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Agonist-Selective Mechanisms of μ-Opioid Receptor Desensitization in Human Embryonic Kidney 293 Cells

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ABSTRACT

The ability of two opioid agonists, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) and morphine, to induce μ-opioid receptor (MOR) phosphorylation, desensitization, and internalization was examined in human embryonic kidney (HEK) 293 cells expressing rat MOR1 as well G protein-coupled inwardly rectifying potassium channel (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 it had no effect on morphine-induced MOR1 desensitization. In contrast, inhibition of protein kinase C (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 bisindolylmaleimide 1 (GF109203X) inhibited MOR1 phosphorylation under basal conditions and in the presence of morphine, but it 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 signaling in HEK293 cells but by different molecular mechanisms; DAMGO-induced desensitization is GRK2-dependent, whereas morphine-induced desensitization is in part PKC-dependent. MORs desensitized by DAMGO activation are then readily internalized by an arrestin-dependent mechanism, whereas those desensitized 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.

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 with 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 (for review, see Connor et al., 2004; Bailey and Connor, 2005). On the one hand, it has been suggested that morphine, because it does not trigger significant MOR internalization, allows the receptor to couple over a prolonged period to intracellular signaling 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 exac-

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ABBREVIATIONS: MOR, μ-opioid receptor; DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; GIRK, G-protein-coupled inwardly rectifying K^+ channel; HEK, human embryonic kidney; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; DMEM, Dulbecco's modified Eagle's medium; GRK, G protein-coupled receptor kinase; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; GF109203X, bisindolylmaleimide 1; PLC, phospholipase C; U-73122, 1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-dione; GPCR, G protein-coupled receptor.

erbated 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 with full MOR agonists such as DAMGO, morphine caused a greater degree of long-term desensitization because 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 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.

Materials and Methods

HEK293 Cells Stably Expressing MOR1. HEK293 cells stably expressing T7-epitope-tagged MOR1 have been described previously (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 of protein (as assessed by [^{3}H]diprenorphine saturation binding). This level of expression approximates to that found in mature brain neurons (Tempel and Zukin, 1987). For the MOR1 phosphorylation studies, we used an HEK293-MOR1 cell line with a higher MOR1 expression level of 1627 ± 41 fmol/mg of protein. Cells were maintained at 37°C in 95% O_2, 5% CO_2, in DMEM, supplemented with 10% fetal bovine serum, 10 U/ml penicillin, and 10 mg/ml streptomycin; for the HEK293-MOR1 cells, 250 μg/ml G418 (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 to 0.5 μg of 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 $CaCl_2$ was added to a final concentration of 0.25 M $CaCl_2$. The mixture was then combined 1:1 with HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM glucose, and 50 mM HEPES, adjusted to pH 6.9 using 1 M NaOH) and added to each well of the plate. Cells were then incubated for 16 to 24 h before use.

Whole-Cell Patch-Clamp Recordings. Nonconfluent 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 visualization of green fluorescent protein (GFP) fluorescence (Nikon, Tokyo, Japan) and superfused (4 ml/min) with extracellular solution at room temperature (22–26°C). The extracellular solution contained 160 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM glucose, and 5 mM HEPES, pH 7.4. Cells that had been successfully transfected with the Kir3.1 and Kir3.2 subunits were identified by their GFP fluorescence. Whole-cell voltage-clamp recordings (V_h = −60 mV) were made using electrodes (3–5 MΩ) filled with 122 mM KCl, 11 mM EGTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 4 mM MgATP, 0.25 mM Na_2GTP, and 5 mM NaCl, pH 7.2. Recordings were filtered at 2 kHz using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) 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 (Fig. 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 because of the inward current raising the intracellular K^+ concentration, thereby reducing the electrochemical drive for further entry of K^+ into the cell, we developed a voltage protocol that minimized 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 to −60 mV for only 60 ms every 5 s. In this way, the current response to an agonist could be measured, whereas the amount of K^+ that entered the cell during a recording was minimized. Barium (1 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 a percentage of the initial peak current. The decay of the current was fitted to a single exponential using Prism software (GraphPad Software Inc., San Diego, CA).

[^{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). In brief, cells were incubated in the presence or absence of agonist for 30 min, washed, and then the cells were resuspended in ice-cold assay buffer (0.2 mM $MgSO_4$, 0.38 mM KH_2PO_4, and 0.61 mM Na_2HPO_4, pH 7.4) and lysed in a hand-held homogenizer. The homogenates were centrifuged at 20,000*g* for 20 min at 4°C, and the pellets were 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. Nonspecific 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 were rapidly filtered through glass fiber 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. HEK293-MOR1 cells were incubated with [^{32}P]orthophosphate (0.15 mCi/ml) in phosphate-free DMEM for 2 h. After exposure to opioid agonists, cells were scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Triton X-100, 40 mM β-glycerophosphate, 1 mM EDTA, 0.1 μM microcystin, 0.5 mM sodium orthovanadate, and protease inhibitor cocktail), nuclei and cell debris were removed by centrifugation, and MOR1 was immunoprecipitated with 1 μg of T7-Tag monoclonal antibody (Novagen, Nottingham, UK) and 20 μl of a 50% slurry of protein A-Sepharose. Immunoprecipitates were washed three times in lysis buffer and subjected to SDS-PAGE. The intensity of the

MOR1 band was determined using a PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

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). In brief, resolved proteins were transferred to Hybond-ECL nitrocellulose membranes and incubated first with a GRK6 rabbit-polyclonal antibody (1:100) that recognizes an epitope of residues 98 to 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 according to the manufacturer's instructions.

MOR1 Internalization. Loss of surface MOR1 as a result of internalization was measured by enzyme-linked immunosorbent assay using a colorimetric alkaline phosphatase assay, as described previously (Bailey et al., 2003). In brief, HEK293-MOR1 cells were first incubated with the primary antibody (anti-T7 monoclonal; 1:5000; Novagen) for 60 min at 37°C to label surface MORs. Cells were then washed and incubated with opioid agonists in DMEM for 30 min at 37°C. Cells were fixed in 3.7% formaldehyde and incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase; 1:1000), a colorimetric alkaline phosphatase substrate (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK) was then added and samples were assayed at 405 nm 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-GFP and arrestin-3-GFP redistribution was assessed as described previously (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 16 to 24 h before use. Cells were then washed three times with phosphate-buffered saline before imaging, and coverslips were 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× 1.40 numerical aperture oil immersion objective. All images were collected on Leica TCS-NT software for two- and three-dimensional image analysis and processed using Photoshop 6.0 (Adobe Systems, Mountain View, CA).

Materials. All cell culture reagents were purchased from Invitrogen (Paisley, UK). All drugs were purchased from Sigma Chemical (Poole, Dorset, UK), except for staurosporine (Tocris Cookson Inc., Bristol, UK), Gö6976 and PKC (19-31) (Calbiochem, Nottingham, UK), and DAMGO (Bachem, St. Helens, Merseyside, UK). [^{35}S]GTPγS (37 MBq/ml) was purchased from PerkinElmer Life and Analytical Sciences (Beaconsfield, Bucks, UK). [^{32}P]Orthophosphate was purchased from GE Healthcare.

Plasmid constructs were obtained as follows: T7 epitope-tagged rat MOR1 (Prof. Volker Höllt, Otto-von-Guericke University, Magdeburg, Germany) and Kir3.1 and Kir3.2 IRES construct (Dr. Andrew Tinker, University College London, UK). The GRK2-K220R DNM, GRK6-K215R DNM and arrestin-GFP constructs were as we have described previously (Mundell et al., 2000; Ghadessy et al., 2003).

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.

Results

Opioid-Activated GIRK Current. In HEK293-MOR1 cells transiently expressing Kir3.1 and Kir3.2 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 (Fig. 1, A and 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

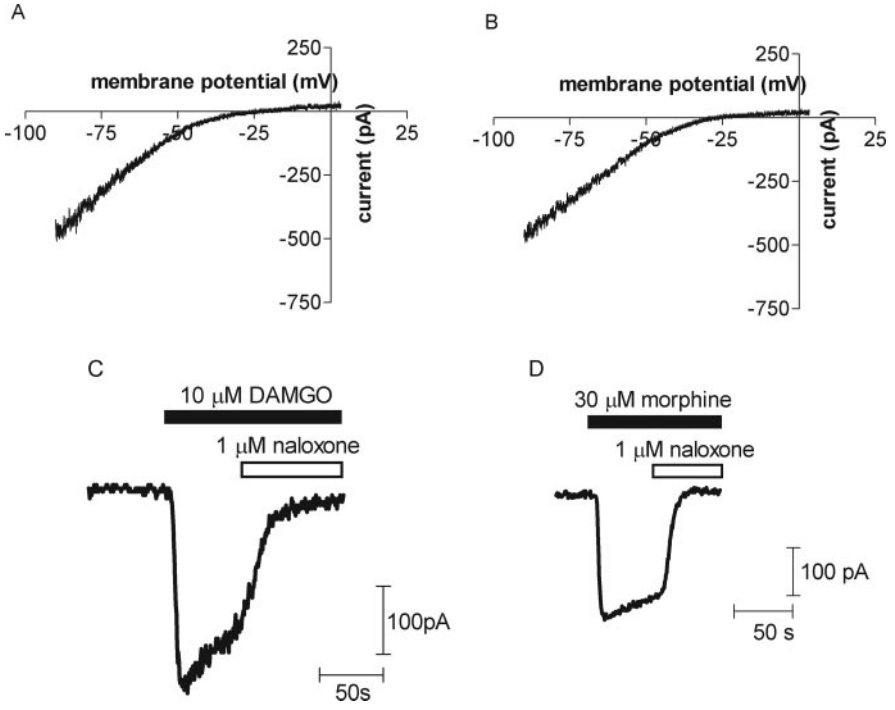

Fig. 1. DAMGO- and morphine-induced GIRK currents. Representative patch-clamp recordings from HEK293-MOR1 cells, transiently expressing the Kir3.1/3.2 channel. A and B, current-voltage relationship of opioid agonist-evoked current. In both traces, the leak current has been subtracted. DAMGO (10 μM) evoked 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 and D, whole-cell currents (V_h = −60 mV). Naloxone inhibited the DAMGO-evoked and the morphine-evoked currents.

($n = 6$), 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 (Bailey et al., 2003, 2004). Naloxone (1 μM) abolished the current evoked by morphine (30 μM) and DAMGO (10 μM) ($n = 3$ for each) (Fig. 1, C and D). Furthermore, pertussis toxin pretreatment (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 (Fig. 2, A and B). The desensitization to DAMGO (10 μM) and morphine (30 μM) was 73 ± 6 and 83 ± 3%, respectively ($n = 4$ for each) (Fig. 2, A–D). The decay of the response to either DAMGO or morphine was well fitted by a single exponential. Although there was no 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^+ buildup inside the cells (see *Materials and Methods*), we measured the inward current only at the beginning and end of the 10 min of opioid agonist application (Fig. 2, E and 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).

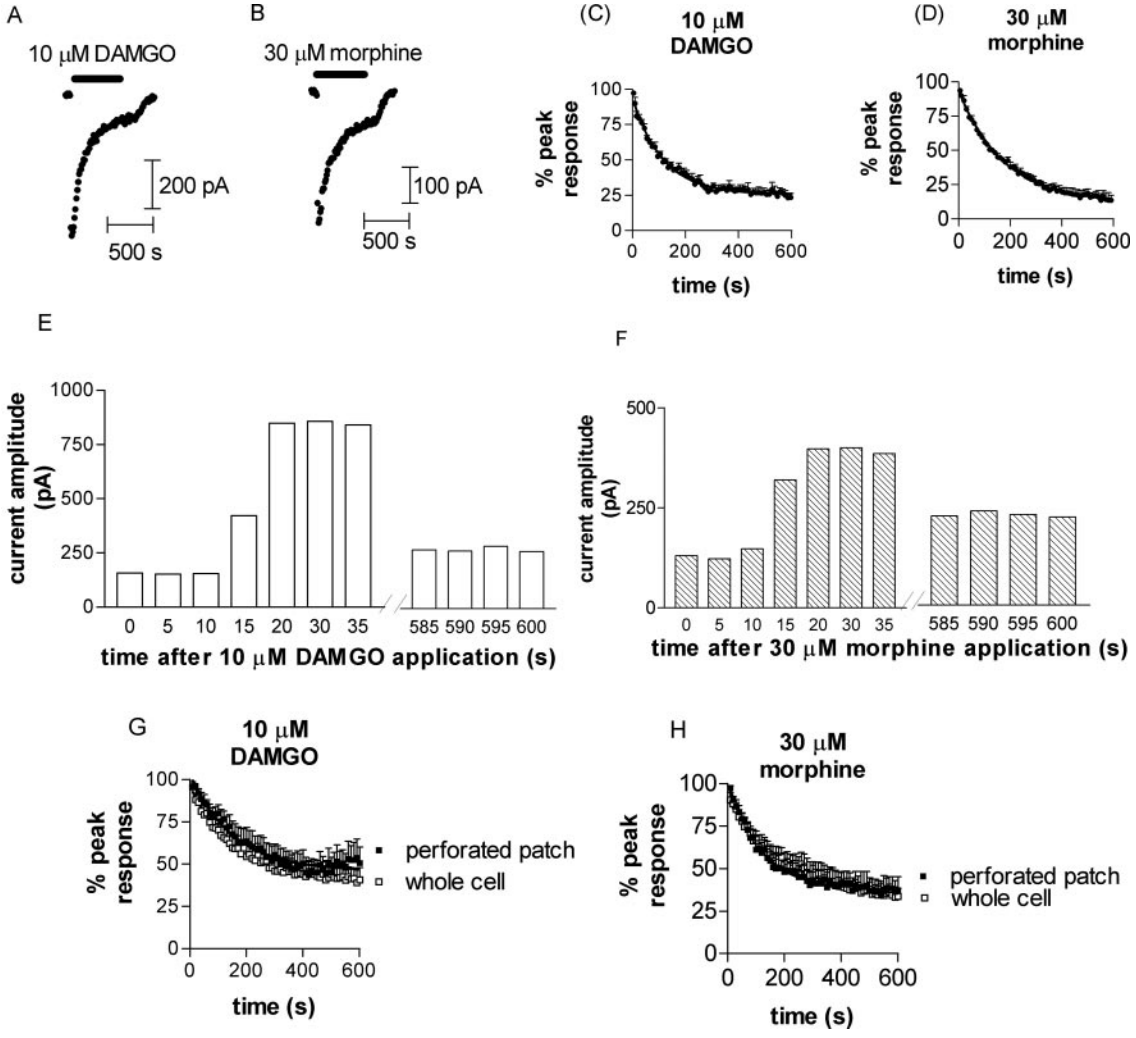

Fig. 2. Desensitization of the DAMGO- and morphine-evoked currents. Patch-clamp recordings from HEK293-MOR1 cells, transiently expressing the Kir3.1/3.2 channel. Cells were held at −25 mV and stepped to −60 mV for 60 ms every 5 s. Receptor saturating concentrations of DAMGO (10 μM) or morphine (30 μM) were applied for 10 min, and the desensitization of the GIRK current response was followed. A and 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 and 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 and 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 (585–600 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 −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 and 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).

To ensure that the desensitization we observed was not the result of a loss of intracellular constituents after intracellular dialysis of the cell in the whole-cell patch-clamp recording mode, we performed perforated patch recordings using amphotericin B (225 μg/ml) 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 (Fig. 2, G and 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, whereas the maximum response to morphine was similar to that of DAMGO (response to 3 μM morphine was 95 $\pm$ 2% of response to 1 μM DAMGO; $n = 4$). Thus, the relative efficacy of morphine seems 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 membranes of MOR1-expressing cells (1 μM DAMGO-stimulated [^{35}S]GTPγS binding was 203 $\pm$ 2, 144 $\pm$ 4, and 126 $\pm$ 8% of basal binding in nonpretreated, DAMGO-pretreated, and morphine-pretreated cells, respectively; 3 μM morphine-stimulated [^{35}S]GTPγS binding was 237 $\pm$ 27, 153 $\pm$ 4, and 119 $\pm$ 9 in nonpretreated, DAMGO-pretreated, and morphine pretreated cells, respectively; values are means $\pm$ 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 overexpressing dominant negative mutants of GRK2 (GRK2-K220R) and GRK6 (GRK6-K215R) (Ghadessy et al., 2003), which lack kinase activity, was investigated. In cells overexpressing GRK2-K220R, the level of desensitization to DAMGO (10 μM) was significantly reduced, whereas the desensitization to morphine was unaffected (Fig. 3, A and B). Overexpressing GRK6-K215R did not inhibit the desensitization induced by either DAMGO or morphine (Fig. 3, C and D). Western blotting confirmed that the GRK6-K125R mutant was being overexpressed 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 used the PKC pseudosubstrate inhibitory peptide [PKC (19-31)]. The peptide was included in the recording pipette solution and allowed to dialyze into the cell for 10 min before applying the opioid agonists. PKC (19-31) (5 μM) significantly reduced the amount of morphine-induced desensitization by ~50%, but it had no effect on the desensitization induced by DAMGO (Fig. 4, A and B). In addition, PKC (19-31) had no effect on the low level of desensitization (~20%) induced by a 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 GF109203X (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 $\pm$ 12% ($n = 3$). Such inhibition of GIRK channels by staurosporine has been reported previously, although the mechanism of the effect remains unclear (Lo and Breitwieser, 1994). However, when staurosporine was applied for 10 min before 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% (Fig. 4, C and D). Pretreatment of cells with the phorbol ester phorbol-12-myristate-13-acetate (1 μM) for 15 min to acti-

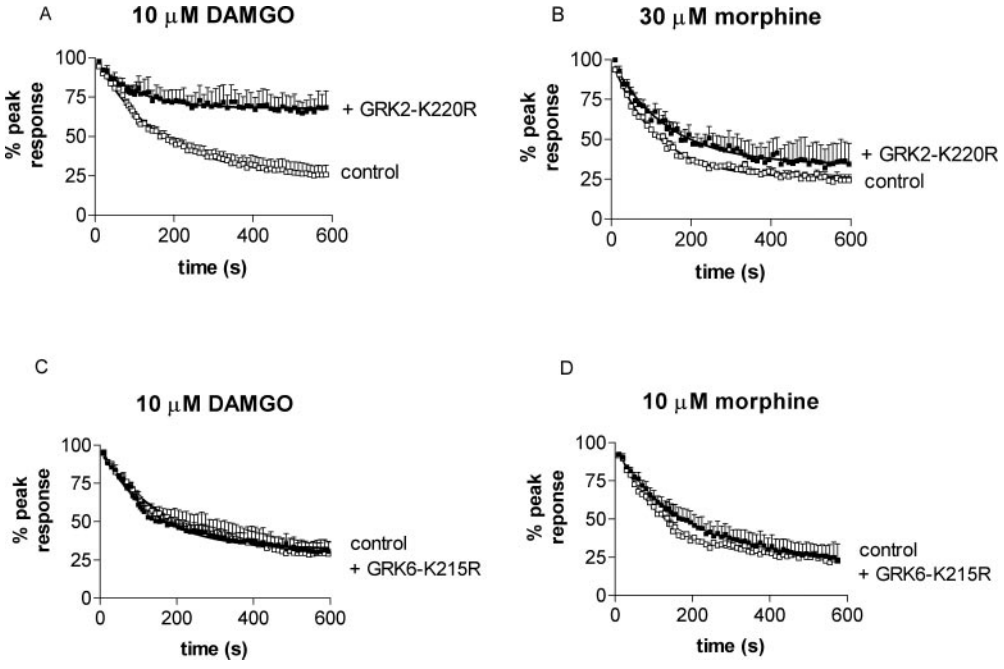

Fig. 3. Effect of GRK2-K220R and GRK6-K215R on MOR1 desensitization. HEK293-MOR1 cells were transfected with DNM GRKs, along with the Kir3.1/3.2 channels 16 to 24 h before recordings were made. Patch-clamp recordings of opioid current decay from individual cells were normalized to the peak response and 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, because 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 ($p > 0.05$; $n = 4$).

vate PKC did not alter either the rate or the extent of the desensitization induced by DAMGO (10 μM) or morphine (30 μM) (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 Gö6976 was investigated. Gö6976 was chosen because it only inhibits the conventional isoforms of PKC (PKCα, PKCβ, and PKCγ) (Martiny-Baron et al., 1993) and PKCμ (Gschwendt et al., 1996). As with staurosporine, Gö6976 inhibited the opioid-activated GIRK current by 49 ± 6% (n = 6). Gö6976 (1 μM) applied for 10 min before and during the application of DAMGO (10 μM) or morphine (30 μM) had no effect on the rate or extent of the DAMGO-induced desensitization but slowed only slightly the rate of morphine-induced desensitization without producing a marked decrease in the amount of desensitization (Fig. 4, E and F).

To determine whether the PKC component of morphine-induced MOR1 desensitization resulted from ongoing PLC activity, either basal activity or enhanced activity as a result of activation of a G_q-coupled receptor, we examined the effect of the PLC inhibitor U-73122 (1 μM) 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 overexpressing T7 epitope-tagged MOR1 were labeled with [^{32}P]orthophosphate. MOR1 was then immunoprecipitated in the presence of phosphatase inhibitors, and the extent of ^{32}P incorporation was measured by PhosphorImager analysis after SDS-PAGE. Under control (basal) conditions, phosphorylated MOR1 could be visualized as a diffuse band at around 80 kDa (Fig. 5, A and B). When cells were exposed to DAMGO (10 μM) for up to 30 min, MOR1 phosphorylation was markedly increased, whereas in cells exposed to morphine (30 μM) for up to 30 min, phosphorylation was enhanced but to a lesser but consistent extent (Fig. 5, A and 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 (1 μM) for 15 min. However, in the presence of 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 (Fig. 6, A and B). In contrast, exposure to morphine (30 μM) for 3 min failed to produce any obvious movement of arrestin-2 from the cytoplasm (Fig. 6, C and 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

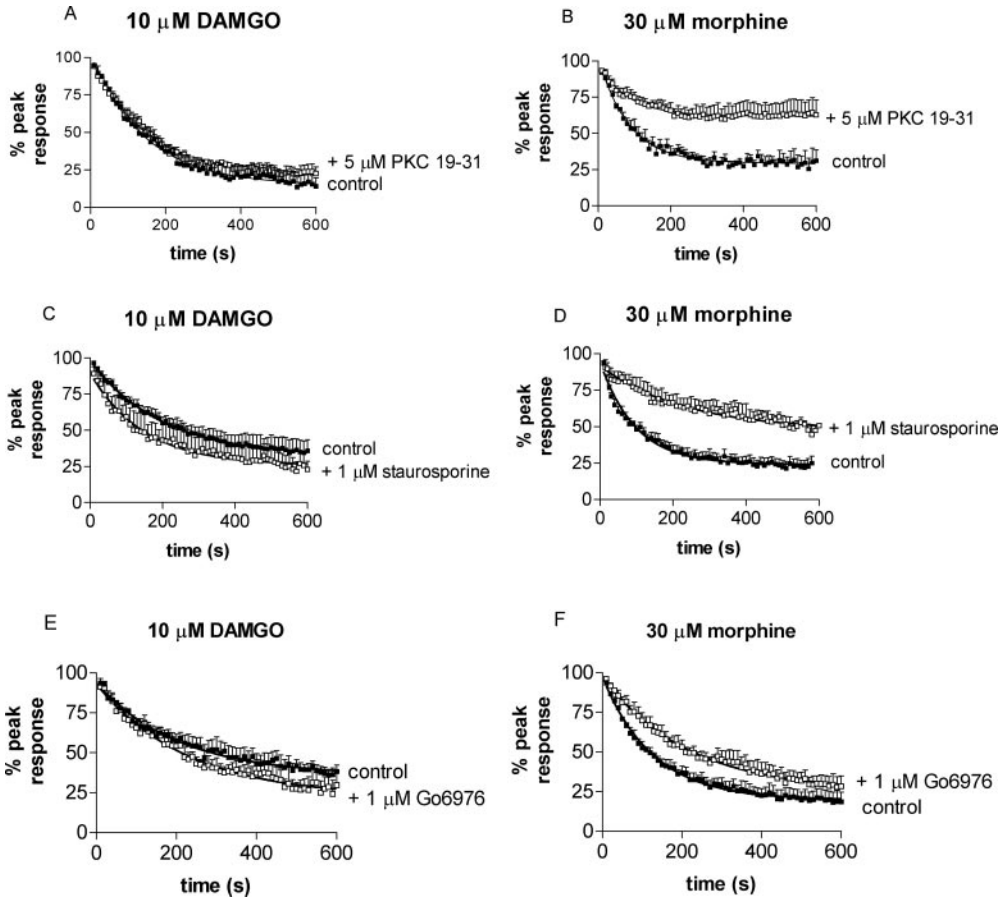

Fig. 4. Effect of PKC inhibition on MOR1 desensitization. The PKC inhibitory peptide PKC (19-31) at 5 μM was added to the solution in the recording electrode and allowed to dialyze into the cells for 10 min before opioid application. Data from individual cells were normalized to each peak response and pooled. Staurosporine or Gö6976 was applied to HEK293-MOR1 cells transiently expressing the Kir3.1/3.2 channel, for 10 min before 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, because 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, Gö6976 had no effect on the extent or rate of DAMGO desensitization after 10 min ($p > 0.05$; n = 4). F, Gö6976 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 Gö6976 ($p < 0.05$).

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 labeling (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 (Fig. 7A). The EC_{50} value for the DAMGO-evoked internalization was 470 nM (150 nM–1.47 μM; 95% confidence limits). The internalization produced by morphine was significantly less (Fig. 7, B, D, and E); it seemed 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 (Fig. 7, C and 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 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; Fig. 7E).

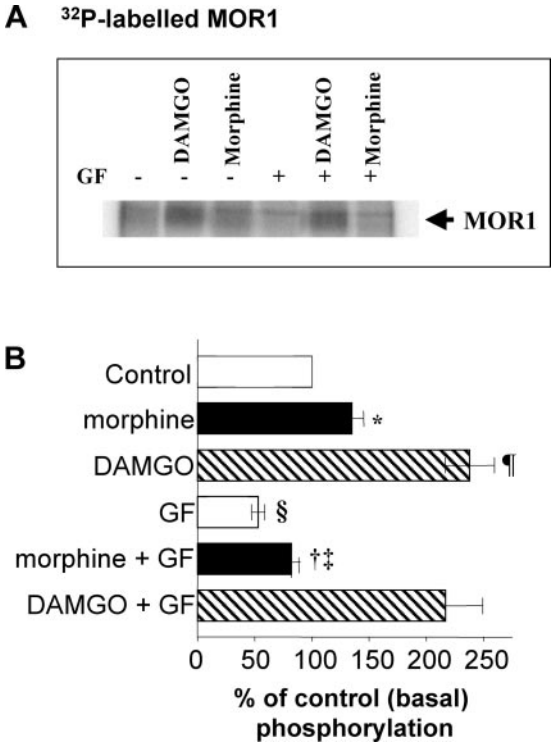

Fig. 5. DAMGO and morphine induced MOR1 phosphorylation. A, PhosphorImage of immunoprecipitated MOR1 from ^{32}P-labeled HEK293-MOR1 cells run on SDS-PAGE. Cells were exposed to DAMGO (10 μM) or morphine (30 μM) for up to 30 min in the absence or presence of GF109203X (1 μM; GF). Cells were pre-exposed to GF109203X for 15 min before the addition of the opioid agonists. B, quantification of up to five experiments of the type shown in A to determine the level of MOR1 phosphorylation. Both DAMGO (10 μM) and morphine (30 μM) induced MOR1 phosphorylation above control. GF (1 μM) 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, the level of phosphorylation induced by morphine was still greater than in the presence of GF alone. Statistical significance ($p < 0.05$) is indicated as follows: *, morphine greater than control; ¶, DAMGO greater than control; §, GF less than control; †, morphine + GF, less than morphine alone; and ‡, morphine + GF, greater than GF alone.

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. In addition, 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 (Kong et al., 1994). The ability of GRK2-K220R to inhibit DAMGO-induced MOR1 desensitization agrees with previous studies implicating the closely related GRKs, GRK2, GRK3, or both, in mediating the phosphorylation,

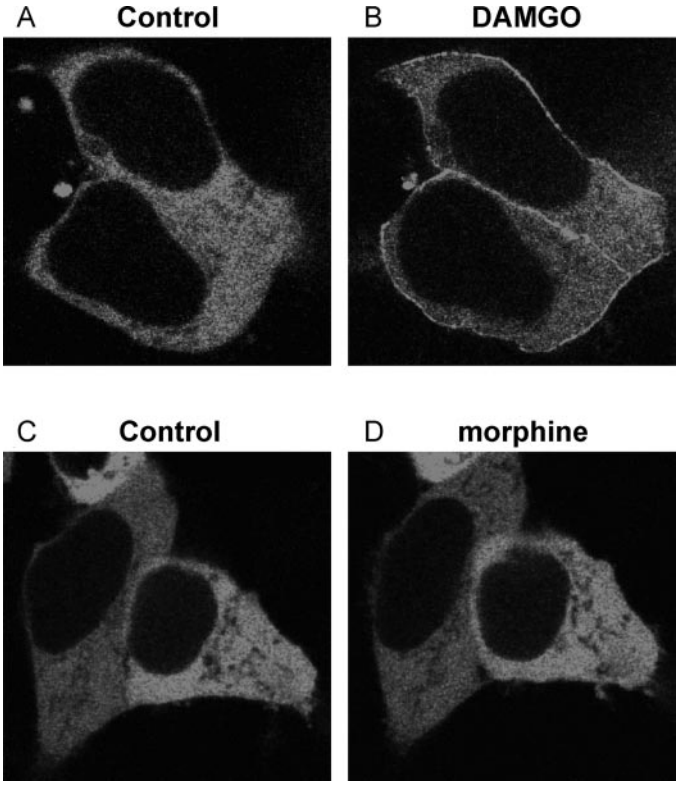

Fig. 6. DAMGO but not morphine promotes arrestin translocation. HEK293-MOR1 cells grown on poly-L-lysine coverslips were transiently transfected with 0.5 μg of pEGFP-N1-arrestin-2-GFP. Before stimulation and viewing, coverslips were mounted in an imaging chamber at 37°C, and arrestin-2-GFP distribution was monitored in real time. A and C, arrestin-2-GFP has a diffuse cytoplasmic distribution in unstimulated cells before 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.

desensitization, and internalization of MORs by full agonists (Zhang et al., 1998; Wang, 2000; Celver et al., 2001, 2004; Li and Wang, 2001; Schulz et al., 2002). 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 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 nonvisual arrestins to the receptor. This can be explained by our present findings, where the morphine-activated MOR1 did not induce significant GRK-dependent desensitization of the receptor, and thus did not trigger 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. It is noteworthy that both staurosporine and the PKC inhibitory peptide inhibited morphine-

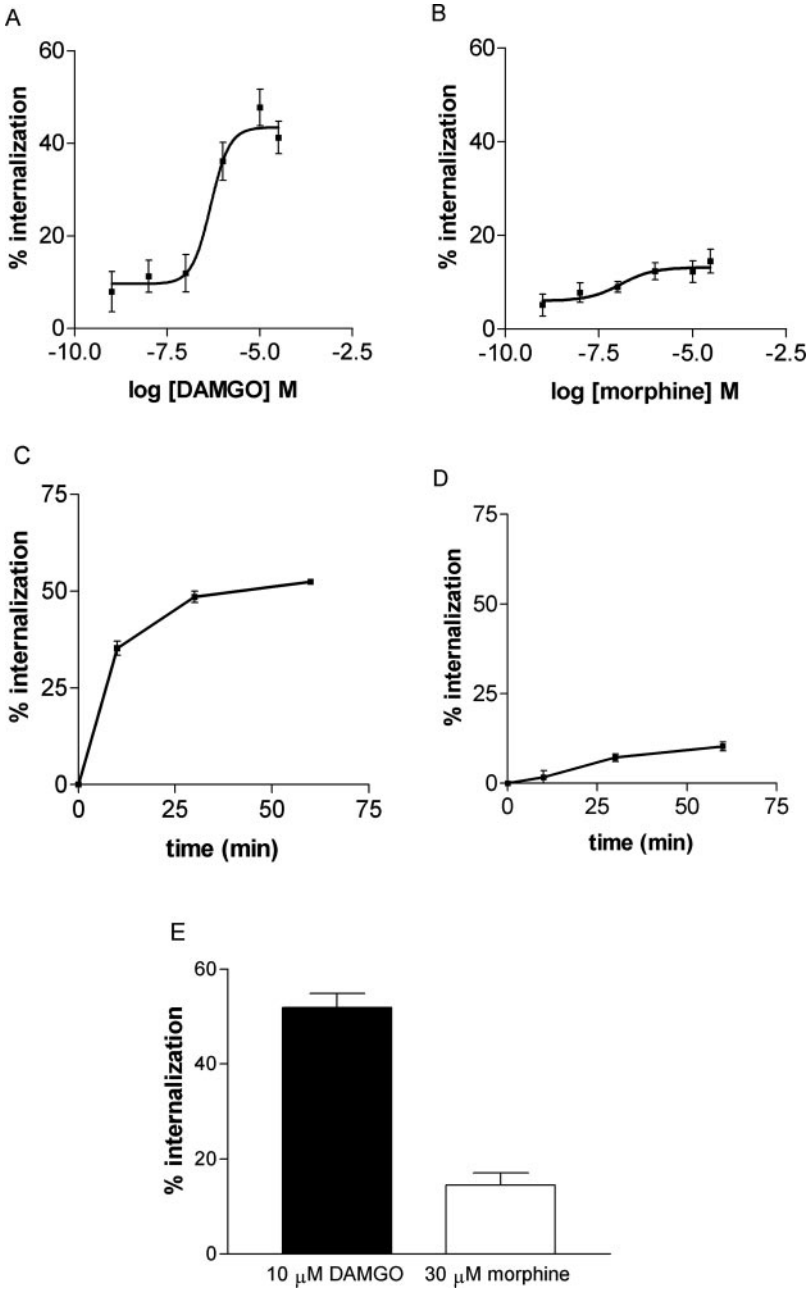

Fig. 7. DAMGO- and morphine-induced MOR1 internalization. Enzyme-linked immunosorbent assays were used to quantify MOR1 internalization in the HEK293-MOR1 cells in response to either DAMGO or morphine. A and B, DAMGO ($n = 4$) and morphine ($n = 4$) produced a concentration-dependent stimulation of MOR1 internalization after 30-min agonist exposure. C and D, time scale 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 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.

induced desensitization to the same degree but did not fully block it, suggesting either that at the concentrations used, these agents did not fully inhibit PKC activity, or that a PKC-independent process also contributes to the morphine-induced desensitization. It was crucial that inhibition of PKC did not affect DAMGO-induced desensitization of MOR1 responsiveness.

Furthermore, Gö6976, a PKC inhibitor with preference for conventional 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. Of course, HEK293 cells endogenously express some conventional (α, βI, and βII but not γ), novel (δ and ε), and atypical PKC isoforms (ξ) (Leaney et al., 2001; Wagey 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 because a PLC inhibitor did not inhibit morphine-induced desensitization. Because in HEK293 cells the MOR is already phosphorylated by PKC, 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 because 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 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.

Although we observed that morphine induced rapid ($t_{1/2} <$ 2 min) desensitization in HEK293 cells, others have reported a slower rate of morphine-induced desensitization occurring (over 1 h) (Koch et al., 2004; Schulz et al., 2004) or no desensitization by morphine (Whistler and von Zastrow, 1998). Although 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 seem to be different from that for endogenous MORs in mature rat brainstem locus ceruleus neurons, there are striking similarities. In HEK293 cells, DAMGO and morphine induced rapid desensitization of GIRK currents (present study), whereas in the locus ceruleus, which expresses predominantly MOR1, DAMGO and other high-efficacy MOR1 agonists were able to induce rapid desensitization of GIRK currents, whereas morphine induced less desensitization (Bailey et al., 2003; Dang and Williams, 2005). In the locus ceruleus, basal PKC activity did not seem to be sufficient to induce MOR desensitization. To reveal PKC-mediated desensitization required activation of PKC through G_q-coupled muscarinic receptors or with the phorbol ester phorbol-12-myristate-13-acetate. This suggests that HEK293 cells have a higher basal level of PKC activity than locus ceruleus neurons. In addition, in locus ceruleus neurons the PKC-mediated component of MOR desensitization was mediated entirely by conventional PKC isoforms because it was completely inhibited by Gö6976 (C. P. Bailey, unpublished observations). 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.

It is noteworthy that 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 it 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 seems 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 (for review, see Kenakin, 2003; Perez and Karnik, 2005), whereas 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. Because morphine-desensitized receptors are not internalized, they will be retained on the plasma membrane in the desensitized form in the continued presence of morphine, 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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