Atrial Natriuretic Factor Receptor on Cultured Leydig Tumor Cells: Ligand Binding and Photoaffinity Labeling†

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ABSTRACT: Atrial natriuretic factor (ANF) is a peptide hormone discovered recently from the heart atrium that possesses potent natriuretic and vasorelaxant activities. Recently we found that ANF markedly stimulates intracellular cGMP and almost completely inhibits cAMP accumulation in testicular interstitial tumor cells [Pandey, K. N., Kovacs, W. J., & Inagami, T. (1985) *Biochem. Biophys. Res. Commun. 133*, 800–806]. These actions of ANF suggest the presence of ANF receptors in testicular interstitial cells. In this study, cultured murine Leydig tumor cells have been shown to contain specific binding sites for ANF. Saturation binding studies indicated a single class of binding sites with a K_d of 5×10^{-9} M at a density of 2×10^6 sites/cell. The binding of mono[^{125}I]iodo-ANF (^{125}I-ANF) was competed by unlabeled ANF in a dose-dependent manner. Hormones unrelated to ANF such as angiotensin I, bovine luteinizing hormone, and human chorionic gonadotropin were not able to compete against ^{125}I-ANF. The binding of ^{125}I-ANF was rapid, reaching maximum levels in 15 min at 4 °C. At 37 °C, the cell-bound ^{125}I label was quickly decreased. Pretreatment of cells with NH_4Cl, chloroquine, or NaN_3 resulted in significant increases in maximum levels of the cell-bound ^{125}I radioactivity. A photoaffinity reagent for ANF receptor was prepared by reacting ANF with succinimido 4-azidobenzoate, and resultant 4-azidobenzoyl- (AZB-) ANF was purified by high-performance liquid chromatography (HPLC). AZB-ANF was radioiodinated by use of chloramine T and purified again by HPLC. Incubation of cultured Leydig tumor cells with pure AZB-^{125}I-ANF at 0 °C followed by photolysis resulted in specific labeling of a 135 000-dalton protein, which was detected in sodium dodecyl sulfate gel electrophoresis as a single radioiodinated band. The labeling was prevented by unmodified ANF but not by atriopeptin I or angiotensins I, II, and III at 1 μM concentrations. Results suggest that a single-chain polypeptide with an apparent M_r of 135 000 is the ANF receptor or its subunit. Data also indicate that, after the binding of ANF to the cell surface receptor, the ligand–receptor complex is rapidly internalized into the cell. ANF is hydrolyzed in the lysosome, and degraded products are released into culture media. The present investigation demonstrates the existence of high-capacity ANF receptor in an established cell line in culture, which will permit detailed studies on the mechanisms of ANF action at cellular and molecular levels.

The cardiac atria contain peptides that stimulate the renal excretion of sodium and water (de Bold et al., 1981) and relaxation of vascular smooth muscle cells (Currie et al., 1983; Grammer et al., 1983). The sequences of both active peptides and the precursor of ANF[1] have been determined by amino acid sequence analysis (Flynn et al., 1983; Misono et al., 1984a,b; Thibault et al., 1984; Currie et al., 1984; Kangawa & Matsuo, 1984) and by cDNA cloning (Maki et al., 1984; Yamanaka et al., 1984; Oikawa et al., 1984), respectively.

The molecular mechanisms of ANF action are not well understood. ANF has been shown to activate membrane-bound guanylate cyclase (Hamet et al., 1984; Waldman et al., 1984; Winquist et al., 1984) and to inhibit adenylate cyclase (Anand-Srivastava et al., 1984), thereby resulting in an increase in intracellular cGMP and a decrease in cAMP in the kidney, adrenal glands, and vascular smooth muscle cells. Among various tissues examined, testis has shown the highest level of stimulation of particulate guanylate cyclase by ANF (Waldman et al., 1984). Recently we found that ANF causes marked elevation of intracellular cGMP and inhibits cAMP levels in cultured Leydig tumor (MA-10) cells (Pandey et al., 1985). Together, these results suggested the presence of ANF receptors in the testicular interstitial cells. In the present work we have studied the binding characteristics of ANF to MA-10 cells and have identified the ANF receptor molecule on these cells by photoaffinity labeling.

Materials and Methods

Materials. Rat ANF IV (Misono et al., 1984a) with the sequence Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr was synthesized by the solid-phase method as described (Sugiyama et al., 1984). Atriopeptin I (Currie et al., 1984), representing the sequence from residue 2 through residue 22 of the above sequence, was synthesized similarly. Bovine luteinizing hormone (bLH) and human chorionic gonadotropin (hCG) were gifts from the National Pituitary Agency. Angiotensins I, II, and III were purchased from Peninsula Laboratories, Inc. (Belmont, CA). Tissue culture supplies were obtained from Gibco Laboratories (Grand Island, NY) and electrophoresis materials from Bio-Rad (Richmond, CA). Succinimido 4-azidobenzoate and triethylamine were from

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[1] Abbreviations: ANF, atrial natriuretic factor; ^{125}I-ANF, mono-[^{125}I]iodo-ANF; AZB, 4-azidobenzoyl; bLH, bovine luteinizing hormone; hCG, human chorionic gonadotropin; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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Pierce Chemical Co. (Rockford, IL). Dimethylformamide was distilled from ninhydrin. Na^{125}I (17 Ci/mg) was obtained from New England Nuclear (Boston, MA). All other chemicals were reagent grade.

Cell Culture. A cloned mouse Leydig tumor cell line, MA-10, was cultured (split ratio 1:6) in 75-cm^2 flasks containing 20 mL of modified Waymouth MB 752/1 medium supplemented with 15% horse serum (growth medium) as described previously (Ascoli, 1981).

Analytical Procedures. Amino acid analyses were carried out by reverse-phase HPLC of (phenylthio)carbamyl amino acid derivatives of peptide hydrolysate (Tarr et al., 1984) with a Waters "Pico-Tag" amino acid analysis system. The amino-terminal analysis was carried out by using dansyl chloride according to the method of Mann and Fish (1972). ^{125}I radioactivity was measured in a Beckman Model 4000 γ counter at an efficiency of 90%.

Radioiodination of ANF. Rat ANF IV (11 μg) was iodinated with ^{125}I (1 mCi; carrier free) by the chloramine T method (Hunter & Greenwood, 1962). The reaction was terminated by dilution with 0.5 mL of distilled water. The entire solution was applied on a Vydac C_{18} column (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile from 0% to 60% over a period of 90 min in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected at intervals of 30 s. ^{125}I-ANF IV was eluted in a predominant radioactivity peak at 46 min after the beginning of the gradient.

Preparation of 4-Azidobenzoyl-^{125}I-ANF IV. ANF IV (1.35 mg, 0.5 μmol) was reacted with 1.3 mg (5 μmol) of succinimido 4-azidobenzoate (Galardy et al., 1974) in 200 μL of dimethylformamide containing 1 μL of triethylamine in the dark at room temperature for 5 h. After the reaction, the peptide was extracted in 3 mL of 1 M acetic acid. The acetic acid layer was washed twice with equal volumes of ethyl acetate and then lyophilized. AZB-ANF IV was purified from this material by HPLC. Conditions are described in the legend to Figure 4a. AZB-ANF IV was aliquotized, dried under vacuum, and stored in the dark at −20 °C. Iodination of AZB-ANF IV (19.5 μg) was carried out in a manner similar to that described above for ANF IV. AZB-^{125}I-ANF IV was purified by HPLC under the conditions described in the legend to Figure 4b. Fractions containing AZB-^{125}I-ANF IV were stored at 4 °C in the dark.

Binding Assay. All binding assays were performed in 6-cm^2 Petri dishes with MA-10 cells cultured for 2–3 days. Prior to the binding assay, cells were washed 3 times (3 mL each) with the assay medium (serum-free Waymouth MB 752/1 containing 0.1% bovine serum albumin). The binding assay was done in 1.5–2 mL of medium with appropriate concentrations of radiolabeled ANF (specific activity, 2.1 μCi/pmol). Hormones were dissolved in 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% bovine serum albumin. Control dishes received 1 × 10^{-7} M unlabeled ANF to determine the nonspecific binding. Twenty minutes was allowed for the binding reaction at 4 °C unless otherwise specified. After the binding reaction, the dishes were quickly washed 4 times with 2 mL each of ice-cold assay medium. Cells were dissolved in 2 mL of 0.5 N NaOH, and the cell-bound ^{125}I radioactivity was counted. Data for the Scatchard plot were analyzed by using the computer program developed by Munson and Rodbard (1980).

Analysis of ANF Internalization. Internalized radioactivity and cell surface bound radioactivity were measured by the following criteria. (1) Total binding and nonspecific surface binding were determined as described above. (2) The cell surface bound radioactivity was determined by allowing binding for the appropriate time for both total and nonspecific binding at 4 °C as described above. For the internalization experiment confluent culture dishes were prepared as described. Cells were washed with assay medium and incubated with ^{125}I-ANF at 4 °C for the appropriate time, after which unbound ^{125}I-ANF was removed by thorough washing as described above and 2 mL of fresh medium was added. In the next step the dishes were divided into two sets; to permit the internalization, one set of dishes was incubated at 37 °C and the second parallel set of dishes was incubated at 4 °C. After the appropriate time of incubation, the media were collected and cells were lysed by 0.5 M NaOH. The radioactivity in the media and cell lysates was determined. Aliquots of the media and cell lysates were also applied to a C_{18} reverse-phase column, and the positions of radioactivity were determined.

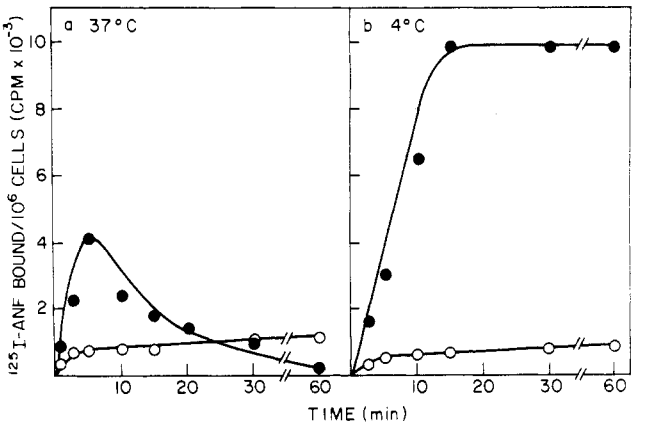

FIGURE 1: Specific binding of ^{125}I-ANF to cultured Leydig tumor cells as a function of time. Cells were incubated with ^{125}I-ANF at 37 (a) or 4 °C (b) in 1.5 mL of binding medium. At the indicated time intervals the cells were washed and cell-bound radioactivity was determined as described under Materials and Methods. The specific binding (●) was obtained by subtracting nonspecific binding (○) determined in the presence of 1 × 10^{-7} M unlabeled ANF from the total binding. Each point is the mean of three determinations.

Photoaffinity Labeling of ANF Receptor. Cells grown in 6-cm^2 Petri dishes were washed and incubated in 2 mL of assay medium containing 1.1 × 10^{-9} M AZB-^{125}I-ANF IV in the presence or absence of unmodified 1 × 10^{-7} M ANF for 10 min at 0 °C in the dark. Following the binding, cells were washed twice with 2 mL each of ice-cold assay medium and then placed in 2 mL of fresh medium. Photolysis was carried out on ice for 10 min, using a 250-W General Electric sunlamp at a distance of 15 cm. Cells were then washed 4 times with 2 mL each of assay medium and then lysed in 1 mL of 0.5% SDS. The cell lysate was incubated with 5 μg/mL deoxyribonuclease for 30 min at 37 °C. Aliquots (equivalent to 2 × 10^5 cells) were used for sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis by the method of Laemmli (1970), using 7.5% gels. Gels were stained with Coomassie Blue, dried, and autoradiographed by use of Kodak XAR-5 films.

RESULTS

The binding of ^{125}I-ANF IV to MA-10 cells at 37 °C was rapid, reaching the maximum levels in 5 min (Figure 1a). The cell-bound ^{125}I radioactivity then declined sharply. At 4 °C, the ligand binding reached the maximum level in 15–20 min (Figure 1b). The maximum amount of the cell-bound radioactivity at 4 °C was 2–3 times higher than that observed at 37 °C. The nonspecific binding was less than 10% of the specific binding. Pretreatment of cells with ammonium

Table I: Amount of Radioactivity Bound to the Cell and Released into Culture Media from Cells Incubated at 37 and 4 °C[a]

temp (°C)	culture medium (cpm)	cell bound (cpm)
37	38098 ± 1734	3072 ± 105
4	12810 ± 162	19549 ± 7743

[a] Confluent cells in 6-cm^2 Petri dishes were allowed to bind ^{125}I-ANF in 1.5 mL of binding medium at 4 °C for 15 min. Cells were washed, and 1.5 mL of fresh medium without radiolabeled ligand was added. The dishes were divided into two groups: one set of cultures was incubated at 4 °C and the other set at 37 °C. After 15 min the media were collected, cells were lysed, and radioactivity in culture media and that in cell lysate were determined as described under Materials and Methods. Values are the mean ± SE of triplicate determinations.

chloride (10 mM), chloroquine (0.2 mM), or sodium azide (5 mM) resulted in an increase in the maximum levels of cell-bound radioactivity at 37 °C as shown in Figure 2a. Chloroquine was the most effective agent, which caused more than a 2-fold increase in the maximum binding and significantly suppressed the subsequent sharp decrease in the cell-bound radioactivity. In order to measure the rate of release of the cell-bound radioactivity, MA-10 cells were initially incubated with ^{125}I-ANF at 4 °C for 15 min to allow the maximum binding. After washing, cells were incubated in the fresh medium at 4 or 37 °C (Figure 2b). During the incubation at 37 °C, cell-bound radioactivity rapidly decreased with a $t_{1/2}$ of 2.5 min. After 30 min 90% of ^{125}I radioactivity originally bound to the cells was released into the medium as shown in Table I. However, at 4 °C, the cell-bound radioactivity decreased only slightly over the same time period. In order to determine the metabolic fate of the cell-bound ^{125}I-ANF after incubation, an aliquot of the medium of the cells incubated at 37 °C was injected onto a C_{18} reverse-phase column. No radioactive peak was found at the position corresponding to ^{125}I-ANF IV. Almost all radioactivity injected was eluted in pass-through fractions. On the contrary, the extract of cells incubated at 4 °C and chromatographed under identical conditions gave a major radioactive peak at the position corresponding to ^{125}I-ANF. An aliquot containing 6000 cpm of radioactivity of the medium of the cells incubated at 37 °C was derivatized directly with phenylisothiocyanate (PTC) and analyzed for amino acids by using a Waters Pico-Tag system. A single radioactive peak was eluted at a position identical with that of the standard monoiodotyrosine derivative, which was eluted slightly earlier than PTC-phenylalanine (data not shown).

The binding of ^{125}I-ANF IV to MA-10 cells at increasing concentrations of the radiolabeled ligand is shown in Figure 3a. Scatchard analysis of these binding data using a one-site model indicated binding sites with a K_d of 5×10^{-9} M at a density (B_{max}) of $1-2 \times 10^6$ sites/cell. The competition binding of ANF IV against a fixed concentration of ^{125}I-ANF IV is shown in Figure 3b. By this method the K_d for ANF IV was calculated to be 1×10^{-9} M. Other peptides unrelated to ANF such as angiotensin II, bLH, or hCG were ineffective in competing against the binding of ^{125}I-ANF to MA-10 cells.

Photoreactive AZB moiety was introduced to the NH_2 terminus of ANF IV by reacting with succinimido 4-azidobenzoate in the dark. Since ANF does not contain Lys residue, the reaction is limited to the NH_2-terminal α-amino group. The reaction products were separated by HPLC as shown in Figure 4a. AZB-ANF IV was eluted in a peak having absorption at both 214 and 313 nm about 48 min after the beginning of the acetonitrile gradient. Amino acid analysis of this material gave a composition identical with that of the parent peptide ANF IV. Amino-terminal analysis using dansyl chloride yielded no detectable dansyl amino acid. The material

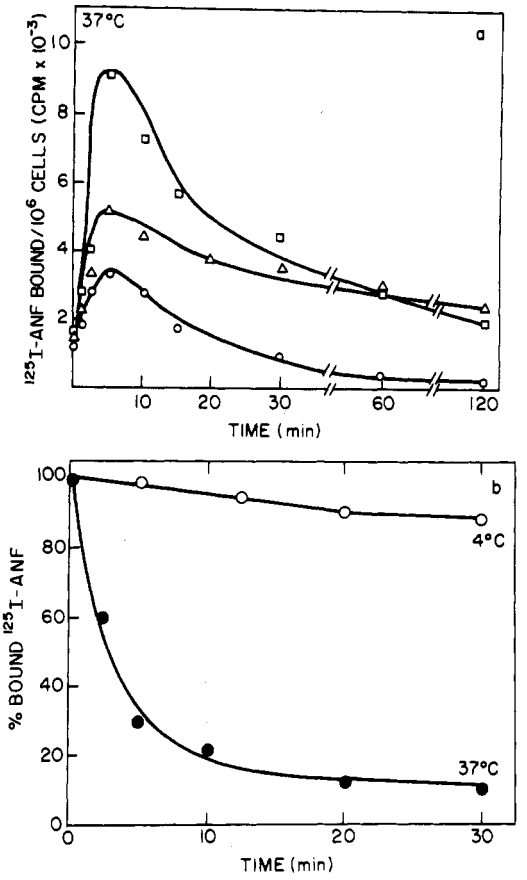

FIGURE 2: (a) Effects of lysomotropic agents on specific binding of ^{125}I-ANF to cultured Leydig tumor cells. The cells were preincubated in the absence (○) or presence of 10 mM NH_4Cl (△) or 0.2 mM chloroquine (□) for 15 min at 37 °C. The medium was removed, and fresh medium containing the lysomotropic agents and ^{125}I-ANF was added. At the indicated time intervals the cells were washed and cell-bound radioactivity was determined. (b) Time course of dissociation of cell-bound ^{125}I-ANF. Leydig tumor cells were allowed to bind ^{125}I-ANF in 1.5 mL of binding medium at 4 °C for 15 min. Cells were washed, and 1.5 mL of fresh medium without radiolabeled ligand was added. The dishes were divided into two groups: one set of cultures was incubated at 4 °C (○) and the parallel set at 37 °C (●). At the indicated time intervals the cells were washed and cell-bound radioactivity was determined as described under Materials and Methods. The results are expressed as the relative percentage of cell-bound radioactivity remaining at the indicated times to that of the initial binding at 4 °C. Each point is the average of three determinations.

showed a UV-absorbance peak at 340 nm that is characteristic to azidobenzoyl compounds. On the basis of the mole residue absorbance coefficient of 300 M^{-1} reported by Galardy et al. (1974) and of the amino acid analysis data, the ratio of azidobenozyl residue to the peptide was estimated to be 1.05, confirming the stoichiometry. By amino acid analysis, the yield of AZB-ANF IV was estimated to be 14%. The peak eluted at 42 min after the beginning of the gradient contained unreacted ANF IV, on the basis of its elution time and amino acid composition. No other peak contained a significant amount of amino acids.

The ^{125}I radiolabel was incorporated into AZB-ANF IV by radioiodination at the COOH-terminal Tyr residue by the chloramine T method. Radioiodinated AZB-ANF IV was purified by HPLC as shown in Figure 4b. The major UV-absorbance peak eluting at about 48 min contained unreacted AZB-ANF IV. A small but discrete UV-absorbance peak eluting at 52 min contained the majority of the radioactivity retained by the column. On the basis of the radioactivity and the amino acid analysis, the specific radioactivity of this

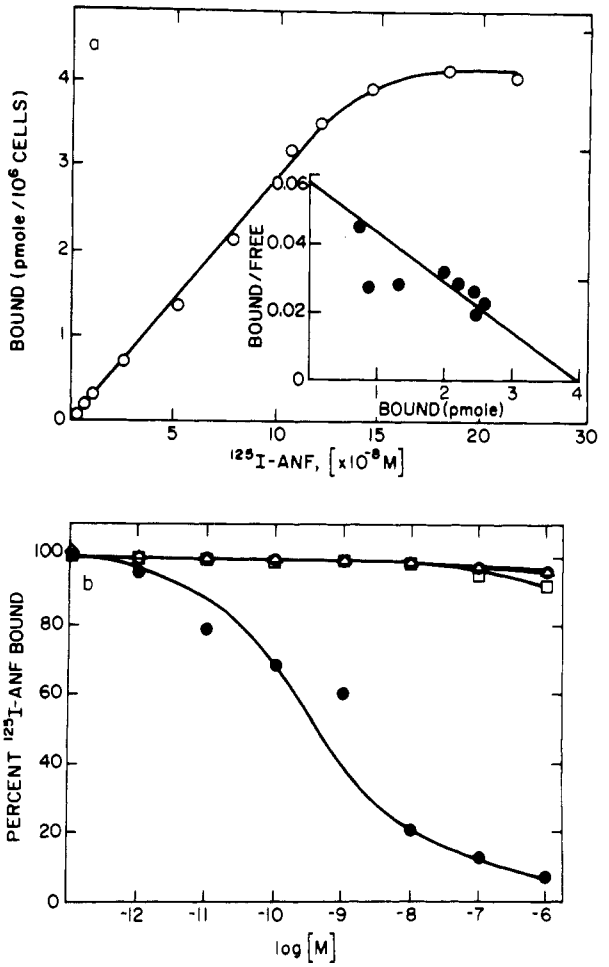

FIGURE 3: (a) Equilibrium saturation binding of ^{125}I-ANF to cultured Leydig tumor cells. The binding was determined by incubating the cells with an increasing amount of radiolabeled ANF. (Inset) Scatchard analysis of the saturation binding. Each point is the mean of two or three determinations. (b) Binding competition of ^{125}I-ANF to Leydig tumor cells by unlabeled ANF (●), angiotensin II (○), bLH (△), and hCG (□). The equilibrium binding was performed in 1.5 mL of medium at 4 °C for 20 min in the absence or presence of the indicated concentrations of unlabeled hormones, while the concentration of radiolabeled ligand was kept constant.

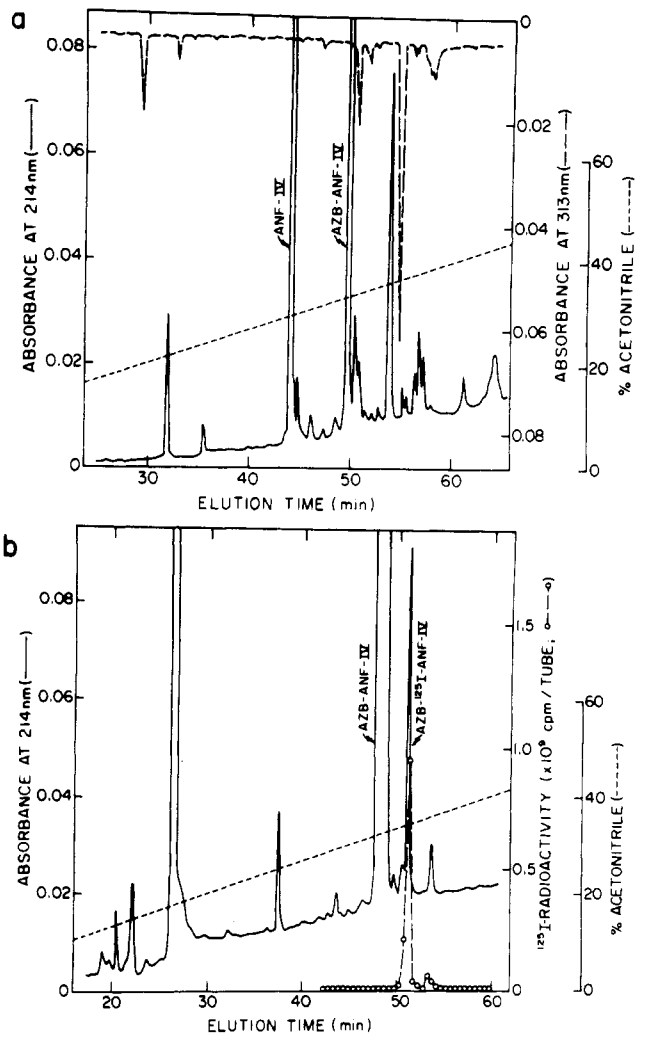

FIGURE 4: (a) Preparation of 4-azidobenzoyl-ANF IV. ANF IV was reacted with succinimido 4-azidobenzoate in the dark. The reaction products were extracted with 1 M acetic acid and chromatographed on a Vydac C_{18} column (10 μM; 0.46 × 25 cm; Separations Group). Elution was carried out in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile from 0% to 60% applied over a period of 90 min at a flow rate of 1 mL/min. Absorbances at both 214 and 313 nm were monitored by two fixed-wavelength detectors connected in a series. (b) Preparation of 4-azidobenzoyl-^{125}I-ANF IV. 4-Azidobenzoyl-ANF IV from (a) was radioiodinated by the chloramine T method (Hunter & Greenwood, 1962). The reaction mixture was chromatographed under conditions identical with those described in (a). Fractions of 0.5 mL (30 s) were collected.

material was estimated to be about 2 μCi/pmol. This specific activity is consistent with a monoiodinated peptide AZB-^{125}I-ANF IV, which has a maximum theoretical specific radioactivity of 2.13 μCi/pmol. The formation of diiodinated AZB-ANF IV apparently was not significant. It appears that the excess of AZB-ANF IV over Na^{125}I (8–10-fold excess) present in the iodination mixture effectively prevented diiodination. The yield of purified AZB-^{125}I-ANF IV ranged from 80 to 150 pmol, on the basis of the radioactivity, representing recoveries of 17–30% of ^{125}I added in the reaction mixture.

The incubation of MA-10 cells with AZB-^{125}I-ANF IV followed by photoactivation yielded essentially a single radiolabeled protein band with an apparent M_r of 135000 as determined by SDS gel electrophoresis and autoradiography (Figure 5a, lane A). The labeling of this band was completely abolished by the inclusion of unmodified ANF IV (1 μM) in the incubation medium (Figure 5a, lane B) In contrast, atriopeptin I (Figure 5a, lane C) or a combination of angiotensins I, II, and III (each 1 μM, lane D) was unable to protect this protein band from labeling. From the radioactivity present in the 135-kDa band cut out from the dried gel, the amount of the ^{125}I-ANF moiety incorporated per 1 × 10^6 cells was estimated to be about 10 fmol. On the basis of the estimation of the B_{max} of 2 × 10^6 sites/10^6 cells, this amount represents an affinity-labeling yield of approximately 0.5–1%.

Discussion

We have presented evidence indicating that testicular Leydig tumor cells contain a high number of specific binding sites for the peptide hormone ANF. The high density of the receptor sites is consistent with the strong response of these cells to ANF. Previously we have shown that ANF stimulated intracellular cGMP of MA-10 cells by 2000-fold and inhibited the accumulation of cAMP by more than 90% (Pandey et al., 1985). Both of these responses occurred within 5 min. Thus the rapid binding of ^{125}I-ANF to MA-10 cells is in agreement with the rapid cellular response of these cells to ANF. The K_d value for ANF IV of 5 × 10^{-9} M obtained by the saturation binding studies is in agreement with the ED_{50} of the peptide on these cells in stimulating cGMP and in suppressing the basal and bLH- or hCG-stimulated accumulation of intra-

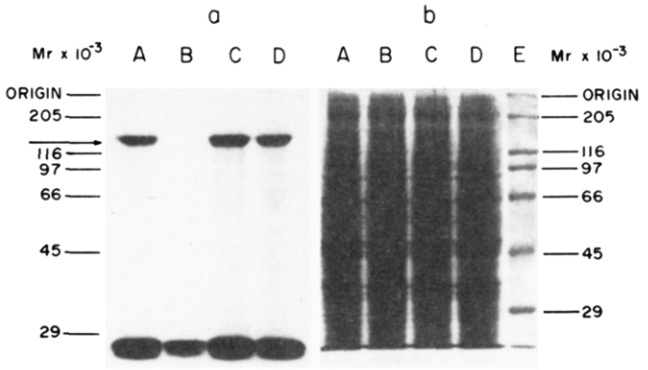

FIGURE 5: (a) Autoradiogram showing photoaffinity labeling of ANF receptor on cultured Leydig tumor cells with 4-azidobenzoyl-^{125}I-ANF IV. AZB-^{125}I-ANF IV was allowed to bind to the cells in the dark in the absence (lane A) or in the presence of unmodified ANF IV (lane B), atriopeptin I (lane C), or angiotensins I, II, and III (lane D), each at 1 μM concentration. After washing with fresh medium, cells were photolyzed. Details are described under Materials and Methods. The arrow indicates the radioactive band specifically labeled. (b) Coomassie Blue stained pattern of the gel used for the autoradiogram shown in (a). Lanes A–D correspond to lanes A–D in (a). Lane E shows electrophoresis of the standard proteins (Sigma, St. Louis, MO) myosin, β-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, and carbonic anhydrase, with their molecular weights indicated on the right margin.

cellular cAMP. The specificity of the binding was indicated by the competition binding of unlabeled ANF IV against ^{125}I-ANF IV. Such competition was not observed with angiotensin II, bLH, or hCG.

A photoaffinity labeling reagent for ANF receptor, AZB-^{125}I-ANF IV, was prepared by reacting ANF IV with succinimido 4-azidobenzoate followed by radioiodination. After each of the above synthetic steps, the peptide derivative was purified by HPLC and characterized. These procedures allowed clear identification of the final product, AZB-^{125}I-ANF IV. The identification of radioiodinated photoaffinity reagents is difficult and often ambiguous in the usual synthetic protocols where both the azido derivatization and radioiodination reactions are carried out in one mixture.

The photoaffinity labeling reaction with AZB-^{125}I-ANF IV on the intact murine Leydig tumor cells yielded essentially a single radiolabeled protein band in SDS gel electrophoresis with an apparent M_r of 135 000. The strong radioactive band coinciding with the dye front is likely to be the photolysis product of AZB-^{125}I-ANF IV which failed to make covalent linkage to the ANF receptor. The specificity of labeling was demonstrated by the protection of the M_r 135 000 protein from the labeling by unmodified ANF IV but not by atriopeptin I or by unrelated peptide angiotensins. The lack of protection by atriopeptin I is consistent with the fact that this peptide has much reduced natriuretic and vasorelaxant activities (Currie et al., 1984; Sugiyama et al., 1984) and weak binding affinity to adrenal ANF receptor (Misono et al., 1985) as compared to larger ANF peptide species.

The minimal levels of nonspecific labeling observed in the present study are in clear contrast to our previous photoaffinity labeling studies (Misono et al., 1985) and those of others (Yip et al., 1985; Vandlen et al., 1985), where significant levels of nonspecific radiolabeling were observed. In those studies, photolysis was carried out without removal of unbound photoaffinity reagents following the incubation with plasma membranes in the dark. The reaction mixtures also contained exogenously added proteins such as bovine serum albumin and bacitracin. Therefore, it is probable that the radiolabeled bands that are not protected from labeling by unmodified ANF have resulted mainly from nonspecific reactions of the photoactivated reagents with those exogenously added proteins acting as scavengers. Nevertheless, the significant levels of nonspecific labeling in these previous studies have left a distinct possibility that a specifically labeled band (or bands) may have been obscured under the nonspecific radiolabeled bands (Meloche et al., 1986). In the present study, on the other hand, AZB-^{125}I-ANF IV that was not bound to MA-10 cells after the incubation in the dark was removed by thorough washing with fresh medium prior to photolysis. This washing evidently eliminated nearly all nonspecific labelings, yielding essentially a single radiolabeled protein species of a high molecular weight. In addition, the yield of the receptor labeling of 0.5–1% is in the range typical of photoaffinity labeling of receptors on intact cells. These results strongly indicate that ANF receptor consists of a single polypeptide chain with an apparent M_r of 135 000.

Photoaffinity labeling studies have shown the specific labeling of a single protein band of ANF receptor in plasma membranes of bovine and rat adrenal cortex with an apparent M_r of about 125 000 (Misono et al., 1985) and of kidney cortex with M_r 140 000 (Yip et al., 1985). These results are consistent with present findings of MA-10 cells where a specific protein band with M_r 135 000 was labeled. In contrast, affinity labelings of additional protein bands, including major radiolabeled protein species with M_r of 60 000–70 000, have been reported by photoaffinity labeling (Vandlen et al., 1985) or affinity cross-linking of ANF receptors in various plasma membrane preparations (Hirose et al., 1985; Vandlen et al., 1985; Meloche et al., 1986) or with intact cultured cells (Schenk et al., 1985). The reason for the discrepancy is not immediately clear. It may have reflected differences in the tissue or species studied or in the method of plasma membrane or cell preparations. Nevertheless, the nearly exclusive labeling of a specific protein band with a M_r of 135 000 observed in the present study strongly indicates that ANF receptor exists mostly in a form of larger M_r species in these cells.

It is intriguing that the binding of ^{125}I-ANF IV to MA-10 cells was very rapid, reaching the maximum in 5 min, as compared to cultured vascular smooth muscle cells, where maximum binding has been reported to occur in 90 min (Hirata et al., 1985). The fast decrease in cell-bound radioactivity during incubation of cells at 37 °C with ^{125}I-ANF IV indicated that the cell surface ANF-receptor complex may be quickly metabolized. Such a decrease was not observed when cells are incubated at 4 °C. To study the nature of the radioactive materials released into medium during the incubation of the cells at 4 and 37 °C, both cell extracts and culture media were analyzed by a reverse-phase column on an HPLC system. Medium of the cells incubated at 37 °C showed no radioactive peak at the position corresponding to ^{125}I-ANF. On the contrary, an extract of the cells incubated at 4 °C gave a major radioactive peak at the position corresponding to ^{125}I-ANF IV. Also, the medium of the cells incubated at 37 °C showed a single radioactive peak that was eluted at a position identical with that of the authentic monoiodotyrosine derivative. Together, these results indicated that at 37 °C most of the cell-bound ^{125}I-ANF is rapidly degraded and the products are released into the medium, while at 4 °C the ligand minimally dissociates from the surface of the cell. Augmentation of cell-bound ^{125}I-ANF by the pretreatment of cells with lysomotropic agents or with inhibitors of metabolic energy production suggests that the turnover of ANF–receptor complex occurs through an energy-dependent endocytotic mechanism involving lysosomal degradation. While this work was in

progress, Hirata et al. (1986) reported similar enhancement of apparent binding of radioiodinated ANF to cultured vascular smooth muscle cells by these types of reagents. These compounds have also been found to inhibit the intracellular degradation of ^{125}I-labeled ligands bound to the receptor in cultured cells (Goldstein et al., 1975; Carpenter & Cohen, 1976). In addition to the lysosomal mechanism, it is possible that the turnover of the ANF–receptor complex may also take place through nonlysosomal mechanism(s) involving endosomal apparatus, which is (are) known to participate in the endocytotic metabolism of ligand–receptor complexes (Wolkoff et al., 1984).

In summary, we have identified specific ANF receptor in testicular interstitial tumor cells, which indicates a new target site for biological action of ANF. Data indicate that, after binding of ANF to the cell surface receptor, the ligand–receptor complex is rapidly internalized into the cell. ANF is hydrolyzed in the lysosome, and degraded products are released into culture media. The presence of high-affinity, specific ANF receptors at a high density coupled with the intriguing action of ANF in this established tumor cell line has provided a unique model system for ongoing research of the molecular mechanism of ANF action and the molecular properties of its receptor molecule.

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Registry No. ANF, 90052-57-6; I-ANF, 105229-66-1; ^{125}I-ANF, 105229-68-3; AZB-ANF, 105229-69-4; AZB-I-ANF, 105229-67-2; AZB-^{125}I-ANF, 105250-93-9; AZB, 53053-08-0; atriopeptin, 85637-73-6.

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