Identification of Apolipoprotein A1 and Immunoglobulin as Components of a Serum Complex That Mediates Activation of Human Sperm Motility†

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ABSTRACT: Serum is superior to other body fluids in activating the progressive motility of human spermatozoa and is used in connection with sperm separation for fertilization in vitro. The major activating capacity is localized to a macromolecular fraction, purified to homogeneity by a four-step protocol with ion-exchange chromatography, chromatofocusing, exclusion FPLC (elution corresponding to a molecular mass of about 250 kDa), and Blue Sepharose chromatography (no binding but elimination of albumin). The pure protein, at a concentration of 20–70 nmol/L, activated the motility to the same extent as serum. SDS–polyacrylamide gel electrophoresis under nonreducing conditions showed one band corresponding to a molecular mass of about 180 kDa. In the presence of mercaptoethanol, two bands are obtained corresponding to 50 kDa and about 25 kDa, respectively. Without the Blue Sepharose step, the sample after reduction revealed an additional band at about 67 kDa, suggesting that the fraction is then in complex also with albumin. Amino acid sequence analysis of the Blue Sepharose eluate identified three protein chains—those of apolipoprotein A1 and immunoglobulin heavy and light chains—suggesting that the preparation is an apolipoprotein A1–immunoglobulin complex. Antiserum raised toward the pure preparation in a rabbit inhibited human sperm motility, when added directly to spermatozoa. Pretreatment of human serum with rabbit antiserum significantly reduced its ability to activate sperm motility. The sperm activating capacity of the protein complex was destroyed by heating at 100 °C for 5 min, suggesting that the activity was dependent on intact protein conformations. Albumin, apolipoprotein A1, and immunoglobulins by themselves had only minor effects on sperm motility. It appears likely that an apolipoprotein A1–immunoglobulin complex, that we tentativly name "sperm activating protein", mediates activation of human sperm motility.

Motile spermatozoa find their way through the uterus and into the Fallopian tubes where fertilization takes place (Clermont, 1966; Austin, 1985). During this passage, sperm progressive motility must be preserved. In some species, spermatozoa even undergo a hyperactivation in the distal part of the Fallopian tube (Austin, 1985). In the human, different ions (Nelson, 1985) affect sperm motility, but little is known about the effects of other compounds present in the fluids of the female genital tract. However, serum has for a long time been known to support human sperm motility and is therefore used in connection with sperm separation for fertilization in vitro. After development of a "standardized model system" for human sperm motility tests (Åkerlöf et al., 1987), we found that the major sperm activating factor is mediated by a specific macromolecular fraction with a molecular mass around 250 kDa (Åkerlöf et al., 1989). As this macromolecule may be important for normal fecundity and for treatment of infertility, we decided to purify it as a step toward understanding its biological function. This report describes the purification, analysis, and identification of the macromolecule named "sperm activating protein" (SPAP).

Materials and Methods

Model for Sperm Motility Test. (A) Semen Samples. Ejaculates and blood sera were collected from six donors and were analyzed within 2 h as described (Fredricsson, 1979). Separation of motile human spermatozoa was achieved by self-migration using a swim-up technique or a Percoll gradient technique followed by translocation of spermatozoa to a defined culture medium (Åkerlöf et al., 1987).

For the swim-up preparation, 0.3 mL of semen was covered with 1 mL of tissue culture medium, RPMI-1640 (Flow), supplemented with 13% (v/v) male human serum, 25 mM Hepes (Sigma), 50 IU/mL penicillin, and 50 μg/mL streptomycein (GIBCO) (buffer B). After incubation at 37 °C for 45 min, the uppermost 0.5 mL, containing motile sperm, was harvested. For the Percoll gradient technique, 1 mL of the ejaculate was layered gently on top of a Percoll gradient. After 3 h at 37 °C (without centrifugation), the upper part was sucked off and discarded, and the lower 3 mL was suspended (Pousette et al., 1986b).

The fractions from 5–10 tubes (separated by swim-up or Percoll) were pooled and added to a pump–filter system (Åkerlöf et al., 1987) in order to translocate motile spermatozoa to buffer A (buffer B without serum).

After dilution to about 3 million spermatozoa/mL, aliquots of 0.5 mL were transferred, and 0.1-mL portions of buffer A (control), serum (control), different additions, or eluates from the purification steps were added. During establishment of the protocol for purification of SPAP, progressive motility was assayed at different time intervals. For routine preparations, motility was measured 4–6 h after addition of the test solutions, since the unspecific effect of albumin is minimized at this time.

(B) Additives. Human serum (used as control in sperm motility tests and as an additive in buffer B) was incubated at 56 °C for 30 min and stored at −20 °C until used. Normal rabbit sera, rabbit anti-SPAP serum, and also immunopre-

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cipitated sera (described below) were added directly or in different combinations to the sperm motility test.

Purified human apolipoprotein A1 (A-9284 Sigma) was dissolved in buffer A to 1.7 g/L and added to the sperm motility test, giving final concentrations of 0.04–0.3 g/L in the test tubes.

Gammonativ (Kabi, Sweden), containing human immunoglobulin and albumin, was submitted to Blue Sepharose chromatography to remove the albumin. The immunoglobulin was freeze-dried, dissolved in water, and added to the sperm motility test.

(*C*) *Motility*. Progressive motility of human spermatozoa was analyzed as described (Pousette et al., 1986a). An aliquot of the sample was placed in a Bürker chamber at room temperature, and the number of spermatozoa passing a specific line, 0.25 mm in length, within 1 min was counted. This procedure was repeated once and the average number of passages calculated. Knowing the concentration, results were expressed as passages per minute per million spermatozoa per milliliter. This value is termed the specific progressive motility (SPM). Sperm motility was assayed by using aliquots from every fraction eluted. The motility was usually assayed 4–6 h after addition of spermatozoa to avoid the initial increase in sperm motility that can be observed after addition of, e.g., albumin.

(*D*) *ATP*. The content of ATP was analyzed in the "standardized model system" as described (Pousette et al., 1986b) and specific ATP defined as content of ATP per spermatozoon.

Purification of SPAP. Sera from male blood donors were incubated at 56 °C for 30 min before storage at −20 °C until used for purifications. Fractions were dialyzed and/or lyophilized before assays for sperm activating capacity in the "standardized model system".

(*A*) *Ion-Exchange Chromatography*. Serum (100 mL) was fractionated on a 100-mL DEAE-Sepharose column (Pharmacia Fine Chemicals) equilibrated in 0.01 M phosphate buffer, pH 7.4. The serum was diluted 2-fold with 0.01 M phosphate buffer, pH 7.4, and added to the column. After the column was washed, a linear gradient of 2 × 300 mL 0–0.25 M NaCl in 0.01 M phosphate buffer, pH 7.4, was applied. Fractions (about 5 mL) were collected, and the absorbancy at 280 nm was monitored. Aliquots of all fractions were then dialyzed against 0.125 M phosphate buffer, pH 7.4, and assayed for ability to stimulate SPM. Fractions able to promote sperm motility were pooled. Usually about 10 fractions (50 mL) recovered at about 0.2 M NaCl in the gradient were used.

(*B*) *Chromatofocusing on PBE 94*. The pooled fractions were lyophilized, dissolved in 5 mL of water (Millipore), dialyzed against the starting buffer (0.025 M histidine hydrochloride, pH 6.0), and added to a 37-mL PBE 94 chromatofocusing column. After the column was washed, Polybuffer 74, pH 4.0, was applied, and fractions were monitored for pH and sperm activating capacity. Samples (1 mL of each fraction) were dialyzed against 0.01 M phosphate buffer, lyophilized, and dissolved in 250 μL of 0.125 M phosphate buffer, pH 7.4, before addition to the test for sperm motility. Fractions showing sperm activating capacity were pooled. Usually, three fractions (15 mL), eluted at about pH 5.1, were obtained.

(*C*) *Fast Protein Liquid Chromatography*. The fractions pooled were dialyzed against 0.001 M phosphate buffer, pH 7.4, and lyophilized, dissolved in 200 μL of distilled water (Millipore), and fractionated on two Superose 12 HR 10/30 columns (serially) equilibrated in 0.125 M phosphate buffer, pH 7.4. Calibrations were performed by using ready-made standards (Pharmacia Fine Chemicals). A part (10 μL) from every fraction (600 μL) was tested for sperm activating capacity. Usually the activity was found in two fractions (1.2 mL) corresponding to a molecular mass of about 250 kDa.

(*D*) *Blue Sepharose Chromatography*. The pooled fractions after FPLC were added to a Blue Sepharose column (10 × 20 mm) equilibrated in 0.125 M phosphate buffer, pH 7.4. The sperm activating capacity did not stick to the gel and was recovered in the fractions corresponding to the void volume and the wash with 0.125 M phosphate buffer, pH 7.4 (total, 30 mL). This material was lyophilized and dissolved in 1.0 mL of water; ten microliters was routinely used for the sperm motility test.

Analytical Methods. Analytical sodium dodecyl sulfate (SDS) disc electrophoresis, in the presence or absence of 2-mercaptoethanol, was performed by using the GE 2/4 vertical system (Pharmacia) with ready-made gel cassette kits, PAA 4/30 (80 × 80 mm), in addition to standards for calibration (Pharmacia Fine Chemicals). Proteins were visualized with Coomassie Brilliant Blue.

Samples for amino acid sequence analysis were degraded in an Applied Biosystems 470 A gas-phase sequencer equipped with a 120 on-line phenylthiohydantoin analyzer.

Quantitation of total protein was performed with the Bio-Rad protein assay (Bio-Rad Laboratories, München, GFR) using bovine serum albumin as standard. Apolipoprotein A1 was analyzed with nephelometry using reagents from Behringwerke (Marburg, GFR).

Immunization and Immunoprecipitation. Two 1-year-old male New Zealand White rabbits were immunized as described (Vaitukaitis et al., 1971) with the pure SPAP protein complex. The protein, 20 μg for one animal and 80 μg for the other, in 1 mL of water was mixed with 1 mL of Freund's complete adjuvant, and the mixture was injected subcutaneously on the back of the animals. After 10 weeks, antiserum was detected in the rabbit given 20 μg of SPAP.

Normal inactivated male serum and rabbit anti-SPAP serum were mixed in proportions ranging from 1:19 to 19:1 (v/v). The mixtures were incubated at 25 °C for 20 h before the tubes were centrifuged at 1400*g* for 25 min. The supernatants were removed by suction, and the pellets formed were stored at −20 °C. The supernatants were tested for sperm motility and analyzed for apolipoprotein A1.

Results

Purification of SPAP. SPAP was purified to homogeneity by using a four-step procedure.

(*A*) *Ion-Exchange* (*DEAE-Sepharose*) *Chromatography*. The sperm activating capacity was eluted at about 0.2 M NaCl.

(*B*) *Chromatofocusing on PBE 94*. The sperm activating capacity was eluted at pH 5.1.

(*C*) *Superose Fast Protein Liquid Chromatography*. The sperm activating capacity was eluted corresponding to a molecular mass of about 250 kDa.

(*D*) *Blue Sepharose Chromatography*. The sperm activating capacity was eluted in the void fraction.

Starting with 100 mL of serum containing about 5 g of total protein, about 50 μg of pure SPAP was recovered.

Homogeneity and Component Composition of SPAP. SDS-polyacrylamide gel electrophoresis under nonreducing conditions showeed one band corresponding to a molecular mass of about 180 kDa (Figure 1). In the presence of mercaptoethanol, this fraction gave two bands corresponding

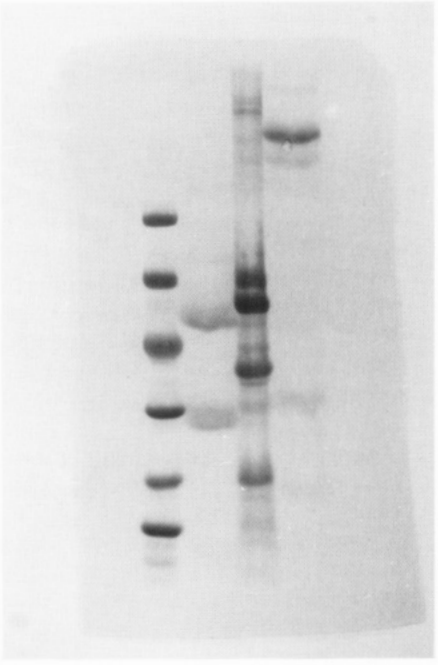


FIGURE 1: SDS–polyacrylamide gel electrophoresis in the presence and in the absence of 2–mercaptoethanol. The arrow denotes the origin. Lane 1: phosphorylase *b* (94000), albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), trypsin inhibitor (20100), and lactalbumin (14400). Lane 2: Blue Sepharose eluate with SPAP fraction (in the presence of 2–mercaptoethanol). Lane 3: Thyroglobulin (330000), ferritin (220000), albumin (67000), catalase (60000), lactate dehydrogenase (36000), and ferritin (18500). Lane 4: Blue Sepharose eluate with SPAP fraction in the absence of 2–mercaptoethanol.

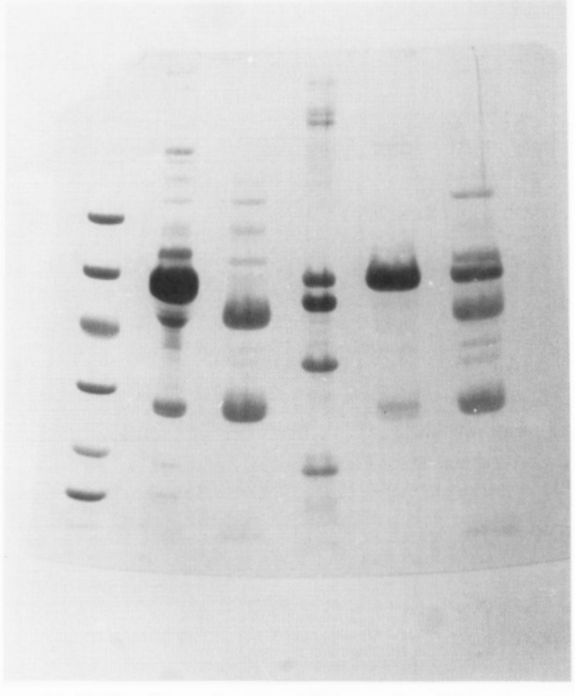


FIGURE 2: SDS–polyacrylamide gel electrophoresis in the presence of 2–mercaptoethanol. Lane 1: same as lane 1 of Figure 1. Lane 2: normal serum. Lane 3: Blue Sepharose eluate with SPAP fraction. Lane 4: same as lane 3 of Figure 1. Lane 5: the albumin fraction of the FPLC eluate. Lane 6: the SPAP fraction of the FPLC eluate.

to 50 kDa (later identified as immunoglobulin heavy chain) and about 25 kDa (later identified as apolipoprotein A1 and immunoglobulin light chain). Without the Blue Sepharose step, the nonreduced complex eluted as a larger component,

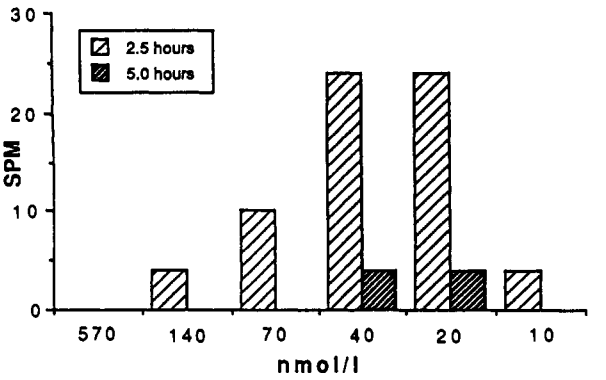
Table I: Amino Acid Sequence Analysis of SPAP[a]

	I		II		III	
cycle	residues	pmol	residues	pmol	residues	pmol
1	Asp	250	(Asp	200)	Glu	200
2	Glu	240	Ile	190	Val	180
3	Pro	200	Val	170	Gln	200
4	Pro	210	Met	170	Leu	200
5	Gln	190	Thr	150	Val	140
6	Ser	150	Gln	130	Glu	130
7	Pro	160	Ser	110	Ser	110
8	Trp	50	Pro	140	Gly	130
9	Asp	150	Val	140	Gly	120
10	Arg	100	Thr	110	(Glu	100)
11	Val	210	Leu	90	Leu	90
12	Lys	170	Ser	100	Val	110
13	Asp	140	(Val	100)	Gln	80
14	Leu	170	Ser	80	Pro	100
15	Ala	150	(Pro	90)	Gly	90
16	Thr	100	(Gly	80)		
17	Val	140	Glu	60		
18	Tyr	130	Arg	50		
19	Val	130	Ala	70		
20	Asp	100	Thr	60		
21	Val	130				
22	Leu	110				
23	Lys	80				
24	Asp	90				
25	Ser	70				
26	Gly	80				

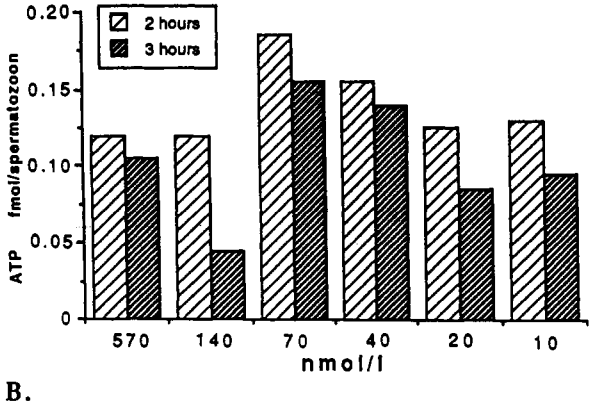
[a] Degradation of two preparations revealed three major N-terminal sequences recovered in average ratios of about 1.4:1.1:1.0 that could be interpreted for 26, 20, and 15 cycles, respectively, before high-background, carry-over, and residue coincidences in the different structures prevented safe assignments. For the triplets identified at each cycle, the assignment as to sequences I, II, and III, respectively, was not always possible from recoveries only, but listing as belonging to I, II, III was readily assigned once combinations were found to fit into three known structures. I corresponds to apolipoprotein A1, II to immunoglobulin light chains (κ), and III to immunoglobulin heavy chains (γ, μ, and α). At a few positions (1, 7, and 11), only two major residues were identified (one in higher yield), reflecting positions where two of the structures appeared to have identical residues. Recoveries are split accordingly in the listing. Assignments considered tentative, mainly because of considerable background, are given within parentheses. Two preparations gave almost identical results, except for slight variation in yields of individual residues, tentative assignments, and lengths of safe sequence tracings.

and after reduction, SDS–polyacrylamide gel electrophoresis revealed an additional band at about 67 kDa (Figure 1), suggesting that the molecule is then in complex with albumin, the latter being removed by the Blue Sepharose step.

Amino Acid Sequence Analysis. Two different pools, each of three preparations of SPAP from the Blue Sepharose step, were analyzed by amino acid sequence degradation. Results obtained were highly similar, establishing reproducibility of the preparations, and revealing the presence of three major N-terminal sequences (Table I), which were identified for about 25 cycles before all were difficult to trace against a high background. The triplets for each position were screened against known structures, revealing that the three protein structures were known before. Thus, one corresponded exactly to human apolipoprotein A1 (Baker et al., 1975), while the other two were identical with the major alternatives known for immunoglobulin light and heavy chains. The light chain corresponds to a typical κ chain, and the heavy chain shows maximal fits with several γ, μ, and α chains, and could well be a typical γ chain. Although the nature of the immunoglobulin chains should not be considered final from a sequence analysis in a mixture, the results nevertheless clearly identify SPAP as composed of three types of protein chain, apolipoprotein A1 and immunoglobulin heavy and light chains,



A.



B.

FIGURE 3: Effect of purified SPAP on SPM (A) and ATP content (B). Sperm were separated by using the Percoll technique and transferred to buffer B. Purified SPAP was added in increasing concentrations to aliquots of the sperm solution, and spermatozoa were assayed for motility, 2.5 and 5 h later, and for ATP content 2 and 3 h after addition of SPAP.

without obvious signs of multiplicity. Stoichiometries are difficult to judge since initial yields in the degradation may be slightly different for the chains, but roughly equimolar amounts of the chains appear possible.

Sperm Activating Capacity of SPAP. The eluates from the different purification steps activate human spermatozoa to the same extent as 16.7% (v/v) serum. The sperm activating capacity of purified SPAP was found to be dependent on the SPAP concentration. The activation could be detected by using SPM as well as ATP (Figure 3). In the "standardized model system" with 2.5 million spermatozoa/mL, the maximum effect was obtained at a concentration of 20–70 nmol/L. Purified SPAP heated to 100 °C for 5 min did not activate sperm motility. Purified albumin, apolipoprotein A1, and immunoglobulins had only minor effects of short duration (a couple hours) on sperm motility (data not shown).

Immunization and Characterization of Anti-SPAP Serum. Ten weeks after immunization, antiserum of sufficient titer was detected in one rabbit. When this antiserum was incubated with normal male sera in proportions of about 9:1 (v/v), a precipitate was formed. Analysis of the supernatant for apolipoprotein A1 showed a low apolipoprotein A1 content compared with that of untreated sera, suggesting that most of the apolipoprotein A1 had been precipitated by the rabbit serum. Compared with untreated human serum, the precipitated human serum showed less capacity (about 25% of untreated serum) to support sperm motility. When rabbit anti-SPAP serum was added directly to spermatozoa in the "standardized model system", the motility was reduced as compared to that seen with the addition of preimmune serum.

Discussion

Our earlier results have shown that human serum is superior both to seminal plasma and to follicular fluid in supporting motility of human spermatozoa. The major sperm activating capacity in serum was previously found to be mediated by a fraction with a molecular mass of about 250 kDa (Åkerlöf et al., 1989). This fraction is now shown to be composed of a complex containing albumin, apolipoprotein A1, and immunoglobulin heavy and light chains. As boiling destroys the activating capacity, it is concluded that the effect is mediated by the macromolecule itself rather than by a small ligand.

The molecular mass of the complex, as judged from exclusion chromatography, was calculated to be about 250 kDa. After chromatography on Blue Sepharose, the band on the SDS–polyacrylamide gel corresponded to about 180 kDa. The difference is about 70 kDa; i.e., it could correspond to the molecular mass of albumin. Stoichiometries are difficult to judge, but a complex between equimolar amounts of the immunoglobulin chains and the apolipoprotein appears possible. The molecular mass of this complex should be about 180 kDa which is similar to the value deduced from gel electrophoresis. Although SPAP without albumin could activate spermatozoa, we believe that the biologically active form of SPAP contains one molecule of albumin and has a molecular mass of about 250 kDa, in agreement with the results of exclusion chromatography. The composition of SPAP was unexpected. Notably, however, covalently linked serum protein–immunoglobulin complexes have earlier been identified in other circumstances (Grubb et al., 1986).

Our studies show that the antiserum raised interacts with the SPAP complex and with immunoglobulins. In order to elucidate the diagnostic use of SPAP, a specific assay has to be developed.

The mechansim by which SPAP induces sperm activation is unknown. Judging from the small amounts of SPAP required for activation, a receptor-mediated or enzyme-linked mechanism appears likely. SPAP also affects sperm ATP content, however, not to the same extent as it influences SPM. It is therefore not clear if SPAP affects motility via an effect on ATP content. In this context, it may be noted that apolipoprotein A1 activates lecithin–cholesterol acyltransferase (LCAT), but we have no evidence that this or any other enzyme is involved in sperm activation.

The novel macromolecular complex is probably present also in other extracellular fluids. It is known that motility is the single most important variable for judging sperm fertilization ability and that different sera and follicular fluids activate sperm nonidentically. SPAP may be useful both for diagnostic and for therapeutic use.

Registry No. ATP, 56-65-5.

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

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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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[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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