Purification of the Neurotensin Receptor from Bovine Brain*

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Ann Millet, Catherine D. Demoliou-Mason, and Eric A. Barnard*
From the Medical Research Council Molecular Neurobiology Unit, Medical Research Council Centre, Cambridge CB2 2QH, Great Britain

We have employed conditions in which highly active neurotensin binding to membranes was assayed in buffer A without Mg²⁺ but containing 10 μM TES-KOH (pH 7.5), 1 mM EGTA-K⁺, 2 mM MgSO₄, 1 mM benzamidine HCl, 0.002% (w/v) soybean trypsin inhibitor, and 250 mM NaCl, was dialyzed against 5 liters of 10 mM TES-KOH (pH 7.5), 1 mM EGTA-K⁺, 2 mM MgSO₄, 1 mM benzamidine HCl and the fractions were assayed immediately for [3,11-tyrosyl-3,5-[³H]neurotensin binding to membranes in buffer A containing 0.06% asolectin and 0.1% (w/v) digitonin (1 h, 20 °C). Bound and free ligand were separated by filtration of aliquots on GF/B filters pretreated with polyethyleneimine (15). Filters were rapidly washed with 3 × 4 ml of ice-cold 50 mM Tris-HCl (pH 7.4), dried, and counted in OptiPhase “Safe” (LKB) scintillation liquid. Protein concentrations were determined as previously described (13).

Affinity Chromatography—Affi-Gel 10 (2 ml), prewashed with 3 volumes of ice-cold water, was gently agitated (3 h, 4 °C) in 2 ml of 20 mM HEPES (pH 7.4) containing 12 μmol of neurotensin and a trace amount of [3,11-tyrosyl-3,5-[³H]neurotensin, followed by a 30-min incubation at 30 °C. Unbound ligand was washed away and the remaining active sites on the gel were blocked by reaction with 1 M ethanolamine HCl, pH 8.0 (1 h, 20 °C). The gel was subsequently washed with 10 volumes of 20 mM HEPES (pH 7.4), followed by 5 volumes of 10 mM TES-KOH (pH 7.5), and stored at 4 °C in the latter buffer containing 0.02% sodium azide. The amount of covalently bound neurotensin was estimated, from the trace label incorporated, to be 4–5 μmol/ml packed gel. The yield of coupling was 30% and did not increase with a longer reaction time.

This neurotensin/Affi-Gel 10 was pre-equilibrated with 5 volumes of buffer A containing 10 μM 1,10-phenanthroline, 0.1% (w/v) digitonin, and 0.005% (w/v) asolectin. Crude solubilized extract (10 ml; 15–20 mg of protein) was adjusted to be in the same medium and loaded at 20–30 ml/h onto a 2-ml column of the gel. This was then washed with 40 volumes (40–50 ml/h) of the equilibration buffer from which the soybean trypsin inhibitor and bacitracin were omitted and the asolectin concentration increased to 0.06%. The bound receptor was eluted with 2.5 volumes of the latter buffer containing also 250 mM NaCl, was dialyzed against 5 liters of 10 mM TES KOH (pH 7.5), 1 mM EGTA-K⁺, 2 mM MgSO₄, 1 mM benzamidine HCl and the fractions were assessed immediately for [3,11-tyrosyl-3,5-[³H]neurotensin binding. After each preparation the column was washed with 5 volumes of 1% NaCl, 0.5 M NaCl, 0.1 M Tris HCl (pH 8.5) and could then be reused.

SDS-PAGE—Protein samples for slab SDS-PAGE (14) were precipitated with chloroform/methanol (16) and boiled for 3 min in 6%}

**The abbreviations used are: TES, N-tri(hydroxymethyl)methyl-2-aminoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylenediamine-(oxyethylene-nitrito)tetraacetic acid.

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‡To whom correspondence and reprint requests should be addressed.

**MATERIALS AND METHODS**

[³H]Neurotensin (40–70 Ci/mmol) was from Du Pont-New England Nuclear and neurotensin was from Cambridge Biochemicals. Other materials, as well as methods not specified, were as given previously (13, 14). Digitonin (Analar) was from BDH; less soluble impurities were removed by dissolving at 100 °C, cooling at room temperature, and filtering.

Membrane Preparation and Solubilization—Using bovine brain cortex (14), membranes were prepared in the presence of protease inhibitors as previously described for rat brain (13). Frozen membranes were thawed and gently agitation (20 °C for 60 min) at 0.5–1.0 mg of protein/ml in buffer A: 10 mM TES-KOH (pH 7.5), 1 mM EGTA-K⁺, 2 mM MgSO₄, 1 mM benzamidine HCl, 0.002% (w/v) digitonin, and 0.002% (w/v) soybean trypsin inhibitor. The membranes were then pelleted at 45,000 × g at 4 °C for 30 min and resuspended in buffer A; digitonin and asolectin were added to give a final concentration of 2% (w/v) and 0.06%, respectively. After centrifugation at 120,000 × g at 4 °C for 60 min, the supernatant was stored at −20 °C for use.

[3,11-tyrosyl-3,5-[³H]Neurotensin Binding Assay—Specific [3,11-tyrosyl-3,5-[³H]neurotensin binding to membranes was assayed in buffer A without Mg²⁺ but containing 10 μM 1,10-phenanthroline and 1 mg/ml bovine serum albumin. Specific [3,11-tyrosyl-3,5-[³H]neurotensin binding (or nonspecific, with added 10⁻⁶ M unlabeled neurotensin) to crude or purified soluble extracts was measured in buffer A containing 0.06% asolectin and 0.1% (w/v) digitonin (1 h, 20 °C). Bound and free ligand were separated by filtration of aliquots on GF/B filters pretreated with polyethyleneimine (15). Filters were rapidly washed with 3 × 4 ml of ice-cold 50 mM Tris-HCl (pH 7.4), dried, and counted in OptiPhase “Safe” (LKB) scintillation liquid. Protein concentrations were determined as previously described (13).

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The binding parameters of the bovine brain neurotensin receptor

The parameters were determined from Scatchard plots of the equilibrium specific binding data (as in Fig. 1). Assay conditions for membranes were 10 mM TES-KOH (pH 7.5), 2 mM Mg++, plus the protease inhibitors at 20°C for 1 h. For crude soluble or purified receptor, conditions were as for membranes but with 0.06% asolectin and 0.1% digitonin also present. Values are the mean ± S.E. of three independent experiments, except that, for the purified samples, due to the variation in the estimated values of the protein content (see "Materials and Methods"), the Bmax values are the highest and lowest obtained.

| Sample       | KD (nM) | Bmax (fmol/mg) |
|--------------|---------|----------------|
| 1. Membranes | 3.3 ± 0.2 | 350 ± 10       |
| 2. Crude soluble | 5.5 ± 0.2 | 250 ± 15       |
| 3. Purified  | 5.5 ± 0.1 | (12.7 ± 10^6) = (7.4 ± 10^6) |

(w/v) SDS, 100 mM dithiothreitol, 8 M urea. Protein bands were visualized by staining with 0.2% Coomassie Blue in ethanol/acetic acid (5:1) or by silver staining (17). The protein content of the purified receptor (200-400 ng) was estimated by gel densitometry (Joyce-Loebel) after staining with Coomassie Blue, using similarly electrophoresed bovine serum albumin (100-500 ng) to construct a linear (r = 0.98) calibration plot.

RESULTS AND DISCUSSION

Purification of the Neurotensin Receptor by Affinity Chromatography—Membranes were incubated with 2 mM Mg++ (13) and then solubilized in a medium containing the detergent digitonin and the phospholipid mixture asolectin. In these conditions, the high-affinity binding of [3,11-tyrosyl-3,5-3H]neurotensin was maintained in solution (Table I, samples 1 and 2).

For the affinity gel used, neurotensin was immobilized on Affi-Gel 10 beads. Since the N terminus of neurotensin is blocked by a pyroglutamyl residue, the N-hydroxysuccinimide reactive group on the spacer arm of Affi-Gel 10 can be expected to react only via the amide chain of Am5 of neurotensin, which is essential for receptor binding (11).

This neurotensin-affinity gel bound 40-70% (range of several experiments) of the receptor activity present in the crude extract applied. The specific neurotensin-binding activity remained tightly bound, even after washing the column extensively. As a result of this tight binding, elution with 10^-5 M neurotensin alone resulted in a low recovery (8%) of receptor activity, since most of the receptor was found in the Na+ cholate wash of the column after each run. Moreover, the prolonged dialysis required for the removal of the eluting neurotensin resulted in losses of receptor activity. Since the SDS-PAGE protein profiles of the neurotensin-eluted and Na+ cholate-eluted protein were found to be the same, complete recovery of the tightly bound receptor was accomplished instead by elution with 250 mM NaCl. We have observed that NaCl strongly decreases (with IC50 = 35 mM) the affinity of the bovine neurotensin receptor.2 In addition to being able to dialyze the salt out quickly, the transition from high to low salt concentrations in the presence of asolectin and detergent during dialysis may stabilize the receptor activity by increasing the phospholipid to detergent ratio in the micelles.

Properties of the Purified Neurotensin Receptor—After the removal of NaCl by dialysis, the binding of [3,11-tyrosyl-3,5-3H]neurotensin to the purified receptor was specific and appeared to be saturable within the same concentration range

2 Mills, A., Demoliou-Mason, C. D., and Barnard, E. A. (1988) J. Neurochem, in press.
ues show that sufficient specificity for full purification is introduced at the stage of the binding to the immobilized neurotensin, together with the affinity change of this receptor on raising the salt concentration.

SDS-PAGE of the purified receptor protein, when denatured in the presence of 100 mM dithiothreitol, showed a single protein band of $M_r$, 72,000 after either Coomassie Blue staining (not shown) or silver staining (Fig. 2, lane 1). Under nonreducing conditions (Fig. 2, lane 2), a single band of $M_r$, 50,000 was instead visible, suggesting the presence of intramolecular disulfide bonds. Under these conditions a larger amount of aggregated protein was observed on the top of the gel, suggesting that there is some aggregation of the receptor protein during precipitation for SDS-PAGE. Increases in apparent size observed here. This may be the result of proteolytic degradation or deglycosylation.

In reality, where the true polypeptide $M_r$ is known from cDNA cloning, the true $M_r$ values in each case of the above mentioned type tend to be in the range of 50,000–55,000. The rest of the masses stated are due either to attached oligosaccharides or to systematic error in the estimation of the $M_r$ of membrane proteins by SDS-PAGE. This knowledge should become available soon for the neurotensin receptor, since the isolation of its subunit permits us to determine some internal protein sequence therein and hence to start its cDNA cloning.

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Fig. 2. SDS-PAGE of the purified neurotensin receptor. The protein eluted from the affinity column was analyzed on a 10% polyacrylamide gel, with silver staining. The receptor, $\approx$350 ng, was first denatured with SDS at 100 °C either with 100 mM dithiothreitol (lanes 1 and 3) or without dithiothreitol (lane 2). Lane 3, an equivalent volume of a sample from a control preparation, in which crude extract was preincubated with $10^{-6}$ M neurotensin (1 h, 20 °C) prior to its application to the affinity gel, was similarly analyzed; all stages were as for the unblocked preparation of lane 1, but the gel was overstained for the detection of any minor bands. The migration positions (kDa) of standard proteins (phosphorylase b, bovine serum albumin, ovalbumin, and soybean trypsin inhibitor) in a parallel track are shown on the left. Coomassie Blue staining of similar gels also showed only the band seen here.
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