Quantification of kappa opioid receptor ligand potency, efficacy and desensitization using a real-time membrane potential assay

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Abstract

We explored the utility of the real-time FLIPR Membrane Potential (FMP) assay as a method to assess kappa opioid receptor (KOR)-induced hyperpolarization. The FMP Blue dye was used to measure fluorescent signals reflecting changes in membrane potential in KOR expressing CHO (CHO-KOR) cells. Treatment of CHO-KOR cells with kappa agonists U50,488 or dynorphin [Dyn (1–13)NH\textsubscript{2}] produced rapid and concentration-dependent decreases in FMP Blue fluorescence reflecting membrane hyperpolarization. Both the nonselective opioid antagonist naloxone and the \(\kappa\)-selective antagonists nor-binaltorphimine (nor-BNI) and zyklophin produced rightward shifts in the U50,488 concentration-response curves, consistent with competitive antagonism of the KOR mediated response. The decrease in fluorescent emission produced by U50,488 was blocked by overnight pertussis toxin pretreatment, indicating the requirement for PTX-sensitive G proteins in the KOR mediated response. We directly compared the potency of U50,488 and Dyn (1–13)NH\textsubscript{2} in the FMP and \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assays, and found that both were approximately 10 times more potent in the cellular fluorescence assay. The maximum responses of both U50,488 and Dyn (1–13)NH\textsubscript{2} declined following repeated additions, reflecting receptor desensitization. We assessed the efficacy and potency of structurally distinct KOR small molecule and peptide ligands. The FMP assay reliably detected both partial agonists and stereoselectivity. Using KOR-selective peptides with varying efficacies, we found that the FMP assay allowed high throughput quantification of peptide efficacy. These data demonstrate that the FMP assay is a sensitive method for assessing \(\kappa\)-opioid receptor induced hyperpolarization, and represents a useful approach for quantification of potency, efficacy and desensitization of KOR ligands.

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CRedit authorship contribution statement

Yuanzi Zhao, Jane V. Aldrich, Thomas F. Murray: Participated in research design, Wrote or contributed to writing of the manuscript. Yuanzi Zhao: Conducted experiments. Anand A. Joshi: Zyklophin analog synthesis. Yuanzi Zhao, Thomas F. Murray: Performed data analysis.

Conflict of interest statement

We declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
Keywords
Opioid; Kappa receptor; Hyperpolarization; Fluorescence; Dynorphin; Efficacy

1. Introduction

The \(\kappa\)-opioid receptor (KOR) is a member of the G-protein-coupled receptor (GPCR) super family, and when activated by agonist ligands couples to heterotrimeric Go\(i/o\) proteins, resulting in inhibition of adenylyl cyclase, decreased calcium conductance or increased potassium conductance [27]. The primary endogenous peptide for the KOR has 17 amino acids (dynorphin A\(1\text{--}17\)); however, the shortened 13- amino acid peptide (dynorphin A\(1\text{--}13\)) is often used in molecular and cellular assays. More recent studies have shown that activation of GPCRs such as the KOR can generate, in addition to G protein-dependent signaling, G protein-independent signaling [7]. G protein-independent signaling occurs following \(\beta\)-arrestin recruitment to the phosphorylated GPCR, where \(\beta\)-arrestin functions as a scaffold enabling signaling through different molecules [12]. This discovery has led to the concept of ligand-directed receptor signaling, also referred to as functional selectivity or biased agonism at GPCRs.

Activation of the KORs produces conformational changes and dissociation of the pertussis toxin–sensitive G-protein alpha subunits, thereby activating G-protein–gated inwardly rectifying potassium channels (GIRKs), which lead to membrane hyperpolarization [42]. Inasmuch as GPCR signaling consists of a series of rapid coupling events, a useful functional assay for GPCR ligand screening and characterization should allow real-time detection of robust signals. Agonist stimulated \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding or adenylyl cyclase inhibition assays have classically been used to characterize the efficacy and potency of opioid receptor small molecule and peptide ligands [1,33]. Agonist binding to GPCRs triggers the formation of the agonist/GPCR/G-protein ternary complex followed by dissociation of GDP. The stimulation of \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) membrane binding therefore detects the initial event following receptor activation in the absence of signal amplification [36]. The performance of the \([^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assay is influenced by the concentrations of GDP, Mg\(^{2+}\) and Na\(^{+}\) ions, and the relative efficacy of partial agonists may be increased by, for example, reducing the concentration of GDP or Na\(^{+}\) [39,43]. Detection of opioid ligand-induced inhibition of adenylyl cyclase activity typically requires the presence of forskolin to elevate cyclic-AMP accumulation [45].

The KOR is currently a therapeutic target for mood and reward-related diseases, including depression and addiction, as well as pain and pruritis [10,13,52]. However, KOR agonists produce adverse effects such as dysphoria, sedation and diuresis which limit the clinical utility of these compounds [52]. KOR antagonists have potential therapeutic applications in the treatment of drug abuse inasmuch as they have been shown to block stress-induced reinstatement of cocaine administration [3]. However, prototypical non-peptide selective KOR antagonists such as nor-BNI and JDTic have extremely long durations of action in vivo, lasting weeks after a single dose, an effect that limits their potential therapeutic development [31]. KOR peptide antagonists such as zyklophin derived from dynorphin A
by modifications in the C-terminal domain exhibit highly selective antagonism of KOR activity [33]. Compared with non-peptide selective KOR antagonists such as nor-BNI, zyklophin demonstrated a much shorter duration of antagonism, lasting less than 12 h after systemic administration, which makes it an ideal pharmacological tool for development of new therapeutic agents [3].

Although electrophysiological studies provide a means to characterize ion channel regulation with temporal resolution capable of tracking millisecond kinetics of activation and inactivation, whole cell patch clamp technologies are not easily amenable to high throughput evaluation of compound libraries for drug discovery. Here we sought to explore the utility of the FLIPR Membrane Potential (FMP) Blue assay as a method to assess KOR-induced hyperpolarization. The FMP Blue dye was used to measure fluorescent signals reflecting changes in membrane potential in KOR expressing CHO (CHO-KOR) cells. The FMP dye is 14-fold faster than the conventional bis-oxonol sensitive probe DIBAC$_{3(3)}$ in response to KCl-induced depolarization, and accordingly the kinetics of fluorescence change of FMP is well correlated with that observed in electrophysiological studies using whole-cell current clamp [5]. We developed a real-time FMP assay to assess small molecule and peptide ligand interaction with KORs. The FMP Blue dye fluorescence intensity was decreased by KOR activation, reflecting the hyperpolarizing influence of KOR agonists. This assay was used to characterize the pharmacological properties of analogs of the peptide zykophin which were designed by modifying the N- and C-terminal domains and cyclic constraint of zyklophin. We demonstrate that this continuous fluorescence assay permitted the characterization of ligand potency, efficacy and KOR desensitization in CHO cells expressing the KOR.

2. Materials and methods

2.1. Materials

U50488 and nor-BNI were purchased from TOCRIS (Minneapolis, MN). Dyn (1–13)NH$_2$, DAMGO and DPDPE were purchased from American Peptide (Sunnyvale, CA). RPMI-1640 medium, naloxone, pertussis toxin (PTX), peptidase inhibitors including bestatin, captopril and L-leucyl-L-leucine, and GDP were purchased from Sigma-Aldrich (St. Louis, MO). [$^{35}$S]GTP$\gamma$S was purchased from Perkin Elmer (Waltham, MA). Penicillin and trypsin were purchased from Life Technologies (Carlsbad, California). FBS was purchased from Atlanta Biologicals (Lawrenceville, GA). Zyklophin and its analogs were synthesized as described previously [22,34].

2.2. Cell culture

CHO cells stably expressing cloned rat kappa opioid receptors (KOR-CHO) were grown in T-75 flasks in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO$_2$ and 95% humidity atmosphere as described previously [6]. One day prior to the experiment, KOR-CHO cells were detached from the flask with trypsin/EDTA and resuspended in 10 mL RPMI-1640 medium with 10% FBS. For FMP assay, the cells were plated at a density of 0.2 x 10$^6$ in a 150-µl volume in 96-well microplates with clear bottom (MIDSCI, St. Louis, MO) and incubated overnight in the CO$_2$ incubator. For [$^{35}$S]GTP$\gamma$S
binding assays, cells were grown in T-75 flasks in RPMI-1640 medium with 10% FBS overnight in the CO\textsubscript{2} incubator.

2.3. FMP blue assay

Membrane potential in the KOR-CHO cell cultures was determined by using the FLIPR Membrane Potential (FMP) Blue assay (Molecular Devices, Sunnyvale, CA) as described previously [17]. FMP Blue dye was used to assess the membrane potential of KOR-CHO cells. FMP dye is a lipophilic, negatively charged, bis-oxonol-based dye, and during membrane depolarization fluorescent signal increases in intensity as the dye follows the positively charged ions inside the cell and binds to intracellular proteins. During membrane hyperpolarization the fluorescent signal decreases in intensity as the cell interior becomes more negative. The dye stock solution (1X) was prepared by adding 10 mL Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 1.0 mM MgCl\textsubscript{2}, 2.3 mM CaCl\textsubscript{2}, 8.6 mM HEPES, 5.6 mM glucose, 0.1 mM glycine, pH 7.4) to the content of each vial. For loading the cells 8-fold diluted stock solutions were used as suggested previously [17,21]. After removing the culture medium, 180 μl of assay buffer was added to the cells, and the plate was incubated at 37 °C in a 5% CO\textsubscript{2} and 95% humidity atmosphere for 30 min. Afterwards, the plate was transferred to a Flex Station™ II (Molecular Devices, Sunnyvale, CA) chamber, and the fluorescence was measured in the cells with excitation at a wavelength of 530 nm and emission recorded at 565 nm. Baseline recording was taken for 60 s at 2 s intervals. After recording the baseline, either 20 μl of U50488, dynorphin A(1–13)NH\textsubscript{2} or vehicle was added to give a final volume of 200 μl at a rate of 26 μl/s, and the fluorescence was monitored for an additional 240 s. For experiments assessing the influence of kappa receptor antagonists these compounds were added and cells were incubated for an additional 10 min prior to agonist addition.

To ensure the veracity of comparisons of IC\textsubscript{50} and the maximum response (E\textsubscript{max}) values of the KOR ligands, all compounds were evaluated in parallel on the same 96-well plate, with the same passage of cells and with identical reagent solutions. This experimental design was used for all drug and peptide comparisons throughout this study. Inasmuch as all assays were performed in the same KOR expressing CHO cell line, we can exclude differences in cellular context as a source of observed differences in drug or peptide potency or efficacy.

2.4. Preparation of KOR-CHO cells membrane

KOR-CHO cells were harvested at confluence and homogenized in ice-cold 25 mM Tris buffer (pH 7.4) and then centrifuged at 18,000 rpm for 25 min. The supernatant was discarded, and the cell pellets were resuspended in Tris buffer, homogenized and centrifuged an additional three times as described above. Protein concentrations of the membranes were determined using the Bradford method with bovine serum albumin as the standard [6].

2.5. \textsuperscript{35}S\textsubscript{GTP\gamma S} binding assay

The binding of the GTP analog \textsuperscript{35}S\textsubscript{GTP\gamma S} to membranes was assayed following the method described previously [41]. Binding was determined in a volume of 500 μl. The assay mixture contains 25 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM magnesium acetate, 3 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 0.7 mg bovine serum albumin per
mL, and approximately 20,000 disintegrations per min (dpm) $[^{35}\text{S}]\Gamma\text{TP} \gamma \text{S} \ (0.1-0.2 \ nM)$. Approximately 15 μg of KOR expressing CHO cell membrane protein was used per tube. Following 90 min incubation at 22 °C, the assay was terminated by filtration under vacuum on a Brandel (Gaithersburg, MD) model M-48R cell harvester using Schleicher and Schuell Inc. (Keene, NH) number 32 glass fiber filters. The filters were rinsed with 4 x 4-mL washes of ice-cold 50 mM Tris HCl, pH 7.4, 5 mM MgCl, at 5 °C, to remove unbound $[^{35}\text{S}]\Gamma\text{TP} \gamma \text{S}$. Filter disks were then placed into counting vials to which 8 mL of Biocount scintillation fluid (Research Products International Corp., Mount Prospect, IL) was added. Radioactivity on filters was determined by liquid scintillation spectrometry (Beckman Instruments, Fullerton, CA) following overnight extraction at room temperature.

2.6. Adenylyl cyclase assay

KOR ligands were evaluated for their ability to inhibit the synthesis of cyclic adenosine monophosphate (cAMP) by adenylyl cyclase (AC) using cloned rat κappa opioid receptors stably expressed in CHO cells as previously described [6]. Cells were washed twice with free F12 medium and then incubated for 4 h in 1 mL of the same media containing 12 μCi $[^{3}H]\text{adenine}$. The cells were then incubated at 37 °C for 40 min in the presence of 50 μM forskolin, peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine), and varying concentrations of ligands (0.1 – 10,000 nM in 10-fold dilutions). Incubations were terminated by the addition of 30 μl of stop solution (2% sodium dodecyl sulfate and 1.3 mM cAMP in water), followed by the addition of 100 μl of concentrated perchloric acid and 750 μl of water. $[^{14}\text{C}]\text{cAMP} \ (500 \ cpm \ in \ 50 \ \mu l)$ was added to each well to correct for recovery. After transferring the contents of the wells to 1.5 mL centrifuge tubes, 12 M KOH was added to neutralize the samples. The resulting precipitates were pelleted by centrifugation at 10,000 g for 10 min cAMP in the supernatants was isolated by sequential chromatography over BioRad AG-50 W-X4 cation exchange resin and neutral alumina columns. The concentrations of $[^{3}H]\text{cAMP}$ and $[^{14}\text{C}]\text{cAMP}$ in the eluants were determined simultaneously by scintillation counting. Counts were corrected for crossover and recovery.

2.7. Data analysis

All concentration-response data were analyzed and graphs generated using GraphPad Prism 7.0 software. IC$_{50}$ and E$_{\text{max}}$ values for decreases in FMP blue fluorescence were determined by nonlinear regression least-squares fitting of a three parameter logistic equation to the agonist concentration-response data. The 95% confidence intervals (CIs) for all IC$_{50}$ and E$_{\text{max}}$ values were used to assess differences in potency and efficacy. The efficacies of KOR ligands are expressed relative to the reference agonist Dyn (1–13)NH$_{2}$.

3. Results

3.1. U50,488 and Dyn (1–13)NH$_{2}$ produce a rapid decrease in FMP fluorescence in CHO-KOR cells

Dyn (1–13)NH$_{2}$ is a fragment of the full length endogenous peptide Dyn (1–17) that acts as a kappa receptor full agonist [2]. U50,488 is a KOR-selective non-peptide full agonist. We assessed U50,488- and Dyn (1–13)NH$_{2}$-induced membrane potential changes in
CHO-KOR cells using the membrane-potential sensitive fluorescence dye, FMP Blue. FMP dye-loaded CHO-KOR cells were recorded in a FlexStation®2 for 300 s. After baseline recording for 60 s, the addition of increasing concentrations of U50,488 (0.01–100 nM) or Dyn (1–13)NH₂ (0.0001–100 nM) produced rapid and concentration-dependent decreases in FMP fluorescence intensity, reflecting membrane hyperpolarization as a consequence of KOR activation of K⁺ channels (Fig. 1, A and B). Nonlinear regression analysis of the concentration-response data for U50,488 (Fig. 1 C) and Dyn (1–13)NH₂ (Fig. 1 D) yielded IC₅₀ values of 0.25 nM for U50,488 (95% CI, 0.10–0.60 nM) and 0.16 nM for Dyn (1–13)NH₂ (95% CI, 0.06–0.43 nM). These data demonstrate that the KOR reference compounds U50,488 and Dyn (1–13)NH₂ are potent, high efficacy agonists in the FMP Blue assay.

3.2. U50,488-induced hyperpolarization is mediated by κ-opioid receptors and requires pertussis toxin sensitive G-proteins

The specificity of the hyperpolarization response to U50,488 and Dyn (1–13)NH₂ was next determined by exposing CHO-KOR cells to a range of concentrations of the δ opioid receptor selective peptide cyclo [D-Pen²,D-Pen⁵]enkephalin (DPDPE, Pen = penicillamine) or the μ-opioid receptor selective peptide [D-Ala²,NMePhe⁴]enkephalin-glyol (DAMGO). Consistent with a KOR-mediated response, neither DPDPE nor DAMGO triggered a decrease in FMP fluorescence intensity in CHO-KOR cells (Fig. 2. A–D). As a first step to confirm the KOR involvement in U50,488-induced membrane hyperpolarization, we tested the ability of the nonselective opioid antagonist naloxone to produce a rightward shift in the U50,488 concentration-response curves. Increasing concentrations of naloxone (10–10,000 nM) produced progressively greater rightward shifts in U50,488 concentration-response curves while having no effect on FMP fluorescence when administered alone (Fig. 3. A). A Schild plot of these data yielded a naloxone Kₐ value of 3.16 nM, which is in good agreement with the affinity of naloxone for KORs [30]. We then tested the ability of both the KOR-selective peptide antagonist zyklophin (300–10,000 nM) and the selective non-peptide antagonist nor-BNI (0.01–10 nM) to shift U50,488 concentration-response curves. Neither zyklophin nor nor-BNI alone influenced FMP fluorescence; however, increasing concentrations of both KOR-selective antagonists produced rightward shifts in U50,488 concentration-response curves. Schild regression analysis revealed Kₐ values of 564 nM and 0.07 nM for zyklophin and nor-BNI, respectively. These Kₐ values for zyklophin and nor-BNI Kₐ are in good agreement with those found in previous studies using, respectively, adenylyl cyclase inhibition or suppression of spontaneous Ca²⁺ oscillation assays [23,34].

The zyklophin and nor-BNI antagonism of U50,488-induced hyperpolarization in CHO-KOR cells confirm the KOR involvement in the hyperpolarization response.

Kappa agonist-induced increases in K⁺ conductance have been shown to be mediated through pertussis toxin-sensitive G proteins [9, 28]. To confirm the G protein involvement in hyperpolarizing action of U50,488, CHO-KOR cells were treated overnight in the absence or presence of pertussis toxin (100 ng/mL) prior to the FMP Blue assay [32]. Pertussis toxin pretreatment completely abolished the U50, 488-induced decrease in the FMP fluorescence (Fig. 4B and C), suggesting that the effect of U50,488 on membrane hyperpolarization requires the involvement of pertussis toxin-sensitive G proteins.
3.3. Comparison of kappa agonist potencies in the FMP and \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assays

Agonist-stimulated binding of \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} to G proteins has been widely used to probe GPCR activation [46]. We therefore sought to compare the potency of KOR agonists in the FMP Blue and \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assays. This comparison was routinely performed in side-by-side experiments on the same day using CHO-KOR cells or a membrane preparation derived from the same CHO-KOR cell culture. One day prior to the assays, CHO-KOR cells were grown either in 96-well plates for the FMP assay or in T-75 flask for membrane isolation for subsequent \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assays. As depicted in Fig. 5A and D, U50,488 and Dyn (1–13)NH\(_2\) produced rapid, real-time decreases in FMP fluorescence with respective IC\(_{50}\) values of 0.13 nM (95% CI, 0.07–0.25 nM) and 0.39 nM (95% CI, 0.24–0.64 nM). Using membranes derived from the same CHO-KOR cultures for the \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assay yielded EC\(_{50}\) values from concentration-response data for U50,488 and Dyn (1–13) NH\(_2\) of 2.13 nM (95% CI, 1.42–3.21 nM) and 4.19 nM (95% CI, 3.07–5.72 nM), respectively. This side by side comparison revealed that both U50,488 and Dyn (1–13)NH\(_2\) were approximately 10 times more potent in the whole cell, real-time FMP fluorescence assay as compared to the fixed-time, isolated membrane \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding assay. While \[^{35}\text{S}]\text{GTP}_{\gamma}\text{S} binding measures the first step in the KOR signal transduction process, additional signal amplification through G-protein beta/gamma subunit activation of potassium channel activity in whole cells with less time for KOR desensitization likely accounts for the enhanced potency in the FMP assay.

3.4. KOR agonist-induced receptor desensitization

Receptor desensitization has been defined by a decrease in receptor-mediated signaling in response to prolonged application of agonists [48]. We reasoned that the real-time FMP fluorescence assay with intact cells would afford the opportunity to assess whether the sensitivity of KOR activation of K\(^+\) channels is reduced following repeated activation of KOR. A series of three acute concentration-response profiles with either U50,488 (0.01–30 nM) or Dyn (1–13)NH\(_2\) (0.001–30 nM) was therefore performed in CHO-KOR cells loaded with FMP dye (Fig. 6. A and B). The initial challenge with either U50,488 or Dyn (1–13)NH\(_2\) produced the expected rapid decreases in FMP fluorescence; however, the magnitude of this response was progressively diminished following the second and third challenge at 6 min intervals (Fig. 6. A and B). Independent analysis of these concentration-response data for each challenge with U50,488 or Dyn (1–13)NH\(_2\) revealed no significant changes in the observed IC\(_{50}\) values, but progressive decreases in the maximum responses (Fig. 6C and D; Table 1). These findings indicate that the real-time FMP assay allows for detection of both peptide and non-peptide agonist-induced KOR desensitization following repeated exposure of CHO-KOR cells.

To ensure that the observed decline in fluorescence was a measure of desensitization and not K\(^+\) channel rundown as a consequence of intracellular ATP depletion during the 18 min total recording time [35], Dyn (1–13)NH\(_2\) was applied to the CHO-KOR cells following two previous 6 min exposures to Locke’s buffer. This experimental design controlled for the presence of cellular changes as a function of incubation time in the desensitization experiment. As shown in Fig. 7 the administration of Dyn (1–13)NH\(_2\) following two 6 min exposures to Locke’s buffer produced a similar reduction in FMP fluorescence (\(E_{\text{max}}\)...
= 11,198, 95% CI = 9182–13,213) as the initial acute response to Dyn (1–13)NH₂ (Eₘₐₓ = 14,276, 95% CI = 11,005–17,548) (Fig. 7. A and C). The Dyn (1–13)NH₂ concentration-response relationships for the initial acute and the delayed challenge after two exposures to Locke’s did not differ significantly (Fig. 7. C and D). These findings indicated that neither K⁺ channel nor cellular rundown contributed significantly to the observed desensitization of the response to Dyn (1–13)NH₂ in CHO-KOR cells.

3.5. Detection of KOR partial agonists and stereoselectivity using FMP assay

We next assessed the fidelity of the FMP assay by testing kappa receptor ligands with known differences in either efficacy or stereoselectivity. Using the FMP assay we compared the IC₅₀ and maximum response values of U50,488 to those of the nonselective partial KOR agonists pentazocine and the stereoisomers of cyclazocine. Both (−)-cyclazocine and pentazocine acted as partial agonists in the FMP assay, with respective maximal decreases of fluorescence intensity of 64% and 44%, respectively, of that produced by the full agonist U50,488, (Fig. 8A, B, D and E). In contrast, the inactive stereoisomer, (+)-cyclazocine, did not affect FMP fluorescence (Fig. 8C, E and Table 2). This stereoselectivity for cyclazocine isomers at KOR correlates well with previous studies in which (+)-cyclazocine did not display agonist activity in a [³⁵S]GTPγS binding assay [38]. The rank order of efficacy and respective potencies of these KOR ligands are provided in Tables 2 and (3).

3.6. Determination of efficacy of novel dyn (1–13)NH₂ analogs using the FMP, [³⁵S]GTPγS binding and adenylyl cyclase assays

We have previously shown that modifications in the “address” domain of Dyn analogues may affect peptide efficacy [33]. Indeed, a cyclic analogue, zyklophin, was found to have minimal efficacy in the adenylyl cyclase inhibition assay, and rather displayed antagonist activity (Kᵦᵦ = 84 nM) at KORs in this assay. Here we have determined the efficacies of additional zyklophin-based peptide analogs that were designed by modifying the N- and C-terminal domains and cyclic constraint of zyklophin to generate the novel cyclic peptide analogs, JVA 3418, JVA 3419, JVA 3413, and the linear peptide JVA 3416 (Fig. 9. A). To determine the efficacies of these peptide analogs we compared them to Dyn (1–13)NH₂ and zyklophin in the CHO-KOR FMP assay. As shown in Figs. 9 and 10, zyklophin did not elicit a significant reduction in FMP fluorescence and the peptide analogs of zyklophin all displayed lower efficacies than the reference full agonist Dyn (1–13)NH₂. Of all the zyklophin analogs tested, the linear peptide derivative JVA 3416 displayed the highest efficacy (relative efficacy = 0.48). The rank order of peptide ligand efficacies in the FMP fluorescence assay was Dyn (1–13)NH₂ > JVA 3416 > JVA 3418 ≥ JVA 3419 ≥ JVA 3418 > zyklophin.

We then compared efficacies determined with the FMP assay to those derived from the agonist-stimulated [³⁵S]GTPγS binding assay in cell membranes from CHO-KOR cells. As shown in the summary in Table 2, the rank order of efficacy profile was similar in the FMP and [³⁵S]GTPγS binding assays. Zyklophin again exhibited negligible efficacy and the linear analog JVA 3416 was the most efficacious of the zyklophin analogs in the [³⁵S]GTPγS binding assay. Similar to the FMP assay, the rank order efficacy profile for stimulation of [³⁵S]GTPγS binding was Dyn (1–13)NH₂ > JVA 3416 > JVA 3418 ≥ JVA 3418 > JVA
3413 = JVA 3419 > zyklophin. Since inhibition of forskolin-stimulated adenylyl cyclase has been commonly used to determine opioid agonist efficacies, we also used this whole cell assay in CHO-KOR cells to assess peptide efficacies. The ability of peptides to inhibit forskolin-stimulated cyclic AMP accumulation yielded a rank order of efficacies of Dyn (1–13)NH₂ = JVA 3416 > JVA 3418 > JVA 3413 = JVA 3419 > zyklophin, indicating that all three assays report similar profiles for opioid peptides. The absolute efficacy values in the adenylyl cyclase assay were somewhat greater than those observed in the FMP and [³⁵S]GTPγS binding assays; this latter difference has been noted previously [16].

4. Discussion

We have demonstrated here that KOR-induced hyperpolarization resulting from coupling to Gᵢₒ was reliably detected using the real-time FMP membrane potential assay. We used the FMP Blue dye to measure fluorescence changes reflecting alteration of membrane potential in CHO-KOR cells. The FMP fluorescence signal decreased rapidly following application of KOR agonists, and these effects of agonists were attenuated by both the nonselective opioid receptor antagonist naloxone, as well as the KOR-selective antagonists zyklophin (peptide) and nor-BNI (non-peptide). The rapid response time of the FMP Blue fluorescence renders it highly suitable for real-time measurement of membrane potential changes. Previous direct comparisons of the kinetics of FMP Blue and the oxonol dye DiBAC₄ demonstrated that the time to half-maximal response was approximately 10-fold faster for FMP Blue than DiBAC₄ [5,49]. In addition to the kinetic superiority, FMP Blue dye is superior to DiBAC₄ with respect to drug-quenching effects, temperature insensitivity and dilution artifacts [47]. Furthermore, a good temporal correlation between FMP fluorescence responses and changes of membrane potential induced by high K⁺ has been reported for current clamped CHO cells [5]. More recently, Fairless et al., [15] demonstrated that the FMP Blue dye reliably reports both depolarization and hyperpolarization events in whole cell assays.

To demonstrate the general applicability of this assay to rigorously assess the pharmacological properties of KOR ligands, we determined the ability of the FMP assay to detect kappa opioid receptor partial agonists and stereoselectivity. To this end the pharmacological profiles of (±)-pentazocine and cyclazocine enantiomers were evaluated. Using U50,488 as a reference full agonist ligand, both (−)-cyclazocine and (±)-pentazocine displayed partial agonist activity, whereas (+)-cyclazocine lacked demonstrable efficacy. The observed cyclazocine stereoselectivity and relative potency and efficacy of pentazocine correlate well with previous studies [18,38]. This establishes this assay as a robust and sensitive method to assess the pharmacological signature of KOR ligands.

Commonly used assays to monitor ligand interaction with GPCRs that couple to Gᵢₒ include agonist-stimulated [³⁵S]GTPγS binding. We therefore directly compared the potency of the reference KOR agonists U50,488 and Dyn (1–13)NH₂ with those in [³⁵S]GTPγS binding assay. Although the EC₅₀ values observed in our [³⁵S]GTPγS binding assays are in good agreement with those of previous studies [40,51], we found both KOR agonists to be approximately 10-fold more potent in the FMP assay. The [³⁵S]GTPγS binding assay requires prolonged incubation followed by cell lysis at a fixed time point (90 min herein). In contrast to real-time assays, this requirement for prolonged incubation in the [³⁵S]GTPγS
assay may decrease the sensitivity to ligands that evoke GPCR desensitization during the incubation.

The real-time FMP assay allowed the assessment of the stability of the hyperpolarization response over time following exposure to either U50,488 or Dyn (1–13)NH₂. For both reference full agonists the peak hyperpolarization response occurred at approximately 30 s after exposure and then waned over the subsequent 210 s of the assay. KOR desensitization was apparent after agonists were applied repeatedly. After a 6 min exposure to KOR, repeated administration of U50,488 and Dyn (1–13)NH₂ produced blunted decreases in the maximum response (Fmax) by 44% and 41%, respectively, in FMP fluorescence, and a third exposure to these KOR agonists for another 6 min further diminished their maximum responses further by 68% and 60%, respectively. These results are in agreement with a previous report that pretreatment of CHO cells expressing the human KOR with 1 μM U50,488 for 15 min reduced the maximal response of U50,488-induced [35S]GTPγS binding without increasing the EC₅₀ value [51]. Similarly, in Xenopus oocytes expressing the rat KOR and Kir3 channel, the U69,593-induced activation of a K⁺ current desensitized by about 20% during a 10 min agonist application [4]. Moreover, in AtT-20 cells transfected with the rat KOR-green fluorescence protein, McLaughlin et al. [29] found that the rat KOR-GFP was desensitized by 0.1 μM U50,488 pretreatment for 1 h, using agonist-enhanced K⁺ current as the functional endpoint.

Endogenous K⁺ channels in CHO-K1 cells are not well defined, and these cells are known to express low levels of these channels [50]. RT-PCR analysis of CHO-K1 cells has failed to demonstrate the presence of mRNA expression of any isoform of GIRK channels (Kir3.x) [26]. CHO cells do however express native K⁺ channels that are sensitive to both membrane potential and intracellular free Ca²⁺ concentration [44]. Elevation of cyclicAMP in CHO cells moreover produces a hyperpolarization due to K⁺ channel activation [24]. Hence one potential mechanism for kappa receptor mediated hyperpolarization is through G-protein beta-gamma subunit modulation of adenylyl cyclase activity. Given that kappa receptors expressed in CHO cells have been reported to elevate intracellular free Ca²⁺ from IP₃ sensitive stores [20], an additional possibility is activation of native Ca²⁺ dependent K⁺ channels. We have previously provided evidence for endogenous Ca²⁺-activated K⁺ channels in CHO-K1 cells heterologously expressing oxytocin receptors [37]. The current lack of a precise mechanism for the KOR-induced hyperpolarization does not detract from the utility of the FMP Blue assay.

Of interest to drug discovery, dynorphin A analogs have been modified to prepare KOR-selective antagonists with increased metabolic stability and durations of action shorter than prototypical non-peptide KOR antagonists. Modifications in the C-terminal “address” domain by cyclization between residues 5 and 8 in the C-terminal sequence to generate [N-BenzylTyr₁,cycloD-Asp⁵,Dap⁸]Dyn (1–11) amide (zyklophin) resulted in loss of efficacy and KOR antagonist activity [34]. Compared with linear peptide analogs such as arodyn, the cyclic KOR antagonist zyklophin exhibited enhanced metabolic stability. It is active after systemic administration and can antagonize KOR agonists in the CNS, displaying a relatively short duration of action compared to the non-peptide antagonist nor-BNI [3]. We have demonstrated here that zyklophin exhibits negligible efficacy to affect the fluorescence
signal in the FMP Blue assay, which is consistent with our \(^{35}\text{S}\)GTP\(\gamma\)S binding studies showing that zyklophin did not stimulate \(^{35}\text{S}\)GTP\(\gamma\)S binding. The findings reported herein further confirm the classification of zyklophin as a KOR antagonist [34].

Zyklophin analogs, including JVA 3413, JVA 3416, JVA 3418 and JVA 3419, were designed and synthesized by modifying the N- and C-terminal domains and cyclic constraint of zyklophin [22]. This study is the first evaluation of the pharmacological properties of these zyklophin peptide analogs by quantifying their relative efficacy in the FMP Blue assay. Further, we provide the first systematic comparison of the FMP Blue, \(^{35}\text{S}\)GTP\(\gamma\)S and adenylyl cyclase assays for zyklophin and its analogs. We have demonstrated zyklophin analogs that are partial agonists compared to the full agonist Dyn (1–13)NH\(_2\) in both the FMP Blue and \(^{35}\text{S}\)GTP\(\gamma\)S binding assays. The rank order of ligand efficacy was similar in both the FMP and \(^{35}\text{S}\)GTP\(\gamma\)S binding assays. The linear zyklophin analog JVA 3416 was the most efficacious among these peptide ligands in both the FMP Blue and \(^{35}\text{S}\)GTP\(\gamma\)S binding assays. The current study also examined the ability of zyklophin and its analogs to inhibit adenylyl cyclase activity. These results differed from the FMP Blue and \(^{35}\text{S}\)GTP\(\gamma\)S binding assays in that the peptides were more efficacious in the adenylyl cyclase assay. Such differences in efficacy in G-protein coupled receptor measurements in amplified assays such as the adenylyl cyclase assay which requires forskolin stimulation versus assays that are presumably less amplified (FMP Blue) are common (Paton et al., 2020).

In conclusion, the FMP Blue assay is a sensitive real-time assay for assessing \(\kappa\)-opioid receptor induced hyperpolarization and receptor desensitization. This approach represents a useful functional measure for quantification of the potency, efficacy and desensitization potential of KOR ligands. We have further characterized the structure-activity relationships of novel analogs of zyklophin. The cyclic analogs of zyklophin JVA 3419, JVA 3418 and JVA 3413 exhibited low efficacy, while the linear peptide JVA 3416 acted as a higher efficacy partial agonist. Whether these cyclic peptide analogs behave like the parent peptide zyklophin with relatively short durations of \(\kappa\)-receptor antagonist activity in vivo remains to be confirmed in future studies.

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Fig. 1. U50488 or Dyn A(1–13)NH₂ produced rapid decreases in the FMP blue fluorescence signals in a concentration-dependent manner in KOR-CHO cells. A and B, representative traces of fluorescent signal changes plotted over 300 s, arrow indicates time of addition of U50488 or Dyn A(1–13) NH₂. C and D, nonlinear regression analysis of the concentration-response data for U50488 and Dyn A(1–13)NH₂ decreased FMP fluorescence yielded an IC₅₀ of 0.25 nM (95% CI, 0.10–0.60 nM) and 0.16 nM (95% CI, 0.06–0.43 nM), respectively. These data are representative of 15 experiments in triplicate.
Fig. 2.
DAMGO (μ opioid receptor agonist) and DPDPE (δ opioid receptor agonist) did not affect FMP blue fluorescence signals in KOR-CHO cells. A and B, representative traces show addition of DAMGO or DPDPE did not cause decreases in the fluorescence signals. C and D, nonlinear regression analysis of the concentration-response data demonstrates DAMGO and DPDPE did not affect FMP blue fluorescence signals.
Fig. 3.
The nonselective opioid antagonist naloxone as well as kappa-selective antagonists nor-BNI and zyklophin attenuated the U50488-mediated decrease in FMP Blue fluorescence signals. A, B and C, dose-response curves for U50488 in the absence and presence of varying concentrations of naloxone (10 nM-10 μM), zyklophin (300 nM-10 μM) or nor-BNI (0.01–10 nM). D, E and F, The $K_B$ values were derived from Schild regressions for naloxone ($K_B=3.16$ nM) zyklophin ($K_B=564$ nM) and norBNI ($K_B=0.07$ nM) antagonism of U50488. Each data point is a representative experiment. The experiment was repeated two to five times in independent cultures.
Fig. 4.
The decrease in fluorescent emission produced by U50488 (A) was blocked by overnight pretreatment of KOR-expressing cells with pertussis toxin (PTX, 100 ng/mL) (B). C, nonlinear regression analysis of the concentration-response data for U50488 demonstrates PTX eliminated the effect of U50488. Each data point represents triplicate determinations in a single experiment. The experiment was repeated three times in independent cultures.
Fig. 5.
Direct comparison of the potency of U50488 or Dyn A(1–13)NH₂ in the FMP Blue and [³⁵S]GTPγS binding assays. A and D, representative traces of FMP blue fluorescence signals after addition of increasing concentrations of U50488 or Dyn A(1–13)NH₂. The IC₅₀ values and 95% Confidence Intervals (CI) generated from nonlinear regression analysis of the concentration-response data for U50488 were 0.13 nM (0.07–0.25) and 2.13 nM (1.42–3.21) in FMP Blue (B) and [³⁵S]GTPγS binding assays (C), respectively. The IC₅₀ values generated from nonlinear regression analysis of the concentration-response data for Dyn A(1–13)NH₂ are 0.39 nM (0.24–0.64) and 4.19 nM (3.07–5.72) in FMP Blue (E) and [³⁵S]GTPγS binding assays (F), respectively. The experiment was repeated three times.
Fig. 6.
Both U50488 and Dyn A(1–13)NH$_2$ can cause desensitization of KOR in KOR-CHO cells. U50488 or Dyn A(1–13)NH$_2$ were applied to the KOR cells after 60 s′ baseline recording at an interval of 360 s for 3 times; arrows indicate the time of compound addition. A and B, representative traces show maximal decreased signal responses to increasing concentrations of U50488 or Dyn A(1–13)NH$_2$ plotted over 1200 s,. C, $E_{\text{max}}$ generated from nonlinear regression analysis of the concentration-response curves for U50488 are 100%, 63% and 44% in first, second and third additions, respectively. D, $E_{\text{max}}$ generated from nonlinear regression analysis of the concentration-response curves for Dyn A(1–13)NH$_2$ are 100%, 66% and 49% in first, second and third additions, respectively. Each data point is from a representative experiment. The experiment was repeated twice in independent cultures.
Cell viability was preserved and FMP Blue dye was stable during the prolonged period of agonist exposure. To ensure the decline in fluorescence in the desensitization assay was not due to the changes in cell viability or instability of FMP Blue dye over the recording time, Dyn A(1–13)NH₂ (A) or Locke’s (B) were applied to the CHO-KOR cells twice at an interval of 360 s, and Dyn A(1–13)NH₂ alone was applied for a third time (A and B). B, \( E_{\text{max}} \) generated from nonlinear regression analysis of the concentration-response curves for Dyn A(1–13)NH₂ are 100%, 67% and 51% in first, second and third additions, respectively. D, Locke’s buffer had no effect on FMP fluorescence and a third addition of Dyn A(1–13)NH₂ still produced rapid concentration-dependent decreases in FMP fluorescence, with an IC₅₀ value and 95% Confidence Interval (CI) of 0.36 nM (0.07–1.93).
Fig. 8. Representative traces of FMP Blue fluorescent signal induced by addition of U50488 (A), (−)-cyclazocine (B), (+)-cyclazocine (C) and pentazocine (D). Concentration-response curves illustrate the differences in agonist and partial agonist potency and efficacy (E).
Fig. 9.

A. The chemical structure of peptide analogs of zyklophin: [N-Benzyl-Tyr$^1$,Dap(Ac)$^8$]Dyn A(1–11) amide (JVA 3416, Dap = 2,3-diaminopropionic acid), [N-Methyl-Tyr$^1$]zyklophin (JVA 3418), [N-Allyl-Tyr$^1$]zyklophin (JVA 3419) and zyklophin-(1–8) (JVA 3413).

Representative traces of the FMP blue fluorescent signal induced by addition of Dyn A(1–13)NH$_2$ (B), JVA3413 (C), JVA 3416 (D), JVA 3418 (E), JVA 3419 (F) and zyklophin (G).
Fig. 10.
Nonlinear regression analysis of the concentration-response curves of JVA 3413 (A), JVA 3416 (B), JVA 3418 (C), JVA 3419 (D) and zyklophin (E) in comparison with Dyn A(1–13)NH_{2}. Each point represents mean ± SEM of triplicate values in a representative experiment. The experiments were repeated five to seven times with triplicate determinations in different independent cultures.
Table 1
Repeated administration of U50,488 or Dyn A(1–13)NH₂ produces KOR desensitization in the FMP Blue membrane potential assay.

|                | U50,488 |            | IC₅₀ (nM) (95% CI) | DynA (1–13)NH₂ |            | IC₅₀ (nM) (95% CI) |
|----------------|---------|------------|--------------------|----------------|------------|--------------------|
|                | Emax * (95% CI) |          |                    | Emax * (95% CI) |          |                    |
| First addition | 16,164 (14,182–18,142) | 0.16 (0.08–0.33) | 18,499 (15,382–21,616) | 0.71 (0.34–1.58) |
| Second addition| 8987 (6392–11,582)  | 0.25 (0.05–1.16) | 10,878 (9002–12,754)  | 0.31 (0.14–0.70) |
| Third addition | 5192 (2802–7582)   | 0.21 (0.02–2.61) | 7326 (5450–9202)     | 0.38 (0.12–1.21) |

* Maximum decrease in FMP Blue fluorescence units.
Table 2
Potency (95% CI) and relative efficacy of U50488, (−)-cyclazocine, pentazocine and (+)-cyclazocine in the FMP Blue assay.

|           | U50,488 (nM)  | (−)-Cyclazocine (IC₅₀ (nM)) | Pentazocine (IC₅₀ (nM)) | (+)-Cyclazocine |
|-----------|---------------|-----------------------------|-------------------------|----------------|
| IC₅₀       | 0.30 (0.13–0.42) | 1.91 (0.48–4.98)            | 26.6 (5.45–114.1)       | –              |
| Efficacy 1.0 | 0.64 ± 0.04   | 0.44 ± 0.04                 | –                       | –              |
Table 3

Efficacies of Dyn A(1–13)NH₂, zyklophin analogs JVA-3413, 3416, 3418, 3419, and zyklophin in the FMP Blue, [³⁵S]GTPγS and adenylyl cyclase assays.

|                  | FMP blue | ³⁵S]GTPγS | Adenylyl cyclase |
|------------------|----------|-----------|-----------------|
| Dyn-A(1–13)NH₂   | 1.0      | 1.0       | 1.0             |
| JVA 3413         | 0.22 ± 0.04 | 0.07 ± 0.14 | 0.35            |
| JVA 3416         | 0.48 ± 0.10 | 0.26 ± 0.06 | 1.0             |
| JVA 3418         | 0.14 ± 0.03 | 0.10 ± 0.10 | 0.58            |
| JVA 3419         | 0.15 ± 0.04 | 0.07 ± 0.17 | 0.33            |
| Zyklophin        | 0        | 0         | 0.23            |