Photoaffinity labelling of atrial natriuretic factor (ANF)-R1 receptor by underivatized ^{125}I-ANF

Involvement of lipid peroxidation

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In bovine adrenal zona glomerulosa, atrial natriuretic factor (ANF) exerts its physiological effect through high-affinity binding to specific membrane receptors. On studying further the molecular properties of the ANF receptor binding domain, we have observed that incubation of intact or solubilized bovine adrenal zona glomerulosa membranes with ^{125}I-ANF-(99–126) followed by u.v. irradiation results in the irreversible labelling of a 130 kDa protein corresponding to the ANF-R1 receptor. This process is time-, protein- and ^{125}I-ANF-dependent. The apparently covalent nature of this complex is documented by its resistance to heat, guanidine hydrochloride, urea and trichloroacetic acid denaturation. Photolabelling with underivatized ^{125}I-ANF is much more efficient with the ANF-R1 than with the ANF-R2 receptor. After photolysis, the covalently linked ^{125}I-ANF is still sensitive to digestion by carboxypeptidase A, suggesting that ANF is linked by its N-terminal end to the receptor upon u.v. irradiation and that its C-terminal end is still freely accessible. Aerobic conditions and lipids are required for the photolabelling, suggesting a role in this process for malondialdehyde, a highly reactive secondary product associated with u.v.-induced lipid peroxidation. This simple method should provide a powerful tool in the accurate characterization of the hormone-binding domain of the ANF receptor.

INTRODUCTION

Atrial natriuretic factor (ANF) is a peptide, secreted mainly by cardiomyocytes, which affects a large variety of target cells by interacting with high-affinity specific membrane receptors [1]. ANF receptors from various tissues have been documented [2–7] and according to biochemical and pharmacological evidence, two subclasses, termed R1 and R2 receptors, have been reported [2,7].

In bovine adrenal zona glomerulosa (BAZG), several studies demonstrated that ANF exerts its inhibitory action on stimulated aldosterone secretion through the R1 receptor subtype [8]. Meloche et al. [8] have investigated the physicochemical properties of the membrane-bound R1 receptor in BAZG. Its apparent molecular mass has been determined to be 130 kDa by SDS/PAGE. This receptor has also been solubilized [8] and affinity-purified to homogeneity [9], and its glycoprotein nature has been demonstrated [10]. In the process of characterizing how ANF and its receptor interact at the molecular level, we needed an appropriate probe to specifically tag the hormone-binding domain of the receptor macromolecule.

In the present study, we document the unexpected ability of underivatized ^{125}I-ANF to be cross-linked at its specific binding site on the ANF receptor by exploiting the photoreactivity of the hormone–receptor complex to u.v. irradiation.

MATERIALS AND METHODS

Materials

Rat ANF-(99–126) obtained from BioMega, Laval, Canada, was radiolabelled with ^{125}I (Amersham) according to the solid-phase method using Iodo-Beads (Pierce Chemical Co., Rockford, IL, U.S.A.) [11]. The usual specific activity of the monoiodinated peptide was 3000 Ci/mmol. Triton X-100 was purchased from Pierce Chemical Co., and L-α-phosphatidylcholine from dried egg yolk was obtained from Sigma. All other reagents were from commercial sources.

Membrane preparation

A complete description of the method used to prepare the membranes has been already published [9]. In brief, a 0.5 mm layer of bovine adrenal cortex corresponding to the zona glomerulosa was homogenized in 20 vol. of a modified ice-cold homogenization buffer [20 mM-NaHCO$_3$, 10 mM-EDTA, 10 μM-aprotinin, 10 μM-leupeptin, 1 μM-pepstatin A and 0.1 μM-phenylmethanesulphonyl fluoride (PMSF)]. After filtration through cheesecloth, the homogenate was first centrifuged at 1000 *g* for 10 min and then the supernatant was centrifuged at 40000 *g* for 15 min. The resulting pellet, washed twice with homogenization buffer, was finally resuspended in 2 ml of buffer (50 mM-Tris/HCl/250 mM-sucrose/0.1 mM-EDTA/1 mM-MgCl$_2$, pH 7.4) per g of initial tissue. Membranes were frozen in liquid N$_2$ and stored at −70 °C until used.

Membrane solubilization

The membranes were resuspended in buffer containing 50 mM-Tris/HCl, 100 mM-NaCl, 20% (v/v) glycerol, 0.1 mM-EDTA, 10 μM-PMSF, 0.1 μM-aprotinin, 1 μM-leupeptin, 1 μM-pepstatin A and 1% Triton X-100 (pH 7.4) at a protein concentration of 4 mg/ml. The suspension was stirred for 60 min at 4 °C and centrifuged at 35000 *g* for 60 min. The supernatant was used for binding and photolabelling.

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^{125}I-ANF binding and photolabelling

The BAZG membranes (20 μg/ml) were incubated in the dark at room temperature for 90 min with ^{125}I-labelled ANF (10 pM) in the presence or absence of unlabelled ANF (0.1 μM) in a total volume of 15 ml of assay buffer [50 mM-Tris/HCl (pH 7.4)/ 0.1 mM-EDTA/5 mM-MnCl$_2$]. After incubation, the membranes were pelleted by centrifugation at 40000 g for 15 min, resuspended in 15 ml of ice-cold assay buffer and photolysed (10 min, 4 °C) at a distance of 6–10 cm from two mercury lamps (54 mW·cm^{-2}, 365 nm) equipped with a 400 nm cut-off filter and a layer of borosilicate glass. Radiation below 300 nm was absorbed by Pyrex glass. After irradiation, the membranes were centrifuged at 40000 g for 15 min and the pellet was solubilized in 200 μl of buffer [10 mM-Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (w/v) glycerol/5% (v/v) 2-mercaptoethanol/0.05% Bromophenol Blue]. After heating at 100 °C for 3 min, the proteins (100 μg) were analysed by electrophoresis on a 7.5% acrylamide gel in the presence of SDS according to the method of Laemmli [12]. Binding of ^{125}I-ANF to the soluble fraction was conducted in similar conditions in buffer containing 50 mM-Tris/HCl, 0.1 mM-EDTA, 0.05% phosphatidylcholine, 0.1% bovine serum albumin and 0.01% Triton X-100 (pH 7.4). At the end of the binding period, the mixture was exposed to u.v. light (10 min, 4 °C), and then the proteins were precipitated with cold 10% trichloroacetic acid. The resulting pellet was processed for electrophoresis as described for membrane preparation. Autoradiographic results were usually obtained after a 2 day exposure of the dried gel at −70 °C to Kodak X-OMAT-RP film with two intensifying screens. The molecular mass of the radiolabelled band was determined using the following molecular mass standards (Bio-Rad): myosin, 200 kDa; β-galactosidase, 116.3 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa.

RESULTS

U.v. irradiation of BAZG membranes which had bound ^{125}I-ANF resulted in the labelling of a single band with an apparent molecular mass of 130 kDa (Fig. 1a, lane 1). Non-specific labelling was evaluated in the presence of unlabelled ANF (0.1 μM) (Fig. 1a, lane 2). The corresponding Coomassie Blue staining pattern is also shown in Fig. 1(b) (lane 2). The molecular mass of this protein is comparable with the value reported previously by several investigators [8,9,13–15]. The apparent covalent nature of the labelled hormone–receptor complex is indicated by its resistance to denaturating treatments such as 6 M-guanidine hydrochloride, 8 M-urea, 10% trichloroacetic acid or boiling in Laemmli sample buffer [12] (Fig. 1a, lanes 3–5). ANF receptor protein was also photolabelled by ^{125}I-ANF following solubilization of BAZG membranes (Fig. 1b, lane 1). Occasionally, a much fainter band was seen, migrating at 60–70 kDa, when the ^{125}I-ANF–receptor complex was precipitated by trichloroacetic acid before electrophoresis (Fig. 1b, lane 1). This band presumably results from acid hydrolysis of the 130 kDa protein as reported previously [8].

The intensity of the 130 kDa band varied with the length of the irradiation period (Fig. 2a). By excising the band from the dry gel and evaluating the corresponding radioactivity, we observed that the amount of covalent hormone–receptor complex was proportional to the duration of the irradiation (results not shown). Under these conditions, the presence of other bands related to proteolytic degradation or to the photoincorporation of ^{125}I-ANF at non-specific sites was negligible, even after 120 min (Fig. 2a). Results from several gels showed that after 10 min of u.v.
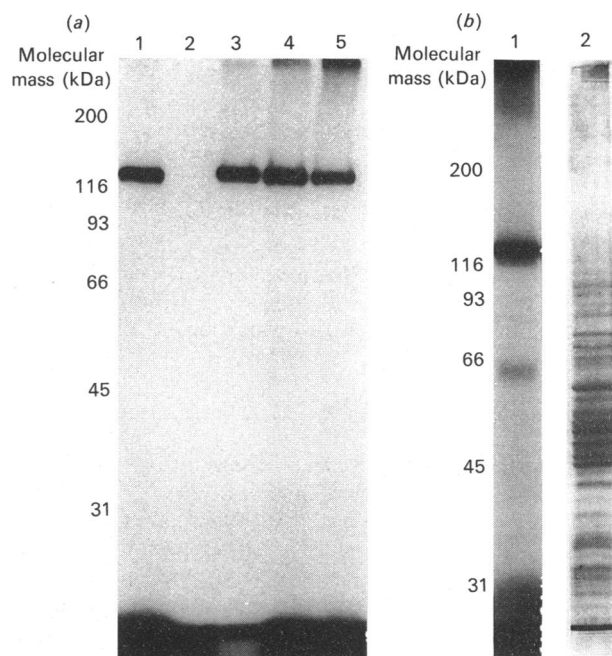

Fig. 1. Photoaffinity labelling of ANF receptors by ^{125}I-ANF

(a) BAZG membranes (200 μg of protein, lane 1) were incubated with 10 pM-^{125}I-ANF for 90 min at room temperature in the dark before exposure to u.v. light. After binding and photoactivation, the membrane proteins were treated for 30 min with various denaturating agents: 6 M-guanidine hydrochloride (lane 3), 8 M-urea (lane 4) and 10% trichloroacetic acid (lane 5). After centrifugation, the samples were solubilized by heating at 100 °C for 3 min in the Laemmli sample buffer [11] and approx. 100 μg of membrane proteins was separated by SDS/PAGE and autoradiographied as described in the Materials and methods section. Non-specific labelling was determined in the presence of an excess of unlabelled ANF (0.1 μM, lane 2). (b) Photolabelling of the soluble membrane fraction by ^{125}I-ANF is shown in lane 1. An example of the Coomassie Brilliant Blue staining of the membrane proteins after electrophoresis is also shown (lane 2).

irradiation, the radioactivity in the 130 kDa band represented 1.55 $\pm$ 0.41% (mean $\pm$ S.E.M., $n = 10$) of the total radioactivity bound to the membranes, reaching levels of 7.5% after 120 min. When added during the binding step, amiloride (0.1 mM) increased the level of hormone–receptor complex and therefore the yield of photolabelled receptor protein (Fig. 2b). The amount of covalent ^{125}I-ANF–receptor complex varied linearly with the amount of receptor employed (Fig. 3) and binding was saturable at high ^{125}I-ANF concentrations (Fig. 4). The specificity of labelling of the 130 kDa protein band was confirmed by the study of the dose-dependent prevention of ^{125}I-ANF photolabelling by unlabelled ANF (Fig. 5). Under these conditions, the K_d estimated for ANF (90 pM) is consistent with the K_d obtained for ANF in typical competition binding analysis (20 pM). The fact that the truncated form ANF-(103–123) is less potent than ANF-(99–126) in preventing photolabelling (Fig. 5) is in agreement with the typical pharmacological specificity of the ANF-R1 receptor subtype [16].

Chemical cross-linking of ^{125}I-ANF to membranes resulted in the labelling of the same protein as with photolabelling (Fig. 6). However, use of the bifunctional chemical cross-linking agent bis(sulphosuccinimidyl)suberate (BS^3) also led to radioactive labelling of high molecular mass aggregates seen at the top of the gel. The subtype specificity of ANF receptor photolabelling was determined by using membranes prepared from LLC-PK1 and NIH-3T3 cell lines [16]. On the same receptor basis, ANF-R1
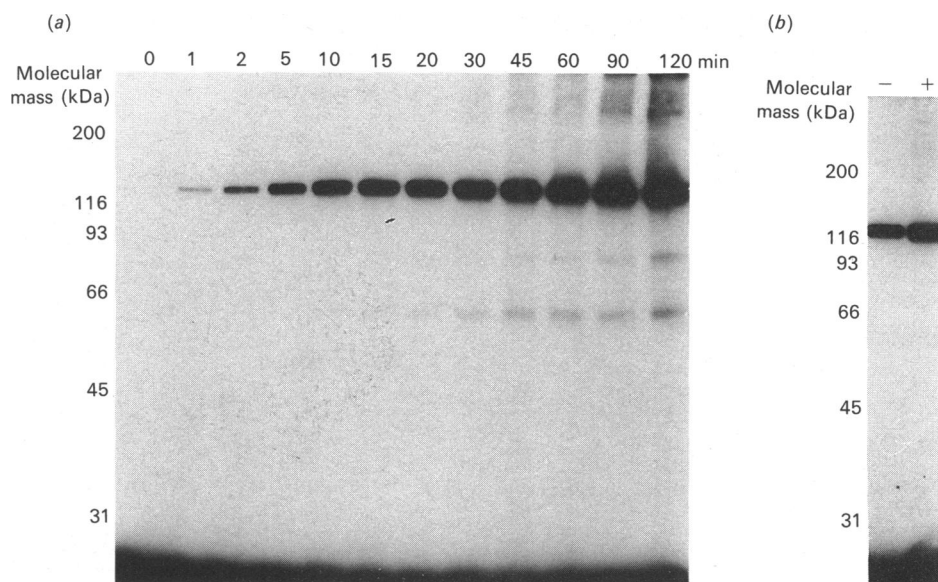
Fig. 2. Photoaffinity labelling of ANF receptor by ^{125}I-ANF: effect of length of irradiation period

(a) BAZG membranes (200 μg of protein) were incubated with ^{125}I-ANF and irradiated with u.v. for increasing periods of time as described in the Materials and methods section. (b) Where indicated (+), binding and photolabelling (10 min) were performed in the presence of amiloride (0.1 mM). The data are representative of three experiments.
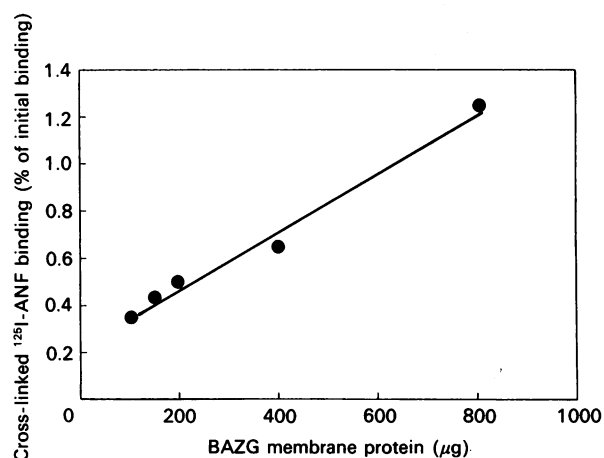

Fig. 3. Influence of proteins on photolabelling of the ANF receptor

Increasing amounts of BAZG membranes were incubated with ^{125}I-ANF and photolabelled as described in the Materials and methods section. After autoradiography, the labelled protein band was excised from the dry gel and the radioactivity in this band was evaluated. The graph shows the percentage of the initial specific binding of ^{125}I-ANF which was covalently incorporated during the u.v. exposure as a function of the amount of BAZG membrane proteins in the assay.
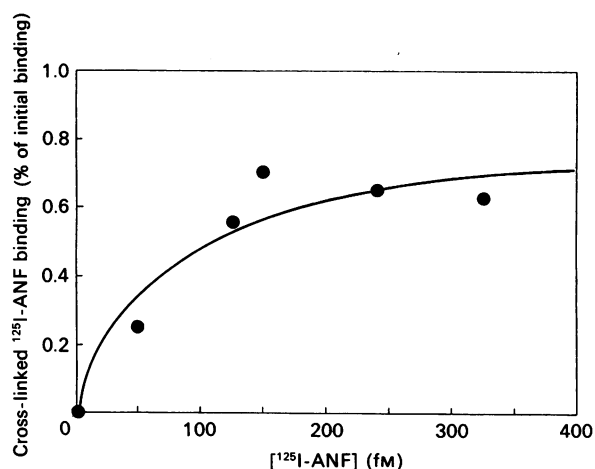

Fig. 4. Effect of increasing concentrations of ^{125}I-ANF on the photoaffinity labelling of ANF receptor

BAZG membranes (200 μg of protein) were incubated with increasing concentrations of ^{125}I-ANF and photolabelled under conditions reported in the Materials and methods section. After autoradiography of the dry gel, the labelled protein band was excised and the radioactivity evaluated. The graph shows the percentage of the initial specific binding of ^{125}I-ANF covalently incorporated during u.v. exposure as a function of the concentration of ^{125}I-ANF used in the assay.

receptor from LLC-PK1 cells was preferentially photolabelled by ^{125}I-ANF when compared with the R2 subtype from NIH-3T3 cells (Fig. 7).

We investigated several possible mechanisms by which the ANF-R1 receptor could be labelled irreversibly by ^{125}I-ANF upon u.v. irradiation. Inclusion during u.v. photolysis of the antioxidant butylated hydroxytoluene (BHT) (0.1–10 mM) (Table 1) or the aqueous scavenger p-aminobenzoic acid (PABA) (100 μM) (results not shown) did not alter the photolabelling of ANF-R1 receptor by ^{125}I-ANF. Moreover, the labelling was not affected by the presence of several protease inhibitors such as soya bean trypsin inhibitor (0.01–0.5%), leupeptin (1 μM),

pepstatin A (0.1 μM), aprotinin (1 μM) and PMSF (100 μM) (Table 1). Under similar photolabelling conditions, 0.1 and 1 mM-EDTA did not interfere with the total ^{125}I-ANF bound to the membranes or with covalent incorporation of ^{125}I-ANF into the 130 kDa band protein. However, 10 mM-EDTA added during the photolysis step slightly decreased the amount of total ^{125}I-ANF bound to the membranes and markedly decreased the labelling intensity of the 130 kDa band protein (Table 1).

In order to demonstrate if this reaction results from the incorporation of free iodine into the receptor molecule, which
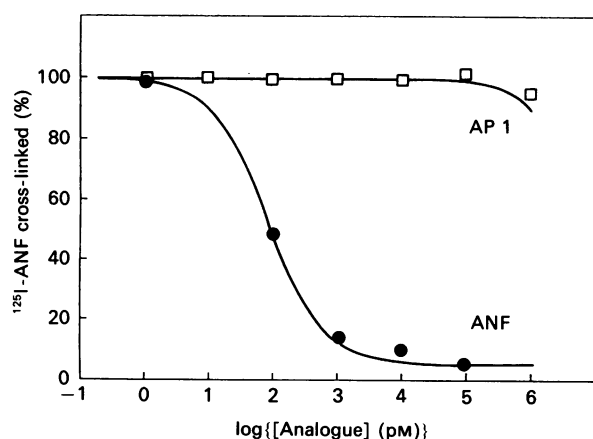
Fig. 5. Inhibition of ^{125}I-ANF photolabelling of the ANF receptor by unlabelled analogues

BAZG membranes (200 µg of protein) were incubated with ^{125}I-ANF as described in the legend to Fig. 1. Binding was performed without addition or in the presence of increasing concentrations of ANF (●) or AP 1 (□). Photolabelling, electrophoresis and autoradiography were conducted as reported in the Materials and methods section. After autoradiography, the labelled band was excised from the dry gel and the radioactivity was evaluated. The graph shows the percentage of ^{125}I-ANF covalently incorporated upon u.v. exposure as a function of the concentration of the unlabelled analogue present in the assay.
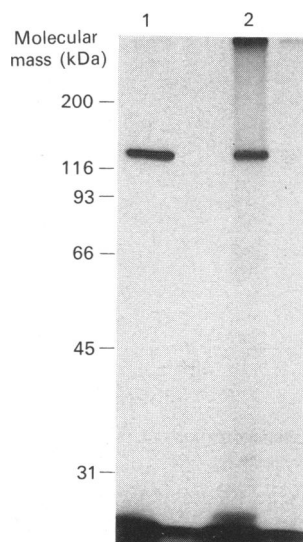

Fig. 6. Comparison between photoaffinity and chemical cross-linking of the ^{125}I-ANF–receptor complex

BAZG membranes (200 µg of protein) were incubated with ^{125}I-ANF as described in the legend to Fig. 1. After binding, photoaffinity labelling of the ANF receptor was conducted as reported in the Materials and methods section (lane 1). Chemical cross-linking of the ligand–receptor complex was performed with 0.1 mM-BS^3 in phosphate buffer (pH 7.4) for 30 min at 4 °C (lane 2). The final membrane pellets were solubilized in Laemmli sample buffer [11] and subjected to electrophoresis. The dry gel was then autoradiographed.

would have been dissociated from the ligand upon u.v. irradiation, we submitted the irradiated ^{125}I-ANF–receptor complex to digestion with the carboxypeptidase A. This resulted in a marked decrease in the intensity of the 130 kDa band (Fig. 8a),
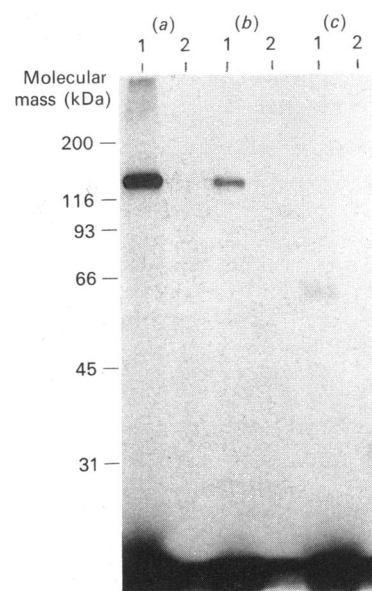

Fig. 7. Photoaffinity labelling of ANF receptor by ^{125}I-ANF

Autoradiogram of the photoaffinity labelled ^{125}I-ANF–receptor complex from BAZG membranes (a), LLC-PK1 (b) and NIH-3T3 cell membranes (c). Non-specific labelling (lanes 2) was evaluated in presence of 0.1 µM unlabelled ANF. Upon binding and photolabelling, 100 µg of protein from each membrane preparation was subjected to electrophoresis and autoradiography as described in the Materials and methods section. The amount of radioactivity (c.p.m.) loaded was: (a) 42570 (lane 1), 3716 (2); (b) 14636 (1), 3766 (2); (c) 16361 (1) and 2852 (2).

Table 1. Effect of BHT, protease inhibitors and EDTA on the photolabelling of the ANF-R1 receptor by ^{125}I-ANF

BAZG membranes were incubated with 10 pM-^{125}I-ANF for 90 min at room temperature. The samples were centrifuged at 40000 g for 15 min and resuspended in 50 mM-Tris/HCl (pH 7.4)/0.1 mM-EDTA before the addition of BHT, protease inhibitors or EDTA. Photolabelling, electrophoresis and autoradiography were performed as reported in the Materials and methods section. The results represent the percentage of the initial specific binding of ^{125}I-ANF covalently incorporated into the labelled band on the dried gel. Results are from one experiment. SBTI, soya bean trypsin inhibitor. The protease inhibitor mix contained 1 µM-leupeptin, 0.1 µM-aprotinin, 0.1 µM-pepstatin A and 100 µM-PMSF.

Conditions	^{125}I-ANF covalently incorporated (% of total binding)
Control	2.0
BHT: 0.1 mM	1.9
1.0 mM	2.0
10.0 mM	2.0
SBTI: 0.01%	1.8
0.10%	2.0
0.50%	1.7
Protease inhibitors mixture:	2.1
EDTA:0.1 mM	2.0
1.0 mM	2.0
10.0 mM	0.8

indicating that the ^{125}I is still linked to the ANF C-terminal tyrosine. Moreover, this also suggests that the radioactive ANF is covalently linked by its N-terminal end to its receptor when photolabelling is performed.

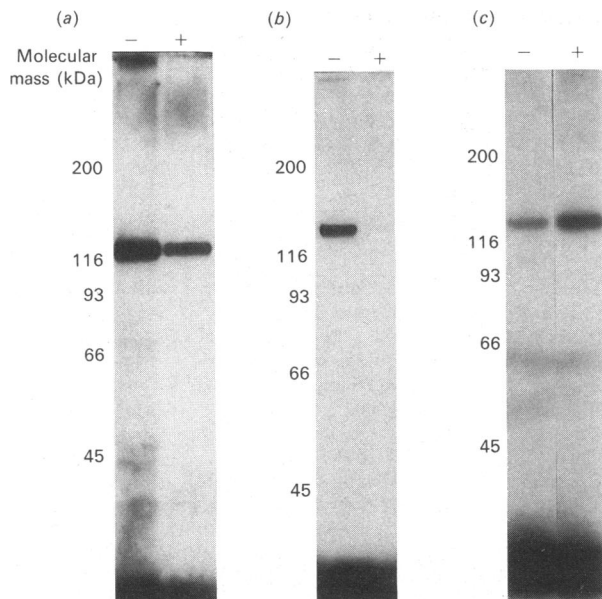The importance of oxygen in this reaction was assessed by u.v.

Fig. 8. Photoaffinity labelling of ANF receptor by ^{125}I-ANF

BAZG membranes (200 μg of protein) were incubated with 10 pM-^{125}I-ANF for 90 min at room temperature. The data are representative of two experiments. (a) Following incubation, the samples were irradiated with u.v. light for 10 min at 4 °C and then centrifuged. The resulting pellets were resuspended in 50 mM-Tris/HCl/0.1 mM-EDTA and treated with (+) or without (−) carboxypeptidase A (5 μg/mg of protein) for 90 min at 37 °C. All samples were centrifuged, electrophoresed and autoradiographed as described in the Materials and methods section. (b) At 10 min before the end of the binding period and during the entire u.v. exposure period, the membrane suspensions were gassed with (+) or without (−) N₂. The samples were subjected to photolabelling, electrophoresis and autoradiography as described in the Materials and methods section. (c) At the end of the binding period, the membrane suspension was centrifuged. The pellet (protein, 2 mg/ml) was solubilized under stirring in buffer containing 50 mM-Tris/HCl, 100 mM-NaCl, 0.1 mM-EDTA, 1% Triton X-100, 20% glycerol, 10 μM-PMSF, 0.1 μM-aprotinin, 1 μM-leupeptin and 1 μM-pepstatin for 60 min at 4 °C. The suspension was centrifuged at 35000 *g* for 15 min and filtered on Millipore 0.22 μm filters. The clear supernatant was applied to a small column of Accell QMA. The ^{125}I-ANF–receptor complex was eluted in the same buffer containing 0.5 M-NaCl. Phosphatidylcholine (0.05%) was added (+) or not (−) to half of the resulting eluate and the samples were exposed to u.v. (10 min, 4 °C). Electrophoresis and autoradiography were conducted as described in the Materials and methods section.

irradiation of the ^{125}I-ANF–receptor complex under anaerobic conditions by gassing with N₂ for 10 min before and during photolysis. As shown in Fig. 8(b), photolabelling of the 130 kDa band under anaerobic conditions was less intense than in the presence of oxygen. In addition to oxygen, lipids are likely to play a major role in this process. To investigate this, membranes were incubated with ^{125}I-ANF and solubilized in 1% Triton X-100, and this soluble fraction was delipidated by passage through an ion-exchange resin (Accell QMA). Finally the resulting material, supplemented or not with exogeneous phospholipids, was exposed to u.v. light. As seen in Fig. 8(c), the labelling of the 130 kDa protein band was almost completely prevented in the absence of lipids.

DISCUSSION

For many biological receptor systems, affinity labelling [17] is currently used to covalently attach a specific label to the active binding site of the receptor molecule. In general, this method-ology is based on the generation of covalent bonds between the receptor and the unmodified specific ligand by addition of a bifunctional agent to ligand–receptor complexes. A disadvantage of the affinity cross-linking technique is that we cannot avoid chemical cross-linking of the receptors with other membrane components by the exogenous cross-linking agent.

Photoaffinity labelling represents a special category of affinity labelling in which a ligand carrying a photosensitive moiety is bound in a specific but reversible manner to the receptor site. During this association state, the ligand may be activated by radiation into a highly reactive intermediate capable of a chemical reaction with its intermediate surroundings and thus forming a covalent ligand–receptor complex. Usually the intermediate formed is a very reactive and short-lived (< 1 ms) radical which is capable of almost indiscriminate insertion reactions [18]. In some unusual cases, however, the use of underivatized hormones for the photolabelling of receptor proteins is advantageous because its natural specificity for its receptor sites is preserved and because no, sometimes awkward, introduction of a photosensitive moiety is needed. Indeed, several reports have indicated successful photoaffinity labelling or photo-cross-linking of receptor systems by radioactive natural ligands. Thyroid hormone [19–22], parathyroid hormone [23], calcitonin [24], cholecystokinin [25] and opiate [26] receptors have been studied using photo-cross-linking of natural peptide hormones or ligands.

The results reported in the present paper show that irradiation with u.v. of underivatized ^{125}I-ANF bound to ANF receptors in their membrane environment or in a solubilized form induced specific apparent covalent cross-linking between the peptide hormone and the R1 subtype form of the ANF receptor. The half-maximal concentration of unlabelled ANF required for prevention of photolabelling (90 pM) was found to be very similar to the previously determined K_d in competition for ^{125}I-ANF binding [8]. Photolabelling of the ANF receptor showed the typical ANF-R1 pharmacological specificity observed for membrane-bound as well as soluble and purified receptors [9,27]. The observation that the photolabelling of this protein can be enhanced by amiloride is also in agreement with its ANF-R1 specificity [16].

The same molecular form of the ANF receptor was labelled in membranes from the LLC-PK1 cell line, which expresses only the ANF-R1 subtype [16]. In contrast, ANF-R2 identified from the NIH-3T3 cell line was not markedly labelled upon photolysis, even though it displays the same affinity for ^{125}I-ANF [27]. This suggests that photolabelling is not exclusively a function of affinity but also depends on the close proximity in the binding site of certain amino acid residues with which the ligand can form a covalent bond.

In order to identify the molecular size of receptors for ANF in several tissues, Koseki *et al.* [15] have also utilized the direct u.v. irradiation method for photoaffinity labelling with biologically active ^{125}I-ANF. They reported labelling of two molecular species for ANF receptors according to the tissues used. However, a closer examination of their autoradiograms revealed several non-specific bands when compared with our results. This discrepancy in labelling patterns can possibly be attributed to several differences in photolysis or binding conditions.

To produce photolysis, we used irradiation at 365 nm to avoid protein inactivation or photodestruction, which occur usually at wavelengths below 300 nm [28]. These irradiation conditions should prevent photolabelling by zero-length protein–protein cross-linking, which does not occur at wavelengths greater than 300 nm [29]. U.v. irradiation is reported to cause homolytic fission of the C–I bond [18,30], resulting in the possible loss of the iodine atom. According to this model, photoaffinity labelling of specific receptor molecules could be attributed only to incor-

poration of free iodine dissociated from the ligand. In our system, the sensitivity to carboxypeptidase A of the photolabelled ANF receptor does not support this theory, but rather suggests that, upon photolysis, the ligand is still bound to the receptor. Furthermore, its C-terminal tyrosine which carries the ^{125}I seems to be still accessible to proteolytic degradation. On the other hand, proteolysis does not appear to be involved in ANF receptor labelling, because during the photolysis period the addition of various protease inhibitors did not alter the photolabelling pattern.

Analogous protein cross-linking reactions are observed during protein aging [31,32]. Reactions of proteins *in vitro* with oxidized lipids produced cross-linked proteins similar to those in aging cells or tissues [32]. In the presence of oxygen, u.v. irradiation is known to promote free-radical and lipid peroxidation [33]. The mechanism by which lipid peroxidation occurs is not completely understood. However, malondialdehyde, a highly reactive secondary product of lipid peroxidation, seems to be directly involved in protein cross-linking reactions [32–34]. Interestingly, malondialdehyde formation is prevented in the presence of a high concentration of EDTA [35,36]. We have provided some evidence suggesting that malondialdehyde could be involved in the photolabelling of ANF-R1 receptors by underivatized ^{125}I-ANF. In fact, although BHT, a potent antioxidant, cannot prevent the photolabelling, the requirement for oxygen and lipids during photolysis and the preferential inhibition by EDTA of ^{125}I-ANF receptor photolabelling all support this hypothesis. The inefficiency of BHT could be attributed to its very poor solubility in the membrane preparation.

Even though its molecular mechanism is not completely known, u.v. photolabelling of the ANF-R1 receptor by underivatized ^{125}I-ANF is particularly valuable for studying the active binding domain, since it preserves fully the receptor characteristics. Covalently labelled functional receptors could also be a very interesting tool in future recombinant experiments.

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