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Europium-Labeled Epidermal Growth Factor and Neurotensin: Novel Probes for Receptor-Binding Studies

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We investigated the possibility of labeling two biologically active peptides, epidermal growth factor (EGF) and neurotensin (NT), with europium (Eu)-diethylenetriaminepentaacetic acid. More specifically, we tested them as probes in studying receptor binding using time-resolved fluorescence of Eu^{3+}. The relatively simple synthesis yields ligands with acceptable binding characteristics similar to isotopically labeled derivatives. The binding affinity (K_d) of labeled Eu-EGF to human A431 epidermal carcinoid cells was 3.6 ± 1.2 nM, similar to the reported K_d values of EGF, whereas the K_d of Eu-NT to human HT29 colon cancer cells (7.4 ± 0.5 nM) or to Chinese hamster ovary (CHO) cells transfected with the high-affinity NT receptor (CHO-NT1) were about 10-fold higher than the K_d values of NT. The bioactivity of the Eu-labeled EGF as determined by stimulation of cultured murine D1 hematopoietic cell proliferation was nearly the same as that obtained with native EGF. The maximal stimulation of Ca^{2+} influx with NT and Eu-NT in CHO-NT1 cells was similar, but the respective $K_{0.5}$ values were 20 pM and 1 nM, corresponding to differences in the binding affinities previously described. The results of these studies indicate that Eu labeling of peptide hormones and growth factor molecules ranging from 10^3 to 10^5 Da can be conveniently accomplished. Importantly, the Eu-labeled products are stable for approximately 2 years and are completely safe for laboratory use compared to the biohazardous radioligands. Thus, Eu-labeled peptides present an attractive alternative for commonly used radiolabeled ligands in biological studies in general and in receptor assays in particular.

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Growth factor and peptide hormone receptors appear on the cell's outer surface in relatively low numbers, usually 10^3–10^6 sites per cell, with binding affinities in the picomolar to nanomolar range. To determine the bioactivity of these receptors, hormone binding or metabolic responses have been widely used in pharmacological, biochemical, and clinical diagnosis. Moreover, the disappearance, overexpression, or malfunctions of these receptors serve as clinical diagnostic tools (1). For instance, overexpression of the epidermal growth factor (EGF)[2] receptor is associated with the prognosis of certain types of breast cancer (2), and the overexpression of neurotensin (NT) receptors has been linked to ductal pancreatic adenocarcinoma and colorectal carcinoma (3, 4).

Receptor binding assays using EGF or NT are commonly performed with labeled ^{125}I or ^{3}H peptides (4, 5) and are considered highly sensitive, permitting the detection of the respective receptors in the femtomolar to picomolar range. However, radioactively labeled ligands have drawbacks including their short shelf life, their high costs, the logistics involved in their handling, and often the need to prepare them shortly before use, as well as the safety hazards involved (6).

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[2] Abbreviations used: EGF, epidermal growth factor; NT, neurotensin; MTT, thiazolyl blue; DTPA, diethylenetriamine-pentaacetic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FU, fluorescence units; TFA, trifluoroacetic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CHO, Chinese hamster ovary; α-MSH, α-melanocyte stimulating hormone.

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To overcome some of these problems, alternative, nonradioactive methods using chemiluminescent or fluorescent markers or enzymes have been employed to label antibodies, hormones, and drugs for immunoassay use. Fluorescent tags using lanthanide chelate (e.g., Eu^{3+}, Sm^{3+}, and Tb^{3+}) labels offer sensitivity comparable to that of radiolabeled ligands. Such lanthanide chelates are exceptionally well-adapted reporter groups for analytical procedures in biomedicine due to their unique fluorescent properties. Among these one can list high quantum yield, extremely large Stoke's shift, narrow emission peaks, and optimal emission and excitation wavelengths (Eu^{3+} 613 nm) for analysis in biological material. The long fluorescent decay time (10–1000 μs) permits time-resolved analysis with high signal-to-noise ratios and high sensitivity. Application of this technique has been adapted to standard microplate readers (7–9). However, the use of lanthanide chelates is not widespread. Thus, drug labeling (e.g., benzodiazepine) (10) and cytokine labeling such as interleukin-8 (11) with Eu^{3+} chelate, and their use in determining of drug-receptor interactions, have seldom been reported.

In this paper we describe EGF and NT labeling with Eu^{3+} chelates and the receptor binding and bioactivity (Ca^{2+} influx and cell proliferation) of the labeled products. Our results indicate that this nonisotopic labeling method is an advantageous alternative for radioactive labeling of these ligands. The sensitivities obtained are similar to those achieved with radioreceptor assays. Moreover, Eu-labeled EGF and NT are stable for over a year with no loss of activity and are safe and inexpensive.

EXPERIMENTAL PROCEDURES

Materials

Enhancement solution for measuring Eu-fluorescence was purchased from Wallac (Turku, Finland). Thiazolyl blue (MTT), human recombinant-EGF, and diethylene triaminepentaacetic acid (DTPA) dianhydride were purchased from Sigma Chemical Co. (St. Louis, MO). NT was obtained from Neosystem (Strasbourg, France). Cell culture media were prepared by the Biological Services Department of the Weizmann Institute and Gibco, France. Europium chloride ($EuCl_3$) was purchased from Aldrich (St. Louis, MO). Fura-2/AM was purchased from Molecular Probes (Interbiotech, France). The labeling reagent *N*-1(*p*-isothiocyanatophenyl)diethylene tri- N^1, N^2, N^3,-tetraacetate chelated with europium (activated Eu-chelate) was kindly provided by Dr. I. Hemmila, Wallac.

Synthesis of Eu-EGF

To a test tube were added 100 μl Na-phosphate (0.1 M, pH 8.5), 50 μl activated Eu-chelate (150 nmol), and 10 μl EGF (100 μg, 17 nmol) and the mixture was incubated overnight at 4°C with constant stirring. To terminate the reaction, 1 μl Tris–Cl (1 M, pH 8.2) was added and the mixture was stirred for 5 min. Next the reaction mixture was loaded and chromatographed on a Bio-Gel P-2 cartridge (11 ml, Bio-Rad) preequilibrated with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and 40 fractions (320 μl) were collected and Eu-fluorescence was determined using a time-resolved fluorimeter (LKB-Wallac, Arcus 1230). Eu-fluorescence eluted in three peaks (fractions 10–15 (11.6%), 19–25 (24.9%), and 25–40 (63.5%)) with a total yield of 70%. Analysis of the fractions by ligand-receptor binding showed Eu-EGF to be present in the first peak only. The two fractions with the highest fluorescence (12 and 13) were used. Repeated chromatography of these fractions under the same conditions recovered the product Eu-EGF with a yield of 70%. Characterization of the Eu-EGF by mass spectrometry using matrix-assisted laser desorption-ionization/time-of-flight Reflex (Bruker Daltonik, Germany) revealed a 1:1 molar ratio of Eu and EGF (experimental mass: Eu-EGF = 6824.3; EGF = 6342.4). The specific activity of the Eu-EGF was estimated to be 8×10^6 fluorescence units (FU)/pmol based on the biological activity of the fraction.

Synthesis of the DTPA-NT Analogue

The DTPA-NT analogue was obtained by reacting DTPA dianhydride with the ε-amino group of Lys^6 of NT (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu) using a procedure similar to that described by Janevik-Ivanovska *et al.* (12). Briefly, 1.8 μmol of NT (220 μl) in Hepes buffer (0.6 M, pH 8.2, filtered through Chelex 100 (Bio-Rad)) was added with stirring to 14.5 μmol of DTPA dianhydride in DMSO (220 μl); the pH was kept at 8.2 until the reaction was completed. After the solvents were evaporated, the crude product was dissolved in water and trifluoroacetic acid (TFA) was added to reach pH 2. Finally the solution was filtered through Chelex 100 (Bio-Rad). The resulting peptide was purified by HPLC C-18 reverse phase chromatography (Nucleosil, Shandon, France) using a linear 15-min gradient (A: H_2O/TFA 0.05%, B: acetonitrile) from 0 to 26% B followed by a linear 20-min gradient from 26 to 30%. The yield of DTPA-NT was 43% with a purity of $\geq$95% as verified by C18 reverse phase chromatography under isocratic conditions (acetonitrile 30%, H_2O TFA 0.05%). The DTPA-NT analogue was further characterized by mass spectrometry using a Nermag R10-10 mass spectrometer with an electrospray ion source equipped with an iron guide (Analytica of Branford), experimental mass: 2048.16, calculated mass: 2048.32.

Preparation of Eu-NT

DTPA-NT (100 μg, 50 nmol) was dissolved in 10 μl of H_2O, and added to 490 μl of citrate buffer (10 mM, pH 5.0) containing freshly dissolved $EuCl_3$ (50 nmol). The reaction mixture was incubated for 1 h at room temperature and neutralized with 5 μl 1 N NaOH. Next, the mixture was loaded on a Sep-Pak C-18 cartridge and washed with 10 ml of H_2O to elute the free Eu. The column was washed with 17.5 ml of 50% MeOH and the Eu-NT was quantitatively eluted as a single peak (fractions 40–45). An additional wash with 100% methanol did not elute any Eu-containing material. The specific activity of this peak was estimated to be 2.5×10^6 FU/pmol or $\sim$1 Eu^{3+} ion per DTPA-NT molecule based on the biological activity of the fraction.

Cell Lines

A431 human epithelial carcinoid cells. A431 cells expressing EGF receptors (5) were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with fetal bovine serum (FBS) (10%), glutamine (2 mM), penicillin (0.06 mg/ml), and streptomycin (0.1 mg/ml).

HT29 human colon carcinoma. HT29 cells expressing high-affinity NT1 receptors (13) were cultured as monolayers in DMEM:F12 (1:1), buffered with Hepes (25 mM, pH 7.4), FBS (10%), glutamine (2 mM), penicillin (0.06 mg/ml), and streptomycin (0.1 mg/ml).

Murine D1 hematopoietic cells. D1 cells expressing EGF receptor (ErbB1, kindly donated by Y. Yarden, Weizmann Institute, Israel) were cultured in suspension as described (14).

Chinese hamster ovary (CHO)-NT1 cells. CHO cells transfected with NT1 receptor were cultured as described earlier (15).

Ligand Binding Assays

Eu-EGF binding. A431 cells (4×10^5 cells/well) cultured in 48-well plates for 48 h were washed once with ice-cold binding medium (DMEM + 0.1% BSA and Hepes 25 mM, pH 7.4). The cells were further incubated for 2 h at 4°C in the same medium with increasing concentrations of Eu-EGF (as indicated in the individual experiments). Binding was terminated by four successive washes with ice-cold binding medium. Enhancement solution (300 μl/well) was then added to the cells and the samples (200 μl) were taken for time-resolved fluorescence determination using an Arcus fluorometer.

Eu-NT binding. HT29 cells (4×10^5 cells/well) cultured in 48-well plates for 48 h were washed once with binding medium (DMEM: F12 + 0.1% BSA and Hepes 25 mM, pH 7.4) and further incubated for 30 min at 37°C in the same medium with increasing concentrations of Eu-NT. All other details were as previously described for binding Eu-EGF to A431 cells.

Calculations. Nonspecific binding was determined for both Eu-EGF and Eu-NT studies in the presence of 1–3 μM unlabeled ligand. The obtained values were subtracted from the total binding values to estimate the net bound ligand. Molar quantities were calculated from the respective specific activities of the relevant Eu-labeled ligands. Experiments in triplicate were repeated at least three times and representative experiments are shown. Dissociation constants were calculated by Scatchard analysis.

^{125}I-NT Binding

^{125}I-NT binding was performed on CHO-NT1 cell membranes as previously described (15). Cell membranes (15–20 mg/ml) were kept frozen at −80°C until used. Binding of ^{125}I-NT was performed with a 10-μg membrane protein/tube in 50 mM Tris–Cl buffer (pH 7.4) containing 5 mM $MgCl_2$, 0.2% BSA, and 0.8 mM 1,10-*o*-phenanthroline, in the presence of 0.5 nM ^{125}I-Tyr^3-NT (2000 Ci/mmol) and increasing concentrations of NT or Eu-NT, for 30 min at room temperature. Bound ^{125}I-NT was determined after filtration (Whatman GFC filters) and washing with 50 mM Tris–Cl containing 0.2% BSA. Nonspecific binding was determined in the presence of 1 μM unlabeled NT. Specific binding was calculated by subtracting nonspecific values. One hundred percent binding was obtained with a tracer alone. The data of the competition, using increasing concentrations of NT or Eu-NT (0.01–500 nM), were analyzed using the EBDA-ligand program to estimate the $K_{0.5}$ for both NT and Eu-NT.

Cell Proliferation Assay for EGF

Murine D1 hematopoietic cells were washed once in RPMI 1640 medium and resuspended to 5×10^5 cells/ml. Portions (100 μl) of the cell suspension were incubated for 24 h in 96-well plates with the indicated concentrations of Eu-EGF or EGF. Cell survival was determined by MTT assay (14). In short, MTT (0.1 mg/ml) was added to the analyzed cells, which were incubated for 2 h at 37°C. The cells were then lysed in acidic isopropyl alcohol and optical density was determined at 570 nm in a microplate reader (Dynex MRX II).

Determination of Intracellular Free Ca^{2+} Concentration

The intracellular Ca^{2+} concentration was measured in response to activation of NT1 receptors using a dynamic imaging microscopy system QuantiCell 700 (Visitech Int. Ltd., UK) with 30–40 CHO-NT1 cells per

field as described earlier (16). The cells were cultured on glass coverslips, washed, and incubated at 37°C for 60 min with PBS–Hepes medium containing 5 μM Fura-2/AM, 5.4 mM KCl, 2 mM Na_2PO_4, 0.8 mM $MgCl_2$, 1.3 mM $CaCl_2$, 20 mM Hepes/Tris (pH 7.4), and 5 mM glucose. Before analysis the cells were washed twice with the same buffer. After background recording for 40 s (20 images), the experiment was initiated by adding 0.001–10 nM of Eu-NT or NT. Fluorescence images were obtained at intervals of 2 s and intracellular Ca^{2+} concentrations were calculated for 200 s from the ratio of the fluorescence intensities at 340 and 380 nm on a pixel basis. The dose–response curve for intracellular calcium concentrations was obtained by integrating the area under the curve (measuring Ca^{2+} transients plotted as a function of time for each field from the addition of the NT or Eu-NT until the end of image recording, 200 s) and averaging the fluorescence from the whole field of cells chosen. The Ca^{2+} stimulation curves were determined for each tested concentration of NT or Eu-NT.

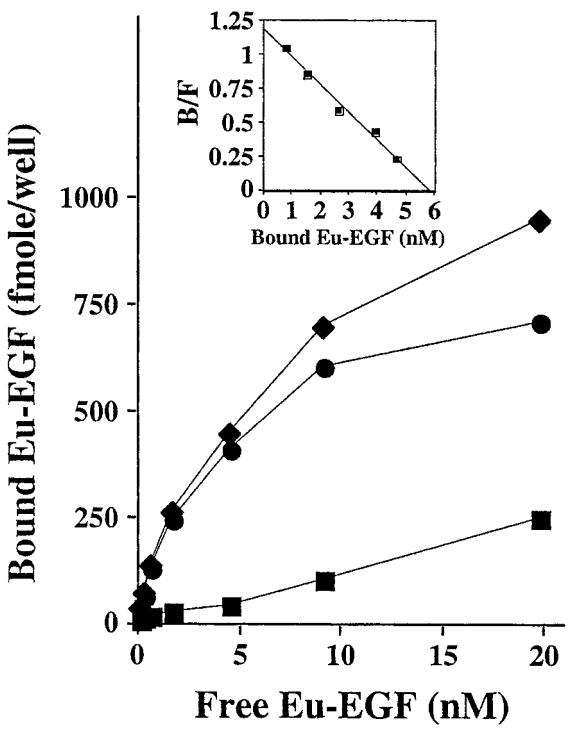

FIG. 1. Binding of Eu-EGF to A431 cells. A431 cells (4×10^5/well) were incubated in the presence of the indicated concentrations of Eu-EGF for 2 h at 4°C and total binding (diamonds) was determined. Nonspecific binding (squares) was determined in the presence of 1 μM unlabeled EGF. Specific binding (circles) was calculated by subtracting nonspecific from total binding. The Scatchard analysis is presented in the inset. All other details are as described under Experimental Procedures.

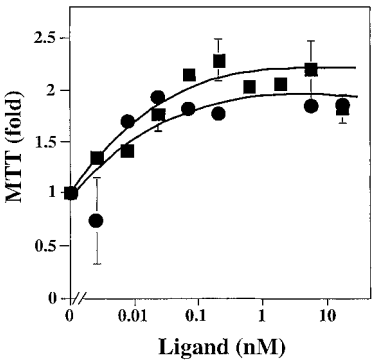

FIG. 2. Stimulation of D1 cell proliferation by Eu-EGF. D1 cells (5×10^4 cells/0.2 ml) were incubated in the presence of the indicated concentrations of Eu-EGF (circles) or EGF (squares). MTT assay was performed 24 h later. Results are expressed as fold stimulation over unstimulated cells. Points represent an average ± SD of triplicates. All other details are as described under Experimental Procedures.

RESULTS

Studies with Eu-EGF

A. Eu-EGF binding to cultured A431 cells. Specific Eu-EGF binding to A431 cells was determined (Fig. 1). Binding of Eu-EGF in the nanomolar range was dose-dependent, with nonspecific binding ≤10% up to ligand concentrations of 5 nM, reaching a maximum of <25% of the total binding at 20 nM. The calculated binding constant (K_d) was (nM) = 3.6 ± 1.2 SEM (n = 4) and maximal binding B_{MAX} = (sites/cell) 1.5 ± 0.2 × 10^6 SEM (n = 4).

B. Induction of proliferation response in D1 cells by Eu-EGF. In order to estimate the biological activity of Eu-EGF, its ability to stimulate proliferation of D1 cells was compared to native EGF. To this end, cells were incubated with increasing concentrations of Eu-EGF. It was found that D1 cell proliferation was stimulated in a dose-dependent manner, with a maximal effect at 1 nM (Fig. 2), a response similar to that obtained with native EGF, suggesting that the bioactivity of the Eu-EGF was preserved during the chemical manipulations involved in its preparation.

Studies with Eu-NT

A. Binding of Eu-NT to HT29 cells. Binding of Eu-NT to HT29 cells showed a concentration-dependent increase in the nanomolar range, reaching saturation at approximately 25 nM (Fig. 3). Nonspecific binding representing <10% was determined in the presence of an excess (3 μM) of unlabeled NT. The binding parameters obtained were K_d (nM) = 7.4 ± 0.5 SEM (n = 5) with maximal binding B_{MAX} = (sites/cell) 1 ± 0.2 × 10^5 SEM (n = 5).

Binding of Eu-NT was also studied in CHO cells transfected with the high-affinity NT receptor using

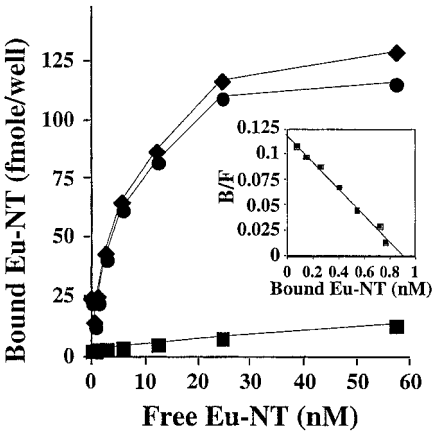

FIG. 3. Binding of Eu-NT to HT29 cells. HT-29 cells (4×10^5/well) were incubated at 37°C with the indicated concentrations of Eu-NT for 30 min and total binding (diamonds) or nonspecific binding in the presence of 3 μM unlabeled NT (squares) was determined. Specific binding (circles) was calculated by subtracting nonspecific from total binding. Scatchard analysis is presented in the inset. All other details are as described under Experimental Procedures.

^{125}I-NT as a tracer. The competition curves indicated a eightfold lower affinity of Eu-NT in comparison with NT, with K_d values of 2.5 and 0.3 nM, respectively (data not shown).

B. Ca^{2+} mobilization in CHO-NT1 cells in response to Eu-NT. The bioactivities of Eu-NT and NT were determined by their ability to induce Ca^{2+} transients in CHO-NT1 cells (Fig. 4). The maximal cytosolic Ca^{2+} concentration induced was similar for both peptides (about 650 nM); however, the potency of Eu-NT ($K_{0.5}$ = 1 nM) was about 50 times lower than that of the native NT ($K_{0.5}$ = 0.02 nM).

DISCUSSION

In this study we synthesized two Eu-labeled peptides and demonstrated their ability to serve as labeled ligands in studying ligand-receptor binding and consequent physiological responses. In the case of EGF the results obtained were similar to published reports obtained with ^{125}I-EGF (5). In the case of Eu-NT the biological activity was markedly affected for reasons discussed below.

Over the past 2 decades, Eu^{3+} labeling was used to replace radioisotopic label of antibodies in immunoassays (9), drugs (10), chemokines (11), or cell cytotoxicity assays (17). The use of time-resolved fluorescence in place of radioactivity has many advantages aside from safety concerns. The low basal Eu concentration in biological samples, combined with the delayed fluorescence measurement, provides a highly sensitive detection of the Eu ions (up to 8×10^{-18} mol per sample) (8).

A431 cells were reported to express two EGF receptor populations, a small fraction (0.1–0.2%) with high affinity (K_d = 70 pM), and a majority with low affinity (K_d = 5 nM) (5). The results obtained in the present study with Eu-EGF yielded a binding constant of K_d = 3.6 nM, likely to represent the larger low-affinity subpopulation of EGF receptors (Fig. 1). The EGF receptor density measured on A431 cells, using Eu-EGF (1.5×10^6 sites per cell), is also in agreement with published data obtained with ^{125}I-EGF for the low-affinity receptor population ($1–3 \times 10^6$ sites per cell) (5). Considering the specific activities of the two labeled EGF ligands, their nearly identical values of K_d, and nonspecific binding on A431 cells one can predict that the binding assays have essentially identical sensitivities. Consequently, high-affinity binding sites of the kind described by Kawamoto *et al.* (5) could be detected with Eu-EGF in our A431 cell colony (if present). As judged by the stimulatory effect on the proliferation of murine D1 hematopoietic cells (Fig. 2), the bioactivity of EGF was also unaffected by conjugation with the Eu-DTPA chelate. The human recombinant EGF used here has two candidate ε-amino groups on Lys residues in positions 28 and 48, as well as an N-terminal amino group that can conjugate with Eu-chelate. These residues appear to reside in regions that apparently play no major role in EGF bioactivity. The calculated specific activity suggests that all three possible positions on the labeled EGF are modified, enabling receptor-binding studies in the picomolar to nanomolar concentration range.

In contrast to EGF, a 54-amino-acid polypeptide, the potency of the smaller NT, a 13-amino-acid peptide, was markedly affected by conjugation of the Eu-DTPA chelate to the ε-amino group of Lys^6. The binding affinity (K_d) of the Eu-NT to HT29 cells was determined to be 7.4 nM (Fig. 3), approximately 10-fold lower than reported earlier for ^{125}I-NT in the same cell line (13). In other experiments, carried out with the same cells (data not shown), we observed that the K_d values for

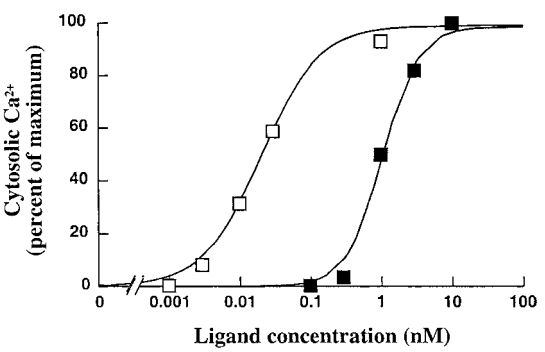

FIG. 4. Intracellular Ca^{2+} mobilization in CHO cells expressing the NT1 receptor. Cells were treated with increasing concentrations of NT (open squares) or Eu-NT (closed squares). Intracellular Ca^{2+} influx was determined for each concentration and is represented as the percentage of the maximal activation. All other details are as described under Experimental Procedures.

indium-DTPA-NT were similar to those obtained with Eu-NT, suggesting that the lower receptor binding activity of Eu-NT can be related to the conjugated DTPA moiety rather than to the character of the chelated atom. In agreement with the impaired binding capacity, intracellular Ca^{2+} mobilization by Eu-NT was even less efficient compared with native NT. The observation that Ca^{2+} mobilization can be obtained at low NT concentrations may be related to the fact that only a small fraction of the high-affinity NT receptors is required to fully activate Ca^{2+} mobilization as previously reported (18). On the other hand, it is possible that the same efficacy of NT and Eu-NT on Ca^{2+} mobilization relates to the amplification process taking place in the endoplasmic reticulum (18). Decline of the ligand-receptor affinity due to peptide modification has been reported in other cases where DTPA labeling of bioactive peptides has been described (19). [^{111}In]-DTPA-MSH(bis-DHP), an α-melanocyte stimulating hormone (α-MSH) homologue, was found to have a 10-fold higher K_d than α-MSH but was found to be a better imaging agent then other analogues with higher receptor affinity. Similarly, Eu-NT remains a useful ligand for such assays: (i) as a tracer in competition receptor binding studies of NT receptor agonists and antagonists, (ii) in the detection and determination of NT in biological fluids using immunoassays with anti NT antibodies, and (iii) for detection of NT receptors in cell and tumor preparations as is routinely performed with [^{111}In]-DTPA-NT having the same properties.

It is expected that modification with Eu-DTPA will induce various effects on the bioactivity of ligands and peptides characteristic to each ligand. Such effects will be difficult to predict; most probably more dominant changes will be seen among small peptides than in macromolecular ligands. As with isotopic labeling, the acceptable range of bioactive change, termed the isotope effect, is determined by the analytical purpose intended. Eu-modified ligands remain stable for up to 2 years when kept under appropriate storage conditions, a great advantage when compared to short-lived radiolabeled peptides, which usually remain active for only a few weeks. The relatively long shelf life of the Eu-labeled ligands allows higher reproducibility of results, since the same stock of labeled ligands can be used for longer periods. Eu labeling can also reduce the costs involved in labeling and handling, often involved in using isotopically labeled materials. The synthesis of the Eu-labeled peptides could be accomplished in a single-step process by conjugation of the Eu-DTPA chelate to the peptide as described in the preparation of EGF or in a two-step process as described for the preparation of Eu-NT, where the synthesis of DTPA-NT was followed by the chelation of Eu into the complex. Attempts to synthesize Eu-NT by the single-step process were unsuccessful.

In conclusion, this work represents a novel alternative for nonradioactive labeling of small peptides such as EGF and NT, which can replace the commonly used radioactive labeling. Eu labeling of ligands presents several valuable advantages over radiolabeling. The introduction of Eu-labeled peptides for binding studies in routine diagnostic tests may also enable their use in automated tasks, which for purely practical reasons was not possible with radioactive homologues. It can be expected that other peptide hormones will be amenable to Eu-labeling, enabling a reduction in the use of radioactivity in routine laboratory work and the introduction of more advanced medical diagnostics.

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