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Analysis of the Adrenal Angiotensin II Receptor with the Photoaffinity Labeling Method†

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ABSTRACT: The angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, AT) receptor of bovine adrenocortex has been investigated with photosensitive analogues of AT. In a first series of experiments, we have shown that isolated cortical cells secrete aldosterone in a permanent and specific manner if they have been photolyzed in the presence of the photolabel [Sar^1,(4′-N_3)Phe^8]AT. This permanent stimulation is in contrast to the smooth muscle assays where under similar conditions a permanent and specific block was always observed. It is assumed that the irreversible occupation of the AT receptor produces this effect. In a second type of experiment, we have shown that the AT binding site on adrenocortical membranes can be specifically and irreversibly occupied under similar conditions and that this occupation can be prevented in a competitive manner by the presence of nonphotosensitive hormone. Using a radioactive label, [Sar^1,(3′-^{125}I)Tyr^4,(4′-N_3)Phe^8]AT, we have identified the AT receptor as a 300-kDa protein by means of gel filtration under nonreducing and nondenaturating conditions. Under reducing and denaturating conditions, a subunit of 60 kDa was found by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration. The AT receptor is proposed to be a 300-kDa protein with one binding subunit of 60 kDa.

The isolation and purification of peptide hormone receptors are the goals of continuing research efforts. Until recently, all the successful isolations [e.g., Jacobs et al. (1977), Welton et al. (1977) and McIntosh et al. (1976)] were of receptors that retained hormone binding ability after solubilization of the cell membrane. This ability permitted the detection of the receptor protein fractions and its purification with affinity-mediated procedures (affinity chromatography, affinity centrifugation). Unfortunately, several peptide hormone receptors lose their binding ability upon total solubilization and cannot be isolated by these straightforward methods. For example, the homogenization, solubilization, and subsequent isolation of the angiotensin II (AT)[1] receptor has often been attempted, but with a few exceptions, preparations from adrenals and blood vessels have always lost AT affinity upon addition of detergent (Devynck et al., 1974; Forget & Heisler, 1979). AT and its receptor have affinities in the range of 10^{-9} M and rapid

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[1] Abbreviations: AT, angiotensin II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; RIA, radioimmunoassay; kDa, kilodaltons; BSA, bovine serum albumin; ^{3}H-AT, [Sar^1,(4′-NH_2,3′,5′-$^3H_2$)Phe^4]AT; N_3-AT, [Sar^1,(4′-N_3)Phe^8]AT; ^{125}I-N_3-AT, [Sar^1,(3′-^{125}I)Tyr^4,(4′-N_3)Phe^8]AT; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

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dissociation kinetics that do not allow the extraction of a preformed hormone–receptor complex.

A technique that circumvents the aforementioned problems is the photoaffinity labeling procedure, a method that allows specific and covalent labeling of the receptor in the native state and subsequent isolation of the labeled hormone–receptor complex. With this method, we have previously carried out pharmacological studies with photosensitive AT analogues on rabbit aorta and other tissue strips and found irreversible effects indicating an irreversible hormone–receptor interaction (Escher et al., 1978; Escher & Guillemette, 1979). Other researchers have found identical results with the same method and have confirmed our results (Galardy & LaVorgna, 1981).

However, attempts to label the AT receptor on vascular tissues with a radioactive photolabel failed because the collagen- and elastin-rich structure of rabbit aorta prevented a successful homogenization and solubilization of this tissue (Bernier, 1980). In this study, we present the effects of photolabile AT analogues on (a) the aldosterone secretion of bovine adrenal glomerulosa cells, (b) the binding of AT to isolated cell membranes of the same cells, and (c) the labeling of these AT receptors with radioactive photolabels and the subsequent analysis of this receptor.

A similar report has already been presented (Capponi & Catt, 1980) where the adrenal and the uterine AT receptor has been identified to be in both tissues a 126-kDa protein with a proposed binding subunit of 64 kDa. However, no pharmacological test on the same tissues except the competitive nature of the labeling permitted identification of the labeled proteins as the AT receptor.

Experimental Procedures

Materials. The peptides used in our experiments were [Sar^1,Val^5]angiotensin II and its analogues (all synthesized in our laboratory). The radioactive peptide [Sar^1,(4′-NH_2-3′,5′-$^3H_2$)Phe^4]AT (^{3}H-AT) was produced by catalytic tritiation of [Sar^1,(3′,5′-I_2-4′-NH_2)Phe^4]AT in France by Dr. J.-L. Morgat, CEN Saclay, as described by Escher et al. (1978). Collagenase (CLS type I) used for the isolation of glomerulosa cells was purchased from Worthington Biochemical Corp., Freehold, NJ. DNase and BSA were purchased from Sigma Chemical Co., St. Louis, MO. Medium 199 (with Earle's salts) used for the incubation of glomerulosa cells was purchased from Flow Laboratories, Mississauga, Ontario. Specific antibodies against aldosterone were obtained from Radioassay System Laboratories Inc., Carson, CA. The irradiation equipment consists of four mercury vapor lamps (Westinghouse JC Par-38, 100 W) capped with Raymaster 5-in. black-light filters (G. W. Gates, Long Island, NY) and are arranged around a thermostated bath. Further filtration through the Pyrex glass and the bath fluid ensures that only light of wavelengths in a narrow band around 365 nm reaches the tissues. For the flash-photolysis experiment, a bath with a built-in tubular black-light filter (type 5040, G. W. Gates) is surrounded with a helical flash lamp (type L-3071, length 74 cm, inner quartz tube diameter 6 mm, mercury-doped xenon filling, pulse width 100 μs, maximal lamp discharge 1000 J). A corresponding power supply of 15-kV charging capacity for single operation was purchased from the same company (ILC Technology, San Carlos, CA). For standard operation, the lamp was fired with 12 kV. Chemicals for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemical used were of reagent grade. Protein molecular weight standards used for calibration of polyacrylamide gels and column chromatography gels (Sephadex G-200 and agarose Bio-Gel A-5m) were purchased from Pharmacia Fine Chemicals, Sweden.

Preparation and Incubation of Adrenal Cells. Bovine adrenal glands were obtained from a nearby slaughterhouse immediately after slaughtering and kept in ice-cold saline until dissected free of adipose tissue. The glomerulosa cell preparation is carried out as described by Douglas et al. (1978). In brief, the adrenals are dissected, and the capsular (glomerulosa) portion is separated from the decapsulated (fasciculata reticularis) portion. The glomerulosa cell suspension is prepared by preincubating the capsules at 37 °C for 15 min in medium 199 containing collagenase (2 mg/mL) and DNase (25 μg/mL). After physical dispersion (Fredlund et al., 1975), the cell suspension is decanted and centrifuged at 100g for 15 min at room temperature. The cell pellets are resuspended in medium 199 with Earle's salts containing BSA (2 mg/mL). Approximately 1 000 000 viable cells (Trypan Blue test) are dispersed from one gland. For studies on aldosterone production, approximately 100 000 cells are incubated for 2 h at 37 °C under 95% O_2–5% CO_2 with different concentrations of AT or analogues. The cells are then sedimented by centrifugation at 1500g for 15 min, and aliquots of the supernatant media are assayed for aldosterone by RIA (Radioassays System Laboratory, Carson, CA). For the photoaffinity labeling experiments, the cells are first incubated in the presence of photosensitive peptide [Sar^1,(4′-N_3)Phe^8]AT (N_3-AT) for 15 min at 37 °C and then irradiated for 30 s with the described equipment and washed 3 times by centrifugation at 100g for 10 min. Finally, the cells are resuspended in medium 199 with BSA (2 mg/mL) and AT or saline for 2 h at 37 °C under an O_2–CO_2 atmosphere. The aldosterone secretion capacity is evaluated by RIA.

Preparation of Subcellular Particles. The preparation of subcellular particles from capsules of bovine adrenal glands was carried out as described by Glossmann et al. (1974). In brief, the glomerulosa layer (see the preceding procedure) is scratched from the capsule with the reverse side of a scalpel blade and homogenized with medium A (sodium bicarbonate, pH 7.0, 20 mM) in a homogenizer (Teflon-coated glass) with two strokes of the tight pestle. The homogenate is stirred for approximately 15 min and subsequently filtered through cheesecloth. The filtered homogenate is spun at 1500g for 10 min and decanted, and the supernatant is spun at 20000g for 30 min. The 20000g pellet is resuspended by gentle homogenization in 0.25 M sucrose buffered with medium B (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 7.2) and layered onto a discontinuous sucrose gradient (31.5%, 38.5% w/w sucrose in medium B) and spun for 2 h in a Beckman SW 28 rotor at 22 000 rpm. After sucrose gradient centrifugation, the band of particulate material is collected at the top of the 31.5% (w/w) sucrose layer, diluted 20-fold with medium A, and sedimented at 40000g for 40 min. All steps are performed between 2 and 4 °C. The microsomal membranes are resuspended in assay buffer (120 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.2% BSA) and used immediately for binding experiments or kept frozen at −25 °C until used.

Synthesis and Purification of [*Sar*^{1},(3′-^{125}I)*Tyr*^{4},(4′-N_3)-*Phe*^{8}]*AT* (^{125}I-N_3-*AT*). Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, Pierce Corp.) in 10 μL of chloroform is pipetted into a 100-μL polyethylene reaction vessel and allowed to evaporate. This leads to a plating of 0.5 μg of iodogen onto the wall of the vessel. To this were added 50 μg of N_3-AT ([Sar^1,(4′-N_3)Phe^8]AT; Escher et al., 1978) in 25 μL of 0.2 M CH_3COOH and 1 mCi of Na^{125}I, and the reaction was allowed to proceed at room temperature for 15 min. The reaction was stopped by adding 25 μL of Na_2SO_3 (1 M),

Table I: Biological Activities of [Sar^1]AT and of N_3-AT[a]

substrate	steroidogenesis			binding		rabbit aorta		
	ED_{50} (M)	α^E	n	K_D (M)	n	ED_{50} (M)	α^E	n
[Sar^1]AT	$(1.4 \pm 0.8) \times 10^{-9}$	1.0	5	$(3.48 \pm 2.45) \times 10^{-9}$	28	$(5.9 \pm 1.2) \times 10^{-10}$	1.0	21
[Sar^1,(4'-N_3)Phe^8]AT	$(2.0 \pm 0.9) \times 10^{-9}$	0.96 ± 0.12	4	$(6.49 \pm 3.24) \times 10^{-9}$	9	$(1.9 \pm 0.4) \times 10^{-9}$	1.0	9

[a] The biological effects of [Sar^1]AT and its photolabile analogue are evaluated on isolated bovine adrenocortical glomerulosa cells for aldosterone production, on purified membranes of the same cells for the binding potency, and on rabbit aorta strips for the myotropic action. ED_{50} is the molar dose that produces half-maximal response, K_D is the affinity constant calculated according to Bennett (1978). α^E is the intrinsic activity as described by Ariens (1964), and n is the number of determinations. Maximal stimulation of steroidogenesis was 215 ± 23 ng/mL of aldosterone; nonstimulated production was 35 ± 10 ng/mL (steroidogenic effect significant, $p < 0.01$).

diluted with 100 μL of KI (1 M) and purified on a HPLC apparatus according to a recently published procedure (Escher, 1983). The fractions corresponding to the elution volume of the monoiodinated analogue are pooled, diluted with 10 mL of saline, and lyophilized. The radioactive peptide was redissolved with water, split in several fractions, and kept frozen until used.

Photoaffinity Labeling with Radioactive Labels. Purified adrenal microsomal membranes, approximatey 2 mg, are incubated in the dark in a 15-mL culture tube at 22 °C in 1 mL of assay buffer (120 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.2% BSA), 100 μL of ^{125}I-N_3-AT (5×10^6 cpm or 5×10^{-12} mmol), and 100 μL of L-4'-aminophenylalanine (10 μg, to act as a scavenger for the radicals generated during the photolysis). Nonradioactive AT (10^{-4} M) is added where appropriate to determine nonspecific binding. After the incubation period, the membrane suspension is diluted with 7 mL of ice-cold saline and exposed for 2 min to UV light. The photolabeled membranes are washed from unbound peptides by dilution with 40 mL of saline (22 °C) and centrifuged for 15 min at 20000*g*. The pellet is washed again with saline, is resedimented, and is ready for solubilization for column chromatography or gel electrophoresis. For SDS–PAGE the pellet is solubilized and reduced in 250 μL of a buffer containing 5 mL of glycerol, 10 mL of 5 mM EDTA, 10 mL of 10% SDS, 2.5 mL of β-mercaptoethanol, 0.5 mg of bromophenol blue, and 10 mL of 50 mM Tris-HCl, pH 8.0, in a final volume adjusted to 50 mL with water. The solubilized proteins are heated for 5 min at 100 °C, cooled, and kept frozen at −25 °C until used. SDS–PAGE of the solubilized membranes is performed on slab gels, by the method of Laemmli (1970), with 7.5% or 10% acrylamide. For gel filtration, the pellet of labeled membranes is solubilized in 100 μL of a buffer containing 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1% Triton X-100, and 0.01% sodium azide. After 30 min at room temperature, the Triton X-100 concentration is diluted to 0.1%, and the proteins are ready to be eluted on a column of 1 cm × 50 cm containing Sephadex G-200 or agarose gel (Bio-Gel A-5m, Bio-Rad). Void volume (V_0) and total volume (V_t) are determined with blue dextran and 3'-nitrotyrosine, respectively. Protein molecular weight standards (Pharmacia) used for calibration of the columns and of SDS–PAGE are the following: ribonuclease A, 13 700; α-lactalbumin, 14 400; trypsin inhibitor, 20 100; chymotrypsinogen A, 25 000; carbonic anhydrase, 30 000; lactate dehydrogenase subunit, 36 000; ovalbumin, 43 000; albumin, 67 000; phosphorylase *b*, 94 000; aldolase, 158 000; catalase, 232 000; ferritin, 440 000; ferritin subunit, 220 000; thyroglobulin, 669 000; thyroglobulin subunits, 330 000. 7.5% SDS–PAGE calibration gave a good linear correlation between 30 000 and 200 000 daltons. 10% SDS–PAGE was linear between 13 000 and 100 000 daltons. Sephadex G-200 was found to be linear between 20 000 and 200 000 daltons and Bio-Gel A-5m between 160 000 and 670 000 daltons. All molecular weights from gel filtrations are understood as molecular sizes, depending on the Stokes radius.

Table II: Influence of Photolabeling on Aldosterone Production[a]

treatment	aldosterone production	
	basal secretion	challenged with 10^{-8} M AT
(1) only washed	24 ± 15	148 ± 22
(2) 10^{-8} M N_3-AT, photolyzed and washed	116 ± 27[b]	129 ± 23
	118 ± 27[c]	158 ± 31
(3) 10^{-8} M N_3-AT, washed	32 ± 19	143 ± 27
(4) 10^{-8} M AT, washed in the presence of 10^{-8} M AT (continuous challenge)		117 ± 31

[a] This represents one typical experiment carried out in triplicate; three additional experiments, each also in triplicate, produced identical results. Dispersed glomerulosa cells are incubated at 37 °C in medium 199 with Earle's salt, BSA (2 ng/mL), and the indicated peptide for 15 min and irradiated. Washings were carried out by three centrifugations (100*g*, 10 min) and resuspensions in the same medium (treatments 1–3 are without peptide). For the aldosterone secretion, aliquots of 350 000 glomerulosa cells are incubated after the above treatments for 2 h in presence or absence of 10^{-8} M AT. The aldosterone contents are evaluated by RIA. [b] Irradiated for 30 s. [c] Flash photolysis.

Results and Discussion

AT and the photosensitive N_3-AT have a similar steroidogenic effect that is readily reversible by washing the glomerulosa cells after incubation, and both peptides have a similar steroidogenic potency with a half-maximal dose in the 10^{-9} M range, as seen in Table I. The maximal secretion level of glomerulosa cells is not changed by UV irradiation up to 1 min; after 5 min, a significant decrease is observed. If the cells are incubated with N_3-AT and irradiated for 30 s or flashed, washed several times, and reincubated with saline, an important aldosterone production is observed. If the cells are (a) incubated with AT, not irradiated, and washed, (b) incubated with prephotolyzed N_3-AT and washed, (c) incubated with N_3-AT and not irradiated but washed, (d) incubated with saline only and not irradiated but washed, or (e) incubated with saline and irradiated, similar low aldosterone production was observed in all cases from (a) to (e) (see Table II). If, however, an identical batch of glomerulosa cells was incubated and washed in constant presence of AT, an aldosterone production is observed that is very similar to the experiment of N_3-AT in presence of UV. If glomerulosa cells were incubated with saline, washed, and then for the first time brought into contact with 10^{-8} M AT, a somewhat higher aldosterone production was observed (see Table II). These results indicate that the irradiation experiments with N_3-AT produce cells that behave like cells that are stimulated in permanence by 10^{-8} M AT. (The only experiment we were not able to carry out was the competitive protection of 10^{-8} M N_3-AT with 10^{-5} M AT during the photolysis because such high concentration of

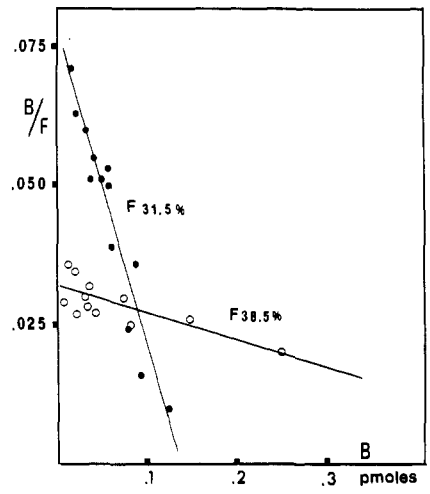

FIGURE 1: Scatchard plot of different adrenal membrane fractions at 22 °C: (full circles) membrane fraction from the 31.5% sucrose layer; (open circles) membrane fraction from the 38.5% sucrose layer. (Ordinate) Specifically bound over free radiolabel ^{3}H-AT; (abscissa) specifically bound radiolabel in pmol/mL. Each point is the mean of three determinations; the lines are calculated linear regressions. (●) $K_1 = 1.4 \times 10^{-8}$ M and $c_1 = 135$ fmol/50 μg of protein; (○) $K_2 = 1.8 \times 10^{-7}$ M and $c_2 = 675$ fmol/300 μg of protein.

AT cannot be washed off sufficiently fast and more washing cycles affect the isolated glomerulosa cells.) Our reasonable explanation is that N_3-AT irradiated on the cells can stimulate continuously the aldosterone production. (Continuous means what is measurable within the limits of such a bioassay.) We attribute this effect to a covalent attachment of N_3-AT to the AT receptor, a claim that is supported by the results shown below.

It is interesting to note that at the adrenal, a secretory system, the permanent occupation of AT receptors results in prolonged secretion as observed earlier with other secretory systems (Galardy et al., 1980; DeGraan & Eberle, 1980; Brandenburg et al., 1980). The same interaction between a photosensitive peptide and the AT receptor on smooth muscle produces a specific block of the myotropic action of AT (Escher et al., 1978; Escher & Guillemette, 1979). This opposite effect could be explained either by the existence of two kinds of AT receptors or by different intracellular hormonal message transduction mechanisms, an explanation which we favor.

Binding studies with purified bovine adrenal microsomes, carried out according to Glossmann et al. (1974), indicate the well-documented presence of two saturable binding sites. In our experiments, a K_{d_1} of $1.15 \pm 0.42 \times 10^{-8}$ M and a K_{d_2} of $3.0 \pm 0.2 \times 10^{-8}$ M was found from eight triplicate determinations. The mean nonspecific binding was found to be 16.15 ± 4.55% from the total binding. With the discontinuous sucrose gradient of 31.5% and 38.5% (see Experimental Procedures), we succeeded in four triplicate experiments to obtain on top of the 31.5% layer a membrane fraction that contained only the high-affinity site and another fraction on top of the 38.5% layer that produced a linear Scatchard plot with a K_d equal to $9.9 \pm 7.1 \times 10^{-8}$ M, corresponding to the low-affinity site. Such a triplicate experiment is presented in Figure 1. In order to evaluate the irreversible effect observed with intact glomerulosa cells, we carried out a similar study on the binding capacity of microsomal membranes. Membranes are photolyzed for 2 min in the presence of N_3-AT (10^{-8} M) together with various amounts of AT (0, 10^{-9}, 10^{-8}, and 10^{-7} M), washed extensively by centrifugation and resuspension, and are incubated with 20000 cpm of ^{3}H-AT. An aliquot of these membranes, not irradiated but washed equally, is capable of binding 445 ± 48 cpm specifically, which is about the same amount as before the washing operations and represents 100% of the binding capacity in this experiment. In the first photoinactivation experiment without AT and only with N_3-AT, 52% of this binding capacity is lost, probably due to an irreversible attachment of N_3-AT. In the presence of 10^{-9} M AT, this loss is reduced to 41%, at 10^{-8} M AT to 20%, and at 10^{-7} M AT to 8.5%. These percentages indicate a progressive protection of the AT receptor by AT from photoinactivation by N_3-AT. No binding-capacity loss was observed if the membranes were either only irradiated for 2 min or only treated with N_3-AT or prephotolyzed N_3-AT and not irradiated.

This second experiment shows that N_3-AT together with UV light can specifically and irreversibly block binding sites of AT, presumably the receptor. Having found an AT-specific, irreversible (or strongly prolonged) effect of the concomitant application of N_3-AT with UV irradiation, we carried out a study with a radioactive photolabel in order to identify the apparent molecular weight of the AT receptor. Purified microsomal membranes are labeled either with 2×10^{-9} M [Sar^1,(4′-N_3-3′,5′-$^3H_2$)Phe^8]AT (Escher & Bernier, 1980) or [Sar^1,(3′-^{125}I)Tyr^4,(4′-N_3)Phe^8]AT (Escher, 1983) in presence of UV irradiation, which should result in the specific and covalent labeling of the membranal receptor. After labeling, solubilization, and reduction of the membranal proteins, the migration of the radioiodinated or tritiated proteins is evaluated on SDS–PAGE. The gel is cut into small slices of equal thickness and assayed for its radioactivity either with liquid scintillation counting for tritium or directly in the gamma counter for iodine-125.

With the tritiated label, a weak peak was observed at 53 ± 4.7 kDa (three experiments on SDS–7.5% PAGE, 920 ± 180 cpm vs. a background of 480 ± 120 cpm). In presence of 10^{-5} M AT during photolysis, no such peak was observed. The higher specific radioactivity of the radioiodinated label permitted one to carry out the same experiment with a much higher yield. An example of a SDS–7.5% PAGE is shown in Figure 2. In five such experiments, a molecular weight of 58.6 ± 2.5 kDa was found. The higher cross-linked SDS–10% PAGE produced in eight experiments a molecular weight of 63 ± 3 kDa, and Figure 3 represented the densitometric evaluation of an example. In all these SDS–PAGE experiments, the membrane proteins were reduced with 5% of β-mercaptoethanol, denatured by heating to 100 °C, and solubilized by 1% SDS. The somewhat broad peak on graph 3 of Figure 3 is due to radiation defocalization resulting from the gel and film thickness (1.5 mm).

The radiolabeled membrane proteins are solubilized without reduction and denaturation for chromatography on agarose and Sephadex gels. on Bio-Gel A-5m, an important peak at 297 ± 21.9 kDa or with a Stokes radius of 6.10 ± 0.45 nm is observed (eight experiments with a K_{av} of 0.39 ± 0.01); one experiment is shown in Figure 4. Sephadex G-200 chromatography shows in five experiments a peak at 275 ± 48 kDa with a much less precise K_{av} of 0.175 ± 0.015. However, if the labeled membrane proteins are reduced and denatured as for electrophoresis, the smaller subunit is found at 64.3 ± 16 kDa with a Stokes radius of 3.45 ± 0.45 nm (four experiments, K_{av} of 0.43 ± 0.01).

These results only partially confirm those obtained earlier by Capponi & Catt (1980) and Paglin & Jamieson (1982). The first have observed an AT binding protein at 65 kDa but also a larger one at 125 kDa from dog adrenal and dog uterus;

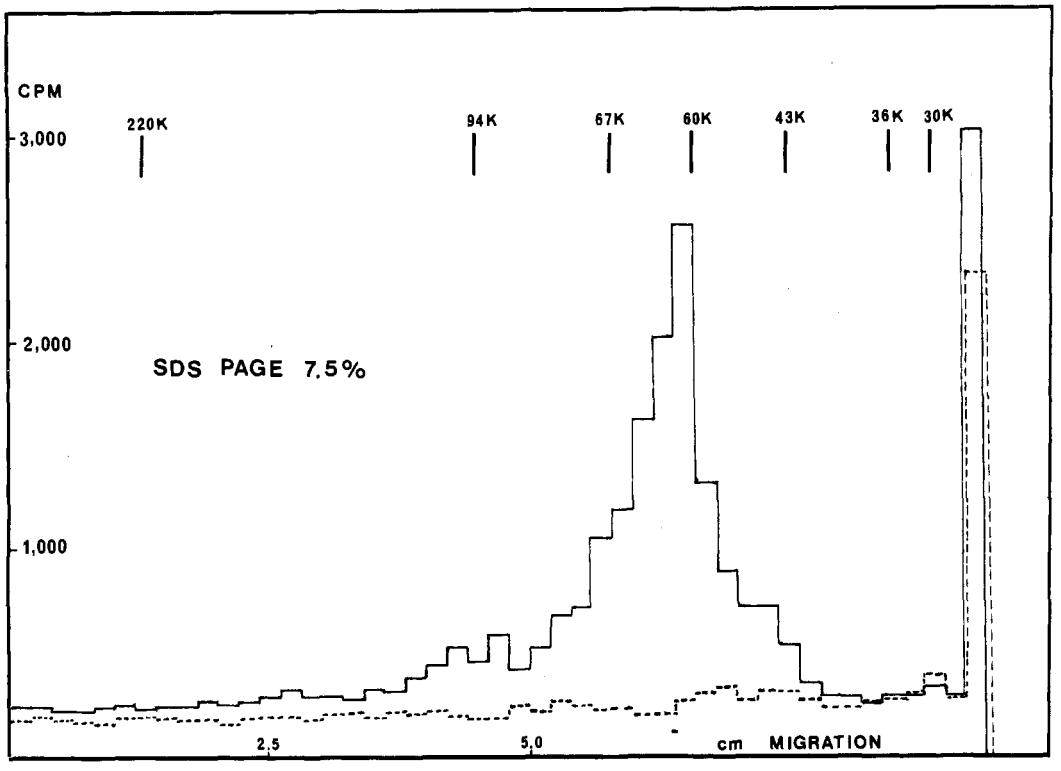

FIGURE 2: Electrophoretic profile of photolabeled membrane protein on SDS–7.5% PAGE. The slab gel is cut into 2-mm slices that are counted, and the radioactivity of the treated membrane proteins is illustrated as a function of migration. The solid line is the photolabeling experiment with ^{125}I-N_3-AT; the dashed line is the same but in the presence of 10^{-4} M AT as competitive protection. The molecular weight standards are indicated in kilodaltons. The peak below 30 kDa corresponds to the unbound low molecular weight photolabel.

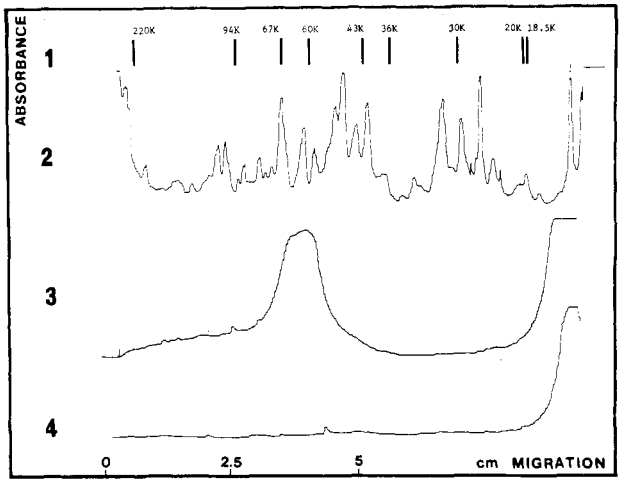

FIGURE 3: Densitometric spectra of the electrophoretic profile on SDS–10% PAGE. Graph 1 represents the standard proteins and molecular weights are expressed in kilodaltons (K). Graph 2 represents the densitometric evaluation of the Coomassie blue staining of the labeled membranes. Graph 3 represents the densitometric evaluation of a SDS–PAGE autoradiography. This graph represents membrane proteins labeled in the absence of 10^{-4} M AT. Graph 4 represents the densitometric evaluation of a SDS–PAGE autoradiography of membranes labeled in presence of 10^{-4} M AT. The peak at the end of the gel represents the free tracer as in graph 3.

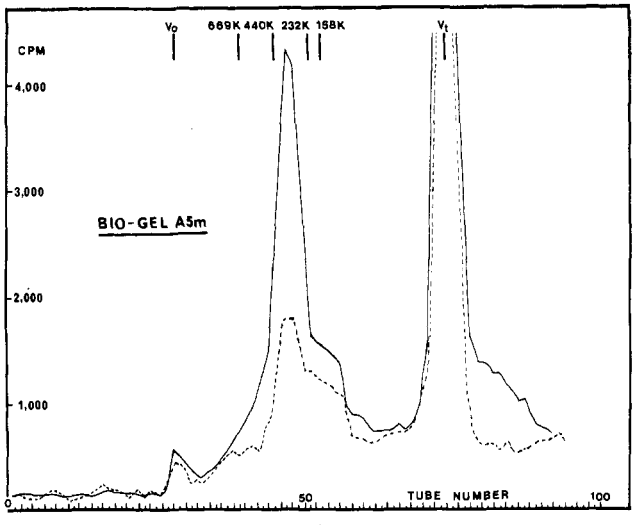

FIGURE 4: Radioactivity elution profile of labeled membranes on agarose gel. The column was 1.0 × 55.0 cm containing Bio-Gel A-5m, eluted with a constant speed of 0.075 mL/min and collected every 10 min. Positions of the standards are indicated between the void volume (V_0) and the total volume (V_t) of the column. The solid line is the photolabeled preparation; the dashed line is the preparation labeled in the presence of 10^{-4} M AT.

the latter report mainly a 116-kDa protein from chemical cross-linking experiments with AT on rat adrenal membranes. We are unable to confirm the 125 or 116-kDa protein, respectively, in any one of our experiments, and we find instead a larger protein, probably the entire AT receptor, at 300 kDa. The reported 125-kDa protein was proposed as a possible dimer of the 65-kDa protein and could therefore still be a part of our larger 300-kDa protein. In order to test this possibility, we have carried out a last type of experiments where the labeled proteins are mildly reduced and denatured (three experiments, 60 °C and 1% β-mercaptoethanol, before chromatography on Bio-Gel A-5m). This procedure is used to reduce ferritin (440 kDa) to its 220-kDa subunit and does not proceed to the complete breakdown to the 18.5-kDa monomer (Bryce & Crichton, 1971)). Under these conditions only two peaks are observed: the complete protein at 300 kDa and a second at about 60 kDa (nonlinear area, K_{av} = 0.74), which would coincide with the fully reduced and denatured protein. This result indicates that the AT receptor of bovine adrenal cortex is a large 300-kDa protein with probably only one AT binding subunit of 60 kDa. Several other peptide hormone

receptors are reported to have molecular weights in this region, e.g., 286 kDa for the thyrotropin receptor (Tate et al., 1975), 280–350 kDa for insulin receptors (Massague et al., 1980), and 270 kDa for nerve growth factor (Costrini et al., 1979). A molecular weight of 300 kDa for the AT receptor is therefore quite reasonable.

Conclusions

Aldosterone secretion experiments with bovine adrenal glomerulosa cells show that AT and N_3-AT produce a significant steroidogenic effect at concentrations in the order of 10^{-9} M. These effects are reversible when the experiments are done in absence of UV light. It is likely that photolabeling of the adrenal glomerulosa cells with N_3-AT (10^{-8} M) produces a covalent link between N_3-AT and the AT receptor; this link results in a prolongated (permanent) secretion of aldosterone suggestive of the Ariens theory of occupation (Ariëns & Beld, 1977). In the smooth muscle tests with the same label (Escher et al., 1979) or similar labels (Galardy & LaVorgna, 1981), always a specific and permanent inhibition of this response was observed, a result that is rather suggestive for the Paton-rate model (Paton, 1961). An answer to these differing results is not yet possible and needs further knowledge of the functional structure of the AT receptor.

Binding studies with purified adrenal vesicular membranes demonstrate the existence of a specific and reversible high-affinity binding site for [Sar^1]AT and for N_3-AT. Photoinactivation experiments with this system and N_3-AT produce a specific and irreversible loss of binding capacity of vesicular membranes for ^{3}H-AT. Photolabeling of the membranes with ^{125}I-N_3-AT followed by solubilization of the membrane proteins permits one to identify the AT receptor by means of gel filtration and polyacrylamide gel electrophoresis as a protein of 300 000 daltons with a binding subunit of 60 000 daltons. When the membrane proteins are mildly denatured and reduced, only two species can be seen (300 000 and 60 000 daltons), demonstrating that the AT receptor contains probably only one 60 000-dalton subunit, presumably the AT binding protein.

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Registry No. AT, 4474-91-3; aldosterone, 52-39-1; [Sar^1,(4′-N_3)Phe^8]AT, 87262-02-0.

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