

Binding and Internalization of Fluorescent Opioid Peptide Conjugates in Living Cells

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ABSTRACT

The dynamics of agonist-stimulated opioid receptor internalization and trafficking have been difficult to study in living cells in part because the available probes were inadequate. To overcome this obstacle, six new fluorescent opioid peptides were developed. Dermorphin (DERM), deltorphin (DELT), TIPP, and endomorphin were conjugated to BODIPY TR or Alexa Fluor 488, two fluorescent dyes with distinct hydrophobic properties. In membrane binding assays the fluorescent conjugates DERM-A488 or -BTR, DELT-A488 or -BTR, and TIPP-A488 displayed good binding affinity and selectivity for μ - and δ -opioid receptor subtypes. Furthermore, the fluorescent conjugates of dermorphin and deltorphin were biologically active as demonstrated by their ability to hyperpolarize locus coeruleus neurons (DERM-A488 or -BTR) and inhibit calcium currents in NG108-15 (DELT-A488). Both of these responses were antag-

onized by naloxone. In conjunction with confocal fluorescent microscopy the trafficking of these fluorescent ligands was monitored in real-time. The internalization of these ligands by μ - and δ -opioid receptors was found to be naloxone-sensitive and temperature-dependent. Interestingly, once these ligands were internalized the fluorescent puncta that formed became distributed in one of two patterns. In Chinese hamster ovary cells heterologously expressing either μ - or δ -opioid receptors the intracellular puncta were concentrated in the perinuclear region of the cell, whereas they were distributed throughout the cytoplasm in cells derived from either NG108-15 or SH-SY5Y cells. In summary, we have demonstrated that these novel, fluorescent opioid peptide conjugates permit real-time visual tracking of receptor-ligand complexes, including their internalization and trafficking, in living cells.

Opiates are powerful analgesic drugs, however, their use in the clinic is limited because repeated administration can lead to the development of tolerance and dependence. It has been proposed that alterations in opioid receptor number and their ability to interact with agonists are responsible, in part, for biochemical and cellular events that contribute to tolerance and dependence (Loh et al., 1988). The dynamic changes in opioid receptor number, distribution, and/or post-translational modifications may be important for various adaptive changes that occur after acute (e.g., desensitization) and chronic (e.g., tolerance and down-regulation) opiate exposure.

Opioid receptors are members of the G protein-coupled receptor family (Kieffer, 1995). When activated these receptors not only couple to and regulate multiple second-messenger pathways but also can become redistributed in the cytoplasm via distinct trafficking pathways (Childers, 1991; Krupnick and Benovic, 1998). Recently, the internalization of

opioid receptors after acute opiate exposure was shown to be differentially regulated depending on the agonist used (Arden et al., 1995; Keith et al., 1996, 1998; Sternini et al., 1996; Zhang et al., 1998; Whistler et al., 1999). For example, morphine was unable to induce internalization, whereas other agonists, such as DAMGO and etorphine, involved receptor internalization and redistribution throughout the cytoplasm.

The dynamic relationship between opioid receptor internalization, desensitization, down-regulation, tolerance, and dependence is poorly understood. In the past, each of these processes had to be studied separately in non-living cells. Typically, ligand-receptor binding and internalization experiments were performed by using either radioactive ligands or fluorescent immunocytochemistry (Dado et al., 1993; Ding et al., 1998). Recently, cell lines expressing epitope-tagged opioid receptors have been used in combination with Flag-tagged antibodies to study receptor trafficking (Arden et al., 1995; Keith et al., 1996, 1998; Sternini et al., 1996; Whistler et al., 1999). Unfortunately, the principle limitation remains; these protocols require fixing of the cells thereby prohibiting a direct correlation between receptor activation, trafficking,

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ABBREVIATIONS: DAMGO, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]-enkephalin; TIPP, Tyr-Tic-Phe-Phe; BTR, BODIPY TR; A488, Alexa Fluor 488; DMSO, dimethyl sulfoxide; DERM, dermorphin; DELT, deltorphin; CHO, Chinese hamster ovary; LC, locus coeruleus; ME, [Met⁵]-enkephalin.

desensitization, and the development of tolerance in living cells. One way to overcome this obstacle would be to probe the ligand-receptor complex with a fluorescently labeled agonist.

Past attempts to make fluorescent opioid peptides have met with mixed success (Kolb et al., 1983; Goldstein et al., 1988; Archer et al., 1992; Baidur and Triggle, 1994; Chang et al., 1996; Emmerson et al., 1997; Gaudriault et al., 1997), in large part because most fluorescent dyes are bulky, often being as large as the ligands themselves and causing steric interference. Therefore, the aim of the present study was to develop fluorescent opioid ligands that retained both their receptor subtype selectivity as well as their biological activity.

For our study we choose three opioid agonists and one opioid antagonist based on their potencies, binding affinities, and μ/δ -receptor selectivities. [Lys⁷]Dermorphin and deltorphin I, originally isolated from frog skin, display the highest reported affinities for μ - and δ -opioid receptors, respectively ($K_i = 0.12$ nM for dermorphin at μ -receptor and $K_i = 0.22$ nM for deltorphin at δ -receptor) (Erspamer, 1992). Endomorphin I, an endogenous μ -opioid agonist isolated from mammalian brain, has been reported to have a high binding affinity ($K_i = 0.36$ nM) and selectivity for μ -opioid receptors (Zadina et al., 1997). In contrast TIPP, a synthetic tetrapeptide antagonist, displays high selectivity for δ -opioid receptors ($K_i = 0.48$ nM) (Tourwe et al., 1998).

When choosing the fluorophores to be conjugated with these peptides we were primarily concerned about their hydrophobic properties, which can have profound effects on the biological activity and selectivity of a given ligand. We were also interested in dyes that possess high fluorescent quantum yields, high photostability, and low sensitivity to pH. BODIPY TR (BTR) and Alexa Fluor 488 (A488) fulfill all of these criteria (Haugland, 1999). BTR is a hydrophobic molecule that has been conjugated to a wide variety of biologically active peptides with mixed success (McGrath et al., 1996). A488 is an interesting alternative because it is hydrophilic, a property that may help eliminate unwanted background.

Here, we describe the development and characterization of six new fluorescent opioid peptides and their utility in studying equilibrium binding to opioid receptors and subsequent internalization in real-time. Our findings suggest that these fluorescent agonists may provide a means by which the visualization of receptor-ligand interactions and internalization can be correlated with functional activation and desensitization in living cells.

Experimental Procedures

Materials. [³H]Diprenorphine (specific activity 36 Ci/mmol) was purchased from Amersham International (Amersham, UK). The unlabeled peptides were custom synthesized by SynPep Corporation (Dublin, CA). BODIPY TR iodoacetamide and Alexa Fluor 488 maleimide (the mixed 5- and 6-carboxy isomers) were obtained from Molecular Probes, Inc. (Eugene, OR). Naloxone, [Met⁵]-enkephalin, bestatin, and thiorphan were purchased from Sigma Chemical Co. (St. Louis, MO). Reagents for tissue cultures were purchased from Life Technologies (Gaithersburg, MD). All other chemicals were of reagent or HPLC grade.

Conjugation of Fluorescent Dyes to Peptides. The peptides were custom ordered from SynPep Corporation and received as gel-filtration grades (<70% purity). All peptides were then further purified on a reversed phase LUNA semipreparative column (C8, 10

μ m, 100 Å, 10 × 250 mm; Phenomenex, Torrance, CA) to obtain the highest purity (>98%) before the conjugations. Reversed phase HPLC was performed on a Waters (Milford, MA) 600E multisolvent delivery system. Generally, a linear gradient of 5 to 65% acetonitrile containing 0.1% trifluoroacetic acid as the aqueous phase in 30 min at 4 ml/min was used. The purified peptide was then reacted with the BTR iodoacetamide or A488 maleimide. The fluorescent dye [1.5 μ mol in 200 μ l of dimethyl sulfoxide (DMSO)] was added into a solution of peptide (1 μ mol in 200 μ l of DMSO) and the mixture was stirred at room temperature for 4 h. The reaction was monitored by analytical HPLC using a LUNA analytical reversed phase column (C8, 5 μ m, 300 Å, 4.6 × 250 mm; Phenomenex) and absorbance at wavelengths 495 and 590 nm for A488 and BTR, respectively. The identity of the peak corresponding to the nonreacted dye was confirmed by comparing the HPLC tracings of the free dye and the crude reaction mixture. Crude mixtures were injected onto the reversed phase LUNA semipreparative column and conjugate peptides were eluted with a linear gradient of 20 to 50% acetonitrile containing 0.1% trifluoroacetic acid as the aqueous phase in 50 min at 4 ml/min. Purity and identity of the conjugate peptides were confirmed by analytical HPLC equipped with a Waters 996 photodiode array detector and mass spectra recorded by FAB mass spectrometer in the positive mode on a Kratos MS50RFTC spectrometer in the Department of Chemistry, Oregon State University (Corvallis, OR).

Spectrofluorometry. Emission spectra were recorded on a PTi spectrofluorometer (Photon Technology International, Monmouth Junction, NJ) in the Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland. The solutions of A488, DERM-A488, and DELT-488 were excited at 450 nm, whereas the solutions of DERM-BTR were excited at 580 nm. Fluorescence emission intensity of A488 and the A488 conjugates was determined at 515 nm and fluorescence emission intensity of DERM-BTR was determined at 615 nm. Both excitation and emission slits were set at 3 nm. Each solution (100 μ l) was placed in a cleaned and dried quartz cell and the data were acquired by Felix software.

Radioligand Receptor Binding Assays. Opioid receptor binding assays were completed as described in detail elsewhere (Bunzow et al., 1995). Competition experiments were performed on membranes derived from CHO cells stably expressing cloned rat μ - and δ -receptors at 25°C for 60 min in a total volume of 500 μ l. [³H]Diprenorphine was used in the assays at 0.4 nM. Nonspecific binding was defined in the presence of 10 μ M naloxone. IC₅₀ values were determined by nonlinear regression analysis to fit a logistic equation to the competition data and K_i values calculated from the IC₅₀ values by the Cheng and Prusoff equation, using K_d values of 0.3 and 1.0 nM for μ - and δ -receptors, respectively.

Electrophysiology. For intracellular recordings, horizontal brainstem slices (300 μ m) containing the locus coeruleus (LC) were prepared from 4- to 10-day-old Sprague-Dawley rats (Charles River, Wilmington, MA) as previously described (Ishimatsu and Williams, 1996). Intracellular recordings of the membrane potential were made with sharp electrodes filled with 2 M KCl (~40 M Ω) using an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). Data were acquired with Chart software, version 3.5 (MacLab System, AD Instruments Pty Ltd., Castle Hill, Australia). Slices were perfused with artificial cerebrospinal fluid containing 126 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 21.4 mM NaHCO₃, 11.1 mM glucose, equilibrated with 95% O₂, 5% CO₂ at 34°C.

For voltage-clamp experiments, coronal brain slices (250 μ m) containing the LC were prepared from adult (1.5–2-month-old) Sprague-Dawley rats as described in Travagli et al. (1996). Currents were recorded under whole-cell configuration and the membrane potential was held at –60 mV. Pipettes were filled with a solution containing 115 mM potassium methylsulfate, 20 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, 10 mM HEPES, 4 mM magnesium-ATP, and 0.4 sodium-GTP, pH 7.3. The resistance was 2 to 3.5 M Ω and the osmolarity was 280 mOsm. Data were recorded with an Axopatch-1D amplifier

(Axon Instruments) and were acquired in Axograph 4.3 (100 Hz) with an ITC-16 Computer Interface (Instrutech Corporation, Long Island, NY). The currents induced by different concentrations of the fluorescent dermorphin were expressed as a percentage of the current induced in the same cell by [Met⁵]-enkephalin (10 μ M). A dose-response curve for dermorphin-A488 was constructed by fitting these experiment data to a logistic equation $y = \max(x^n/(x^n + EC_{50}^n))$, where y is the amplitude of the response induced by x concentration of agonist, \max is the maximum amplitude of response, EC_{50} is the concentration of agonist that induced half-maximal response, and n is the slope (index of cooperativity).

μ -Opioid agonists were diluted to the desired final concentration from stock solutions just before being tested. The peptidase inhibitors bestatin (10 μ M) and thiorphan (2 μ M) were included in the solution. To minimize the amount of fluorescent peptides used during a prolonged perfusion, a peristaltic pump was used in some experiments to recycle the solution containing the fluorescent peptide and thereby reperfusing continuously the brain slice.

The NG108-15 (mouse N18 neuroblastoma \times rat C6 glioma) cell line was used to determine biological function of δ -opioid agonists. These cells were maintained in Ham's F-12 medium supplemented with 10% heart extract, and then were differentiated for 7 to 12 days in Ham's F-12 medium supplemented with 10% heart extract and 1 mM dibutyl cAMP (Morikawa et al., 1998). Barium currents through voltage-gated calcium channels were recorded at room temperature in whole-cell configuration and voltage-clamp mode (Axopatch 1D amplifier; Axon Instruments). Pipettes (1–3 M Ω) were filled with internal solution containing 125 mM CsCl, 1 mM MgCl₂, 5 mM HEPES, 5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, 4 mM magnesium-ATP, 0.4 mM sodium-GTP, 14 mM phosphocreatine, pH adjusted to 7.3 with HCl, osmolarity = 290 mOsmol. The external solution contained 4 mM BaCl₂, 145 mM NaCl, 10 mM glucose, 5 mM HEPES, pH adjusted to 7.4. Barium currents were elicited every 20 s by applying 100-ms voltage step to 0 mV from a holding potential of –60 mV. Data were digitized with ITC-16 Computer Interface (Instrutech Corporation) and acquired at 10 kHz with Axograph 4.2.

Fluorescent-Ligand Binding and Internalization Studies. CHO cells stably expressing cloned rat μ - or δ -opioid receptors (Bunzow et al., 1995) were maintained in Ham's F-12 medium containing 10% fetal calf serum and G418 (Life Technologies; 700 μ g/ml). Confluent cells were detached with a PBS solution containing 0.05% trypsin and 0.53 mM EDTA, harvested, and after diluting 1:10 cells were plated on circular glass microscope coverslips (25 mm in diameter; Fisher Scientific, Pittsburgh, PA) in 35 \times 10-mm culture dishes (Falcon Plastics, Oxnard, CA). Cells were grown in the incubator for 24 to 48 h before fluorescent-ligand receptor binding.

NG108-15 and SH-SY5Y cells (subcloned of SK-N-SH human neuroblastoma) were obtained from the tissue culture facility of the Vollum Institute. NG108-15 cells were maintained in Ham's F-12 medium plus 10% heart extract and was cultured in monolayers on the circular coverslips 2 to 3 days before fluorescent binding experiments. SH-SY5Y cells were maintained in F-12/Dulbecco's minimal essential medium (1:1) medium plus 10% fetal calf serum and then were plated and allowed to grow on the coverslips overnight before the experiments.

All fluorescent-ligand receptor binding and internalization experiments were observed concurrently during incubation while cells were vital. Before binding experiments cells were washed twice and left in Ham's F-12 medium plus 10% fetal calf serum at room temperature and then observed their autofluorescence to establish an initial emission background level. The medium was then replaced with a solution of fluorescent ligand in Ham's F-12 medium containing bestatin (10 μ M), thiorphan (1 μ M), and 10% fetal calf serum. Cells were incubated for various times at 4°C for binding study and 35°C for internalization study, as noted in the figure legends. Incubation was terminated by removing the medium and washing the cells 5 to 10 times with Ham's F-12 medium plus 10% fetal calf

serum. To determine nonspecific binding of the fluorescent ligands, cells were pretreated with naloxone (10 μ M) and subsequently incubated in a solution of fluorescent ligand plus naloxone (10 μ M).

Confocal Laser Microscopy. Cells were visualized using a krypton-argon laser coupled with a Bio-Rad MRC-1000. The confocal head was attached to an Optiphot II Nikon microscope with a Plan Apo 40 \times objective lens (1.4 NA) or a Plan Apo 60 \times objective lens (1.4 NA). The filters used for scanning A488 were 488 nm for excitation, and 522 nm for emission. For BTR fluorophore, the filter set was 568 nm for excitation and 585 nm for emission. Acquisition parameters were four frames per image, 30% laser power, Kalman filter, motor step size 0.5 μ m, and iris 3.0 (\sim 0.5 μ m). Images were processed in Adobe Photoshop 4.0 (Adobe Systems, Mountain View, CA).

Results

Conjugation of Fluorescent Dyes to Opioid Peptides

The opioid peptides [Lys⁷]dermorphin, deltorphin I, TIPP, and endomorphin I were chosen for this study because of their high potency and receptor subtype selectivity. Each peptide was modified by adding a cysteine residue to the C terminus. The thiol group on the side chain of this residue was the target site of the conjugation reaction. Thiol-reactive dyes selectively reacted with this modified C terminus. To minimize interference of the bulky fluorescence moiety with the binding properties of the peptides, BTR iodoacetamide and A488 maleimide with an extended arm linkage between the fluorescent moiety and the site of attachment of $n = 11$ and $n = 8$, respectively, were used. Conjugation was carried out in a nonaqueous system of DMSO and was initiated by adding BTR- or A488-reactive dyes to the peptide solution. The reaction was monitored from 1 to 4 h by analytical HPLC equipped with a photodiode array detector. Generally, the reaction was completed within 1 h. After conjugation with BTR, analytical reversed phase HPLC of the crude mixture demonstrated two peaks when monitored at 590 nm. The first peak corresponded to the fluorescent conjugated peptide, whereas the other was the unreacted dye. The conjugation of A488 to the peptides resulted in a pair of product peaks because A488 maleimide exists as two isomers.

Dye-conjugated peptides were further purified by reversed phase HPLC to a purity of $\geq 95\%$ with a yield of 30 to 50%. FAB mass spectrometry and molecular mass ($M+H$) analysis of the products further confirmed the chemical structure of the conjugated peptides (Table 1).

Spectrofluorometric Properties

Emission spectra of the conjugated peptides were prepared to determine possible changes in the spectrofluorometric properties of the fluorescent moiety due to attachment to the peptide. No shift in the emission spectra was observed, however, the emission intensity of the conjugated peptides was decreased in all cases. In addition, the emission intensity varied among the different conjugates. For example, the fluorescent emission of dermorphin-Alexa 488 (DERM-A488) was 2-fold lower than that of deltorphin-Alexa 488 (DELT-A488; Fig. 1A).

Interestingly, the fluorescence emission from DERM-BTR was pH-sensitive (Fig. 1B). DERM-BTR fluorescence increased as the pH decreased, suggesting the potential of additional uses for this fluorescent peptide. Conversely, the fluorescence intensity of DERM-A488 and DELT-A488 was slightly decreased as the pH of the solution was decreased.

DERM-A488 fluorescence emission was less sensitive to pH, compared with the free dye or DELT-A488.

Binding Affinity and Selectivity

Standard radioligand binding assays were used to assess the affinity and selectivity (μ/δ -affinity constant ratio) of the fluorescent opioid peptides. The data, summarized in Table 2, show that dermorphin-BODIPY TR (DERM-BTR) and DERM-A488 exhibited high binding affinity and moderate subtype selectivity for μ - over δ -opioid receptors. DERM-BTR and DERM-A488 bound μ -opioid receptors with a K_i of 2.52 and 2.34 nM, respectively (Table 2). These K_i values were about 20-fold higher than that of [Lys⁷]dermorphin. The conjugated μ -ligands displayed lower K_i values for δ -opioid receptors (2.3- and 7-fold decrease) compared with [Lys⁷]dermorphin. Thus, both fluorescent dermorphins exhibited a decreased selectivity for μ - over δ -receptors.

Deltorphan-BODIPY TR (DELT-BTR) exhibited high affinity binding to δ -opioid receptors with a K_i value of 3.08 nM, an increase of 14-fold compared with native deltorphin I. Interestingly, the binding of DELT-BTR at μ -receptors was considerably improved ($K_i = 49.3$ nM) compared with native deltorphin I ($K_i > 1$ μ M). Thus, DELT-BTR retained DELT-like affinity but lost its selectivity for δ -receptors. When this peptide was conjugated to A488, its K_i for δ -receptors was increased to 78 nM, a value 350-fold greater than unconjugated deltorphin I. DELT-A488 did, however, retain some selectivity for δ over μ because its binding affinity for μ -receptor was still extremely poor ($K_i > 10,000$; Table 2).

The conjugation of A488 to the shorter peptide TIPP resulted in a 250-fold decrease in this peptide's affinity for δ -receptors. Furthermore, this fluorescent antagonist exhibited a higher binding affinity at μ -receptors compared with the unconjugated TIPP. However, it still retained selectivity for δ - over μ -receptors.

In contrast to native endomorphin, endomorphin-A488 displayed very low binding affinities for both μ - and δ -opioid

receptors heterologously expressed in CHO cells with K_i values greater than 1 μ M. Thus, modifying endomorphin with A488 destroyed the peptide's affinity and selectivity for μ - and δ -opioid receptors.

Biological Activity

To determine whether the fluorescent opioid peptides retained their ability to activate functionally coupled μ - and δ -opioid receptors to physiological responses, membrane properties were evaluated in tissue slices prepared from the LC that express μ -opioid receptors and cultured NG108-15 cells that express δ -opioid receptors.

Activation of μ -opioid receptors in LC neurons increases a potassium conductance that hyperpolarizes the membrane potential of these cells (Williams et al., 1982). In this study, both the outward current and the hyperpolarization induced by the fluorescent dermorphins and [Met⁵]-enkephalin (ME) were tested. The outward current evoked by different concentration of DERM-A488 was recorded and normalized to the current induced by a saturating concentration of ME (10 μ M; current mean amplitude = 128 ± 11 pA; $n = 21$) (Fig. 2A). The experimental data were plotted in a dose-response curve and fitted to a logistic equation (Fig. 2C). The estimated EC_{50} value was 29.5 ± 0.5 nM and the estimated maximum DERM-A488 effect was $100.2 \pm 0.6\%$ ($R^2 = 0.999$). These experiments showed that DERM-A488 activates μ -opioid receptors in a concentration-dependent manner and it does behave as a full agonist at these receptors.

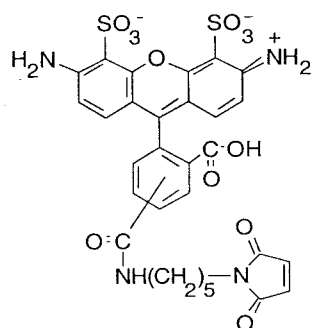
The voltage dependence of the DERM-A488-activated current is shown in Fig. 2B. The net current induced by DERM-A488 flowed outwards when the cell was held at -60 mV and it reversed at the estimated equilibrium potential for potassium in this condition (-110 mV). A similar relationship was observed for the ME-induced current (data not shown), as well as the characteristic inward rectification displayed by opioid activated potassium current (Williams et al., 1988).

The effect of high concentrations of DERM-BTR was tested

TABLE 1
Molecular structure of fluorescent peptide conjugates and their molecular weights

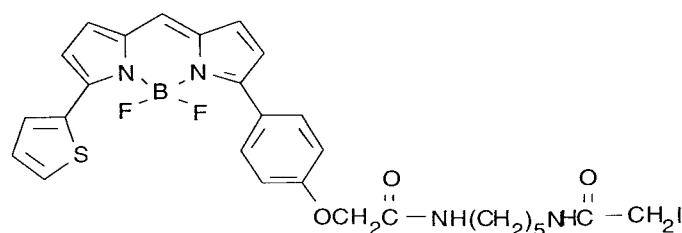
Fluorescent Ligands	Structure	M + H (calculated)	M + H (found)
Dermorphin-Alexa 488	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-Cys(R ¹)-NH ₂	1668 (+Na)	1669 (+Na)
Dermorphin-BODIPYTR	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Lys-Cys(R ²)-NH ₂	1495	1496
Deltorphan-Alexa 488	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-Cys(R ¹)-NH ₂	1592 (+Na)	1593 (+Na)
Deltorphan-BODIPYTR	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-Cys(R ²)-NH ₂	1419	1420
TIPP-Alexa 488	Tyr-Tic-Phe-Phe-Cys(R ¹)-NH ₂	1457 (+Na)	1458 (+Na)
Endomorphin-Alexa 488	Tyr-Pro-Trp-Phe-Cys(R ¹)-NH ₂	1412	1413

R¹ =



Na⁺

R² =



and compared with the maximal effects achieved by DERM-A488, ME, and dermorphin on the membrane potential of LC neurons. In horizontal brain slices, a maximal hyperpolarization of ~ 20 mV was reached by dermorphin (300 nM) and ME (10 μ M) (Fig. 3A, upper traces, and B). Both DERM-BTR and DERM-A488 at a concentration of 1 μ M hyperpolarized the membrane potential of LC neurons by 15.5 ± 1.2 and 16.0 ± 3.7 mV, respectively (Fig. 3B, $n = 5$). The effect of ME on the membrane potential was completely reversible upon washing, whereas the dermorphin-induced hyperpolarization was washed out very slowly. Similar results have been observed with another metabolically stable opioid agonist DAMGO (data not shown). In all cases, naloxone (10 μ M) reversed the effect of the dye-conjugated and nonconjugated agonists, demonstrating that the hyperpolarization was mediated via opioid receptors.

The ability of DELT-A488 to inhibit calcium channels through the activation of δ -opioid receptors was tested on differentiated NG108-15 cells. DELT-A488 (1 μ M) inhibited $35 \pm 6\%$ of the barium current, whereas a maximal concentration of ME (10 μ M) inhibited $53 \pm 7\%$ of the current (Fig. 4B). The inhibition was reversed after washing DELT-A488 and it was blocked by naloxone (1 μ M, Fig. 4B), indicating that the inhibition was opioid receptor mediated.

Visualization of Binding and Internalization

CHO Cells. Confocal microscopy was used to study equilibrium binding of the fluorescent opioid peptides to heterologously expressed μ - and δ -opioid receptors in the membrane of transfected CHO cells. At a concentration 10 to 30 times the K_i value DELT-BTR (100 nM) and DERM-BTR (25 nM) selectively stained the membrane surface of CHO cells heterologously expressing δ - and μ -receptors, respectively (Fig. 5, B and E). Binding was observed within 1 min after the incubation began and was completely prevented in the presence of the opioid antagonist naloxone (10 μ M). At 35°C fluorescent puncta became distinguishable in the cytoplasm during the first 3 to 5 min of incubation. Intracellular fluorescence was more clearly visible after the fluorescent peptide was washed from the extracellular medium (Fig. 5, C and F). The fluorescent puncta were initially distributed along the intracellular surface of the plasma membrane and after 10 to 15 min became clustered in the perinuclear region where they remained for the duration of the experiment (60 min).

Interestingly, when CHO cells heterologously expressing δ -opioid receptors were initially incubated with DELT-A488 (700 nM, 5 min, at 35°C), the green fluorescence labeling of the plasma membrane, as well as the distribution of intracellular fluorescence, was similar to what was observed with the red agonist DELT-BTR (Figs. 5, E and F, and 6B). Indeed, after washing the fluorescent conjugated peptide for 10 min, a dense green fluorescence was observed in the perinuclear region (Fig. 6B). If DELT-BTR (100 nM) was then added to the same cells and incubated for another 5 min (at 35°C) the red-fluorescent puncta was initially distributed in the cytoplasm but near the plasma membrane and then gradually was observed to merge with the perinuclear green fluorescence within 10 to 15 min (Fig. 6, C and D), giving a yellow color.

Similar experiments were performed using CHO cells that heterologously expressed μ -opioid receptors, however, no co-

localization of the fluorescent agonists was seen in the perinuclear region. Although DERM-A488 stained the plasma membrane of μ -receptor-expressing cells, fluorescent intracellular puncta were rarely observed after a 10-min incubation (Fig. 7, B and C). Even after prolonged incubation time (up to 25 min) only weak perinuclear staining was observed with this ligand (data not shown). Subsequent incubation of the same cells with DERM-BTR produced a dense and bright accumulation of fluorescence at the perinuclear region, demonstrating that the cells were capable of internalization (Fig. 7D).

The binding and internalization of these fluorescent li-

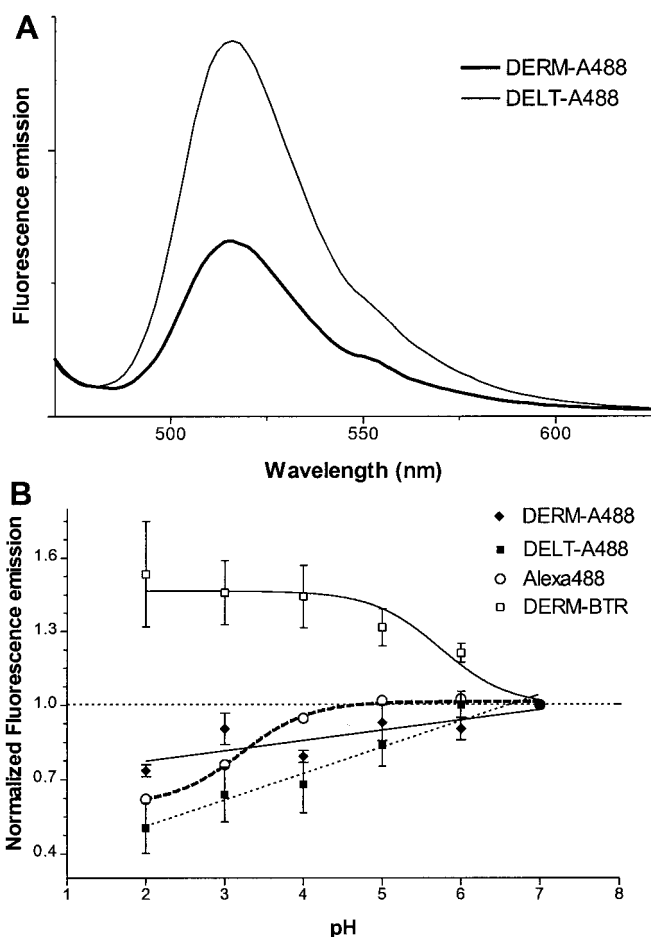


Fig. 1. A, emission spectra of DERM-A488 and DELT-A488. Each peptide was tested in a 1 μ M solution at pH 7. Triplicate measurements of the emission spectra were taken using an excitation wavelength of 450 nm. B, pH sensitivity of the fluorescence emission. The fluorescent emission of the fluorescence-conjugated peptides and the fluorescent dye were examined at different pHs. Three to five measurements were determined at each pH and each fluorescent emission value was normalized to the emission obtained at pH 7. Symbols and bars represent the average and standard error of the normalized measurements. Data were fit to a sigmoidal equation, for DERM-BTR and A488; and to a linear regression, in the case of DERM-A488 and DELT-A488. The sigmoidal fit for DERM-BTR was used to determine that the fluorescence emission increased from 1 ± 0.13 to 1.46 ± 0.06 when the pH was changed from 7 to 4. The half-maximum increase (23%) was observed at $\text{pH } 5.7 \pm 0.6$. The correlation coefficient for the fit (R^2) was 0.33, $n = 5$. For Alexa 488, the fluorescence emission changed from 1 ± 0.01 to 0.59 ± 0.01 . At $\text{pH } 3.2 \pm 0.05$ the A488 fluorescence decreased 20% (half of the maximum change). The R^2 value for this fit was 0.98, $n = 5$. The fluorescence emission of DERM-A488 and DELT-A488 was insensitive to pH (DERM-A488, slope = 0.04 ± 0.01 , y-intercept = 0.69 ± 0.06 , $R^2 = 0.43$, $n = 3$; and DELT-A488: slope = 0.1 ± 0.01 , y-intercept = 0.30 ± 0.09 , $R^2 = 0.57$, $n = 4$).

TABLE 2
Binding affinity and selectivity of the fluorescent peptide conjugates at μ - and δ -opioid receptors.

Fluorescent Ligands	K_i^a		K_i ratio (μ/δ)
	μ	δ	
	nM		
[LYS] ⁷ Dermorphin ^b	0.12 \pm 0.01	1,105 \pm 194	0.0001
Dermorphin-Alexa 488	2.34 (0.767–7.15)	420 (175–1010)	0.00557
Dermorphin-BODIPY TR	2.52 (0.983–6.46)	143 (99.5–207)	0.0176
Deltorphan I ^b	1,985 \pm 224	0.22 \pm 0.05	9,000
Deltorphan-Alexa 488	>10,000	78.0 (52.6–116)	>128
Deltorphan-BODIPY TR	49.3 (35.1–69.3)	3.08 (1.85–5.12)	16
TIPP ^c	>10,000	0.48 \pm 0.07	>20,000
TIPP-Alexa 488	>10,000	119 (67.6–209)	84
Endomorphin I ^d	0.36 \pm 0.08	1,506 \pm 174	0.0002
Endomorphin-Alexa 488	>1000	>1000	1

^a 95% confidence intervals are shown in parentheses, $n = 2$.

^b Data from Erspamer, 1992.

^c Data from Tourwe et al., 1998.

^d Data from Zadina et al., 1997.

gands were naloxone-sensitive. Coincubation of naloxone (10 μ M) and DERM-BTR blocked membrane and intracellular fluorescent staining (Fig. 8A). Similar results were obtained with DERM-A488, DELT-BTR, and DELT-A488.

Both binding and internalization of the fluorescent peptides were also sensitive to coincubation with unconjugated opioid agonists. DAMGO (10 μ M) prevented binding and internalization of both A488- and BTR-conjugated dermorphin ($n = 2$, data not shown). In addition, etorphine (1 μ M) blocked both A488 and BTR deltorphin binding and internalization ($n = 2$, data not shown).

The temperature dependence of the binding and the internalization process were also studied. At low temperatures (4–8°C) the fluorescent agonists bound the plasma membrane of CHO cells expressing μ - or δ -opioid receptors but intracellular fluorescent puncta were not observed, even after a prolonged incubation (30 min) with the fluorescent agonists (Fig. 8C). Raising the temperature to 32–35°C increased the rate of binding equilibrium and internalization was seen within 3 to 5 min after the incubation was initiated (Fig. 8D).

Depending on the conjugated peptide, different amounts of background fluorescence and nonspecific staining were observed. In general the BTR peptides were more difficult to wash from the cells than the A488 peptides. They also tended to adhere to cellular debris and dead cells that were present in the cultures.

As expected, internalization was only induced by opioid agonists. The incubation of CHO- δ cells with the fluorescent δ -opioid antagonist TIPP-A488 (200 nM or 1 μ M) resulted in plasma membrane staining with no subsequent intracellular fluorescent puncta observed (Fig. 9, A and B). Subsequent incubation of the same cells with the agonist DELT-BTR demonstrated that the cells were capable of internalization (Fig. 9, C and D). These experiments also suggested that receptor activation was required for the internalization process.

Neuroblastoma Cells. Binding to receptor and internalization of these fluorescent opioid ligands were also studied in neuroblastoma cell lines. The NG108-15 cell line expresses mouse δ -opioid receptors. When incubated with DELT-A488 (700 nM), the fluorescence associated with the plasma membrane of these cells was not as intense as that observed on δ -CHO cells (Fig. 10A). However, fluorescent intracellular

puncta were clearly observed after a 20-min incubation and were distributed evenly throughout the cytoplasm with no distinct perinuclear accumulation (Fig. 10B). Similar results were observed for the SH-SY5Y cell line that predominantly expresses the human μ -receptor subtype. Incubation with 25 nM DERM-BTR (10-fold K_i) resulted in weak but clear staining of the membrane surface of these cells, whereas increasing the concentration to 150 nM (60-fold K_i) improved visualization of both membrane binding and internalization (Fig. 10, C and D). After 20 to 25 min of incubation, internalization had taken place with no perinuclear accumulation (Fig. 10D). In both cell lines, the binding of DELT-A488 and DERM-BTR was inhibited by naloxone (data not shown).

Discussion

Fluorescent peptide ligands, in conjunction with confocal microscopy, have been used previously to study binding and internalization of G protein-coupled receptors (Beaudet et al., 1998). In the specific case of the opioid receptors the development of receptor antibodies, Flag-tagged antibodies for epitope-tagged receptors and fluorescent peptides have been reported (Kolb et al., 1983; Arden et al., 1995; Keith et al. 1996; Sternini et al., 1996; Gaudriault et al., 1997; Whistler et al., 1999). However, until now these visualization studies were performed on fixed cells, and thus the real-time dynamics of binding and internalization could not be explored. The goal of this study was, therefore, to develop fluorescent opioid peptides that could be used to probe for ligand-receptor trafficking in living cells.

The conjugation of nonpeptide moieties to peptides is usually established via the formation of an amide bond at either the N terminus or the side chain of a lysine residue. For opioid peptides it has been well documented that the N-terminal region is necessary for receptor recognition and activation. Therefore, to minimize the loss of biological activity, the conjugation of some fluorescent dyes was attempted at the C terminus. In the past, modification of C-terminal residues was accomplished by adding a lysine or an amino-alkyl linker and this extended amine became the site of conjugation for amine-reactive dyes (i.e., isothiocyanate or succinimidyl ester) (Kolb et al., 1983; Goldstein et al., 1988; Gaudriault et al., 1997). Unfortunately, the amine-reactive dyes can also conjugate to the peptide's N terminus or any

other free amine in the peptide sequence. This can result in a mixture of fluorescent products requiring extensive post-synthesis purification (Gaudriault et al., 1997). In contrast, the peptides in this study were modified by the addition of a C-terminal cysteine residue such that the conjugation event selectively occurs between this amino acid side chain and the thiol-reactive dyes (i.e., BTR iodoacetamide or A488 maleimide). Adopting this strategy considerably simplified the purification and identification processes.

The conjugation of a fluorophore to the opioid peptides resulted in some changes in the pharmacological and spectrofluorometric properties of the products. BTR-conjugated dermorphin and deltorphin retained their affinities for μ -

and δ -opioid receptors, respectively; however, they both displayed a reduction in receptor subtype selectivity. Similar results have been reported for other BODIPY analogs conjugated to dermorphin via an amide bond (Gaudriault et al., 1997), suggesting that this effect was not a consequence of exploiting the thioether linkage. When the hydrophilic fluorophore A488 was conjugated to the opioid peptide dermorphin and deltorphin, both retained their high affinity and receptor subtype selectivity. Nonspecific staining was also lower with A488-conjugated peptides compared with BTR peptides, suggesting that the hydrophilic properties of the fluorescent dyes can significantly influence the pharmacological properties of the conjugated opioid peptides.

With respect to the physicochemical properties of the fluorescent peptide conjugate, it has been reported that BODIPY dyes are pH-insensitive and therefore useful over a wide range of physiological pHs (Karolin et al., 1994). In this study, the fluorescent emission of DERM-BTR increased as the pH value decreased from 7 to 4. This property of DERM-BTR makes it a potentially useful probe of receptor trafficking through acidic compartments. In contrast, DERM-A488 and DELT-A488 became less sensitive to pH compared with the free dye. Because the fluorescent emissions of both con-

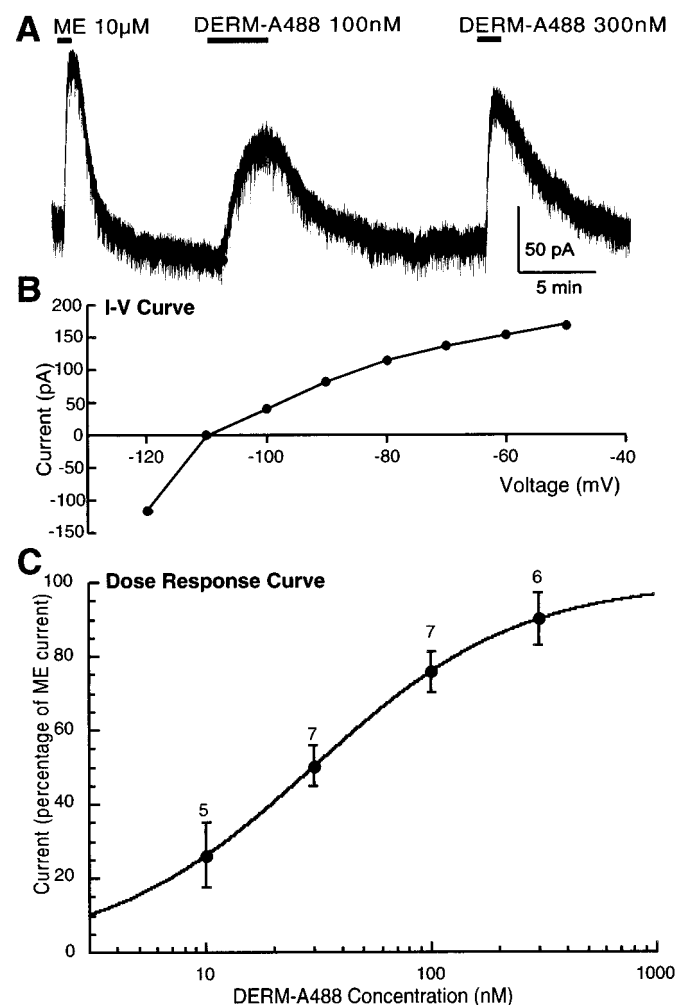


Fig. 2. DERM-A488 activates μ -opioid receptors and increases potassium conductance. **A**, representative recordings from a voltage-clamp experiment. The currents were induced by ME (10 μ M), DERM-A488 (100 nM), and DERM-A488 (300 nM) in an LC neuron at holding potential of -60 mV. **B**, voltage dependence of the DERM-A488-activated current. The symbols (●) represent the net current induced by 300 nM DERM-A488 at each voltage step (100-ms steps from -50 to 120 mV in -10-mV increments). In each case, the net current was obtained by subtracting the total current recorded in the absence of DERM-A488 to the current recorded during DERM-A488 perfusion. **C**, dose-response curve for DERM-A488. Symbols (●) and bars represent the mean and standard error for the effect of DERM-A488 on LC neurons expressed as a percentage of the maximal effect induced by 10 μ M ME. The numbers on top of each symbol represent the number of recorded cells at each concentration. The solid line is the curve that fits the experimental data. The estimated parameters were $n = 0.94 \pm 0.012$, $EC_{50} = 29.8 \pm 0.5$ nM, maximum = 100.2 ± 0.6 ; and $R^2 = 0.999$.

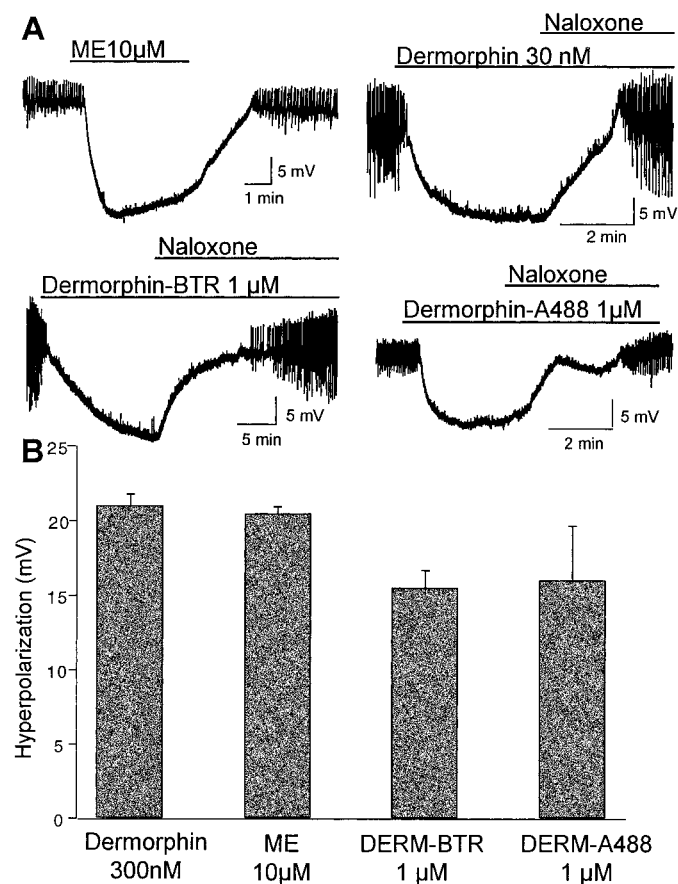


Fig. 3. Biological activity of BTR- and A488-dermorphin conjugates. **A**, intracellular recordings of the membrane potential of LC neurons in horizontal slices of brainstem. The opioid peptides were perfused in the presence of peptidase inhibitors bestatin (10 μ M) and thiorphan (2 μ M). Naloxone (10 μ M) was perfused to reverse opioid receptor-mediated hyperpolarizations. **B**, summary of the results comparing the amplitude of the hyperpolarizations caused by each peptide agonist. Columns and bars represent the mean hyperpolarization and standard error, respectively. $n = 3-5$.

jugates showed a linear relationship as a function of pH, this phenomenon is not due to the protonation of the fluorescent moiety but rather to the influence of physicochemical properties of the peptides.

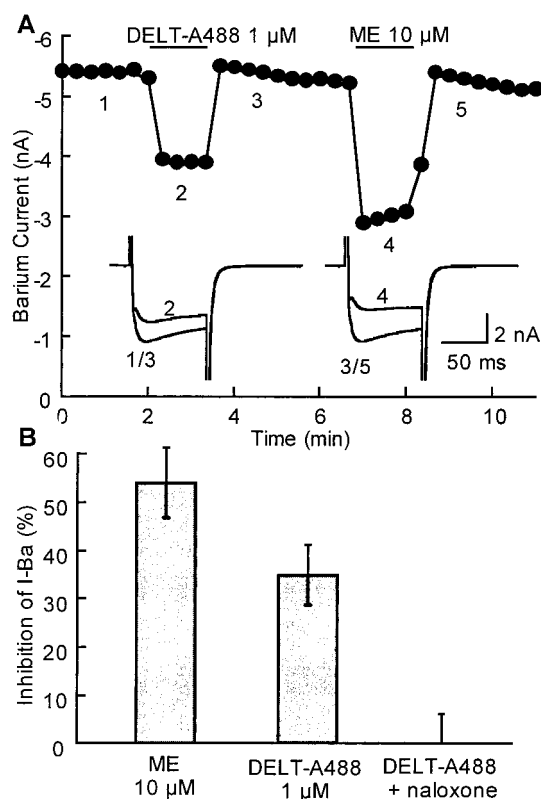


Fig. 4. Biological activity of deltorphin conjugate. A, ME (10 μ M) and DELT-A488 (1 μ M) inhibited the barium currents (I-Ba) through voltage-gated calcium channels recorded in differentiated NG108-15 cells in culture. Barium currents were measured every 20 s by stepping to 0 mV from a holding potential of -60 mV. B, summarized data of barium current inhibition (mean and standard errors) exerted by ME, DELT-A488, and DELT-A488 + naloxone ($n = 5-8$).

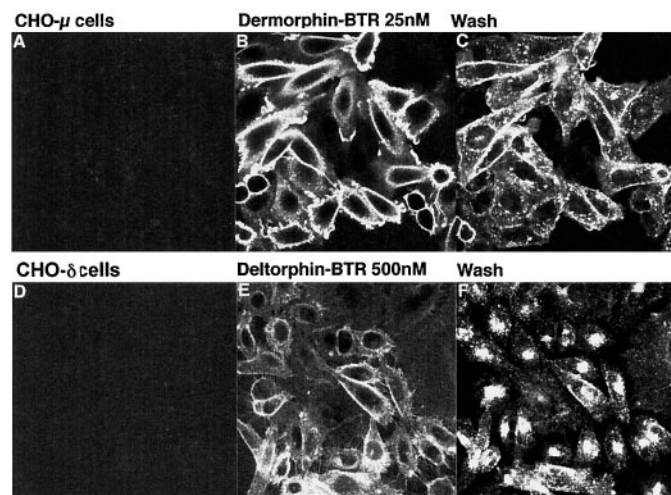


Fig. 5. Binding and internalization of BTR conjugates. Top, representative confocal images of CHO- μ cells obtained before incubation with DERM-BTR (25 nM) (A), during the 5-min incubation (B), and after washing (C). Bottom, representative confocal images of CHO- δ obtained before incubation with DELT-BTR (100 nM) for 5 min at room temperature (D), during the incubation (E), and after washing (F).

The retention of biological activity was a key objective as we designed and evaluated these pharmacological probes. Electrophysiological studies were chosen to evaluate functionality because with this technique dynamic physiological responses can be followed in real-time and eventually, when combined with confocal microscopy, could allow a direct correlation between desensitization and internalization of opioid ligand-receptor complexes. Both DERM-BTR and DERM-A488 activated μ -opioid receptors and hyperpolarized the membrane potential of LC neurons in brain slice preparations. Although their potencies might be slightly reduced compared with native dermorphin (Chiu et al., 1990), both were capable of causing a maximal effect. These conjugated peptides are also metabolically stable due to the inclusion D-amino acids and the C-terminal amidation. The long half-life of dermorphin, DERM-BTR, and DERM-A488 probably accounts for the prolonged washout time compared with ME. Furthermore, the hydrophobicity of the fluorophores themselves probably contributes to the prolonged washout times observed. Similarly, the conjugation of deltorphin to A488 did not disrupt the ability of this ligand to activate δ -receptors expressed in NG108-15 cells. DELT-A488 potentially inhibited calcium currents with an equal efficacy to ME and its response was also reversed by naloxone.

Real-time visualization of binding and internalization of the fluorescent peptide conjugates was successfully achieved in CHO cells as well as neuroblastoma cells. Binding to the plasma membrane and subsequent internalization were clearly observed in CHO- μ or - δ cells and occurred from the

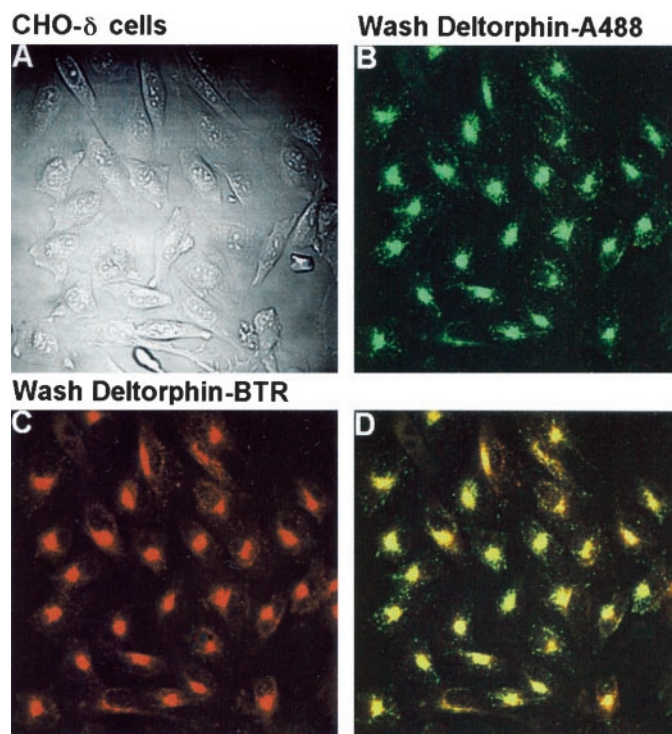


Fig. 6. Colocalization of DELT-A488 and DELT-BTR intracellular fluorescence. A, bright field image of the CHO- δ cells in culture. B, intracellular green fluorescence is revealed after 5-min incubation of DELT-A488 (700 nM) at room temperature. C, subsequent incubation of the same cells with DELT-BTR (100 nM), 5 min at room temperature, revealed intracellular red fluorescence. D, superimposed B and C images reveal in yellow the degree of colocalization of the intracellular green and red fluorescence.

onset of incubation. When NG108-15 or SH-SY5Y cells were used, the fluorescent staining of the plasma membrane was not as intense as in CHO cells. One explanation for this observation is that there may be significantly fewer receptors on the surface of neuroblastoma cells compared with the transfected CHO cells. Interestingly, different patterns of fluorescent intracellular distribution were also observed between CHO- μ or - δ and neuroblastoma (NG108-15 and SH-SY5Y) cells. In CHO- μ or - δ cells the fluorescent puncta were concentrated in the perinuclear region, whereas in NG108-15 and SH-SY5Y cells these puncta were patchy throughout the cytoplasm. This different distribution may be due to trafficking characteristics that could vary between CHO cells, which are epithelial, and neuroblastomas, which display more neuronal characteristics. Therefore, cell type-specific differences must be considered when designing and interpreting any trafficking study in tissue culture cells.

Besides the inherent differences between cell lines, it is well documented that trafficking is influenced by the choice of ligand. Interestingly, in the current study, we found that even the same ligand (dermorphin) can display very different properties depending on the fluorophore to which it is conjugated. A488 is a negatively charged hydrophilic molecule, whereas BTR is an uncharged, more hydrophobic fluorophore. Both DERM-A488 and DERM-BTR bound and activated μ -opioid receptors heterologously expressed on the surface of CHO cells (Table 2; Figs. 2, 3, 5, and 7) with high affinity and in a naloxone-reversible manner. However, by the time the punctate fluorescent emissions from intracellular DERM-BTR were detected, the fluorescence from DERM-A488 was much less apparent (Fig. 7, C and D).

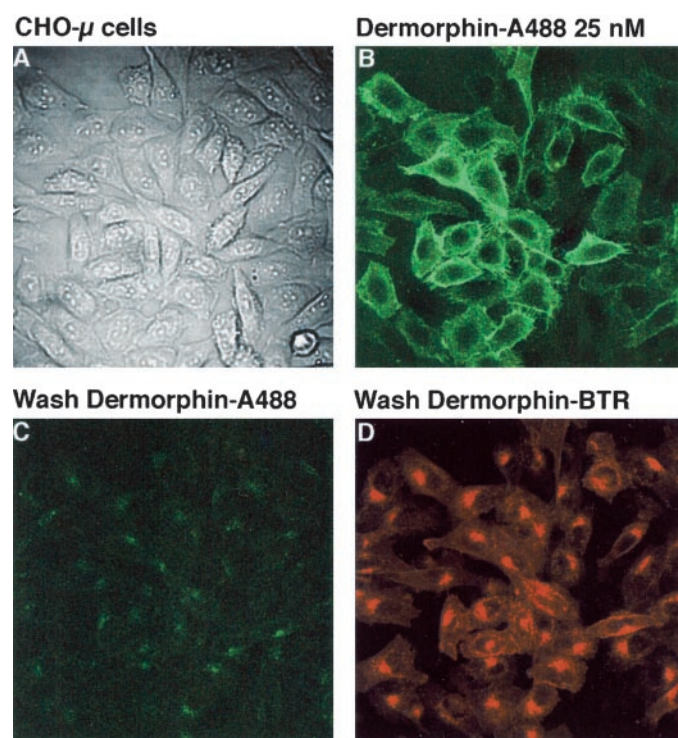


Fig. 7. A, bright field image of the CHO- μ cells in culture. B, green fluorescence is mainly localized to the plasma membrane after 2-min incubation with DERM-A488 (25 nM) at room temperature. C, no intracellular fluorescence after washing a DERM-A488 (25 nM). D, subsequent incubation of the same cells with DERM-BTR (25 nM) for 5 min reveals red intracellular fluorescence after wash.

This observation can be interpreted in at least two ways. Either the DERM-A488 ligand-receptor complex is being internalized and its fluorescence is being quenched or the DERM-A488- μ -opioid receptor complex is not being trafficked in the same way as the DERM-BTR- μ -opioid receptor complex. Our data (Fig. 1B) suggested that the quenching of the DERM-A488 is not due to a low pH environment, such as would be encountered in acidic endosomes. Furthermore, in CHO cells heterologously expressing the rat δ -receptor intracellular DELT-A488 gave bright fluorescent emissions (Fig. 6). Another possibility we considered is that the local environment of the ligand within the ligand-receptor complex may have been altered during internalization such that emissions from A488 were quenched. If this were the case one would expect that after DERM-A488 dissociated from the μ -receptor the fluorescent emission of A488 would resume. However, this was not observed even 1 h after the excess DERM-A488 had been removed from the medium.

Given the lack of data supporting the quenching hypothesis it may be worth reconsidering the possibility that DERM-A488, unlike DERM-BTR, fails to, or has a significantly reduced ability to trigger μ -receptor internalization. In this way, DERM-A488 may be acting like morphine, a μ -opioid receptor agonist, that can couple the μ -opioid receptor to various physiological processes but cannot rapidly induce its internalization (Keith et al., 1996; Sternini et al., 1996; Whistler et al., 1999). However, an important distinction between the two is that DERM-A488 consists of a pharmacophore (dermorphin) that is expected to directly interact with the

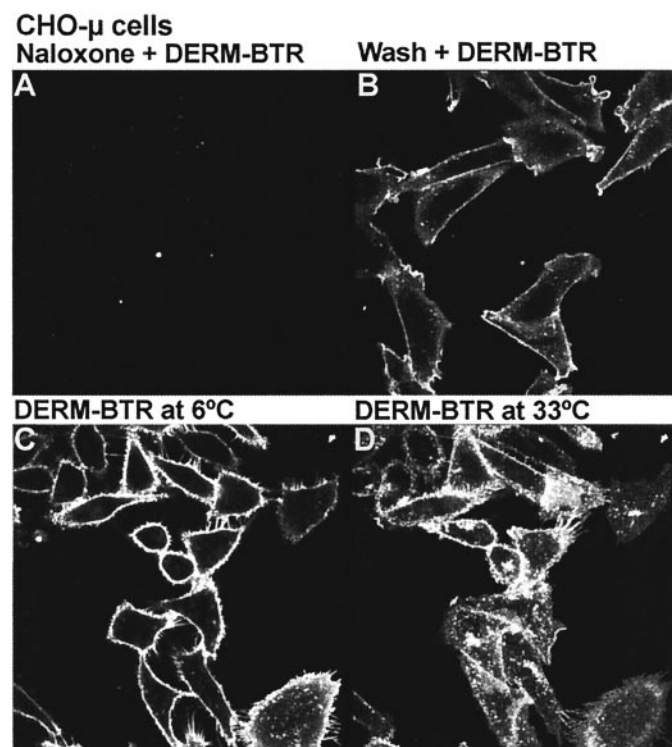


Fig. 8. Specificity and temperature dependence of the internalization process. A, confocal image obtained after washing CHO- μ cells coincubated with naloxone (10 μ M) and DERM-BTR (25 nM). B, subsequent incubation of the same cells with DERM-BTR (25 nM) in the absence of naloxone. C, incubation of CHO- μ cells with DERM-BTR (25 nM) at 6°C during 15 min. D, the same cells after raising the temperature to 33°C for 5 min.

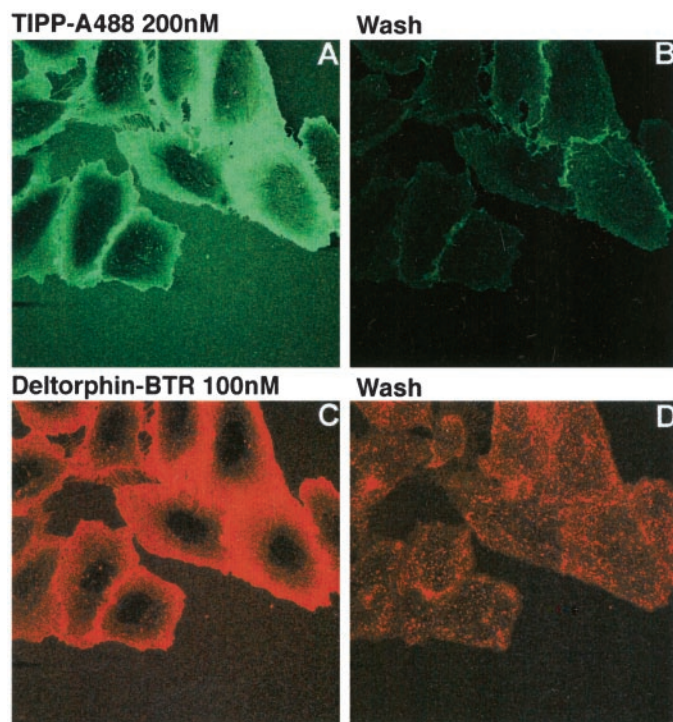


Fig. 9. Binding and internalization properties of receptor antagonist TIPP-A488. A, incubation of δ -CHO cells with 200 nM TIPP-A488 at room temperature reveals plasma membrane staining. B, after washing TIPP-A488 no intracellular fluorescence is observed. C, subsequent incubation of the same cells with the receptor agonist DELT-BTR (100 nM) stains the plasma membrane. D, after washing DELT-BTR, intracellular red fluorescence is observed.

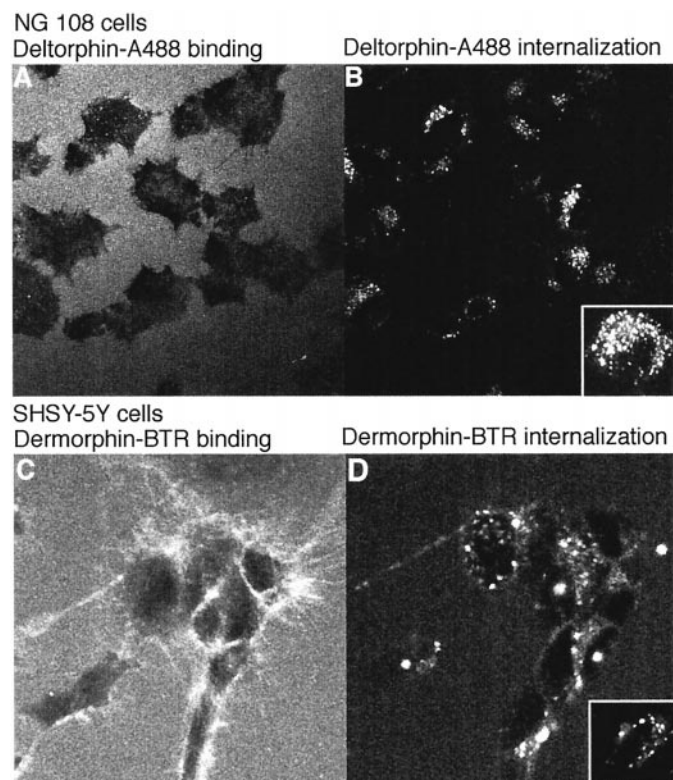


Fig. 10. Binding and internalization of opioid peptide conjugates in NG108-15 and SH-SY5Y cells in cultures. A, incubation of NG108-15 cells with DELT-BTR (700 nM) for 10 min at 33°C. B, wash DELT-A488 after 20-min incubation. C, incubation of SH-SY5Y cells with DERM-BTR (160 nM) for 10 min at 33°C. D, wash DERM-BTR after 20-min incubation.

receptor and a fluorophore (A488) that does not. Although we do not have a molecular explanation for how the fluorophore A488 could prevent the internalization of the DERM-A488-receptor complex, it may be worth noting that A488 is more hydrophilic than BTR, due in part to the two negative charges ($-\text{SO}_3^-$) it carries at physiological pH. Therefore, a detailed structure-activity analysis of A488 and DERM-A488 should provide some insight into whether these charges play a role in preventing internalization of the DERM-A488-receptor complex. If it can be convincingly demonstrated that DERM-A488 fails to cause the internalization of the μ -opioid receptor, this unique fluorescent agonist may be of potential use in probing the dynamic relationship between the molecular determinants on the ligand and receptor that mediate receptor internalization and perhaps acute desensitization.

In summary, a set of novel fluorescent opioid peptides has been developed and used to follow real-time, ligand-receptor binding and internalization in living cells. These fluorescent probes retained their ability to stimulate opioid activity and may serve as useful tools in studies of ligand-receptor interactions, receptor activation, desensitization, and the development of tolerance.

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