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Quantitative encoding of a partial agonist effect on individual opioid receptors by multi-site phosphorylation and threshold detection

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Abstract

Many drugs act as partial agonists of seven-transmembrane signaling receptors when compared to endogenous ligands. Partial agonism is well described as a 'macroscopic' property manifest at the level of physiological systems or cell populations, but it is not known whether partial agonists encode discrete regulatory information at the 'microscopic' level of individual receptors. We addressed this question by focusing on morphine, a partial agonist drug for μ -type opioid peptide receptors, and combining quantitative mass spectrometry with cell biological analysis to investigate morphine's reduced efficacy for promoting receptor endocytosis when compared to a peptide full agonist. We show that these chemically distinct ligands produce a complex, and qualitatively similar mixture of phosphorylated opioid receptor forms in intact cells. Quantitatively, however, the agonists promote markedly disproportional production of multi-site phosphorylation involving a specific Ser/Thr motif, whose modification at more than one residue is essential for efficient recruitment of the adaptor protein β -arrestin to clathrin-coated pits that mediate subsequent endocytosis of MORs. These results reveal quantitative encoding of agonist-selective endocytosis at the level of individual opioid receptors, based on the conserved biochemical principles of multi-site phosphorylation and threshold detection.

INTRODUCTION

Morphine has been used for its therapeutic and euphoric properties since before the time of Hippocrates, and remains among the most important drugs in modern medicine (1). Morphine is a plant-derived alkaloid that acts as a partial agonist of μ -type opioid receptors (MOP-Rs or MORs), seven-transmembrane signaling receptors (7TMRs) whose endogenous agonists are opioid neuropeptides such as enkephalin. The discovery that morphine and

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opioid peptides activate the same receptors contributed to the molecular mimicry hypothesis of drug action (2). Despite compelling evidence that morphine mediates its main biological effects through MORs (3), it differs significantly from opioid peptide agonists in various receptor-linked regulatory processes. A long-standing question is precisely how such 'agonist-selectivity' of opioid action is determined (4–10). 7TMRs comprise the largest family of signaling receptors and therapeutic drug targets (11, 12), and many 7TMR-acting drugs are traditionally considered partial agonists (8). Accordingly, the question of how morphine's regulatory effects are discriminated is relevant not only to opioid biology, but also to the cellular basis of drug action more generally.

Morphine is classified as a MOR partial agonist because, even when present at saturating concentrations that achieve full receptor occupancy, it drives various receptor-mediated signaling and regulatory processes less strongly than peptide full agonists (13, 14). A clear example is the regulated endocytosis of MORs. MORs internalize within minutes following activation by opioid peptide agonists and some highly efficacious non-peptide drugs, but even high doses of morphine promote MOR internalization inefficiently in cultured cell models (15–18) and in various neuronal populations (*e.g.*, (19–25)). Regulated endocytosis of MORs, by initiating subsequent receptor trafficking through specific recycling or degradative membrane pathways, is thought to be a critical event determining cellular opioid responsiveness under conditions of prolonged or repeated drug exposure (5, 26). Moreover, differential endocytic regulation of MORs affects physiological measures of opioid tolerance and dependence in rodent models, thus suggesting relevance to neuroadaptive processes that limit the clinical utility of opioid drugs *in vivo* (27–31).

The mechanistic basis of morphine's reduced endocytic activity is reasonably well understood, when considered at the 'macroscopic' level of the overall cellular complement of MORs. Regulated endocytosis of MORs is mediated by clathrin-coated pits (17). MORs engage this conserved endocytic machinery by recruiting β -arrestins (also called non-visual arrestins), which function as regulated endocytic adaptors to drive concentration of various 7TMRs into coated pits (32, 33); this process is promoted by agonist-induced phosphorylation of receptors (21, 22, 34). It has been known for some time that morphine stimulates overall MOR phosphorylation relatively inefficiently, when compared to highly efficacious agonists (14, 16, 34, 35). Further, morphine-induced endocytosis of MORs is enhanced by experimental manipulations that increase agonist-induced receptor phosphorylation (21, 22, 36), including phosphorylation specifically of the MOR C-tail (37). Thus, when examined at the macroscopic level of the overall MOR population, the determination of agonist-selective endocytic activity involves differential phosphorylation of the MOR C-tail.

What is not known is whether morphine's reduced endocytic activity is also specified at a 'microscopic' level; *i.e.*, at the level of individual receptors. A traditional view of partial agonism suggests that agonist-selective differences in MOR internalization are determined entirely at the macroscopic level, based on reduced phosphorylation of the overall receptor population occurring in direct proportion to a single dimension of relative agonist efficacy (14, 38). An alternative possibility, consistent with emerging concepts of ligand bias or functional selectivity (6, 8, 10), is that agonist-selective endocytosis of MORs might also involve some sort of biochemical encoding at the level of individual receptors. If so, one might expect agonists differing in relative endocytic efficacy to disproportionately produce particular phosphorylated form(s) of the C-tail, which in turn control the ability of MORs to engage the endocytic machinery.

Determining whether or not such receptor-intrinsic discrimination occurs is fundamental to understanding the cellular basis of opioid drug action, as well as partial agonism more

generally, and hinges on two key experimental questions: First, do morphine and opioid peptide drive differential MOR phosphorylation in a strictly proportional manner, or are there particular phosphorylated receptor form(s) whose production is favored disproportionately by one of these agonists? Second, if disproportional phosphorylation of MORs does occur, are there particular phosphorylated species that 'encode' agonist-selective differences in receptor engagement with the endocytic machinery? These key questions, while simple to pose in principle, have proven remarkably difficult to answer in practice. Indeed, previous efforts to do so, based on correlative approaches or analysis of MOR phosphorylation averaged over the overall cellular receptor population, have failed to reach a consensus (*e.g.*, (14, 37–40)). In the present study we combine quantitative mass spectrometry with detailed cell biological analysis, to resolve discrete phosphorylated MOR species and their effects on receptor engagement of the arrestin/clathrin endocytic machinery, thus addressing both experimental questions directly.

RESULTS

Phosphopeptide mapping of MOR phosphorylation from intact cells by MALDI MS

The reduced endocytic efficacy of morphine relative to the highly efficacious enkephalin analogue [D-Ala², N-MePhe⁴, Gly-ol]-enkephalin (DAMGO) is clearly established in human embryonic kidney (HEK293) cells expressing Flag epitope-tagged MORs (15, 17). Differences in the endocytic effects of various opioids established in this cell model have proven to be relevant to differential regulation occurring in native neurons (*e.g.*, (19, 20, 23, 25, 28)). We thus focused on HEK293 cells as a model for investigating agonist effects on MOR phosphorylation by mass spectrometry, after rapid immunopurification of receptors (Fig. 1A). Flag-tagged MORs were efficiently isolated from cell extracts, and exhibited electrophoretic mobility (Fig. 1B) consistent with that of the full-length receptor glycoprotein produced in cultured cells and native brain tissue (17, 41).

To focus specifically on differences in agonist efficacy rather than affinity (10), we applied each agonist at a saturating concentration (10 μ M) sufficient to drive maximal receptor occupancy (42). Phosphorylation and endocytic trafficking of MORs through the recycling pathway occur dynamically and iteratively in the presence of opioid peptide (43), and considerable agonist-selectivity of net MOR internalization can be observed within 20 minutes after application in HEK293 cells (17); accordingly, we chose this time point for initial comparison. We first surveyed tryptic phosphopeptides covering the entire C-tail (Fig. 1C) by MALDI mass spectrometry (Fig. 1D). Metal ion affinity chromatography (IMAC), used to enrich phosphorylated peptides from the mixture (44), revealed prominent signals representing phosphorylated forms of a proximal region of the C-tail ('region 1' corresponding to phosphorylated EFCIPTSSSTIEQQNSAR) as well as a middle region ('region 2' corresponding to phosphorylated IRQNTREHPSTANTVDR) (Fig. 1E). The distal portion of the C-tail ('region 3' corresponding to TNHQLLENLEAETAPLP, or DRTNHQLLENLEAETAPLP, using AspN), in contrast, was not detectably phosphorylated.

Quantitative analysis of C-tail phosphorylation using SILAC and LC-MS techniques

We quantified agonist effects on the abundance of phosphorylated species by LC-MS and stable isotope labeling in cell culture (SILAC) techniques (45), using isotope-tagged arginines (Fig. 2A) to achieve complete coverage of the C-tail (Fig. 2B, see Table S1 for corresponding *m/z* values). Additionally, this approach allowed us to assure co-elution of isoptomers (peptides containing the three different isotopic labels) in the LC separation step preceding peptide ionization (45). Two singly phosphorylated (+1Pi) forms of region 1 (peptides 1 and 2) were resolved by LC (Fig. 2C), and differed in whether phosphorylation was present in Ser363 (peptide 1, fig. S1) or in the 354TSST357 Ser/Thr cluster (peptide 2,

fig. S2). Calculation of the isotope ratios for peptide 1 indicated relative abundances near unity for morphine vs no drug (M/ND), DAMGO vs no drug (D/ND), and DAMGO vs morphine (D/M) comparisons (Fig. 2D, Table S2), indicative of constitutive phosphorylation. By contrast, peptide 2, containing single phosphorylation in the 354TSST357 motif, was agonist-dependent (Fig. 2E), since its abundance was markedly increased by exposure of cells to both morphine (M/ND isotope ratio = 4.6) and DAMGO (D/ND = 9.3). Single phosphorylation in this region was also moderately agonist-selective, because DAMGO increased phosphorylated peptide abundance more effectively than morphine (D/M = 2.0). We also detected dual (+2Pi) phosphorylation in region 1, and phosphopeptides containing this modification were isolated from cells under all treatment conditions (peptide 3, Fig. 2F). Production of this species was agonist-dependent (M/ND = 2.8; D/ND = 2.9), but not agonist-selective (D/M = 1). In summary, none of the phosphorylated forms of region 1 was unique to a particular treatment condition, and the degree of agonist-selectivity observed in the production of any particular species was moderate (D/M = 2).

Region 2 of the C-tail was represented by two overlapping proteolytic fragments ([368–382] and [372–383], Fig. 3A), suggesting that phosphorylation of a fraction of receptors occurred next to the protease cleavage site (46). MS/MS analysis of the singly phosphorylated [368–382] species (peptide 4) verified phosphorylation specifically at Thr370 (fig. S3). The abundance of this species was moderately increased by both morphine (M/ND = 1.4) and DAMGO (D/ND = 2.5), with DAMGO having a stronger effect (D/M = 1.8) (Fig. 3B). Single phosphorylation was also detected in the 375STANT379 Ser/Thr motif (peptide 5, Fig. 3C), revealing similar fragmentation patterns under the three different treatment conditions (fig. S4). The abundance of single phosphorylation in this motif was increased by morphine (M/ND = 1.6) and DAMGO (D/ND = 2.7), with DAMGO again having a somewhat stronger effect (D/M = 1.7). Dual phosphorylation in the fragment containing both Thr370 and the 375STANT379 Ser/Thr motif (peptide 6, Fig. 3D) was also observed under all treatment conditions. Quantification indicated that the abundance of this species was markedly increased by both morphine (M/ND = 3.5) and DAMGO (D/ND = 9.2), again with DAMGO having a moderately stronger effect (D/M = 2.6). Strikingly, the only phosphorylated species that was absolutely agonist-dependent (*i.e.*, not detectable in untreated cells) included dual phosphorylation within the 375STANT379 Ser/Thr cluster (peptide 7, Fig. 3E). Further, this phosphopeptide species was the most highly agonist-selective in its production by DAMGO compared to morphine (D/M = 4.3). Thus, higher-order phosphorylation in region 2, involving the 375STANT379 Ser/Thr cluster, differentiates the effects of morphine and DAMGO in a disproportional manner relative to other phosphorylations observed.

Persistent agonist-selective phosphorylation in the 375STANT379 motif

We carried out a separate analysis to independently verify agonist-selective phosphorylation in region 2, and to examine if skewed production of higher order phosphorylation in this region could also be observed after longer periods of agonist exposure. We were particularly interested in a longer time point because morphine's duration of therapeutic action typically exceeds 20 min, and because agonist-selective trafficking is evident in cultured cells even after prolonged agonist exposure (16, 47). Thus, we carried out a separate labeling experiment comparing agonists after continuous application for 3 hours, approximating the clinical duration of morphine action in humans (47). To assure independent analysis, we focused on a distinct proteolytic fragment that includes the 375STANT379 motif of interest, but is generated by alternate cleavage at the proximal Arg residue in region 2 (Fig. 4A). Production of single phosphorylation (+1Pi) in this peptide (peptide 8) was moderately agonist-selective (D/M = 2.7, Fig. 4B), and the degree of agonist-selectivity increased

progressively for production of higher-order phosphorylated forms (peptides 9 and 10, representing +2Pi and +3Pi species, respectively; Fig. 4C and D). The triple (+3Pi) phosphorylated form was completely undetectable in untreated cells and exhibited very pronounced agonist-selectivity in its production, as shown by a relative abundance ratio (D/M) of 14.8-fold between morphine and opioid peptide exposure (Fig. 4D, Table S2). These results independently verify agonist-selective phosphorylation in region 2, show that this phenomenon persists with longer agonist exposure, and emphasize the remarkable degree to which distinct agonists can drive disproportional higher-order phosphorylation of this region.

Multi-site phosphorylation in the 375STANT379 motif regulates endocytosis

We next investigated whether phosphorylation in either Ser/Thr cluster (354TSST357 or 375STANT379) present in C-tail regions 1 and 2, respectively, is functionally relevant to the control of MOR endocytosis. Mutating all phosphorylatable residues in the proximal cluster in region 1 (354AAAA357) had no detectable effect on steady state receptor localization or on agonist-selective internalization, as visualized by fluorescence microscopy. However, mutating the more distal cluster in region 2 (375AAANA379) resulted in a visibly reduced ability of DAMGO to induce receptor internalization (Fig. 5A). Complementary fluorescence flow cytometry experiments quantitatively verified the specificity and reproducibility of these results (Fig. 5B). Thus, MOR endocytosis is controlled specifically by phosphorylation within the C-tail involving the 375STANT379 Ser/Thr cluster, which, notably, represents a sequence motif conserved in MORs across vertebrates (48).

Because the SILAC-LC/MS data revealed the most pronounced agonist selectivity in production of higher-order phosphorylation in the C-tail region that includes the 375STANT379 motif, we investigated whether the endocytic activity conferred by this motif specifically requires its phosphorylation at more than one residue. To do so, we mutated each potential phosphorylation site individually, and examined the effect on regulated endocytosis across multiple clones of stably transfected cell lines selected for similar surface receptor levels in untreated cells. Interestingly, mutating any individual Ser/Thr residue in the 375STANT379 motif retained the plasma membrane localization of receptors (Fig. 6A) and inhibited regulated endocytosis (Fig. 6B) to a similarly large degree as mutating all three in combination. These results suggest that full endocytic activity conferred by the 375STANT379 motif requires its phosphorylation at more than one residue.

β -arrestin recruitment to clathrin-coated pits functions as a specific detector of higher-order phosphorylation involving the 375STANT379 motif

We next investigated the mechanism by which multi-site phosphorylation is interpreted to produce differential endocytosis of MORs. We focused on arrestins because they are known to associate with preferentially with phosphorylated 7TMRs, including MORs, and act as regulated endocytic adaptors by binding both to the 7TMR and the assembled coat structure of clathrin-coated pits (32, 33). Previous studies have clearly established that regulated endocytosis of MORs is arrestin-dependent (22, 49, 50), and manipulations that increase phosphorylation of the MOR C-tail in intact cells enhance both arrestin recruitment and receptor endocytosis (21, 22, 37). However, it is not known whether agonist-selective engagement of this endocytic adaptor is determined simply by a proportional difference in net phosphorylation of the overall receptor pool, or if arrestins discriminate activated MORs according to specific higher-order phosphorylation in the C-tail.

To address this question, we investigated the potential role of 375STANT379 phosphorylation in controlling recruitment of β -arrestin-2 to discrete clathrin-coated pits. DAMGO-induced activation of MORs robustly recruits GFP-tagged β -arrestin-2 to coated

pits, while morphine-induced recruitment is below the detection limit of this assay (22, 34, 51). To investigate this process with high spatiotemporal resolution, we thus focused on DAMGO and imaged a GFP-tagged version of β -arrestin-2 in living cells by total internal reflection fluorescence microscopy (TIR-FM). Activation of wild type MORs using 10 μ M DAMGO elicited rapid and pronounced recruitment of β -arrestin to diffraction-limited spots on the plasma membrane (Fig. 7A, top row), shown previously to represent discrete clathrin-coated pits containing receptor-arrestin complexes (43, 52). Mutating all three phosphorylatable residues in the 375STANT379 motif (375AAANA379 mutant) visibly inhibited β -arrestin recruitment (Fig. 7A, bottom row), implicating the 375STANT379 motif in regulating this critical endocytic adaptor. This represented an effective blockade, rather than a kinetic delay, because β -arrestin clustering was reduced in continuous image series (Fig. 7B) spanning the typical surface residence time of individual coated pits (45, 47). This result was verified quantitatively by TIR-FM analysis across multiple fixed specimens, and was specific to the 375STANT379 motif because mutating all four residues in the 354TSST357 cluster did not prevent β -arrestin clustering (Fig. 7C). Interestingly, mutating any individual phosphorylatable residue in the 375STANT379 motif caused a pronounced inhibition of arrestin recruitment that was indistinguishable from mutating all three residues together (Fig. 7C), fully mirroring the effect of the same mutations on MOR endocytosis (Fig. 6). Thus, arrestins appear to function as a 'decoding' device for agonist-selective endocytosis of MORs, by selectively recognizing multi-phosphorylated receptor species involving the second Ser/Thr cluster that are produced in markedly disproportional amount by morphine relative to opioid peptide.

DISCUSSION

In the present study, we applied mass spectrometry-based analytical methods to resolve discrete phosphorylated forms of MORs isolated from intact cells. We used this approach to investigate the nature of agonist-selective regulation of opioid receptor endocytosis by morphine, a partial agonist drug, in comparison to DAMGO, a highly efficacious peptide full agonist. The cellular pool of MORs was found to exist as a remarkably heterogeneous mixture of phosphorylated species under all conditions tested. Despite this complexity, the present mass spectrometric approach allowed resolution and quantitative comparison of discrete phosphorylated MOR species produced in cells in the presence of saturating concentrations of each agonist. Morphine and DAMGO differed most markedly (up to ~15-fold) in driving higher-order phosphorylation in a region of the C-tail containing a specific, and conserved, Ser/Thr-rich motif (375STANT379). By contrast, agonist-selective differences in phosphopeptide abundance were far less evident in lower-order phosphorylation of the same region, or in phosphorylation detected elsewhere in the MOR C-tail. Here, morphine and DAMGO produced various phosphopeptide species in similar (D/M~1) or only modestly different (D/M ~2) relative abundance. Next we established, using site-directed mutagenesis combined with quantitative analysis of receptor trafficking, that multi-phosphorylation specifically involving the 375STANT379 motif is required for efficient endocytosis of MORs. Finally, and consistent with the previously established endocytic adaptor function of β -arrestins, we found that multi-phosphorylation involving the 375STANT379 motif is specifically required for the efficient recruitment of GFP-tagged β -arrestin-2 to resolved clathrin-coated pits.

In essence, our results support the hypothesis that agonist-selective differences in MOR regulation are determined not only by net phosphate incorporation in the receptor population as a whole, but by individual receptors achieving a critical number of phosphorylated residues in a specific region of the C-tail. Importantly, in addition, our results indicate that differential production of particular multi-phosphorylated species effectively encodes agonist-selective endocytic information at the level of discrete opioid receptors. Our results

also indicate that this agonist-selective regulatory information is subsequently decoded by β -arrestins, which selectively recognize receptors that have achieved a critical multi-phosphorylation threshold, thereby promoting efficient endocytosis by clustering (together with receptors (52)) in clathrin-coated pits. Similar principles of biochemical encoding / decoding by multi-phosphorylation and threshold detection are widely deployed in other cellular contexts to generate non-linear or ultrasensitive responses (53, 54). In the case of MORs and arrestins, these principles evidently function to disproportionally amplify and transduce differences in the regulatory effects of discrete agonists, and at the level of individual phosphorylated receptors.

Ser363 was previously found, using a phospho-selective antibody, to undergo constitutive (agonist-independent) phosphorylation (40); our results confirm this observation with high precision (Fig. 2D). Using a similar strategy, Thr370 phosphorylation was previously reported to be stimulated by DAMGO but not by morphine, motivating the hypothesis that these agonists produce qualitatively different phosphorylated receptor species (40). The present mass spectrometric analysis verified Thr370 phosphorylation stimulated by DAMGO, but also clearly identified Thr370 phosphorylation in morphine-treated cells at moderately reduced relative abundance (Fig. 3B). This new finding illustrates the analytical power of mass spectrometry for achieving precise and sensitive interrogation of a complex phosphorylated receptor mixture and, importantly, supports the fundamentally different hypothesis that agonist-selective phosphorylation of MORs is primarily quantitative rather than qualitative in nature. Further supporting this view, we observed a similar degree of agonist-selectivity in phosphorylation of the proximal 354TSST357 Ser/Thr cluster. Our results are also consistent with the previous conclusion that Ser375 is phosphorylated in response to both morphine and DAMGO (40, 55, 56), and that Ser375 contributes to the regulation of MOR endocytosis (55, 56). Significantly, our results also suggest that Ser375 functions as part of a multi-site motif, whose higher-order phosphorylation by distinct agonists occurs in a markedly disproportional manner. We propose that this disproportionality is the basis for biochemical encoding that is subsequently decoded by β -arrestins into agonist-selective endocytic activity. We believe that the present identification of quantitative, receptor-intrinsic encoding and decoding of agonist-selectivity by multi-site phosphorylation represents a significant conceptual advance in our present understanding of opioid drug action.

An important strength of the present study is that it examined MOR phosphorylation in intact cells, and in the presence of a full complement of cellular kinases and phosphatases expressed at endogenous levels. A logical next step is to identify particular kinases and phosphatases that contribute to the formation of particular agonist-selective phosphorylated receptor species under these native conditions, by combining the present analytical methods with chemical inhibition or specific gene knockout/knockdown approaches. Likely candidates for phosphorylation of the 375STANT379 motif include G protein-coupled receptor kinase (GRK) family members, which are known to be remarkably sensitive to the conformational state of their 7TMR substrates (57). Further, it was shown previously that experimentally increasing GRK2 abundance (21, 22) or activity (58) enhances morphine-induced endocytosis of MORs, and that GRK2 affects the phosphorylation state of Ser375 (56) in the 375STANT379 motif. Other candidates include protein kinase C family members and MAP kinases, both of which have been implicated in the regulation of MOR signaling and trafficking (59, 60). The human kinome contains ~500 members, most of which have not been tested for effects on MOR endocytosis; thus, in principle, there are many additional possibilities. Another logical extension of the present approach is to examine other clinically important opioid drugs, such as fentanyl and methadone, which produce differential MOR regulatory effects in cultured cells and *in vivo* (14, 20, 61, 62). A third future direction is toward exploring other cellular regulatory consequences linked to MOR phosphorylation,

such as functional desensitization of MOR signaling and control of the specificity of signal regulation, which appear to involve modification of cytoplasmic residues distinct from, or in addition to, the 375STANT379 motif (18, 49, 55, 63, 64).

To our knowledge, the present results are the first to establish multi-site phosphorylation as a receptor-intrinsic basis for discriminating a biologically important 7TMR regulatory mechanism at the level of individual MORs, in intact cells, and under conditions of saturating concentration. However, we note that multi-phosphorylation has been shown previously by other methods to influence several other aspects of 7TMR regulation (65). Multi-site phosphorylation of the light-activated 7TMR rhodopsin, in a conserved C-tail Ser/Thr cluster, increases the temporal precision with which individual rhodopsin molecules inactivate after photo-isomerization of bound retinal, thereby enhancing reproducibility of the single-photon response (66). Differential phosphorylation of the β_2 -adrenergic receptor, in discrete locations (third cytoplasmic loop or C-tail) and by different kinases, can distinguish functional regulation of the cellular receptor complement according to overall degree of agonist occupancy (67, 68). Phosphorylation of muscarinic acetylcholine receptors, in different regions of the third cytoplasmic loop, contributes to differential regulation of the cellular receptor pool in distinct cell or tissue backgrounds (69). Finally, we note that many important 7TMR-active drugs are chemically distinct from endogenous ligands and classified as partial agonists, and that agonist-selective production of higher-order phosphorylated species has also been observed for the β_2 -adrenergic receptor (70, 71). Thus, the presently described mechanism of encoding and decoding agonist-selective control may not be unique to morphine, MORs, or regulated endocytosis. Instead, we anticipate that it likely contributes to the cellular basis of partial agonism more broadly, and to manifestations of functional selectivity or ligand-bias at 7TMRs as a class.

MATERIALS AND METHODS

Materials

^{13}C -labeled Arginine (^{13}C L-Arg:HCl) and $^{13}\text{C}^{15}\text{N}$ -labeled Arginine ($^{13}\text{C}^{15}\text{N}$ -L-Arg:HCl) were from Cambridge Isotope Laboratories (Andover, MA). Dulbecco's modified Eagle's medium (DME) and dialyzed FBS were from Invitrogen. Dulbecco's modified Eagle's medium (DME) deficient in L-Arginine was from Athena ES (Baltimore, MD). L-Arginine (L-Arg:HCl) and n-Dodecyl b-D-maltoside (DDM) were from Sigma (St. Louis, MO). Sequencing grade endoproteases Asp-N and modified bovine trypsin were purchased from Roche Applied Science (Indianapolis, IN). Handee minispin columns were from Pierce, and the microcon centrifugal filter devices and C18 zip tips were from Millipore (Bedford, MA). Reversed-phase packing, Oligo R3, and POROS 50 R2 were purchased from Applied Biosystems. All other chemicals, unless indicated otherwise, were obtained from Sigma (St. Louis, MO).

cDNA constructs

The Flag-tagged murine MOR1 cDNA was described previously (17). MOR mutants were generated by oligonucleotide-directed mutagenesis (Quikchange system, Stratagene), and verified by dideoxy sequencing (Elim Biochemical). The rat β -arrestin-2-GFP construct was described previously (72).

Cell culture, transfection and isotope labeling

Stably transfected HEK293 cells from a single clone expressing amino-terminally Flag-tagged murine μ -opioid receptor (MOR1), as described previously (17), were propagated in Dulbecco's modified Eagle's medium (DME) deficient in L-Arginine containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 ug/ml), and supplemented with L-

Arginine, ^{13}C -labeled Arginine, or $^{13}\text{C}^{15}\text{N}$ -labeled Arginine, to a final concentration of 0.398 mmol/L. Cells were maintained in specific isotope conditions for a minimum of 6 doublings with frequent medium changes. Uniform isotopic labeling of receptors was confirmed at >99% by MALDI-TOF-MS of Arg-containing peptides. The stably transfected clone was selected on the basis of fluorescence intensity. This clone exhibited robust internalization to DAMGO, and normal growth rate and morphology typically seen in Flag-MOR transfected HEK293 cells.

For cell biological analysis of mutant receptors, HEK293 cells were transfected with the indicated mutations of Flag-MOR cloned in pcDNA3, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. For assays of transiently transfected cells, experiments were carried out 48 hours later. For experiments with stably transfected cells, clones were selected in the presence of 50 $\mu\text{g}/\text{ml}$ G418 conditions (Geneticin, Invitrogen). Clones used on subsequent studies were chosen based on similar levels of receptor surface levels, as assessed by fluorescence flow cytometry.

Agonist stimulation and cell harvest for mass spectrometry samples

Cells were grown to ~70% confluence in 15cm round cell culture dishes containing 20ml of appropriate culture medium. For non-SILAC experiments, 6 dishes of HEK293 cells were used for each agonist condition (10 μM morphine or 10 μM DAMGO). For SILAC experiments, two dishes were used for each condition. Cells were grown in arginine-depleted media which was supplemented with either regular arginine, ^{13}C arginine, or $^{13}\text{C}^{15}\text{N}$ arginine. Regular arginine supplemented cells acted as the control. ^{13}C arginine supplemented cells were treated with morphine. $^{13}\text{C}^{15}\text{N}$ arginine supplemented cells were treated with DAMGO. Dishes were incubated with agonist for 20 minutes at 37°C, the medium was removed and replaced with ice-cold PBS with 1 mM EDTA and incubated on ice for 10 min. Intact cells were harvested by gentle aspiration while on ice. The cell suspension of each condition was pelleted for 2 minutes at 500 $\times$ g at 4°C, and aspirated pellets were quickly frozen at -80°C. To allow for efficient incorporation of isotopic arginine, cells were allowed to double 5–6 times prior to agonist application. Subsequent analysis of arginine levels showed that approximately 99 percent of arginines within a peptide contained the heavy isotope.

Receptor purification

The protocol for Flag-MOR purification was adapted from procedure described previously for isolation of Flag-tagged adrenergic receptors (71). For non-isotopically-labeled experiments, each cell pellet from a specific agonist condition was purified separately. For isotopically labeled experiments, cell pellets from three different isotope agonist conditions to be compared were solubilized together in buffer A (20 mM Tris pH 7.4, 100 mM NaCl) and 1% n-dodecylmaltoside containing protease inhibitors (Complete cocktail, Roche), 2.5 mg/ml leupeptin and phosphatase inhibitors (50 mM NaF, 0.1 mM sodium orthovanadate, 80 mM glycerol-2-phosphate) and 10 μM Naloxone to stabilize the solubilized receptor. Pellets were processed in a Dounce homogenizer and extracts were supplemented with 1 mM CaCl_2 and centrifuged at 18000 rpm in a SS34 centrifuge rotor for 30 minutes. Clarified supernatant was applied to M1-Flag affinity resin (Sigma) equilibrated in low salt washing buffer (buffer A + 0.1% n-dodecylmaltoside) supplemented with 1 mM CaCl_2 . The volume of resin used was 0.05 ml/ml extract. The column was washed three times with 3 column volumes of low salt washing buffer, and then washed with 5 sets of alternating high salt buffer (20 mM Tris pH 7.4, 500 mM NaCl, 0.1% n-dodecylmaltoside, 1 mM CaCl_2) and low salt washing buffer. After an additional three column volume washes with low salt washing buffer, the receptor was eluted with buffer A supplemented with 1mM EDTA and 200 $\mu\text{l}/\text{ml}$ Flag peptide (Sigma). Receptor-containing fractions were identified by Bradford assay,

combined with SDS-PAGE sample buffer, and electrophoresed on 4–20% polyacrylamide gels. SYPRO Ruby staining reagent (Molecular Probes) was applied per the manufacturer's instructions, and imaged using a Typhoon 9400 (Amersham Biosciences) with excitation at 610 nm to detect the SYPRO signal. Images were processed and staining intensities quantified using ImageQuant software (Amersham Biosciences).

Sample preparation for mass spectrometric analyses of proteolytic digests

To separate Flag peptide (DYKDDDDK) from the MOR, gel filtration was performed using Handee minispin columns filled with Sephadex G-50 fine resin, essentially as described previously (71). Briefly, purified receptor (~10 pmol) was loaded and eluted from the resin using a Tris buffer (20 mM, pH 7.5) containing NaCl (100 mM). Reduction, alkylation (iodoacetamide), and in solution proteolytic digests (with modified-trypsin and Asp-N) of the MOR was accomplished using minor modifications of methods described previously in greater detail (73). All samples were depalmitoylated on the microconcentrator membrane prior to enzymatic digestion by adding 1 M hydroxylamine (pH to ~8 with NaOH) and leaving samples in the dark for ~20 min at room temperature. Reduction of disulfides with tris(carboxyethyl)phosphine (TCEP) pH 7 were carried out for ~20 min at room temperature instead of 37°C to minimize receptor aggregation. IMAC procedures for phosphopeptide enrichment were carried out using the manufacturer's protocol (Phosphopeptide kit, Pierce Biotechnology) except that an extra elution step with 1 M EDTA pH 8 was included. Phosphopeptides were subsequently purified using a step-elution reversed-phase procedure [Oligo R3: POROS R2 (1:2) loaded on top of zip tips] (71, 74). For LC-ESI MS procedures, samples were dried using a SpeedVac concentrator (Thermo Electron) and redissolved in 0.1% formic acid.

MALDI mass spectrometry

Comprehensive analysis of available proteolytic peptides pre and post-IMAC enrichment were analyzed using the MALDI orthogonal time-of-flight instrument prOTOF 2000 (PerkinElmer Life Sciences) as previously described (75). The MALDI matrix used was 4-hydroxy- α -cyanocinnamic acid (4HCCA) mixed with a mixture of trifluoroacetic acid (0.1%), acetonitrile (33%) and water (67%). All MALDI- prOTOF 2000 spectra for preparation of figures used the program M-over-Z data (Genomic Solutions, Ann Arbor, MI).

Nano-LC-ESI-QqTOF mass spectrometry

IMAC-enriched or non-enriched peptide digests were separated using a 75-mm $\times$ 15-cm reversed-phase C18 column (LC Packings) at a flow rate of 350 nL/min, running a 3–32% acetonitrile gradient in 0.1% formic acid over 1 h on nano-HPLC equipped with an autosampler (Eksigent). The LC eluent was coupled to a Nanospray II source attached to a QSTAR Elite mass spectrometer running Analyst QS 2.0 software (Applied Biosystems). The mass spectrometer was set up to an MS survey scan for 500 milliseconds followed by up to 12 MS/MS spectra at fixed m/z values corresponding to peptides observed from the MOR (200 milliseconds per MS/MS). The Q1 precursor selection resolution was set to "low", with a full width half maximum of approximately five Da. Collision-induced dissociation energies were calculated using an empirically determined ramp based upon the optimal collision energies for a standard set of peptides. ESI peptides were identified using Protein Prospector version 5.2 (prospector.ucsf.edu). For quantification on the QSTAR, extracted ion chromatograms were generated for selected fragment ions. The MS/MS data was manually submitted to A-score (www.ascore.med.harvard.edu) for assessing the identified sites of phosphorylation for each peptide.

LC-ESI QTRAP mass spectrometry

IMAC-enriched or non-enriched peptides were separated using a 75-mm × 15-cm reversed-phase C18 column (LC Packings) at a flow rate of 350 nl/min, running a 3–32% acetonitrile gradient in 0.1% formic acid over 1 h on nano-HPLC equipped with an autosampler (Eksigent). The LC eluent was coupled to a Nanospray II source attached to a QTRAP 4000 triple quadrupole mass spectrometer running Analyst QS 1.4 software (Applied Biosystems). Selective reaction monitoring (SRM) transitions were based upon informative fragment ions observed in the QTOF experiments. The collision energies were identical to those used on the QTOF. Individual SRM measurements were measured for 20 msec each. For precursor ion isolation, the quadrupole resolution was set to “low”.

Quantification of MS data

To examine agonist effects on MOR phosphorylation, light (i.e. ^{12}C and ^{14}N) and two different heavy MOR cell preparations (representing three different treatment conditions) were mixed in equal amounts prior to protein purification to ensure identical handling. After separation and analysis with LC-MS, we calculated peptide abundance ratios using the MS signal intensity areas for each treatment condition. These signal intensity areas were calculated by first averaging the mass signals at each LC elution time. Next, the total number of signal counts of 2–3 monoisotopic peak tops per peptide was summed per condition. Subsequently, these areas were adjusted by normalizing all MS signal counts with an internal unmodified MOR proteolytic peptide to account for small variations in mixing. The counts from the MS signal of the Asp-N MOR peptide, [164–176], [DRYIACHPVAL], fulfilled this role as the internal standard for quantitation experiments (both Trypsin and Asp-N digests). For determining quantitative ratios when there was overlap between light and heavy or the different heavy (^{13}C , ^{14}N vs ^{13}C , ^{15}N) isotope distributions, an isotopic correction factor was applied as described previously by Ong et al (76).

Quantification of receptor internalization by fluorescence flow cytometry

Endocytosis of Flag-tagged MOR either stably or transiently transfected HEK293 cells in response to DAMGO or morphine was assessed by fluorescence flow cytometry with minor modifications to a previously described method (77). Briefly, cells were incubated in the absence or presence of the indicated agonist for 20 minutes at 37°C, then quickly chilled on ice. Cells were dissociated and surface-accessible receptors were labeled with Alexa 488-conjugated M1 antibody, and surface receptor immunoreactivity was measured using a FACScan instrument (BD Biosciences). 10,000 cells were collected for each sample. Triplicate samples were analyzed for each condition in each experiment.

Visualization of receptor trafficking by fluorescence microscopy

Flag-tagged MOR constructs were visualized by an antibody-feeding assay previously described (22). Briefly, HEK293 cells either stably or transiently transfected with the indicated construct(s) (as indicated) were plated on glass coverslips. Surface-accessible receptors were labeled in intact cells by addition to the culture medium of Alexa 488 – conjugated M1 anti-Flag antibody (2 µg/ml) for 30 minutes at 37°C, then cells were incubated for an additional 20 minutes in the presence or absence of 10 µM morphine or 10 µM DAMGO as indicated. After a quick wash with PBS, cells were fixed in 4% formaldehyde in PBS for 15 minutes, then washed 4 more times with PBS and mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA). Epifluorescence microscopy was performed with an inverted Nikon Diaphot microscope equipped with a 60X/numerical aperture (NA)1.4 objective, mercury arc lamp illumination, and standard dichroic filter sets (Omega Optical). Images were collected with a CCD camera (Princeton Instruments) interfaced to a PC running Micromanager software (www.micro-manager.org).

TIR-FM imaging of β -arrestin-2 recruitment in HEK293 cells

Total internal reflection fluorescence microscopy (TIR-FM) was carried out using a system described previously (52) based on a Nikon TE-2000 inverted microscope equipped with a 60x / NA 1.49 objective. Evanescent illumination was generated by focusing a fiber-coupled 488 nm argon ion laser (Melles Griot, Rochester, NY) onto the outer back focal plane of the objective using a micrometer-guided illuminator (Nikon). Temperature was controlled at 37°C using a thermoelectric stage (Bioscience Tools, San Diego, CA) and objective warmer (Bioptechs, Butler, PA). Time-lapse sequences were collected in living cells using a deep-cooled electron-multiplying CCD camera (Andor) operated in frame transfer mode, and in the linear range of detection. Image analysis was carried out using ImageJ (imagej.nih.gov). For analysis across multiple cells and experiments, specimens were fixed 7 min after agonist application using 4% formaldehyde freshly dissolved in phosphate-buffered saline (PBS) supplemented with 150 mM sucrose. Samples were washed in PBS after exposure to fixative for 10 min, and TIRF imaging was carried out in PBS.

Statistical analysis

The significance of differences between experimental groups was analyzed using Student's t-test or ANOVA with Bonferroni's multiple comparison test, depending on whether two or more groups were being compared, calculated using Prism software (GraphPad, La Jolla CA), using $p < 0.05$ as threshold for significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Overview of MS Analyses

(A) Experimental scheme for processing MOR for analyses by mass spectrometry (MS) with or without phospho-enrichment via immobilized metal ion affinity chromatography (IMAC). HEK293 cells expressing WT MOR were treated with 10 μ M morphine, 10 μ M DAMGO, or left untreated (No Drug) for 20 minutes at 37°C. (B) Receptors were purified from cell pellets as described in *Materials and Methods*, subjected to SDS-PAGE, and detected with SYPRO Ruby total protein stain. (C) Sequence of MOR C-Terminal tail. Highlighted in blue are the potential phosphorylation sites. (D) MS of MOR tryptic peptides. Full coverage of Ser and Thr residues in the C-tail of MOR are observed unphosphorylated: [349–365] m/z = 1967.92

(calc m/z = 1967.92); [372–382] m/z = 1226.58 (calc m/z = 1226.58); [383–398] m/z = 1776.88 (calc m/z = 1776.88). (E) MS of phosphopeptides after enrichment using IMAC procedure, reveals MOR phosphorylation for Region 1: [349–365]+1Pi m/z = 2047.86 (calc m/z = 2047.88); [349–365]+2Pi m/z = 2127.86 (calc m/z = 2127.85) and Region 2: [368–382]+1Pi m/z = 1805.79 (calc m/z = 1805.79); [366–382]+1Pi m/z = 2074.97 (calc m/z = 2074.98); [366–382]+2Pi m/z = 2154.93 (calc m/z = 2154.95); [366–382]+3Pi m/z = 2234.91 (calc m/z = 2234.91). Region 3 is not observed phosphorylated.

Figure 2. LC-ESI MS of phosphorylated MOR region 1 under three different conditions after 20 minutes of agonist exposure

(A) Schematic diagram of experiment. Use of stable isotope labeling of MOR allows all three conditions (ND= No Drug with ^{12}C , ^{14}N -Arg; M= +Morphine with ^{13}C , ^{14}N -Arg; D= +DAMGO with ^{13}C , ^{15}N -Arg) to be purified, handled and analyzed simultaneously.

Adjusted isotope ratios were calculated by summing signal count areas for 2–3 mass isotope peaks, adjusted for isotope overlap, and calibrated using the internal MOR reference peptide multipliers (1.1x for (M); 1.4x for (D)). (B) MOR c-terminal tail peptide sequence, specifying region 1 as the phosphorylated peptides of EFCIPT**S**STIEQQNSAR. (C) Using LC-ESI MS, we observed two separate elutions of singly phosphorylated EFCIPT**S**STIEQQNSAR, at 29.3 min and 30.5 min. Peptide 1 with phosphorylation site at Ser363 eluted at 29.3 min. Peptide 2 with phosphorylation localized within the 354**TSST**357 region eluted at 30.5 min. (D) At elution time 29.3 min, monoisotopic signals were observed at m/z = 1024.5, 1027.5, and 1029.5 corresponding to $[\text{M}+2\text{H}]^{+2}$ of peptide 1 under the three

agonist conditions ND, M, and D, respectively. Adjusted isotope ratios calculated for peptide 1 include $M/ND = 1.1$, $D/ND = 1.0$ and $D/M = 0.9$. (E) At elution time 30.5 min, monoisotopic signals were observed at $m/z = 1024.4$, 1027.5 , and 1029.5 corresponding to $[M+2H]^{+2}$ of peptide 2 under the three agonist conditions ND, M, and D, respectively. Adjusted isotope ratios calculated for peptide 2 include $M/ND = 4.6$, $D/ND = 9.3$, and $D/M = 2.0$. (F) At elution time 31 min, peptide 3 elutes as a single peak on the LC with monoisotopic signals observed at $m/z = 1064.4$, 1067.4 , and 1069.4 corresponding to $[M+2H]^{+2}$ of peptide 3 under the three agonist conditions ND, M, D, respectively. Adjusted isotope ratios calculated for peptide 3 include $M/ND = 2.8$, $D/ND = 2.9$, and $D/M = 1.0$.

Figure 3. LC-ESI MS of phosphorylated MOR region 2 under three different conditions after 20 minutes of agonist exposure

(A) Using LC-ESI MS, we observed phosphorylated region 2 in two different overlapping sequences, QNTREHPSTANTVDR and EHPSTANTVDR. Adjusted isotope ratios were calculated by summing signal count areas for 2–3 mass isotope peaks, adjusted for isotope overlap, and calibrated using the internal MOR reference peptide multipliers (1.1x for (M); 1.4x for (D)). (B) Peptide 4 with phosphorylation site at Thr370 eluted at 15.9 min. Monoisotopic signals were observed at $m/z = 602.6$, 606.6 , and 609.3 corresponding to $[M+3H]^+3$ of peptide 4 under the three agonist conditions ND, M, D, respectively. Adjusted isotope ratios calculated for peptide 4 include $M/ND = 1.4$, $D/ND = 2.5$ and $D/M = 1.8$. (C) At elution time 15.9 min, monoisotopic signals were observed at $m/z = 653.8$, 656.8 , and 658.8 corresponding to $[M+2H]^+2$ of peptide 5 under the three agonist conditions ND, M, D, respectively. Similar to peptide 4, adjusted isotope ratios calculated for peptide 5 include $M/ND = 1.6$, $D/ND = 2.7$, and $D/M = 1.7$. (D) At elution time 16.5 min, monoisotopic signals were observed at $m/z = 629.3$, 633.3 and 635.9 corresponding to $[M+3H]^+3$ of peptide 6 under the three agonist conditions ND, M, D, respectively. Adjusted isotope ratios calculated for peptide 6 include $M/ND = 3.5$, $D/ND = 9.2$, and $D/M = 2.6$. (E) At elution time

16.5min, monoisotopic signals were observed at $m/z=696.8$, and 698.8 corresponding to $[M+2H]^{+2}$ of peptide 7 under the two agonist conditions M and D, respectively. No signal above noise was observed for ND treatment of peptide 7 at $m/z= 692.8$ and therefore the adjusted isotope ratio calculated was $D/M= 4.3$.

Figure 4. Independent LC-ESI MS analysis of phosphorylated MOR region 2 after 180 minutes of agonist exposure

An independent series of peptides spanning region 2, generated by variation of trypsin cleavage and representing single (+1Pi), double (+2Pi) and triple (+3Pi) receptor phosphorylation in this region. Adjusted isotope ratios were calculated by summing signal count areas for 2–3 mass isotope peaks, adjusted for isotope overlap, and calibrated using the internal MOR reference peptide multipliers (1.5x for (M); 3.3x for (D)). (A) Singly phosphorylated (+1Pi) peptide eluted at 13.4 min. Monoisotopic signals were observed at $m/z = 692.3$, 698.3 , and 702.3 corresponding to $[M+3H]^+3$ of peptide 8 under the three agonist conditions ND, M, D, respectively. Adjusted isotope ratios (D/M value is also indicated in the figure): M/ND= 1.1; D/ND= 2.9; and D/M= 2.7 (B) Doubly phosphorylated (+2Pi) peptide eluted at 14.4 min. Monoisotopic signals were observed at $m/z = 725.0$, 729.0 , corresponding to $[M+3H]^+3$ of peptide 9 under the two agonist conditions M and D, respectively. No signal above noise was observed for ND at $m/z = 718.9$. Adjusted isotope ratio: D/M= 4.5. (C) Triply phosphorylated (+3Pi) peptide eluted at 14.6 min. Monoisotopic signals were observed at $m/z = 751.6$ and 755.6 corresponding to $[M+3H]^+3$ of peptide 10 under the three agonist conditions M and D, respectively. No signal above noise was observed for ND at $m/z = 745.6$. Adjusted isotope ratio: D/M= 14.8.

Figure 5. Effects of 354AAAA357 and 375AAANA379 mutations on regulated endocytosis of MORs

(A) HEK293 cells stably transfected with the indicated Flag epitope-tagged MOR constructs were incubated in the absence of agonist (ND) or exposed to 10 μ M DAMGO (D) or morphine (M) for 20 minutes at 37°C. Cells were then chemically fixed, and receptors detected and localized with Alexa 488-conjugated M1 antibody as described in *Materials and Methods*. (B) Quantification of endocytic effects by fluorescence flow cytometry, based on agonist-induced reduction in surface receptor immunoreactivity as described in *Materials and Methods*. In each experiment, and for each condition, triplicate analyses were carried out using 10,000 cells / analysis. For each receptor construct, at least two independently isolated cell clones were tested. Bars represent averaged internalization values across independent experiments (WT n=10; 354AAAA357 n=4; 375AAANA379 n=4 experiments). Error bars represent S.E.M. and p-values were calculated using Student's t-test.

Figure 6. Fine structure analysis of endocytic activity conferred by the 375STANT379 motif
(A) HEK293 cells stably transfected with the indicated Flag epitope-tagged MOR constructs were analyzed as in Figure 5. **(B)** Flow cytometric quantification of mutational effects on DAMGO-induced endocytosis (WT n=10; S375A n=6; T376A n=4; T379A n=4; 375AAANA379 n=4 experiments; each condition in triplicate.). Values are expressed as mean $\pm$ S.E.M., with p-values calculated by Student's t-test.

Figure 7. Contribution of the 375STANT379 motif to receptor-mediated recruitment of β -arrestin to clathrin-coated pits

(A) HEK293 cells co-expressing β -arrestin-2 (arrestin 3)-GFP and the indicated receptor construct were imaged live by TIR-FM. Representative frames from 0, 2 min, or 4 min after 10 μ M DAMGO addition are shown. For clarity of presentation, fluorescence intensity values were pseudo-colored using the gradient shown. (B) Magnified frames 12 s apart from the representative areas delineated by the squares in panel A are shown. The time course illustrates the appearance of arrestin 3-GFP puncta from time of agonist addition through a plateau of maximum fluorescence intensity. (C) Quantification of arrestin localization in HEK293 cells co-expressing arrestin 3-GFP and the indicated receptor construct, and fixed 7 min after bath application of 10 μ M DAMGO. Values for each condition (expressed as mean $\pm$ S.E.M.) represent number of puncta visualized per unit surface area in multiple cells (WT n=27 cells; S375A n=20; T376A n=24; T379A n=16; 375AAANA379 n=50; 354AAAA357 n=17; p-values were calculated by Student's t-test and are only indicated for p<0.05).