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RAPID COMMUNICATION

RADIOIMMUNOASSAY OF ATRIAL NATRIURETIC FACTOR (ANF) IN RAT ATRIA

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Abstract. We describe a solid phase radioimmunoassay for atrial natriuretic factor (ANF) and its application for measurement of this peptide in homogenates of rat atria. The method uses a synthetic 26 amino-acid fragment (8-33 ANF) of the native peptide. Sample (or standard) are incubated with the rabbit anti-8-33 ANF antiserum in peptide (8-33 ANF)-coated wells. Then an excess of I^{125} goat anti-rabbit IgG is added. The radioactivity bound is directly proportional to the amount of ANF present. The concentration of immunoreactive ANF has been found to be about 4 times higher in the right atrium than in the left atrium of the rat.

Introduction. It is now well established that mammalian cardiac atria contain a biologically active peptide capable of producing natriuresis, diuresis (1-3), and vasorelaxation (4,5), which has been called atrial natriuretic factor (ANF). Recently, this peptide has been purified, sequenced and synthesized (3,6,7). A synthetic fragment (8-33 ANF), composed of the 26 amino acids from residues 8 to 33 of ANF, has been found to have the same biological activity as the native peptide. We now report a radioimmunoassay for ANF using the synthetic 8-33 ANF fragment and its application in the measurement of ANF in rat atria.

Materials and Methods. The synthetic 8-33 ANF peptide has been generously supplied by Dr. R.F. Nutt (Merck, Sharp and Dohme Lab., West Point, PA).

Sprague-Dawley rats weighing 200-250 g were decapitated and the hearts quickly removed. After washing in cold 0.1 M PBS buffer, pH 7.4, the atria were carefully dissected. The two atria from one rat or the right and left atrium separately were homogenized in 2 ml of 1 M acetic acid for 10 seconds, and centrifuged for 20 min at 30,000 rpm. The supernatant was lyophilized overnight. The lyophilized atria were dissolved in 500 µl 0.01 M ammonium acetate buffer, pH 5.0, and 100-µl aliquots were stored frozen at -20°C until assayed. Dilutions of 1:10 in 0.1 M PBS containing 1% ovalbumin were used for the assay.

Production of antisera to 8-33 ANF:

For immunization, 8-33 ANF was coupled to bovine thyroglobulin using carbodiimide (8). One hundred µg of this conjugate in 1 ml saline emulsified with 1 ml of complete Freund's adjuvant was injected intradermally at multiple sites on the shaved backs of New Zealand white rabbits (1.5 kg). The animals were reimmunized at monthly intervals with 100 µg 8-33 ANF-thyroglobulin conjugate in incomplete Freund's adjuvant and bled by ear artery 10 days after the injection. Two of three rabbits produced high titer antisera. For all our studies the fourth bleeding from one rabbit was used (17/4).

Preparation of labelled goat anti-rabbit:

Anti-rabbit γ-globulin were produced in goats. Goat anti-rabbit IgG was purified by affinity chromatography on Protein A-sepharose (Pharmacia Inc., Uppsala, Sweden) (9).

Fifty µg of anti-rabbit IgG was labelled with I^{125} using the Chloramine-T method (10). Purification of radiolabelled tracer was performed on an anion exchange resin (AG-1-X8, 100-200 mesh, Chloride form, Bio-Rad).

Preparation of the standard curve:

The synthetic 8-33 ANF peptide was used as a standard. A stock solution of 1 mg/ml in 0.01 M ammonium acetate buffer, pH 5.0, was fractionated in 10-

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µl aliquots and stored frozen at -20°C. The stock solution (10 µl) was diluted before use in 0.1 M PBS buffer, pH 7.4, 1% ovalbumin to a concentration of 1000 ng/ml, the most concentrated point of the standard curve. Serial two-fold dilutions were then prepared with the assay buffer to final concentrations ranging from 7.8 to 1000 ng/ml. One hundred µl of standards were used for preparing the standard curve.

Radioimmunoassay procedure:

The radioimmunoassay was performed in Removawells (Dynateck Laboratories Inc., Alexandra, VA). The wells were coated with 200 µl of 1 µg 8-33 ANF/ml in 0.005 M glycine buffer, pH 9.5, for 2 hr at room temperature. The peptide solution was then discarded and the wells were exposed for 30 min at room temperature to 400 µl of 0.1 M PBS buffer containing 1% ovalbumin to saturate the plastic in order to minimize the non-specific binding of radioactivity. The wells were then washed in 0.15 M NaCl containing 0.025% Tween 20. The standards or samples (100 µl) and the antibody against 8-33 ANF diluted 1:32,000 (100 µl) were added and the wells were incubated 2 hr at room temperature. The wells were next washed in 0.15 M NaCl 0.025% Tween 20 and 200 µl of I^{125} anti-rabbit IgG diluted in PBS buffer with 1% ovalbumin was added to the well. Non-specific binding was determined in the wells coated only with PBS buffer 1% ovalbumin or the wells coated with peptide 8-33 ANF. After an overnight incubation at 4°C the wells were washed in Tween 20 buffer and the bound radioactivity was determined in an LKB gamma counter. Results were expressed as B/Bo where B = cpm of bound radioactivity − cpm non-specifically bound. Bo = cpm bound in absence of standard − cpm non-specifically bound.

Results. To determine the optimal conditions for the assay, the effects of concentration, pH, time and temperature for coating the wells with 8-33 ANF and for the incubations with anti-ANF antibody and I^{125} goat anti-rabbit IgG were studied. To establish coating conditions, concentrations of 8-33 ANF from 0.01 to 2 µg/ml (200 µl/well) diluted in buffer from pH 6.0 to 10 were incubated at 4°C or room temperature. Binding was determined using excess anti-ANF antibody. The appropriate dilution of anti-ANF was then determined by incubating at either 4°C or room temperature doubling dilutions, from 1000-fold to 128,000-fold (in the assay buffer pH 7.4, 1% ovalbumin), with either the most concentrated ANF standard 100 ng/well or diluent. Based on these preliminary experiments, the coating conditions chosen consisted in a 2-hour incubation at room temperature with 8-33 ANF at a concentration of 1 µg/ml (200 µl/well). Prolonged incubation did not increase the binding. Varying the incubation time from 30 min to 8 hr at 4°C and room temperature for the first antibody before adding tracer demonstrated that 2 hr at room temperature is optimal. The second immunological reaction was slower and I^{125} anti-rabbit IgG had to be incubated for at least 16 hr at 4°C to achieve the maximum binding.

The standard curve obtained in these conditions is shown in Figure 1. The detection limit, defined as the lowest concentration of ANF that gives a binding which differs significantly from Bo at the 95% confidence interval, was 0.78 ng/well which corresponds to the lowest concentration of the standard. The working range extends from 0.78 to 100 ng/well.

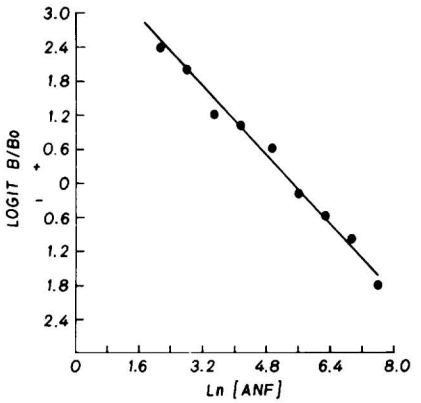

Fig. 1. Standard curve for radioimmunoassay of 8-33 ANF.

The anti-8-33 ANF used in this study showed cross-reactivity with different fragments of native ANF such as 3-33 ANF (95%), 10-33 ANF (99%) and propeptide, composed of 73 amino acids (23%). These peptides were isolated from rat atrial homogenates during the purification of ANF.

The within and between-run precision were assessed by measuring two rat atria homogenates with different concentrations of ANF. The mean values for 10 determinations in a single run were 151 ± 15.4 ng/ml and 20.5 ± 4.0 ng/ml. The between assay CV for the same samples assayed in duplicate in four different runs ranged from 9.5% to 15%.

The analytical recovery, as determined by addition of 1 µg/ml of 8-33 ANF peptide to 6 rat atria homogenates, was between 75% to 100% (mean ± SD 87 ± 14.2%).

The different dilutions of homogenates of rat atria showed parallelism with the standard curve.

This assay was applied for measurement of ANF in homogenates of rat atria. We have found under these conditions about 4 times higher concentrations of immunoreactive ANF in right rat atrium (means ± SD 0.74 ± 0.14 µg/atria) than in left atrium (0.17 ± 0.08 µg/atria). When both atria were extracted together from each rat (n = 10), the concentration of 2.2 µg/left and right atria of immunoreactive ANF was found.

Discussion. To our knowledge the present paper is the first report of a radioimmunoassay of atrial natriuretic factor. This method is sensitive enough to determine the content of immunoreactive ANF in rat atria. The range for measurement of ANF by the described method is 0.78 to 100 ng and is therefore broad enough to measure accurately a wide range of ANF concentrations in rat atria. The procedure requires only a 2-hour incubation of anti-ANF antibody and sample in previously prepared antigen-coated wells followed by an overnight incubation with the labelled second antibody. The wells in several plates can be coated simultaneously and the plates stored at 4°C for at least one week.

The determination of immunoreactive ANF by radioimmunoassay have shown much higher concentrations (about 4 times) in the right than in the left atrium. This potent atrial natriuretic factor has been found to be stored in the specific atrial granules, which are present in atrial muscle of mammals (11). These specific granules are of three different types A, B, and D, and have morphological and histochemical properties similar to the storage granules of polypeptide hormones (13). The number of these specific granules determined by quantitative ultrastructural and cytochemical methods is significantly greater in the right atrium of rat than in the left (12,13).

The difference in concentration of immunoreactive ANF between the rat atria may be related to the differences in left and right atrial pressure or to yet unidentified reflex mechanisms operating in the atria, including the described granules and stretch or osmoreceptors.

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