Receptor-induced Internalization of Selective Peptidic \( \mu \) and \( \delta \) Opioid Ligands*

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The binding and internalization of radiodinated and fluorescent \( \mu \) and \( \delta \) opioid peptides in mammalian cells were quantitatively studied by biochemical techniques and directly visualized by confocal microscopy. The labeled peptides were prepared by inserting either a \(^{125}\text{I}\)-Bolton-Hunter group or a fluorescent probe into the C-terminal part of 5-aminoptylamide derivatives of deltorphin-I and [Lys\(^7\)]dermorphin. The purified derivatives kept most of their specificity and selectivity toward \( \delta \) and \( \mu \) opioid receptors, respectively. Biochemical and confocal microscopy data showed that both \( \mu \) and \( \delta \) opioid peptides were internalized in mammalian cells transfected with the corresponding opioid receptor according to a receptor-mediated mechanism. The internalization process was time- and temperature-dependent and was completely blocked by the endocytosis inhibitor phenylarsine oxide. Internalization of both \( \delta \) and \( \mu \) ligands occurred from a single large cap at one pole of the cell, indicating that polymerization of ligand-receptor complexes preceded internalization. Finally, green and red fluorescent analogues of deltorphin-I and [Lys\(^7\)]dermorphin, respectively, were found to internalize through partly distinct endocytic pathways in cells co-transfected with \( \mu \) and \( \delta \) receptors, suggesting that each of these receptors interacts with distinct proteins mediating intracellular sorting and trafficking.

The pharmacological, behavioral, and binding properties of \( \mu \), \( \delta \), and \( \kappa \) opioid receptors have been extensively studied. By comparison, much less is known about the intracellular routing and addressing of opioid receptors either before or after agonist binding. Yet, understanding the cellular regulation of this class of receptors is of prime importance, since it is at least partly involved in the mechanisms of tolerance and physical dependence (1). There is a considerable amount of \textit{in vitro} pharmacological evidence to suggest that both \( \mu \) (2, 3) and \( \delta \) (4–7) opioid receptors may undergo rapid down-regulation following exposure to agonists. Whether, and under which condition, such down-regulation involves internalization of receptor-ligand complexes remains a matter of debate. Thus, while biochemical studies have reported on either the occurrence (5, 8) or absence (9) of internalization of the tritiated enkephalin agonist \(^{3}\text{H}\)-AdAla, \( \delta \)-Leu-enkephalin in cultured neuroblastoma cells, morphological studies clearly failed to observe internalization of a fluorescent derivative of enkephalin in the same cell system (10–12). More recently, confocal microscopic studies carried out on transfected cells have shown a rapid endocytosis of \( \mu \) (13, 14) and \( \delta \) (13) antigenic epitope-tagged receptors following exposure to enkephalins, but not to morphine. Whether this endocytosis occurs in conjunction with that of the bound ligand, however, remains unclear. In the present study, we have reinvestigated the fate of receptor-bound opioid peptides using newly developed radioactive and fluorescent derivatives of the selective \( \mu \) and \( \delta \) opioid agonists dermorphin and deltorphin.

Dermorphin, isolated from the skin of the frog \textit{Phylomedusa sauvagei} (15), was the first natural peptide described as having high affinity and selectivity for the \( \mu \) opioid receptor. Another unusual property of dermorphin is the \( \delta \) configuration of the alanine residue in position 2, which is responsible for its strong resistance to enzymatic degradation and for its good opioid binding site recognition. The most interesting analogue of this peptide is [Lys\(^7\)]dermorphin (16), which possesses the highest affinity for the \( \mu \) opioid receptor (less than \( 10^{-10} \) M) together with the highest \( \mu/\delta \) selectivity (10,000-fold more specific for the \( \mu \) than for the \( \delta \) receptor). Two other peptides having also a \( \alpha \)-alanine in position 2 have been purified from the skin of another frog, \textit{Phylomedusa bicolor} (17). They have the same first three amino acids (Tyr-d-Ala-Pho) but differ in their C-terminal sequence (Asp-Val-Val-Gly for deltorphin-I and Glu-Val-Val-Gly for deltorphin-II). Deltorphin-I was chosen for our study because of its superior affinity and specificity for the \( \delta \) opioid receptor.

Sequences of [Lys\(^7\)]dermorphin and deltorphin-I may be divided into two parts, the same first three amino acids (Tyr-d-Ala-Pho) confirming something like an opioid master key and the last four being responsible for the \( \mu/\delta \) selectivity and affinity. The only site that could be modified without changing the properties of these peptides is the C-terminal end. We have thus incorporated radiolabeled and fluorescent groups into C-terminal extensions of deltorphin-I and dermorphin and used these specific tools to compare the cellular distribution and fate of specifically labeled \( \mu \) and \( \delta \) opioid receptors. Radioactive compounds were used to quantitatively assess the binding and internalization of the opioid derivatives, while fluorescent compounds were used to study their distribution in the confocal microscope, an approach that has been successfully resorted to for a variety of other neuropeptides, including fluorescent analogues of cholecystokinin (18), gastrin-releasing peptide (19), neurotensin (20), thyrotropin-releasing hormone (21, 22), substance P (23, 24), and somatostatin (25).
MATERIALS AND METHODS
Preparation of Peptide Precursors
DLT-I 5APA\(^1\) and (K\(7\))DRM 5APA were prepared as described previously (26). Briefly, deltorphin-I and dermorphin were assembled by stepwise solid phase synthesis using a β-totyroxycarbylon-benzyl strategy. The aminopentyl group was then grafted on the C-terminal amino acid by aminolysis of the peptide resin with 1,5-diaminopentane.

Preparation of Radioactive Peptides (27)
Bolton-Hunter reagent was iodinated with Na\(^{125}\)I and purified as described previously (28). DLT-I 5APA or (K\(7\))DRM 5APA (40 nmol) was incubated with iodinated Bolton-Hunter reagent (0.5–2 nCi, 2000 Ci/nmol) in 50 μl of borate/phosphate buffer (50 mM/50 mM) for 2 h at pH 8.5. The incubation mixture was injected on a C18 reverse phase HPLC (Merck, lichrocart), and the different products were eluted in 0.1% trifluoroacetic acid, 0.05% triethylamine by a linear gradient of pH 8.5. The incubation mixture was counted on filters presoaked in 0.3% polyethyleneimine-Cl, pH 7.5. Filters were rinsed with PBS, phosphate-buffered saline.

Fluorescent Binding Experiments—
Peptide precursors were reacted with the N-hydroxy succinimide esters of Bodipy 503/512 or Bodipy 576/589 (Molecular Probes). NHS-Bodipy (5 μmol in 400 μl of dimethyl sulfoxide) was incubated with DLT-I 5APA or (K\(7\))DRM 5APA (2 μmol) in a final volume of 1 ml of boric acid (50 mM), sodium phosphate (50 mM) buffer, pH 8.5, for 3 h at 4°C. The different derivatives were purified by reverse phase HPLC on a C18 Ultrasphere ODS column (10 x 250 mm, Beckman) eluted in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile from 20 to 60% during 60 min. Fluorescent peaks were tested for their ability to displace the specific binding of e-BH* DLT-I 5APA and e-BH* (K\(7\))DRM 5APA to the μ and δ opioid receptors, respectively (27). Seven fluorescent peaks were found to have a high binding activity and were further characterized by Edman degradation.

Preparation of Receptor-encoding Plasmids
Rat μ (MOR) (29) and δ (DOR) (30) opioid receptor cDNAs were amplified by PCR, cDNA fragments specific to oligonucleotides. Polymerase chain reaction products were subcloned in pcDNAI. pcDNAI, pcDNAI-MOR, and pcDNAI-DOR were transfected into COS cells by the DEAE-Dextran method (31).

Binding of Fluorescent Peptides to COS-7 Cell Membranes
Binding properties of fluorescent derivatives were determined by displacement of e-BH* DLT-I 5APA and e-BH* (K\(7\))DRM 5APA-specific binding to membranes of cells transfected with pcDNAI-MOR (32). The concentration of ligand was 50–200,000 cell/ml. Total binding was determined by reverse phase HPLC analysis of a fraction of ligand recovered at the end of the incubation. Fractions collected after HPLC were counted and compared with initial solutions.

Fluorescent Binding Experiments—Cells transfected with pcDNAI-DOR and pcDNAI-MOR were incubated for 0–60 min at 37°C in binding buffer containing 10 nmol e-BH* DLT-I 5APA and e-BH* (K\(7\))DRM 5APA (0.1–10 nM, 2000 Ci/nmol), respectively, in the presence or absence of 10 μM of the endocytosis inhibitor, phenylarsine oxide. The incubation was terminated by adding 3 ml of hypertonic acid buffer (Earle-HEPES, pH 4, acetic acid, 0.4 M NaCl) or of control buffer (Earle-HEPES at neutral pH) for 2 min, after which the cells were filtered on GF/C filters presoaked in GFC filters and rinsed three times with 3 ml of binding buffer. Cell-bound ligand was determined by counting the radioactivity retained on filters with a γ counter. Nonspecific binding was determined by carrying the incubation in the presence of 10 μM naloxone. Temperature sensitivity was verified by incubating additional cells for 60 min at 0°C with 0.2 μM radioactive ligand. Stability of the ligands was determined by reverse phase HPLC analysis of a fraction of ligand recovered at the end of the incubation. Fractions collected after HPLC were counted and compared with initial solutions.

Concomitant labeling of δ and μ opioid receptors was carried out on COS cells co-transfected with the pcDNAI-DOR and pcDNAI-MOR plasmids. Co-transfected cells were incubated at 37°C for 90 min with a mixture of 10 nmol of e-Bodipy 503/512 DLT-I 5APA (green) and (K\(7\))DRM 5APA (red). At the end of the incubation, cells were centrifuged at 2000 rpm during 1 min, deposited in 10 μl of Earle-HEPES on glass microscope slides, air-dried, and examined by confocal microscopy.

Confocal Microscopy—Labeled COS cells were examined under a Leica confocal laser scanning microscope configured with a Leica Diplan inverted microscope equipped with an argon/krypton laser with an output power of 2–50 mW (Leica, St. Laurent, Canada). Images of cells were acquired as single midcellular optical sections and averaged over 32 scans/frame. For double labeling experiments, δ and μ ligand images were acquired in the green and red channels, respectively.

RESULTS
Biochemical Studies—The binding and internalization of the selective opioid agonists deltorphin-I and dermorphin were first assessed quantitatively in COS-7 cells transfected with cDNA encoding δ and μ opioid receptors, using \(^{125}\)I-labeled Bolton-Hunter derivatives of deltorphin-I (e-BH* DLT-I 5APA) and dermorphin (e-BH* (K\(7\))DRM5APA), respectively. These derivativess have been documented to selectively bind to δ and μ opioid receptors with respective K\(_d\) values of 0.7 and 0.14 nM (27). Binding and internalization kinetics were established for each of these compounds by incubating whole cells at 37°C. As can be seen in Fig. 1, in which total specific binding (open symbols) corresponds to the sum of acid wash-resistant specific binding (corresponding to internalized ligand; closed symbols) and of acid-washable specific binding (corresponding to membrane-bound ligand; not shown), total binding kinetics were very rapid (K\(_{d}\) of about 1 min), whereas internalization kinetics were about 10 times slower (K\(_{d}\) = 13 and 19 min for δ and μ receptors, respectively). The mean diffusion step was about 60 min at 37°C. Two opioid systems was the maximal proportion of internalizable ligand, which represented approximately 55 and 25% of the total specific binding in cells transfected with δ and μ receptors, respectively. As shown in Fig. 2, this proportion was not very sensitive to ligand concentration as increasing the concentration of each radioligand by a factor of 100 (from 0.1 to 10 nM) at 37°C in binding buffer (Earle-HEPES buffer, pH 7.4, supplemented with 0.09% glucose and 0.2% of bovine serum albumin) at a final concentration of 50–200,000 cell/ml.

Radioactive Binding Experiments—Cells transfected with pcDNAI-DOR and pcDNAI-MOR were incubated for 0–60 min at 37°C with 0.1 μM e-BH* DLT-I 5APA and e-BH* (K\(7\))DRM 5APA (0.1–10 nM, 2000 Ci/nmol), respectively, in the presence or absence of 10 μM of the endocytosis inhibitor, phenylarsine oxide. The incubation was terminated by adding 3 ml of hypertonic acid buffer (Earle-HEPES, pH 4, acetic acid, 0.4 M NaCl) or of control buffer (Earle-HEPES at neutral pH) for 2 min, after which the cells were filtered on GF/C filters presoaked in GFC filters and rinsed three times with 3 ml of binding buffer. Cell-bound ligand was determined by counting the radioactivity retained on filters with a γ counter. Nonspecific binding was determined by carrying the incubation in the presence of 10 μM naloxone. Temperature sensitivity was verified by incubating additional cells for 60 min at 0°C with 0.2 μM radioactive ligand. Stability of the ligands was determined by reverse phase HPLC analysis of a fraction of ligand recovered at the end of the incubation. Fractions collected after HPLC were counted and compared with initial solutions.

Fluorescent Binding Experiments—Cells transfected with pcDNAI-DOR and pcDNAI-MOR were reincubated as above and incubated for 15–90 min at 37°C in binding buffer containing 10 nmol e-Bodipy 503/512 DLT-I 5APA or e-Bodipy 503/512 (K\(7\))DRM 5APA in the presence or absence of (total binding) of 10 μM naloxone. In some experiments, the incubation was carried out in the presence of phenylarsine oxide, to prevent ligand endocytosis. At the end of the incubation, cells were washed with either hypertonic or isotonic neutral buffers as above, centrifuged at 2000 rpm during 1 min, deposited in 10 μl of Earle-HEPES on glass microscope slides, air-dried, and examined by confocal microscopy.

Concomitant labeling of δ and μ opioid receptors was carried out on COS cells co-transfected with pcDNAI-DOR and pcDNAI-MOR plasmids. Co-transfected cells were incubated at 37°C for 90 min with a mixture of 10 nmol of e-Bodipy 503/512 DLT-I 5APA (green) and (K\(7\))DRM 5APA (red). At the end of the incubation, cells were centrifuged at 2000 rpm, deposited on glass slides, and air-dried for confocal microscopy examination.

Confocal Microscopy—Labeled COS cells were examined under a Leica confocal laser scanning microscope configured with a Leica Diplan inverted microscope equipped with an argon/krypton laser with an output power of 2–50 mW (Leica, St. Laurent, Canada). Images of cells were acquired as single midcellular optical sections and averaged over 32 scans/frame. For double labeling experiments, δ and μ ligand images were acquired in the green and red channels, respectively.
enhanced maximal internalization by a factor of less than 2. Thus, independent of ligand concentration, the internalization process was approximately twice as efficient for the δ as for the μ ligand.

Internalization of both δ and μ ligands was almost totally inhibited by blocking endocytosis with phenylarsine oxide or by lowering the temperature of incubation, as reflected by the reduction in size of the acid wash-resistant fraction (Fig. 3). Somewhat surprisingly, the addition of phenylarsine oxide also markedly reduced the total binding of 125I-labeled deltorphin to cells transfected with the δ receptor (Fig. 3A), but not that of 125I-labeled dermorphin to cells transfected with the μ receptor (Fig. 3B). In both systems, lowering the temperature of incubation to 0°C resulted in large losses of total specific binding (35 and 55% of the binding observed at 37°C for δ and μ systems, respectively). Finally, neither binding nor internalization of either 125I-deltorphin or 125I-dermorphin were observed in COS-7 cells transfected with the noncorresponding opioid receptor.

Biochemical Characterization of Fluorescent Analogues—In order to visualize at the cellular level the interaction of δ and μ opioid ligands with their respective receptors, we have synthesized two different fluorescent derivatives of deltorphin-I and [Lys3]dermorphin. These two heptapeptides are the most potent and selective δ and μ agonists currently known. The synthesis was carried out in two steps. First, an aminopentyl group was grafted on the C-terminal carboxyl function of dermorphin and deltorphin-I. Second, peptide precursors DLT-I 5APA and [K7]DRM 5APA were reacted with the N-hydroxysuccinimide esters of green (Bodipy 503/512) or red (Bodipy 576/589) fluorescent probes. The four different mixtures resulting from the reaction of the two peptide precursors with the two fluorescent reagents were fractionated by reverse phase HPLC. Elution profiles illustrated in Fig. 4 show that it was always possible to separate the unmodified peptide (N) from its fluorescent derivatives. Peaks numbered 1–6 in Fig. 4 were selected for further characterization on the basis of their ability to compete for binding to the δ (peaks 1 and 2) or to the μ (peaks 3–6) opioid receptor. Amino acid analysis and UV-visible spectra of each fraction (not shown) indicated that fluorescent peptides 1–6 contained a single fluorescent group per mol of peptide. The position of the fluorophore into each peptide sequence was determined by Edman degradation (Table I). The sequences of peaks 1 and 2 were identical to that of deltorphin-I, indicating that the green (peak 1) and red (peak 2) Bodipy fluorophores had been incorporated on the ε amine function of the 5-aminopentylamide group in DLT-I 5APA. In the same way, peaks 4 and 6 were identified as the red and green ω-substituted analogues of [K7]DRM 5APA, because their sequences were indistinguishable from that of [K7]DRM. By contrast, the seventh cycle of sequencing of fractions 3 and 5 did not give a PTH-Lysine, indicating that the ε-amino group of Lys 7 has
been modified. Therefore, fractions 3 and 5 were identified as the green and red e-labeled analogues of [K7]DRM 5APA.

The binding properties of the six fluorescent peptides were then evaluated by measuring their ability to displace the specific binding of 125I-labeled analogues of DLT-I 5APA and [K7]DRM 5APA to the δ and μ opioid receptors transiently expressed in COS cells. The two fluorescent analogues of DLT-I 5APA interacted with the δ opioid receptor with high affinity (K<sub>0.5</sub> = 2 nM) and retained much of their selectivity since their affinity for the μ receptor was lower by at least two orders of magnitude (Table II). The four fluorescent derivatives of [K7]DRM 5APA bound to the μ opioid receptor with affinities in the nanomolar range. However, their selectivity was not as specific because of their higher selectivity for the μ receptor as compared with derivatives 3 and 5.

Confocal Microscopic Studies—Confocal microscopic examination of COS-7 cells transfected with a cDNA encoding the δ opioid receptor and incubated for 15–90 min with 10 nM ω-Bodipy 503/512 [K7]DRM 5APA revealed selective fluorescent labeling of approximately 30% of the cells, in keeping with the documented transfection yield of this cell line (32) (Fig. 5A).

This labeling was specific in that it was no longer detected when the incubation was carried out in the presence of 10 μM naloxone (Fig. 5D) or with cells transfected with an empty plasmid (Fig. 5C). It was selective for δ opioid receptor-expressing cells, since cells transfected with cDNA encoding the μ site were totally devoid of fluorescent labeling (Fig. 5B).

Similarly, approximately 30% of COS-7 cells transfected with a cDNA encoding the μ opioid receptor and incubated with the μ-selective ligand ω-Bodipy 503/512 [K7]DRM 5APA exhibited intense fluorescent labeling (Fig. 6A). Here again, this labeling was specific in that it was displaced by naloxone (Fig. 6D) and absent from cells transfected with an empty plasmid (Fig. 6C). It was also selective for μ receptor-transfected cells as it was no longer apparent in cells transfected with cDNA encoding the δ opioid receptor (Fig. 6B).

At all time intervals examined, the bulk of DLT-I 5APA fluorescent labeling was intracellular, as attested by its intracytoplasmic distribution in single optical sections passing through the core of the cells (Figs. 5A and 7A) and by its resistance to hypertonic acid wash (Fig. 7C). Internalization of the fluorescent δ agonist was totally prevented by addition of the endocytosis inhibitor, phenylarsine oxide, in which case the bound fluorescent molecules remained clustered at the periphery of the cells (Fig. 7B). This excentric labeling pattern corresponded to cell surface labeling as it completely disappeared after hypertonic acid wash (Fig. 7D).

Similarly, a sizeable fraction of ω-Bodipy 503/512 labeling of μ receptor-expressing cells was found to be acid wash-resistant, i.e. intracellular, at all times examined (Figs. 8, A and C). In keeping with our biochemical results, this fraction was smaller overall than in the case of δ labeling. As with the δ ligand, incubation in the presence of phenylarsine oxide prevented internalization of the μ opioid agonist and resulted in an acid-washable (Fig. 8D), cell surface clustering of the bound fluorescence (Fig. 8B).

The intracellular distribution of ω-Bodipy 503/512 DLT-I 5APA and ω-Bodipy 503/512 [K7]DRM 5APA, specifically bound to δ opioid- and μ opioid-transfected cells, respectively, varied markedly as a function of time. After 15 min of incubation, both fluorescent markers were clustered at one pole of the cell, onto and/or immediately beneath the plasma membrane (Fig. 9, A and B). At 30 min, they were detected in the form of small fluorescent particles excentrically clustered in the cytoplasm of the cells (Fig. 9, C and D). By 60 min, δ and μ opioid labeling remained highly punctate, but entirely filled the cytoplasm of the cells, sparing the nucleus (Fig. 9, E and F). However, by that time, the dermorphin-labeled fluorescent particles were on average larger and less numerous than the dermorphin-labeled ones and stood out less clearly against a greater intracytoplasmic background labeling.

In order to compare the distributional pattern of δ and μ

FIG. 4. Purification of fluorescent analogues of deltorphin and dermorphin by HPLC. Incubation mixtures obtained after reaction between DLT-I 5APA and NHS-Bodipy 503/512 (A), DLT-I 5APA and NHS-Bodipy 576/589 (B), [K7]DRM 5APA and NHS-Bodipy 503/512 (C), and [K7]DRM 5APA and NHS-Bodipy 576/589 (D) were injected on a C18 ultrasphere ODS column and eluted with a linear gradient of acetonitrile. Elutions were followed by measurement of optical density at 230 nm. The arrows indicate the elution time of the native (N) peptide and of fluorescent peptides 1–6.

Table I

Sequences of fluorescent peptides

Peaks 1–6 were submitted to Edman degradation and the PTH-derivative characterized for each cycle of sequence are shown. Cycle no. 7 of peaks 3 and 5 gave no known PTH-amino acid and corresponded to the PTH-derivative of lysine labeled on its side chain by Bodipy 503/512 and Bodipy 576/589, respectively.

| Fluorescent peak no. | Result of cycle no. |
|---------------------|---------------------|
| 1                   | Tyr                 |
| 2                   | Tyr                 |
| 3                   | Tyr                 |
| 4                   | Tyr                 |
| 5                   | Tyr                 |
| 6                   | Tyr                 |

| Result of cycle no. |
|---------------------|
| Ala                 |
| Phe                 |
| Asp                 |
| Val                 |
| Val                 |
| Gly                 |
| Tyr                 |
| Pro                 |
| Pro                 |
| Lys                 |
| Lys                 |
TABLE II  
Binding properties of fluorescent derivatives of DLT-I 5APA and [K7]DRM 5APA

Membranes from cells transfected with pcDNAI-DOR or pcDNAI-MOR were incubated with their specific 125I-radiolabeled ligand (0.2 nM ω-BH+ DLT-I 5APA or ε-BH+ [K7]DRM 5APA, respectively) and increasing concentrations of fluorescent peptides. Binding experiments were terminated by filtration. IC₅₀ is the concentration of fluorescent peptide that induces 50% displacement of the bound radio-active ligand.

| No. | Fluorescent peptide | IC₅₀ (nM) | δ (%) | μ (%) |
|-----|---------------------|-----------|-------|-------|
| 1   | ω-Bodipy 503/512 DLT-I 5APA | 2.0 | 1400 |       |
| 2   | ω-Bodipy 576/589 DLT-I 5APA | 2.0 | 450  |       |
| 3   | ε-Bodipy 503/512 [K7]DRM 5APA | 2.6 | 0.7  |       |
| 4   | ω-Bodipy 503/512 [K7]DRM 5APA | 10.0 | 0.6  |       |
| 5   | ε-Bodipy 576/589 [K7]DRM 5APA | 14.0 | 1.8  |       |
| 6   | ω-Bodipy 576/589 [K7]DRM 5APA | 21.0 | 1.0  |       |

opioid agonists internalized within the same cells, COS-7 cells were co-transfected with the pcDNAI-DOR and pcDNAI-MOR plasmids and co-incubated with the green fluorescent analogue ω-Bodipy 503/512 DLT-I 5APA and the red fluorescent analogue ω-Bodipy 576/589 [K7]DRM 5APA for 90 min at 37 °C. As shown in Fig. 10, A and B, and at higher magnification in Fig. 10, A’ and B’, images acquired through distinct red and green excitation/emission channels showed labeling patterns comparable with those obtained in singly transfected cells confirming the efficiency of the co-transfection procedure. Superimposition of the two images (Figs. 10, C and C’) showed only partial overlap of the red and green fluorescent clusters, indicating that the ω and δ fluorescent ligands were sequestered partly in the same and partly in distinct compartments. This partial overlap could not be attributed to a bleed-through of one of the fluorophores into the other channel since Bodipy 567/589 and Bodipy 503/512 derivatives gave no signal in the green and red channels, respectively. Interestingly, the bulk of distinct green and red particles were small and predominated at the periphery of the cell (Fig. 10C). By contrast, double-labeled endosome-like particles (in yellow, Fig. 10C) were larger and mainly concentrated within the cytoplasmic core.

DISCUSSION

In the present study, the binding and internalization of μ and δ opioid peptides in mammalian cells were quantitatively studied by means of biochemical techniques and directly visualized by confocal microscopy. The radiolabeled and fluorescent analogues developed for this purpose were synthesized by inserting either an 125I-labeled Bolton-Hunter group or a fluores-
ers, since insertion of a Bolton-Hunter group into the ging probably resulted from the steric hindrance of the mark-reduced affinity observed after radioactive or fluorescent tag-thanthose of ligand binding (results not shown). Scale bar, 10 μm.

![Image](75x529 to 281x729)

**FIG. 8.** Internalization of ω-Bodipy 503/512 [K7]DRM 5APA in µ receptor-transfected cells. COS-7 cells transfected with pcDNAI-MOR were incubated for 90 min at 37 °C with ω-Bodipy 503/512 [K7]DRM 5APA in the absence (A, C) or in the presence (B, D) of 10 μM phenylarsine oxide. A and B, total binding; C and D, residual binding after hypertonic acid wash. Optical sections acquired through the nuclear plane. In A and C, the labeling is mainly intracellular and confined to intracytoplasmic organelles (arrow). This ring corresponds to cell surface labeling as attested by its disappearance after acid wash (D). Scale bar, 10 μm.

**FIG. 9.** Kinetics of ω-Bodipy 503/512 DLT-I 5APA and ω-Bodipy 503/512 [K7]DRM 5APA internalization in COS-7 cells. COS-7 cells transfected with pcDNAI-DOR (left) and pcDNAI-MOR (right) were incubated for 15 (A, B), 30 (C, D), and 60 (E, F) min at 37 °C with 10 nM ω-Bodipy 503/512 DLT-I 5APA or ω-Bodipy 503/512 [K7]DRM 5APA, respectively. Confocal optical sections acquired through the nuclear plane. Note the similarity in the intracellular mobilization patterns of the two fluorescent probes. Also note that at 60 min the intracytoplasmic fluorescent clusters are both smaller and more distinct in cells labeled with δ than with µ ligands (arrow). Scale bar, 10 μm.

Biochemical and confocal microscopic data showed that both µ and δ opioid ligands were internalized in mammalian cells according to a time- and temperature-dependent process. This internalization was clearly receptor-mediated, since it was no longer observed in nontransfected cells or in cells transfected with a receptor not specifically recognized by the ligand. Furthermore, it was completely prevented by incubation with the non selective opioid antagonist naloxone. The kinetics of internalization of µ and δ opioid ligands were considerably slower than those of ligand binding (t½ of 10 and 13 min for µ and δ ligands, respectively), but within the same range as reported for bradikinin receptor (33) and vasopressin V2 receptor (34) complexes (t½ = 9 and 13 min, respectively). Slightly faster receptor-mediated internalization kinetics have been described for peptides bound to vasopressin V1 (35, 36), angiotensin II 1a and 1b (37), substance P (23), gastrin-releasing peptide (38), and neurotensin (39, 40) receptors, with t½ values ranging between 3 and 5 min. The similarity between the internalization kinetics of these different neuropeptide-receptor complexes suggests that they may be controlled by a common rate-limiting step.

Despite the fact that formation of receptor-ligand complexes was clearly critical for the initiation of ligand internalization, the percentage of internalized ligand molecules was in no way proportional to the degree of receptor occupancy. Indeed, cells transfected with the δ receptor and incubated with concentrations of 125I-labeled deltorphin-I ranging from 0.025 to 10 nM internalized between 40 and 70% of the bound radiolabeled peptide, whereas receptor occupancy varied from 3.5 to 94%. The disproportion was even greater in the case of the µ opioid ligand, which proportionally internalized about 2-fold less than its δ counterpart. Variable internalization capacities have been reported for other peptidergic systems. Ratios of internalized to bound ligand ranging between 50 and 80% have been reported for substance P (23), vasopressin (34, 35), angiotensin II (37, 41), or neurotensin (39, 40) and of 100% for gastrin-releasing peptide (38) and bradykinin (33). The low proportion (20–35%) of 125I-labeled dermorphin internalized by COS cells trans-
**μ and δ Opioid Internalization**

![Concomitant internalization of δ and μ opioid ligands in a cell cotransfected with pcDNAI-MOR and pcDNAI-DOR.](image)

COS-7 cells cotransfected with pcDNAI-DOR and pcDNAI-MOR were co-incubated for 90 min at 37°C with 10 nM α-Bodipy 503/512 DLT-I 5APA and α-Bodipy 576–589 [K7]DRM 5APA. Specific μ labeling is imaged through the red channel (A, A’) and δ labeling through the green channel (B, B’). Superimposition of the two signals reveals only partial overlap (in yellow, arrows) of the two fluorophores (C, C’). A’, B’, and C’ are higher magnifications of A, B, and C, respectively.

Internalization of both μ and δ opioid ligands in transfected COS-7 cells was totally prevented in the presence of the endocytosis inhibitor, phenylarsine oxide (PAO), indicating that the internalization process is endocytic in nature. Accordingly, internalized ligand molecules were seen by confocal microscopy to be concentrated within small, endosome-like organelles. Current models of receptor-mediated internalization call for internalization of receptor-ligand complexes into clathrin-coated pits, followed by their mobilization into early and then late endosomes (42). These endosomes eventually fuse into multivesicular bodies and lysosomes, while dissociated receptors are either recycled back to the membrane or degraded (42).

The progressive shift in size and intracellular mobilization of fluorescent organelles observed in the present study are consistent with this pattern. Although the present approach did not allow a direct visualization of receptors themselves, the similarity of the punctate labeling seen here after internalization of fluorescent dermorphin with that observed by immunohistochemistry in cells expressing an epitope-tagged μ opioid receptor (14) and exposed to the selective μ agonist Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol strongly suggests that the internalization involves ligand-receptor complexes. A similar mechanism has been invoked to account for the internalization of tritiated δ-Ala2, δ-Leu5enkephalin in neuroblastoma cells (5, 8) and may play a role in the agonist-induced down-regulation of δ opioid receptors documented in several cell lines (6, 7, 43). Our results are at odds, however, with the reported lack of internalization of a rhodamine-tagged Met-enkephalin derivative in cultured neuroblastoma cells (10–12). This discrepancy may be due to differences in receptor behavior dependent on the ligand utilized. Mu opioid receptors were indeed shown to internalize following exposure to Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol, but not to morphine (14).

In addition to blocking receptor-mediated internalization, phenylarsine oxide markedly decreased total specific binding of the 125I-labeled deltorphin-I analogue to cells transfected with the δ receptor without affecting that of the dermorphin analogue to μ sites. This decrease cannot be imputed to direct effects of the drug on the plasma membrane, as it was no longer observed when the binding experiments were performed on COS-7 cell membranes, as opposed to whole cells (not shown). It is therefore likely that the loss of specific deltorphin binding observed in whole cells is the result of cellular traffic blockade by PAO, implying that μ and δ opioid receptors are differentially distributed within the cells at steady state. Specifically, our results suggest that μ receptors are predominantly localized on the cell membrane, since they are freely accessible to radioligand even in the presence of PAO, whereas a significant fraction (65%) of δ receptors are located inside the cell, within vesicular structures that can no longer be recruited to the membrane in the presence of PAO. The ability of PAO to inhibit not only endocytosis but also exocytosis has been documented in various reports dealing with trafficking properties of glucose transporters (44) and transferrin receptors (45) or with secretory mechanisms of the RBL-2H3 mast cell line (46). Furthermore, such differential distribution of μ and δ receptors in COS cells would be consistent with the results of light and electron microscopic localization studies in the central nervous system, which have shown μ opioid receptors to be predominantly associated with the plasmalemma (14, 47, 48) and δ receptors to be almost equally distributed between the plasma membrane and intracellular stores (48–52). Comparable differences in the subcellular distribution of two homologous G-protein-coupled receptors have been observed previously within the family of adrenergic receptors. Thus, the β2- and α2C10-adrenergic receptors expressed in transfected fibroblasts are mainly localized in the plasma membrane at steady state, whereas the α2C4-adrenergic receptor is found both in the plasma membrane and in a population of intracellular vesicles (53).

A striking feature of both μ and δ labeling patterns was their clustering into a single large cap at one pole of the cell. Although comparable clustering patterns have been observed after ligand binding to insulin (54) and muscarinic cholineric (55) receptors, most hormone- or neuropeptide-receptor (18, 19, 23, 39) complexes have a tendency to aggregate into small, distinct patches distributed all over the cell membrane. In fact, earlier fluorescence studies have reported rhodamine-tagged enkephalins to form multiple small clusters at the surface of neuroblastoma cells (10). It may be that the extent of receptor clustering is controlled by the relative rates of ligand binding and internalization and that it varies from one type of cell to the other. When the rate of internalization is equal to (or faster...
than) the binding step, as observed for example in the case of neurotensin-receptor complexes (39), internalization begins as soon as small aggregates are formed, preventing the formation of large caps. By contrast, if the internalization proceeds at a much slower pace than ligand binding, as is the case for opioid receptors, polymerization of the complexes is allowed to proceed for longer times leading to the formation of large macromolecular aggregates. In any event, our kinetic results clearly show that internalization of both \( \mu \) and \( \delta \) ligands occurs only from a single polar cap, corroborating the idea that polymerization of the ligand-receptor complexes is a necessary step for internalization.

A major result of the present work is the demonstration that \( \mu \) and \( \delta \) receptors co-expressed in the same cells internalize through partly distinct endocytic pathways. Thus, during the early phase of internalization, \( \mu \) and \( \delta \) receptor-ligand complexes appeared to be mainly localized within mutually exclusive endosomal populations, as there was little overlap between the small, peripheral red and green fluorescent particles that likely correspond to early endosomes. Co-localization of the two fluorophores occurred only in larger organelles that were observed deeper in the core of the cells and probably correspond to late endosomes or lysosomes (56). To our knowledge, the present results provide the first direct evidence that different receptor subtypes co-expressed in the same cell may be sorted via different endocytic vesicles. A selective internalization of the different receptor complexes is allowed to proceed in transfected COS-7 cells as a consequence of their selective interaction with \( \mu \) and \( \delta \) opioid receptors, respectively. For each ligand, the internalization is likely to be deeply in the core of the cells and probably correspond to late endosomes or lysosomes (56). To our knowledge, the present results provide the first direct evidence that different receptor subtypes co-expressed in the same cell may be sorted via different endocytic vesicles. A selective internalization of the different receptor complexes is allowed to proceed in transfected COS-7 cells as a consequence of their selective interaction with \( \mu \) and \( \delta \) opioid receptors, respectively. For each ligand, the internalization is likely to be.

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**μ and δ Opioid Internalization**