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# A real time, fluorescence-based assay for measuring μ-opioid receptor modulation of adenylyl cyclase activity in Chinese Hamster Ovary cells.

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**ABSTRACT**

Inhibition of adenylyl cyclase (AC) activity is frequently used to measure μ-opioid receptor (MOR) activation. We sought to develop a simple, rapid assay of AC activity in whole cells that could be used to study MOR signalling. Chinese hamster ovary cells expressing human MOR (CHO-MOR cells) were grown in 96-well plates and loaded with membrane potential-sensitive fluorescent dye. CHO-MOR cells were treated with the AC activator forskolin (FSK), with or without simultaneous application of MOR agonists, and the resulting change in fluorescence was measured. CHO-MOR cells hyperpolarised in response to application of FSK ($p$EC$_{50}$ 7.3) or calcitonin ($p$EC$_{50}$ 9.4). A submaximally effective concentration of FSK (300 nM) caused a 52±2% decrease in fluorescence. Simultaneous application of the opioids DAMGO ($p$EC$_{50}$ 7.4, E$_{max}$ 56%), morphine ($p$EC$_{50}$ 7.0, E$_{max}$ 61%) and buprenorphine ($p$EC$_{50}$ 8.6, E$_{max}$ 24%) inhibited the FSK response in a dose-dependent manner, while having no effect by themselves. The effects of DAMGO were blocked by pertussis toxin. This assay represents a simple, robust method for real-time observation of AC inhibition by MOR in CHO cells. It represents an appealing alternative to end-point assays that rely on cAMP accumulation and can avoid potential confounds associated with rapid desensitisation of MOR signalling.

Keywords: opioid, membrane potential, high throughput, cAMP, calcitonin

## INTRODUCTION

Opioid analgesics are the most widely prescribed drugs in the treatment of moderate to severe pain. Despite their powerful analgesic effects, the use of opioids is limited due to the number of associated adverse affects such as respiratory depression, sedation, constipation and nausea, as well as the development of tolerance. Over time, the development of opioid tolerance and physical or psychological dependence may require 10-fold escalations in dose in order to maintain adequate pain relief.[1] Both the analgesic and adverse effects of opioid analgesics are mediated via the μ-opioid receptor (MOR) subtype.[2] MOR mediate their effects via downstream mechanisms including inhibition of adenylyl cyclase (AC) activity via $G\alpha_{i/o}$ subunits, inhibition or activation of ion channels via Gβγ subunits and activation of MAPK signalling via β-arrestin.[3]

One of the hallmarks of MOR receptor activation is the inhibition of AC activity, leading to a decrease in the production of cAMP. Changes in cAMP-dependent signalling are also hallmarks of processes associated with chronic opioid receptor activation.[4,5] cAMP is an important cellular second messenger, mediating a diverse range of physiological processes via activation of cAMP-dependent protein kinase A (PKA), exchange protein directly regulated by cAMP (EPAC) as well as directly via cAMP-gated ion channels.[6,7] The measurement of cAMP accumulation is frequently used as a sensitive endpoint assay in studies of both acute and chronic MOR signalling. A number of techniques have been developed for quantifying cAMP accumulation in recent years, particularly in the rapidly growing field of high-throughput screening (HTS). AC activity can be measured in a number of ways including measurement of the accumulation of [$^3$H]-cAMP, by [$^3$H]-cAMP binding displacement assays, by reporter gene assays utilizing cAMP-dependent transcription factors, by ELISA assays that measure cAMP-like immunoreactivity, and through measurements of

cAMP-dependent protein/protein interactions using FRET.[8] These assays often require cell lysis, are usually single time-point, and require the addition of multiple reagents or transfection of reporter constructs. Importantly, many assays of AC activity require significant incubation times to allow appropriate cAMP accumulation.[9, 10] In studies of MOR signalling, prolonged incubation times can pose a significant and underappreciated problem as MOR signalling undergoes rapid desensitization followed by receptor internalization during agonist exposures as short as 5 - 10 minutes.[11] As the incubation periods in most cAMP assays are at least 10 – 20 minutes, during which time opioid exposure is sustained, these assays are likely to be measuring the combined effects of receptor activation, desensitization, internalization and even resensitization.[11,12]

In this study, we sought to develop a simple, rapid assay of AC modulation. Here we report an assay of MOR inhibition of AC in intact CHO cells using a proprietary membrane potential sensitive dye. The assay is rapid, real-time, robust and requires minimal preparation. This assay should also obviate the problem of MOR receptor desensitization during baseline measurements of AC inhibition.

## MATERIALS AND METHODS

### *MOR transfection and cell culture*

Flp-In T-Rex Chinese hamster ovary (CHO) cells were created as follows. CHO Flp-In cells were grown in minimal essential medium alpha (Invitrogen, Melbourne, Australia) containing 5% fetal bovine serum (FBS) and 100 µg/ml zeocin. They were transfected with pcDNA6TR (tet-repressor plasmid) using Fugene 6 reagent (Promega, Alexandria, Australia) and selected with 10 µg/ml blasticidin and 100 µg/ml zeocin. Six individual clones were isolated and transiently transfected with the plasmid pcDNA5-FRT-TRPV1, which encodes the Transient Receptor Potential vanilloid 1 receptor (TRPV1) under control of a tetracycline-sensitive repressor. Clones were then tested for successful induction with 1 µg/ml tetracycline using a plated-based calcium assay of TRPV1 receptor activation.[13] One CHO-FRT-TR cell line was chosen and stably transfected with a pcDNA5 construct encoding the haemagglutinin-tagged human µ-opioid receptor cDNA together with the pOG44 (Flp recombinase plasmid) using the transfectant Fugene (Promega). The HA-tagged human µ-opioid receptor was synthesised by Genscript (Piscataway, New Jersey, USA). Cells expressing MOR were selected using hygromycin B (500 µg/mL) and grown to confluency. The selected cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 100U penicillin/streptomycin and 500 µg/mL hygromycin B up to passage 5. Hygromycin concentration was reduced to 200 µg/mL beyond passage 5. Cells were passaged at 80% confluency as required. Assays were carried out on cells up to 30 passages. Cells for assays were grown in 75 $cm^2$ flasks and used at greater than 80% confluence. The day before the assay cells were detached from the flask with trypsin/EDTA (Sigma) and resuspended in 10 ml of Leibovitz's L-15 media supplemented with 1% FBS, 100U penicillin/streptomycin and 15 mM glucose. hMOR receptor expression was induced with 2 µg/mL tetracyclin 20 hrs

prior to the assay. The cells were plated in a volume of 90 µl in black walled, clear bottomed 96-well microplates (Corning) and incubated overnight at 37 $^{o}$C in ambient $CO_2$.

***Membrane potential assay***

Membrane potential was measured using a FLIPR Membrane Potential Assay kit (blue) from Molecular Devices. The dye was reconstituted with assay buffer containing (in mM), NaCl 145, HEPES 22, $Na_2HPO_4$ 0.338, $NaHCO_3$ 4.17, $KH_2PO_4$ 0.441, $MgSO_4$ 0.407, $MgCl_2$ 0.493, $CaCl_2$ 1.26, glucose 5.56 (pH 7.4, osmolarity 315 ± 5). Prior to the assay, cells were loaded with 90 µl/well of the dye solution without removal of the L-15, giving an initial assay volume of 180 µl/well. Plates were then incubated at 37°C at ambient $CO_2$ for 60 minutes. Fluorescence was measured using a FlexStation 3 (Molecular Devices) microplate reader with cells excited at a wavelength of 530 nm and emission measured at 565 nm. Baseline readings were taken every 2 seconds for at least 2 minutes, at which time either drug or vehicle was added in a volume of 20 µl. Further additions were made in volumes of 20 µl, as indicated. The background fluorescence of cells without dye or dye without cells was negligible. Changes in fluorescence were expressed as a percentage of baseline fluorescence after subtraction of the changes produced by vehicle addition. The final concentration of DMSO was not more than 0.1%, and this concentration did not produce a signal in the assay.

***Drugs and Chemicals***

Unless otherwise noted, tissue culture reagents and buffer salts were from Invitrogen or Sigma. Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol acetate (DAMGO), was purchased from Auspep (Tullamarine, Australia). Morphine was a kind gift from the Department of Pharmacology,

University of Sydney. Buprenorphine was from the National Measurement Institute (Lindfield, Australia). Rp-8-(4-chlorophenylthio)adenosine- 3', 5'- cyclic monophosphorothioate, (Rp-8-CPT-cAMPS), Sp-8-(4-chlorophenylthio)adenosine- 3', 5'-cyclic monophosphorothioate (Sp-8-CPT-cAMPS) and 8-(4-chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, acetoxymethyl ester (8-CPT-2Me-cAMP) were from Biolog (Bremen, Germany). Membrane potential dye (blue) was from Molecular Devices (Sunnyvale, California, USA). Calcitonin was from Bachem (Bubendorf, Switzerland). Nigericin was from Enzo Life Sciences (Farmingdale, NY, USA). Forskolin, naloxone, H89, staurosporine, glibenclamide and 4-aminopyridine were from Ascent Pharmaceuticals (Bristol, UK). 1,9-dideoxyforskolin and tetraethylammonium (TEA) were from Sigma Aldrich (Castle Hill, Australia). Pertussis toxin (PTX) and VU-591 were from Tocris Bioscience (Bristol, UK). Charybdotoxin (CHX) was from Alexis Biochemicals (San Diego, US). KT-5720 was from Cayman Chemicals (Michigan, US).

***Data***

Unless otherwise noted, data is expressed as mean ± s.e.m. of at least 5 determinations made in duplicate or triplicate. Concentration response curves were fit with a 4 parameter logistic equation using Graphpad Prism (Graphpad). Statistical comparisons were made with an unpaired Student's T-test, unless otherwise noted. $P < 0.05$ was considered significant. All channel and receptor nomenclature is consistent with the British Journal of Pharmacology Guide to Receptors and Channels. [14]

To calculate Z', a measure of the robustness of the assay and indication of its suitability for HTS, the assay was performed on three separate occasions using assay buffer as the minimum response and either 300 nM FSK or 300 nM FSK with 3 μM DAMGO as the

maximum response in 96-well plates. The Z' factor was calculated as outlined in Zhang *et al*.[15]

## RESULTS

### *Hyperpolarisation of CHO cells by calcitonin and forskolin*

CHO cells express calcitonin receptors, $G_s$-coupled G protein-coupled receptors (GPCR) which stimulate AC.[16] In CHO-MOR cells loaded with the proprietary membrane potential dye, addition of rat calcitonin produced an immediate decrease in fluorescence, consistent with hyperpolarisation of the cells (Figure 1A). The fluorescent signal continued to slowly decrease for 10 min after the addition of calcitonin, after which fluorescent signals remained stable for the remainder of the assay. The decrease in fluorescence was concentration dependent, with $E_{max}$ of 46 ± 3% and $pEC_{50}$ of 9.4 ± 0.1 (n=5, Figure 1B). Addition of forskolin (FSK) to CHO cells loaded with membrane potential dye produced a similar decrease in fluorescence to that observed with calcitonin, with the fluorescent signal stabilising 5 min after the addition of FSK (Figure 1A). The decrease in fluorescence for FSK was concentration dependent, with a maximal effect ($E_{Max}$) of 52 ± 2% and $pEC_{50}$ of 7.3 ± 0.1 (n=6, Figure 1B). The AC-inactive FSK analogue 1,9-dideoxyforskolin (1 μM) did not produce a change in fluorescence (n=5, Figure 3C, $P > 0.1$).

### *Opioid inhibition of adenylyl cyclase*

Application of opioids alone did not affect the membrane potential of CHO-MOR cells. However, when the MOR agonist DAMGO was added together with a sub-maximally effective concentration of FSK (300 nM), DAMGO inhibited FSK induced membrane hyperpolarisation in a concentration dependent manner, consistent with inhibition of cAMP

generation (Figure 2A). This response was blocked by naloxone (1 μM) (Figure 2A). We measured the effects of opioids on the FSK-induced hyperpolarisation 5 minutes after co-application of the drugs. DAMGO inhibited the FSK-induced hyperpolarisation with a $pEC_{50}$ of 7.4 ± 0.1 with a maximum inhibition of 56 ± 3%. Maximum DAMGO inhibition of FSK-induced hyperpolarisation was reduced to 40 ± 2% ($p < 0.02$) when measured 10 minutes after FSK addition, while the potency was unchanged. Pretreatment of cells overnight with pertussis toxin (200 ng/ml) significantly reduced the inhibition of the FSK response by DAMGO (1 μM), inhibition was 81 ± 12% in control, and 11 ± 0.5 % after PTX treatment ($P < 0.01$, n=3). The change in fluorescence produced by FSK was unaffected by PTX treatment (46 ± 2% in control; 45 ± 5% after PTX, $P = 0.73$). FSK-induced hyperpolarisation was observed in CHO-MOR cells where MOR expression had not been induced by tetracycline, however the hyperpolarisation was not inhibited by opioids (data not shown). DAMGO (1 μM) also reduced membrane hyperpolarisation produced by 10 nM calcitonin from 40 ± 1.6% to 23 ± 1.2% ($P < 0.01$).

We assessed the capacity of this assay to reliably detect agonists of differing efficacy by examining the effects of morphine and buprenorphine (Figure 2B). Morphine and buprenorphine have previously been shown to have partial agonist activity at MOR.[17,18] Both agonists inhibited FSK-stimulated AC activation. Morphine had a similar efficacy as DAMGO, with $E_{max}$ of 61 ± 7%, and $pEC_{50}$ of 7.0 ± 0.2. Buprenorphine showed lower efficacy with $E_{max}$ of 24 ± 4%, and $pEC_{50}$ of 8.6 ± 0.5. Addition of increasing concentrations of naloxone produced a parallel shift in the concentration response curve for morphine, with a $pA_2$ of -8.5 ± 0.1, (2.9 ± 0.5 nM, n=3), a value consistent with the reported binding affinity of naloxone at human MOR (3 nM)[19] (Figure 2C).

*Mechanism of forskolin induced hyperpolarisation of CHO-MOR cells*

We sought to determine the mechanism by which AC activation caused membrane hyperpolarisation in CHO-MOR cells. The increase in cAMP resulting from AC activation can lead to activation of PKA, EPAC, or cAMP-gated ion channels.[6-7, 20] The membrane permeable cAMP analogue Sp-8-CPT-cAMPs (100 μM), a direct activator of PKA, mimicked the FSK response, producing a 44 ± 4% decrease in fluorescence (n=5, Figure 3A). Rp-8-CPT-cAMPs, a cAMP analogue that inhibits activation of PKA, did not produce a change in membrane fluorescence. The hyperpolarisation produced by Sp-8-CPT-cAMPs was not affected by DAMGO (Figure 3B), consistent with Sp-8-CPT-cAMPs producing its effects downstream of AC, potentially by acting directly on PKA. However, moderate concentrations of PKA inhibitors H89 (100 nM - 1 μM), KT5270 (100 nM – 1 μM) and staurosporine (1 μM) did not affect the hyperpolarization produced by forskolin, and at higher concentrations (10 μM and above) the protein kinase inhibitors produced substantial hyperpolarizations by themselves. The cAMP analog 8-CPT-2Me-cAMP (100 μM), which selectively activates EPAC and not PKA, did not significantly affect cellular fluorescence (n=4, Figure 3C, $P > 0.5$).

Membrane hyperpolarisation usually occurs by efflux of K ions from cells. The non-specific K channel blockers tetraethyl ammonium chloride (TEA, up to 10 mM) or 4 aminopyridine (4-AP**,** up to 1 mM) did not inhibit the FSK-induced hyperpolarisation (Figure 4B). Additionally, the hyperpolarization produced by FSK was not affected by the more selective $K_{ATP}$ channel blocker glibenclamide (10 μM), the renal outer medullary potassium channel ($K_{ir}$ 1.1) blocker VU-591 (10 μM) or charybdotoxin (10 μM)**,** a blocker of high-conductance Ca activated K channels ($K_{Ca}$1.1). To determine if FSK-induced membrane hyperpolarisation

was due to K efflux, the extracellular K concentration ($[K]_{Ex}$) was increased to 30 mM and 75 mM in order to make the reversal potential ($K_e$) for K less negative. This will reduce K efflux and associated membrane potential changes when K channels are opened. $[K]_{Ex}$ was adjusted by dissolving the membrane potential dye in HBSS where NaCl was substituted by KCl. The effects of a maximally effective concentration of FSK (10 μM) was reduced by about 50% in 30 mM $[K]_{Ex}$, and almost completely when $[K]_{Ex}$ was 75 mM (Figure 4A), suggesting FSK-induced membrane hyperpolarisation is mediated by efflux of K from cells. The hyperpolarizations produced by 100 nM, 1 μM and 10 μM FSK in the 3 concentration of $[K]_{Ex}$ were analysed by 2 Way ANOVA and found a main effect of FSK ($P < 0.003$) and $[K]_{Ex}$ ($P < 0.0001$). Subsequent analysis of the effect of $[K]_{Ex}$ on each concentration of FSK using Tukey's multiple comparison test indicated that the hyperpolarization produced by each concentration of FSK differed for each concentration of $[K]_{Ex}$ ($P < 0.05$ for each). The changes in fluorescence produced by altering the membrane K permeability were independently assessed by incubating CHO-MOR cells with nigericin, a potassium selective antibiotic ionophore.[21] A maximally effective concentration of nigericin (1 μM) produced a decrease in fluorescence signal of 70 ± 3%, which was not decreased any further upon the addition of 10 μM FSK (Figure 4C). The fluorescent signal observed after nigericin incubation may reflect the signal when the membrane potential of the cells is driven to $E_K$.[21]

We assessed the suitability of this assay for HTS by calculating the Z' factor, a measure of the assay robustness. An assay with a Z' factor of between 0.5 and 1 is an appropriate assay in terms of signal-to-noise ratio and data reproducibility.[15] The Z' factor for this assay was calculated for both the 300 nM FSK response and for inhibition of the FSK response with 3 μM DAMGO in multiple experiments. The Z' values for FSK alone were 0.7, 0.7, and 0.8, and for FSK + DAMGO were 0.7, 0.7 and 0.7, indicating that the assay is suitable for HTS.

## DISCUSSION

In this study, we have developed a real time, no wash, fluorescence based assay for MOR-mediated inhibition of AC in intact CHO-K1 cells. We used a proprietary membrane potential sensitive dye from Molecular Devices to measure membrane hyperpolarisation following FSK stimulated AC activation.[22] Activation of endogenous calcitonin receptors in CHO-K1 cells[16] produced a reduction in fluorescence similar to that seen following FSK application, and in both cases this reduction was less than that produced by application of the K-selective ionophore nigericin[21]. The fluorescent signal rapidly and reliably decreased after FSK application, and this decrease in signal was inhibited by the simultaneous application of opioid agonists.

CHO cells are frequently used in assays of opioid inhibition of AC activity. The elevation of cAMP in CHO cells following FSK application, as well as the ability of opioids to inhibit this elevation of cAMP is well documented.[16, 23-24] In this assay, opioid inhibition of FSK-stimulated membrane hyperpolarisation is consistent with opioid inhibition of AC, one of the hallmarks of MOR activation.[3] CHO cells have been shown to express AC subtypes VI and VII.[25] Opioid modulation of AC activity is isozyme specific, with acute opioid treatment shown to inhibit $G_s$-stimulated AC-VI activity and potentiating $G_s$-stimulated AC-VII.[26] Because AC-VII has been reported to be insensitive to FSK,[32] we chose to use FSK rather than calcitonin as the AC-stimulating agent, as this should avoid the confounding effects of opposite MOR modulation of the $G_s$-mediated stimulation of AC-VI and AC-VII.

Morphine and buprenorphine are lower efficacy agonists at MOR[17,18], however the measured efficacy of a compound depends on the assay being used. In the present assay, morphine

acted as a full agonist, with an $E_{max}$ similar to that of DAMGO. This likely reflects the relatively low receptor occupancy required for AC inhibition, particularly as we were stimulating AC with a submaximally effective concentration of forskolin. Many previous studies have reported morphine to be a full agonist in assays of AC inhibition.[4,28]

The measurement of MOR inhibition of AC is often performed using end-point assay techniques with lengthy inhibition times. As shown in our assay, some sensitivity would be lost using this approach. Inhibition of the FSK-stimulated membrane hyperpolarisation peaked at approximately 5 minutes after the addition of FSK and opioid. After this time, the fluorescent signal gradually decreased further. A single time-point measurement of cAMP accumulation after 10 minutes in our assay shows a reduction in the efficacy of DAMGO in AC inhibition, with $E_{max}$ decreased from 56% to 40%, possibly reflecting receptor desensitisation. While we chose to measure at a time point when the FSK-induced hyperpolarization signal had plateaued, it would be a simple matter to measure at any time point after addition of the drugs, enabling the virtually instantaneous measurement of cell responses after the addition of FSK and/or opioid. Depending on the agonist employed, MOR rapidly desensitises and/or internalises, with up to 50% of MOR internalized within 5 minutes of agonist exposure in some cells.[11-12,20] AC assays using single time point measurement of cAMP accumulation after incubation times of up to 20 minutes are measuring the summed output of signalling from activated, desensitized and internalized MOR, and thus give little insight into the real-time dynamics of the effects of MOR activation on cAMP mediated signalling.

The measurement of real-time AC inhibition has previously only been achievable by the use of complex techniques such as transfection of reporter genes or proteins with cAMP binding

domains, or bioluminescence resonance energy transfer (BRET) assays.[8, 29-32] These assays can be very useful for studying cAMP signalling, although each has its potential weaknesses as well as strengths.[8] Bioluminescence assays such as the Glosensor (Promega) can provide a continuous reading of cAMP levels, allowing kinetic studies and repeated drug applications[30]. However, this assay requires transfection of a luciferase sensor construct with cAMP binding domains and use of specialized reagents, in addition to any stable transfections of the receptors of interest. For the kind of studies described in this paper – inhibition of cAMP accumulation by a $G_i/G_o$-coupled GPCR, the developers of the Glosensor assay recommend a 5-10 minute pre-incubation with agonist before the addition of FSK, which is likely to be unsuitable for studies of rapidly desensitizing GPCR such as MOR.[30] The assay described here is simpler than these assays, requiring transfection of the receptor of interest only and addition of a single assay reagent. The drug responses can be observed immediately following agonist addition. The Z' calculated for this assay were similar to those reported for the Glosensor assay.[31,32] However, as the plate reader format means that we are unable to study recovery of AC activity following wash of agonists, and the kinetic and stoichiometric relationship between the FSK-induced rise in AC levels and hyperpolarization of the CHO cells is unknown.

Activation of the endogenous $G_s$-coupled CTR receptors in CHO cells caused membrane hyperpolarisation similar to that produced by the AC activator FSK. Membrane hyperpolarisation in CHO cells by AC activation has not been shown previously, so we sought to determine the mechanism by which membrane hyperpolarisation occurs. Endogenous K channels are not well defined in CHO cells, and CHO cells are frequently used as heterologous expression systems for recombinant K channels due to the apparent low levels of native K channel activity.[33] The non-specific K channel inhibitors TEA and 4-AP

did not inhibit FSK induced membrane hyperpolarisation, nor did the more specific K channel inhibitors glibenclamide, VU-591 or charybdotoxin. However, when the reversal potential for K was made less negative by increasing $[K]_{Ex}$, membrane hyperpolarisation was reduced, and hyperpolarization was essentially abolished when $[K]_{Ex}$ was increased to 75 mM. Furthermore, the FSK induced membrane hyperpolarisation was mimicked and occluded by the addition of the K-selective ionophore nigericin. This suggests that FSK induced membrane hyperpolarisation is due to the movement of K ions through native K channels in CHO cells.

Membrane hyperpolarisation resulting from application of FSK and calcitonin is due to the activation of AC.[16,22] The resulting elevation of cAMP levels in the cell leads to the activation of PKA, as well as EPACs. The cAMP analogue Sp-8-CPT-cAMPs, a direct activator of PKA, mimicked the effects of FSK, while Rp-8-CPT-cAMPs, an inhibitor of cAMP activation of PKA, was inactive. 8-CPT-2Me-cAMP, a selective activator of EPAC, also produced no effect. Together these data are consistent with the idea that membrane hyperpolarisation is occurring via PKA activation. However, we were unable to inhibit the effects of FSK with modest concentrations of protein kinase inhibitors, and higher concentrations of these drugs themselves hyperpolarized the CHO cells. Novel cAMP-dependent signal transduction pathways are still being discovered,[34] and it may be that the observed hyperpolarization of CHO cells is mediated by such a mechanism.

Assays of AC inhibition represent one of the most straightforward ways of studying Gα (as opposed to Gβγ) signalling in a high throughput environment. The assay described here offers a novel approach for measuring MOR-mediated AC inhibition in intact CHO cells, and has the advantages of being both real-time and reflecting the naturalistic coupling of MOR to

the signalling pathway. The lack of a defined mechanism for the hyperpolarization does not detract from the utility of the assay for acute studies, however, the assay may not be suitable for studies of more complex signalling cascades such as those potentially underlying agonist-induced receptor regulation. Nevertheless, our results show the membrane potential assay to be a rapid, reliable and inexpensive method for assessing opioid activation of MOR in CHO cells, and may be scaled up to enable high-throughput screening. Coupled with our recent description of a HTS-appropriate membrane potential assay of Gβγ signalling in AtT-20 cells[35], it is clear that multiple aspects of GPCR signalling can be studied in a relatively simple, non-invasive and efficient manner using simple reagents which report changes in basic cellular properties such as membrane potential.



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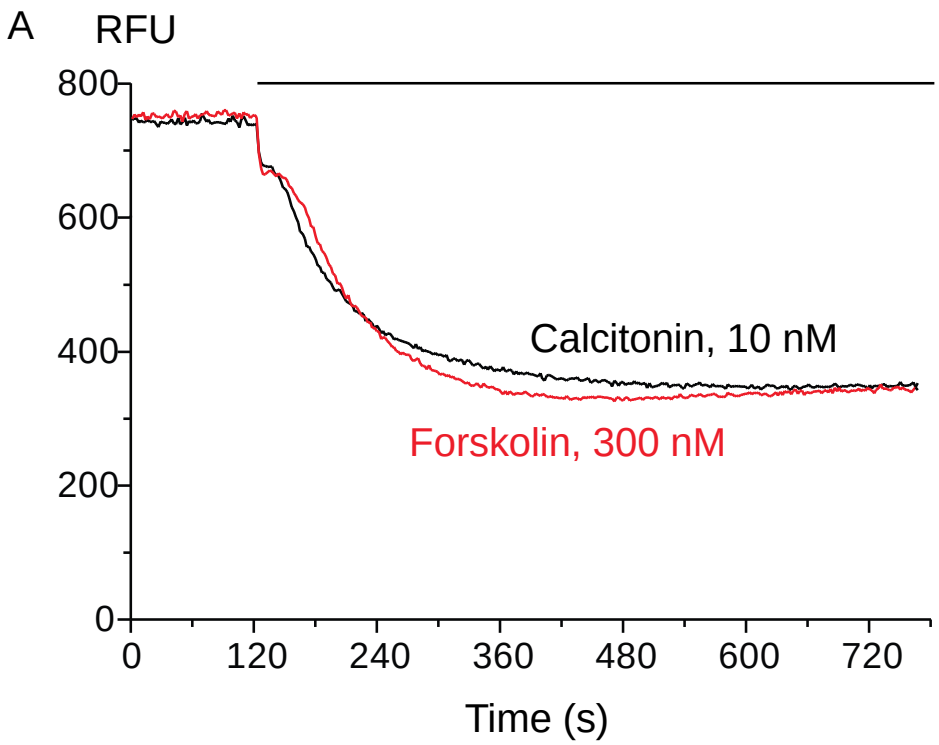
**Figure 1: Stimulating adenylyl cyclase hyperpolarises CHO cells**. The membrane potential of CHO cells was determined as outlined in the Methods. Both calcitonin and forskolin hyperpolarise CHO-MOR cells in a concentration dependent manner. A) Representative traces showing the raw fluorescence (RFU) from individual wells of a 96-well plate. Calcitonin (10 nM) or forskolin (1 μM) were added to CHO-MOR cells for the duration of the bar. B) Concentration response curves illustrating the effects of calcitonin and forskolin on the membrane potential of CHO-MOR cells. The calcitonin $E_{max}$ was 46 ± 3% and $pEC_{50}$ was 9.4 ± 0.1. Forskolin $E_{max}$ 52 ± 2% and $pEC_{50}$ was 7.3 ± 0.1. Each point represents the mean ± s.e.m. of at least 5 experiments performed in triplicate.

**Figure 2: Opioids inhibit forskolin-stimulated membrane hyperpolarisation in CHO cells.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** Example traces showing the effects of forskolin (FSK, 300 nM), FSK and DAMGO (1 μM) applied simultaneously and FSK and DAMGO added in the presence of naloxone (NAL, 1 μM) which had been added 10 minutes earlier. FSK and DAMGO were added for the duration of the bar. **B)** Concentration-response curves morphine, buprenorphine and DAMGO inhibition of the hyperpolarization produced by FSK (300 nM). DAMGO inhibition of FSK stimulated AC activation was concentration-dependent, with an $E_{max}$ of 56 ± 3 %, and $pEC_{50}$ of 7.4 ± 0.1, morphine had an $E_{max}$ of 61 ± 7% and $pEC_{50}$ of 7.0 ± 0.2, Buprenorphine showed partial agonist activity with $E_{max}$ of 24 ± 4% and $pEC_{50}$ of 8.6 ± 0.5. **C)** Preincubation of the cells with increasing concentrations of naloxone (3, 10, 100 nM) produced a parallel shift in the concentration-response curve of morphine, with a $pA_2$ of -8.5 ± 0.1, (2.9 ± 0.5 nM, n=4).

**Figure 3: Protein kinase A activators mimic the effects of forskolin.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** The cAMP analog Sp-8-CPT-cAMPs (100 µM, black trace), but not Rp-8-CPT-cAMPs (blue trace) mimicked the effect of FSK (100 nM, red trace). **B)** The hyperpolarisation produced by 30 µM Sp-8-CPT-cAMPs (black trace) was not inhibited by the simultaneous addition of 1 µM DAMGO (blue trace). **C)** The AC-inactive FSK analogue 1,9-ddFSK (1 µM) and the EPAC selective cAMP analogue 8-CPT-2Me-cAMP (100 µM) had little effect on CHO-MOR membrane potential when compared with 1 µM FSK. Bars represent the mean ± s.e.m of 4-5 determinations in triplicate.

**Figure 4: The forskolin-induced hyperpolarization is dependent on extracellular K concentration.** The membrane potential of CHO-MOR cells was determined as outlined in the Methods. **A)** The effects of forskolin (FSK) were reduced as the extracellular K concentration was changed from 5 mM to 30 mM or 75 mM. K was replaced with equimolar Na. The hyperpolarizations produced by 100 nM, 1 µM and 10 µM FSK in the 3 concentration of $[K]_{Ex}$ were analysed by 2 Way ANOVA and found a main effect of FSK ($P < 0.003$) and $[K]_{Ex}$ ($P < 0.0001$). **B)** Membrane hyperpolarisation produced by FSK (300 nM) was not affected the non-specific $K^+$ channel blockers tetraethylammonium chloride (TEA) or 4-aminopyridine (4-AP), the selective $K_{ATP}$ channel blocker glibenclamide (Glib), charybdotoxin (CHTX), a blocker of high-conductance Ca activated K channels and the renal outer medullary K channel channel blocker VU-591. **C)** The K selective ionophore nigericin (1 µM) produced a decrease in fluorescence that occluded the effects of a high concentration of FSK (10 µM).

Figure 1


A
RFU
800
600
400
200
0
0
120
240
360
480
600
720
Time (s)
Calcitonin, 10 nM
Forskolin, 300 nM

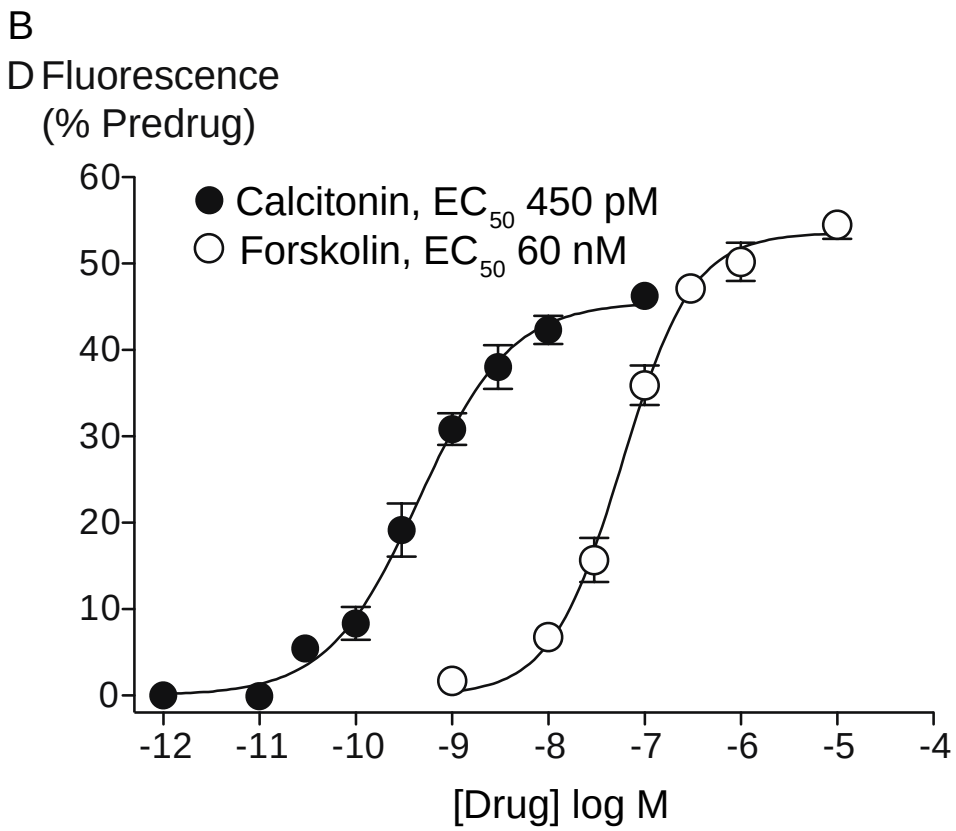
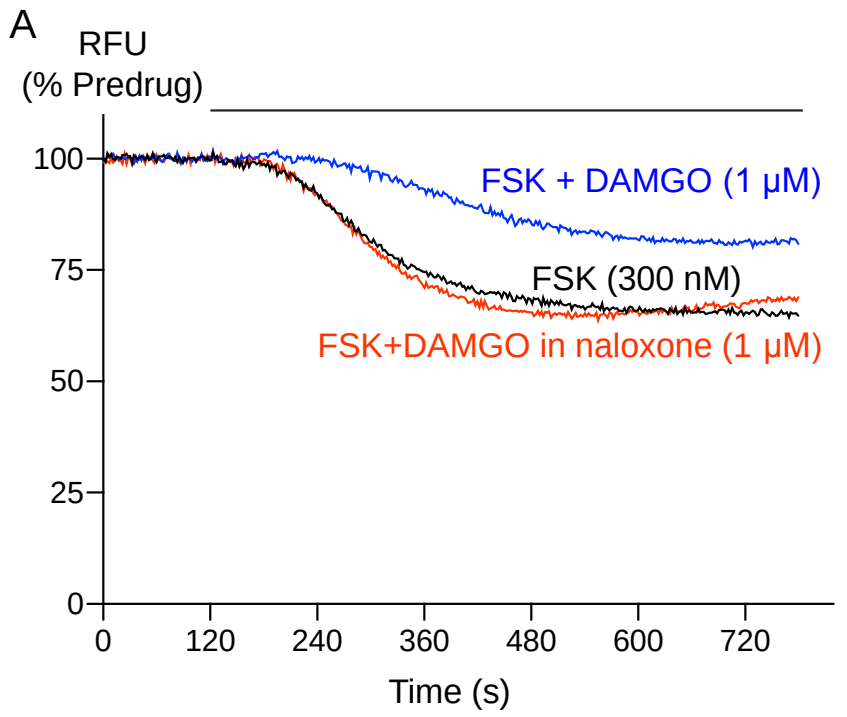
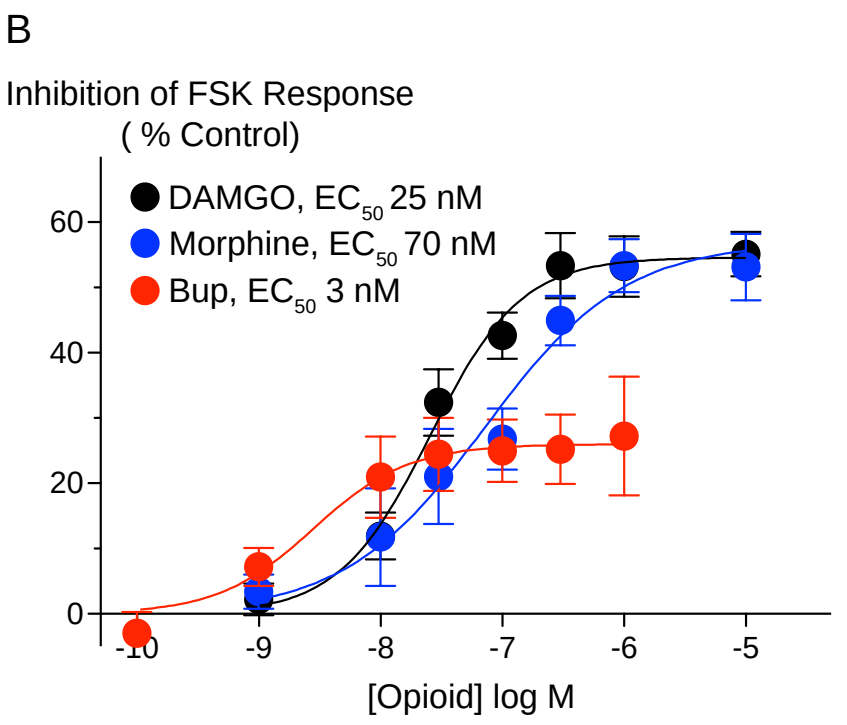

B
D Fluorescence
(% Predrug)
60
50
40
30
20
10
0
-12
-11
-10
-9
-8
-7
-6
-5
-4
[Drug] log M
Calcitonin, EC50 450 pM
Forskolin, EC50 60 nM

Figure 2


A
RFU
(% Predrug)
100
75
50
25
0
FSK + DAMGO (1 μM)
FSK (300 nM)
FSK+DAMGO in naloxone (1 μM)
0
120
240
360
480
600
720
Time (s)


B
Inhibition of FSK Response
( % Control)
DAMGO, EC50 25 nM
Morphine, EC50 70 nM
Bup, EC50 3 nM
60
40
20
0
-10
-9
-8
-7
-6
-5
[Opioid] log M

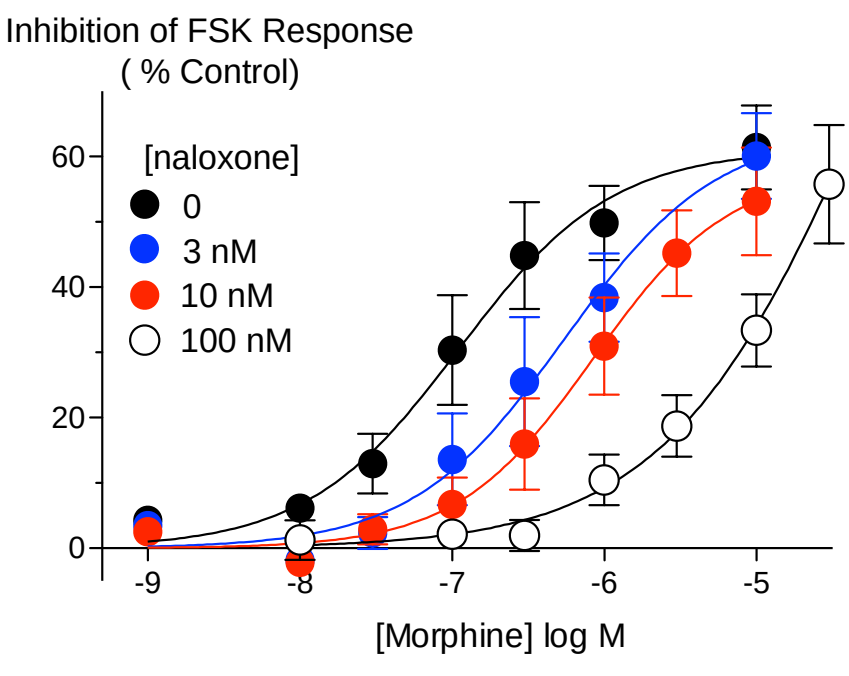
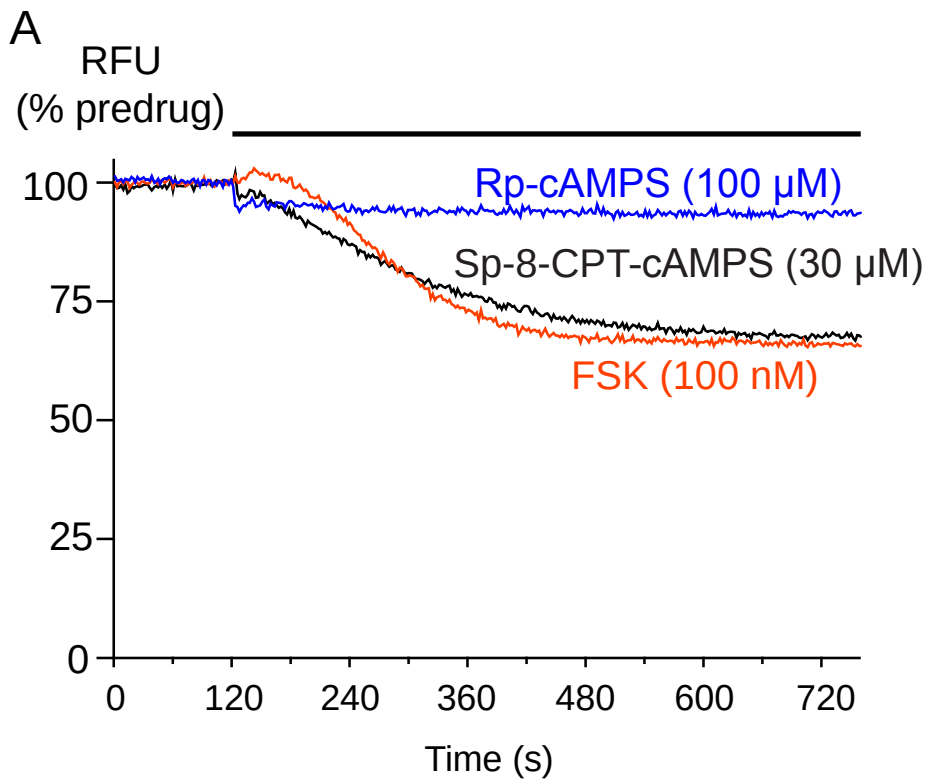
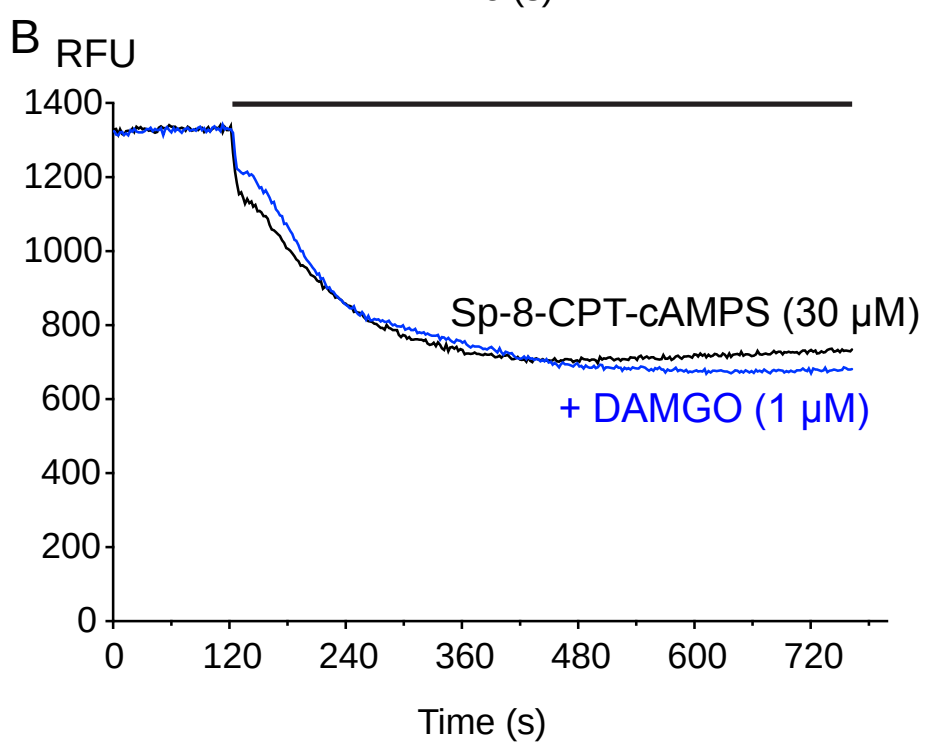

C
Inhibition of FSK Response
( % Control)
[naloxone]
0
3 nM
10 nM
100 nM
60
40
20
0
-9
-8
-7
-6
-5
[Morphine] log M

Figure 3


A
RFU
(% predrug)
100
75
50
25
0
Rp-cAMPS (100 µM)
Sp-8-CPT-cAMPS (30 µM)
FSK (100 nM)
0
120
240
360
480
600
720
Time (s)


B
RFU
1400
1200
1000
800
600
400
200
0
Sp-8-CPT-cAMPS (30 µM)
+ DAMGO (1 µM)
0
120
240
360
480
600
720
Time (s)

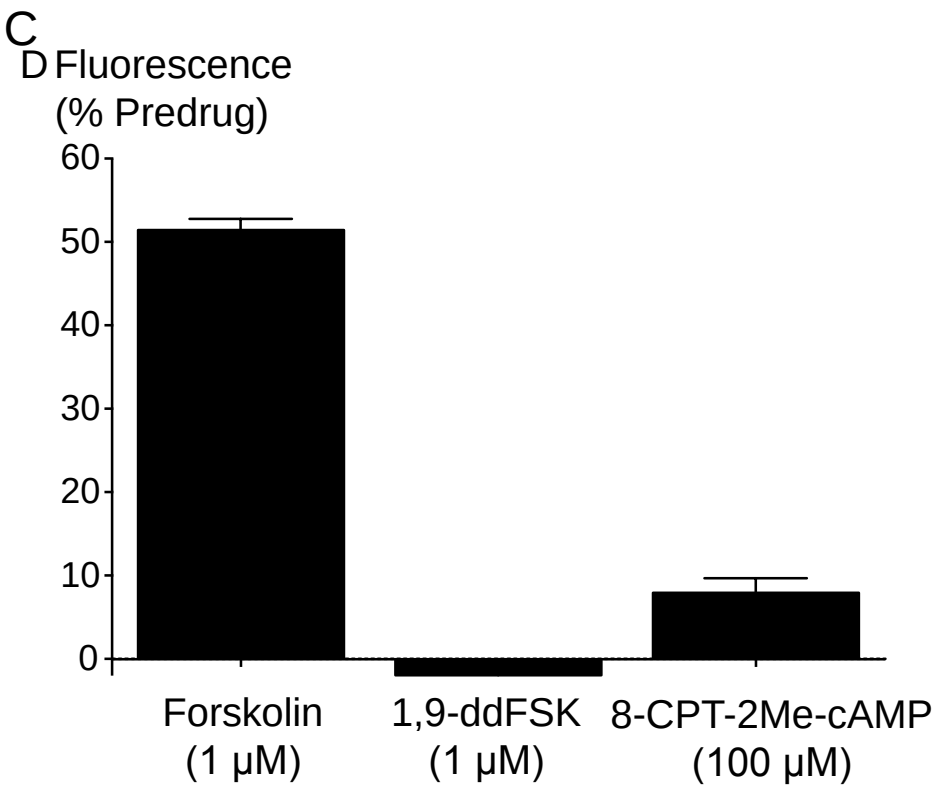
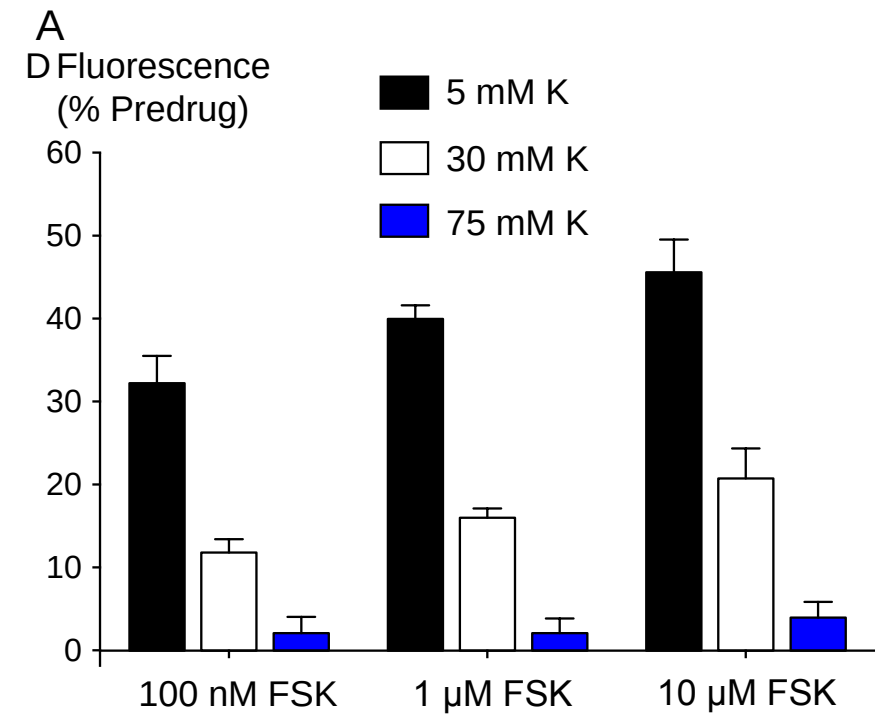
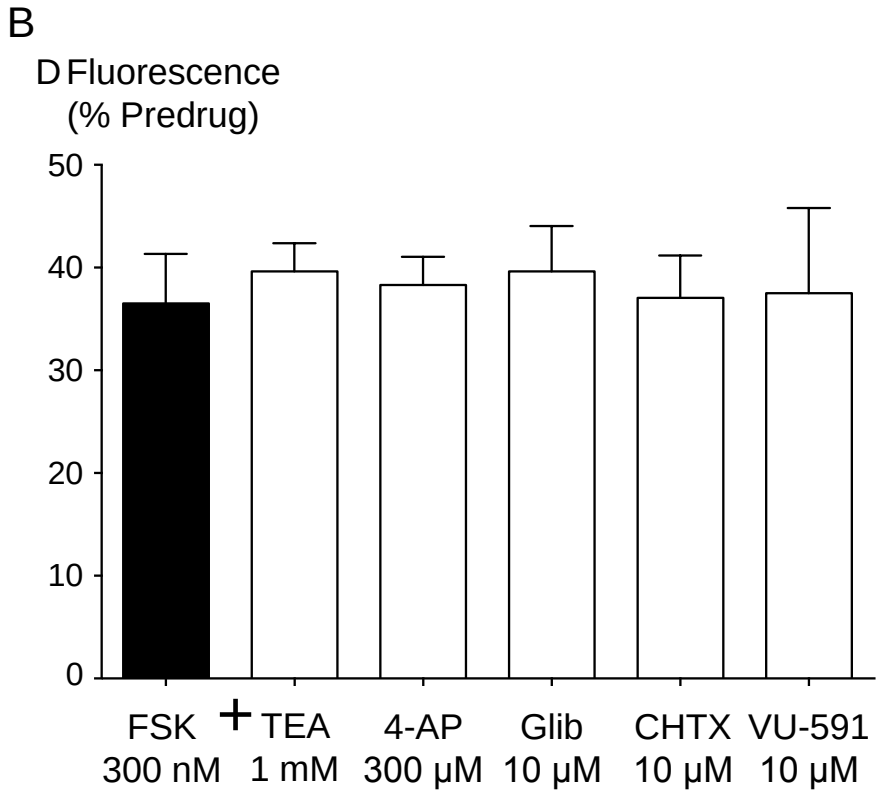
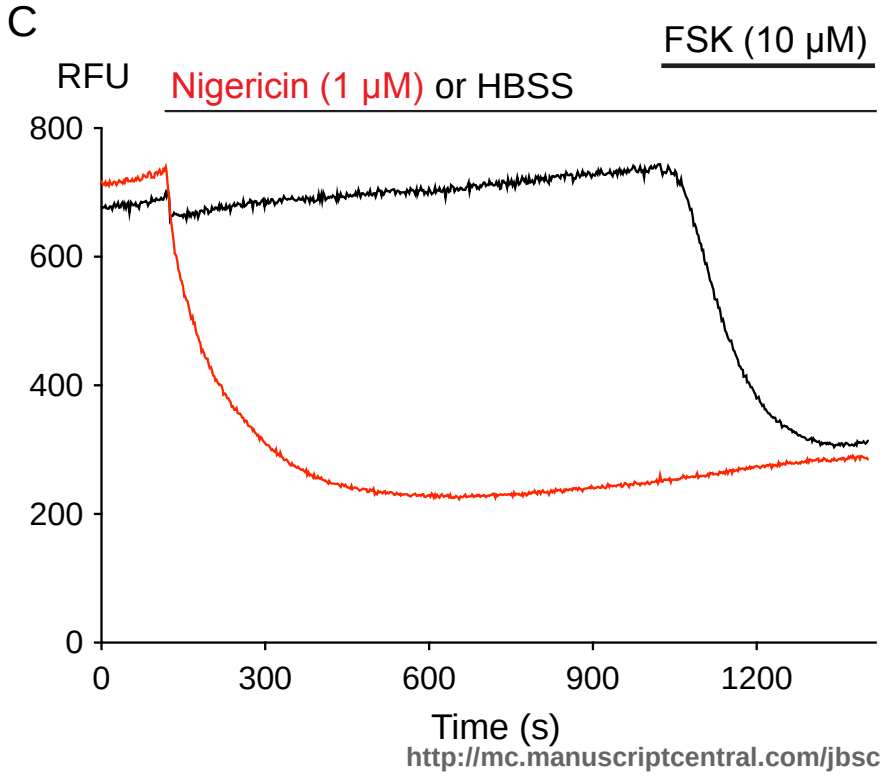

C
D Fluorescence
(% Predrug)
60
50
40
30
20
10
0
Forskolin
(1 µM)
1,9-ddFSK
(1 µM)
8-CPT-2Me-cAMP
(100 µM)

Figure 4


A
D Fluorescence
(% Predrug)
5 mM K
30 mM K
75 mM K
60
50
40
30
20
10
0
100 nM FSK
1 μM FSK
10 μM FSK


B
D Fluorescence
(% Predrug)
50
40
30
20
10
0
FSK
300 nM
+
TEA
1 mM
4-AP
300 μM
Glib
10 μM
CHTX
10 μM
VU-591
10 μM


C
FSK (10 μM)
RFU
Nigericin (1 μM) or HBSS
800
600
400
200
0
0
300
600
900
1200
Time (s)