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BOMBESIN-LIKE PEPTIDES IN RAT BRAIN: QUANTITATION AND BIOCHEMICAL CHARACTERIZATION

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SUMMARY: A highly specific and sensitive radioimmunoassay was developed to measure the regional distribution of bombesin-like peptides in rat brain. Extracts derived from the hypothalamus had the highest levels of bombesin-like immunoreactivity. Intermediate levels of bombesin-like immunoreactivity were observed in the thalamus and midbrain; low levels in the parietal cortex, striatum, hippocampus and medulla pons, whereas few, if any, bombesin-like peptides were detected in the cerebellum and pituitary. Using gel filtration techniques, one major and two minor peaks of immunoreactivity were derived from the hypothalamus, thalamus and midbrain. These endogenous bombesin-like peptides may play an important neuroregulatory role in the central nervous system.

INTRODUCTION:

BN,[1] a tetradecapeptide isolated from anuran skin (1), is biologically active in both the central nervous system and gastrointestinal tract. After injection into the brain, BN induces hypothermia (2), hyperglycemia (3) and analgesia (4) with a well-defined structure-activity relationship. Several amino acid residues near the C-terminal, in particular Trp^{8}, Val^{10} and His^{12} are essential for high affinity binding and biological potency (5). Also, BN induces gastrin (6) and cholecystokinin (7) release from the gastrointestinal tract as well as amylase release from acinar cells (8).

BN-like immunoreactivity has been observed in the dog intestine (9), porcine intestine (10), human foetal lung (11) and ovine (12) as well as rat brain (13). This communication describes the preparation of antibodies in rabbits which recognize numerous amino acid residues near the C-terminal

[1] Abbreviations used: BN, bombesin.

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of BN. Using the antiserum we developed a highly sensitive and specific radioimmunoassay for endogenous BN-like peptides. We report here the biochemical characterization and regional distribution of endogenous BN-like peptides in rat brain.

METHODS:

Antibody Elicitation. Immunogens were prepared using the method of Goodfriend et al. (14). Five mg of synthetic [Lys^3]BN (Peninsula Laboratories, San Carlos, CA) was coupled to 10 mg of bovine serum albumin using 100 mg of ethyl-3-(3-dimethyl-amino propyl) carbodimide hydrochloride. After a 15 min. incubation unconjugated peptide and excess carbodimide were removed by dialysis against water.

The conjugated peptide was emulsified in an equal volume of Freund's complete adjuvant (Calbiochem-Behring Corp., La Jolla, CA) and the rabbits were injected in the dermis of the back at multiple sites. The rabbits were boosted at monthly intervals with conjugated peptide emulsified with Freund's incomplete adjuvant. Three months after the initial injection the rabbits were boosted with 100 mg of aluminum oxide (ICN Pharmaceuticals, Inc., Cleveland, OH) which was precoated with 0.5 mg of BN. Sera which contained antibodies were frozen at -80^{o}C and thawed just prior to use.

Iodination of Peptide. [^{125}I-Tyr^4]BN was prepared monthly using stoichiometric amounts (0.5 nmol) of [Tyr^4]BN (Peninsula) and ^{125}I (Amersham Corp., Arlington Heights, IL) but a 10-fold molar excess of chloramine T (15). Radiolabeled peptide was separated from free ^{125}I using the gel filtration techniques described previously (5).

Radioimmunoassay. The radioimmunoassay was performed using the procedure of Margules et al. (16). Synthetic peptides or brain extracts in radioimmunoassay buffer (50 mM potassium phosphate, 0.25% bovine serum albumin and 0.5% β-mercaptoethanol (pH 7.4) were incubated with 200 µl antiserum at 1:100,000 dilution for 2 hours. Four fmol of [^{125}I-Tyr^4]BN (5000 cpm) were added and the mixture incubated for 16 hours at 4^{o}C; the total volume was 400 µl. Free peptide was then adsorbed by the addition of 100 µl of charcoal suspension (0.6 g charcoal in 10 ml of radioimmunoassay buffer). After 5 min. the charcoal was pelleted by centrifugation at 1000 x g for 10 min. and 300 µl of the supernatant, which contained [^{125}I-Tyr^4]BN antibody complex, assayed for radioactivity in a Beckman gamma counter.

Preparation of Brain Extracts. Brain extracts were prepared using the protocol of Rossier et al. (17). Adult male Sprague-Dawley rats (150-200 g) were decapitated and the brain regions dissected rapidly, frozen on dry ice, weighed and boiled in three volumes of 2 N acetic acid to inactivate endogenous proteases. After 15 min. the samples were chilled in ice, homogenized using a Brinkmann Polytron and centrifuged at 18,000 x g for 10 min. The supernatant was then frozen and lyophilized. The off-white lyophilized material was resuspended in a minimal volume of 50 mM Tris-HCl and the pH adjusted to 7.0 with concentrated Tris base. The solution was then centrifuged for 2 min. in a Brinkmann microfuge and the supernatant assayed for BN-like immunoreactivity.

Radioreceptor Assay. Brain regions were dissected from rats, weighed and membranes prepared as described previously (5). The binding assay was performed at 4^{o}C and the membrane-bound [^{125}I-Tyr^4]BN was separated from free radiolabeled peptide by filtration. Membrane protein concentration was determined using the method of Lowry et al. (18).

TABLE I

Specificity of Antiserum for Bombesin†

PEPTIDE	% CROSS REACTIVITY
[Lys^3]BN	115
BN	100
[Tyr^4]BN	75
[N-Ac-Gly^5]BN	62
[D-Met^{14}]BN	8.0
[Ala^{14}]BN	4.0
Ranatensin	<0.1
Litorin	<0.1
Substance P	<0.1
[Des-(Leu^{13},Met^{14})]BN	<0.1

Other peptides which cross-reacted minimally with the antibody (<0.1%) include eledosin, physalaemin, α-MSH, somatostatin, thyrotropin releasing factor, β-endorphin, Met^5-enkephalin and vasoactive intestinal polypeptide.

†(p-Glu-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2).

RESULTS:

Upon completion of the immunization scheme, several rabbits produced significant amounts of antibody which bound [^{125}I-Tyr^4]BN. One rabbit had a titre of 1:100,000 and this serum was used in all subsequent experiments. The specificity of this antiserum was determined using numerous BN analogues and other neuropeptides. Table I shows that the antibody cross-reacts strongly with [Lys^3]BN, [Tyr^4]BN and BN. Also, it cross-reacts strongly with [Ac-Gly^5]BN, which lacks the N-terminal tetrapeptide, but not with [des-Leu^{13},Met^{14}]BN, which lacks the C-terminal dipeptide. These data indicate that the antibody recognizes the C-terminal of BN.

Figure 1 shows the results of a typical radioimmunoassay. In the absence of competitor 1350 cpm remain in the supernatant, whereas in the presence of 1 pmol of BN or in the absence of antiserum, 400 cpm remain in

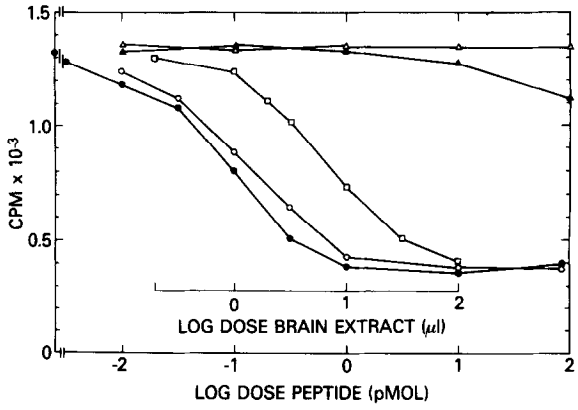

Figure 1: Amount of [^{125}I-Tyr^{4}]BN bound to antibody as a function of increasing doses of BN (●), [Ac-Gly^{5}]BN (o), Substance P (▲) and [des-(Leu^{13},Met^{14}]-BN (Δ). Also, the dose response curve is shown using a crude extract derived from 3 g of medulla pons (□). The extract was resuspended in 3 ml of 50 mM Tris/HCl, the pH adjusted to 7.0 with Tris base and assayed for BN-like immunoreactivity. Each point in the figure represents a mean of two determinations.

the supernatant. In this experiment 950 cpm of [^{125}I-Tyr^{4}]BN bound specifically to the antibody and the limit of sensitivity was 5 fmol. Also, Figure 1 shows that the dose response curve [Ac-Gly^{5}]BN is shifted slightly to the right of the BN curve, whereas the dose response curves for Substance P and [des-(Leu^{13},Met^{14})]BN are shifted at least 3 log units to the right of BN. Similar data was obtained if the radioimmunoassay was performed using goat anti-rabbit antibody instead of charcoal.

Appreciable amounts of immunoreactive material were extracted from rat brain using 2N HAc. Figure 1 shows that the immunoreactive components extracted from the medulla/pons yielded a dose-response curve parallel to that of BN; the extract from 6 mg of wet tissue was sufficient to displace half of the [^{125}I-Tyr^{4}]BN bound to the antibody. The dose response curve using hypothalamus and thalamus extracts had a similar shape but were displaced to the left of the medulla/pons curve (data not shown).

In Figure 2 the regional distribution of BN receptors and BN-like peptides is compared. The receptor density and concentration of BN-like immunoreactivity is greatest in the hypothalamus, intermediate in the thalamus

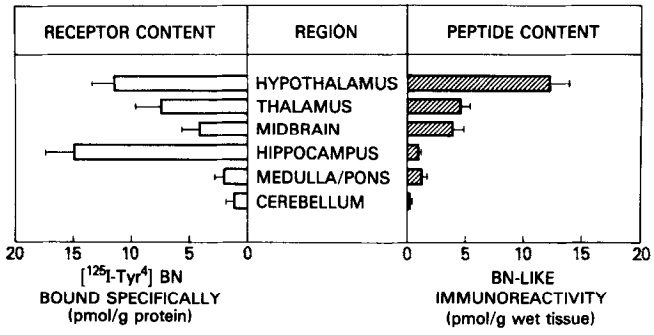

Figure 2: Comparison of the BN-receptor content and the content of BN-like immunoreactivity in various brain regions. Mean values ± S.E. for at least four determinations are indicated.

as well as midbrain, and low in the medulla/pons as well as cerebellum. While there was a close correlation between the receptor and peptide content in these brain regions, the hippocampus had a high density of receptors but a low concentration of BN-like immunoreactivity. Also, the parietal cortex, striatum, lung, heart, spinal cord and blood had low levels of immunoreactivity whereas the pituitary, liver, kidney and spleen had no detectable BN-like peptides (data not shown).

The brain extracts were fractionated using the gel filtration techniques described previously (5). Figure 3 shows that most of the u.v. absorbing material present in the extract eluted in the void and included volumes. In comparison, three peaks of BN-like immunoreactivity eluted just behind the void volume. The major peak of immunoreactivity eluted just behind [^{125}I-Tyr^{4}]BN and, therefore, may have a molecular weight similar to that of BN. Minor peaks of immunoreactivity eluted slightly before and behind the main peak of immunoreactivity. The former may represent a high molecular weight precursor and the latter a low molecular weight metabolite of endogenous BN-like peptide. Similar gel filtration profiles were obtained using extracts from the hypothalamus, thalamus and hippocampus. We were not able to demonstrate that the main peak of rat-immunoreactive material in our extracts has the ability to inhibit the specific binding of [^{125}I-Tyr^{4}]BN to its receptor.

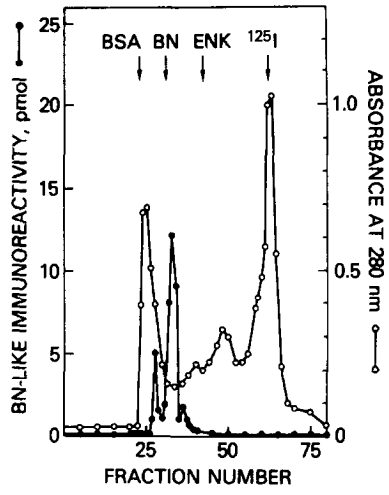

Figure 3. Gel filtration profile of an extract derived from 4.3 g of midbrain. The lyophilized extract was resuspended in 2 ml of MeOH/HAc/H_2O (10/2/1), centrifuged and the supernatant applied to a 50 x 1.5 cm Sephadex LH20 column. The column was eluted with MeOH/HAc/H_2O; fraction size was 1.3 ml. Fractions were assayed for u.v. absorbance, diluted in H_2O, frozen and lyophilized. The lyophilized material was suspended in 50 mM Tris/HCl and the pH adjusted to 7.0. The solution was then assayed for BN-like peptide content using the radioimmunoassay. The elution positions of bovine serum albumin, [^{125}I-Tyr^4]BN, [^{3}H-Leu]enkephalin and ^{125}I are indicated.

DISCUSSION:

This study demonstrates that the rat brain contains endogenous components that cross-react with an antibody that recognizes BN. The main immunoreactive component elutes at approximately the same position as does BN using gel filtration techniques and interacts with CM-Sephadex cation exchange resin at neutral pH (unpublished data). Therefore, the main immunoreactive component in rat brain may have a molecular weight and isoelectric point similar to that of BN.

This endogenous BN-like component, however, cannot be BN. When midbrain, thalamus and hypothalamus extracts were fractionated using HPLC techniques, the main peak of immunoreactivity eluted before BN as well as Substance P (unpublished data). This endogenous BN-like component represents a neuropeptide of undetermined structure which may be more hydrophilic than BN or Substance P.

While the hippocampus has a high receptor density but very low levels of BN-like immunoreactivity (Fig. 2), other data (not shown) indicate that hippocampus extracts contain appreciable amounts of material active in our brain radioreceptor assay. The extract derived from 100 mg of hippocampus inhibits 50% of the amount of [^{125}I-Tyr^{4}]BN bound to rat brain membranes, equivalent to 10 pmol of BN. By contrast, extracts of liver and cerebellum prepared simultaneously as described are essentially inactive. Hippocampus radioreceptor-active material appears to have a molecular weight much greater than that of BN for it elutes in the void volume using the gel filtration techniques described in Fig. 3. The nature of this brain radioreceptor-active material, which is also active on the BN receptors of pancreatic acinar cells (8), remains to be determined. In conclusion, immunoreactive BN-like peptides of several molecular weights are present in high concentrations in the hypothalamus whereas high molecular weight radioreceptor-active, radioimmuno-inactive material is present in the hippocampus and other receptor-rich regions of brain. This heterogeneity must be considered in further purification of the endogenous ligand for the BN receptor.

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