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Allosteric Modulation by ATP of the Bovine Adrenal Natriuretic Factor R_1 Receptor Functions†

Louise Larose,‡ Normand McNicoll, Huy Ong, and André De Léan*,§

Laboratory of Molecular Pharmacology, Clinical Research Institute of Montreal, Department of Pharmacology and Faculty of Pharmacy, University of Montreal, Montreal, Quebec, H2W 1R7 Canada

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ABSTRACT: Atrial natriuretic factor (ANF-R_1) receptor is a 130-kDa protein that contains a cytoplasmic guanylate cyclase domain. We report that ATP interacts in an allosteric manner with the ANF-R_1 receptor, resulting in reduced ANF binding and enhanced ANF-stimulated guanylate cyclase activity. The modulatory properties of various nucleotides indicate a preference for the adenine family with a rank order of potency of ATP > App(NH)p ≥ ADP ≥ AMP while cyclic and guanine nucleotides except GTP are inactive. The negative modulation by ATP of ANF binding is specific for the ANF-R_1 receptor subtype since the amount of ANF bound by the guanylate cyclase uncoupled ANF-R_2 subtype is increased in the presence of ATP. Furthermore, the effects of ATP on ANF-R_1 receptor binding function are still observed with the affinity-purified ANF-R_1 receptor, suggesting an allosteric binding site for ATP on the ANF-R_1 receptor. In intact membranes, limited proteolysis of the ANF-R_1 receptor with trypsin dose-dependently prevents the ATP-induced decrease in ANF binding concomitantly with the formation of a membrane-associated ANF-binding fragment of 70 kDa. These results confirm the direct modulatory role of ATP on hormone binding activity of ANF-R_1 receptor and suggest that the nucleotide regulatory binding site is located in the intracellular domain vicinal to the protease-sensitive region.

Atrial natriuretic factor receptor (ANF-R_1)[1] selectively binds only biologically active forms of the hormone (Fêthière et al., 1989) and consists of a single 130-kDa subunit including both the hormone binding and guanylate cyclase activities. Previous studies from our laboratory have shown that in bovine adrenal glands, ANF binding to its receptor is reduced by ATP (De Léan, 1986). Kurose et al. (1987) have, however, reported that in rat liver membranes ATP enhanced the ANF-stimulated guanylate cyclase activity but did not alter the binding of ANF. In similar studies, Chang et al. (1990) have demonstrated that ATP activates guanylate cyclase from rat lung membranes and potentiates the effect of ANF on the enzyme.

Recently, molecular cloning (Chinkers et al., 1989; Lowe et al., 1989) studies have revealed an internal domain in the primary structure of the ANF-R_1 receptor that shares significant homology with the protein tyrosine kinase domain of the PDGF receptor. In this respect, the ATP modulation of the ANF binding and catalytic functions of the ANF-R_1 receptor could be supported by the direct interaction of ATP with this receptor domain.

We report here that the ANF binding function of both crude and affinity-purified ANF-R_1 receptor can be regulated by ATP. The modulatory effects of adenine nucleotides on the ANF-R_1 receptor seem to be due to an allosteric modification mediated by the binding of ATP within a cytoplasmic domain that is also sensitive to proteolysis by trypsin.

Experimental Procedures

Materials. ATP, ADP, AMP, App(NH)p, GTP, and Gpp(NH)p were purchased from Boehringer Mannheim. GDP, cGMP, cAMP, theophylline, IBMX, protease inhibitors, trypsin (TPCK-treated trypsin, type XIII), phosphatidylcholine, creatinine phosphate, and creatinine phosphokinase were obtained from Sigma Chemical Co. (St. Louis, MO). ANF was rat ANF(99–126) from Institut Armand Frappier, Laval, Canada. Triton X-100 and Iodo-Beads were purchased

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* Author to whom correspondence should be addressed at Laboratoire de Pharmacologie Moléculaire, Institut de Recherches Cliniques de Montréal, 110 Ouest, Avenue des Pins, Montréal, Québec, Canada, H2W 1R7.
‡ Recipient of a studentship from the Medical Research Council of Canada.
§ Scientist of the Medical Research Council of Canada.

[1] Abbreviations: ANF, atrial natriuretic factor; ATP, adenosine triphosphate; PDGF, platelet-derived growth factor; ADP, adenosine diphosphate; AMP, adenosine monophosphate; App(NH)p, adenylyl imidophosphate; GTP, guanosine triphosphate; Gpp(NH)p, guanylyl imidophosphate; GDP, guanosine diphosphate; cGMP, guanosine cyclic 3′,5′-monophosphate acid; cAMP, adenosine cyclic 3′,5′-monophosphate acid; BAZG, bovine adrenal zona glomerulosa; IBMX, 3-isobutyl-1-methylxanthine; $t_{1/2}$, half-time for dissociation; ED_{50}, median effective dose; PMSF, phenylmethanesulfonyl fluoride.

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from Pierce Chemical Co. (Rockford, IL). The antiserum to cyclic GMP was kindly provided by Dr. Alain Bélanger, Laval University Hospital Center, Québec, Canada. ^{125}I-ANF(99–126) was prepared by radioiodination according to the solid-phase Iodo-Beads method (Ong et al., 1987) and purified by HPLC. The specific activity of the monoiodinated peptide was typically 3300 Ci/mmol. Carrier-free Na^{125}I was from Amersham Corp. (Oakville, Canada). All other reagents were from commercial sources.

Preparation of Membranes. Bovine adrenal glands were obtained from the slaughterhouse. Adrenal zona glomerulosa membranes were prepared as described previously (Meloche et al., 1986) and stored frozen at −70 °C until used. Confluent LLC-PK1 and NIH-3T3 cells were used to prepare the membranes according to Fêthière et al. (1989).

Preparation of the Pure ANF-R_1 Receptor. Bovine adrenal zona glomerulosa membranes were used as crude receptor preparation. The ANF-R_1 receptors were purified by affinity chromatography with use of ANF–agarose as previously described (Meloche et al., 1988).

Receptor Binding Experiments. The adrenal zona glomerulosa (16 μg/mL), LLC-PK1 (32 μg/mL), or NIH-3T3 (20 μg/mL) membranes were incubated with 10 pM ^{125}I-ANF-(99–126) for 90 min at 25 °C or overnight at 4 °C in 50 mM Tris-HCl, pH 7.4/5 mM $MnCl_2$/0.1 mM EDTA/0.1% bovine serum albumin. Bound ^{125}I-ANF was separated from free ligand by filtration on Whatman GF/C filters precoated with 1% poly(ethylenimine). The bound hormone was determined in a γ counter (LKB 1272, Clinica Gamma). Nonspecific binding was evaluated in the presence of 0.1 μM unlabeled ANF(99–126). When the purified ANF-R_1 receptor (15 fmol) was used, the binding buffer was supplemented with 0.05% phosphatidylcholine and 0.01% Triton X-100. Prior to filtration, an equal volume of 20% poly(ethylene glycol) prepared in 50 mM Tris-HCl, pH 7.4/0.1 mM EDTA was added and the tubes were kept at 4 °C for 30 min. In all conditions, each sample was assayed in triplicate and the data were expressed as specific ^{125}I-ANF bound in counts per minute per assay. ATP or the other nucleotides were incubated during the ^{125}I-ANF binding step.

To study the ^{125}I-ANF association rate, the bovine adrenal zona glomerulosa membranes were incubated with 10 pM ^{125}I-ANF for the time desired in the presence or absence of ATP (1 mM). For the determination of the ^{125}I-ANF dissociation rate, the BAZG membranes were incubated with 10 pM ^{125}I-ANF for 90 min under the conditions already described and the dissociation was initiated by the addition of unlabeled ANF(99–126) at 0.1 μM in the presence or absence of 1 mM ATP.

Cyclic GMP Determination. The adrenal zona glomerulosa membranes (10 μg) were incubated during 15 min at 37 °C in 50 mM Tris-HCl Tris-HCl, pH 7.6, with 10 mM theophylline, 2 mM IBMX, 10 mM creatine phosphate, 10 units of creatine kinase, 1 mM GTP, and 4 mM $MgCl_2$. When ANF was included, the final concentration was 10 nM and the studies were conducted in the absence or in the presence of ATP. Cyclic GMP produced was separated from GTP by chromatography on alumina and evaluated by radioimmunoassay, according to the second antibody precipitation technique (Steiner et al., 1972). The assay was performed in acetate buffer, pH 6.2, as previously reported (Fêthière et al., 1989). The cyclic GMP production for each membrane sample was assayed in triplicate, and the data were expressed as picomole of cGMP produced per minute per milligram of protein.

Table I: Effects of Adenosine and Guanosine Nucleotides on the ^{125}I-ANF Binding to BAZG Membranes[a]

nucleotides (1 mM)	^{125}I-ANF bound[b] (%)	nucleotides (1 mM)	^{125}I-ANF bound[b] (%)
–	100	GMP	94.3 ± 8.7
ATP	57.8 ± 1.4*	GDP	94.9 ± 3.6
AppNHp	73.0 ± 3.6*	GppNHp	95.0 ± 4.8
ADP	81.1 ± 3.8*	cAMP	98.1 ± 3.6
GTP	82.2 ± 2.5*	cGMP	102.1 ± 2.3
AMP	89.5 ± 37*		

[a] The binding was performed as described in the Experimental Procedures section. [b] Specific binding obtained in the presence of each nucleotide is expressed as percentage of ^{125}I-ANF bound in the absence of the nucleotide. The results are the mean ± SEM of at least three experiments. The asterisk indicates a value different from without nucleotides (at least $p \leq 0.05$).

ATPase Assay. ATP hydrolysis was monitored at 24 °C by using an enzyme-linked NADH oxidation procedure (Schwartz et al., 1971). Affinity-purified ANF-R_1 receptor was assayed at a final concentration of 0.15 nM and Na,K-ATPase (dog kidney) (0.1 unit) was used as a positive control.

Limited Proteolysis by Trypsin. Bovine adrenal zona glomerulosa membranes were incubated with varying concentrations of trypsin for 30 min at 25 °C. Proteolysis was stopped by the addition of 1 mM PMSF, and then the membranes were separated by centrifugation. The membranes were incubated with ^{125}I-ANF under the same conditions as previously described for the binding studies. At the end of the binding period, the mixture was exposed to UV light for 10 min at 4 °C (Larose et al., 1990) and then centrifuged. The final membrane pellets were dissolved in Laemmli buffer (Laemmli, 1970) and submitted to electrophoresis and autoradiography. The molecular masses of the standards (in daltons) were myosin (200 000), β-galactosidase (116 250), phospholipase *b* (92 500), bovine serum albumin (66 200), and ovalbumin (45 000). The membranes were also assayed for ^{125}I-ANF binding functions, and the inhibitory effect of ATP on the binding activity was evaluated under conditions the same as those described in the section for the receptor binding experiments.

Data Analysis. Data presented in the Figures are representative of at least two experiments. Results are expressed as means ± SEM and are analyzed by the Student's *t* test for unpaired values. The results were considered significant when at least $p \leq 0.05$. In the kinetic studies, the data were fitted by nonlinear least-squares curve fitting according to the multiexponential equation

$$Y = C - \sum_{i=1}^{n} A_i e^{-B_i t}$$

where Y is the measured ^{125}I-ANF bound, C is Y at equilibrium, A_i is the size of component i, B_i is the time constant of the component i, t is time, and n is the number of exponential components. A model involving two exponentials was retained when the fit was statistically better, as evaluated by a partial F test (De Lêan et al., 1978). The $t_{1/2}$ values were calculated by use of $(\ln 2)/B_i$. Binding data and the dose–response curve for cGMP production were analyzed with the ALLFIT program, based on a four-parameter logistic equation (De Lêan et al., 1978).

Results

Our former observations on the effects of ATP on ANF binding indicated that this nucleotide could regulate receptor function. Since other nucleotides (e.g., GTP) regulate many

Table II: Effect of ATP on the Kinetic Characteristics of ^{125}I-ANF Binding to the ANF Receptor of the Bovine Adrenal Zona Glomerulosa Membranes[a]

	A. Association (n = 3) proportion (%)		half-life (min)	
	fast	slow	fast	slow
−ATP	24.7 ± 3.1	75.3 ± 3.1	8.6 ± 2.7	55.7 ± 7.4
+ATP	100*	−	13.0 ± 3.0	−
	B. Dissociation (n = 4) proportion (%)		half-life (min)	
	fast	slow	fast	slow
−ATP	40.6 ± 5.8	59.4 ± 5.8	45.0 ± 9.5	ND
+ATP	26.3 ± 6.1*	73.7 ± 6.1*	8.3 ± 3.3*	155.2 ± 51.3

[a] The results are the mean ± SEM, and n represents the number of separate experiments. ND = not determined. The asterisk indicates a value different from without ATP (at least $p \leq 0.05$).

other receptors, we investigated the specificity of the effect of ATP on ANF receptor. The ^{125}I-ANF binding to the BAGZ membranes was reduced by the presence of adenosine nucleotides (Table I). The most potent nucleotides were ATP and its triphosphate nonhydrolyzable analogue App(NH)p. Cyclic nucleotides and the guanosine nucleotides did not alter ANF binding activity in bovine adrenal membranes. The exception was GTP, which, however, was significantly less potent than ATP. These results indicate that, in contrast to G-protein-coupled receptors, ANF receptor is preferentially regulated by adenine nucleotides.

In order to determine whether the interaction of ATP with the ANF-R_1 receptor was reversible, we tested the effect of removing ATP by washing the ATP-treated BAZG membranes prior to evaluating their ANF binding properties. The interaction of ATP with the ANF-R_1 receptor was completely reversible under these conditions, compatible with an allosteric mechanism (data not shown).

We have previously reported that ATP reduces the high-affinity component of ANF binding (De Léan, 1986). In order to further characterize the effect of ATP on the binding characteristics of ANF-R_1 receptor, we have studied the kinetic properties of ^{125}I-ANF binding to the ANF receptor from BAZG membranes in the presence or absence of ATP. As shown in Figure 1A, in the presence of ATP the association of ^{125}I-ANF with the BAZG ANF receptor reached equilibrium earlier and the amount of ^{125}I-ANF bound at the plateau was greatly reduced. The analysis of the association curve for ^{125}I-ANF binding in the absence of ATP revealed the existence of a fast and a slow component in this reaction (Table II, part A). However, when ATP is added, the association kinetics presented only the fast component. Figure 1B shows the effect of ATP on the ^{125}I-ANF dissociation kinetics obtained following binding of the radioligand in the absence of the nucleotide. The overall rate of dissociation of the radioligand was increased in the presence of ATP, and this change was apparent at the earliest time measured. When the data obtained in the absence of ATP were analyzed, the bound ^{125}I-ANF was evaluated to dissociate following a fast and a slow component (Table II, part B). However, the slow component was too slow for the experimental time frame to provide a reliable $t_{1/2}$ estimate. In the presence of ATP, the dissociation kinetics of the radioligand still present two components but both components have their half-lives reduced (Table II, part B).

Since the effect of ATP on ANF-sensitive guanylate cyclase has been reported in rat liver (Kurose et al., 1987) and lung membranes (Chang et al., 1990), we proceeded to confirm these results in bovine adrenal ANF-R_1 system. ATP (1 mM)

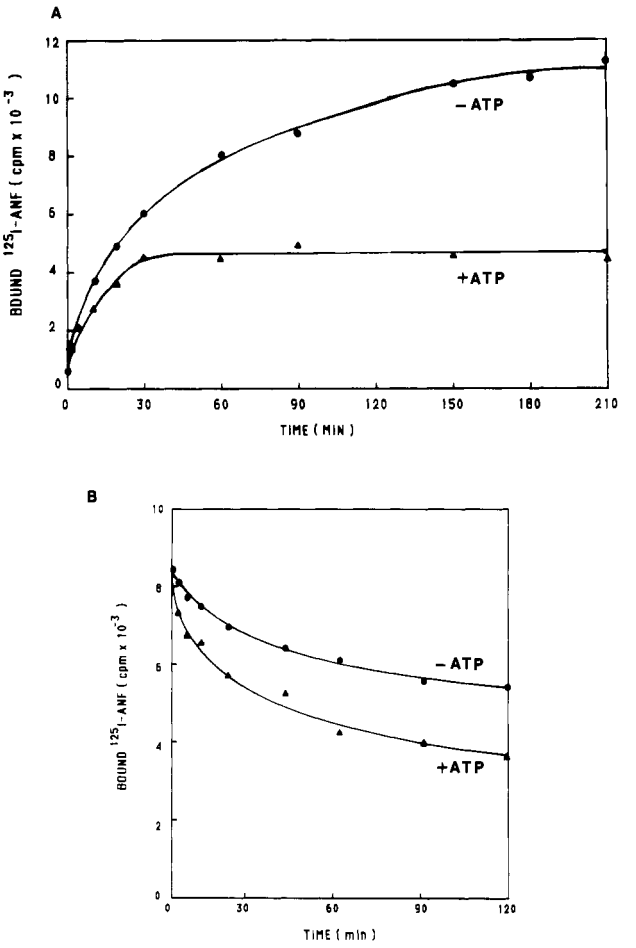

FIGURE 1: Effect of ATP on the association and dissociation rates of ^{125}I-ANF to BAZG membranes. (A) Membranes were incubated with 10 pM ^{125}I-ANF for the indicated times in the presence (▲) or absence (●) of ATP (1 mM). (B) Membranes were incubated for 90 min with 10 pM ^{125}I-ANF, and then the dissociation was initiated by the addition of unlabeled ANF(99–126) at 0.1 μM in the presence (▲) or absence (●) of 1 mM ATP. Data shown are typical of three experiments for the association and four for the dissociation and were fitted by computer analysis.

did not have any effect on basal guanylate cyclase activity (basal, 0.81 ± 0.01 pmol of cGMP per min per mg of protein; ATP, 0.71 ± 0.08), but when added at the same time as ANF (10 nM), it potentiates the hormone-stimulated cGMP production (ANF, 1.75 ± 0.52; ANF + ATP, 3.20 ± 0.67; ANF vs basal and ANF + ATP vs ANF, at least $p \leq 0.05$). Both effects of ATP on the ANF-R_1 receptor hormone binding and catalytic activities were dose-dependent and occurred within the same range of ATP concentrations, sharing the same ED_{50} value of 190 μM (Figure 2).

Two ANF receptor subtypes have been documented and are distinguished by differential pharmacological and biochemical properties (Fêthière et al., 1989). The ANF-R_1 receptor includes a large cytoplasmic domain that contains guanylate cyclase catalytic activity and is also selectively sensitive to amiloride. The ANF-R_2 receptor subtype does not include a large cytoplasmic domain and is insensitive to amiloride. We therefore tested the differential properties of ATP on ANF receptor subtypes using the epithelial cell line LLC-PK1 and the fibroblast cell line NIH-3T3, which exclusively express the ANF-R_1 or ANF-R_2 receptor, respectively (Fêthière et al., 1989). The results indicate that both ANF receptor subtypes are modulated by the presence of ATP, however, in an opposite manner. In fact, ATP reduces the binding of ANF to the R_1 subtype (1459 ± 134 cpm of ^{125}I-ANF bound to 620

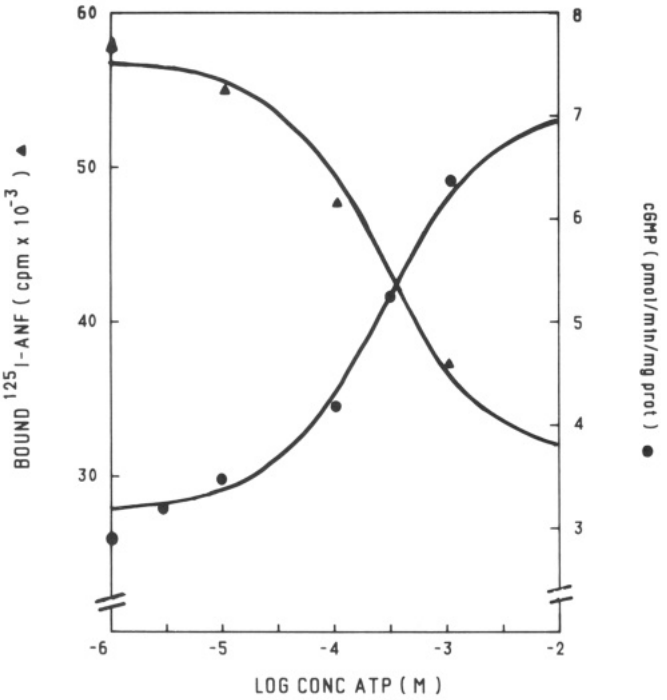

FIGURE 2: Dose-dependent effect of ATP on the BAZG membranes ANF-binding and ANF-induced cGMP production. The ^{125}I-ANF binding and the guanylate cyclase activities were evaluated as described in the Experimental Procedures section, and data shown are typical experiment.

± 1, at least $p \leq 0.05$) and increases the ANF bound by the R_2 subtype (1269 ± 76 to 1633 ± 38, at least $p \leq 0.05$).

In order to further investigate these modulatory effects of ATP on ANF-R_1 receptor functions and to study whether they involve the receptor itself or other associated protein, we used pure ANF-R_1 receptor. The atrial natriuretic factor ANF-R_1 receptor from bovine adrenal zona glomerulosa was purified 13 000-fold to apparent homogeneity, by sequential chromatography on ANF–agarose and steric-exclusion high-performance liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining of the purified receptor preparation in the absence or presence of dithiothreitol revealed a single protein band of M_r 130 000. An amount of 250 ng of purified receptor was applied to the gel for silver staining, and the limit of sensitivity of the method was below 10 ng of protein so that a single contaminant of less than 5% of the total protein could be detected (Meloche et al., 1988). ^{125}I-ANF binding to the affinity-purified ANF-R_1 receptor was still modulated by ATP (72.7 ± 4.9%, percentage of ^{125}I-ANF bound in the absence of nucleotide, at least $p \leq 0.05$). In the pure ANF-R_1 receptor preparation, App(NH)p seems to slightly reduce ANF binding but this is not statistically significant (94.4 ± 1.2). GTP and Gpp(NH)p failed to alter the ANF binding (96.1 ± 2.2, 96.3 ± 2.2), and Gpp(NH)p did not impair the effect of ATP when added simultaneously (79.7 vs 72.7 ± 4.9).

Since guanine nucleotide regulatory proteins coupled to receptors not only bind GTP but also display GTPase activity, we next investigated whether purified ANF-R_1 receptor protein might hydrolyze ATP. The pure ANF-R_1 receptor preparation is devoid of any ATPase activity (data not shown).

We have previously documented that limited digestion with trypsin of the ANF-R_1 receptor from bovine adrenal zona glomerulosa membranes resulted in the conversion of the native 130-kDa ANF receptor into a single membrane-associated proteolytic fragment of 70 kDa still competent in ANF binding

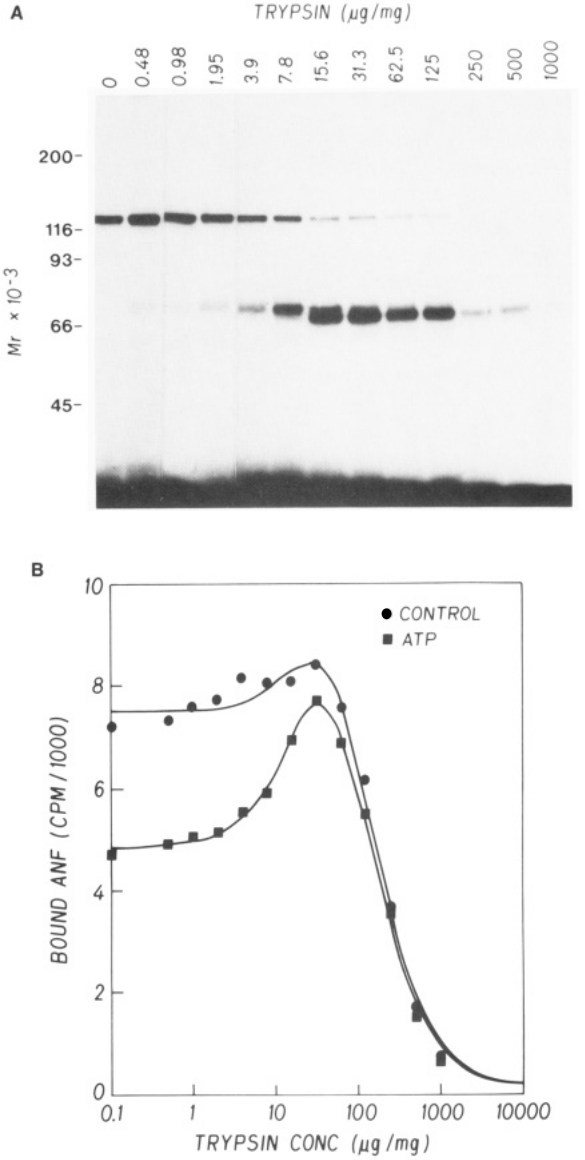

FIGURE 3: Proteolysis of BAZG membranes by trypsin. (A) Effect on the photoaffinity labeling by the ^{125}I-ANF. (B) Effect of the ATP modulation of the ^{125}I-ANF binding.

(Liu et al., 1989). Since this 70-kDa fragment is devoid of any guanylate cyclase activity (basal or ANF-stimulated) and still associated with the membrane, we have estimated that the protease-sensitive region in the ANF-R_1 molecule is in the intracellular part, just distal to the membrane-spanning sequence that separates the extracellular ANF binding domain and the cytoplasmic guanylate cyclase domain. An interesting point is that this intracellular region adjacent to the plasma membrane is similar in sequence to protein tyrosine kinases of growth factor receptors (Chinkers et al., 1989; Lowe et al., 1989) and such protein kinases domain are known to be competent in the binding of ATP. In order to determine which domain of the ANF-R_1 receptor interacts with ATP relative to this protease-sensitive region, we studied the effect of limited proteolysis on the ATP modulating effects that we have documented. Following limited proteolytic treatment with trypsin, a 70-kDa truncated receptor can still be photolabeled by ^{125}I-ANF (Figure 3A). The maximal formation of the 70-kDa fragment was observed between 16 and 35 μg of trypsin/mg of protein, and the inhibitory effect of ATP on ANF binding to BAZG membranes was sensitive to limited proteolytic digestion and appeared to be almost totally abol-

ished at a trypsin concentration above 30 μg/mg of protein (Figure 3B).

Discussion

This study supports the interaction of ATP with an intracellular regulatory site on the ANF-R_1 receptor from bovine adrenal zona glomerulosa. We demonstrate that ATP modulates ^{125}I-ANF binding to both crude membranes and to affinity-purified ANF-R_1 receptor. The effect of ATP on the ANF-R_1 receptor does not seem to involve a phosphorylation reaction but rather the occupation of an allosteric site as suggested by its reversibility when ATP is removed and by the fact that the nonhydrolyzable analogue App(NH)p is able to mimic the effect of ATP. This regulation of the membrane ANF-R_1 receptor binding activity by nucleotides appears specific for adenine nucleotides, even though GTP is able to reduce ANF binding in the same membrane preparation. The fact that Gpp(NH)p, a nonhydrolyzable GTP analogue, is without effect on ANF binding to BAZG membranes would tend to rule out the hypothesis that GTP directly interacts with the ANF-R_1 receptor at the allosteric regulatory site the same as that proposed for ATP. Moreover, this is also supported by the results obtained with pure ANF-R_1 receptor where GTP and Gpp(NH)p fail to alter the ^{125}I-ANF binding. Interestingly, Gpp(NH)p does not modify the ATP effect when it is added simultaneously. The effect of GTP might rather be explained by a nucleotide interconversion activity in our membrane preparation. As reported for microtubules and membranes of HL-60 leukemic cells (Batra et al., 1987; Seifero et al., 1988), the presence of nucleoside mono- and diphosphate kinase activities in membrane proteins can generate ATP from ADP or AMP in the presence of GTP. In our membrane preparation, it is possible that the resulting ATP produced by nucleotide interconversion of GTP into ATP might support the small regulatory effect on ANF binding function observed in the presence of GTP.

It has been previously reported that ATP, which inhibits ^{125}I-ANF binding, alters the proportion of the high- and low-affinity components of the ANF binding to BAZG membranes with little change in their reciprocal affinity (De Lêan, 1986). In addition, our data on the effect of ATP on the kinetic parameters of ^{125}I-ANF binding to the BAZG membranes indicate that occupation by ATP of an allosteric binding site on the ANF receptor reduces the overall interaction of the radioligand with its receptor by an alteration of the radioligand association rates leading to destabilization of the high-affinity form of ANF binding but also of the low-affinity form. Whether those two forms of the receptor correspond to a monomeric and a dimeric form of the receptor as we have suggested (Meloche et al., 1987) remains to be clarified.

The interaction of ATP with the ANF-R_1 receptor did not result only in the regulation of the activity of the binding domain. In the intact BAZG membranes, the ANF-stimulated guanylate cyclase catalytic activity of the ANF-R_1 receptor was also modulated by ATP. In the presence of ATP, the ANF-stimulated guanylate cyclase activity is greatly enhanced and ATP by itself was without effect on the enzyme activity. These results suggest that ATP can exert its effect on the guanylate cyclase activity only after activation of the ANF-R_1 receptor by ANF. Similar results were published concerning the EGF receptor (Basu et al., 1986), and it was suggested that ATP can exert its effect on the EGF receptor only after EGF binding because in the absence of EGF the regulatory site that binds ATP is hindered.

The ability of ATP to reduce ANF binding and to enhance ANF-stimulated guanylate cyclase activity in the same range of concentrations with similar ED_{50} values seems compatible with a common mechanism involved in the two processes. This suggests that ATP interacts with a single regulatory site to exert its multiple regulatory effects on ANF-R_1 receptor. Therein, the fact that the ^{125}I-ANF binding to the affinity-purified ANF-R_1 receptor is still reduced by ATP suggests that ATP interacts directly with a regulatory site on the ANF-R_1 receptor to exert its effects on the ANF binding activity. Moreover, the very low amount of protein contaminant in our purified receptor preparation (<5%; Meloche et al., 1988) argues against the fact that an accessory protein still associated with the receptor can mediate the 25% inhibition of the ^{125}I-ANF binding observed with this preparation. In rat liver membranes, Kurose et al. (1987) have suggested that an allosteric ATP binding site on the ANF-R_1 receptor is also responsible for the potentiation of the guanylate cyclase activation by ATP. In contrast, Chang et al. (1990) have proposed that the effect of ATPγS on the ANF-stimulated guanylate cyclase activity from rat lung membranes might be mediated by accessory proteins or some cofactors since it was lost following extensive membrane washing. However, if we looked carefully at their results, the modulation by ATPγS of the ANF-stimulated guanylate cyclase activity in the washed membranes was not totally abolished. After the washing, the activation of the guanylate cyclase by ANF is about 40% of that observed in the unwashed membranes and the modulatory effect of ATPγS on the ANF-stimulated guanylate cyclase activity is still observed. It is possible that further membrane washing could result in a loss of adenine regulatory accessory proteins involved in the regulation of the activation of the ANF-sensitive guanylate cyclase. However, the persistent residual adenine nucleotide regulatory activity of the ANF-stimulated guanylate cyclase in the washed rat lung membranes suggests that an intrinsic allosteric ATP binding site can also exist in the guanylate cyclase domain of the receptor. As the modulation by ATP of the guanylate cyclase activation is concerned, this following assumption cannot be experimentally tested with the pure ANF-R_1 receptor since purification has always resulted in a loss of guanylate cyclase activation by ANF.

Recently, for the first time, it was reported that the ANF-sensitive particulate guanylate cyclase can be Triton-solubilized from bovine adrenal cortex membranes without major alterations in the kinetic properties of the enzyme (Ivanova et al., 1990). Under these conditions, the enzyme activity retained its sensitivity toward ANF; however, the ATP effect was no longer observed, suggesting that a cofactor is required for the modulation by ATP of the enzyme activity. Clarification of the existence and role of accessory protein or cofactor in the adenine nucleotide modulation of the ANF-stimulated guanylate cyclase activity will have to await successful reconstitution experiments.

The finding that only the ANF bound to the ANF-R_1 receptor from epithelial cell line LLC-PK1 and not the ANF bound to the ANF-R_2 receptor from fibroblast cell line NIH-3T3 was inhibited by the presence of ATP documents that this type of nucleotide regulation is specific to the ANF-R_1 subtype. Interestingly, the ANF-R_2 dimeric receptor protein is devoid of the cytoplasmic protein kinase and guanylate cyclase domains present in the ANF-R_1 subtype (Fuller et al., 1988). However, in the presence of ATP, ^{125}I-ANF binding to the NIH-3T3 membranes appears slightly enhanced. This effect of ATP could be supported by an indirect modulation of the amount of ANF bound to the ANF-R_2 receptor by chelation of the free Mn^{2+} in the incubation medium. Since

it has already been reported that increasing concentrations of $MnCl_2$ from 0 to 10 mM decreased by 50% the ^{125}I-ANF bound to the NIH-3T3 membranes (Fêthière et al., 1989), the presence of ATP at 1 mM could contribute to reduce the free Mn^{2+} and consequently to increase the binding of ANF to the NIH-3T3 membranes.

Limited tryptic digestion of BAZG membranes containing the ANF-R_1 receptor with increasing concentrations of trypsin (Figure 3A) results in the progressive yield of a membrane-associated ANF-binding fragment of 70 kDa as documented by Liu et al. (1989). A fragment of similar size was reported by Pandey et al. (1988) following tryptic proteolysis of LLC-PK1 cells previously photoaffinity labeled by the 4-azidobenzoyl-^{125}I-ANF. On the basis of the size of the fragment after proteolysis, the loss of guanylate cyclase activity, and the association of the residual fragment with the membranes, Liu et al. (1989) have proposed that trypsin cleaves the ANF-R_1 receptor at a site 70–90 residues distal to the membrane-spanning domain. In this study, our results indicate that the ATP binding domain on the ANF-R_1 receptor is either fragmented or absent in the 70-kDa proteolytic fragment that contains the functional ANF binding domain and the membrane-spanning region (Liu et al., 1989). Therefore, the nucleotide binding activity is associated with the intracellular domain of the receptor that represents the protein kinase homology domain (Chinkers et al., 1989; Lowe et al., 1989).

Interestingly, in spite of striking differences between the hormonal activation of adenylate cyclase and guanylate cyclase, these two systems present an attractive analogy. This analogy is based on the fact that adenylate cyclase activation following the challenge of the G-protein-coupled receptor is fully dependent on guanine nucleotides and that maximal activation of the ANF-sensitive guanylate cylase requires adenine nucleotides.

In summary, our results support the hypothesis that ATP interacts in an allosteric manner with the ANF-R_1 receptor subtype to modify the ANF binding activity. The significance of ATP modulation of the ANF-R_1 receptor to physiological functions of ANF is still unclear. In view of the very high affinity and slow reversibility of ANF binding to its receptor sites in isolated membrane preparations, this regulatory effect by ATP might promote dynamic recycling of the ANF-activated receptor form by favoring dissociation of the hormone.

Added in Proof

In the course of the review of this article, Chinkers et al. (1991) have reported that adenine nucleotides are required for activation of rat atrial natriuretic peptide receptor/guanylyl cyclase expressed in a Baculovirus system.

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Registry No. ATP, 56-65-5; App(NH)p, 25612-73-1; ADP, 58-64-0; AMP, 61-19-8; GTP, 86-01-1; atriopeptin, 85637-73-6.

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