Alternative Splicing Determines the Post-endocytic Sorting Fate of G-protein-coupled Receptors

Received for publication, August 25, 2008, and in revised form, October 14, 2008. Published, JBC Papers in Press, October 20, 2008, DOI 10.1074/jbc.M806588200

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Mu-type opioid receptors are physiologically important G-protein-coupled receptors that are generally thought to recycle after agonist-induced endocytosis. Here we show that several alternatively spliced receptor variants fail to do so efficiently because of splice-mediated removal of an endocytic sorting sequence that is present specifically in the MOR1 variant. All of the recycling-impaired receptor variants were found to undergo proteolytic down-regulation more rapidly than MOR1, irrespective of moderate differences in endocytic rate, indicating that alternative splicing plays a specific role in distinguishing the trafficking itinerary of receptors after endocytosis. The recycling-impaired MOR1B variant was similar to MOR1 in its ability to mediate opioid-dependent inhibition of adenylyl cyclase, and to undergo opioid-induced desensitization in intact cells. Functional recovery (resensitization) of MOR1B-mediated cellular responsiveness after opioid removal, however, was significantly impaired (4-fold reduction in rate) compared with MOR1. To our knowledge the present results are the first to establish a role of alternative RNA processing in specifying the post-endocytic sorting of G-protein-coupled receptors between divergent and functionally distinct membrane pathways.

Opioid receptors mediate the diverse physiological effects of endogenously produced opioid peptides, and are primary targets of a wide variety of therapeutic and abused drugs (1). Mu-type opioid receptors represent particularly important members of this family and, in the mouse, it is clear that all functional Mu opioid receptors are encoded by a single structural gene (Oprm1) (2). It is increasingly evident that alternative splicing of the Oprm1 transcript can generate multiple receptor isoforms (3). The physiological significance of such splice-mediated variation, however, remains incompletely understood.

Mu opioid receptors are regulated by agonist-induced endocytosis both in native neurons and in heterologous cell models (4–7). Following their initial endocytosis, receptors can traverse divergent post-endocytic pathways that confer distinct effects on cellular signaling. Recycling of internalized receptors to the plasma membrane typically promotes rapid recovery of cellular responsiveness to subsequent agonist exposure (8–11). Trafficking of internalized receptors to lysosomes promotes receptor proteolysis, contributing to down-regulation of cellular receptor number and prolonged attenuation of cellular responsiveness (11). Studies of cultured cell models have established that the specificity with which receptors traffic via these distinct pathways is determined in large part by molecular sorting operations occurring shortly after endocytosis (12). There is increasing evidence that such post-endocytic sorting decisions contribute to determining the long term effects of opioids in vivo (13–15).

Efficient recycling of MOR14 (also called the MOP-1 receptor), the first Mu opioid receptor whose cDNA was cloned, requires a specific sequence present in the receptor’s distal C-terminal cytoplasmic domain. This so-called MOR1-derived recycling sequence (MRS) is sufficient to promote efficient recycling when fused to a distinct GPCR, defining this sequence as a bona fide endocytic sorting determinant (16). MOR1 is encoded by exons 1–4 of the murine Oprm1 gene, and essential residues of the MRS are encoded entirely by exon 4. Although the exon 4-encoded MRS is conserved in all known MOR1 homologs, early studies of Oprm1 expression in human (17), rat (18), and mouse (19) discovered the existence of Oprm1 transcripts that include exons 1–3 but lack exon 4 due to alternative RNA processing. At present a large number of Oprm1 splice variants lacking exon 4 (and hence the MRS) have been identified, and there is convincing evidence that at least some of these variants are naturally expressed in the central nervous system (3, 18).

None of the known exon 4-lacking opioid receptor variants represents a simple deletion of the MRS, however, and all such variants identified to date contain other C-terminal sequences distinct from the MRS. This observation suggests two opposing hypotheses regarding the possible significance of alternative RNA processing to the post-endocytic sorting of receptors. One possibility is that exon 4-lacking splice variants contain alter-

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1 This work was supported, in whole or in part, by National Institutes of Health grants (to M. E.) from the National Institute on Drug Abuse. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.
3 Supported by a Kirschstein Individual National Research Service Award from the National Institute on Drug Abuse.
4 Received postdoctoral fellowship support from the American Heart Association.
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nate cytoplasmic sequence(s) that possess recycling activity similar to the MRS, effectively complementing the lack of this sequence in receptor variants. A second possibility is that alternative cytoplasmic sequences present in exon 4-lacking variants do not complement the MRS, resulting in significant differences among opioid receptor variants in post-endocytic sorting.

Distinguishing these hypotheses is important, because each has fundamentally different functional and mechanistic implications. If the first hypothesis is correct, this could identify divergent recycling sequences encoded by the same opioid receptor gene. Although biochemically distinct recycling sequences have been recognized previously by comparing GPCRs encoded by separate structural genes (15), we are not aware of any precedent for the existence of distinct recycling sequences encoded by alternate exons of the same receptor gene. If the second hypothesis is correct, this could establish a function of alternative RNA processing in dictating the post-endocytic sorting itinerary of receptors. Although alternative RNA processing has been shown previously to alter various aspects of GPCR function and regulation, including the ability of various ligands to promote initial endocytosis and desensitization of opioid receptor variants (10, 20), we are not aware of any previous evidence for RNA processing determining the post-endocytic sorting of receptors between functionally divergent pathways.

The present study investigated these hypotheses using several previously identified murine Oprm1 splice variants expressed in HEK293 cells, a well-established experimental model in which sequence-dependent recycling of MOR1 was first established (16). Our findings clarify functional differences among Oprm1 splice variants and reveal a novel role of alternative RNA processing in specifying the post-endocytic sorting fate of GPCRs.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—A FLAG-tagged version of the cloned murine MOR1 (or MOP-1 receptor) variant cDNA was described previously (16). Additional splice variants were generated by modification of this construct using oligonucleotide-directed mutagenesis and PCR (Vent polymerase, New England Biolabs). All cDNAs were cloned into pcDNA3 (Invitrogen) and sequence-verified (Elim Biopharmaceuticals Inc., Hayward, CA) before study.

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (University of California San Francisco Cell Culture Facility). Cells grown in 6-cm culture dishes were transfected using a calcium phosphate co-precipitation method as described previously (16). For studies of receptor trafficking in transiently transfected cells, cells were plated onto glass coverslips 24 h after transfection, and experiments were conducted 24–48 h thereafter. Clonal populations of stably transfected cells expressing epitope-tagged receptors were studied 24–48 h after plating.

**Fluorescence Microscopy**—Endocytic trafficking of receptors initially labeled in the plasma membrane was visualized by fluorescence microscopy using a previously described “antibody feeding” method (21). Briefly, cells plated on glass coverslips (Corning) were specifically labeled for surface receptors by incubation for 30 min in the presence of M1 anti-FLAG antibody (2.0 μg/ml, Sigma). Surface-labeled cells were subsequently incubated (at 37 °C for 30 min) in the presence of 10 μM [d-Ala²,Me-Phe³,Glyol⁵]enkephalin (DAMGO) to drive agonist-induced internalization. Following this incubation, cells were either fixed immediately, or were washed twice in DMEM and subsequently incubated (at 37 °C for 45 min) in the presence of an excess concentration of the opioid antagonist (10 μM naltrexone). Cells were fixed in 4% paraformaldehyde freshly prepared in phosphate-buffered saline (PBS, pH 7.4) for 15 min then quenched with three washes of Tris-buffered saline (pH 7.5) supplemented with 1 mM CaCl₂. Specimens were permeabilized with 0.1% Triton X-100 (Sigma) in a blocking solution (3% dry milk in Tris-buffered saline plus 1 mM CaCl₂) and incubated with fluorescein isothiocyanate-conjugated donkey antimouse secondary antibody (1:500 dilution, Jackson ImmunoResearch) for 30 min to detect antibody-labeled receptors. Fluorescence microscopy was carried out in the wide field using a Nikon 60×, numerical aperture 1.4, objective and standard interference filter sets (Omega Optical). Images were collected using a 12-bit cooled charge-coupled device camera (Princeton Instruments) interfaced to a computer running Metamorph software (Molecular Dynamics).

**Fluorescence Ratio Imaging**—To quantify recycling of variant receptors in individual cells, a dual-label version of the antibody feeding method was applied. This variation specifically labels the internalized and recycled pools of receptors with distinct fluorochromes, based on the pronounced calcium dependence of anti-FLAG antibody binding, and has been described in detail previously (16). Fractional recycling of antibody-labeled receptors was calculated in individual cells exactly as described in this previous work. In each experiment, 20–30 cells were imaged at random per construct. Recycling values reported represent mean percentages calculated across four to five independent experiments.

**Fluorescence Flow Cytometry**—Internalization and recycling of epitope-tagged receptors were estimated using fluorescence flow cytometry of stably transfected cells to measure changes in the relative amount of FLAG-tagged receptors present in the plasma membrane after surface labeling with Alexa488-conjugated M1 antibody, as described previously (22). Triplate samples (2 × 10⁴ cells/sample) were analyzed for each condition and in each experiment. The mean fluorescence values for each experiment (n = 5 experiments) were averaged, and the ±S.E. was calculated across all experiments. Internalization of receptors was determined by fractional reduction in surface receptor fluorescence, and recycling was determined by fractional surface receptor recovery, calculated as described previously (16).

**Surface Biotinylation and Assays of Receptor Proteolysis**—Immunoblotting and cell surface biotinylation assays were carried out as described previously (23, 24).

**Radioligand Binding Assay**—Cell monolayers were lifted with PBS supplemented with 2 mM EDTA, washed twice with PBS by centrifugation (200 × g for 5 min), and subjected to a
freeze/thaw cycle in PBS containing a protease inhibitor mixture (leupeptin, aprotinin, pepstatin, and phenylmethylsulfonyl fluoride) followed by four passes using a tight-fitting Dounce homogenizer. Large particulates and nuclear material were removed by centrifugation at 500 × g for 5 min, and a crude membrane and cytosolic fraction was isolated. Binding assays were conducted in 120 μl of 25 mM Tris-Cl, 1 mM EDTA, pH 7.4. Assay tubes contained 50–100 μg of the crude membrane preparation, determined using the Bradford reagent (25) purchased from Bio-Rad and bovine serum albumin as standard. Suspensions were incubated with 20 nM [3H]diprenorphine (Amersham Biosciences) for 30 min at room temperature prior to vacuum filtration through glass fiber filters (Packard Instruments) and repeated washes with ice-cold Tris-buffered saline, pH 7.4. Bound radioactivity was determined by scintillation counting (Scintiverse, Fisher) using a Beckman LS 6500 instrument. Bound counts represented ≤10% of input radioligand. Nonspecific binding, defined by assays conducted in the presence of 10 μM naloxone, was ≤10% of total counts recovered from washed filters. All assays were conducted in triplicate.

Assay for cAMP Accumulation—Cells were seeded onto poly-L-lysine-coated 48-well cultures dishes in DMEM supplemented with 10% fetal bovine serum. For acute determination of opioid receptor-mediated signaling, the medium was replaced with 0.5 ml of serum-free media containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 10 μM forskolin with or without the indicated concentration of DAMGO. The cells were then incubated for 15 min at 37 °C, and the reaction was terminated with rapid agonist-induced endocytosis of receptors. Redistribution of MOR1E, although clearly visible by microscopy, was less pronounced than that observed for the other variants (Fig. 2, B, E, H, and K). The internalized pool of MOR1 returned to a largely plasma membrane distribution within 45 min after removing DAMGO from the culture medium (Fig. 2C), consistent with rapid and efficient recycling of this receptor as shown

Results

Oprm1 Splice Variants Lacking Exon 4 Recycle Inefficiently after Ligand-induced Endocytosis—cDNAs encoding FLAG-tagged versions of four previously identified murine Oprm1 splice variants possessing divergent fourth exons, MOR1, MOR1B, MOR1D, and MOR1E, were examined (Fig. 1). The steady-state subcellular localization of receptors expressed in HEK293 cells was visualized using anti-FLAG immunostaining and fluorescence microscopy. For all variants tested, the N-terminal epitope tag was clearly detected by surface labeling of non-permeabilized cells, confirming successful targeting of receptor variants to the plasma membrane (Fig. 2, A, D, G, and J). Exposure of cells to the opioid peptide agonist DAMGO (10 μM) for 30 min produced a pronounced redistribution of MOR1, MOR1B, and MOR1D to intracellular vesicles, consistent with rapid agonist-induced endocytosis of receptors. Redistribution of MOR1E, although clearly visible by microscopy, was less pronounced than that observed for the other variants

FIGURE 1. C-terminal sequence variation among Oprm1 splice variants. The diagram shows amino acid sequence variation in the C-terminal cytoplasmic domain of the Oprm1-encoded Mu opioid receptor variants studied. A, overall location of the sequence variation in the context of the intact receptor protein. B, amino acid sequences distinguishing the C-terminal cytoplasmic domains of variant receptors. The core MRS sequence, which is present exclusively in the MOR1 variant, is indicated in bold text.

FIGURE 2. Localization of receptor variants by fluorescence microscopy. Representative epifluorescence micrographs showing the localization of Oprm1 splice variants visualized in fields of HEK293 cells after labeling receptors present in the plasma membrane selectively with anti-FLAG monoclonal antibody. Surface-labeled cells expressing the indicated receptor variant were incubated for 30 min in the absence of opioid (A, D, G, and J), exposed to DAMGO for 30 min (B, E, H, and K) or exposed to DAMGO for 30 min, stripped of antibody bound to residual surface receptors with PBS/EDTA and incubated in fresh media containing naloxone for 45 min (C, F, I, and L). The scale bar in the lower right panel represents 20 μm. 
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To pursue quantitative comparison of variant receptor trafficking, and to exclude possible secondary effects of differences between variants in receptor expression level, we generated stably transfected cells expressing each receptor construct in similar amount, as quantified by radioligand binding analysis of total membrane fractions (Table 1). Importantly, this range of receptor expression has been shown previously not to saturate the post-endocytic sorting machinery (23). We then used fluorescence flow cytometry to estimate receptor endocytosis and recycling by measuring ligand-induced changes in surface receptor immunoreactivity in large cell populations (16). DAMGO produced a rapid and pronounced reduction in relative surface receptor number in cell clones expressing MOR1, MOR1B, and MOR1D, confirming robust agonist-induced endocytosis of these variants, whereas internalization of MOR1E was detectable but significantly reduced (Fig. 3A). Surface MOR1 immunoreactivity recovered almost completely within 60 min after removal of DAMGO from the culture medium (Fig. 3B, left bar), as expected from the efficient recycling of this receptor variant shown previously. In contrast, parallel analysis of cell clones expressing MOR1B and MOR1D revealed significantly reduced surface receptor recovery (Fig. 3B, second and third bars). Time course analysis revealed that, although all variants tested exhibited some degree of rapid recycling, the extent to which MOR1B and MOR1D recycled was markedly reduced when compared with MOR1 (Fig. 3C). It was not possible to determine the recycling efficiency of MOR1E accurately using the flow cytometric assay, because it internalized poorly compared with the other variants. Another limitation of the flow cytometric assay, which is potentially applicable to all variants, is that this assay does not distinguish endocytic recycling from surface receptor delivery occurring via non-endocytic route(s) such as the biosynthetic pathway (26).

As an independent means to quantify receptor recycling, which is more sensitive than the flow cytometric assay and measures only surface delivery from the endocytic pathway, a previously established fluorescence ratio assay based on specific labeling of previously internalized receptors was applied (16). Reduced recycling of MOR1B, MOR1D, and MOR1E relative to MOR1 was visually evident from the increased immunoreactive signal (Alexa488 fluorescence) representing internalized receptors that failed to recycle 60 min after agonist washout (example micrographs are shown in Fig. 4A). Quantification of this assay across multiple cells and independent experiments confirmed the significantly reduced recycling of MOR1B and MOR1D relative to MOR1 (Fig. 4B). This method also established reduced recycling of MOR1E, despite its relatively low level of agonist-induced internalization that precluded accurate quantification by the flow cytometric assay. Together, the present results clearly refute the hypothesis that

### TABLE 1
Pharmacological characterization of stably transfected cell clones used in this study

|       | MOR1 | MOR1B | MOR1D | MOR1E |
|-------|------|-------|-------|-------|
| $B_{max}$ (fmol/µg) | 0.755 | 2.74  | 5.06  | 2.49  |
| $K_d$ (nM)    | 0.43  | 0.61  | 0.45  | 0.48  |

Saturation binding analysis was carried out on a total membrane fraction using $[^3H]$diprenorphine, as described under “Experimental Procedures.” $B_{max}$ and $K_d$ values were estimated by fitting data to a one-site binding model using GraphPad Prism software.

FIGURE 3. Flow cytometric quantification of variant receptor internalization and recycling. Stably transfected cells expressing FLAG-tagged MOR1, MOR1B, MOR1D, or MOR1E were analyzed using fluorescence flow cytometry to measure ligand-dependent changes in surface receptor immunoreactivity. A, agonist-induced internalization of receptors was determined by measuring the reduction in surface receptor immunoreactivity produced by incubation of cells for 30 min in the presence of 10 μM DAMGO. Bars represent mean reduction in surface receptor fluorescence (relative to untreated control) derived from five independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent ±S.E. calculated across experiments. MOR1E exhibited a statistically significant decrease in DAMGO-induced internalization compared with MOR1 ($p < 0.005$ by Student’s t test). B, recycling of receptors after agonist-induced internalization (induced by 10 μM DAMGO × 30 min as in panel A) was determined by measuring the fractional recovery of surface receptor immunoreactivity after agonist washout and incubation of cells in the presence of 10 μM naloxone (to prevent residual agonist effects) for the indicated time period. Each point represents mean fractional surface recovery measured in five independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent ±S.E. calculated across experiments, and statistical significance is indicated (*, $p < 0.005$ compared with MOR1 by Student’s t test). C, recycling of the indicated receptor variants was measured as in panel B, except at varying time points after agonist washout. Each point represents mean fractional surface recovery measured in five independent experiments, with 20,000 cells analyzed per condition in each experiment. Error bars represent ±S.E. calculated across experiments.

Previously (16). In marked contrast, a substantial fraction of labeled MOR1B, MOR1D, and MOR1E remained in intracellular vesicles, suggesting impaired recycling of these receptor variants (Fig. 2, C, F, I, and L).
alternatively spliced carboxyl termini of the tested splice variants contain a recycling sequence comparable to the MRS. Instead, they strongly support the hypothesis that alternative splicing dictates the post-endocytic sorting fate of receptors by including or removing the MRS.

Enhanced Proteolytic Down-regulation of Recycling-defective Oprm1 Splice Variants—To begin to investigate the potential functional significance of post-endocytic sorting differences, we examined the ability of continuous incubation of cells with DAMGO to promote proteolytic degradation of opioid receptor variants. Because mutational deletion of the MRS from MOR1 effectively re-routes endocytosed receptors from the recycling pathway to lysosomes (16), we reasoned that naturally occurring variants lacking the MRS might exhibit enhanced proteolytic down-regulation compared with MOR1. Immunoblotting revealed that all receptor variants exhibited heterogeneous electrophoretic mobility by SDS-PAGE (Fig. 5A), as observed previously in studies of MOR1 (16, 27). To compare variants, we focused on immunoreactive species corresponding in electrophoretic mobility to the complex glycosylated form of each receptor variant that represents the major species detected in the plasma membrane by surface biotinylation (arrow in Fig. 5A). Following incubation of cells in the continuous presence of 10 μM DAMGO, significant differences in the stability of individual splice variants were apparent (Fig. 5B). MOR1 was relatively stable, with substantial immunoreactivity detected on blots even after 8 h of continuous agonist exposure (Fig. 5B, top panel). The recycling-defective variants (lower panels) had visibly decreased stability in the presence of DAMGO relative to MOR1. MOR1B and MOR1D were considerably less stable, with a pronounced decrease in full-length receptor immunoreactivity visible within ~2 h of DAMGO exposure (panels 2 and 3 from top). MOR1E was also less stable
that MOR1, as indicated by loss of immunoreactivity corresponding to full-length receptor protein and a relative shift of receptor immunoreactivity toward lower apparent molecular mass (panel 4 from top). To quantify differences among receptor variants in agonist-induced degradation, we applied a previously established radioligand binding assay to measure total cellular receptor number using the high affinity antagonist [³H]diprenorphine (28). All of the receptor variants exhibited significant down-regulation following incubation of cells in the presence of 10 µM DAMGO for 8 h, as indicated by reduced B_max relative to that estimated in membranes prepared from cells not previously exposed to DAMGO. The magnitude of agonist-induced down-regulation observed at this time point was significantly greater for each of the recycling-defective receptor variants, however, when compared with MOR1 (Fig. 5C). Time course analysis (Fig. 5D) revealed that MOR1 down-regulated with an estimated t½ of ~9 h. In contrast MOR1B, MOR1D, and MOR1E down-regulated with estimated t½ values of 4.7, 4.9, and 5.7 h, respectively. These quantitative pharmacological data are fully consistent with the biochemical observations and, together, argue strongly that inhibited recycling of variant receptors after acute endocytosis is indeed associated with their enhanced proteolytic degradation in the prolonged presence of agonist.

**Effects on Functional Opioid Signaling**—We next asked if trafficking differences among opioid receptor variants are associated with functional effects on cellular signaling via adenylyl cyclase. We focused on MOR1B as an example, and compared its signaling properties to those of the efficiently recycled MOR1. MOR1B was chosen because natural expression of this receptor variant has been verified in the intact nervous system at both the mRNA and protein levels (18). Further, previous studies of inbred mice suggest that both MOR1 and MOR1B contribute significantly to physiological opioid responsiveness in vivo (29). When forskolin-stimulated cAMP accumulation was measured after acute (15 min) exposure to DAMGO, both variants were closely similar in maximal response (Fig. 6A) and dose dependence (Fig. 6B). We then compared the opioid responsiveness of previously naive cells to that of cells pre-exposed for 1 h to 10 µM DAMGO. Opioid pre-exposure caused a significant right shift in the normalized dose-response curve measured for both receptor variants (Fig. 6C). By comparing calculated EC50 values (legend to Fig. 6), it was established that DAMGO pre-exposure reduced subsequent opioid potency ~20-fold for both receptor variants. Thus, under these experimental conditions, both MOR1 and MOR1B exhibited similar acute opioid signaling and agonist-induced desensitization.
To investigate possible differences between variants in functional recovery (resensitization) of cellular opioid responsiveness after agonist-induced desensitization, MOR1- and MOR1B-expressing cells were desensitized by pre-exposure to DAMGO as above, washed, and incubated in the absence of opioid for a variable recovery period and then challenged with 100 nM DAMGO. This concentration was chosen, because it falls in the most sensitive part of the dose-response curve of both opioid-naive and DAMGO-desensitized cells (see Fig. 6C), thereby allowing reliable detection of shifts in agonist potency. Cells expressing MOR1 recovered responsiveness to 100 nM DAMGO considerably more rapidly than cells expressing MOR1B (Fig. 6D). Importantly, because opioid pre-exposure of cells even for 60 min produced a detectable increase in cAMP accumulation measured in response to a subsequent forskolin challenge (supplemental Fig. S1B), DAMGO plus forskolin re-challenge values measured at each time point after initial opioid washout were normalized to the corresponding forskolin-alone values measured from parallel dishes of cells exposed to the same pretreatment. Exponential fitting of the recovery data averaged over multiple experiments estimated that MOR1 resensitized ~4-fold more rapidly than MOR1B (t_{1/2} = 6.9 min and 25.8 min, respectively). Together, these results indicate that MOR1 and MOR1B are similar in their ability to mediate acute opioid signaling and to undergo rapid desensitization following opioid pre-exposure, but that variant-specific differences in post-endocytic sorting are associated with a pronounced difference in the rate of functional recovery of cellular signaling responsiveness.

**DISCUSSION**

In the present study we demonstrate that alternative splicing of the Oprm1 transcript is sufficient to specify the post-endocytic sorting fate of variant opioid receptors between divergent, and functionally distinct, downstream membrane pathways. Alternative splicing is known to affect a number of aspects of GPCR function and regulation, including the rate at which agonist-activated receptors are initially internalized (e.g. Refs. 10, 30 and Fig. 3A of the present study). We are not aware, however, of previous evidence that alternative splicing determines the sorting of opioid receptors (or any other GPCR) after endocytosis. Therefore, we believe that the present results are the first to establish a role of alternative RNA processing in specifying the post-endocytic sorting fate of GPCRs. We show, further, that distinct sorting fates are associated with significant differences in the functional regulation of receptor number and signaling activity in intact cells.

The present findings are consistent with previous evidence identifying an essential role of the MRS in promoting efficient recycling of MOR1 (16), and with the hypothesis that receptor recycling contributes to functional resensitization of cellular opioid signaling (13, 31). Nevertheless, we anticipated that alternatively spliced variants, at least MOR1B, would be found to contain distinct cytoplasmic sequences with recycling activity comparable to the MRS. The reason for this expectation was that previous studies reported highly efficient recycling of the rat MOR1B. Further, cellular opioid signaling mediated by MOR1B was reported to undergo reduced net desensitization relative to MOR1, and this was proposed to reflect increased (rather than reduced) functional resensitization of MOR1B (18, 32). Because much of this previous work was also carried out using HEK293 cells, and variant-specific cytoplasmic sequences are identical between mouse and rat, we devoted considerable effort to investigating key discrepancies with respect to the present findings.

First, with regard to recycling of MOR1B, a measurable fraction of internalized receptors indeed returned to the plasma membrane rapidly after agonist washout (Figs. 3 and 4). The proportion of internalized MOR1B that rapidly recycled was small, however, and similar in magnitude (~30%) to that observed previously for recycling of other lysosomally sorted receptors such as the DOR1 delta opioid (also called DOP-1) GPCR (23) as well as the epidermal growth factor receptor tyrosine kinase (33, 34). Such fractional recycling is thought to occur by a bulk flow process, and to reflect some degree of inefficiency in the post-endocytic sorting mechanism (15, 35, 36). The present results thus establish that rapid recycling of MOR1B, while detectable, is not the predominant post-endocytic trafficking itinerary of this receptor variant.

Second, with regard to desensitization and resensitization of opioid signaling, we note that previous studies (18, 32) measured changes in cellular cAMP accumulation elicited by a relatively high concentration of DAMGO (1 μM) in the re-challenge incubation. This concentration is clearly in the saturating range, as indicated by previous determinations of agonist affinity (37) and by the EC_{50} values for adenyl cyclase inhibition estimated in our experiments (Fig. 6, B and C). Further, it is not evident that the previous work accounted for superactivation of adenyl cyclase occurring during the opioid pre-exposure period. Nevertheless, superactivation of adenyl cyclase can play a major role in determining cellular concentrations of cAMP following prolonged opioid pre-exposure (38, 39). When we analyzed the present cAMP accumulation data by normalizing to forskolin-stimulated cAMP accumulation measured in opioid-naive cells, we indeed observed an apparent reduction of opioid-induced inhibition (supplemental Fig. S1A). The magnitude of this effect was similar to that reported at the corresponding time points in the initial comparison of rat MOR1 and MOR1B (18), although somewhat less than reported for MOR1 in a subsequent study (compare *left set of bars* in supplemental Fig. S1A to Fig. 1 in Ref. 32). Such differences in absolute magnitude notwithstanding, we noted that the apparent reduction of maximal inhibition produced by DAMGO pre-exposure in both MOR1- and MOR1B-expressing cells occurred in the context of a significant increase in the “baseline” value of cAMP accumulation, as determined by parallel assay of cAMP accumulation in the presence of forskolin alone (i.e. after DAMGO washout from pre-exposed cells). Indeed, an increase in forskolin-stimulated accumulation was detectable even after the earliest time period (1 h) of DAMGO pre-exposure tested (supplemental Fig. S1B). When cAMP accumulation measured upon subsequent DAMGO re-challenge was normalized to this appropriately matched (i.e. DAMGO pre-exposed) baseline value, rather than to the value measured in opioid-naive cells, we failed to observe a significant attenuation of opioid response upon opioid re-challenge applied after either 1 or 2 h of...
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It is interesting to note that, whereas functional recovery of cellular opioid responsiveness (assessed by re-challenge with a sub-saturating concentration of DAMGO) was significantly slower for MOR1B than for MOR1, nearly complete recovery did occur for both receptor variants after a sufficiently long incubation period after agonist washout (Fig. 6D). Further study will be required to determine if this slower process of MOR1B resensitization requires endocytic trafficking of receptors, reflects delivery of new receptors from the biosynthetic pathway, or might reveal trafficking-independent regulation of receptor activity as suggested in an elegant study of cellular opioid signaling in locus coeruleus neurons (40).

The present results, while they establish that the MRS plays a major role in distinguishing the post-endocytic sorting of Oprm1 splice variants in a well established model cell system, do not exclude the possibility that variant-specific cytoplasmic sequences produce additional effects on opioid receptor signaling or regulation physiologically. Another important goal of future studies, therefore, is to determine how variant-specific differences in opioid receptor trafficking are manifest in cells and tissues that naturally express the corresponding receptor variants. In particular, it will be interesting to investigate whether the principles identified in the present study contribute to previously identified differences among brain regions in functional regulation of opioid signaling (e.g. Ref. 41).

In conclusion, our findings identify a novel role of alternative RNA processing in controlling the post-sorting fate of a physiologically important GPCR. They also demonstrate that variant-specific differences in post-endocytic sorting are associated with significant effects on receptor number and signaling activity. Therefore, we propose that variant-specific programming of post-endocytic sorting fate represents a fundamental mechanism for generating diversity and specificity in the cellular regulation of GPCRs.

Acknowledgment—We thank Dr. Vu Dang for valuable discussion and critical comments on the manuscript.

REFERENCES

1. Evans, C. J. (2004) Neuropharmacology 47, Suppl. 1, 293–299

2. Matthes, H. W., Maldonado, R., Simonin, F., Valverde, O., Slowe, S., Kitchen, I., Befort, K., Dierich, A., Le, M. M., Dolle, P., Tzavara, E., Hansone, J., Roques, B. P., and Kieffer, B. L. (1996) Nature 383, 819–823

3. Pasternak, G. W. (2001) Trends Pharmacol. Sci. 22, 67–70

4. Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Monteleit, A. G., Stewart, P. L., Evans, C. J., and von Zastrow, M. (1998) Mol. Pharmacol. 53, 377–384

5. Sternini, C., Brecha, N. C., Minnis, J., D’Agostino, G., Balestra, B., Fiori, E., and Tonini, M. (2000) Neuroscience 98, 233–241

6. Trafjen, J. A., Abbadie, C., Marek, K., and Basbaum, A. I. (2000) J. Neurosci. 20, 8578–8584

7. Haberstock-Debic, H., Wein, M., Barrot, M., Colago, E. E., Rahman, Z., Neve, R. L., Pickel, V. M., Nestler, E. J., von Zastrow, M., and Svingos, A. L. (2003) J. Neurosci. 23, 4324–4332

8. Lefkowitz, R. J., Pitcher, J., Krueger, K., and Daaka, Y. (1998) Adv. Pharmacol. 42, 416–420

9. Ferguson, S. S., Zhang, J., Barak, L. S., and Caron, M. G. (1998) Life Sci. 62, 1561–1565

10. Koch, T., Schulz, S., Pfeiffer, M., Klotzny, M., Schroder, H., Kahl, E., and Holtt, V. (2001) J. Biol. Chem. 276, 31408–31414

11. Law, P. Y., and Loh, H. H. (1999) J. Pharmacol. Exp. Ther. 289, 607–624

12. Tsao, P., and von Zastrow, M. (2000) Curr. Opin. Neurobiol. 10, 365–369

13. Koch, T., and Holtt, V. (2007) Pharmacol. Ther. 117, 199–206

14. Kim, J. A., Bartlett, S., He, L., Nielsen, C. K., Chang, A. M., Kharaizova, V., Waldhofer, M., Ou, C. J., Taylor, S., Ferwerda, M., Cado, D., and Whistler, J. L. (2008) Curr. Biol. 18, 129–135

15. Hanayoglu, A. C., and von Zastrow, M. (2008) Annu. Rev. Pharmacol. Toxicol. 48, 537–568

16. Tanowitz, M., and von Zastrow, M. (2003) J. Biol. Chem. 278, 45978–45986

17. Bare, L. A., Mansson, E., and Yang, D. (1994) FEBS Lett. 354, 213–216

18. Zimmer, A., Simon, T., and Holtt, V. (1995) FEBS Lett. 359, 142–146

19. Pan, Y. X., Xu, J., Bolen, E., Abbadie, C., Chang, A., Zuckerman, A., Rossi, G., and Pasternak, G. W. (1999) Mol. Pharmacol. 56, 396–403

20. Pasternak, G. W. (2004) Neuropearmacology 47, Suppl. 1, 312–323

21. Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A., and von Zastrow, M. (1999) Nature 401, 286–290

22. Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001) J. Biol. Chem. 276, 44712–44720

23. Tsao, P., and von Zastrow, M. (2000) J. Biol. Chem. 275, 11130–11140

24. Tanowitz, M., and von Zastrow, M. (2002) J. Biol. Chem. 277, 50219–50222

25. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254

26. Koenig, J. A., and Edwardson, J. M. (1997) Trends Pharmacol. Sci. 18, 276–287

27. Whistler, J. L., and von Zastrow, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9914–9919

28. Law, P. Y., Hom, D. S., and Loh, H. H. (1982) Mol. Pharmacol. 72, 1–4

29. Narita, M., Imai, S., Ozaki, S., Suzuki, M., and Suzuki, T. (2003) Eur. J. Neurosci. 18, 3193–3198

30. Kokkedaa, J., Claeyesen, S., Becamel, C., Dumuis, A., and Marin, P. (2006) Cell Tissue Res. 326, 553–572

31. Martini, L., and Whistler, J. L. (2007) Curr. Opin. Neurobiol. 17, 556–564

32. Koch, T., Schulz, S., Schröder, H., Wolf, R., Rauf, E., and Holtt, V. (1998) J. Biol. Chem. 273, 13652–13657

33. Babst, M., Ordorizzi, G., Estepa, E. J., and Erm, S. D. (2000) Traffic 1, 248–258

34. Raiborg, C., Malerod, L., Pedersen, N. M., and Stenmark, H. (2000) Exp. Cell Res. 314, 801–813

35. Maxfield, F. R., and McGraw, T. E. (2004) Nat. Rev. Mol. Cell. Biol. 5, 121–132

36. Bache, K. G., Slagvold, T., and Stenmark, H. (2004) EMBO J. 23, 2707–2712

37. Raynor, K., Kong, H., Chen, Y., Yasuda, K., Yu, L., Bell, G. I., and Reisine, T. (1994) Mol. Pharmacol. 45, 330–334

38. Sharma, S. K., Klee, W. A., and Nirenberg, M. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3365–3369

39. Nestler, E. J. (1993) Crit. Rev. Neuropathy 7, 23–39

40. Arttamangkul, S., Torrecilla, M., Kobayashi, K., Okano, H., and Williams, J. T. (2006) J. Neurosci. 26, 4118–4125

41. Sim, L. J., Selley, D. E., Dworkin, S. I., and Childers, S. R. (1996) J. Neurosci. 16, 2684–2692