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Involvement of N_i protein in the functional coupling of the atrial natriuretic factor (ANF) receptor to adenylate cyclase in rat lung plasma membranes

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In the presence of 1 μM atrial natriuretic factor (ANF) and low (0.1 mM) Mg^{2+} concentrations, the initial rate of binding of [^{3}H]guanosine 5′-[β,γ-imido)triphosphate ([^{3}H]p[NH]ppG) to rat lung plasma membranes was increased twofold to threefold. ANF-dependent stimulation of the initial rate of [^{3}H]p[NH]ppG binding was reduced at high (5 mM) Mg^{2+} concentrations. Preincubation of membranes with p[NH]ppG (5 min at 37 °C) eliminated the ANF-dependent effect on [^{3}H]p[NH]ppG binding whereas ANF-dependent [^{3}H]p[NH]ppG binding was unaffected by similar pretreatment with guanosine 5′-[β-thio]diphosphate (GDP[βS]). An increase in ANF concentration from 10 pM to 1 μM caused a 40% decrease in forskolin-stimulated or isoproterenol-stimulated adenylate cyclase activities (IC_{50} 5 nM) in rat lung plasma membranes. GTP (100 μM) was obligatory for the ANF-dependent inhibition of adenylate cyclase, which could be completely overcome by the presence of 100 μM GDP[βS] or the addition of 10 mM Mn^{2+}. Reduction of Na^{2+} concentration from 120 mM to 20 mM had the same effect. Pertussis toxin eliminated ANF-dependent inhibition of adenylate cyclase by catalyzing ADP-ribosylation of membrane-bound N_i protein (41-kDa α subunit of the inhibitory guanyl-nucleotide-binding protein of adenylate cyclase). The data support the notion that one of the ANF receptors in rat lung plasma membranes is negatively coupled to a hormone-sensitive adenylate cyclase complex via the GTP-binding N_i protein.

Atrial natriuretic factors (ANF) are small peptides that have been shown to be synthesized, stored and secreted by mammalian atrial tissue [1]. They exhibit natriuretic and diuretic activities [1] as well as regulating blood pressure and vascular tonicity [2 – 4]. They also play a role in the regulation of aldosterone [5], renin [4, 8] and vasopressin [7] secretion. The diverse and numerous effects of ANF and the large number of tissues that contain receptors for ANF suggest that the mechanism of the ANF action may involve different ANF receptors and intracellular second messengers.

It has been shown that the ANF-dependent increase in cGMP levels that occurs in diverse tissues (kidney, lung, aorta, intestine, liver, testes, adrenal cortex) [3, 8 – 11] can be correlated with the activation of a particulate guanylate cyclase. Analysis of ANF receptors from different cells, using an affinity cross-linking technique, has revealed at least two types of the ANF receptor with molecular masses of 66 kDa and 130 kDa [12 – 17]. The 66-kDa receptor is not coupled to particulate guanylate cyclase, and it is unknown on which second messenger system this receptor operates.

ANF has also been shown to inhibit adenylate cyclase in various target tissues such as vascular smooth muscle, adrenal cortex and pituitary gland [18 – 21]. The mechanism of this coupling of the ANF receptor to adenylate cyclase is unclear. Here we report that ANF-dependent inhibition of adenylate cyclase also exists in rat lung plasma membranes. This signal of ANF receptor coupling is transduced to the adenylate cyclase complex via the inhibitory GTP-binding protein (N_i protein).

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Abbreviations. ANF, atrial natriuretic factor; N_i, inhibitory guanyl-nucleotide-binding protein of adenylate cyclase; p[NH]ppG, guanosine 5′-[β,γ-imido]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate; IC_{50}, half-maximal inhibitory concentration.

EXPERIMENTAL PROCEDURE

Materials

[α-^{32}P]ATP (3000 Ci/mmol) and [8-^{3}H]guanosine 5′-[β,γ-imido)triphosphate ([^{3}H]p[NH]ppG) (15 Ci/mmol) were obtained from Amersham. [*adenylate*-^{32}P]NAD, 500 Ci/mmol (NEN Products), human ANF (Nova Biochem), forskolin (Calbiochem), ATP, GTP p[NH]ppG, NAD, GDP[βS], creatine kinase (all Boehringer), creatine phosphate (disodium salt), cAMP (Sigma) were used. Pertussis toxin, purified as described [22], was kindly supplied by Dr V. O. Rybin (Laboratory of Molecular Endocrinology, CRC).

Preparation of plasma membranes and cytosolic fraction from rat lungs

Lungs were rapidly excised from 8 – 10 Wistar rats (280 – 300 g) after decapitation, washed with ice-cold buffer A containing 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and stripped of vasculature and bronchi. All subsequent procedures were conducted at 4 °C. Lungs were minced with scissors and homogenized in five volumes of buffer A with a Polytron homogenizer (three times for 15 s with 30-s intervals at maximal speed). The

homogenate was filtered through two layers of medical gauze and centrifuged for 60 min at 25000 rpm (SW-27 rotor Beckman). The lipid-free portion of the supernatant cytosol was aspirated and 1–2-ml aliquots were frozen in liquid nitrogen and stored at −80°C. The pellet was rinsed with buffer A and resuspended in five volumes of the same buffer containing 0.25 M sucrose. 15 ml buffer A containing 0.8 M sucrose was gently layered under the crude membrane preparation (20 ml) with a syringe, and samples were centrifuged for 60 min at 25000 rpm. The plasma membrane fraction floating between 0.25 M and 0.8 M sucrose was aspirated, diluted with four volumes of buffer A and centrifuged for 90 min at 25000 rpm. The resulting pellet was resuspended in buffer A to give a final concentration of 3–5 mg protein/ml, frozen in liquid nitrogen and stored at −80°C.

[^{3}H]p[NH]ppG binding assays

[^{3}H]p[NH]ppG binding to rat lung plasma membranes was determined in a reaction mixture (final volume 100 µl) containing 25 mM Hepes/NaOH (pH 8.0 at 30°C), 0.1 mM $MgCl_2$, 100 mM NaCl, 1 µM [^{3}H]p[NH]ppG, 8–15 µg membrane protein. Incubations were carried out at 30°C for 2 min. Non-specific binding was determined in the presence of 1 mM unlabelled p[NH]ppG. Bound [^{3}H]p[NH]ppG was separated from free ligand by filtration through GF/C filters (Whatman). The filters were washed three times with 4 ml buffer containing 20 mM cold Tris/HCl (pH 7.5 at 4°C) and 100 mM NaCl. Radioactivity retained on the filters was measured in a liquid scintillation counter.

Adenylate cyclase assay

The reaction mixture (final volume 100 µl) contained 50 mM Tris/HCl (pH 7.5 at 37°C), 1 mM cAMP, 10 mM creatine phosphate (disodium salt), 0.25 mg/ml creatine kinase, 2 mM $MgCl_2$, 100 mM NaCl, 0.1 mM [α-^{32}P]ATP ($1.0-2.0\times10^6$ cpm/assay), 2.5 mg/ml cytosolic protein, and 30–50 µg membrane protein. Incubations were carried out for 10 min at 37°C. Under the given assay conditions adenylate cyclase activity was linear with respect to both time and protein concentrations and ATP levels were maintained at 98% of the original [determined by poly(ethyleneimine)-cellulose chromatography]. Reactions were terminated by the addition of 200 µl 0.5 M HCl followed by immersion in boiling water for 7 min. Samples were neutralized by adding 200 µl 1.5 M imidazole. cAMP was quantified by the method of White [23]. Experiments were performed in triplicate at least three times for each preparation of membranes. Standard deviation was generally less than 5%.

ADP-ribosylation of rat lung plasma membranes by pertussis toxin

Reaction mixture (final volume 1 ml) contained 50 mM Tris/HCl (pH 7.5 at 37°C), 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 1 mM ATP, 10 µM GTP, 10–100 µM NAD, 0.02 mg/ml pertussis toxin and 1 mg/ml membrane protein. Incubation was carried out for 60 min at 37°C.

SDS-PAGE and protein measurement

SDS/polyacrylamide gel electrophoresis was performed according to the method of Laemmli [24] using a 12% polyacrylamide slab gel. Protein concentrations were determined [25] using bovine serum albumin as a standard.

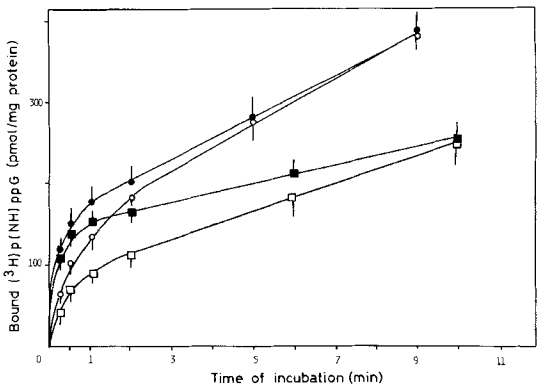

Fig. 1. *Kinetics of [^{3}H]p[NH]ppG binding to rat lung plasma membranes.* The binding assay was carried out as described under Experimental Procedure without ANF (○, □) and with 1 µM ANF (●, ■). The reaction mixture contained 0.1 mM (□, ■) or 5 mM $MgCl_2$ (○, ●)

Data analysis

Unless otherwise indicated, data are given as mean values ± standard deviation from at least four separate experiments (performed in triplicate) for each experimental series. Statistical analysis was performed using Student's *t*-test for unpaired data.

RESULTS

ANF (1 µM) in the presence of 0.1 mM Mg^{2+} increased the initial rate of [^{3}H]p[NH]ppG binding to rat lung plasma membranes twofold to threefold (Fig. 1). Raising the concentration of Mg^{2+} from 0.1 mM to 5 mM in the incubation mixture increased the initial rate of [^{3}H]p[NH]ppG binding and dramatically reduced the amplifying effect of ANF on this process. ANF-dependent stimulation of the initial rate of [^{3}H]p[NH]ppG binding to lung plasma membranes was abolished by preincubation of membranes with 10 µM p[NH]ppG but unaffected in membranes precincubated with 10 µM GDP[βS] (Table 2). For these experiments (Table 1) [^{3}H]p[NH]ppG binding was measured after 2-min incubation periods since this disequilibrium time interval was optimal (Fig. 1) for determining the ability of ANF to promote guanine-nucleotide exchange.

An increase in ANF concentration from 10 pM to 10 µM resulted in a dose-dependent inhibition of adenylate cyclase activity in rat lung plasma membranes (Fig. 2). Half-maximal inhibition of the enzyme activity was observed at 5 nM ANF. Maximal inhibition (40%) occurred between 0.1–1 µM ANF. This inhibitory effect of ANF was GTP-dependent and was not observed in GTP-free incubations or when performed in the presence of GDP[βS] instead of GTP (Table 2). GTP-dependent inhibition of adenylate cyclase by ANF was apparent only in the presence of adenylate cyclase activators, isoproterenol or forskolin, and there was no inhibitory effect of ANF on basal adenylate cyclase activity (Table 3). The addition of either 10 mM $MnCl_2$ or the reduction of Na^+ concentration from 120 mM to 20 mM abolished ANF-dependent inhibition of forskolin-stimulated adenylate cyclase in rat lung plasma membranes (Table 3). The inhibition of

Table 1. *Effect of rat lung plasma preincubation with p[NH]ppG or GDP[βS] on ANF-dependent binding of [^{3}H]p[NH]ppG*
Rat lung plasma membranes (1.0 mg/ml) were preincubated in medium containing 25 mM Hepes/NaOH (pH 8.0), 100 mM NaCl, 0.1 mM $MgCl_2$, and the indicated guanine nucleotide for 5 min at 37°C. Then the suspension was diluted with 29 vol. of cold washing buffer containing 25 mM Hepes/NaOH (pH 8.0), 100 mM NaCl, 0.1 mM $MgCl_2$, and centrifuged for 60 min at 25000 rpm (SW-27 rotor). The pellet was washed twice by resuspension in washing buffer and centrifugation as above. The final pellet was resuspended in buffer A, and [^{3}H]p[NH]ppG binding assay in the absence or presence of 1 μM ANF was carried out as described under Experimental Procedure. Asterisks indicate a significant difference ($P < 0.01$) in [^{3}H]p[NH]ppG binding between the absence and presence of 1 μM ANF

Conditions of [^{3}H]p[NH]ppG binding	Bound [^{3}H]p[NH]ppG in membranes preincubated		
	without added guanine nucleotides	with 10 μM GDP[βS]	with 10 μM p[NH]ppG
	pmol/mg protein		
Without ANF	353 ± 64	398 ± 44	331 ± 32
In the presence of 1 μM ANF	550 ± 10*	581 ± 51*	345 ± 40

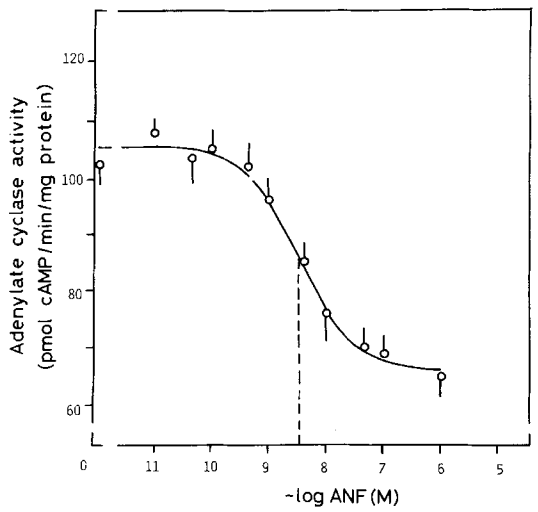

Fig. 2. *Concentration-dependent effect of ANF on adenylate cyclase activity in rat lung plasma membranes.* Adenylate cyclase activity was determined as described in Experimental Procedure. Incubations contained 100 μM GTP and 100 μM forskolin. (- - - -) The half-maximal effective concentration of ANF

Table 2. *Effect of guanine nucleotides on ANF-dependent inhibition of adenylate cyclase in rat lung plasma membranes*
Assays were performed as described in Experimental Procedure. The reaction mixtures contained 100 μM forskolin, and guanine nucleotides were included as indicated. The asterisk indicates a significant difference ($P < 0.001$) in enzyme activities between the absence and presence of 1 μM ANF

Guanine nucleotide	Adenylate cyclase activity	
	without ANF	with 1 μM ANF
	pmol cAMP min^{-1} mg protein^{-1}	
Without nucleotide	115 ± 2	108 ± 6
100 μM GTP	158 ± 1	104 ± 2*
100 μM GDP[βS]	110 ± 1	111 ± 6

Table 3. *Effects of isoproterenol, forskolin, Mn^{2+}, and Na^+ on ANF-dependent inhibition of adenylate cyclase in rat lung plasma membranes*
The reaction mixtures contained 100 μM GTP, and unless otherwise indicated, the Na^+ concentration was 120 mM. Assays were performed as described in Experimental Procedure with various additions as indicated. Asterisks indicate significance of difference in enzyme activities between the absence and presence of 1 μM ANF; * $P < 0.01$, ** $P < 0.001$

Additions	Adenylate cyclase activity	
	without ANF	with 1 μM ANF
	pmol cAMP min^{-1} mg protein^{-1}	
Without additions	19.1 ± 1.6	22.3 ± 1.8
10 μM isoproterenol	39.1 ± 3.0	27.1 ± 2.1*
100 μM forskolin	87.6 ± 1.6	59.2 ± 1.1**
100 μM forskolin plus 10 mM $MnCl_2$	460 ± 11	448 ± 14
100 μM forskolin with low Na^+ (20 mM)	350 ± 40	341 ± 22

isoproterenol-stimulated or forskolin-stimulated adenylate cyclase activities by ANF was not observed after preincubation of membranes with pertussis toxin (Table 4). Pertussis toxin preincubation of membranes in the presence of [^{32}P]NAD resulted in the ADP-ribosylation of the 41-kDa protein as shown by [^{32}P]ADP incorporation data after SDS/polyacrylamide gel electrophoresis and autoradiography (Fig. 3).

DISCUSSION

Hormone-dependent exchange of tightly bound GDP for exogenous GTP on N protein induces dissociation of N protein from the hormone-receptor complex and the acquisition by the receptor of lowered affinity for its hormone. Conversely hydrolysis of GTP to GDP and P_i by N protein results in reassociation between N protein and the hormone-receptor complex and the reacquisition by the receptor of a high affinity of its hormone [26–30]. Such an affinity heterogeneity within one molecular types of receptor has been established for muscarinic cholinergic, β- and α-adrenergic, histamine and glucagon receptors [31–39] and is a function of coupling between receptors and membrane-bound GTP-binding proteins [31–39].

Heterogeneity in ANF-receptor affinities has also been previously demonstrated for bovine adrenal zona glomerulosa [40] and intact cultured smooth muscle cells [41], although

Table 4. *Effect of preincubation of lung plasma membranes with pertussis toxin on ANF-dependent inhibition of adenylate cyclase*
Preincubation with pertussis toxin in the presence of 100 μM NAD was carried out as described under Experimental Procedure. Control preincubations were conducted in pertussis-toxin-free medium. After 60 min preincubation control and ADP-ribosylated membranes were diluted with 19 volumes of ice-cold buffer containing 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA and sedimented by centrifugation (SW-27 rotor) for 60 min at 25000 rpm. The pellets were resuspended in buffer A and adenylate cyclase activity was determined immediately. Asterisks indicate a significant ($P < 0.01$) inhibitory effect of ANF on either isoproterenol, or forskolin-stimulated adenylate cyclase

Effectors	Adenylate cyclase activity	
	control membranes	membranes preincubated with pertussis toxin
	pmol cAMP min^{-1} mg $protein^{-1}$	
10 μM isoproterenol	24.7 ± 3.7	78.4 ± 6.1
10 μM isoproterenol plus 1 μM ANF	14.8 ± 0.9*	89.0 ± 3.3
100 μM forskolin	55.5 ± 0.8	91.6 ± 3.4
100 μM forskolin plus 1 μM ANF	45.0 ± 2.8*	107.0 ± 4.7

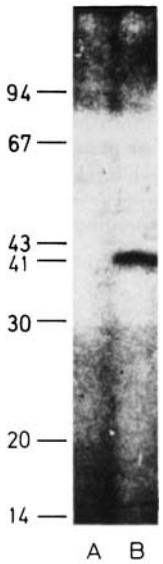

Fig. 3. *ADP ribosylation of the 41-kDa peptide by pertussis toxin in rat lung plasma membranes.* The reaction was carried out as described under Experimental Procedure in the presence of 10 μM [^{32}P]NAD (10000 cpm/pmol) in a final volume of 20 μl. A control preincubation was carried out in pertussis-toxin-free medium. After 60 min incubation the samples were diluted with electrophoresis sample buffer to a final volume of 100 μl, immersed in boiling water for 5 min and analyzed by SDS/polyacrylamide (12%) gel electrophoresis under reducing conditions followed by autoradiography. Lane A, autoradiogram of control membranes. Lane B, autoradiogram of the membranes treated with pertussis toxin. A low-molecular-mass protein calibration kit (Pharmacia) was used as a marker standard (molecular masses shown in kDa)

this may be due to the presence of at least two different molecular species of ANF receptors [11–17]. Nevertheless, that in rat lung plasmalemma ANF receptors are coupled to N protein is supported by our observation of ANF-promoted [^{3}H]p[NH]ppG binding in these membranes. We have made similar observations for plasma membranes isolated from rat kidney cortex and cultured rat aortic smooth muscle cells (data not shown). Hormone-dependent binding of GTP or its analogs to membrane-bound N protein has been observed in plasma membranes from diverse tissues [26, 36, 42–44]. This is due to hormone-receptor complex promoted exchange of N-protein-bound GDP for exogenous GTP or its analog [26–30, 33]. The GDP/GTP exchange process on N protein is Mg^{2+}-dependent, and in the presence of high concentrations of Mg^{2+} the necessity for prior hormone-receptor complex formation is circumvented [45, 46]. Thus, our observations of a reduced influence of ANF on initial [^{3}H]p[NH]ppG binding rates in the presence of high Mg^{2+} (5 mM) clearly indicates that guanine-nucleotide exchange on N protein coupled to the ANF receptor proceeds according to the accepted Mg^{2+}-dependent mechanism [27, 30, 33].

ANF-dependent binding of [^{3}H]p[NH]ppG was eliminated in membranes preincubated with p[NH]ppG but preserved in membranes preincubated with GDP[βS], indicating that ANF-stimulated exchange of guanine nucleotides on N protein was possible only when GDP or its analog GDP[βS] was present in the nucleotide-binding site of the protein. Such data are in good agreement with the currently accepted scheme for nucleotide exchange on N proteins. Namely, that the formation of the hormone-receptor/N-protein complex and consequent hormone-dependent exchange of guanine nucleotides on N protein is possibly only in the presence of GDP, but not GTP, at the nucleotide-binding site of N-protein [27–30, 33].

In an attempt to understand further the intracellular consequences of ANF-receptor/N-protein coupling we examined the effect of ANF on membrane-associated adenylate cyclase since inhibition of this enzyme by ANF has been demonstrated for several different tissues [18–21, 41]. Rat lung plasma membrane adenylate cyclase was inhibited at low concentrations of ANF ($IC_{50} \approx 5$ nM) indicative of receptor-mediated hormone action. According to the current model for the regulation of adenylate cyclase activity by hormones in eucaryotic cells, receptor-dependent inhibition of enzyme activity is promoted by coupling between adenylate cyclase and the inhibitory GTP-binding protein (N_i protein) [27, 30]. We investigated the involvement of N_i protein in ANF-dependent inhibition of adenylate cyclase in rat lung plasma membranes by application of standard procedures developed to test N_i-mediated hormone inhibition of adenylate cyclase [47–55]. The following data confirm that N_i protein is involved in ANF-dependent inhibition of adenylate cyclase: (a) the enzyme inhibition by ANF occurred in the presence of GTP and was blocked by GDP[βS]; (b) ANF-dependent inhibition of adenylate cyclase was significant only in the simultaneous presence of the enzyme activators such as isoproterenol and forskolin; (c) adenylate cyclase inhibition by ANF was abolished by 10 mM Mn^{2+} or by decreasing the Na^{2+} concentration from 120 mM to 20 mM; (d) ADP ribosylation of the 41-kDa α subunit of N_i protein by pertussis toxin blocked adenylate cyclase inhibition by ANF.

In conclusion the data presented here unequivocally demonstrate that ANF-dependent inhibition of adenylate cyclase activity in rat lung plasma membrane is mediated via N_i protein coupling and may provide a general mechanism for transducing the ANF signal into eucaryotic cells.

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