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Recycling and Resensitization of Delta Opioid Receptors

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Abstract

Exposure to opioids results in the activation of opioid receptors; this is follow ed by receptor endocytosis. Previously, we showed that delta opioid receptors undergo rapid agonist-mediated internalization and that mutations in the C-tail result in a substantial loss of agonist-mediated internalization. In this study, we investigated the fate of receptors following rapid internalization. We found that the majority of the wild type receptors recycled back to the surface after acute agonist treatment. The kinetics of internalization and recycling of the receptor were virtually identical to the kinetics of internalization and recycling of the radiolabeled agonist. In contrast, the kinetics of internalization and recycling of a C-tail mutant receptor were substantially altered, suggesting an involvement of the C-tail in the recycling process. It is possible that in addition to agonist-mediated internalization, opioid receptors undergo constitutive, agonist-independent internalization. We directly examined this possibility using an antibody-prebinding assay. The wild type delta opioid receptors exhibited agonist-independent internalization via the clathrin-coated pit pathway. We also examined the role of receptor internalization and recycling in the modulation of its function by quantitating the level of opioid-stimulated phosphorylation of MAP kinase (MAPK) under conditions of receptor internalization and recycling. We found that agonist treatment caused a rapid increase in the level of phosphorylated MAPK that was rapidly desensitized. The removal of the agonist, which results in receptor recycling, led to the resensitization of the receptor, as evidenced by the agonist's ability to reinduce MAPK phosphorylation. Mutant receptors that underwent rapid recycling exhibited enhanced resensitization, suggesting a role for receptor recycling in the re-sensitization process. Taken together, these results indicate that agonist-mediated internalization and recycling modulate opioid receptor function and that the receptor C-tail plays an important role in both processes.

INTRODUCTION

The binding of an opioid agonist to opioid receptors initiates the activation of the associated G proteins, followed by the induction of a number of second-messenger systems (Herz, 1993; Zaki *et al.*, 1996; Smart and Lambert, 1996). This action is accompanied by rapid agonist-induced internalization of the receptor. By regulating the number of receptors on the cell surface, receptor endocytosis plays an important role in modulating the biologic actions of opioids.

The agonist-induced internalization of the opioid receptor is a rapid and selective event (Jordan and Devi, 1998). Mu and delta opioid receptors are internalized with a $t_{1/2}$ of 5 to 10 min, and only high-efficacy selective agonists induce receptor internalization (Keith *et al.*, 1996, 1998; Koch *et al.*, 1998; Trapaidze *et al.*, 1996). Morphine, a low-efficacy agonist, does not induce internalization of mu or delta opioid receptors in transfected cells or in

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neurons endogenously expressing the receptor (Arden *et al.*, 1995; Keith *et al.*, 1996, 1998; Sternini *et al*., 1996). Interestingly, etorphine, a high-efficacy universal opioid agonist, is able to preferentially internalize mu and delta but not kappa opioid receptors, suggesting that internalization of opioid receptors also exhibits receptor type specificity (Chu *et al.*, 1997; Jordan *et al.*, in press). Opioid receptor internalization is blocked by agents that disrupt clathrin-coated pit formation (Keith *et al.*, 1996; Trapaidze *et al.*, 1996). This finding, as well as studies with colocalization of opioid receptors within the transferrin-containing compartment (Keith *et al.*, 1996), indicates that the clathrin-coated pit-mediated endocytic pathway is used for agonist-mediated internalization of opioid receptors.

Internalization of G protein-coupled receptors (GPCRs) is initiated by the agonist-induced phosphorylation of the receptor by G-protein receptor kinases (GRKs). Adapter proteins such as beta-arrestin recruit GPCRs to the clathrin-coated pit pathway by binding to the phosphorylated receptor as well as clathrin with high affinity (Gurevich *et al.*, 1995; Goodman *et al.*, 1996). Both GRK-mediated phosphorylation and beta-arrestin binding have been shown to be crucial for internalization of certain GPCRs but not others (Tsuga *et al.*, 1994; Ferguson *et al.*, 1996; Zhang *et al.*, 1996). For instance, internalization of m2-muscarinic cholinergic receptor is enhanced by GRK overexpression and decreased by a dominant-negative mutant (Tsuga *et al.*, 1994). Similarly, internalization of a sequestration-defective beta-adrenergic receptor mutant is rescued by overexpression of GRK (Ferguson *et al.*, 1996), whereas internalization of angiotensin II 1A receptor is not substantially affected by overexpression of GRK, beta-arrestin, or dynamin (Zhang *et al.*, 1996). In the case of opioid receptors, the agonist-mediated internalization appears to be a GRK- and beta-arrestin-mediated phenomenon (Zhang *et al.*, 1998; Schulz *et al.*, 1999; Whistler and Von Zastrow, 1998).

Although a number of studies have examined the mechanisms of agonist-mediated endocytosis of opioid receptors, only a few have explored agonist-independent endocytosis or receptor recycling. A study examining the trafficking of muopioid receptor mutants has found that the truncated receptors are constitutively internalized and recycled at a rapid rate (Segredo *et al.*, 1997). In the present study, we have examined opioid receptor recycling and correlated it with the recycling of the ligand. We found that delta receptors recycle following rapid endocytosis. Receptor mutants that did not undergo rapid agonist-mediated internalization underwent robust agonist-independent internalization and recycling. Receptor recycling plays a role in receptor resensitization, as examined using the opioid-mediated phosphorylation of MAP kinase (MAPK).

MATERIALS AND METHODS

Generation of cell lines expressing wild type and mutant delta opioid receptor

Chinese hamster ovary (CHO) cells stably expressing N-terminally Flag-epitope (ADDDDKYD)-tagged wild type delta opioid receptor or mutant receptors Δ C15, Δ C37, and T353A (see Fig. 1 for the schematic of the C-tail) were generated and characterized for their binding affinities, coupling to adenylyl cyclase, internalization, and downregulation as described previously (Cvejic *et al.*, 1996; Trapaidze *et al.*, 1996). For the generation of N18-DOR cells, the N18TG2 cells maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were stably transfected with epitope-tagged wild type delta receptors and characterized. The binding affinities and coupling properties of these cells are similar to those of CHO cells stably expressing Flag-tagged delta receptors. The NG108-15 cells were maintained in DMEM containing hypoxanthine, aminopterin, and thymidine (HAT) and 10% FBS.

Detection of agonist-mediated receptor internalization by confocal microscopy

The N18TG2 cells stably transfected with delta receptors were grown on coverslips and were treated without or with 100 nM agonist for 30 min or 24 h. Following incubation, cells were washed with ice-cold 50 mM Tris Cl, pH 7.5, containing 150 mM NaCl and 1 mM $CaCl_2$ (TBS) and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). Fixed cells were washed with TBS, permeabilized, and blocked with 0.1% Triton X-100 in Blotto (3% nonfat dry milk in 50 mM Tris Cl, pH 7.5). Cells were incubated for 1 h at room temperature with primary antibody (anti-Flag M1; Sigma) 10 μg/ml diluted in Blotto, then washed with TBS, incubated for 30 min with fluoresceine isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Vector Laboratories) 2 μg/ml diluted in Blotto, washed with TBS, and mounted on glass slides using Permount. Cells were examined using an oil-immersion objective and standard fluorescein epifluorescence optics, and confocal fluorescence microscopy was performed using a laser-scanning microscope.

Receptor internalization and recycling

Receptor internalization was measured by flow cytometry as described previously (Trapaidze *et al.*, 1996; Cvejic and Devi, 1997). For quantifying receptor recycling, the CHO or N18 cells expressing wild type delta opioid receptors were pretreated with 10 μ M cycloheximide for 1 h to block protein synthesis and then exposed to a single dose of 100 nM DADLE for 30 min. The agonist was removed by extensive washing and incubated for various periods of time with buffer without the agonist in the absence or presence of 100 μ M monensin; treatment of cells with 100 μ M monensin blocks > 50% of receptor recycling. At the end of the incubation, cells were chilled to 4°C, washed three times with 0.5 ml of PBS, and incubated for 1 h at 4°C with primary antiserum 10 μ g/ml in PBS containing 50% FBS. Cells were washed with 1% FBS in PBS and incubated with FITC-conjugated goat anti-mouse IgG 5 μ g/ml for 1 h. Cells were washed with 1% FBS in PBS followed by a PBS wash, collected from the wells with 1 mM EDTA in PBS, and analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Inc). Live cells were gated by light scatter or exclusion of propidium iodide, and 5000 to 10,000 cells were acquired for each time point. The mean fluorescence of all live cells, minus the mean fluorescence of cells stained only with FITC-conjugated second antibody, was used for the calculation (Trapaidze *et al.*, 1996).

Internalization and recycling of the ligand-[^{3}H] DPDPE

CHO cells expressing the Flag-tagged wild-type delta receptors or T353A mutant delta receptors and NG108-15 cells expressing endogenous delta receptors were plated ($1–2 \times 10^5$ cells/well) in 24-well plates. After 24 h, the medium was removed, and cells were incubated with 2 nM [^{3}H]-DPDPE in Krebs Ringer–HEPES buffer, pH 7.4 (buffer A) in a final volume of 300 μ l. Incubation was carried out for different time periods, after which cells were chilled at 4°C, washed in 50 mM Tris Cl, pH 7.5, and collected to measure the total binding. The amount of ligand internalized was determined by washing a parallel set of wells with ice-cold 0.2 M sodium acetate, pH 4.8, containing 500 mM sodium chloride (acid buffer); a wash with this buffer has been previously shown to remove cell-surface binding (Sorokin *et al.*, 1989). The acid-washed cells were collected to determine the amount of internalized radiolabeled agonist. To quantify ligand recycling, the acid buffer-washed cells were washed with 50 mM Tris Cl, pH 7.5 and incubated at 37°C for various time periods. The spent medium was collected, and the cells were washed again in acid buffer ("acid wash"). The spent medium + the acid wash samples were combined, and the radioactivity was determined using BioSafe scintillation fluid. The radioactivity remaining in the cells was determined by dissolving them in 1 N NaOH, neutralizing with 1 N HCl, and measuring in scintillation fluid.

Detection of constitutive internalization by confocal microscopy

Cells expressing Flag-tagged wild type receptors were grown on coverslips. Cells were incubated for 1 h at 4°C to label cell-surface receptors or for 30 min and 60 min at 37°C to enable antibody uptake with primary antibody 5 μ g/ml diluted in Blotto containing TBS. To examine the effect of sucrose on receptor internalization, cells were labeled with primary antiserum 5 μ g/ml at 4°C, washed, and warmed for 60 min in the absence or presence of 0.65 M sucrose. To examine the effect of sucrose on antibody uptake, cells were incubated for 30 min at 37°C with 0.65 M sucrose followed by 60 min at 37°C with M1 antibody in 0.65 M sucrose. After treatment, cells were washed with ice-cold TBS. In order to visualize only the internalized receptor, the antibody bound to the cell surface receptors was removed by washing three times with ice-cold PBS containing 5 mM EDTA (stripped). Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in Blotto. The internalized receptors were visualized by incubation for 60 min with FITC-conjugated goat anti-mouse IgG 2 μ g/ml (Vector Laboratories) diluted in Blotto.

MAPK phosphorylation

To determine the level of opioid-mediated increase in the phosphorylation of MAPK, CHO cells expressing either wild-type or mutant receptors were plated in a 24-well plate and grown to 80% confluency. Cells were incubated in serum-free medium for 2 h before the addition of ligands and treated with 100 nM DPDPE for 30 min (conditions of internalization). This was followed by the removal of the agonist. A second 5-min treatment with 100 nM DPDPE was used to determine the extent of opioid-mediated phosphorylation following receptor internalization. Cells not pretreated with agonists but exposed to a 5-min pulse of 100 nM DPDPE were used as the control. To examine opioid-mediated phosphorylation during recycling, cells were treated with 100 nM DPDPE for 30 min (to induce internalization). The cells were then washed and incubated in the medium without the agonist for various periods of time (recycling). The cells were extracted by lysing in 100 μ l/well of 2% SDS in 50 mM Tris Cl, pH 6.8, and ~10 μ g of protein was subjected to SDS-PAGE on an 8% gel. The protein concentration of the lysates was determined using BCA protein assay reagent (Pierce). To detect the phosphorylated MAPKs, proteins were transferred to a nitrocellulose membrane (MSI Inc.) and incubated overnight with 5% milk in 50 mM Tris Cl, pH 7.4, and 150 mM NaCl, 0.1% Tween 20 (TBST). Membranes were incubated with a 1:2000 dilution of p44/42 phospho-ERK antibody E10 (New England Biolabs) in 5% milk/TBST for 1 h and washed with TBST four times for 15 min each. This was followed by incubation with a 1:3000 dilution of horseradish peroxidase-conjugated anti-mouse IgG (Vector Laboratories) in 5% milk in TBST for 1 h. The membranes were washed with TBST four times for 15 min each, and the signal was detected with enhanced chemiluminescence SuperSignal® West Pico Chemiluminescent Substrate as described by the manufacturer (Pierce). To confirm equal loading and quantitation, membranes were stripped by incubation in 0.1 M glycine, pH 2.6, for 20 min at room temperature. The membranes were incubated with a 1:2000 dilution of monoclonal tubulin antibody (Sigma), and the signal was detected as described above. For densitization of the blots, a LaCie Silverscanner attached to a Macintosh Quadra 950 running NIH Image software was used. Typically, two or three exposures of each membrane were scanned, and only the values in the linear range of the film were used.

RESULTS

We have previously shown that opioid receptors expressed in fibroblast cell lines are internalized rapidly upon acute exposure to agonists (Trapaidze *et al.*, 1996). Chronic (prolonged) exposure to agonists results in the degradation of the receptor (Cvejic *et al.*, 1996; Trapaidze *et al.*, 2000a). In this study, we examined the fate of the receptor following

internalization by acute (single) exposure to agonists. Because neuroblastoma cell lines are a more appropriate model than the previously used fibroblast cell line, we first examined the trafficking of delta receptors in the N18TG2 neuroblastoma cell line. We found that in cells not treated with the agonist, delta receptors were localized primarily on the plasma membrane (Fig. 2A). Acute treatment with 100 nM DADLE (30 min) resulted in the redistribution of the receptor to an intracellular location (Fig. 2B), whereas chronic treatment (24 h) resulted in a substantial loss of the receptor fluorescence from these cells (Fig. 2C). These results are consistent with the rapid internalization of the receptor followed by the degradation of the receptor on chronic treatment; these properties are similar to the reported properties of delta receptors in neuronal cells expressing endogenous or exogenous receptors (Law *et al*., 1984; Trapaidze *et al*., 1996; Cvejic *et al*., 1996; Afify *et al*., 1998).

Next, we quantified the extent of receptor internalization by flow cytometry. Approximately 50% of the receptors had been internalized by about 10 min of agonist treatment, and more than 60% of the receptors were internalized by about 30 min both in neuroblastoma cells and in CHO cells expressing delta receptors (Fig. 3). Receptor internalization exhibited agonist selectivity in that treatment with delta-selective peptide agonists (100 nM DADLE or DPDPE) or a universal opioid agonist (30 nM etorphine) caused substantial internalization, whereas treatment with the muselective agonist 100 nM DAMGO did not (Table 1).

The cells expressing wild type receptors were treated with 10 μM cycloheximide for 1 h and with the various agents (at 100 nM except etorphine at 30 nM) for 30 min. The cells were washed and incubated in medium without the ligand (but with cycloheximide) for 60 min. The cells were stained and analyzed by flow cytometry as described (Trapaidze *et al*., 1996). The data represent the mean and SEM of triplicate determinations.

We next examined receptor recycling. For this, the cells were treated with cycloheximide (to block protein synthesis) and exposed to a single dose of 100 nM DADLE for 30 min (to cause internalization of ~60% of the surface receptors), and the level of cell-surface receptors at various times after agonist removal was examined. We found a rapid increase in the cell-surface fluorescence intensity on removal of agonist; within 30 min of agonist removal, approximately 50% of the internalized receptors had been recycled, and by about 90 min, the majority of the receptors were recycled back to the surface (Fig. 3). The receptor recycling was substantially reduced by monensin. We found that the receptors internalized in response to selective as well as nonselective agonists recycled to about the same extent (Table 1). Because the treatment of cells with 100 to 1000 μM morphine (low-efficacy alkaloid agonist) did not induce internalization of the receptor (not shown), we did not examine receptor recycling in cells treated with morphine. Blocking protein synthesis by treatment of the cells with cycloheximide did not affect the extent of receptor recycling, suggesting that new receptor synthesis does not contribute to the level of surface receptors seen during recycling. Taken together, these results suggest that the rapid internalization of the delta opioid receptor is not a cell-line-specific phenomenon and that N18 neuroblastoma as well as the CHO fibroblast cell lines are well suited for studies of delta receptor trafficking.

We next compared the time course of receptor internalization/recycling with the time course of ligand internalization/recycling. We took advantage of the availability of NG108-15 cells that express endogenous mouse delta receptors to examine the time course of ligand internalization/recycling. The findings were compared with the time course of ligand internalization and recycling in CHO cells transfected with delta receptors. For this, confluent cells in 24-well plates were incubated with 2 nM [^{3}H]-DPDPE for different periods. At the end of the incubation, the cells were washed with mild acid buffer to remove surface-bound ligand. The amount of ligand released into the acid wash and that remaining

in the cells were quantified independently. A substantial increase in internalization as well as recycling of the agonist was detected both in NG108-15 cells and in CHO cells expressing wild type delta receptors (Fig. 4). We have previously shown that the delta receptor mutants with mutations in the C-tail exhibit deficient agonist-induced internalization (Trapaidze *et al.*, 1996). The extent of ligand internalization in these cells is lower than in wild type cells (Fig. 4A). Interestingly, the internalized ligand was rapidly recycled, and the extent of ligand recycling was significantly higher in cells expressing mutant receptor (Fig. 4B), suggesting that both endocytosis and recycling of these receptors are affected. It is possible that the apparent lack of agonist-mediated internalization of the mutant receptors observed previously (Trapaidze *et al.*, 1996) is attributable to an increase in the rate of constitutive internalization, as well as an increase in the kinetics of recycling of these receptors.

To examine agonist-independent internalization directly, we used an antibody-prebinding assay and confocal microscopy to visualize the receptors and flow cytometry to quantitate the extent of receptor internalization. At 4°C, the receptor fluorescence was primarily on the cell surface (Fig. 5A), whereas warming the cells to 37°C for 30 or 60 min, resulted in a cytoplasmic localization of these receptors (Fig. 5B, C). These results suggest that delta receptors undergo agonist-independent internalization. Pretreatment of cells with hypertonic medium is thought to disrupt the clathrin-mediate dendocytic pathway (Heuser and Anderson, 1989), and under these conditions, the receptor fluorescence was primarily on the cell surface even on incubation for 60 min at 37°C, suggesting that the constitutive internalization was significantly reduced by treatment with sucrose (Fig. 5D). To further explore this possibility, we carried out antibody uptake studies in the absence or presence of hypertonic medium. We found that in the absence of the hypertonic medium, the receptor fluorescence was seen mainly as punctate staining within the cytoplasm (Fig. 5G) and on treatment was mainly on the cell surface (Fig. 5E, H). These results suggest involvement of clathrin-coated pits in the agonist-independent internalization process. Treatment of cells with 1 μM naloxone alone did not affect agonist-independent internalization (not shown). Taken together, these results suggest that the delta receptors undergo slow constitutive endocytosis via the classic clathrin-coated-pit endocytic pathway.

Internalization and recycling have been implicated in the re-sensitization and thus modulation of function of a number of GPCRs (Lefkowitz, 1998). In order to examine the role of internalization and recycling in the modulation of opioid receptor function, we used the opioid activation of the MAPK pathway as a measure of receptor activity and examined the relative level of phosphorylated MAPK under the conditions of internalization and recycling. Several reports have shown that many GPCRs, including opioid receptors, activate the MAPK pathway (Polakiewicz *et al.*, 1998; Li and Chang, 1996; Daaka *et al.*, 1998). We found that both the p44 and p42 forms of MAPKs were phosphorylated within 5 min of agonist treatment of wild-type receptor-expressing cells (Fig. 6). After 30 min of agonist treatment, the receptors were desensitized, as a second dose of agonist was not able to induce phosphorylation of these MAPKs. Under the conditions of recycling (incubation in the absence of the agonist following internalization), the receptors were resensitized, as they were able to respond to a second dose of the agonist, leading to phosphorylation of the MAPK. Treatment of cells with monensin (a known blocker of recycling) that caused substantial reduction in receptor recycling (see Fig. 3) also caused a substantial reduction in the extent of resensitization of the receptor (Fig. 6). When the T353A mutant receptor was examined for the kinetics of MAPK phosphorylation under conditions of recycling, we found that both the rate and the extent of phosphorylation were significantly enhanced in these receptors compared with the wild type receptors (Fig. 6). These results are consistent with the notion that receptor recycling plays a role in the resensitization of the opioid receptors. Taken together, these results suggest that the rapid internalization and recycling of

the delta opioid receptor play an important role in the desensitization/resensitization of the receptor.

DISCUSSION

In this study, we characterized the internalization and recycling of the delta opioid receptors, as well as the receptor-selective ligand DPDPE, in fibroblast or neuronal cells expressing exogenous or endogenous receptors. We found that the kinetics of internalization and recycling of the receptor and ligand were similar in these cells and consistent with the kinetics reported by others (Afify *et al.*, 1998; Law *et al.*, 1984). These results, taken together with those of other studies that have used these cells to examine GPCR endocytosis, indicate that CHO cells contain the machinery for endocytosis of this and other GPCRs and thus are suitable for studies on GPCR trafficking.

Using an antibody-prebinding assay to examine receptor internalization, we found that opioid receptors undergo slow constitutive internalization via the classic clathrin-coated pit pathway. It is possible that the constitutive internalization and recycling of opioid receptors maintains a steady-state level of active receptors on the cell surface. Previous studies have shown that a number of cell-surface receptors undergo constitutive internalization via the classic clathrin-coated pit-mediated pathway (Watts, 1985; Moore *et al.*, 1995; Brown and Greene, 1991; Tan *et al.*, 1993). Constitutive internalization is thought to play an active role in the process of mating-type switching in the case of alpha-mating factor receptor (Tan *et al.*, 1993) by constantly clearing the surface receptors so that after a mating-type switch, the older receptors can be replaced by newly synthesized ones. Constitutive internalization is also thought to lead to polarized distribution of other GPCRs (Jackson *et al.*, 1991). Thus, it is possible that agonist-independent constitutive internalization is an additional mechanism that is involved in the modulation of opioid receptor function.

Internalized GPCRs colocalize with the transferrin receptor in an endosomal pathway characterized by the presence of rab5 (Moore *et al.*, 1995; Koenig and Edwardson, 1997). The internalized receptor is processed in the endosomes, which is thought to be necessary for the resensitization of the receptor. This process could include dissociation of the ligand–receptor complex in the acidified pH of the endosomes, dephosphorylation of the receptor, dissociation of arrestins, and recycling of the receptor to the plasma membrane (Lefkowitz, 1998). It appears that structurally related receptors such as the D1 and D2 dopamine receptors can be selectively endocytosed to distinct endocytic compartments by dynamin-dependent and -independent mechanisms (Vickery and von Zastrow, 1999). After this initial segregation, both these receptors recycle back to the plasma membrane. Thus, it appears that the differential endocytic processes, rather than targeting the receptors to lysosomes, may in fact physically segregate structurally homologous receptors. The fate of the endocytosed peptide ligand is less clear because of the complications of peptide degradation by proteases. Significant recycling of a stable enkephalin analog or somatostatin analog in a neuroblastoma cell line has been demonstrated and shown to be the factor limiting the amount of radiolabeled agonist retained inside the cells (this study; Law *et al.*, 1984; Koenig and Edwardson, 1997).

We found that the endocytosed receptor recycles back to the cell surface and that the receptors lacking the C-tail are able to recycle better than wild type receptors. The previously observed lack of agonist-mediated internalization of these receptors could be attributable to rapid agonist-independent internalization and recycling that would mask the agonist-induced internalization. The results from the ligand internalization and recycling studies support such a view. Segredo *et al.* (1997) have shown that a muopioid receptor C-tail deletion mutant exhibits rapid constitutive internalization and recycling. The fact that

mutations in the C-tail affect both constitutive and agonist-mediated internalization suggests that multiple regulatory elements within the C-tail govern receptor trafficking; the removal of negative regulatory elements would allow increased constitutive endocytosis, and the receptor would rapidly be recycled back to the cell surface. In naive cells, the majority of the wild type receptors exist as dimers and monomers (Cvejic and Devi, 1997) that are transiently retained on the cell surface, presumably via interaction of the C-tail with intracellular proteins. In the absence of the agonist, there would be a slow dissociation of these factors, leading to a low level of receptor internalization. Agonist binding could lead to changes in the conformation of the receptor, resulting in the monomerization of the delta dimers (Cvejic and Devi, 1997). This change would be accompanied by an increased rate of dissociation of these proteins that could allow the phosphorylation of the receptor or binding of adapter proteins such as β-arrestin, thus leading to rapid endocytosis.

One of the signal transduction pathways that is activated by opioids appears to be the MAPK pathway. Although many studies have used the activation/inhibition of adenylate cyclases or phospholipase C as a measure of GPCR activity, a relatively small number of studies have used the activation of MAPK to examine receptor activity. Furthermore, opioid-mediated desensitization/resensitization of the MAPK pathway has not been well explored. We have found that activation of all three opioid receptor types (mu, delta, and kappa) by agonists results in the rapid phosphorylation of MAPKs (this study; Trapaidze *et al.*, 2000b; Jordan *et al.*, 2000). Furthermore, on receptor internalization, the level of MAPK phosphorylation is significantly reduced in the case of mu and delta receptors (Trapaidze *et al.*, 2000b). These results suggest a role for receptor internalization in the desensitization of MAPK pathways. Recent findings with rat kappa receptors further support such a model: these receptors do not exhibit substantial agonist-mediated internalization, and they do not exhibit a significant reduction in the level of MAPK phosphorylation even on prolonged (120–240-min) exposure to agonists (Jordan *et al.*, in press). Taken together, these results support a role for opioid receptor internalization/recycling in the desensitization/resensitization of receptor function.

In summary, we have demonstrated that the wild type delta opioid receptor undergoes agonist-independent internalization and recycling. The receptor C-tail is involved in this process, as mutations of the C-tail result in increased recycling. Receptor internalization/ recycling are important for the recovery of receptor activity.

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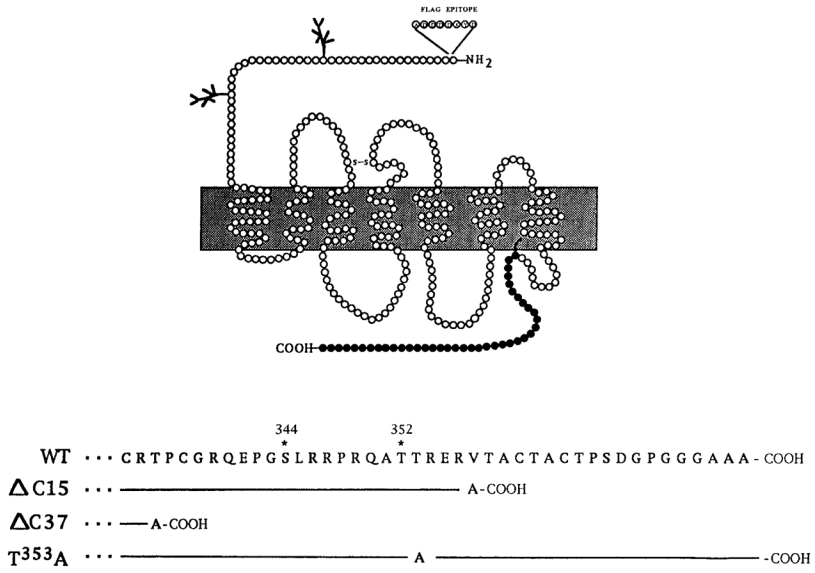

FIG. 1.

Schematic representation of the C-terminal tail of wild type and mutant delta opioid receptors. The C-terminal tail residues 333–372 of the wild type receptor are in single-letter amino acid code. The numbering is according to Evans *et al.* (1992); the amino acid sequence of the mutants identical to the wild type is represented by a line, and changes are as indicated.

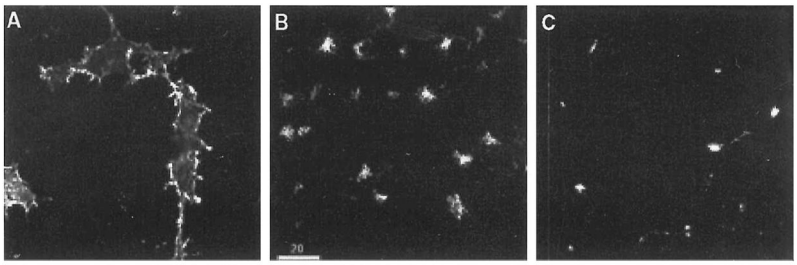

FIG. 2.

Neuroblastoma cells expressing delta opioid receptors. The N18TG2 cells expressing wild type mouse delta opioid receptors were incubated in the absence (**A**) or presence of 100 nM DADLE for 30 min (**B**) or 24 h (**C**). Fixation, permeabilization, staining, and confocal microscopy of receptors with the anti-Flag antibody were carried out as described in the text. Bright staining of the plasma membrane is seen in (**A**), while prominent intracellular staining is seen inside the cells in (**B**) and (**C**).

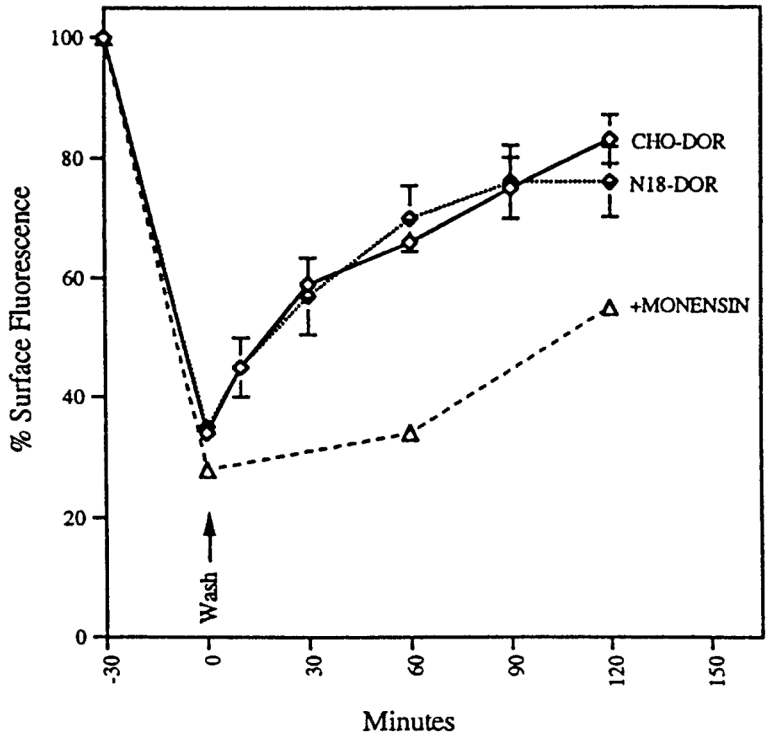

FIG. 3.

Time course of recycling of epitope-tagged mouse delta opioid receptors. CHO cells or N18G2 cells expressing epitope-tagged delta opioid receptor (CHO-DOR and N18-DOR) were treated with 10 μ M cycloheximide for 1 h, followed by incubation with 100 nM DADLE at 37°C for 30 min. The cells were washed and incubated for various periods of time in buffer without the agonist in the absence or presence of 100 μ M monensin. Cycloheximide was included in all the incubations. The cells were stained with M1 antibody followed by FITC-conjugated second antibody. Cell-surface fluorescence was measured by flow cytometry as described (Trapaidze *et al.*, 1996). The mean fluorescence, after subtracting autofluorescence of cells (stained with second antibody alone) without DADLE treatment, was taken as 100%. The data represent mean ± SEM from three independent experiments.

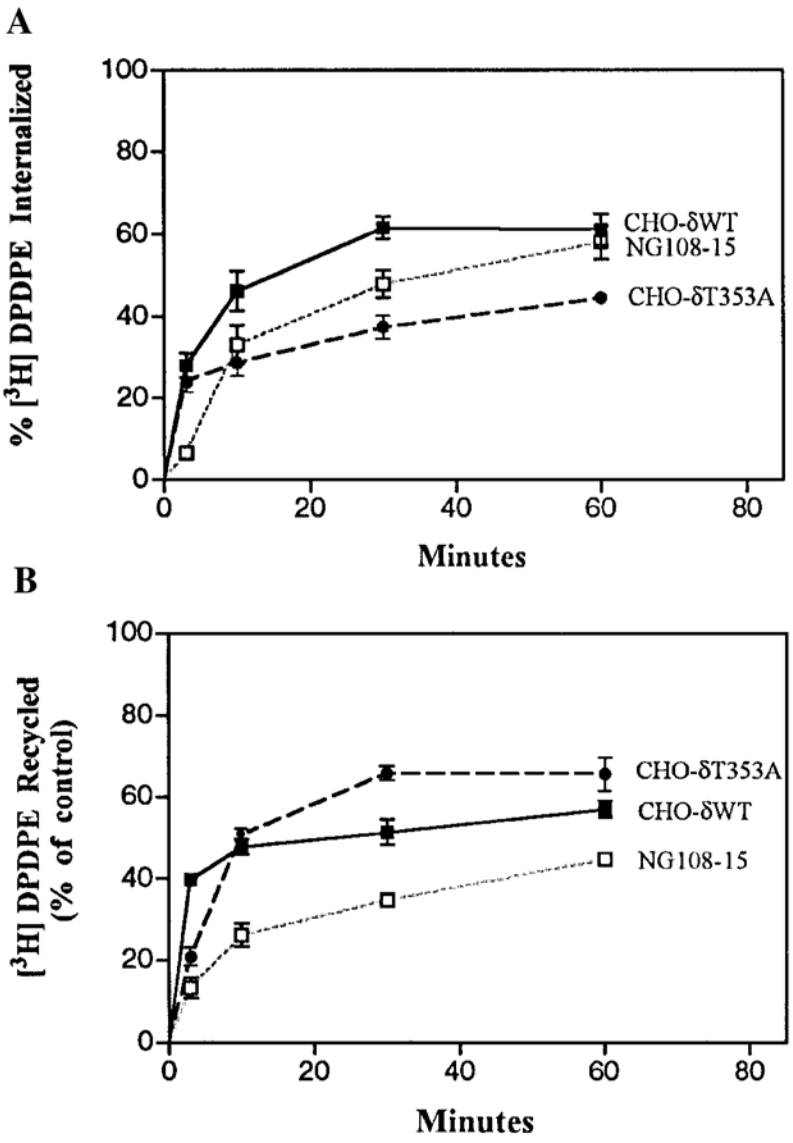

FIG. 4.

Internalization and recycling of opioid ligand. (**A**) Internalization. CHO cells expressing wild type or T353A receptors or NG108-15 cells expressing endogenous receptors were incubated at 37°C with 2 nM [^{3}H]-DPDPE for different time intervals. Cells were acid washed to remove surface-bound ligand, and the radiolabel associated with the cells was determined as described in Materials and Methods. (**B**) Recycling. Cells were incubated for 30 min with 2 nM [^{3}H]-DPDPE, acid washed to remove surface-bound ligand, and incubated in buffer without radioactivity for various periods of time. The radioactivity in acid-washed cells after 30 min of incubation with radiolabeled agonist is taken as 100%. The data represent mean ± SEM of three to five experiments.

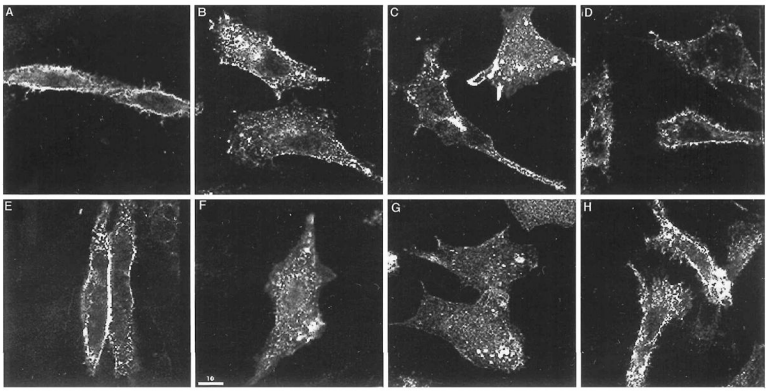

FIG. 5.

Confocal immunofluorescence microscopy of epitope-tagged wild type delta opioid receptors expressed in CHO cells after constitutive internalization. Cells expressing wild type delta opioid receptors were incubated for 1 h with M1 antibody at 4°C, washed, and warmed at 37°C for 0 (**A**), 30 min (**B**), or 60 min either in the absence of sucrose (**C**) or in the presence of 0.65 M sucrose (**D**). Cells were incubated with antibody at 37°C in the absence of sucrose for 30 min (**F**) or 60 min (**G**) or in the presence of 0.65 M sucrose for 60 min (**H**). Cells incubated with 0.65 M sucrose prior to staining with M1 antibody are shown in (**E**). To visualize both the cell-surface and internal receptors, cells were subjected to acid wash to remove primary antibody from the cell-surface receptors in the experiments shown in panels **B, C, F,** and **G**, then fixed and stained with FITC-conjugated second antibody following permeabilization. Immunofluorescence staining of the receptors with the monoclonal antibody against the epitope tag was as described in Materials and Methods. Cells were imaged by confocal fluorescence microscopy using a plane of focus adjusted 3 to 6 mm above the surface of the coverslip. This produces a cross-section through the center of the cell. Bright staining of the plasma membrane is apparent in panels **A, D, E,** and **H**. Prominent intracellular staining within the cytoplasm is seen in **B, C** and **F, G**.

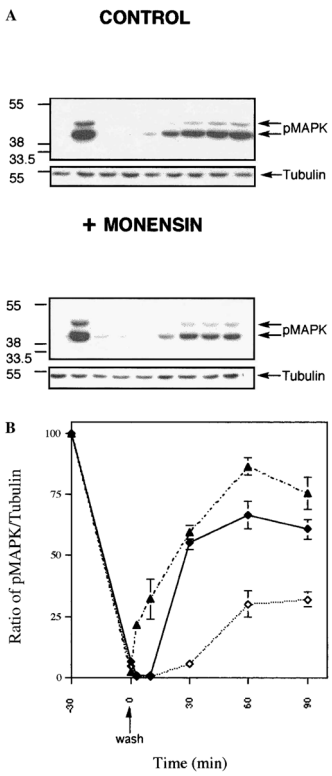

FIG. 6.

Phosphorylation of MAPK under conditions of internalization and recycling. (**A**) CHO cells expressing wild type or T353A mutant receptors were exposed to ligand for various periods of time (for internalization studies) or for 30 min. (**B**) The cells were washed and incubated without the agonist for various periods of time in the absence or presence of 100 μM monensin (for recycling studies). The level of phosphorylated MAPK was determined by immunoblotting using phospho-MAPK (pMAPK) antibodies as described in the text. The blots were reprobed with tubulin antibody for standardization. A representative figure from three independent experiments is presented.

Table 1

Effect of Various Ligands on Delta Opioid Receptor Internalization and Recycling

	% Surface receptors	
Ligand	**Internalization**	**Recycling**
DADLE	34 ± 1.5	66 ± 0.9
DPDPE	43 ± 2.1	83 ± 5.6
DSLET	49 ± 3.1	74 ± 4.2
Etorphine	50 ± 1.4	59 ± 5.6
DAMGO	102 ± 4.5	93 ± 5.3