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Short communication

Specific G protein activation and μ-opioid receptor internalization caused by morphine, DAMGO and endomorphin I

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Abstract

Previous studies have shown that the agonist [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO) but not morphine induces μ-opioid receptor internalization [Arden, J.R., Segredo, V., Wang, Z., Lameh, J., Sadee, W., 1995. J. Neurochem. 65, 1636–1645]. In the present study we investigated the relationship between internalization of the μ-opioid receptor and the specific G proteins activated following treatment with morphine, DAMGO and endomorphin I (Tyr–Pro–Trp–Phe–NH_2) (a putative endogenous μ-opioid receptor agonist) in human embryonic kidney (HEK) cells. Endomorphin I and DAMGO, but not morphine, caused μ-opioid receptor internalization. Morphine, DAMGO and endomorphin I each activated $Gi_1\alpha/Gi_2\alpha$, $Go\alpha$ and $Gi_3\alpha$ to a similar extent, but not $Gq\alpha/G11\alpha$ or $Gs\alpha$ in HEK membranes. Therefore, the three ligands tested differed in their ability to internalize μ-opioid receptors even though they were similar in activating individual G proteins. © 1998 Elsevier Science B.V.

Keywords: μ-Opioid receptor; Internalization; Endomorphin I; G protein

1. Introduction

Internalization of G protein-coupled receptors, following agonist exposure, is one mechanism by which G protein-coupled receptors decrease responsiveness to further administration of drugs. Agonist-mediated desensitization and internalization of the β_2-adrenoceptor has been extensively studied (Lohse et al., 1990; Ferguson et al., 1996; Goodman et al., 1996). However, the mechanism underlying agonist-mediated internalization of other G protein-coupled receptors is less clear. Early mutagenesis studies suggested a correlation between receptor-G protein coupling and internalization (Cheung et al., 1989). However, more recently it has become apparent that internalization of some G protein-coupled receptors can take place without detectable G protein activation (Petrou et al., 1997; Segredo et al., 1997).

The potential role of specific G protein subtypes in agonist-mediated G protein-coupled receptor internalization has not been well characterized. It has become increasingly evident that G protein-coupled receptors can couple to multiple G protein subtypes. Further, measurement of specific effector responses does not necessarily indicate the G protein types activated, as several G proteins can mediate the same second messenger response, via release of their α or βγ subunits (Yatini et al., 1988; Birnbaumer et al., 1990; Gerhardt and Neubig, 1991; Clapham and Neer, 1997).

The μ-opioid receptor internalizes via clathrin-coated vesicles following exposure to specific agonists such as [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO) and etorphine. In contrast, morphine does not cause internalization despite the fact that all of these agonists modulate similar second messenger pathways (Arden et al., 1995; Keith et al., 1996; Segredo et al., 1997). The possible existence of multiple receptor conformations induced by different agonists could account for these observations.

Recently, a potent and selective endogenous peptide agonist for the μ-opioid receptor, endomorphin I (Tyr–Pro–Trp–Phe–NH_2), was isolated from bovine brain (Zadina et al., 1997). In the present study, we investigated internalization of the μ-opioid receptor and activation of specific G proteins following treatment with morphine, DAMGO and endomorphin I, in human embryonic kidney (HEK) cells, to test whether these chemically diverse ligands differentially activate specific G proteins and whether these G proteins might play a role in μ-opioid

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receptor internalization. The present study shows that endomorphin I and DAMGO, but not morphine, caused internalization of μ-opioid receptors in HEK cells, whereas morphine, DAMGO and endomorphin I activated $Gi_1\alpha/Gi_2\alpha$, $Go\alpha$ and $Gi_3\alpha$ with a similar efficacy.

2. Materials and methods

2.1. Materials

[^{35}S]Guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTPγS) and the G protein antisera to $Gi_1\alpha/Gi_2\alpha$ (AS/7), $Gi_3\alpha$ (EC/2), $Go\alpha$ (GC/2), $Gq\alpha/G11\alpha$ (QL) and $Gs\alpha$ (RM/1) were from New England Nuclear (Boston, MA). Protein G Sepharose was from Pharmacia Biotech (Sweden). Normal rabbit serum and Nonidet P-40 were from Calbiochem (La Jolla, CA). Cy3-labelled goat anti-mouse secondary antibody was from Amersham Corp. (Arlington Heights, IL). All other reagents were from Fisher Scientific (Fair Lawn, NJ).

2.2. Cell culture and membrane preparation

Human embryonic kidney (HEK-293) cells stably transfected with cDNA encoding either recombinant rat μ-opioid receptors (HEK-μ) or the rat μ-opioid receptor tagged at the N-terminal with the epitope tag sequence EYMPME (HEK-μEE) (Arden et al., 1995) (both expressing approximately 4 pmol [^{3}H]diprenorphine sites/mg protein), were grown in T-175 cm^3 tissue culture flasks containing media (a 1:1 mix of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with 10% foetal bovine serum and 200 μg/ml G418). Crude membranes were prepared as described previously (Lazareno et al., 1993) and frozen at −70°C at a protein concentration of 5–7 mg/ml.

2.3. Confocal microscopy

HEK-μEE cells were visualized by confocal microscopy as described previously (Tolbert and Lameh, 1996). Briefly, HEK-μEE cells were grown on chamber slides to ~50% confluency. Media was removed and replaced with media containing agonist (for 30 min). After agonist treatment, cells were fixed for 10 min with 3.7% paraformal-dehyde in phosphate-buffered saline (PBS) and permeabilized for 1 h in PBS containing 0.25% fish gelatin, 0.04% saponin and 0.05% NaN_3. Cells were labelled with anti-EE monoclonal antibody for 1 h, followed by a 30-min incubation with Cy3-labelled goat anti-mouse secondary antibody. Slides were visualized with a BioRad MRC 600 laser scanning confocal microscope with a yellow high sensitivity filter block.

2.4. [^{35}S]Guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTPγS) binding to G proteins

[^{35}S]GTPγS binding to G proteins and subsequent immunoprecipitation was conducted as a modification of a method previously described (Freidman et al., 1993; Wang et al., 1995). Aliquots of frozen cell membranes were diluted in assay buffer (10 mM HEPES, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4) to yield a final protein concentration of approximately 35 μg/50 μl. Cell membranes (50 μl) were then added to 1.5 ml microcentrifuge tubes containing 50 μl of assay buffer with various reagents and a final assay concentration of 1 nM [^{35}S]GTPγS (1250 Ci/mmol). Incubations were conducted in a waterbath at 30°C for 1 min and terminated by addition of ice-cold assay buffer (500 μl) and transfer of the microcentrifuge tubes to iced water. The cell membranes were pelleted (16,000 g for 5 min, at 4°C) and the supernatants removed. Pellets were then solubilized in 50 μl of ice-cold solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% (v/v) Nonidet P-40, pH 7.4) containing 0.2% (w/v) sodium dodecyl sulphate (SDS). The tubes were vortex-mixed and an equal volume of solubilization buffer was added (without SDS).

2.5. Immunoprecipitation of [^{35}S]GTPγS bound G proteins with antisera to specific Gα proteins

The solubilized cell membranes were pre-cleared with normal rabbit serum (1:100 dilution) and 20 μl of a protein G bead suspension (solubilization buffer (without SDS) containing 20% (v/v) protein G beads, 2% (w/v). Bovine serum albumin and 0.1% (w/v) NaN_3) for 1 h at 4°C. The protein G beads and unsolubilized fraction of the cell debris were pelleted (16 000 × g for 5 min at 4°C) and 100 μl of the supernatant was carefully transferred to tubes containing 40 μl of protein G suspension and 1:200 final dilution of specific G protein antisera. Samples were vortex-mixed and rotated overnight at 4°C. Protein G beads were then pelleted (16 000 × g for 5 min at 4°C), the supernatant removed and the protein G beads washed and pelleted twice in 500 μl of solubilization buffer at 4°C. After the final wash, the supernatant was removed and the protein G beads were resuspended in scintillation cocktail (1 ml) and vortex-mixed. Radioactivity was detected by liquid scintillation spectrometry. Non-specific binding was defined in the presence of 10 μM GTPγS (approximately 50 dpm/tube) and was subtracted from the dpm value for each tube.

3. Results

3.1. Confocal microscopy of agonist-treated HEK-μEE cells

Confocal microscopy studies showed that a 30 min incubation with DAMGO (10 μM) and endomorphin I (10

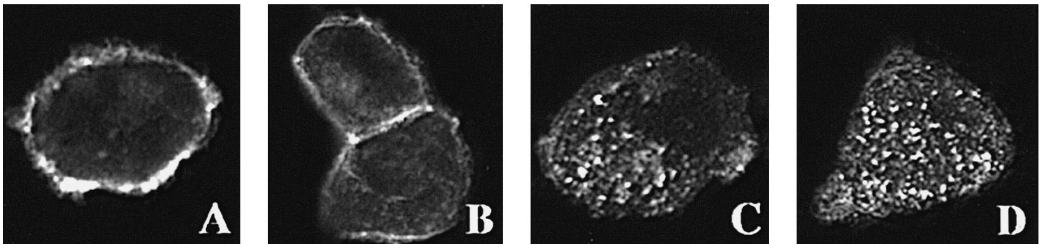

Fig. 1. Confocal microscopy images showed that μEE receptors in HEK-μEE cells were distributed at the cell surface in the absence of agonist (A) and in the presence of morphine (B). Treatment with DAMGO (C) or endomorphin I (D) caused a redistribution of μEE receptors into intracellular vesicles. Data are representative of 3 separate experiments.

μM) caused a redistribution of epitope-tagged μ-opioid receptors from the cell surface (Fig. 1A) into intracellular vesicles (Fig. 1C and D), whereas morphine (10 μM) did not cause internalization (Fig. 1B).

3.2. Agonist-stimulated G protein activation in HEK-μ cell membranes

Agonist-stimulated [^{35}S]GTPγS binding and subsequent immunoprecipitation with specific G protein antisera was conducted as described. No agonist-stimulated G protein activation was detected following immunoprecipitation with Gqα/G11α or Gsα antisera, either at 1 min (Fig. 2) or at later time periods, for any of the ligands studied (data not shown). In contrast, HEK-membranes expressing either m1 muscarinic receptors or melanocortin receptors produced large agonist-stimulated increases in [^{35}S]GTPγS binding to Gqα/G11α (12-fold above basal) and Gsα (22-fold above basal), respectively ($P < 0.05$ by Student's *t*-test), suggesting that the assay was sensitive enough to detect activation of these G proteins.

Morphine (1 μM) maximally increased [^{35}S]GTPγS binding to Gi$_1\alpha$/Gi$_2\alpha$, Goα and Gi$_3\alpha$ by 3.9-fold, 2.5-fold and 3.7-fold above basal, respectively ($P < 0.05$ by Student's *t*-test) (Fig. 2). DAMGO (1 μM) and endomorphin I (1 μM) produced activation of these G proteins with a similar efficacy to that of morphine (Fig. 2). No agonist (10 μM)-stimulated G protein activation was observed in untransfected HEK cell membranes (data not shown), or in HEK-μ membranes from cells pretreated with pertussis toxin (100 ng/ml, 20 h) (data not shown).

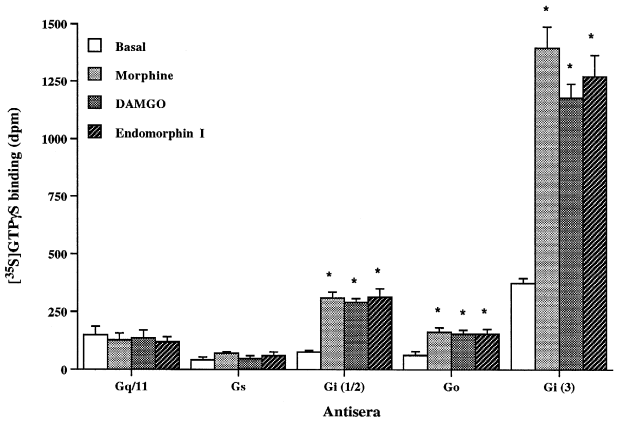

Fig. 2. Agonist-stimulated [^{35}S]GTPγS binding (in dpm) following immunoprecipitation with specific G protein antisera (*x*-axis) in HEK-μ membranes. Data are represented as the mean $\pm$ S.E.M. of three experiments each performed in duplicate. * Significantly greater ($P < 0.05$) compared to basal [^{35}S]GTPγS binding.

4. Discussion

In this study we have demonstrated that endomorphin I (a putative endogenous agonist peptide for the μ-opioid receptor) causes internalization of μ-opioid receptors in HEK-μEE cells. Previous studies have shown that DAMGO and etorphine similarly cause rapid internalization of the μ-opioid receptor, whereas the prototypical agonist morphine does not (Arden et al., 1995; Keith et al., 1996). These results suggested the possibility that these ligands may also differentially activate specific G proteins. However, analysis of the G protein coupling specificity of the μ-opioid receptor following activation with morphine, DAMGO and endomorphin I, showed that each of these agonists activated Gi$_1\alpha$/Gi$_2\alpha$, Goα and Gi$_3\alpha$ with similar efficacy, but did not cause activation of Gqα/G11α or Gsα in HEK-μ membranes.

Previous studies in our laboratory have shown that μ and μEE receptors behave in a similar manner with regards to ligand binding affinity, total G protein activation measured by [^{35}S]GTPγS binding and second messenger responses (Segredo et al., 1997). Therefore, introduction of the epitope-tag (EE) should not have affected the comparisons between HEK-μ membranes for G protein activation and HEK-μEE cells for confocal studies.

The present study strongly suggests that Gqα/G11α and Gsα are not involved in mediating μ-opioid receptor internalization, but we cannot rule out that other G proteins, not investigated in the present study, may play a role. The fact that morphine activated G proteins with the same profile as DAMGO and endomorphin I, but unlike the latter agonists, failed to mediate μ-opioid receptor internalization, suggests that activation of Gi$_1\alpha$/Gi$_2\alpha$, Goα and Gi$_3\alpha$ is insufficient to cause μ-opioid receptor internalization. This is supported by evidence that pertussis

toxin failed to inhibit DAMGO-stimulated μ-opioid receptor internalization in HEK-μEE cells (Segredo et al., 1997). However, Chakrabarti et al. (1997) recently observed that DAMGO-mediated internalization of the μ-opioid receptor, in $Neuro_{2A}$ cells, was reversed by pertussis toxin pretreatment, suggesting that internalization pathways may differ among cell types.

In summary, it appears that μ-opioid receptor activation leads, on the one hand, to G protein activation, and on the other, to receptor internalization by a mechanism distinct from G protein activation. Thus, different agonists may induce differences in activated receptor conformation, leading to similar G protein activation but distinct patterns of receptor trafficking.

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