemExpress MCE, Monmouth Junction NJ), [D-Ala², N-Me-Phe⁴, Gly⁵-ol]-Enkephalin acetate salt (DAMGO) (Sigma-Aldrich, St. Louis MO), morphine (Henry Schein, Melville NY), carbachol (Montana Molecular, Bozeman MT), Adenosine-5’ triphosphate (ATP) (Sigma-Aldrich, St. Louis MO) Ser-Phe-Leu-Leu-Arg-Asn-amide trifluoroacetate salt (PAR1-AP) (Sigma-Aldrich, St. Louis MO), Pertussis toxin (PTX) (Sigma-Aldrich, St. Louis MO).

Animals

All animal procedures were conducted at the University of Michigan according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of Institutional Animal Care and Use Committee (IACUC) at the University of Michigan. Wild type C57BL/6 mice purchased from Envigo (Indianapolis, IN), and from an in-house breeding colony were used for these studies. PLCβ3-/- mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were bred on a C57BL/6 background. Mice were housed with a maximum of five animals per cage in clear polypropylene cages with corn cob bedding and Nestlets as enrichment. Animals were housed in specific pathogen–free rooms maintained between 68°F and 72°F and between 30% and 70% humidity and a 12-hour light/dark cycle (lights on at 7 A.M. and lights off at 7 P.M.) light/dark cycle with free access to food (Lab Diets, St. Louis, MO; 5L0D) and water. Experiments were conducted in the housing room during the light cycle. All mice
were used between 8 and 15 weeks of age and weighed 19–26 g. A combination of male and female mice was used in gallein experiments, but male mice were used for myrGq-inhibitor experiments. Mice were tested only once with a single dose of drug, and all analyses are between-subject.

Electrophysiology studies were done at Oregon Health & Science University (OHSU). These studies used male and female wildtype C57BL/6 mice and PLCβ3−/− mice and wildtype littermates. Mice were group housed with unlimited access to food and water. Lights were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.). Mice were sacrificed and cellular recordings were conducted during the light phase of this cycle. The Institutional Animal Care and Use Committee at Oregon Health & Science University approved all experimental procedures. Experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

*Maintenance of HEK293 cell culture and Stable MOR-FLAG HEK293 cell culture*

Human Embryonic Kidney (HEK293) cells were grown in DMEM medium (Corning, Corning NY) with 4.5 g/L glucose, L-glutamine and sodium pyruvate with 100U penicillin/streptomycin and research grade 10% Fetal Bovine Serum (FBS) (ThermoFisher scientific, Pittsburgh, PA). Cells were maintained in a 5% CO2 humid atmosphere at 37°C. HEK293 cells stably expressing MOR-FLAG tagged receptor were obtained from the Puthenveedu laboratory at the University of Michigan and were maintained with addition of 50mg/mL Geneticin(G418 Sulfate) (ThermoFisher Scientific, Rochester NY).
Transduction of diacylglycerol (DAG) fluorescent biosensor and M1 muscarinic acetylcholine receptor in HEK293 cells

HEK293 cells were used for these studies. Green fluorescent up DAG assay kit (#U0300G) and CAAX-Green downward DAG kit (#D0331G) were purchased through Montana Molecular (Bozeman, MT). HEK293 cells were incubated (8-24hrs) with a viral transduction reaction including DAG Sensor BacMam, sodium butyrate, and M1 muscarinic acetylcholine BacMam (receptor control was not added for endogenous Gq-coupled receptor experiments). A 96-well black plate with transparent bottom (Corning, Corning NY) was used (50µL of 500,000 cells/mL per well) with BacMam transduction reaction (100µL per well).

Activation of DAG sensor and collection of data

Assays were conducted with a Hamamatsu μCell FDSS plate reader. Agonists were loaded into a 96-well plastic conical bottom source plate (Thermo Fisher Scientific, Rochester NY) prior to transfer by the instrument. Before placing the cells in the plate reader, transduction media was exchanged with 120µL of warmed Gibco Dulbecco’s Phosphate Buffered Saline (DPBS) with Ca\(^{2+}\) and Mg\(^{2+}\) (Life Technologies Co, Grand Island, NY). Cells at 80% confluency were kept in the dark inside the plate reader and incubated in DPBS for 10m before agonist was added. Baseline fluorescence at 540 nM was measured each second for 30s followed by 15 µL of agonist added simultaneously to each well of the plate. Fluorescence intensity measurements were aquired every second for 230s. Data are normalized to baseline fluorescence (ΔF/Fo=1) in each well and...
the change in fluorescence in each well relative to baseline is monitored over time. Each condition was tested in 3-4 wells in at least 3 different sets of experiments.

**Transient transfection of MOR in HEK293 cells**

HEK293 cells were transiently transfected with flag-MOR cDNA using Lipofectamine 2000 in a 10 cm plate at 70% confluency one day before BacMam transduction. After 24 h cells were transferred to a 96 well plate at 80% confluency and incubated for 24h before the assay.

**Immunocytochemistry (ICC)**

Transfected cells and stable MOR-FLAG cells were plated in a 20 mm glass bottom cell culture dish (Wuxi NEST Biotechnology, China). Cells were allowed to adhere and then fixed with 4% PFA for 15 min and then incubated with 10% normal goat serum in PBS containing 0.1% Triton X100 (PBS-T) for 1 hr at room temperature. Anti-FLAG primary antibody DYKDDDK tag polyclonal antibody (Invitrogen, Rockford IL) was incubated at a dilution of 1:1000 in 2% goat serum in PBS-T overnight at 4C°. After three washes with PBS-T, cells were incubated with secondary antibody goat anti-rabbit Alexa Fluor 568 (Life Technologies, Carlsbad CA) at a dilution of 1:1000 in PBS-T for 1.5 hr at room temperature. After three washes with PBS-T cells were imaged using confocal microscopy at 63 x.

**Electrophysiological recordings.** Mice (postnatal day >25) were anesthetized with isoflurane, brains were removed, and brain slices containing the vlPAG were cut with a vibratome (180–
220 µm thick) in sucrose cutting buffer containing the following: 75 mM NaCl, 2.5 mM KCl, 0.1 mM CaCl₂, 6 mM MgSO₄, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, 2.5 mM dextrose, 50 mM sucrose and placed in a holding chamber with artificial cerebral spinal fluid (ACSF) containing the following: 126 mM NaCl, 21.4 mM NaHCO₃, 11.1 mM dextrose, 2.5 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgCl₂, and 1.2 mM NaH₂PO₄, pH 7.35, and equilibrated with 95% O₂/5% CO₂ until moved into a recording chamber. In experiments using gallein and myrGαq-CT inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitor before recording. Recordings were made with electrodes pulled to 2–4 MOhm resistance with an internal solution consisting of the following: 140 mM CsCl, 10 mM HEPES, 10 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.3 mM CaCl₂, 4 mM MgATP, and 3 mM NaGTP, pH 7.4. Junction potentials of 5 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Neurons were voltage-clamped at -70 mV. Miniature inhibitory post-synaptic currents (mIPSCs) were collected in the presence of tetrodotoxin (500 nM). Evoked inhibitory postsynaptic currents (eIPSCs) were stimulated with bipolar stimulating electrodes placed ~50-100 µm away from recording site. A paired-pulse stimulation paradigm was used (two pulses (2 ms) at 50-100 ms intervals) and paired-pulse ratios (PPRs= Pulse 2/Pulse 1) were determined. Data were collected with Axopatch 200B microelectrode amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph data acquisition software and analyzed using AxoGraph (Axograph Scientific).

*Intracerebroventricular (i.c.v.) pre-treatment injection*
I.c.v. injection was done after baseline withdrawal latencies were taken and before morphine injection. Hamilton syringes of 10µL with a 26 G (catalog #7804-03; Point #4, 12° bevel) needle were used, with a custom-made stopper that allowed 4mm of the needle to enter the skull. Mice were anesthetized using isoflurane until they were no longer responsive to noxious stimuli and breathing slowed down to one inhale per second. Injection is free handed utilizing the ears and eyes for orientation to target the lateral ventricles of the brain. The needle was inserted through the skull using published methods (Laursen and Belknap, 1986). Immediately after the experimental procedure, mice are euthanized to confirm injection site. When the needle enters the skull, 3µL of solution is injected into the ventricles, then after 30 seconds the needle is carefully removed. Mice were placed back in their home cage to recover from isoflurane anesthesia. Following recovery from anesthesia, mice were able to move and groom in a normal manner. Gallein was administered 30 min prior to morphine; MyrGαq-CT peptide was administered 60 min prior to morphine.

*Warm Water Tail Withdrawal*

Withdrawal latencies were determined by briefly placing a mouse into a cylindrical plastic restrainer and immersing 2–3 cm of the tail tip into a water bath maintained at 55°C. The latency to tail withdrawal or rapidly flicking the tail back and forth was recorded with a maximum cut-off time of 15 seconds to prevent tissue damage; baseline latencies, 2-3s for 55°C, were consistent for each assay. Mice were briefly habituated to handling and restrainer, injected with saline (i.p.), and 30 min later withdrawal latencies were recorded (BL, baseline withdrawal latency). Thirty min after i.c.v. injections, withdrawal latencies were recorded, and
then mice were injected with 3.2 mg/kg morphine (i.p.). Withdrawal latencies were recorded 30, 60, 90, and 120 min post-morphine injection.

**Data analysis**

For HEK cell experiments, mean and standard error of the mean (SEM) were calculated for each data set. For animal experiments, mean and standard deviation (SD) were calculated for each data set. Where indicated, unpaired student’s two-tailed t-tests, one-way ANOVAs with Tukey’s or Dunnett’s multiple comparisons tests, or two-way ANOVAs with Sidak’s multiple comparisons tests were conducted for all analyses involving the comparison of group means as indicated in the figure legends. Concentration dependent curves were fitted using non-linear regression. AUC calculations are after baseline subtraction (vehicle). % Max Response was calculated as a percentage of the maximal response to PDBu of separately analyzed wells in the same plate after vehicle subtraction. All analyses were performed using Prism 9 (Graphpad, San Diego, CA). Statistical significance was accepted at P < 0.05.

Each electrophysiological recording from a single neuron is treated as an individual observation because the vlPAG contains heterogeneous cell populations; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists, and peak drug effects were measured as an increase in current from the average of baseline and washout or the presence of antagonists. Differences between groups were assessed using Student’s t-test or ANOVA when appropriate (significance is denoted as *p < 0.05). All data are expressed as mean and standard error of the mean (SEM) except figure 6.
where data are mean and standard deviation (SD). Data were analyzed with Prism 9 (GraphPad Software).
Results

**MOR does not activate PLCβ in HEK293 cells.**

To measure activation of PLC in cells, HEK 293 cells were transduced with a protein kinase C-based fluorescent reporter that detects PLC-dependent diacylglycerol (DAG) production as an increase in overall fluorescence intensity (Green UP DAG assay, Montana Molecular, (Tewson et al., 2016). The sensor was expressed efficiently in the cytoplasm of the cells (Fig. 1A). Addition of the phorbol ester Pbd as a positive control that binds directly to C1 domain of the sensor produced a strong sustained increase in fluorescence (Fig. 1A). To measure GPCR-dependent PLC activation we transduced cells with the Gq-coupled M1 muscarinic acetylcholine receptor and stimulated cells with the muscarinic agonist carbachol. After establishing baseline fluorescence, addition of carbachol produced a strong time-dependent increase in fluorescence (Fig 1B). To measure MOR-dependent activation of PLC activity, cells transduced with the reporter were transfected with N-terminally flag tagged MOR. MOR was detected at the plasma membrane (PM) by immunocytochemistry in the majority of the cells (Fig 1C). Surprisingly, addition of saturating concentrations of DAMGO or morphine produced no detectable PLC activation (Fig. 1D).

We considered the possibility that the DAG sensor needs to be targeted to the PM to detect local PLC activation and DAG production (Halls et al., 2016). To target the DAG sensor to the PM, a PM targeting CAAX sequence was fused to the C-terminus of a DAG sensor that responds with decreasing fluorescence intensity upon DAG binding (CAAX-Green Down DAG assay, Montana Molecular). Localization to the PM was confirmed by monitoring GFP fluorescence of the sensor transduced into HEK293 cells (Fig 1E). Activation of transfected MOR
with either DAMGO or morphine produced no detectable change in reporter fluorescence, while transduced M1 muscarinic receptors produced robust DAG accumulation (Fig. 1F).

Synergistic stimulation of PLC activity by muscarinic Gq-coupled receptors and MOR in HEK293 cells

Synergistic activation of PLCβ3 has been implicated in cross-talk between Gi and Gq coupled receptor-dependent activation of PI hydrolysis in cells (Pfeil et al., 2020; Rebres et al., 2011). To test if low level stimulation of Gαq signaling could reveal MOR-dependent stimulation of DAG production downstream of PLC activity, we co-expressed the M1-muscarinic receptor with MOR in HEK293 cells and stimulated with either a subsaturating concentration (100 nM) of carbachol alone, or carbachol with saturating concentrations of either DAMGO (100 nM) or morphine (1 μM). As before, treatment of cells with either DAMGO or morphine did not activate the DAG reporter while 100 nM carbachol led to a small increase in DAG production. When cells were co-stimulated with DAMGO and carbachol, or morphine and carbachol together, DAG production was strongly increased relative to the signal with carbachol alone (Fig 2A). Traces were quantified and data plotted in Figure 2B. Since DAMGO and morphine gave no response on their own, anything greater than the carbachol alone response is greater than additive and thus synergistic. To confirm that DAMGO and Morphine components of the synergistic responses were Gi-dependent, cells were pretreated with pertussis toxin (PTX), followed by addition of agonists. Treatment with PTX eliminated the DAMGO or morphine-dependent components of the response without affecting the response to carbachol (Fig 2 C, D and E).
Cooperation of MOR with Gq-coupled receptors in PLC activation is generalizable.

To explore synergy with endogenous Gαq-coupled receptors we used HEK293 cells with stable expression of MOR but without transfected M1-muscarinic receptor. HEK293 cells have been reported to endogenously express the M3 muscarinic receptor (Atwood et al., 2011). Stimulation with saturating concentrations of carbachol (50 μM) gave a barely detectable signal likely due to low level endogenous expression of the M3 receptor (Fig. 3A and B). However, co-stimulation with carbachol and DAMGO resulted in strong PLC activation (Fig 3A and B). We performed concentration response analysis for both DAMGO and morphine in the presence of a fixed 50 μM concentration of carbachol (a representative experiment for DAMGO is shown in figure 3C). EC50s for DAMGO and morphine were calculated from multiple experiments (Fig 3D). HEK293 cells have also been reported to endogenously express other Gq coupled receptors including P2Y11 and P2Y12 purinergic receptors and the protease activated receptor F2R (PAR-1) (Atwood et al., 2011). Stimulation of HEK293 cells with either the purinergic agonist ATP (100 μM) or the PAR-1 agonist PAR-1 activating peptide (PAR1AP)(3 μM) resulted in very low levels of detectable DAG production that was strongly enhanced in the presence of DAMGO (Fig 3, E-H). Both PAR and purinergic receptors can also couple to other G proteins, thus it remains possible that other G proteins, including Gi and G12/13, could be involved in this process. Nevertheless, the most straightforward interpretation of the data, consistent with prior literature, is a model where low level Gq activation, regardless of the nature of the activating receptor, synergizes with MOR to reveal MOR-dependent PLC activation in HEK293 cells.
DAMGO-mediated inhibition of GABA release in the PAG is greater in PLCβ3 KO mice or with blockade of Gαq or Gβγsignaling.

To examine the role of synergistic PLCβ activation via Gq and MOR-dependent Gi/Gβγsignaling in a physiological setting, we blocked each of these components individually in PAG brain slices. Inhibition of GABA release by presynaptic MORs in the PAG produces antinociception in the descending pain pathway (Lau and Vaughan, 2014; Morgan et al., 2020; Vaughan et al., 1997). Since the only PLCβ isoform that is synergistically regulated by Gαq and Gi/Gβγis PLCβ3, and since MOR-mediated antinociception is enhanced in PLCβ3−/− mice, we first tested whether MOR-dependent inhibition of GABA release was potentiated in vIPAG slices from these mice. Evoked GABAergic inhibitory postsynaptic currents (eIPSCs) were isolated in the presence of NBQX, an inhibitor of AMPA glutamate receptor-mediated synaptic currents (Fig. 4A). The concentration-response curve for DAMGO-mediated inhibition of the GABAergic eIPSCs was shifted to the left in recordings from PLCβ3−/− slices compared to recordings from PLCβ3+/+ slices (Fig. 4B). Thus, lower concentrations of DAMGO were sufficient to inhibit GABAergic eIPSCs when PLCβ3 was deleted.

To test whether inhibition of Gβγsignaling could potentiate MOR-dependent inhibition of GABA release, we incubated slices from WT mice with gallein (Fig. 6C). Gallein is an inhibitor of Gβγthat selectively blocks activation of a subset of effectors including PLCβ3 (Bonacci et al. 2006). We have previously demonstrated that gallein enhances the antinociceptive potency of morphine in mice (Bianchi et al., 2009; Bonacci et al., 2006; Campbell and Smrcka, 2018; Lehmann et al., 2008; Mathews et al., 2008) supporting the idea that gallein inhibits PLCβ3 activation by Gβγwithout inhibiting interaction of Gβγwith other targets relevant to MOR
actions including $\text{Ca}^{2+}$ and $\text{K}^+$ channels. Gallein (10 µM) potentiated inhibition at various concentrations of DAMGO leading to a left shift in the DAMGO concentration-response curve with a minor effect on efficacy (Fig 4C). Gallein also potentiated the ability of DAMGO (50 nM) to inhibit the frequency of miniature inhibitory postsynaptic currents (mIPSCs) (43 ± 4%, $n = 6$) compared to control (6 ± 4%, $n = 5$), without changing mIPSC amplitude (2 ± 5%) indicating a presynaptic effect of gallein on MOR signaling. Gallein had no effect in slices from PLCβ3−/− mice (Fig 4D) indicating that gallein enhances MOR-dependent inhibition of GABA release through blockade of Gβγ-dependent regulation of PLCβ3. This provides evidence for a synaptic mechanism underlying gallein’s ability to enhance the nociceptive potency of morphine in mice.

To examine the role of $\text{G}_{\alpha_q}$ signaling in the PAG we used a myristoylated peptide from the C terminus of $\text{G}_{\alpha_q}$ (myrG$\alpha_q$-CT) that competes for $\text{G}_{\alpha}$ subunit interactions with endogenous GPCRs to prevent G protein activation (Gilchrist et al., 2002). Slices pretreated with myrG$\alpha_q$-CT revealed DAMGO-dependent inhibition of eIPSCs at 50 nM DAMGO to an extent similar to treatment with gallein (Fig 5A). A similar potentiation was produced after incubating slices in a small molecule inhibitor of $\text{G}_{\alpha_q}$ YM-254890 (500 nM) (Schmitz et al., 2014). The paired pulse ratio (PPRs) for eIPSCs in the presence of DAMGO compared to baseline were changed in both inhibitors (myrG$\alpha_q$-CT: $t(5) = 5.4$, $p = 0.003$; YM-254890: $t(5) = 3.1$, $p = 0.03$) indicating that the DAMGO-mediated inhibition is via presynaptic MORs. Neither gallein nor myrG$\alpha_q$-CT had any effect on DAMGO dependent inhibition of eIPSCs in slices from PLCβ3−/− mice (Fig 5B) or in the absence of DAMGO stimulation (Fig 5C). These results indicate that blocking either Gβγ or $\text{G}_{\alpha_q}$ is sufficient to enhance MOR inhibition of GABA release at low concentrations of a MOR agonist, and that these G proteins dampen MOR signaling in vPAG terminals via PLCβ3.
Since either G\(\alpha_q\) or G\(\beta\gamma\) inhibition alone is sufficient to enhance MOR potency, these data together indicate that signaling via both subunits simultaneously is required to maintain inhibition of MOR-dependent regulation of neurotransmitter release via PLC\(\beta3\).

\textit{Gq signaling and antinociception in mice.}

Since simultaneous activation of PLC\(\beta3\) by G\(\alpha_q\) and G\(\beta\gamma\) is required for inhibition of MOR-dependent regulation of GABA release in the PAG we examined whether either inhibition of G\(\alpha_q\) or G\(\beta\gamma\) is sufficient to enhance MOR-dependent antinociception. As discussed above, we have previously demonstrated that G\(\beta\gamma\) inhibition with gallein enhances morphine-dependent antinociception (Bonacci et al., 2006; Mathews et al., 2008). To test whether Gq inhibition \textit{in vivo} in the PAG would enhance MOR-dependent antinociception, we injected mice i.c.v. with either myrG\(\alpha_q\)-CT or control myrG\(\alpha_q\)-scrambled peptide, or with gallein as a reference, and measured morphine effects (3.2 mg/kg) in the WWTW assay. At this dose, morphine alone had very little, if any, effect on tail withdrawal latencies as compared with baseline (BL) and Post-ICV withdrawal latencies. As previously described (Mathews et al., 2008), gallein (100 nmoles) had no effect on antinociception in the absence of morphine (post-ICV on graph), but strongly increased the effects of morphine, in terms of magnitude and duration of antinociception (Fig. 6A). Similarly, mice injected with myrG\(\alpha_q\)-CT did not have altered withdrawal latencies compared with BL latencies and compared with myrG\(\alpha_q\)-scrambled control peptide alone, but showed enhanced morphine-induced antinociception compared to DMSO (vehicle) or myrG\(\alpha_q\)-scrambled peptide injected mice (Fig. 6B).
These data, together with prior data demonstrating that MOR-dependent antinociception is enhanced in PLCβ3−/− mice, support the idea that Gαq signaling in cooperation with Gβγ signaling via PLCβ3 in the CNS, opposes MOR-dependent antinociception (Fig. 7). This model explains how blockade of any of these components enhances morphine-dependent antinociception in vivo.
Discussion

Previous work identified negative regulatory effects of PLCβ3 on opioid antinociception (Xie et al., 1999). Our prior studies showed that inhibitors of Gβγ (M119 and gallein) enhance opioid-mediated antinociception (Bonacci et al., 2006; Hoot et al., 2013; Mathews et al., 2008) and that the effects of M119 were occluded in mice with PLCβ3 deletion supporting the idea that gallein and M119 block Gβγ-PLCβ3 interactions (Bonacci et al., 2006). Based on this information we proposed that Gβγ released from Gi-coupled MORs activates PLCβ3 which opposes MOR-stimulated analgesia. Importantly, our results presented here show that opioids do not appreciably activate PLC, and subsequent DAG production, on their own unless there is coincident signaling from Gq-coupled receptors. Cross-talk between Gq coupled receptors and MOR has previously been described for regulation of Ca2+ signaling in MOR expressing cell lines, but the mechanism for this cross-talk, and its role in MOR biology has not been clearly defined (Connor and Henderson, 1996; Samways and Henderson, 2006; Samways et al., 2003; Werry et al., 2003; Yeo et al., 2001). Here we provide evidence for coincident regulation of PLC activation by Gq and Gi/Gβγ signaling MOR in HEK293 cells, and show that this synergistic pathway operates in PAG synapses, a critical brain region involved in MOR-dependent antinociception (Fig. 7). Finally we show that Gβγ and Gq signaling both oppose MOR-dependent antinociception in mice. These data, together with previous data from PLCβ3−/− mice implicate PLCβ3 as a source of Gq-MOR cross-talk in the CNS for MOR-dependent antinociception.

PLCβ3, but not PLCβ1 or PLCβ4, is activated by Gβγ, and the vast majority of effector regulation by Gβγ subunits occurs downstream of Gi-coupled receptors (Campbell and Smrcka,
PLCβ3 is unique in that it is strongly synergistically regulated by Gαq and Gβγ and it was proposed that this could serve as a coincidence detector for cells to respond to simultaneous signals from Gi and Gq coupled receptors (Philip et al., 2010; Rebres et al., 2011). This was initially demonstrated in detailed in vitro biochemical reconstitution experiments and later confirmed downstream of Gq and Gi coupled receptors in bone marrow derived macrophages and in NIH3T3 cells. A recent study confirmed and extended these observations with a broader range of receptors (Pfeil et al., 2020).

In the HEK cell-based studies we tested several examples of Gq-coupled receptors and observed synergistic activation of PLC indicating that the negative regulation exerted on MORs originates from the biochemical properties of PLCβ3. Thus, we propose that any Gq-coupled receptor would synergize with MOR in this system. The myrGαq-CT used as an inhibitor of Gq signaling in these studies does not inhibit Gαq directly, but rather competes for interactions between Gq-coupled GPCRs and Gαq preventing activation of Gαq by GPCRs. The effectiveness of this inhibitor in our experiments indicates that tonic Gq coupled receptor activation in the PAG is limiting MOR-mediated analgesia via this mechanism. Future experiments will determine the nature of this receptor or possibly multiple receptors. A recent C. elegans screen identified GPR139 as a Gq-coupled GPCR that opposes opioid analgesia (Wang et al., 2019), and is one possible candidate.

Contrasting with our results, Halls et al. reported that morphine, but not DAMGO, activated a plasma membrane targeted PKC sensor, pmCKAR, without a requirement for coincident Gq activation (Halls et al., 2016). We see robust responses to both DAMGO and morphine, in the presence of a Gq stimulus, regardless of the localization of the sensor. It is
possible that DAG sensor used in our study is less sensitive than pmCKAR. CKAR is a PKCα based FRET reporter which contains both Ca\(^{2+}\) and DAG binding sites that interact cooperatively which may sensitize CKAR to local generation of DAG in the presence of elevated Ca\(^{2+}\). It is also possible that in the HEK cell line used in that study there is a tonic Gq signal that does not translate across different HEK cell lines. Thus, while MOR may stimulate low level PLC activation in the absence of Gq signaling, coincident Gq activation results in robust MOR-dependent PLC activation that we demonstrate to have physiological relevance.

One strategy to avoid development of opioid tolerance for treatment of chronic pain and to reduce the potential for addiction would be to lower the doses of morphine needed to produce analgesia. We and others have previously shown that inhibition of G\(\beta\gamma\) subunits increases the antinociceptive potency of morphine without enhancing side effects such as constipation and respiratory depression (Hoot et al., 2013) suggesting that inhibition of a subset of effectors downstream of G\(\beta\gamma\) is a possible strategy to reduce morphine doses required for pain management (Campbell and Smrcka, 2018). Targeting G\(\alpha_q\) signaling is a possible alternative strategy. Since G\(\alpha_q\) signaling appears to be tonically activated by a yet to be unidentified Gq-coupled GPCR, either an antagonist or inverse agonist targeting this receptor would likely enhance the antinociceptive effects of morphine.

Highly potent MOR agonists such as fentanyl already exist, and are very dangerous drugs. Since these drugs target MOR itself, their potency with respect to causing side effects severely limits their usefulness. MOR-dependent G protein activation is relatively cell context independent, while signaling downstream of G protein activation is highly cell context dependent. G\(\beta\gamma\) signaling depends on the cell type specific expression of G\(\beta\gamma\)-regulated
effectors and cell-type specific responses to regulation of those receptors (Campbell and Smrcka, 2018; Fisher et al., 2020; Lin and Smrcka, 2011). Thus, targeting PLCβ3 or its regulators may enhance the potency of MOR antinociceptive effects relative to side effects because the neurons responsible for antinociception may have different downstream signaling responses that are more sensitive to PLCβ3 than the neurons responsible for respiratory depression or constipation. The signaling mechanisms downstream of PLCβ3 that oppose opioid analgesia have not yet been identified. One possibility is through PKC-dependent phosphorylation of key targets such as MOR itself. Both of these issues will be the subject of further investigation.
Author Contributions:

Participated in research design: Smrcka, Jutkiewicz, and Ingram.

Conducted experiments: Sanchez, and Ingram

Performed data analysis: Sanchez, Smrcka, Jutkiewicz, and Ingram

Wrote or contributed to the writing of the manuscript: Sanchez, Smrcka, Jutkiewicz, and Ingram
References


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Figure Legends

Figure 1. MOR activation alone does not stimulate detectable DAG production. A) field of HEK293 cells showing transduction of the DAG reporter. HEK293 cells were transduced with a fluorescent DAG reporter (DAG-up, Montana Molecular) and M1 muscarinic acetylcholine receptor (M1R). B) At the dotted line, DPBS vehicle (Veh), PDBu or 50 μM carbachol (Carb) were added and the change in fluorescence intensity across the entire well of a 96 well plate relative to baseline F0 was monitored. C) Cells were transduced with the DAG reporter, transfected with flag-MOR. Cells were fixed and stained with an anti-flag antibody. C) Cells transduced with the DAG reporter and transfected with flag-MOR were treated with DPBS (veh), 1 μM DAMGO or 10 μM Morphine (Morph) as in B. D) Cells were transduced with the CAAX-DAG reporter (DAG-down, Montana Molecular) and M1R, and transfected with MOR. Shown is a field of live cells showing plasma membrane localization of the reporter. F) Cells expressing CAAX-DAG reporter, M1R and MOR were treated with the indicated agonists and fluorescence intensity was measured. For B, D and F, Each trace is the mean +/-SEM of 3-4 separately transduced wells in a 96 well plate, representative of at least 3 separate experiments. All baseline traces were normalized to 1. The initial downward deflections at the dotted line in traces in F are artifacts associated with compound/vehicle addition.

Figure 2. Coactivation of MOR and Gq-coupled muscarinic receptors reveals synergistic PLC activation. A) HEK293 cells stably expressing flag-MOR were transduced with the DAG reporter and M1 receptor as in Fig 1A and B. Cells were treated (compounds added at the dashed verticle line) with DPBS, 100 nM Carbachol, 500 nM DAMGO, 1 μM Morphine,
Carbachol+DAMGO, or Carbachol+morphine at the same concentrations. B) To quantify these responses the area under the curve (AUC) with vehicle subtracted for each curve was calculated; one way ANOVA F(4,10)=11.5, P=0.0009. C and D) Stable MOR, HEK293 cells transduced, transfected and treated as in A were treated for 16h without (C) and with 100 ng/mL PTX for 16h (D). E) Peak DAG production at each concentration of agonist relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments, each performed with four replicates. All data are +/- SEM. P values were calculated with an ordinary one-way ANOVA with Tukey’s post hoc test and two-way ANOVA with Sidak’s post hoc test. *P<0.05 and **P<0.005.

Figure 3. Gq synergy with MOR for PLC activation is independent of the nature of the stimulating Gq-coupled GPCR. A) HEK cells stably expressing MOR, and transduced with the DAG reporter, without transduction of the M1 muscarinic receptor, were treated with vehicle, 50 μM Carbachol, or 50 μM Carbachol+100 nM DAMGO. Data are mean +/-SEM from one representative experiment. B) Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in A; unpaired t-test t(4)=12.03, P=0.0003. C) HEK293 cells stably expressing MOR were as in A treated with vehicle, 50 μM carbachol, or carbachol + varying concentrations of DAMGO. Representative traces from 1 experiment with 4 replicates each condition. D) Peak DAG production at each concentration of agonist relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells for each concentration. Non-linear regression curve fitting; morphine
EC₅₀ = 17nM [95%CI 7-27nM]; DAMGO EC₅₀ = 3.8nM [95%CI 1.6-7.7nM] E) Experiments were performed as in A except 100 μM ATP instead of carbachol was used as the agonist for activation of Gq. Data are mean +/-SEM from one representative experiment. F) Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in E; unpaired t-test t(4)=3.7, P=0.02. G) Experiments were performed as in A except 3 μM PAR1AP (SSFLRN) was used as the agonist for activation of Gq. Data are mean +/-SEM from one representative experiment. H) Peak DAG production relative to maximum PDBu-dependent DAG production was calculated from 3 independent experiments each performed in four independent wells as in G; unpaired t-test t(4) =4.45, P=0.01. *P<0.05, ***P<0.0005.

Figure 4. DAMGO inhibition of GABAergic eIPSCs is potentiated in slices from PLCβ₃⁻/⁻ mice and with Gβγ inhibition. A) Representative eIPSCs from a recording from a slice from a WT mouse showing the effect of DAMGO (1 μM) and reversal with naloxone. B) Concentration-response curves for DAMGO-mediated inhibition in slices from WT compared to PLCβ₃⁻/⁻ mice. The EC50 for DAMGO is shifted to the left in slices from PLCβ₃⁻/⁻ mice (238 nM; 95% CI 140-376 nM) compared to WT mice (1.3 μM; 95% CI 732nM- 2.8 μM; F(1,79) = 22.8, p < 0.0001. Recordings were from 4-6 cells from at least 3 mice per data point. C) Gallein shifted the DAMGO concentration response curve in slices from PLCβ₃⁺/+ mice (control: 0.8 μM [95%CI 0.52-1.54 μM], gallein: 0.12 μM [95%CI 0.08-0.18 μM]). D) Gallein does not shift the DAMGO concentration-response curve in slices from PLCβ₃⁻/⁻ mice. EC50 = 162 nM (95% CI 81 – 367
nM). PLCβ3+/+ and PLCβ3−/− curves are the same as in B. Recordings were from 4-6 cells from at least 3 mice per data point. Data are mean +/-SEM.

**Figure 5.** Gq inhibition potentiates DAMGO inhibition of GABAergic eIPSCs in PAG slices. A) Bar graph comparing effects of Gβγ and Gq inhibitors on inhibition of eIPSCs produced by a single dose of DAMGO (50 nM). All samples were preincubated with either vehicle control (DMSO) or the indicated inhibitors at 10 μM for 30 min, followed by addition of DAMGO. One way ANOVA, F(3, 21) = 14.4, p < 0.0001; Dunnett’s multiple comparisons, **p< 0.01, ****p<0.0001. B) Gallein (10 μM) and mGq-CT (10 μM) had no effect on DAMGO-dependent inhibition of eIPSCs in PAG slices isolated from PLCβ3−/− mice. Experiments were performed as in A except PAG slices from PLCβ3−/− mice were used. Symbols denote number of recordings and numbers in bars denote number of animals. C) Gallein (10 μM) and mGq-CT (10 μM) had no effect on eIPSCs in the absence of MOR activation. eIPSCs were measured before and after addition of the indicated inhibitors without addition of DAMGO. Data are mean +/-SEM of all recordings, Numbers in bar graphs indicate the number of animals tested.

**Figure 6.** Gβγ and Gq inhibition enhance morphine-induced antinociception in mice. A) Mice were injected i.c.v. with gallein (100 nmoles) or DMSO (8 mice per condition) and allowed to recover for 30 min and post-ICV tail flick latency was measured. 3.2 mg/kg morphine was then injected at time 0 and tail flick latency was measured at the indicated times; mixed effects two way ANOVA with Sidak’s multiple comparisons, significant interaction of time X pretreatment effect F(5,69)=9.2 P=<0.0001 B) Same as A except mGαq-CT (5 male mice) or myrGαq-scrambled
(4 male mice) were injected i.c.v. at 30 nmoles each; mixed effects two way ANOVA with Sidak’s multiple comparisons, significant interaction of time X pretreatment effect F(5,35)=2.78, P=0.03. Data was analyzed with a mixed effects ANOVA followed by Sidak’s multiple comparisons test. *P<0.05, **P<0.005, ****P<0.0001 at each time point comparing treatment to control (gallein vs. DMSO) or (mGαq-CT vs. Scr peptide). Data are mean +/-SD.

Figure 7. Model for mechanism of MOR -Gq coincidence detection in feedback inhibition of MOR-dependent antinociception in presynaptic PAG input neurons in the descending pain pathway. Pictured is a GABAergic synapse between a PAG input and output neuron. The boxed inset shows the anatomic location of the PAG in the rodent brain with inputs from the cortex, and outputs to the spinal cord. MOR activation in the presynaptic neuron inhibits GABA release resulting in activation of output neurons that ultimately suppress afferent pain transmission in the spinal cord. PLCβ3 activation suppresses MOR actions in presynaptic neuron, and activation of PLCβ3 requires inputs from both Gi/βγ from MOR and Gq from an unknown Gq-coupled receptor. Since PLCβ3 activation requires simultaneous Gαq and Gβγ binding, blockade of either Gαq or Gβγ is sufficient to relieve the PLC-dependent inhibition of MOR signaling leading to enhanced MOR potency and increased antinociception. Figure created with Biorender.com.
Figure 1

A

B

C

D

E

F
Figure 2
Figure 3

[Graphs showing fluorescence changes over time for different treatments: Vehicle, Carbachol, DAMGO+Carb, ATP, ATP+DAMGO, PAR1AP, PAR1AP+DAMGO, and [DRUG] (M) for Morphine and DAMGO.]

These graphs illustrate the effects of various treatments on fluorescence changes over time, highlighting the responsiveness to different pharmacological agents.

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Figure 4
**Figure 5**

Panel A: Graph showing % inhibition of eIPSCs. Treatments include control, gallein, mGq-CT, and YM. 7, 5, 4, and 6 data points are indicated, respectively.

Panel B: Graph showing % inhibition of eIPSCs. Treatments include PLCβ3, gallein/PLCβ3, mGq-CT/PLCβ3. 3, 4, and 4 data points are indicated, respectively.

Panel C: Graph showing % change in eIPSCs. Treatments include Gallein alone, mGq-CT alone. 3 and 4 data points are indicated, respectively.
Figure 6
Figure 7