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Agonist-selective mechanisms of μ-opioid receptor (MOR) desensitization in HEK293 cells

by

Elizabeth A. Johnson, Sue Oldfield, Ellen Braksator, Ana Gonzalez-Cuello, Daniel Couch, Kellie J. Hall, Stuart J. Mundell, Chris P. Bailey, Eamonn Kelly & Graeme Henderson

Department of Pharmacology,

University of Bristol

Bristol, BS8 1TD UK

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Running title: Mechanism of MOR desensitization is agonist-dependent

Author for correspondence:

Graeme Henderson
Department of Pharmacology
University of Bristol
Bristol, BS8 1TD
UK
Tel: 44-117-9287629
FAX: 44-117-9250168
Email: graeme.henderson@bris.ac.uk.

Text Pages 35
Figures 7
References 40
Abstract 248 words
Introduction 430 words
Discussion 1386 words

The abbreviations used are: DAMGO, [D-Ala^2,N-MePhe^4,Gly-ol^5]enkephalin; DMEM, Dulbecco's Modified Eagle's Medium; GF109203X, bisindolylmaleimide 1; GIRK, G-protein-coupled inwardly rectifying K^+ channel; GFP, Green fluorescent protein; Go6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; GRK2, G-protein-coupled receptor kinase 2; GRK2-K220R, kinase deficient dominant negative mutant of GRK2; GRK6, G-protein-coupled receptor kinase 6; GRK6-K215R, kinase deficient dominant negative mutant of GRK6; HEK293-MOR1 cells, HEK293 cells stably expressing rat MOR1; MOR, μ-opioid receptor; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; RGS protein, Regulator of G-protein signalling protein; SDS-PAGE, SDS polyacrylamide gel electrophoresis; U 73122, 1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione.

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Abstract

The ability of two opioid agonists, DAMGO and morphine, to induce μ-opioid receptor (MOR) phosphorylation, desensitization and internalization was examined in HEK293 cells expressing rat MOR1 as well GIRK channel subunits. Both DAMGO and morphine activated GIRK currents but the maximum response to DAMGO was greater than that of morphine indicating that morphine is a partial agonist. The responses to DAMGO and morphine desensitized rapidly in the presence of either drug. Expression of a dominant negative mutant G protein-coupled receptor kinase 2 (GRK2), GRK2-K220R, markedly attenuated the DAMGO-induced desensitization of MOR1, but had no effect on morphine-induced MOR1 desensitization. In contrast, inhibition of PKC either by the PKC inhibitory peptide, PKC (19-31) or staurosporine reduced MOR1 desensitization by morphine, but not that induced by DAMGO. Morphine and DAMGO enhanced MOR1 phosphorylation over basal. The PKC inhibitor GF109203X inhibited MOR1 phosphorylation under basal conditions and in the presence of morphine, but did not inhibit DAMGO-induced phosphorylation. DAMGO induced arrestin-2 translocation to the plasma membrane and considerable MOR1 internalization whereas morphine did not induce arrestin-2 translocation and induced very little MOR1 internalization. Thus DAMGO and morphine each induce desensitization of MOR1 signalling in HEK293 cells but by different molecular mechanisms; DAMGO-induced desensitization is GRK2-dependent whereas morphine-induced desensitization is PKC-dependent. MORs desensitised by DAMGO activation are then readily internalised by an arrestin-dependent mechanism whereas those desensitised by morphine are not. These data suggest that opioid agonists induce different conformations of the MOR that are susceptible to different desensitizing and internalization processes.

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Introduction

Activation of μ-opioid receptors (MORs) underlies the rewarding and analgesic effects of morphine, however the efficacy of morphine is limited by rapidly developing tolerance to the drug (Inoue and Ueda, 2000). The mechanisms underlying tolerance are poorly understood, but it is clear that in some systems morphine causes greater tolerance than other higher efficacy MOR agonists such as etorphine and fentanyl (Duttaroy and Yoburn, 1995). In addition, compared to full agonists, morphine produces little loss of cell surface MORs by internalization (Keith et al., 1996). These findings have been interpreted in different ways to explain the phenomenon of tolerance (reviewed in Connor et al., 2004; Bailey and Connor, 2005). On the one hand, it has been suggested that morphine, since it does not trigger significant MOR internalization, allows the receptor to couple over a prolonged period to intracellular signalling systems that induce marked tolerance (Finn and Whistler, 2001). On the other hand, it has been suggested that the poor ability of MORs to internalize in response to morphine means that morphine-desensitized receptors accumulate at or near the cell surface. This implies that internalization is a crucial first step in resensitization, followed by dephosphorylation and recycling back to the plasma membrane, thus, tolerance may be exacerbated when desensitized receptors cannot internalize and resensitize (Schulz et al., 2004). Further evidence for the latter view comes from a very recent study showing that in comparison to full MOR agonists such as DAMGO, morphine caused a greater degree of long term desensitization since the morphine activated receptor was unable to internalize, and hence recycle and resensitize effectively (Koch et al., 2005).

In the present study we examined the coupling and desensitization of MOR-activated GIRK currents in HEK293 cells stably expressing the MOR1 subtype of MOR at relatively low, near physiological levels and transiently transfected with Kir3.1 and Kir3.2 GIRK channels. By

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recording the agonist-activated GIRK current we can obtain a real-time measure of MOR activation. The agonists used were DAMGO, a full agonist at MORs, and morphine, which in most systems behaves as a partial agonist at this receptor. We found that DAMGO and morphine activated GIRK currents in these cells, and that both agonists produced rapid and quantitatively similar degrees of desensitization of GIRK currents. However, the mechanism underlying the desensitization was agonist specific, with DAMGO-induced desensitization being GRK2-dependent, and morphine-induced desensitization being partly PKC-dependent. Furthermore, although the degree of desensitization by DAMGO and morphine was similar, DAMGO induced arrestin-2 translocation to the plasma membrane whereas morphine did not, and DAMGO induced a far greater degree of MOR1 internalization.

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Materials and Methods

HEK293 cells stably expressing MOR1

HEK293 cells stably expressing T7-epitope-tagged MOR1 have been previously described (Bailey et al., 2003). For the electrophysiological experiments, [^{35}S]GTPγS assays, MOR1 internalization and arrestin translocation studies we used a stable cell line (HEK293-MOR1) with a MOR1 expression level of 175 ± 28 fmol/mg protein (as assessed by [^{3}H]diprenorphine saturation binding). This level of expression approximates to that found in mature brain neurones (Tempel and Zukin, 1987). Whereas, for the MOR1 phosphorylation studies we used a HEK293-MOR1 cell line with a higher MOR1 expression level of 1627 ± 41 fmol/mg protein. Cells were maintained at 37 °C in 95 % O_2 / 5 % CO_2, in DMEM, supplemented with 10 % foetal bovine serum, 10 U ml^{-1} penicillin and 10 mg ml^{-1} streptomycin; for the HEK293-MOR1 cells, 250 μg ml^{-1} geneticin was also added to the culture medium. For transient transfections, cells were plated out at ~50 % confluence on glass coverslips in 24 well plates and were transfected with 0.3 - 0.5 μg cDNA using calcium phosphate precipitation. For transient transfections, cDNAs were inserted into pcDNA3 (dominant negative mutants of GRK2 and GRK6) or pEGFP-N (arrestin2-GFP and arrestin3-GFP). Plasmid DNA was diluted in sterile water and 2.5 M $CaCl_2$ added to a final concentration of 0.25 M $CaCl_2$. The mixture was then combined 1:1 with HEPES buffered saline (280mM NaCl, 10mM KCl, 1.5mM Na2HPO4, 12mM glucose, 50mM HEPES adjusted to pH 6.9 using 1 M NaOH) and added to each well of the plate. Cells were then incubated for 16-24 h before use.

Whole-cell patch-clamp recordings

Non-confluent monolayers of HEK293-MOR1 cells grown on glass coverslips were mounted in a perfusion chamber (1.9 ml) on an inverted phase contrast microscope also equipped for

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visualization of Green Fluorescent Protein (GFP) fluorescence (Nikon) and superfused (4 $ml.min^{-1}$) with extracellular solution at room temperature (22-26 ºC). The extracellular solution contained (in mM): NaCl 160; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1; glucose 11 and HEPES 5; pH 7.4. Cells that had been successfully transfected with the Kir 3.1 and 3.2 subunits were identified by their GFP fluorescence. Whole-cell voltage-clamp recordings (V_h=-60mV) were made using electrodes (3-5 MΩ) filled with (mM) KCl 122; EGTA 11; $CaCl_2$ 1; $MgCl_2$ 2; HEPES 10; MgATP 4; Na_2GTP 0.25 and NaCl 5; pH 7.2. Recordings were filtered at 2kHz using an Axopatch 200B amplifier (Axon Instruments, USA) and saved to a computer for subsequent analysis. All drugs were applied in known concentrations in the superfusing solution.

To enhance the amplitude of the MOR1-evoked GIRK currents we recorded inward current through these inwardly rectifying channels (Figure 1). The K^+ concentration of the extracellular bathing solution was raised to 50 mM and the NaCl concentration reduced to 115 mM. Furthermore, to ensure that currents evoked by prolonged exposure to agonists did not decline due to the inward current raising the intracellular K^+ concentration thus reducing the electrochemical drive for further entry of K^+ into the cell, we developed a voltage protocol that minimised the amount of K^+ entry into the cell during the application of the drug. Cells were initially held at a membrane potential of -60 mV and, when the buffer was changed from low [K^+] to high [K^+], the membrane potential was stepped to -25 mV, which is the reversal potential for the GIRK channel under these recording conditions (i.e. the potential at which no K^+ ions would flow into or out of the cell). To measure GIRK channel activation in response to morphine or DAMGO, the membrane potential was then stepped from –25 mV to -60 mV for only 60 ms every 5 s. In this way, the current response to an agonist could be measured, whilst the amount of K^+ that entered the cell during a recording was minimised. Barium (1

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mM), a blocker of Kir channels, abolished the DAMGO and morphine-evoked currents. No opioid-evoked current was observed in HEK293-MOR1 cells that were not transfected with Kir3.1 and Kir3.2 subunits.

Desensitization of the MOR1-evoked GIRK current was quantified by expressing the current amplitude as as a percentage of the initial peak current. The decay of the current was fitted to a single exponential using Graph Pad Prism.

[^{35}S]GTPγS assay

The binding of [^{35}S]GTPγS to membranes of MOR1-expressing cells was based on the assay described previously (Harrison and Traynor, 2003). Briefly, cells were incubated in the presence or absence of agonist for 30 min, then washed and the cells resuspended in ice-cold assay buffer (0.2 mM $MgSO_4$, 0.38 mM KH_2PO_4, 0.61 mM Na_2HPO_4, pH 7.4) and lysed in a hand-held homogeniser. The homogenates were centrifuged at 20,000 x g for 20 min at 4^{o}C and the pellets resuspended in 50 mM Tris-HCl buffer, pH 7.4. Aliquots of membrane (~ 30 μg of protein) were then incubated with 200 pM [^{35}S]GTPγS and assay buffer containing 3 μM GDP and DAMGO/morphine as indicated (final volume 400 μl) for 1 h at room temperature. Non-specific binding in all cases was determined by the addition of 10 μM GTPγS to the assay. Binding was stopped by the addition of 2 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4, and the samples rapidly filtered through glass fibre filters using a Brandel Cell Harvester, using 50 mM Tris-HCl buffer, pH 7.4 as filtering buffer. The amount of [^{35}S]GTPγS bound to membranes on individual filters was then determined by liquid scintillation counting.

MOR1 phosphorylation

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HEK293-MOR1 cells were incubated with [^{32}P] orthophosphate (0.15mCi ml^{-1}) in phosphate-free DMEM for 2 h. Following exposure to opioid agonists, cells were scraped into ice-cold lysis buffer (50mM Tris-HCl pH7.5, 120mM NaCl, 1% Triton X-100, 40mM β-glycerophosphate, 1mM EDTA, 0.1μM microcystin, 0.5mM sodium orthovanadate and protease inhibitor cocktail), nuclei and cell-debris removed by centrifugation and MOR1 immunoprecipitated with 1μg T7-Tag monoclonal antibody (Novagen) and 20μl of a 50% slurry of protein A-sepharose. Immunoprecipitates were washed 3 times in lysis buffer and subjected to SDS-PAGE. The intensity of the MOR1 band was determined using a Molecular Dynamics phosphorimager.

Confirmation of GRK6 dominant negative mutant overexpression

The expression of GRK6-K215R in HEK293-MOR1 cells was verified using SDS-PAGE and Western blotting as described previously (Ghadessy et al., 2003). Briefly, resolved proteins were transferred to Hybond-ECL nitrocellulose membranes and incubated first with a GRK6 rabbit-polyclonal antibody (1:100) that recognises an epitope of residues 98-136 of human GRK6 (Ghadessy et al., 2003). Membranes were then incubated with horseradish peroxidase conjugated secondary antibody (1:1000) to enable protein detection by enhanced chemiluminescence (ECL) according to the manufacturer's instructions.

MOR1 internalization

Loss of surface MOR1 due to internalization was measured by ELISA using a colorimetric alkaline phosphatase assay, as described previously (Bailey et al., 2003). Briefly, HEK293-MOR1 cells were first incubated with the primary antibody (anti-T7 monoclonal, Novagen, UK; 1:5000) for 60 min at 37 ^{o}C to label surface MORs. Cells were then washed and incubated with opioid agonists in DMEM for 30 min at 37 ^{o}C. Cells were fixed in 3.7 %

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formaldehyde and incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase; 1:1000), a colorimetric alkaline phosphatase substrate (Biorad, UK) was then added and samples were assayed at 405nm with a microplate reader. Background was subtracted by simultaneous assay of HEK293 cells not expressing MOR1. Percentage surface receptor loss was calculated by normalizing data from each treatment group to corresponding control surface receptor levels determined from cells not exposed to opioid agonists. All experiments were performed in triplicate.

Arrestin translocation

Arrestin-2-Green Fluorescent Protein (arrestin-2-GFP) and arrestin-3-GFP redistribution was assessed as previously described (Mundell et al., 2000). Briefly HEK293-MOR1 cells grown on poly-L-lysine coverslips were transiently transfected with 0.5 μg of peGFP-N1-arrestin-2- or 3-GFP and incubated for incubated for 16-24 h before use. Cells were then washed 3 times with PBS prior to imaging and coverslips mounted in a heated imaging chamber through which media and drugs could be added. Cells were examined by microscopy on an inverted Leica TCS-NT confocal laser scanning microscope attached to a Leica DM IRBE epifluorescence microscope with phase-contrast and a Plan-Apo 40 x 1.40 NA oil immersion objective. All images were collected on Leica TCS-NT software for 2D and 3D image analysis and processed using Adobe Photoshop 6.0.

Materials

All cell culture reagents were purchased from Invitrogen, UK. All drugs were purchased from Sigma (UK) except for staurosporine (Tocris, UK), Go6976 and PKC (19-31) (Calbiochem, UK), and DAMGO (Bachem, UK). [^{35}S]GTPγS (37 MBq ml^{-1}) was purchased from PerkinElmer, UK. [^{32}P]-orthophosphate was purchased from Amersham.

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Plasmid constructs were obtained as follows: T7 epitope tagged rat MOR1 - Prof Volker Höllt (Otto-von-Guericke University, Magdeburg, Germany); Kir3.1 and 3.2 IRES construct - Dr Andrew Tinker (University College London, UK). The GRK2-K220R DNM, GRK6-K215R DNM and arrestin-GFP constructs were as we have previously described (Ghadessy et al., 2003; Mundell et al., 2000).

Data analysis

All data are expressed as means ± S.E.M.s, or means and 95% confidence intervals. Unpaired two-tailed Student's t-test or one-sample t-test were used as appropriate to assess statistical significance.

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Results

Opioid activated GIRK current

In HEK293-MOR1 cells transiently expressing Kir3.1 and 3.2 and superfused with an extracellular solution containing raised [K^+] (50 mM), DAMGO (10 μM) and morphine (30 μM) evoked currents that displayed a high degree of inward rectification (Figure 1A & B). The reversal potential for the DAMGO- and morphine-evoked currents was -25.2 ± 2 mV (n=3), and -25.3 ± 0.3 mV (n=3) respectively, which agrees closely with the calculated equilibrium potential for K^+ of -25 mV under these recording conditions. At a holding potential of -60 mV the maximum current evoked by a receptor saturating concentration of DAMGO (10 μM) was 624 ± 132 pA (n=6) whereas the maximum current evoked by a receptor saturating concentration of morphine (30 μM) was smaller and had an amplitude of 299 ± 37 pA (n=6; $p<0.05$), indicating that morphine is a partial agonist at MOR1 receptors (see also Bailey et al., 2003; 2004). Naloxone (1 μM) abolished the current evoked by morphine (30 μM) and DAMGO (10 μM) (n=3 for each) (Figure 1C & D). Furthermore Pertussis toxin pre-treatment (0.1 μg/ml for 16 h) abolished the ability of DAMGO and morphine to evoke any current (n=4) (data not shown).

MOR1 desensitization

When DAMGO (10 μM) or morphine (30 μM) was applied for 10 min they evoked an inward current that reached a peak and then declined in the continued presence of the agonist (Figure 2A & B). The desensitization to DAMGO (10 μM) and morphine (30 μM) was 73 ± 6 % and 83 ± 3 % respectively (n=4 for each) (Figure 2A, B, C & D). The decay of the response to either DAMGO or morphine was well fitted by a single exponential. Although there was no

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significant difference between the amount of desensitization in response to DAMGO or morphine ($p>0.05$) the response to DAMGO decayed slightly faster ($t_{1/2}$, DAMGO = 79 s, 95 % confidence limits 72 – 87 s; $t_{1/2}$, morphine = 109 s, 95% confidence limits 99 – 121 s). To demonstrate further that the desensitization was not due to K^+ build-up inside the cells (see Methods), we measured the inward current only at the beginning and end of the 10 min of opioid agonist application (Figure 2E & F). Under these conditions the opioid-evoked currents still desensitized by similar amounts (DAMGO desensitization 80 ± 11 %; morphine desensitization 82 ± 5 %, n=4 for each).

To ensure that the desensitization we observed was not the result of a loss of intracellular constituents following intracellular dialysis of the cell in the whole cell patch clamp recording mode, we performed perforated patch recordings using amphotericin B (225 μg ml^{-1}) in the pipette to perforate the region of the cell membrane under the tip, permitting electrical access to the interior of the cell, without dialysis of the interior of the cell. In perforated patch mode the desensitization by DAMGO (10 μM) and morphine (30 μM) was unchanged (Figure 2G & H).

Morphine-induced MOR1 desensitization could also be observed at the level of G-protein activation using a [^{35}S]GTPγS binding assay. Both DAMGO and morphine stimulated [^{35}S]GTPγS binding to membranes of HEK293-MOR1 cells, with EC_{50} values of 45 and 83 nM respectively, whilst the maximum response to morphine was similar to that of DAMGO (response to 3 μM morphine was 95 ± 2 % of that due to 1 μM DAMGO, n=4). Thus the relative efficacy of morphine appears to change when different readouts of agonist action are used. Pretreatment of intact cells for 30 min with 1 μM DAMGO or 3 μM morphine led to significant desensitization of both DAMGO- and morphine-stimulated [^{35}S]GTPγS binding to

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membranes of MOR1 expressing cells (1 μM DAMGO-stimulated [^{35}S]GTPγS binding was 203 ± 2, 144 ± 4 and 126 ± 8 % of basal binding in non-pretreated, DAMGO-pretreated and morphine pretreated cells respectively; 3 μM morphine-stimulated [^{35}S]GTPγS binding was 237 ± 27, 153 ± 4 and 119 ± 9 in non-pretreated, DAMGO-pretreated and morphine pretreated cells, respectively; values are means ± S.E.M., n =3 in each case).

Involvement of GRKs in DAMGO-induced MOR1 desensitization

To investigate the role of G-protein receptor kinases in MOR1 desensitization, the effect of over-expressing dominant negative mutants of GRK2 (GRK2-K220R) and GRK6 (GRK6-K215R) (Ghadessy et al., 2003), which lack kinase activity, was investigated. In cells over-expressing GRK2-K220R the level of desensitization to DAMGO (10 μM) was significantly reduced whereas the desensitization to morphine was unaffected (Figure 3A & B). Over-expressing GRK6-K215R did not inhibit the desensitization induced by either DAMGO or morphine (Figure 3C & D). Western blotting confirmed that the GRK6-K125R mutant was being over-expressed in HEK293-MOR1 cells (data not shown).

Involvement of PKC in morphine-induced MOR1 desensitization

To investigate whether PKC is involved in the desensitization of morphine-induced GIRK currents as proposed by Bailey *et al*., (2004), we employed the PKC pseudosubstrate inhibitory peptide (PKC 19-31). The peptide was included in the recording pipette solution and allowed to dialyse into the cell for 10 min prior to applying the opioid agonists. PKC 19-31 (5 μM) significantly reduced the amount of morphine-induced desensitization by ~50 %, but had no effect on the desensitization induced by DAMGO (Figure 4A & B). In addition, PKC 19-31 had no effect on the low level of desensitization (~20 %) induced by a

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concentration of the full agonist DAMGO (100 nM) that evoked a similar sized GIRK current to a receptor saturating concentration of the partial agonist morphine (30 μM) (data not shown).

It is possible that PKC 19-31 did not completely block morphine-induced desensitization because its activity had been reduced by degradation of the peptide by intracellular peptidases. We therefore examined the effect of membrane permeable PKC inhibitors. Unfortunately the PKC inhibitors GF 109203X (1 μM) and chelerythrine (1 μM) abolished the opioid-activated GIRK current and thus prevented their use. The broad spectrum protein kinase inhibitor staurosporine (1 μM), reduced the opioid-current by 67 ± 12 % (n=3). Such inhibition of GIRK channels by staurosporine has been previously reported, although the mechanism of the effect remains unclear (Lo and Breitwieser, 1994). However, when staurosporine was applied for 10 min prior to and then during the application of DAMGO (10 μM) or morphine (30 μM) it did not affect the desensitization induced by DAMGO but it inhibited the desensitization induced by morphine by ~60 % (Figure 4C & D). Pretreatment of cells with the phorbol ester, PMA (1μM) for 15 min to activate PKC did not alter either the rate or the extent of the desensitization induced by DAMGO (10 μM) or morphine (30μM)(n = 3 for each; data not shown).

To determine which isoforms of PKC are involved in MOR1 desensitization by morphine, the effect of the PKC inhibitor Go6976 was investigated. Go6976 was chosen as it only inhibits the conventional isoforms of PKC (PKCα, PKCβ & PKCγ) (Martiny-Baron *et al*, 1993) and PKCμ (Gschwendt *et al*, 1996). As with staurosporine, Go6976 inhibited the opioid-activated GIRK current by 49 ± 6 % (n=6). Go6976 (1 μM) applied for 10 min prior to and during the application of DAMGO (10 μM) or morphine (30 μM) had no effect on the rate or extent of

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the DAMGO-induced desensitization but slowed only slightly the rate of morphine-induced desensitization without producing a marked decrease in the amount of desensitization (Figure 4E & F).

To determine whether the PKC component of morphine-induced MOR1 desensitization resulted from ongoing PLC activity, either basal activity or enhanced activity due to activation of a Gq-coupled receptor, we examined the effect of the PLC inhibitor U-73122 (1μM) included in the recording pipette solution on morphine-induced desensitization. When U-73122 was allowed to diffuse from the pipette into the cell for up to 20 min before applying morphine it did not inhibit either the rate or the extent of morphine-induced MOR1 desensitization (n = 4; data not shown).

Agonist-induced MOR1 phosphorylation

To study MOR1 phosphorylation HEK293 cells over-expressing T7 epitope tagged MOR1 were labelled with [^{32}P]-orthophosphate. MOR1 was then immunoprecipitated in the presence of phosphatase inhibitors and the extent of ^{32}P incorporation measured by phosphoimager analysis following SDS-PAGE. Under control (basal) conditions phosphorylated MOR1 could be visualised as a diffuse band at around 80 kDa (Figure 5 A & B). When cells were exposed to DAMGO (10μM) for up to 30 min MOR1 phosphorylation was markedly increased whereas in cells exposed to morphine (30μM) for up to 30 min phosphorylation was enhanced but to a lesser but consistent extent (Figure 5 A & B). These results are in agreement with those of Schulz et al., 2004. Both basal phosphorylation of MOR1 and that in the presence of morphine were reduced by pre-exposing the cells to the broad spectrum PKC inhibitor, GF109203X (1μM) for 15 min. However, in the presence of

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GF109203X morphine was still able to induce further MOR1 phosphorylation. DAMGO-induced MOR1 phosphorylation was not reduced by GF109203X.

Agonist-induced arrestin translocation

In cells transiently transfected with arrestin-2-GFP exposure to DAMGO (10 μM) for 3 min produced a marked translocation of arrestin-2 to the plasma membrane (Figure 6A & B). In contrast, exposure to morphine (30 μM) for 3 min failed to produce any obvious movement of arrestin-2 from the cytoplasm (Figure 6C & D). Similar results were obtained with arrestin-3-GFP (data not shown).

Agonist-induced MOR1 internalization

First, the amount of constitutive MOR1 internalization that occurred in cells was determined by comparing the density of surface MORs on control cells incubated in medium for 30 min to the receptor density of cells that were fixed in formaldehyde immediately after antibody labelling (i.e. not incubated for 30 min). Over 30 min, constitutive internalization accounted for 8 ± 2 % of receptor loss.

DAMGO produced a concentration-dependent enhancement of MOR1 internalization (Figure 7A). The EC_{50} for the DAMGO-evoked internalization was 470 nM (150 nM – 1.47 μM - 95% confidence limits). The internalization produced by morphine was significantly less (Figure 7B, D & E); it appeared to be concentration-dependent but we have been unable to fit a sigmoidal curve to the data. To determine the time course for MOR1 internalization, cells were incubated with receptor saturating concentrations of DAMGO or morphine for periods up to 60 min (Figure 7C & D). Internalization could be detected after 10 min exposure to DAMGO (10 μM) or morphine (30 μM) and had reached a maximum by 30 min of agonist

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exposure. A receptor saturating concentration of DAMGO (10 μM) produced 52 ± 3 % MOR1 internalization over 30 min, whereas a receptor saturating concentration of morphine (30 μM) produced only 15 ± 3 % internalization (n=7 for both; Figure 7E).

Discussion

The key finding of this work is that the mechanisms underlying the desensitization and internalization of MOR1 are agonist-dependent. Although DAMGO and morphine application induced similar levels of desensitization of MOR-mediated GIRK currents in HEK293 cells, they did so by different molecular mechanisms. Hence, whereas the full agonist DAMGO induced desensitization by a GRK2-dependent mechanism, morphine induced desensitization by a GRK2-independent mechanism involving PKC. Also, DAMGO caused marked arrestin translocation and MOR internalization whereas morphine did not cause measurable arrestin translocation and induced much less MOR internalization.

The agonist-induced homologous desensitization of many GPCRs is thought to be mediated by phosphorylation of the receptor, in numerous cases by the family of G protein-coupled receptor kinases. HEK293 cells express GRK2 endogenously (Iwata et al., 2005), and DAMGO-induced desensitization of MOR1-stimulated GIRK currents was markedly reduced by a dominant negative mutant of GRK2, GRK2-K220R. The ability of GRK2-K220R to inhibit DAMGO-induced MOR1 desensitization agrees with previous studies implicating the closely related GRKs, GRK2 and/or GRK3, in mediating the phosphorylation, desensitization and internalization of MORs by full agonists (Zhang et al., 1998; Wang 2000; Li and Wang, 2001; Celver et al., 2001; Schulz et al., 2002; Celver et al., 2004). HEK293 cells also express GRK6 endogenously (Iwata et al., 2005), but a dominant negative mutant of GRK6 was unable to modify DAMGO-induced MOR1 desensitization, indicating the specificity of action

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of GRK2-K220R. In contrast to the desensitization induced by DAMGO, neither GRK2-K220R nor GRK6-K215R inhibited the morphine-induced desensitization of MOR1, indicating that GRKs are probably not responsible for the morphine-induced desensitization of MOR1 responses.

We and others (Whistler and von Zastrow, 1998) have observed that morphine-activated MORs were unable to recruit non-visual arrestins to the receptor. This can be explained by our present findings, where the morphine-activated MOR1 does not induce significant GRK-dependent desensitization of the receptor, thus not triggering measurable arrestin association with the receptor. These findings however should be tempered by those recently made by Bohn et al. (2004) who suggested that in HEK293 cells recruitment of endogenous arrestins to morphine-activated MORs could potentially prevent the assessment of arrestin-GFP translocation. They also observed that in mouse embryonic fibroblasts cells transfected with the MOR and either arrestin-2-GFP or arrestin-3-GFP but lacking both endogenous arrestins morphine induced detectable translocation of arrestin-3-GFP but not arrestin-2-GFP.

In contrast to DAMGO, morphine-induced desensitization of MOR1 responses was partially inhibited by the PKC inhibitory peptide, PKC 19-31 which acts as a pseudosubstrate for PKC as well as by the broad-spectrum kinase inhibitor staurosporine. Interestingly, both staurosporine and the PKC inhibitory peptide inhibited morphine-induced desensitization to the same degree but did not fully block it, suggesting either that at the concentrations employed these agents did not fully inhibit PKC activity, or that a PKC-independent process also contributes to the morphine-induced desensitization. Crucially, inhibition of PKC did not affect DAMGO-induced desensitization of MOR1 responsiveness.

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Furthermore, Go6976, a PKC inhibitor with preference for classical PKC isoforms (Way et al., 2000), produced much less inhibition of morphine-induced desensitization than the PKC inhibitory peptide and staurosporine. This suggests that a combination of conventional and other PKC isoforms are involved in MOR1 desensitization in HEK293 cells. Certainly HEK293 cells endogenously express some classical (α, βI, βII but not γ), novel (δ, ε) and atypical PKC isoforms (ξ) (Wagey et al., 2001; Leaney et al., 2001).

We have observed that in HEK293 cells the MOR is phosphorylated by PKC even in the absence of morphine. This is in line with previous work that has shown activation of PKC with a phorbol ester to induce direct phosphorylation of MORs in the absence of an opioid agonist (Zhang et al., 1996; El Khouhen et al., 1999) and may indicate a high level of basal PKC activity in HEK293 cells. This high basal level of PKC activity cannot be due to ongoing activation of PLC as a PLC inhibitor did not inhibit morphine-induced desensitization. Since in HEK293 cells the MOR is already phosphorylated by PKC then morphine does not need to activate PKC and does not induce PKC phosphorylation of the MOR, rather, when morphine binds to MORs already phosphorylated by PKC the receptor rapidly desensitizes either by subsequently adopting a desensitized conformation or by facilitating the binding of some secondary accessory protein that induces desensitization. Morphine has previously been reported to cause translocation of PKC to the plasma membrane in SH-SY5Y cells but only after 2 h of agonist exposure (Kramer and Simon, 1999). Such translocation is unlikely to contribute to the desensitization observed in HEK293 cells since desensitization occurs during the first 1 to 2 min of exposure to morphine.

In the presence of a PKC inhibitor we observed a small amount of morphine-induced phosphorylation of MOR1. Other workers have previously reported that morphine is able to

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induce MOR1 phosphorylation (Zhang et al., 1996; Yu et al., 1997; Schulz et al., 2004). This and the large amount of DAMGO-induced phosphorylation of MOR1 are likely to be mediated by GRK2 (Zhang et al., 1998) and may be responsible for the small amount of MOR1 internalization observed with morphine.

While we observed that morphine induced rapid ($t_{1/2}$ < 2min) desensitization in HEK293 cells others have reported a slower rate of morphine-induced desensitization occurring (over 1 hour) (Koch et al., 2004; Schulz et al., 2005) or no desensitization by morphine (Whistler & von Zastrow, 1998). Whilst we cannot offer a definitive explanation for these different results, one important difference between the studies is the level of MOR expression. We have used cells expressing MORs at a level close to those reported to occur physiologically.

Although the pattern of desensitization of MOR1 responses in HEK293 cells may appear to be different from that for endogenous MORs in mature rat brainstem locus coeruleus neurons there are striking similarities. In HEK293 cells DAMGO and morphine induced rapid desensitization of GIRK currents (present study), whereas in the locus coeruleus, which expresses predominantly MOR1, DAMGO and other high efficacy MOR1 agonists were able to induce rapid desensitization of GIRK currents, whilst morphine induced less desensitization (Bailey et al., 2003; Dang & Williams, 2005). In the locus coeruleus basal PKC activity did not appear to be sufficient to induce MOR desensitization. To reveal PKC-mediated desensitization required activation of PKC through Gq-coupled muscarinic receptors or with the phorbol ester, PMA. This suggests that HEK293 cells have a higher basal level of PKC activity than locus coeruleus neurons. Also, in locus coeruleus neurons the PKC-mediated component of MOR desensitization was mediated entirely by classical PKC isoforms since it was completely inhibited by Go6976 (Bailey, unpublished observations).

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Morphine has also been shown to induce profound desensitization of MORs responsiveness in AtT20 cells (Borgland et al., 2003; Celver et al., 2004) but unfortunately the involvement of PKC in this desensitization was not investigated.

Importantly, the present results suggest that the different mechanisms of DAMGO- and morphine-induced MOR1 desensitization in HEK293 cells cannot be explained simply by the lower efficacy of morphine at MOR1, but is likely instead to result from different conformations of MOR1 being stabilized by the two agonists, which consequently recruit different regulatory elements to the receptor. There is some evidence to support this. DAMGO, but not morphine, activation of MOR1 increased the activity of phospholipase D2 (Koch et al., 2003), which appears to be important for DAMGO-induced MOR1 internalization (Koch et al., 2004). There is increasing evidence that ligand-selective GPCR conformations are functionally relevant for a number of GPCRs (reviewed in Kenakin et al., 2003; Perez and Karnik, 2005), whilst a recent study showed that differential phosphorylation of the COOH-terminus of β2-adrenoceptors was induced by receptor agonists (Trester-Zedlitz et al., 2005), which could lead to activation of different regulatory pathways.

Our results indicate that agonist-dependent mechanisms of desensitization and internalization occur for MOR1 receptors. Since morphine-desensitized receptors are not internalized then, in the continued presence of morphine they will be retained on the plasma membrane in the desensitized form. Whereas, high efficacy MOR agonists induce a GRK-dependent MOR desensitization and in the presence of these agonists the desensitized MORs can be rapidly internalized, dephosphorylated, resensitized and recycled back to the plasma membrane. The lack of recycling of morphine-desensitized receptors may explain why morphine induces a greater degree of tolerance than high efficacy opioid agonists (Koch et al., 2005).

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Footnotes

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Reprint requests should be sent to:

Graeme Henderson

Department of Pharmacology

University of Bristol

Bristol, BS8 1TD

UK

Email: Graeme.Henderson@bris.ac.uk

Present addresses:

AG-C, Departmento de Farmacología, Facultad de Medicina,Universidad de Murcia, Murcia, Spain.

DC, Institut des Sciences du Végétal, CNRS, Avenue de la terrasse, Bat 23 91198, Gif-sur-Yvette, France

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Figure Legends

Figure 1: DAMGO- and morphine-induced GIRK currents.

Representative patch clamp recordings from HEK293-MOR1 cells, transiently expressing the Kir3.1/3.2 channel. **A & B**: Current-voltage relationship of opioid agonist-evoked current. In both traces, the leak current has been subtracted. DAMGO (10 μM) evoked an inwardly rectifying current that had a reversal potential of -25.1 mV. Morphine (30 μM) evoked an inwardly rectifying current with a reversal potential of -25.7 mV. **C & D**: Whole cell currents (V_h –60 mV). Naloxone inhibited the DAMGO-evoked and the morphine-evoked currents.

Figure 2: Desensitization of the DAMGO- and morphine-evoked currents

Patch clamp recordings from HEK293-MOR1 cells, transiently expressing the Kir3.1/3.2 channel. Cells were held at –25mV and stepped to –60 mV for 60 ms every 5s. Receptor saturating concentrations of DAMGO (10 μM) or morphine (30 μM) were applied for 10 min and the desensitization of the GIRK current response was followed. **A & B**: Representative traces showing that the currents evoked by DAMGO and morphine were not maintained throughout the period of drug application, but declined (desensitized). **C & D**: To determine the rate of desensitization, the current amplitude at each time point was normalized to the amplitude of the peak current response for each cell. Desensitization kinetics for both DAMGO and morphine were then fitted to single-exponential decays; the R^2 value for the morphine curve is 0.95 and the R^2 value for DAMGO is 0.92. **E & F**: In these experiments membrane potential was held at –25 mV and 60 ms pulses to –60 mV (to measure the amplitude of the opioid-evoked current) were only applied at the beginning (15 – 35 s) and end (585 to 600 s) of the 10 min of opioid agonist application (n=3). The responses to DAMGO and morphine desensitized by the same extent as in experiments in which pulses to

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–60 mV were applied repeatedly throughout the period of agonist application confirming that desensitization was not the result of a build up of K^+ inside the cell. **G & H**: DAMGO and morphine desensitization was measured using whole cell recording and perforated patch recording. Data from individual cells were normalized to the peak response and pooled. Desensitization kinetics were then fitted to a single exponential decay. The rate and extent of desensitization induced by DAMGO and morphine were the same under each recording condition (n = 3 for all)

Figure 3: Effect of GRK2-K220R and GRK6-K215R on MOR1 desensitization

HEK293-MOR1 cells were transfected with DNM GRKs, along with the Kir3.1/3.2 channels 16-24 h before recordings were made. Patch clamp recordings of opioid current decay from individual cells were normalized to the peak response and pooled. Desensitization kinetics for both DAMGO and morphine were then fitted to single-exponential decays. **A**: GRK2-K220R significantly reduced the extent of DAMGO-induced desensitization ($p<0.05$; n=4) but did not alter the rate of desensitization, as the $t_{1/2}$ was 111 s (96-132 s) under control conditions and 69 s (44-158 s) in cells expressing GRK2-K220R. **B**: GRK2-K220R had no effect on the extent or rate of morphine-induced desensitization ($p>0.05$; n=4). **C**. GRK6-K215R expression had no effect on the extent or rate of DAMGO-induced desensitization ($p>0.05$; n=4). **D:** GRK6-K215R expression had no effect on extent or rate of morphine-induced desensitization ($p>0.05$; n=4).

Figure 4: Effect of PKC inhibition on MOR1 desensitization

The PKC inhibitory peptide PKC 19-31 at 5 μM was added to the solution in the recording electrode and allowed to dialyse into the cells for 10 min prior to opioid application. Data from individual cells were normalized to each peak response and pooled. Staurosporine or

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Go6976 was applied to HEK293-MOR1 cells transiently expressing the Kir3.1/3.2 channel, for 10 min prior to and during the 10 min application of DAMGO or morphine. Desensitization kinetics for both DAMGO and morphine were then fitted to single-exponential decays. **A:** PKC 19-31 had no effect on the extent or rate of DAMGO desensitization. **B:** PKC 19-31 significantly reduced the extent of morphine desensitization ($p<0.05$; n=6) but did not affect the rate of desensitization, as the $t_{1/2}$ was 59 s (54-63 s) under control conditions and 58 s (52-65 s) in the presence of PKC 19-31.**C:** Staurosporine had no effect on the extent or rate of DAMGO desensitization ($p>0.05$; n=4). D: Staurosporine significantly reduced the extent of the morphine desensitization ($p<0.05$) and increased the $t_{1/2}$ from 67 s (59-77 s; n=4) to 161 s (113-276 s; n=3) ($p<0.05$). **E**: Go6976 had no effect on the extent or rate of DAMGO desensitization after 10 min ($p>0.05$; n=4). **F:** Go6976 had no effect on the level of morphine desensitization after 10 min ($p>0.05$), but the rate of desensitization was slower; the $t_{1/2}$ was increased from 88 s (78-102 s; n=3) under control conditions to 147 s (121-187 s; n=4) in the presence of Go6976 ($p<0.05$).

Figure 5: DAMGO and morphine induced MOR1 phosphorylation and PKC translocation. A: Phosphorimage of immunoprecipitated MOR1 from ^{32}P labelled HEK293-MOR1 cells run on SDS-PAGE. Cells were exposed to DAMGO (10μM) or morphine (30μM) for up to 30 min in the absence or presence of GF109203X (1μM; GF). Cells were pre-exposed to GF 109203X for 15 min prior to the addition of the opioid agonists. B: quantification of up to 5 experiments of the type shown in A to determine the level of MOR1 phosphorylation. Both DAMGO (10μM) and morphine (30μM) induced MOR1 phosphorylation above control. GF 109203X (1μM; GF) reduced the level of phosphorylation in control and morphine-treated cells but did not significantly reduce the phosphorylation induced by DAMGO. In the presence of GF 109203X the level of phosphorylation induced by

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morphine was still greater than in the presence of GF 109203X alone. Statistical significance ($p<0.05$) is indicated as follows: * morphine greater than control; ¶ DAMGO greater than control; § GF 109203X less than control; † morphine + GF 109203X less than morphine alone; ‡ morphine + GF 109203X greater than GF 109203X alone.

Figure 6: DAMGO but not morphine promotes arrestin translocation.

HEK293-MOR1 cells grown on poly-L-lysine coverslips were transiently transfected with 0.5 μg of peGFP-N1-arrestin-2-GFP. Prior to stimulation and viewing, coverslips were mounted in an imaging chamber at 37 °C and arrestin-2-GFP distribution was monitored in real time. **A & C**: Arrestin-2-GFP has a diffuse cytoplasmic distribution in unstimulated cells prior to agonist exposure. **B:** In cells exposed to morphine (30 μM) for 3 min the diffuse cytoplasmic distribution of arrestin-2-GFP is maintained. D: In cells exposed to DAMGO (10 μM) for 3 min significant agonist-induced translocation of arrestin-2-GFP to the cell membrane is visible. Data shown are representative of three independent experiments.

Figure 7: DAMGO- and morphine- induced MOR1 internalization

ELISA assays were used to quantify MOR1 internalization in the HEK293-MOR1 cells in response to either DAMGO or morphine. **A & B:** DAMGO (n=4) and morphine (n=4) produced a concentration-dependent stimulation of MOR1 internalization after 30 min agonist exposure. **C & D**: The timescale for MOR1 internalization in response to DAMGO (10 μM; n=5) and morphine (30 μM; n=5), internalization was measured after 10, 30 or 60 min of agonist exposure. **E:** Comparing the level of DAMGO (10 μM) and morphine (30 μM) induced MOR1 internalization after 30 min of agonist treatment revealed that DAMGO produced significantly more internalization than morphine ($p<0.05$; n=4). The amount of

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constitutive receptor internalization occurring after 30 min was 8 ± 2 % (n=3). The value for constitutive internalization was subtracted from that of the agonist-induced internalization in each experiment.

Figure 1

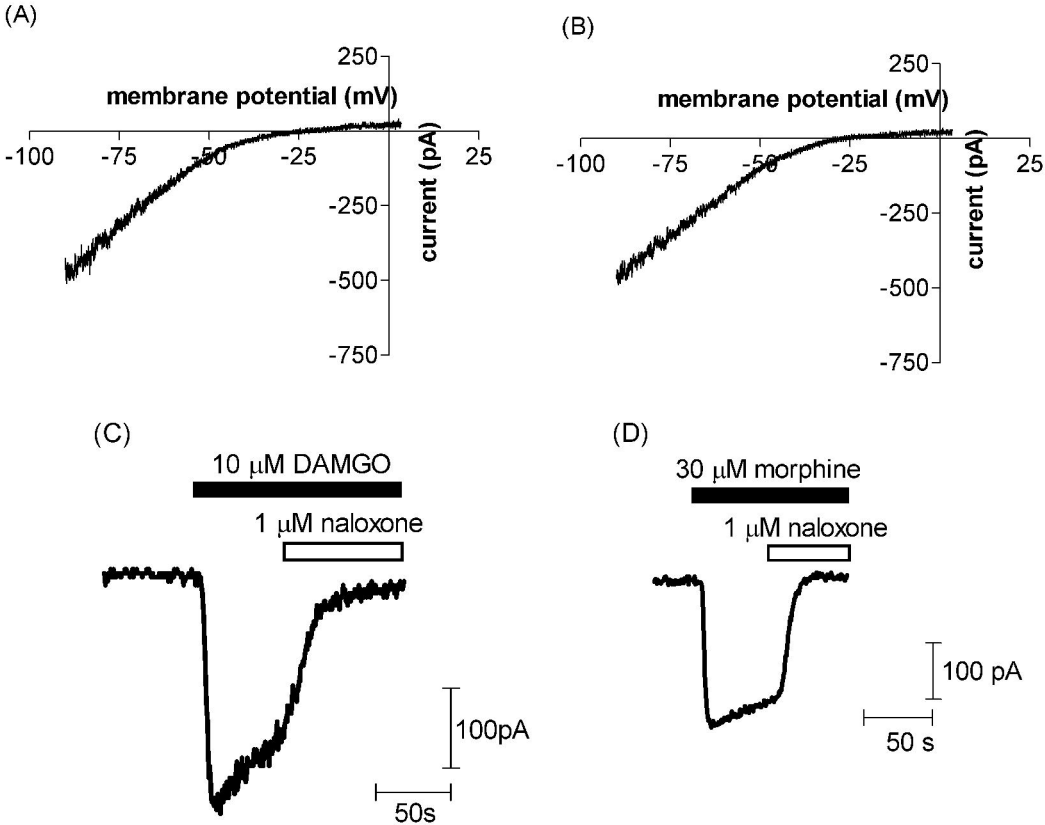

(A)
membrane potential (mV)
250
-100
-75
-50
-25
25
-250
-500
-750
current (pA)
(B)
membrane potential (mV)
250
-100
-75
-50
-25
25
-250
-500
-750
current (pA)
(C)
10 μM DAMGO
1 μM naloxone
100pA
50s
(D)
30 μM morphine
1 μM naloxone
100 pA
50 s

Figure 2

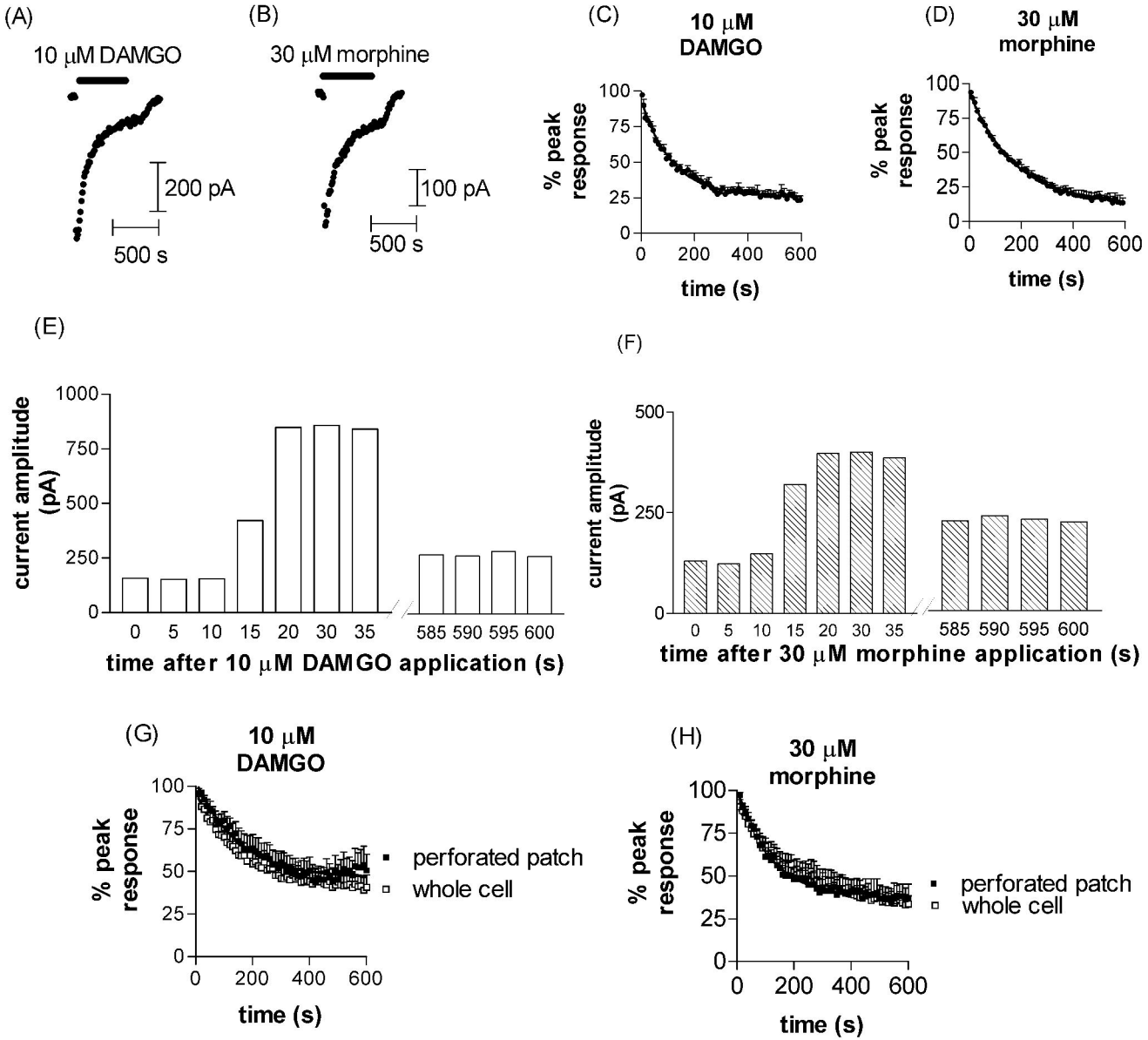
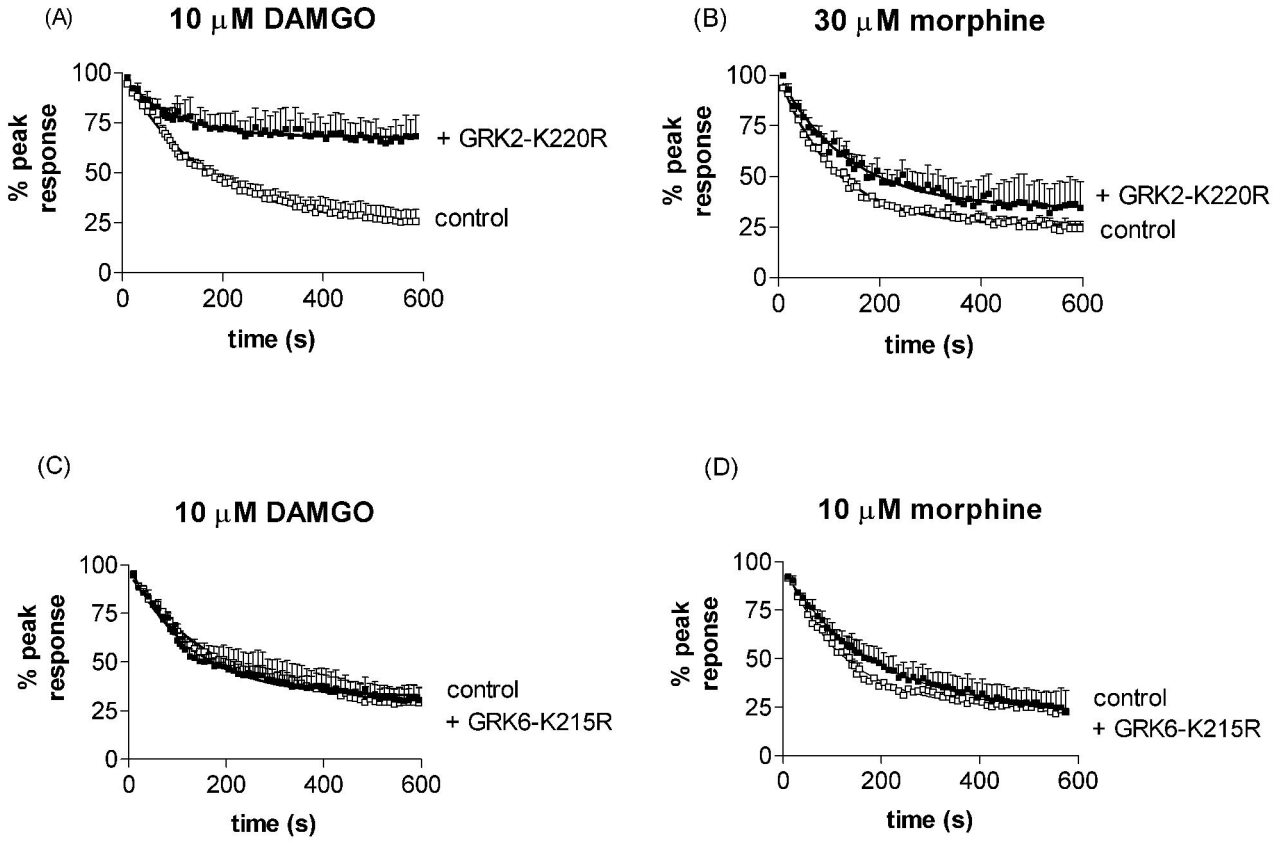

(A)
10 μM DAMGO
200 pA
500 s
(B)
30 μM morphine
100 pA
500 s
(C)
10 μM DAMGO
% peak response
time (s)
(D)
30 μM morphine
% peak response
time (s)
(E)
current amplitude (pA)
time after 10 μM DAMGO application (s)
(F)
current amplitude (pA)
time after 30 μM morphine application (s)
(G)
10 μM DAMGO
% peak response
perforated patch
whole cell
time (s)
(H)
30 μM morphine
% peak response
perforated patch
whole cell
time (s)

Figure 3


(A)
10 μM DAMGO
% peak response
100
75
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25
0
0
200
400
600
time (s)
+ GRK2-K220R
control
(B)
30 μM morphine
% peak response
100
75
50
25
0
0
200
400
600
time (s)
+ GRK2-K220R
control
(C)
10 μM DAMGO
% peak response
100
75
50
25
0
0
200
400
600
time (s)
control
+ GRK6-K215R
(D)
10 μM morphine
% peak reponse
100
75
50
25
0
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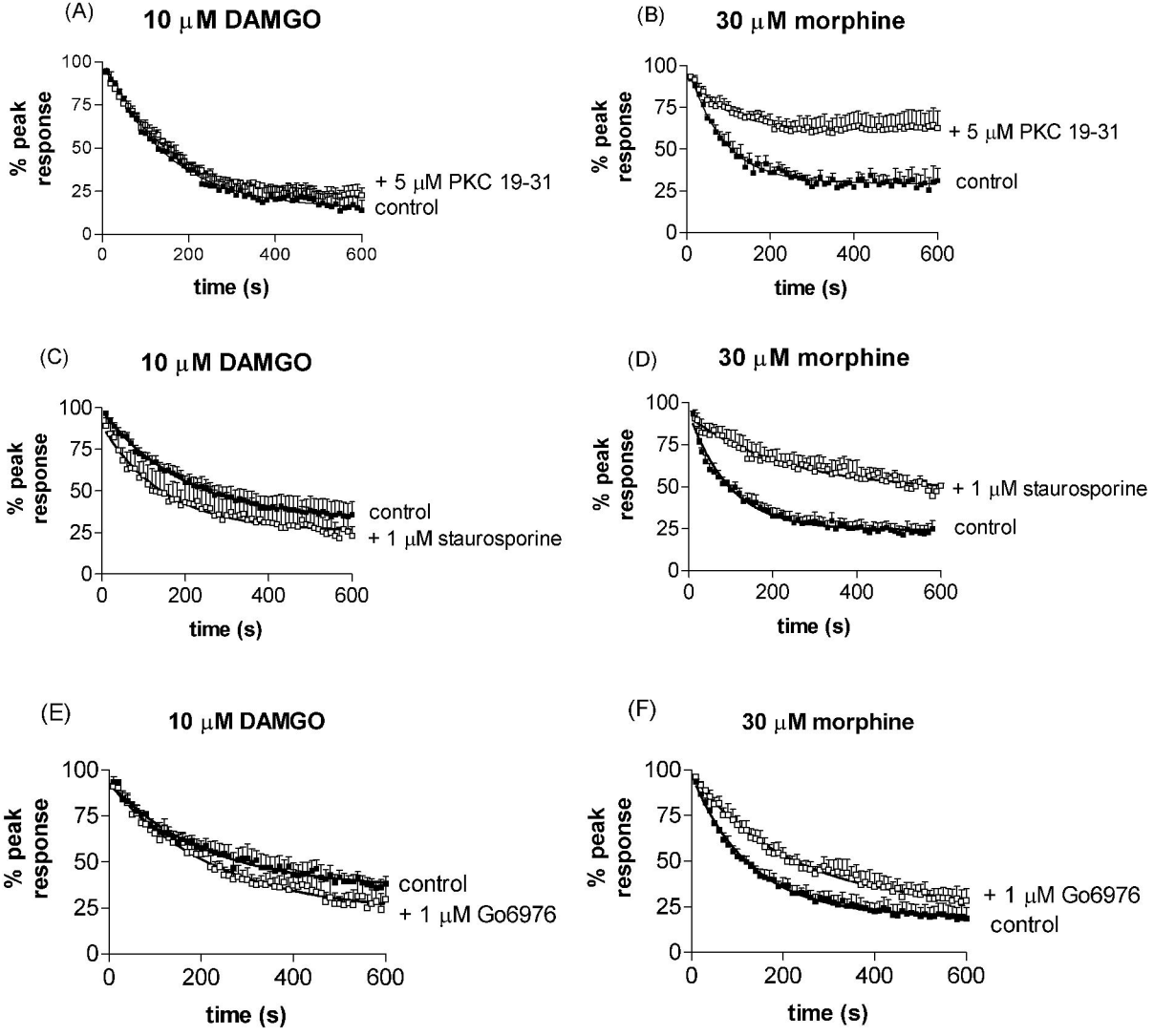
200
400
600
time (s)
control
+ GRK6-K215R

Figure 4


(A)
10 μM DAMGO
% peak response
100
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time (s)
+ 5 μM PKC 19-31
control
(B)
30 μM morphine
% peak response
100
75
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600
time (s)
+ 5 μM PKC 19-31
control
(C)
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time (s)
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+ 1 μM staurosporine
(D)
30 μM morphine
% peak response
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time (s)
+ 1 μM staurosporine
control
(E)
10 μM DAMGO
% peak response
100
75
50
25
0
0
200
400
600
time (s)
control
+ 1 μM Go6976
(F)
30 μM morphine
% peak response
100
75
50
25
0
0
200
400
600
time (s)
+ 1 μM Go6976
control

Figure 5

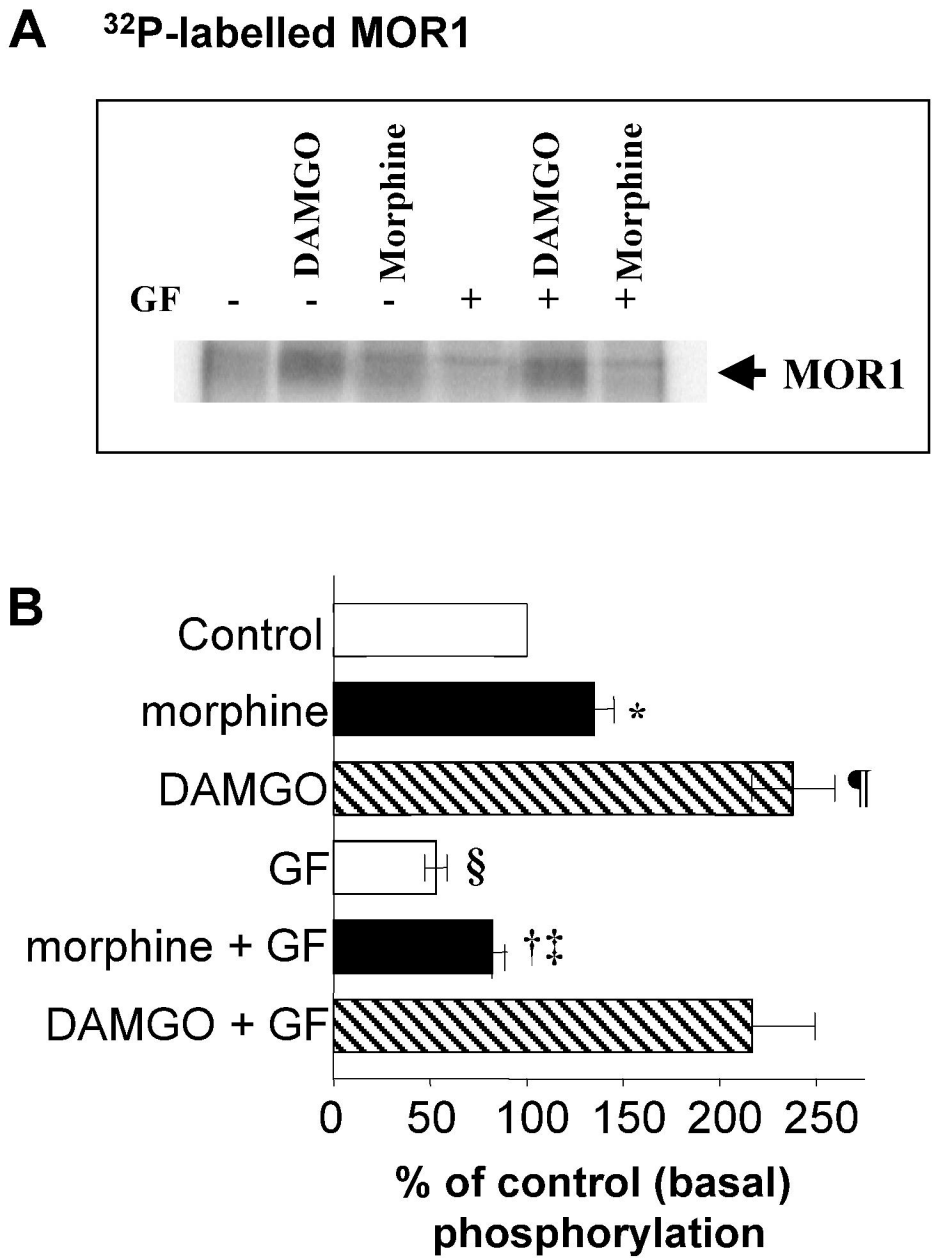

A
32P-labelled MOR1
GF
-
- DAMGO
- Morphine
+
+ DAMGO
+ Morphine
MOR1
B
Control
morphine
DAMGO
GF
morphine + GF
DAMGO + GF
*
¶
§
†‡
0
50
100
150
200
250
% of control (basal) phosphorylation

Figure 6

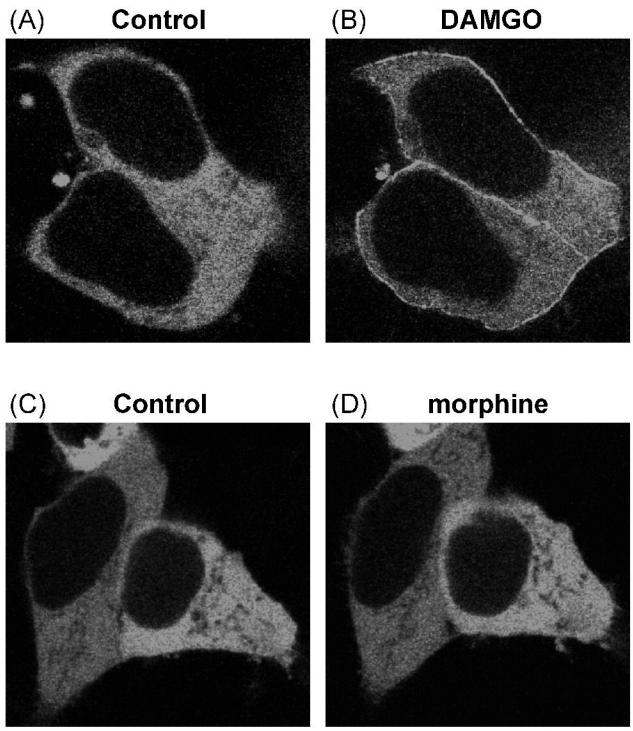
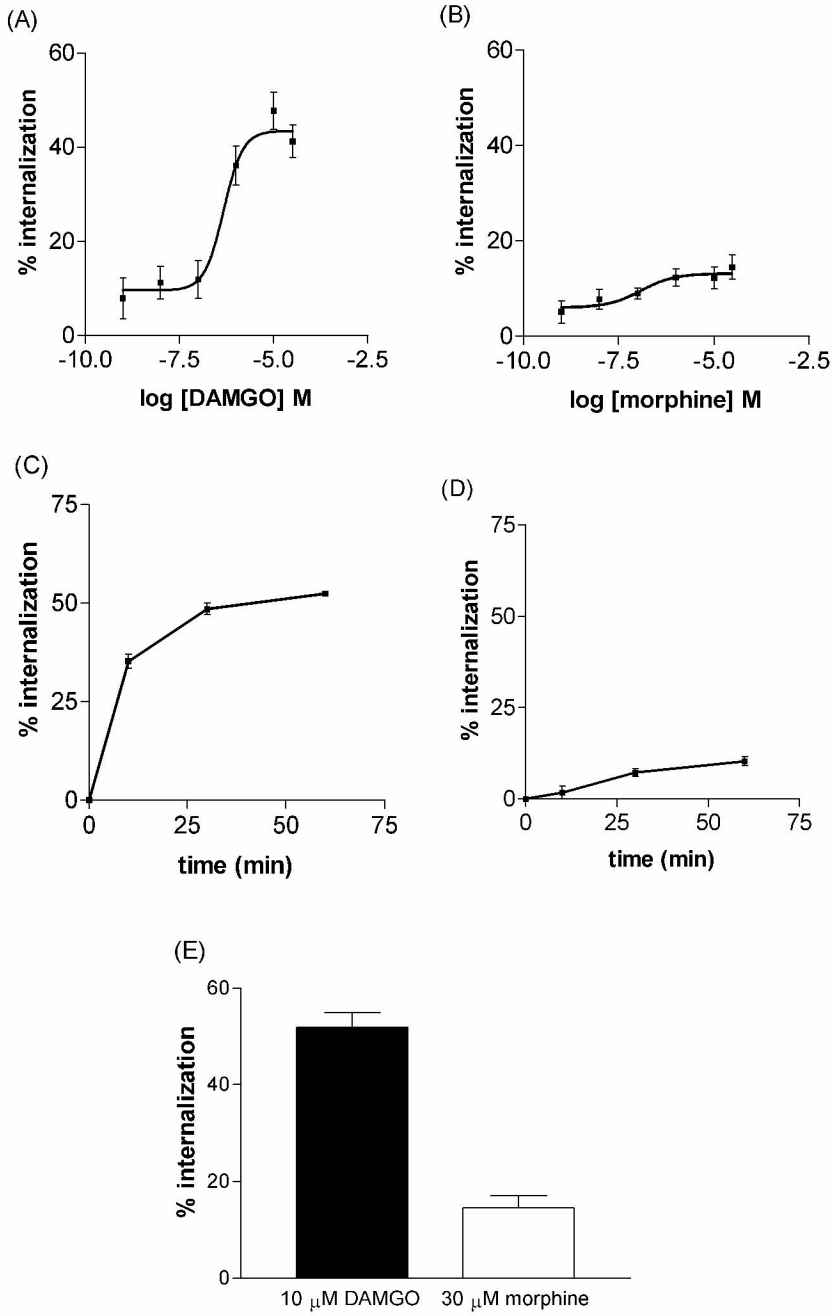


Figure **7**


(A)
% internalization
60
40
20
0
-10.0
-7.5
-5.0
-2.5
log [DAMGO] M
(B)
% internalization
60
40
20
0
-10.0
-7.5
-5.0
-2.5
log [morphine] M
(C)
% internalization
75
50
25
0
0
25
50
75
time (min)
(D)
% internalization
75
50
25
0
0
25
50
75
time (min)
(E)
% internalization
60
40
20
0
10 μM DAMGO
30 μM morphine