Endocytic Profiles of δ -Opioid Receptor Ligands Determine the Duration of Rapid but Not Sustained cAMP Responses[§]

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ABSTRACT

Traditional assays that monitor cAMP inhibition by opioid receptor ligands require second-messenger accumulation over periods of 10–20 minutes. Since receptor regulation occurs within a similar time frame, such assays do not discriminate the actual signal from its modulation. Here we used bioluminescence resonance energy transfer to monitor inhibition of cAMP production by δ -opioid receptor (DOR) agonists in real time. cAMP inhibition elicited by different agonists over a period of 15 minutes was biphasic, with response buildup during the first 6 to 7 minutes, followed by a second phase of response decay or of no further increment. The rate at which the cAMP response disappeared was correlated with operational parameters describing ligand efficiency [$\log(\tau/K_A)$] to promote $G\alpha_i$ activation, as well as with ligand ability to promote internalization during the time course of the assay. Thus, ligands that displayed low signaling efficiency and poor sequestration (SB235863 [$[8R-(4bS^*, 8a\alpha, 8a\beta, 12b\beta)]7,10$ -dimethyl-1-methoxy-

11-(2-ethylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]isoquinoline hydrochloride), morphine) had minimal or no response decay. On the other hand, the decay rate was pronounced for deltorphin II, [D-Pen(2), D-pen(5)]-enkephalin, met-enkephalin, and SNC-80 ((+)-4-[(αR)- α -(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide), which displayed high signaling efficiency and internalization. Moreover, inhibition of internalization by dynasore reduced or abolished response decay by internalizing ligands. Unlike acute responses, endocytic profiles were not predictive of whether an agonist would induce prolonged cAMP inhibition over sustained (30–120 minutes) DOR stimulation. Taken together, the data indicate that ligand ability to evoke G-protein activation or promote endocytosis was predictive of response duration over short, but not over sustained periods of cAMP inhibition.

Introduction

Opioid receptors belong to the subfamily of G_i/o -coupled receptors, and their stimulation results in canonical responses such as Kir3 channel activation, Cav2 channel inhibition, and reduction in cAMP production (McDowell, 2003; Audet et al., 2010; Nagi and Pineyro, 2011; Richard-Lalonde et al., 2013). All these responses are subject to regulation via a series of events that not only determine the duration of acute pharmacological responses but may also contribute to the development of analgesic tolerance (Williams et al., 2013). The earliest of these regulatory steps is the recruitment of G protein-coupled

receptor kinase to the activated receptor. It starts at the same time and progresses within the same time frame (seconds) as G-protein stimulation (Breton et al., 2010), achieving maximal phosphorylation of the receptor during the first 2 minutes of agonist binding (Doll et al., 2011). β -Arrestin (β arr) is subsequently recruited with a half-life of approximately 2 minutes (Molinari et al., 2010; Audet et al., 2012), whereas internalization progresses at a much slower pace, with half-lives of 10–15 minutes in the case of δ -opioid receptors (DORs) expressed in human embryonic kidney (HEK) cells (Archer-Lahlou et al., 2009; Audet et al., 2012).

Elegant studies assessing Kir3 activation by highly efficacious μ -opioid receptor (MOR) agonists have shown that ligand-induced currents decay within a similar time frame as β arr recruitment and do so without the intervention of receptor endocytosis (Arttamangkul et al., 2006, 2008). Much less is known about the early time course of cAMP inhibition by opioids and the way internalization regulates their initial cyclase response. Indeed, most of the assays that have been

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ABBREVIATIONS: ANOVA, analysis of variance; ARM390, N,N-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide; β arr, β -arrestin; BRET, bioluminescence resonance energy transfer; DBC, DeepBlue C Coelenterazine 400a; DMEM, Dulbecco's modified Eagle's serum; DOR, δ -opioid receptor; DPDPE, [D-Pen(2), D-pen(5)]-enkephalin; HEK, human embryonic kidney cell line; HRP, horseradish peroxidase; IBMX, 3-isobutyl-1-methylxanthine; mcp-TIPP, H-Mcp-Tic-Phe-Phe-OH; met-ENK, met-enkephalin; MOR, μ -opioid receptor; OD, optical density; PBS, phosphate-buffered saline; RLuc, *Renilla* luciferase; SB235863, [$[8R-(4bS^*, 8a\alpha, 8a\beta, 12b\beta)]7,10$ -dimethyl-1-methoxy-11-(2-ethylpropyl)oxycarbonyl 5,6,7,8,12,12b-hexahydro-(9H)-4,8-methanobenzofuro[3,2-e]pyrrolo[2,3-g]isoquinoline hydrochloride; SNC-80, (+)-4-[(αR)- α -(2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-N,N-diethylbenzamide; YFP, yellow fluorescent protein.

used to monitor inhibition of cAMP production require accumulation of the second messenger over 15–20 minutes and therefore cannot discriminate the actual response from its rapid regulation. The observation that counter-regulatory superactivation of the cyclase pathway is less pronounced after sustained exposure to internalizing than noninternalizing ligands has led to the notion that endocytosis actively limits cyclase inhibition (Finn and Whistler, 2001; He et al., 2009). However, these observations refer to regulation of sustained, and not rapid, responses.

Here we used bioluminescence resonance energy transfer (BRET) to monitor DOR modulation of cAMP production with a time resolution of seconds. We observed that acute cAMP inhibition by DOR ligands involved two phases, one of response buildup that was completed within the first 340–450 seconds (depending on the drug) of receptor stimulation and another of response decay or of no further increment that went on for the rest of the experiment (15 minutes). Ligand ability to promote G-protein activation was a good predictor of the decay rate of the acute cAMP response, although there were exceptions. Ligand ability to induce internalization also contributed to signal decay in acute responses, but the endocytic profile of an agonist did not allow us to predict whether it would induce sustained cAMP inhibition over prolonged exposure.

Materials and Methods

[D-Pen(2), D-Pen(5)]-enkephalin (DPDPE), morphine, forskolin, 3-isobutyl-1-methylxanthine (IBMX), mouse anti-FLAG M1 antibody, anti-FLAG M2 antibody, horseradish peroxidase (HRP) substrate *o*-phenylenediamine dihydrochloride, and dynasore were all purchased from Sigma-Aldrich (St. Louis, MO). HRP-conjugated anti-mouse antibody was purchased from GE Healthcare (Franklin Lakes, NJ). Deltorphin II and met-enkephalin (met-ENK) were purchased from AnaSpec (Freemont, CA). SNC-80 ((+)-4-[(α R)- α -(2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl]-3-methoxybenzyl]-*N,N*-diethylbenzamide) was from Tocris Cookson (Ellisville, MO). DeepBlue C Coelenterazine 400a (DBC) from Cedarlane (Burlington, ON, Canada), Coelenterazine H from PerkinElmer (Waltham, MA), polyethylenimine was from Polyscience (Warrington, PA), and Lipofectamine and anti-mouse Alexa 488 antibody were from Life Technologies (Grand Island, NY). Mcp-Tic-Phe-Phe-OH [Mcp, 4-(methyl-carboxamido) phenylalanine; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid] (mcp-TIPP) was synthesized by Dr. P. W. Schiller (Université de Montréal, QC, Canada), and SB235863 ([8*R*-(4*bS**,8*a* α ,8*a* β ,12*b* β)]7,10-dimethyl-1-methoxy-11-(2-ethylpropyl)oxycarbonyl 5,6,7,8,12,12*b*-hexahydro-(9*H*)-4,8-methanobenzofuro[3,2-*e*]pyrrolo[2,3-*g*]isoquinoline hydrochloride) was a generous gift from Dr. L. Gendron (Beaudry et al., 2009) (University of Sherbrooke, QC, Canada).

DNA Constructs

Plasmids encoding yellow fluorescent protein (YFP) fused at the N terminus of human G γ 2 were obtained by subcloning the human G γ 2 coding sequence into YFP vectors (Gales et al., 2005). The recombinant plasmid encoding for human G α_{i1} -Luc91 was prepared using a flexible linker to insert the coding sequence of humanized *Renilla* luciferase (RLuc; PerkinElmer Life Sciences) into that of human G α_{i1} between residues Leu91 and Lys92 (Audet et al., 2008). The GFP10-Epac-Rluc BRET²-cAMP biosensor was constructed in three steps as described by (Leduc et al., 2009). First, GFP10, the linker GSAGT-(Acc65I/*Hind*III)-KLPAT, and Rluc were inserted in pcDNA3.1/Zeo (Invitrogen, Carlsbad, CA) (Hamdan et al., 2007). Part of human Epac1 (residues 144–881) was then amplified by polymerase chain reaction, digested by Acc65I/*Hind*III, and inserted using the same

restriction sites between GFP10-GSAGT and KLPAT-Rluc3 in pcDNA3.1/Zeo. Finally, the substitutions T781A and F782A were introduced by polymerase chain reaction to remove the Rap1-binding site of Epac1. Murine DORs tagged with the Flag epitope at the N terminus were a generous gift from Dr. M. von Zastrow (University of California at San Francisco, San Francisco, CA).

Cell Culture and Transfection

HEK cells stably expressing murine FLAG-DORs were produced using Lipofectamine (Invitrogen) to transfect 6 μ g of DNA/100-mm Petri dish followed by selection with geneticin (G418) (500 μ g/ml) as previously described (Audet et al., 2012). For BRET-based cAMP assays, stable cell lines expressing the GFP10-Epac-RlucII construct were prepared as previously described (Audet et al., 2012). For cAMP assays, these cells were seeded at a density of 3.5×10^6 cells in 100-mm Petri dishes, cultured for 24 hours, and transiently transfected with 10 μ g FLAG-DOR using polyethylenimine as indicated by the manufacturer. In experiments in which mutant β arr1 (318–419) was used, the minigene or its corresponding vector (pcDNA3) was used at a concentration of 12 μ g/Petri dish. Twenty-four hours after transfection, cells were transferred (100,000–150,000 cells/well) onto 96-well plates coated with poly (D-lysine) and left at 37°C for additional 24 hours. For BRET assays assessing G-protein activation, cells were transfected with G β_1 (4 μ g), FLAG-DOR (5 μ g), YFP-G γ_2 (8 μ g), and G α_{i1} -Luc91 (1 μ g) (values for 100-mm Petri dish) and used 48 hours post-transfection. Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, at 37°C in humidified atmosphere at 95% air and 5% CO₂ (Audet et al., 2012).

Evaluation of DOR Internalization in HEK Cells

Measurement of surface-expressed receptors and quantification of receptor internalization was done using an enzyme-linked immunosorbent assay-based method adapted from a previously published protocol (Archer-Lahlou et al., 2009). Briefly, HEK cells stably expressing FLAG-DOR were seeded at a density of 2.5×10^5 cells/well and grown on 24-well poly(D-lysine)-coated plates for 48 hours. Two types of enzyme-linked immunosorbent assays were carried out: an assay in which cells were treated with a fixed concentration of agonist for 15 minutes (10 μ M), and dose-response curves in which cells were exposed to increasing concentration of selected agonists (100 nM–100 μ M) for 30 minutes. At the end of either assay, internalization was stopped by the addition of cold phosphate-buffered saline (PBS). After two washes, cells were fixed in paraformaldehyde (3%, 15 minutes, 4°C) and nonspecific binding was blocked by incubation with PBS/bovine serum albumin 1%/CaCl₂ 1 mM at room temperature for 30 minutes. Cells were subsequently incubated with anti-FLAG M1 antibody (1:1000) for 1 hour (room temperature), washed three times, and incubated with HRP anti-mouse antibody (1:8000) for 30 minutes. After extensive washing, HRP substrate *o*-phenylenediamine dihydrochloride (200 μ l) was added to each well. The reaction was allowed to proceed for 8 minutes and stopped using 50 μ l of 3N HCl; 200 μ l of the mix was then transferred to a 96-well plate for optical density (OD) evaluation at 492 nm. Experiments in which dynamin function was blocked were similarly performed, except that dynasore (80 μ M) or vehicle (0.8% dimethylsulfoxide) was introduced into the medium 30 minutes before exposure to agonist. The amount of surface receptors that were internalized after agonist treatment was calculated by subtracting OD obtained in the presence of agonist from the one obtained in its absence. Results were expressed as the percent of receptor initially present at the membrane according to the following calculation:

$$100 \times (\text{OD}^{\text{Basal}} - \text{OD}^{\text{Stimulated}}) / (\text{OD}^{\text{Basal}}),$$

where OD^{Basal} and OD^{Stimulated} correspond to the signal obtained in absence or presence of agonist, respectively (Audet et al., 2012).

Monitoring cAMP Inhibition by Means of a BRET-Based Biosensor

Kinetic Experiments. Cells were incubated at 37°C with DMEM/HEPES (20 mM) for 15 minutes, after which they were washed with PBS and kept in Tyrode's solution. DBC coelenterazine was added to a final concentration of 5 μ M, and 9 minutes after its introduction, cells were treated with 10 μ M forskolin plus 10 μ M of selected ligands or vehicle. BRET2 measures were taken at 20-second intervals for a period of 15 minutes immediately after the introduction of DOR ligands. Readings were taken at 37°C with a Victor 3 plate reader from PerkinElmer Life Sciences, which allows the sequential integration of signals detected in the 440–480-nm (RLuc emission), 520–550-nm (YFP emission; BRET1) and 515–530-nm (GFP emission; BRET2) windows. BRET2 readings were expressed as the ratio of light emitted by GFP (515–530 nm) over the light emitted by RLuc (440–480 nm). When dynasore was used, cells were kept in the incubator and exposed to DMEM/HEPES (20 mM) + dynasore (80 μ M) for 30 minutes before adding DBC and completing the kinetic assay as described herein.

Sustained cAMP Responses. Cells were kept in the incubator, where they were exposed to Tyrode's solution containing forskolin (10 μ M) and IBMX (250 μ M) for 120 minutes. During this period, selected agonists (10 μ M) were added to the medium at various time points (120, 60, 30, and 0 minutes) before taking BRET2 measures.

Dose-Response Curves. Cells were incubated with DMEM/HEPES (20 mM) and then transferred to Tyrode solution as described, before introduction of DBC to a final concentration of 5 μ M. Five minutes after DBC, forskolin and IBMX were introduced into the medium to respective final concentrations of 20 and 750 μ M, and 4 minutes after that, cells were exposed to increasing concentrations of different DOR agonists. BRET readings were taken 5 minutes after the agonist addition.

Monitoring G-Protein Activation Using a BRET-Based Biosensor

The effect of agonists on DOR–G protein interaction was assessed using a method that we previously developed and validated for detection of ligand-induced conformational changes within DOR–G-protein complexes (Gales et al., 2005, 2006; Audet et al., 2008, 2012; Richard-Lalonde et al., 2013). Two days after transfection, HEK293 cells expressing the *Gai1*-Luc91/YFP-G γ_2 BRET pair plus accessory subunits and DORs were washed with PBS and distributed into 96-well plates at a concentration of 1 to 2 mg/ml. Cells were then incubated for 3 minutes with coelenterazine H (1 μ g/ml) before the addition of different ligands at increasing concentrations. BRET1 readings were taken 2 minutes after ligand introduction, and BRET ratios were corrected (net BRET) by subtracting the background signal detected when the RLuc-tagged construct was expressed without acceptor (Richard-Lalonde et al., 2013).

Immunofluorescence and Confocal Microscopy

HEK cells stably expressing FLAG-DOR were seeded on poly(D-lysine)-coated coverslips and grown as described already herein. Surface Flag-DORs were labeled with mouse anti-FLAG M2 antibody (1:100, 30 minutes, 37°C). Cells were then stimulated with agonist (10 μ M) for increasing periods, after which they were washed with PBS, fixed with paraformaldehyde (3%, 20 minutes, room temperature), and permeabilized with 0.1% Triton X-100 (20 minutes). Cells were then washed in PBS-1% bovine serum albumin and incubated with anti-mouse antibody Alexa 488 (1:1000) for 1 hour. At the end of incubation, cells were extensively washed and coverslips were mounted onto slides using mounting medium. Immunofluorescence microscopy was performed using a Leica LSM510 microscope; images were analyzed using LSM Image Browser software (Leica, Wetzlar, Germany).

Curve-Fitting

Concentration response curves describing G-protein activation, modulation of cAMP production, and DOR internalization were obtained for seven different agonists. Curves were analyzed with the operational model of agonism to obtain efficacy (τ) and functional affinity (K_A) values that were then used to calculate $\log(\tau/K_A)$ transduction coefficients, which describe drug efficiency to produce a response (Kenakin et al., 2012). Curve-fitting was done with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using the equation:

$$y = basal + (E_{max} - basal) / 1 + [(10^{\log K_A} + x) / (10^{\log t} + x)]^n,$$

where *basal* corresponds to the response observed in the absence of agonist; E_{max} is the maximal response of the system; K_A denotes agonist affinity for the receptor state(s) mediating the response; τ describes agonist efficacy; x corresponds to the logarithm of molar concentration of agonist; and n is the slope of the function that links occupancy to response. DPDPE was used as the standard since it was the only ligand that behaved as full agonist in all three assays. K_A and τ values for the standard as well as system parameters (n and E_{max}) were estimated in depletion assays that were carried out by generating DPDPE dose-response curves in cells that expressed progressively lower amounts of receptors (Prism 5 operational model: “receptor depletion” mode). The only constraint imposed for curve fitting was that n and E_{max} be shared by all curves, obtaining the following estimates: 1) for G-protein activation, $n = 0.4$ and $E_{max} = -115$; 2) for cAMP assays, $n = 0.34$ and $E_{max} = 121$; and 3) for internalization assays, $n = 0.5$ and $E_{max} = 125$. These values were then used as system parameters to simultaneously fit curves for all ligands using the “partial agonist” mode in GraphPad 5. Pooled standard error values were calculated as (Gregory et al., 2012):

$$\text{pooled S.E.} = \sqrt{(\text{S.E.}_1)^2 + (\text{S.E.}_2)^2}.$$

Results

Monitoring Real-Time Inhibition of cAMP Production by DOR Agonists. Monitoring inhibition of cAMP production by opioid receptor ligands has typically relied on methods that require extended periods of receptor activation for second-messenger levels to accumulate to detectable levels. Because of this restriction, it has not been possible to separate fully the cAMP signaling response from regulatory adaptations that take place within the time frame of the assay. The development of genetically encoded RET biosensors offer a solution to this limitation by providing real-time measures of second-messenger level (Edwards et al., 2012). Here we assessed acute (15 minutes) and prolonged (30 minutes to 2 hours) modulation of cAMP production by different DOR agonists by using a previously validated BRET-based biosensor (Leduc et al., 2009; Audet et al., 2012), which undergoes conformational changes upon cAMP binding (Masri et al., 2008; Salahpour et al., 2012). In particular, cAMP binding draws apart the C- and N-terminal ends of the biosensor, separating respective donor (RLuc) and acceptor (GFP10) moieties of the BRET pair, such that increases in cAMP production were captured as a decrease in basal energy transfer. In HEK cells coexpressing the biosensor and DORs, the basal net BRET signal was 0.30 ± 0.01 , and exposure to forskolin (10 μ M) reduced energy transfer by $32\% \pm 1\%$, reaching steady-state levels with a half-life of 2.5 ± 0.2 minutes ($n = 10$; Fig. 1A). The amount of cAMP produced by

Fig. 1. Monitoring real-time inhibition of cAMP production by DOR agonists. (A) HEK293 cells expressing DORs and a BRET-based biosensor that detects cAMP production as a loss in basal energy transfer (0.30 ± 0.01) were incubated with forskolin ($10 \mu\text{M}$) in the presence or absence of different DOR agonists ($10 \mu\text{M}$) and BRET measures taken every 20 seconds for a period of 15 minutes (inset) at 37°C . Results correspond to drug-induced changes in basal BRET and represent the mean $\pm$ S.E.M. of 3–10 independent experiments. Data were analyzed by two-way analysis of variance (ANOVA), which indicated an effect of drug ($P < 0.0001$) and time ($P < 0.0001$) with no interaction. Details of comparisons are given in Table 1. (B) Histograms correspond to mean $\pm$ S.E.M. of the areas (arbitrary units) defined by the kinetic curve of forskolin and those produced by forskolin in the presence of the indicated ligands ($10 \mu\text{M}$) and represent cAMP inhibition in presence of each ligand. Areas were compared by one-way ANOVA with Bonferroni's correction, and results are shown in the figure. (C) Data represent incremental changes in ligand-induced cAMP inhibition, represented by the area between forskolin and forskolin/agonist curves over consecutive intervals of 112 seconds each. Parameters describing time dependent changes in cAMP production appear in Table 2.

forskolin over the first 15 minutes of its application was estimated by calculating the area between the curve and the x-axis, yielding a value of 69 ± 4 , measured in arbitrary units. The addition of maximal effective concentrations ($10 \mu\text{M}$) of different DOR agonists reduced the rate and/or steady-state production of cAMP (Fig. 1A; Table 1). The ability to modify forskolin-induced cAMP production at steady-state allowed the definition of three distinct groups of drugs: 1) met-ENK and DPDPE, which after 15 minutes had reached control levels of cAMP production; 2) morphine, SNC-80, and deltorphin II, whose response was stabilized at intermediate cAMP levels; and 3) SB235863 and mcp-TIPP, whose presence resulted in the greatest inhibition of second-messenger levels (Table 1). Interestingly, if the same kinetic experiment was completed in the presence of IBMX ($750 \mu\text{M}$), differences among ligands disappeared

with kinetic responses by different ligands, collapsing to a curve similar to the one produced by mcp-TIPP in Fig. 1A (not shown).

Parameters given in Table 1 represent the combined kinetics of agonist and forskolin effects. To estimate the inhibition of cAMP production by each of the ligands, we calculated the areas comprising the kinetic curve generated by forskolin and curves produced in presence of forskolin plus each ligand. SB235863 produced the largest inhibition, defined by an area of 29 ± 3 arbitrary units ($n = 7$). Relative areas for each of the other agonists are shown in Fig. 1B. In addition, to obtain a better idea of how the cAMP response progressed over time, we divided each of the corresponding areas into eight consecutive intervals of 112 seconds' duration and estimated ligand-induced cAMP inhibition within of each of these intervals. Data obtained from these calculations are shown in Fig. 1C, where it is possible to observe

TABLE 1

Kinetics of cAMP production after exposure to forskolin (10 μ M) in the presence or in absence of different δ -opioid receptor agonists

	BRET Change (Steady-State) Mean $\pm$ S.E.M.	$t_{1/2}$ (min) Mean $\pm$ S.E.M.
Control		
Forskolin ($n = 10$)	-0.096 ± 0.002	2.5 ± 0.2
Met-enkephalin ($n = 8$)	-0.094 ± 0.003	3.7 ± 0.4
DPDPE ($n = 7$)	$-0.096 \pm 0.006^*$	$4.3 \pm 0.7^{a,b}$
Morphine ($n = 8$)	$-0.078 \pm 0.002^{**}$	2.8 ± 0.3
SNC-80 ($n = 8$)	$-0.073 \pm 0.003^{***}$	2.9 ± 0.4
Deltorphin II ($n = 3$)	$-0.080 \pm 0.003^{**}$	$4.2 \pm 0.4^{c,d}$
mcp-TIPP ($n = 6$)	$-0.059 \pm 0.002^{***}$	2.3 ± 0.4
SB235863 ($n = 7$)	$-0.052 \pm 0.002^{***}$	1.9 ± 0.4
Dynasore		
Forskolin ($n = 4$)	-0.070 ± 0.002^e	2.7 ± 0.1
DPDPE ($n = 3$)	$-0.062 \pm 0.004^{e,*}$	6.3 ± 0.1
Met-enkephalin ($n = 4$)	$-0.057 \pm 0.003^{e,***}$	4.3 ± 0.1
Morphine ($n = 3$)	$-0.050 \pm 0.002^{e,***}$	3.1 ± 0.1^f
Deltorphin II ($n = 3$)	$-0.041 \pm 0.003^{e,***}$	5.0 ± 0.1^g
SNC-80 ($n = 4$)	$-0.034 \pm 0.004^{e,***}$	4.4 ± 0.2
mcp-TIPP ($n = 6$)	$-0.031 \pm 0.004^{e,***}$	4.6 ± 0.2
SB235863 ($n = 6$)	$-0.024 \pm 0.001^{e,***}$	2.3 ± 0.3^h

Curves generated by different agonists were further analyzed by simultaneous curve-fitting indicating:

^a $t_{1/2}$ DPDPE versus $t_{1/2}$ morphine, $P < 0.01$.

^bSteady-state DPDPE versus morphine, $P < 0.01$.

^c $t_{1/2}$ Deltorphin II versus $t_{1/2}$ SB235863, $P < 0.001$.

^dSteady-state deltorphin II versus SB235863, $P < 0.01$.

^eSteady-state values obtained in control and dynasore-treated cells were compared by two-way analysis of variance (ANOVA), which showed the effect of drugs ($P < 0.0001$) and treatment ($P < 0.0001$). Post hoc comparisons indicated that BRET changes by all drugs were smaller in dynasore than in control condition.

^fPost hoc comparisons following two way ANOVA analysis of kinetics curves shown in Fig. 4B indicated that curves by all agonists were different from that of forskolin. Curves generated by different agonists were further analyzed by simultaneous curve fitting which showed: $t_{1/2}$ DPDPE versus $t_{1/2}$ morphine, $P < 0.002$.

^gSteady-state DPDPE versus deltorphin II, $P < 0.005$.

^hSteady-state deltorphin II versus SB235863, $P < 0.0001$.

Post hoc comparisons after two-way ANOVA of kinetics curves shown in Fig. 1 indicated that curves produced by all agonists except Met-enkephalin were different from that of forskolin: $^{}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

that cAMP inhibition by DPDPE, met-enkephalin, deltorphin II, SNC-80, and morphine increased to its maximum within the first 336 seconds, then declined for the remainder of the experiment. The two remaining ligands that were tested, mcpTIPP and SB235863, attained maximum cAMP inhibition 112 seconds later than the rest, and their response did not significantly decline from this point on. Rates of increase and decrease of ligand-induced cAMP responses were calculated from the slopes of the graphs in Fig. 1C and appear in Table 2. The rate at which cAMP responses increased over time indicated that second-messenger inhibition by deltorphin II and SB235863 reached their maximum faster than most of the other ligands (Table 2). Decay slopes also allowed identification of different groups of agonist. Thus, SB235863 and mcp-TIPP response did not significantly decay over time, and DPDPE and met-enkephalin showed the fastest decay rates, whereas that of deltorphin II, SNC-80, and morphine were intermediate among the other two groups.

Signaling Efficacy as a Determinant of Time Course of cAMP Inhibition by DOR Agonists. Given the distinct time course of acute cAMP responses, it was of interest to characterize factors contributing to these differences. Thus, in a first series of experiments, we examined whether increasing and decreasing rates of cAMP inhibition were related to agonist ability to signal. Ligand signaling efficacy was assessed at two consecutive levels of the cAMP pathway, namely, G-protein activation and inhibition of cAMP production. Activation of the G protein was also assessed by means of a BRET-based biosensor (Gales et al., 2005), which we had previously shown to attain steady-state readings within the first 2 minutes of drug exposure (Audet et al., 2012; Richard-Lalonde et al., 2013). Consistent with previous observations,

the $G\alpha_i$ -Luc91/YFP-G γ 2 biosensor produced a basal net BRET signal (0.395 ± 0.011) that was reduced after DOR activation by agonist (Audet et al., 2008). DPDPE, which was used as the standard, reduced this value by $13\% \pm 1\%$ ($n = 6$), and dose-response curves for all agonists were normalized to this maximum (Fig. 2A). Corresponding operational parameters of efficacy (τ), functional affinity (K_A), and transduction coefficients $\log(\tau/K_A)$ (Black and Leff, 1983; Kenakin and Christopoulos, 2013) are given in Table 3. Rank order of transduction coefficients describing ligand efficiency to activate the G protein were as follows: deltorphin II = DPDPE = met-ENK $\geq$ mcp-TIPP = SNC-80 $\geq$ SB 235863 = morphine (Table 3). Interestingly, the magnitude of transduction coefficients for $G\alpha_{i1}$ activation was directly correlated with the rate of decay of the cAMP response (Fig. 2A, inset). No correlation was observed between transduction coefficients and initial rate of increase in cAMP inhibition ($r^2 = 0.0030$; $P = 0.9064$).

In a second series of experiments, we compared cyclase modulation by DOR agonists. Given that the presence of IBMX erased kinetic differences among ligands, the use of the phosphodiesterase inhibitor was seen as an advantage to avoid kinetic confounders during completion of concentration-response curves. For this reason, curves were obtained in cells that were exposed to forskolin (10 μ M) and IBMX (750 μ M) 4 minutes before introducing increasing concentrations of the different agonists. BRET measures were taken 6 minutes after initial drug exposure, corresponding to the point in time at which agonists had attained maximal response (Fig. 1B). The net BRET signal that resulted from cAMP accumulation in the presence of forskolin and IBMX was of 0.154 ± 0.002 and at maximal concentration DPDPE increased this value by $15\% \pm 2\%$ ($n = 13$). Moreover, in these experimental conditions,

TABLE 2

Rates of increase and decrease in the cAMP response induced by different ligands DOR in control cells and in cells exposed to dynasore

	Rate of Response Increase Mean $\pm$ S.E.M.	Rate of Response Decay Mean $\pm$ S.E.M.
	<i>arbitrary area U/min</i>	
Control*:**		
Deltorphan II ($n = 3$)	0.47 ± 0.01^a	-0.087 ± 0.02^b (0.0023)
SB235863 ($n = 7$)	0.46 ± 0.02	-0.0008 ± 0.02 (0.9712)
mcp-TIPP ($n = 6$)	0.40 ± 0.02^c	-0.022 ± 0.01 (0.1491)
SNC-80 ($n = 8$)	0.34 ± 0.03	-0.048 ± 0.01^d (0.0015)
Morphine ($n = 8$)	0.33 ± 0.02	-0.034 ± 0.01^e (0.0399)
DPDPE ($n = 7$)	0.31 ± 0.02	-0.13 ± 0.02 ($P < 0.0001$)
Met-enkephalin ($n = 8$)	0.27 ± 0.01	-0.11 ± 0.01 ($P < 0.0001$)
Dynasore***:****		
Deltorphan II ($n = 3$)	0.51 ± 0.03 ($P < 0.001$)	0.00 ± 0.01 ($P = 0.83$) ^{f,g}
SB235863 ($n = 6$)	0.50 ± 0.04 ($P < 0.001$) ^h	0.01 ± 0.01 ($P = 0.66$) ⁱ
mcp-TIPP ($n = 6$)	0.59 ± 0.04 ($P < 0.001$) ^j	0.00 ± 0.01 ($P = 0.92$) ^k
SNC-80 ($n = 4$)	0.56 ± 0.03 ($P < 0.001$)	0.01 ± 0.02 ($P = 0.47$) ^l
Morphine ($n = 3$)	0.30 ± 0.02 ($P < 0.001$)	-0.01 ± 0.01 ($P = 0.29$)
DPDPE ($n = 3$)	0.42 ± 0.04 ($P < 0.001$)	-0.08 ± 0.02 ($P = 0.0017$)
Met-enkephalin ($n = 4$)	0.33 ± 0.03 ($P < 0.001$)	-0.05 ± 0.01 ($P = 0.0005$) ^g

^aDeltorphan II versus SNC-80, $P < 0.001$.

^bDeltorphan II versus SB 235863, $P < 0.05$.

^cmcp-TIPP versus met-enkephalin, $P < 0.0001$. Decrease rate ($P < 0.0001$).

^dSNC-80 versus met-enkephalin, $P < 0.001$.

^eMorphine versus met-enkephalin, $P < 0.01$.

^fSB235863 versus met-enkephalin, $P < 0.05$.

^gDecay rates obtained in control and dynasore-treated cells were compared by two-way ANOVA, which showed an effect of treatment ($P < 0.01$).

Post hoc comparisons indicated that decay rate for met-enkephalin and deltorphan II were significantly reduced by dynasore treatment ($P < 0.05$).

^hSB235863 versus met-enkephalin, $P < 0.01$.

ⁱmcp-TIPP versus DPDPE, $P < 0.01$.

^jmcp-TIPP versus DPDPE, $P < 0.01$. Decrease rate ($P < 0.0001$).

^kDPDPE versus Deltorphan II, $P < 0.05$.

^lMet-enkephalin versus SNC-80, $P < 0.05$.

*All slopes for response increase were different from zero as calculated by linear regression ($P < 0.0001$); ** P values corresponding to slopes for response decrease are indicated in brackets. Differences in increase and decrease rates were established by one way ANOVA indicating: increase rate ($P < 0.0001$); ***Slopes for response increase rate were different from zero as calculated by linear regression ($P < 0.001$); **** P values for slopes of response decay are indicated in parentheses. Differences in increase and decrease rates were established by one-way ANOVA indicating the following: increase rate ($P < 0.0001$).

maximal response did not significantly differ among agonists (one-way analysis of variance; $P = 0.7405$; $n = 5-13$). Dose-response curves for all agonists are shown in Fig. 2B, and corresponding operational parameters for these curves appear in Table 3. Transduction coefficients for inhibition of cAMP production displayed the following rank order: deltorphan II = DPDPE $\geq$ mcpTIPP = SNC-80 = met-ENK $\geq$ SB235863 = morphine. Interestingly, the correlation observed between decay rate of cAMP inhibition and G_{ai} protein activation was no longer evident for cAMP signaling coefficients (Fig. 2B, inset). As described, no correlation was observed between transduction coefficients and the initial rate of cAMP inhibition ($r^2 = 0.0030$; $P = 0.9064$).

Internalization Determines the Time Course of Rapid cAMP Responses by DOR Agonists. Ligand efficacy to trigger signaling and internalization may not necessarily parallel one another (Keith et al., 1998), and ligand-specific patterns of receptor sequestration have been proposed as possible determinants of the duration of different opioid-mediated responses, including analgesia (Martini and Whistler, 2007; Pradhan et al., 2009). To assess whether or not the time course of rapid cAMP modulation is influenced by ligand ability to promote endocytosis, we completed dose-response curves to determine ligand efficiency to induce internalization. These curves appear in Fig. 3A, where it is evident that internalization by morphine, SB235863, and mcp-TIPP was minimal and precluded meaningful curve-fitting. Curves for the other agonists were fit by means of the operational model, and corresponding parameters are shown in Table 4. Transduction

coefficients (Kenakin et al., 2012) indicated that despite producing similar maximal responses, deltorphan II was more efficient than met-ENK and met-ENK was more efficient than SNC-80 in producing internalization (Table 4). Log (τ/K_A) coefficients for these four agonists were unrelated to rates of increase ($P = 0.3413$) or decrease (Fig. 3A, inset) of their cAMP responses, indicating that for this group of ligands, sequestration could not account for the differences in their kinetic profiles.

In an alternative approach to relate the time course of cAMP response to DOR endocytosis, we assessed the kinetics of receptor sequestration at a maximal effective concentration of agonist (10 μ M). As shown in Table 4, at steady-state sequestration by DPDPE, SNC-80, deltorphan II, and met-enkephalin was 63–67% of receptors present at the membrane before treatment. Morphine and SB235863 did not induce a significant response, whereas mcp-TIPP produced a small (9%) but significant increase in sequestration. Internalization half-life was ~ 15 minutes for highly internalizing agonists and 21 minutes for mcp-TIPP. Thus, internalization values at half-life (values for morphine and SB235863 were arbitrarily fixed at zero) were correlated to the decay rate in cAMP production. As shown in Fig. 3B, inset, there was a clear polarization in which noninternalizing ligands displayed marginal or no loss of response, whereas decay rates for internalizing ones were considerably more pronounced (also compare decay rates shown in Table 2).

Interfering with Sequestration Enhances cAMP Inhibition by “Internalizing” Agonists. To characterize more clearly whether endocytosis influences the decline of

Fig. 2. Dose-response curves for G-protein activation and inhibition of cAMP production by DOR agonists. (A) HEK293 cells were transfected with DORs and a BRET-based biosensor ($G\alpha i1$ -Rluc91-YFP- $G\gamma 2 + \beta 1$) that detects G-protein activation as a decrease in basal energy transfer (0.395 ± 0.011). On the day of the experiment, cells were exposed to increasing concentrations of ligands. Results are expressed as the percent of maximal BRET change produced by DPDPE and correspond to mean $\pm$ S.E.M. of three or four independent experiments. Inset shows correlation between transduction coefficients $\log(\tau/K_A)$ describing drug efficiency to activate $G\alpha i1$ and the rate of decrease in cAMP response. Curves were analyzed by two-way analysis of variance (ANOVA), which indicated an effect of drug, concentration, and an interaction ($P < 0.0001$). Further comparisons appear in Table 3. (B) Dose-response curves were obtained as described herein, in cells expressing DORs and the cAMP-EPAC biosensor. Results are expressed as described and correspond to 5–13 independent experiments. Two-way ANOVA analysis indicated effects of drug and concentration ($P < 0.0001$). Further comparisons appear in Table 3. Inset shows lack of correlation between transduction coefficients $\log(\tau/K_A)$ describing drug efficiency to inhibit cAMP production and the rate of decrease in cAMP response.

cAMP responses generated by DOR agonists, we determined whether interfering with internalization modified inhibition of cAMP production by different ligands. Internalization was blocked with dynasore, which inhibits dynamin activity and prevents sequestration of clathrin-coated vesicles formed at the membrane (Macia et al., 2006; Thompson and McNiven, 2006; Patierno et al., 2011). Pretreatment with dynasore (80 μ M; 30 minutes) had no effect on the total amount of receptors present at the membrane in nonstimulated cells (surface immunoreactivity: controls: 1.7 ± 0.1 ; dynasore: 1.7 ± 0.1 ; $P = 0.512$; $n = 6$) but reduced ligand-induced internalization. In particular, dynasore reduced by ~one-third the internalization induced by 15-minute exposure to DPDPE, met-enkephalin, and SNC-80 (Fig. 4A). On the other hand, it had minimal effect on sequestration induced by deltorphin II, a characteristic that was maintained throughout longer periods of receptor stimulation (Supplemental Fig. 1).

The effect of this same dynasore treatment was then tested in cAMP assays (Fig. 4B). The basal BRET signal in cells that were exposed to dynasore was 0.34 ± 0.01 ($n = 7$). This value

was higher than basal values of nontreated controls (0.30 ± 0.01 ; $n = 10$; $P = 0.015$), which is consistent with dynasore enhancing the constitutive inhibition of cAMP production by DORs. Exposure of dynasore-treated cells to forskolin (10 μ M) resulted in a $21\% \pm 0.1\%$ ($n = 4$) decrease in basal BRET, an effect that was smaller than the $32\% \pm 1\%$ reduction observed in controls ($P < 0.0001$; $n = 10$; Table 1). In addition, dynasore shifted to higher levels of energy transfer kinetic curves for all agonists (Fig. 4B; Table 1). To distinguish whether these changes were distinct for different ligands or were simply a generalized consequence of reduced cAMP production after dynasore, we compared overall cAMP inhibition (an area delimited by kinetic curves for forskolin and ligand) by each ligand in control and dynasore-treated cells (Fig. 5A). For noninternalizing ligands like morphine and SB235863, inhibition of cAMP production was similar in control and dynasore-treated cells (Fig. 5A). Although we cannot exclude the lack of dynasore effect being related to a maximal SB235863 response in controls, the fact that morphine maintained similar submaximal response in both conditions

TABLE 3

Operational parameters describing δ -opioid receptor agonist response in $G\alpha_{i1}$ protein activation and cAMP inhibition assays

Transduction coefficients were analyzed by one-way analysis of variance ($P < 0.0001$) followed by post hoc comparisons with Bonferroni's correction. Operational parameters were calculated using DPDPE as a standard full agonist.

	Log(K_A) Mean ± S.E.M.	Log(τ) Mean ± S.E.M.	Log (τ/K_A) Mean ± S.E.M.
$G\alpha_{i1}$ activation			
Deltorphin II ($n = 3$)	-8.35 ± 0.30	1.00 ± 0.19	9.35 ± 0.35 ^a
DPDPE ($n = 4$)	-6.17 ± 0.55	2.87 ± 0.53	9.04 ± 0.77
mcp-TIPP ($n = 4$)	-7.90 ± 0.57	0.71 ± 0.23	7.19 ± 0.62 ^b
Met-enkephalin ($n = 4$)	-7.12 ± 0.37	1.31 ± 0.28	8.43 ± 0.46
Morphine ($n = 3$)	-5.78 ± 0.53	-0.99 ± 0.26	4.79 ± 0.5 ^c
SNC-80 ($n = 3$)	-5.09 ± 0.52	1.47 ± 0.43	6.56 ± 0.68
SB235863 ($n = 3$)	-4.98 ± 0.55	0.10 ± 0.35	5.08 ± 0.65 ^d
cAMP inhibition			
Deltorphin II ($n = 8$)	-8.75 ± 0.27	0.96 ± 0.17	9.71 ± 0.32
DPDPE ($n = 13$)	-7.77 ± 0.32	1.75 ± 0.27	9.52 ± 0.42
mcp-TIPP ($n = 6$)	-7.08 ± 0.52	0.96 ± 0.36	8.04 ± 0.63
SNC-80 ($n = 9$)	-6.57 ± 0.45	0.99 ± 0.32	7.56 ± 0.55 ^e
Met-enkephalin ($n = 7$)	-6.41 ± 0.67	1.12 ± 0.49	7.53 ± 0.83
SB235863 ($n = 6$)	-5.22 ± 0.81	0.98 ± 0.69	6.20 ± 1.07
Morphine ($n = 5$)	-4.13 ± 0.71	0.95 ± 0.59	5.08 ± 0.92 ^f

^aDeltorphin II versus SNC-80, $P < 0.001$.

^bmcp-TIPP versus Deltorphin II, $P < 0.01$.

^cMorphine versus mcpTIPP, $P < 0.001$.

^dSB235863 versus mcpTIPP, $P < 0.01$.

^eDPDPE versus SNC-80, $P < 0.05$. Only comparisons for log (τ/K_A) were included.

^fMorphine versus Met-enkephalin, $P < 0.05$.

(Fig. 5A) is consistent with the notion that inhibition of internalization does not affect cAMP modulation by this noninternalizing ligand. In contrast, dynasore increased by ~60–65% cAMP inhibition by met-ENK, DPDPE, and SNC-80, whose sequestration had been reduced by pre-exposure to the dynamin inhibitor. The effect of dynasore on cAMP modulation by deltorphin II was smaller (23%) and failed to reach significance, which is not surprising since dynasore also failed to reduce DOR sequestration significantly by this agonist. Finally, the cAMP response induced by mcpTIPP, a ligand with small but significant internalizing properties, was increased by a similar margin as that of deltorphin II (Fig. 5A). Taken together, these data indicate that internalization contributes to modulating the early stages of cyclase inhibition by DOR ligands.

To evaluate the influence of dynasore on the decline of cAMP response, we determined whether pretreatment with this internalization blocker modified response decay rates (Fig. 5B). Dynasore reduced the decay rate of cAMP response induced by all internalizing agonists (Table 2). For ligands like DPDPE and met-ENK, whose decay slope was largest in controls, dynasore reduced but did not abolish the rate at which the cAMP response declined. For SNC-80, whose control cAMP response showed slight but significant decrease over time, dynasore reduced internalization, abolishing decay and transforming the descending portion of the kinetic curve into a plateau. Surprisingly, despite its lack of effect on deltorphin II internalization, dynasore also abolished the decay phase of its cAMP response. Finally, for poor internalizing ligands, response decay was minimal or nonexistent in controls and remained that way after exposure to dynasore (Fig. 5B; Table 2).

To corroborate that interference with internalization potentiated cAMP inhibition by internalizing DOR agonists while sparing responses by noninternalizing ones, we monitored the time course of SNC-80 and morphine responses in cells that were transfected with a minigene encoding for the C-terminal portion of β arr1 (318–419). This construct blocks internalization by interfering with binding of endogenous β arr to clathrin (Krupnick et al., 1997) and, in the conditions used in this study, it inhibits internalization induced by 15-minute exposure to SNC-80 by ~15% (Charfi et al., 2013). The basal BRET signal in cells that were transfected with mutant β arr (0.272 ± 0.012) or the corresponding vector pcDNA3 (0.266 ± 0.008) were not different ($P = 0.6751$; $n = 6$), and neither were changes induced by 15-minute exposure to forskolin (controls: 24 ± 4%; mutant β arr: 27 ± 4%; $P = 0.6154$). (Fig. 6, A and B). In keeping with observations obtained with dynasore, mutant β arr enhanced cAMP inhibition by SNC-80 by ~20% but left morphine response unchanged (Fig. 6C).

Sustained cAMP Modulation by DOR Ligands. The endocytic profile of opioid receptor ligands has been associated with differential ability to promote tolerance (Martini and Whistler, 2007; Pradhan et al., 2009), presumably because sequestration may interfere with the production of proanalgesic signals or because its absence could prolong signals that lead to tolerance (Martini and Whistler, 2007). Since prolonged inhibition of cAMP production is considered a signal that may lead to cellular tolerance (Williams et al., 2013), it was of interest to compare ligand modulation of cAMP levels over a time frame in which the endocytic response of ligands like deltorphin II, DPDPE, SNC-80, and met-enkephalin was fully developed. Thus, cAMP inhibition by different agonists was assessed within a time frame of sustained receptor activation. Functional experiments were carried out in the presence of forskolin (10 μ M) and IBMX (250 μ M) to maintain cAMP production throughout the experiment. Each of these reagents was introduced in the incubation medium at the beginning of the experiment and remained present for its entire duration (2 hours). DOR agonists were either introduced together with cAMP modulators or at progressively shorter periods before taking BRET measures. Net BRET values obtained in cells that were exposed only to forskolin/IBMX for 120 minutes were 0.146 ± 0.002 ($n = 10$). Figure 7A shows modification of this basal signal by different ligands, and Fig. 7B shows the subcellular distribution of the receptor at the corresponding time intervals. Morphine and SB235863 produced the largest increases in energy transfer, corresponding to 9% ± 1% of net BRET after 60-minute exposure to each ligand. Interestingly, and despite their differences in internalization (Figs. 3B and 7B), cAMP inhibition by SB235863 was not different from inhibition produced by deltorphin II, with BRET increases by both ligands greater than background variability at all times assessed (Fig. 7A). Conversely, despite sharing a similar internalization and distribution profile with deltorphin II (Figs. 3B and 7B), cAMP inhibition by met-enkephalin had already decayed within the first 30 minutes of treatment. The cAMP response produced by DPDPE and SNC-80 was intermediate between these two examples, and mcp-TIPP did not reach significance at any of the times assessed. Subcellular distribution of DORs after internalization with these four ligands is shown in Supplemental Fig. 2. Taken together, these data indicate that internalization profiles per se did not predict whether the cAMP response would persist or disappear over sustained DOR stimulation.

Fig. 3. Characterization of DOR sequestration by different agonists. HEK cells expressing FLAG-DORs were used to assess ligand-induced DOR sequestration. (A) Dose-response curves were obtained by incubating cells with increasing ligand concentrations for 30 minutes. Results are expressed as the percent of maximal internalization induced by DPDPE and correspond to 9–12 independent experiments. Curves were analyzed by two-way analysis of variance (ANOVA), which indicated an effect of drug, an effect of concentration, and an interaction ($P < 0.0001$). Inset shows lack of correlation between transduction coefficients $\log(\tau/K_A)$ describing drug efficiency to induce internalization and the rate of decrease of cAMP response. (B) HEK293 cells stably expressing FLAG-DORs were exposed to different agonists ($10 \mu\text{M}$) for the indicated times. Internalization was expressed as percent of receptors present at the membrane before exposure to agonist and represent mean $\pm$ S.E.M. of 9–11 independent experiments. Statistical analysis by two-way ANOVA revealed an effect of drug ($P < 0.0001$), an effect of time ($P < 0.0001$), and an interaction ($P < 0.0001$). Post hoc comparisons indicated that deltorphin II, SNC-80, and met-enkephalin did not differ from DPDPE, but the last of these was different from morphine, SB235863, and mcp-TIPP ($P < 0.0001$). Inset shows correlation between sequestration corresponding to the first 15 minutes of drug exposure and decay rate of cAMP response.

Discussion

In this study, we used real-time monitoring of intracellular cAMP levels to characterize cyclase modulation by DOR ligands. Temporal resolution that was afforded by the use of a conformational BRET-based biosensor allowed us to divide the initial stage (15 minutes) of ligand-mediated cAMP

inhibition into a first phase of progressive buildup of the inhibitory response and a second phase characterized by response decay. Data obtained indicated that the decay rate of this early response was correlated with ligand efficiency [$\log(\tau/K_A)$] to activate $G\alpha_{i1}$ signaling and with maximal endocytosis attained within the time frame of the kinetic experiment

TABLE 4

Operational and kinetics parameters describing δ -opioid receptor internalization by different ligands

Transduction coefficients were analyzed by one-way analysis of variance ($P < 0.0001$), followed by post hoc comparison with Bonferroni's correction. Operational parameters were calculated using DPDPE as a standard full agonist.

	Operational Parameters			Kinetic Parameters	
	$\log(K_A)$ Mean $\pm$ S.E.M.	$\log(\tau)$ Mean $\pm$ S.E.M.	$\log(\tau/K_A)$ Mean $\pm$ S.E.M.	Plateau Internalization Mean $\pm$ S.E.M.	$t_{1/2}$ (min) mean $\pm$ S.E.M.
Deltorphin II ($n = 12$)	-7.98 ± 0.13	0.57 ± 0.06	8.56 ± 0.14^a	Deltorphin II ($n = 9$)	$67 \pm 2^{**}$
Met-enkephalin ($n = 11$)	-6.58 ± 0.11	0.35 ± 0.05	6.93 ± 0.12^b	Met-enkephalin ($n = 7$)	$63 \pm 3^{**}$
DPDPE ($n = 9$)	-6.10 ± 0.36	0.58 ± 0.56	6.68 ± 0.67	DPDPE ($n = 8$)	$63 \pm 2^{**}$
SNC-80 ($n = 9$)	-4.39 ± 0.17	0.87 ± 0.13	5.27 ± 0.22	SNC-80 ($n = 5$)	$66 \pm 2^{**}$
Morphine ($n = 10$)	N/A	N/A	N/A	Morphine ($n = 3$)	N/A
mcp-TIPP ($n = 8$)	N/A	N/A	N/A	mcp-TIPP ($n = 3$)	$9 \pm 2^*$
SB235863 ($n = 10$)	N/A	N/A	N/A	SB235863 ($n = 3$)	N/A

N/A, not applicable.

^aDeltorphin II versus met-enkephalin, $P < 0.05$.

^bMet-enkephalin versus SNC-80, $P < 0.05$.

*For each time course in Fig. 3B, maximal internalization observed at 90 minutes was compared with internalization observed at 5 minutes by mean of student's t test;

** $P < 0.001$; * $P < 0.05$.

Fig. 4. Inhibition of internalization by interfering with dynamin function modifies kinetics of cAMP inhibition by DOR agonists. (A) HEK cells expressing FLAG-DORs were pretreated with dynasore (80 μ M; 30 minutes) and then exposed to 10 μ M of the indicated ligands. Internalization was expressed as the percent of receptors present at the membrane before exposure to agonist and represent mean $\pm$ S.E.M. of six independent experiments. Data were analyzed by one way analysis of variance (ANOVA) ($P < 0.0001$) followed by post hoc comparisons with Bonferroni's correction $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (B) HEK cells transfected with DORs and the cAMP biosensor were treated with dynasore as described herein and then exposed to forskolin (10 μ M) in the presence or absence of different DOR agonists (10 μ M) before taking BRET measures. Results correspond to drug-induced changes in basal BRET and represent the mean $\pm$ S.E.M. of three to six independent experiments. Data were analyzed by two-way ANOVA, which indicated an effect of drug ($P < 0.0001$) and time ($P < 0.0001$) with no interaction. Details of comparisons are given in Table 1.

(15 minutes). These last findings were in contrast with those obtained over prolonged periods of DOR stimulation, where the time course of cAMP inhibition could not be predicted from the sequestration profile of the different agonists.

The role of endocytosis as a determinant of the duration and magnitude of opioid responses is not completely understood. In vivo studies indicate that a single administration of the internalizing DOR agonist SNC-80 induces acute analgesic tolerance and loss of G-protein signaling that is not observed after similar treatment with its noninternalizing metabolite (ARM390; *N,N*-diethyl-4-(phenyl-piperidin-4-ylidenemethyl)-benzamide) (Pradhan et al., 2009). These observations led to the notion that internalizing DOR agonists are more prone to tolerance than noninternalizing ones (Pradhan et al., 2009; Williams et al., 2013). However, since SNC-80 produces DOR phosphorylation that is absent after administration of ARM390, it is possible that the rapid loss of function by

SNC-80 could also have resulted from uncoupling mechanisms driven by phosphorylation (Pradhan et al., 2010). In the case of MORs, internalization has been equated with shorter cAMP responses (Finn and Whistler, 2001; Martini and Whistler, 2007) but also with recovery from desensitization (Raehal et al., 2011) and presumably prolonged signaling. Studies that have assessed the time course of K⁺ currents elicited by MOR activation have clearly shown that endocytosis is not necessary for rapid modulation of channel-mediated opioid responses (Arttamangkul et al., 2006). Here we characterized the time course and modulation of acute enzymatic responses by DOR ligands in HEK cells. Data show that inhibition of cAMP production rises to a maximum within ~6–7.5 minutes of receptor activation, which is considerably slower than cAMP stimulation by β_2 -adrenergic receptors (Violin et al., 2008). Since both studies were carried out at 37°C, it is unlikely that the observed differences are of kinetic

Fig. 5. Dynasore increases and prolongs cAMP inhibition by internalizing ligands. (A) Histograms represent overall cAMP inhibition induced by different ligands in control and dynasore-treated cells. This magnitude was estimated from data presented in Figs. 1 and 4 and represents the area between the curves produced by forskolin in the absence and presence of different ligands. They correspond to mean $\pm$ S.E.M. (arbitrary area units) of at least three independent experiments. Data were analyzed by two-way analysis of variance, which revealed the effect of the drug ($P < 0.001$), treatment ($P < 0.001$), and an interaction ($P < 0.001$). Post hoc comparisons allowed us to establish differences in the magnitude of cAMP inhibition by different ligands, as indicated. $*P < 0.001$. (B) Data represent incremental changes in ligand-induced cAMP inhibition assessed in the presence of dynasore. Values were derived from areas defined in A that were divided into eight intervals of 112 seconds each. Parameters describing time-dependent changes in cAMP production and their comparison with those obtained in controls appear in Table 2.

nature but could be rather related to the fact that the response of DOR agonists was estimated from forskolin-stimulated and not basal cAMP levels. Rates of increase and decrease of the inhibitory cAMP response produced by DOR agonists were independent of one another, and factors that influenced the latter were unrelated to the former. Thus, deltorphin II, an agonist that was highly efficient in inducing signaling and internalization, reached maximal cAMP inhibition at the same rate as SB235863, whose signaling efficiency was among the lowest and whose internalization was minimal. On the other hand, ligand signaling ability and endocytosis promoted over the time course of the experiment were predictive of the rate of decline in of the initial cAMP response. We previously showed that DOR ligands display different internalization profiles in HEK cells and neurons (Charfi et al., 2013). It would therefore be of interest to corroborate whether the time course of cAMP responses follow the distinct internalization profiles displayed by different ligands in HEK cells and neurons.

Different observations point to endocytosis as a modulator of acute (15-minute) cAMP inhibition by DOR agonists: 1) the decay rate of cAMP inhibition was fastest for ligands that produced measurable internalization within the time frame of the experiment, and this rate was reduced by the internalization blocker dynasore; 2) the decay of the cAMP response started with a delay that is compatible with the time course of internalization; 3) internalizing ligands whose endocytic trafficking was susceptible to inhibition by dynasore displayed an increase of greater than 50% in the magnitude of their cAMP response after incubation with this internalization blocker; 4) ligands that triggered minimal internalization or those whose endocytic response was not significantly affected by dynasore underwent

reduced or no increment of their cAMP responses. In contrast, it was not possible to relate any of these aspects of the cAMP response to actual measures of ligand efficiency to promote endocytosis ($\log(\tau/K_A)$). Transduction coefficients combine efficacy (τ) and functional affinity (K_A) values as calculated by fitting experimental data with the operational model of Black and Leff (1983) and have been proposed as an overall descriptor of ligand “efficiency” to promote response (Black and Leff, 1983; Kenakin and Christopoulos, 2013). The fact that morphine, SB235863, and mcpTIPP did not generate meaningful internalization dose-response curves precluded estimation of sequestration efficiency for these ligands. However, for ligands like deltorphin II, DPDPE, met-enkephalin, and SNC-80, whose internalization $\log(\tau/K_A)$ coefficients were available, these were unrelated to the rate of decline of cAMP inhibition. Thus, although there is a clear polarization indicating that decline of cAMP response is faster for DOR agonists that produced internalization within the time frame of the experiment, sequestration efficiency values did not allow to predict the decline of cAMP inhibition by internalizing ligands.

In addition to endocytosis, the decay rate of cAMP responses was correlated to transduction coefficients [$\log(\tau/K_A)$] for $G\alpha_{i1}$ activation. Our data indicate that agonist efficiency to promote G-protein activation is predictive of the decay rate of cAMP signaling. These observations are consistent with those obtained by (McPherson et al., 2010), who showed that for most MOR agonists the ability to promote regulatory events, such as phosphorylation and arrestin recruitment, was correlated with ligand ability to activate G proteins. Unlike $G\alpha_{i1}$ activation coefficients, $\log(\tau/K_A)$ values describing ligand efficiency to inhibit cAMP production were not correlated with

Fig. 6. Inhibition of internalization by interfering with β arr function modifies the kinetics of cAMP inhibition by DOR agonists. HEK cells were transfected with DORs and the cAMP biosensor together with pcDNA3 vector (A) or mutant β arr (318–419). (B) On the day of the experiment, BRET measures were taken, as in Fig. 4, in cells pre-exposed to forskolin and DOR agonists as indicated. Results correspond to drug-induced changes in basal BRET and represent the mean $\pm$ S.E.M. of six independent experiments. Data were analyzed by two-way analysis of variance (ANOVA), which indicated an effect of drug ($P < 0.0001$) and time ($P < 0.0001$) with no interaction both in control cells and those transfected with mutant β arr. In the latter, post hoc comparisons revealed a significant difference between SNC-80 and morphine ($P < 0.0001$) that was absent in controls. (C) Histograms represent overall cAMP inhibition induced by different ligands in controls and cells transfected with mutant β arr. Values were estimated from data presented in A and B and correspond to mean $\pm$ S.E.M. (arbitrary area units) of six independent experiments. Data were analyzed by two-way ANOVA, which revealed an effect of drug ($P < 0.020$), of mutant ($P < 0.035$), and no interaction. Post hoc comparisons allowed us to establish differences in the magnitude of cAMP inhibition by SNC-80. * $P < 0.05$.

the decay rate for this response. This is not altogether surprising given that the further downstream in the signaling pathway modulatory factors that are independent of receptor–ligand interaction are progressively recruited. As a consequence, downstream readouts such as cAMP levels are expected to be weaker predictors of the regulation that takes place at the receptor level (Dang and Christie, 2012).

Inhibition of second-messenger production was also assessed over prolonged treatment periods, within a time frame that allowed internalization by deltorphin II, DPDPE, SNC-80, or met-enkephalin to attain its maximum, whereas that of morphine and SB235863 remained unchanged. A cAMP response was observed both for internalizing and

noninternalizing ligands, indicating that poor endocytosis is not a prerequisite for prolonged cyclase inhibition. Moreover, since mechanisms that trigger internalization are also involved in functional uncoupling of the receptor from the G protein (Lefkowitz, 1998; Ferguson, 2001; Violin and Lefkowitz, 2007; Zheng et al., 2010), it is not strictly possible to conclude that poor endocytosis was the only cause for prolonged cAMP signaling by morphine or SB235863. Indeed, morphine fails to produce DOR phosphorylation (Navratilova et al., 2005) and β arr2 recruitment to the receptor (Zhang et al., 1999), both of which participate in desensitization as much as they may contribute to endocytosis. On the other hand, DOR ligands that effectively trigger internalization also

Fig. 7. cAMP inhibition after sustained DOR activation by different agonists. (A) HEK293 cells expressing DORs and a BRET-based biosensor that detects cAMP production were incubated with forskolin and IBMX for 2 hours, together with different DOR agonists that were introduced into the incubation medium at progressively shorter intervals before taking BRET readings (inset). Results are expressed as the difference with respect to the mean basal BRET value for all experiments (0.146 ± 0.002) and represent mean $\pm$ S.E.M. of 7–10 independent experiments. Agonist effects were compared by means of a two-way analysis of variance that revealed an effect of drug ($P < 0.0001$) and time ($P < 0.0001$). Post hoc comparisons indicated that SB235863 ($P < 0.01$) and deltorphin II ($P < 0.05$) produced significant effects after 30, 60, and 120 minutes of incubation, whereas the effects of DPDPE and morphine were significant at 30 and 60 minutes ($P < 0.05$) and that of SNC-80 only at 60 minutes ($P < 0.05$). (B) FLAG-DORs were labeled at the membrane as described in the experimental section of this article. They were then exposed to deltorphin II, met-enkephalin, or SB235863 (10 μ M) for the indicated times before fixing, permeabilizing, and labeling for visualization. Throughout treatment with SB235863, DORs were localized predominantly at the membrane as in controls (white arrows). In the case of deltorphin II and met-enkephalin, DORs accumulated in the intracellular compartment (white arrowheads).

induce adaptations that lead to functional desensitization (Pradhan et al., 2010). Hence, if 60–120 minutes of treatment with deltorphin II internalizes 70% of membrane receptors and those remaining at the surface undergo desensitization, how can sustained exposure to deltorphin II and SB235863 produce similar levels of cAMP inhibition? A possible explanation is that deltorphin II is $\sim 20,000$ more efficient than SB235863 at activating signaling (see Table 3 transduction coefficients for G-protein activation) and that this difference compensates for the loss of functional membrane receptors. If this were the case, met-ENK, which is ~ 2000 -fold

more efficient than SB235863, would not be expected to lose all activity within the first 30 minutes of sustained stimulation. Assessment of the subcellular distribution of DORs by immunofluorescence indicated that receptors internalized by met-enkephalin, deltorphin II, DPDPE, and SNC-80 accumulated in the cytoplasm over the 2-hour period of the experiment. Thus, a simple and plausible alternative is that internalizing ligands that produce sustained cAMP inhibition do so via intracellular signaling. In support of this notion is the fact that G protein-coupled receptors, heterotrimeric G proteins, and adenylyl cyclase have been localized to endosomes,

where they function as a signaling unit (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009; Irannejad et al., 2013).

In conclusion, this study shows that ligand ability to evoke G-protein activation is a fairly good predictor of the time course of rapid cAMP inhibition by DOR agonists. Results also showed that internalization influences the time course of short-term cAMP responses, but the endocytic profile does not predict whether DOR agonists will display sustained cAMP inhibition.

Authorship Contributions

Participated in research design: Pineyro.

Conducted experiments: Tudashki, Robertson.

Contributed new reagents or analytic tools: Schiller.

Performed data analysis: Tudashki, Robertson, Pineyro.

Wrote or contributed to the writing of the manuscript: Tudashki, Robertson, Pineyro.

References

- Archer-Lahlou E, Audet N, Amraei MG, Huard K, Paquin-Gobeil M, and Pineyro G (2009) Src promotes delta opioid receptor (DOR) desensitization by interfering with receptor recycling. *J Cell Mol Med* **13**:147–163.
- Arttamangkul S, Quillinan N, Low MJ, von Zastrow M, Pintar J, and Williams JT (2008) Differential activation and trafficking of micro-opioid receptors in brain slices. *Mol Pharmacol* **74**:972–979.
- Arttamangkul S, Torrecilla M, Kobayashi K, Okano H, and Williams JT (2006) Separation of mu-opioid receptor desensitization and internalization: endogenous receptors in primary neuronal cultures. *J Neurosci* **26**:4118–4125.
- Audet N, Archer-Lahlou E, Richard-Lalonde M, and Piñeyro-Filpo G (2010) [Functional selectivity of opioid receptors ligands]. *Med Sci (Paris)* **26**:734–739.
- Audet N, Charfi I, Mnie-Filali O, Amraei M, Chabot-Doré AJ, Millicamps M, Stone LS, and Pineyro G (2012) Differential association of receptor-G $\beta\gamma$ complexes with β -arrestin2 determines recycling bias and potential for tolerance of δ opioid receptor agonists. *J Neurosci* **32**:4827–4840.
- Audet N, Galés C, Archer-Lahlou E, Vallières M, Schiller PW, Bouvier M, and Pineyro G (2008) Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *J Biol Chem* **283**:15078–15088.
- Beaudry H, Proteau-Gagné A, Li S, Dory Y, Chavkin C, and Gendron L (2009) Differential noxious and motor tolerance of chronic delta opioid receptor agonists in rodents. *Neuroscience* **161**:381–391.
- Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220**:141–162.
- Breton B, Lagacé M, and Bouvier M (2010) Combining resonance energy transfer methods reveals a complex between the alpha2A-adrenergic receptor, Galphai1-beta1gamma2, and GRK2. *FASEB J* **24**:4733–4743.
- Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, Persani L, and Lohse MJ (2009) Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol* **7**:e1000172.
- Charfi I, Nagi K, Mnie-Filali O, Thibault D, Balboni G, Schiller PW, Trudeau LE, and Pineyro G (2013) Ligand- and cell-dependent determinants of internalization and cAMP modulation by delta opioid receptor (DOR) agonists. *Cell Mol Life Sci*, in press.
- Dang VC and Christie MJ (2012) Mechanisms of rapid opioid receptor desensitization, resensitization and tolerance in brain neurons. *Br J Pharmacol* **165**:1704–1716.
- Doll C, Konietzko J, Pöhl F, Koch T, Höllt V, and Schulz S (2011) Agonist-selective patterns of μ -opioid receptor phosphorylation revealed by phosphosite-specific antibodies. *Br J Pharmacol* **164**:298–307.
- Edwards HV, Christian F, and Baillie GS (2012) cAMP: novel concepts in compartmentalised signalling. *Semin Cell Dev Biol* **23**:181–190.
- Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53**:1–24.
- Ferrandon S, Feinstein TN, Castro M, Wang B, Bouley R, Potts JT, Gardella TJ, and Vilardaga JP (2009) Sustained cyclic AMP production by parathyroid hormone receptor endocytosis. *Nat Chem Biol* **5**:734–742.
- Finn AK and Whistler JL (2001) Endocytosis of the mu opioid receptor reduces tolerance and a cellular hallmark of opiate withdrawal. *Neuron* **32**:829–839.
- Galés C, Rebois RV, Hogue M, Trieu P, Breit A, Hébert TE, and Bouvier M (2005) Real-time monitoring of receptor and G-protein interactions in living cells. *Nat Methods* **2**:177–184.
- Galés C, Van Durm JJ, Schaak S, Pontier S, Percherancier Y, Audet M, Paris H, and Bouvier M (2006) Probing the activation-promoted structural rearrangements in preassembled receptor-G protein complexes. *Nat Struct Mol Biol* **13**:778–786.
- Gregory KJ, Sexton PM, Tobin AB, and Christopoulos A (2012) Stimulus bias provides evidence for conformational constraints in the structure of a G protein-coupled receptor. *J Biol Chem* **287**:37066–37077.
- Hamdan FF, Rochdi MD, Breton B, Fessart D, Michaud DE, Charest PG, Laporte SA, and Bouvier M (2007) Unraveling G protein-coupled receptor endocytosis pathways using real-time monitoring of agonist-promoted interaction between beta-arrestins and AP-2. *J Biol Chem* **282**:29089–29100.
- He L, Kim JA, and Whistler JL (2009) Biomarkers of morphine tolerance and dependence are prevented by morphine-induced endocytosis of a mutant mu-opioid receptor. *FASEB J* **23**:4327–4334.
- Irannejad R, Tomshine JC, Tomshine JR, Chevalier M, Mahoney JP, Steyaert J, Rasmussen SG, Sunahara RK, El-Samad H, and Huang B et al. (2013) Conformational biosensors reveal GPCR signalling from endosomes. *Nature* **495**:534–538.
- Keith DE, Anton B, Murray SR, Zaki PA, Chu PC, Lissin DV, Monteillet-Agius G, Stewart PL, Evans CJ, and von Zastrow M (1998) mu-Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol Pharmacol* **53**:377–384.
- Kenakin T and Christopoulos A (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat Rev Drug Discov* **12**:205–216.
- Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci* **3**:193–203.
- Krupnick JG, Santini F, Gagnon AW, Keen JH, and Benovic JL (1997) Modulation of the arrestin-clathrin interaction in cells: characterization of beta-arrestin dominant-negative mutants. *J Biol Chem* **272**:32507–32512.
- Leduc M, Breton B, Galés C, Le Guillou C, Bouvier M, Chemtob S, and Heveker N (2009) Functional selectivity of natural and synthetic prostaglandin EP4 receptor ligands. *J Pharmacol Exp Ther* **331**:297–307.
- Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* **273**:18677–18680.
- Macia E, Ehrlich M, Massol R, Boucrot E, Brunner C, and Kirchhausen T (2006) Dynasore, a cell-permeable inhibitor of dynamin. *Dev Cell* **10**:839–850.
- Martini L and Whistler JL (2007) The role of mu opioid receptor desensitization and endocytosis in morphine tolerance and dependence. *Curr Opin Neurobiol* **17**:556–564.
- Masri B, Salahpour A, Didriksen M, Ghisi V, Beaulieu JM, Gainetdinov RR, and Caron MG (2008) Antagonism of dopamine D2 receptor/beta-arrestin 2 interaction is a common property of clinically effective antipsychotics. *Proc Natl Acad Sci USA* **105**:13656–13661.
- McDowell TS (2003) Fentanyl decreases Ca²⁺ currents in a population of capsaicin-responsive sensory neurons. *Anesthesiology* **98**:223–231.
- McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C, Dewey WL, Bailey CP, Rosethorne EM, and Charlton SJ et al. (2010) μ -opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol Pharmacol* **78**:756–766.
- Molinari P, Vezzi V, Sbraccia M, Grò C, Riitano D, Ambrosio C, Casella I, and Costa T (2010) Morphine-like opiates selectively antagonize receptor-arrestin interactions. *J Biol Chem* **285**:12522–12535.
- Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, and Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. *Nat Chem Biol* **5**:428–434.
- Nagi K and Pineyro G (2011) Regulation of opioid receptor signalling: implications for the development of analgesic tolerance. *Mol Brain* **4**:25.
- Navratilova E, Eaton MC, Stropova D, Varga EV, Vanderah TW, Roeske WR, and Yamamura HI (2005) Morphine promotes phosphorylation of the human delta-opioid receptor at serine 363. *Eur J Pharmacol* **519**:212–214.
- Patierno S, Anselmi L, Jaramillo I, Scott D, Garcia R, and Sternini C (2011) Morphine induces μ opioid receptor endocytosis in guinea pig enteric neurons following prolonged receptor activation. *Gastroenterology* **140**:618–626.
- Pradhan AA, Becker JA, Scherrer G, Tryoen-Toth P, Filliol D, Matifas A, Massotte D, Gavériaux-Ruff C, and Kieffer BL (2009) In vivo delta opioid receptor internalization controls behavioral effects of agonists. *PLoS ONE* **4**:e5425.
- Pradhan AA, Walwyn W, Nozaki C, Filliol D, Erbs E, Matifas A, Evans C, and Kieffer BL (2010) Ligand-directed trafficking of the δ -opioid receptor in vivo: two paths toward analgesic tolerance. *J Neurosci* **30**:16459–16468.
- Raehal KM, Schmid CL, Groer CE, and Bohn LM (2011) Functional selectivity at the μ -opioid receptor: implications for understanding opioid analgesia and tolerance. *Pharmacol Rev* **63**:1001–1019.
- Richard-Lalonde M, Nagi K, Audet N, Sleno R, Amraei M, Hogue M, Balboni G, Schiller PW, Bouvier M, and Hébert TE et al. (2013) Conformational dynamics of Kir3.1/Kir3.2 channel activation via δ -opioid receptors. *Mol Pharmacol* **83**:416–428.
- Salahpour A, Espinoza S, Masri B, Lam V, Barak LS, and Gainetdinov RR (2012) BRET biosensors to study GPCR biology, pharmacology, and signal transduction. *Front Endocrinol (Lausanne)* **3**:105.
- Thompson HM and McNiven MA (2006) Discovery of a new 'dynasore'. *Nat Chem Biol* **2**:355–356.
- Violin JD, DiPilato LM, Yildirim N, Elston TC, Zhang J, and Lefkowitz RJ (2008) beta2-adrenergic receptor signaling and desensitization elucidated by quantitative modeling of real time cAMP dynamics. *J Biol Chem* **283**:2949–2961.
- Violin JD and Lefkowitz RJ (2007) Beta-arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* **28**:416–422.
- Williams JT, Ingram SL, Henderson G, Chavkin C, von Zastrow M, Schulz S, Koch T, Evans CJ, and Christie MJ (2013) Regulation of μ -opioid receptors: desensitization, phosphorylation, internalization, and tolerance. *Pharmacol Rev* **65**:223–254.
- Zhang J, Ferguson SS, Law PY, Barak LS, and Caron MG (1999) Agonist-specific regulation of delta-opioid receptor trafficking by G protein-coupled receptor kinase and beta-arrestin. *J Recept Signal Transduct Res* **19**:301–313.
- Zheng H, Loh HH, and Law PY (2010) Agonist-selective signaling of G protein-coupled receptor: mechanisms and implications. *IUBMB Life* **62**:112–119.

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