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# Participation of adenosine 5′-triphosphate in the activation of membrane-bound guanylate cyclase by the atrial natriuretic factor

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The addition of ANF to Percoll-purified liver plasma membranes produced a slight activation of guanylate cyclase; the ANF-stimulated cyclase activity was further increased upon the addition of ATP to the enzyme assay mixture. The effect of ATP to potentiate the cyclase activation was concentration-dependent, required $Mg^{2+}$ as a divalent cation, and was seen with membranes from various tissues and cells. ATP increased the maximal velocity of the cyclase without a change in the affinity for GTP or ANF. Phosphorylation by ATP might not be involved since ANF-stimulated guanylate cyclase was enhanced by non-phosphorylating ATP analogues as well. Thus, an allosteric ATP binding site is suggested to participate in ANF-induced regulation of membrane-bound guanylate cyclase.

Atrial natriuretic factor; Guanylate cyclase; ATP

## 1. INTRODUCTION

Atrial natriuretic factor (ANF) secreted from cardiac atria increases cellular cGMP probably as a result of activation of guanylate cyclase in particulate fractions [1,2], although the activation of enzyme is usually not so marked as the increment in the cyclic nucleotide observable in intact cells [3–5]. We will describe that ATP enhances ANF-induced activation of guanylate cyclase in membranes from various tissues.

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## 2. MATERIALS AND METHODS

### 2.1. *Preparation of liver plasma membranes*

Plasma membranes were prepared from rat liver at 0–4°C by the method of Seyfred and Wells [6] with slight modifications as follows. Liver tissues excised from Donryu rats (body weight 200–300 g) were homogenized in 20 mM Hepes (pH 7.5)/5 mM EDTA/250 mM sucrose/aprotinin by means of a Polytron homogenizer (PT 10-20-3500). The homogenates were pelleted by centrifugation (30000 × *g* for 15 min), washed twice (i.e., 2 cycles of suspension in 5 mM Hepes (pH 7.5)/1 mM EGTA/250 mM sucrose/aprotinin by a Teflon-pestled homogenizer followed by centrifugation), and finally suspended in 25 mM Hepes (pH 7.5)/250 mM sucrose/aprotinin. The concentration of aprotinin was 50 U/ml in these media. The suspension was then mixed with Percoll and centrifuged at 10000 × *g* for 20 min to obtain the plasma membrane fraction at the top of

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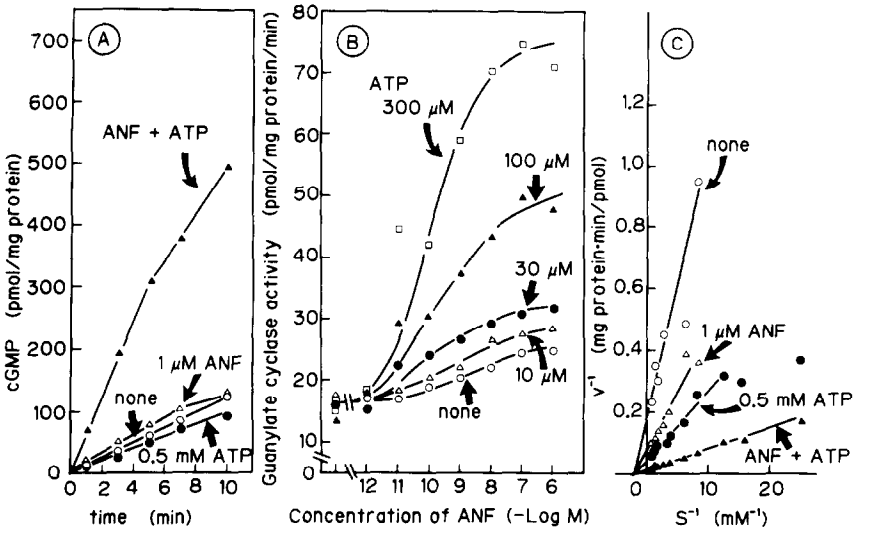


Fig.1. Enhancement by ATP of ANF-induced activation of liver membrane guanylate cyclase. (A) Time courses of cGMP accumulation as a result of guanylate cyclase activity in the absence (○) or presence of 1 μM ANF (△), 0.5 mM ATP (●), 1 μM AFN + 0.5 mM ATP (▲); (B) guanylate cyclase activities plotted against increasing concentrations of ANF in the absence (○) or presence of 10 μM (△), 30 μM (●), 100 μM (▲) or 300 μM (□) ATP; (C) Lineweaver-Burk plots for guanylate cyclase activities as a function of GTP in the absence (○) or presence of 1 μM ANF (△), 0.5 mM ATP (●) or 1 μM ANF + 0.5 mM ATP (▲).

the centrifuge tubes. The fraction was washed and stored at −80°C until use.

### 2.2. *Assay of guanylate cyclase activity*

The thawed membrane fraction was washed in 50 mM Tris (pH 7.5) and assayed for guanylate cyclase activity at 30°C in the reaction mixture (total volume of 0.1 ml) consisting of 50 mM Tris (pH 7.5), 4 mM $MgCl_2$, 10 mM theophylline, 10 mM phosphocreatine, 100 units/ml of creatine phosphokinase and 1 mM GTP, unless otherwise specified in fig.1C. The mixture was further supplemented with ANF, the preparation (consisting of 31 amino acid residues) purified from rat atrium [7], and ATP used in the concentrations indicated in the figures. The assay was started by the addition of the membrane fraction, continued for 5 min except in fig.1A and stopped by the addition of 0.1 ml of 0.2 N HCl followed by boiling for 2 min. The supernatant obtained by centrifugation at 1500 × *g* for 10 min was assayed for cGMP generated from added GTP [8] to calculate the guanylate cyclase activity. Protein was determined by the method of Lowry et al. [9] using bovine serum albumin as standard.

### 2.3. *$^{125}$I-ANF binding to liver plasma membranes*

Liver plasma membranes were incubated with 0.2 nM $^{125}$I-ANF and various concentrations of ATP for 10 min at 20°C in the same medium as used for the guanylate cyclase assay. Incubation was terminated by the addition of 5 ml of ice-cold 50 mM Tris (pH 7.5) containing 5 mM $MgCl_2$ and 100 mM NaCl, and the mixture was quickly poured onto a glass fiber Whatman GF/C (2.4 cm diameter) which had been soaked in 0.1% (w/v) polyethyleneimine (pH 10.0) [10]. The filter was rapidly rinsed twice with 5 ml of the same solution and then analyzed for the retained radioactivity by the use of a gamma counter. Specific binding assessed from the difference between bindings in the absence and presence of 1 μM non-radioactive ANF is shown on the ordinate.

## 3. RESULTS AND DISCUSSION

Plasma membrane rich-fractions from rat liver were incubated with ANF (fig.1). There was a slight increase in guanylate cyclase activity in response to ANF (fig.1A). The ANF-induced increase in guanylate cyclase activity was markedly

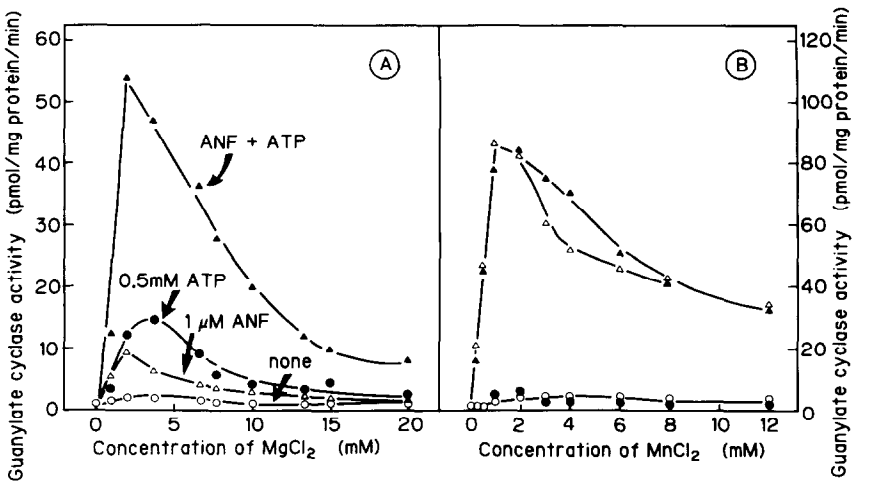


Fig.2. Guanylate cyclase activity of liver plasma membranes as supported by $MgCl_2$ or $MnCl_2$. Guanylate cyclase activity was measured without (○) or with 1 μM ANF (△), 0.5 mM ATP (●) or 1 μM ANF + 0.5 mM ATP (▲) in the medium containing the indicated concentrations of $MgCl_2$ (A) or $MnCl_2$ (B) as described in section 2.

enhanced by ATP, though ATP by itself was without effect on the enzyme activity or caused only slight effects which were different from preparation to preparation (e.g., cf. fig.1C with fig.1A). The guanylate cyclase activity was plotted against concentrations of ANF in the presence of ATP at concentrations from 10 to 300 μM (fig.1B). ATP enhanced the maximal response of the cyclase to ANF, without causing a significant change in the half-maximally effective concentration of ANF. The cyclase activities as a function of the concentration of substrate, GTP, in the presence or absence of ANF and/or ATP are depicted as the Lineweaver-Burk plots in fig.1C. The effects of ATP and ANF were to increase the maximal velocity without a change in the $K_m$ value for the substrate. Thus, $V_{max}$ and $K_m$ for liver guanylate cyclase were 6.35 pmol/mg protein per min and 0.52 mM, respectively, in the absence of ANF and ATP. $V_{max}$ increased 2.3- (to 14.9 pmol/mg protein per min), 3.5- (to 22.3 pmol/mg protein per min) and 15.4- (to 98.0 pmol/mg protein per min) fold upon the addition of ANF, ATP and ANF + ATP, respectively.

Table 1

Effects of ANF and/or ATP on guanylate cyclase activity in particulate or plasma membrane fractions prepared from various tissues and cells

| Tissues | Guanylate cyclase activity (pmol/mg protein per min) | | | |
|---|---|---|---|---|
| | None | ANF | ATP | ANF + ATP |
| Kidney | 0.75 | 6.48 | 0.65 | 34.4 |
| Lung | 1.36 | 15.91 | 6.85 | 121.6 |
| Heart | 0.67 | 1.36 | 2.94 | 6.9 |
| Brain | 1.92 | 4.70 | 3.06 | 12.8 |
| Adipocyte | 1.70 | 18.59 | 4.65 | 118.9 |
| NG108-15 | 0.59 | 1.02 | 0.71 | 9.1 |

Particulate fractions from kidney, lung and heart were prepared as described in the legend to fig.1 with the centrifugation step using Percoll being omitted. The membrane preparations from brain, adipocytes and NG108-15 hybrid cells were obtained by the methods of Katada et al. [13], Cushman and Wardzala [14] and Kurose et al. [15], respectively. Guanylate cyclase activity was then measured with 1 μM ANF and/or 0.5 mM ATP

Crude or partially purified membrane fractions from various tissues and cells exhibited various degrees of guanylate cyclase activity (table 1). These guanylate cyclases were activated by ANF alone 2- to 12-fold, and activated 6- to 90-fold by combining ANF with ATP, which was by itself essentially ineffective in some cases or activated the enzyme activity 1.6- to 5-fold in other cases. In any case, the activation of membrane guanylate cyclase by ANF was greater in magnitude in the presence of ATP than in its absence in all the

Table 2

Effects of adenosine triphosphate, adenine nucleotide analogues and adenosine on guanylate cyclase activity in the presence or absence of ANF

| Additions | Guanylate cyclase activity (pmol/mg protein per min) | |
|---|---|---|
| | None | ANF |
| None | 1.76 | 6.50 |
| ATP | 2.03 | 41.43 |
| AppNHp | 2.82 | 24.85 |
| $ApCH_2pp$ | 3.47 | 25.78 |
| UTP | 3.43 | 7.33 |
| CTP | 1.99 | 6.39 |
| ADP | 1.75 | 18.42 |
| ADP$\beta$S | 2.22 | 11.98 |
| AMP | 1.75 | 6.55 |
| Adenosine | 2.18 | 7.51 |

Plasma membranes partially purified from liver were incubated as described in the legend to fig.1 without or with 1 $\mu$M ANF in the presence of nucleotides or adenosine. The concentration of nucleotides and adenosine was 0.5 mM. The GTP-regenerating system was omitted from the assay mixture to minimize phosphorylation of ADP or AMP

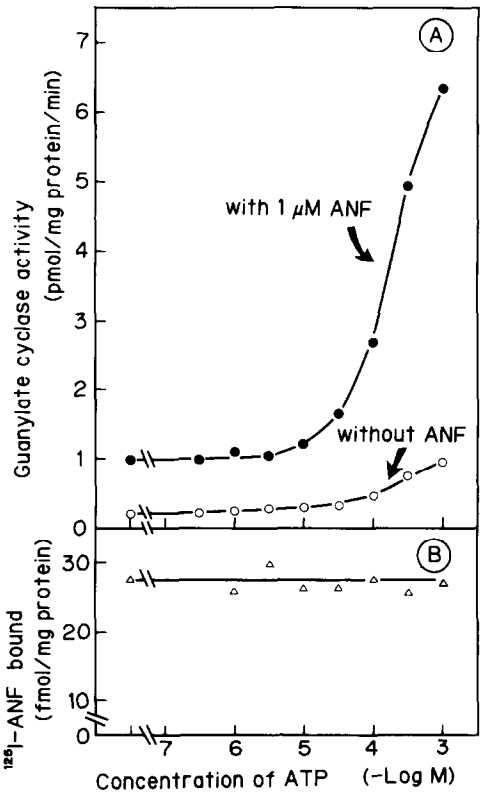


Fig.3. Failure of ATP to affect $^{125}$I-ANF binding to liver plasma membranes. (A) Guanylate cyclase activities of liver plasma membranes were measured with (●) or without (○) 1 $\mu$M ANF in the presence of the indicated concentrations of ATP. (B) Binding of $^{125}$I-ANF to liver plasma membranes under the same conditions.

tissues so far studied.

$Mg^{2+}$ (or $Mn^{2+}$) was essential for liver membrane guanylate cyclase; no enzymic activity was detected in the $Mg^{2+}$- or $Mn^{2+}$-free medium regardless of whether or not the medium was fortified with ANF and/or ATP (fig.2A). The striking difference between $MgCl_2$ and $MnCl_2$, however, was found for the effect of ATP; ATP did not increase guanylate cyclase activity measured with $MnCl_2$ in either the presence or absence of ANF (fig.2B). Thus, the unique effect of ATP to potentiate ANF-induced activation of guanylate cyclase was not observed unless the nucleotide was added to the assay medium as a complex with $Mg^{2+}$.

ANF binds with a high affinity ($K_d$ = 148 pM) to specific receptors on liver membranes as revealed by the Scatchard analysis (not shown). Since essentially the same $K_d$ value was obtained when the binding experiment was carried out in the enzyme assay mixture (i.e. the medium containing GTP and the nucleotide-regenerating system), the effects of ATP on both guanylate cyclase and $^{125}$I-ANF binding were then studied concurrently in fig.3, in which the reaction mixture was incubated at 20°C with 200 pM $^{125}$I-ANF to obtain the high-affinity binding of ANF. Under these conditions, ATP at concentrations higher than 50 $\mu$M enhanced ANF-induced activation but did not alter the binding of ANF to membranes. Therefore, ATP promoted the ANF-stimulated activity by a different mechanism from the GTP binding protein in the adenylate cyclase system. AppNHp, which does not serve as a substrate of kinases or a phosphate donor, enhanced ANF-induced activation of membrane guanylate cyclase, though the degree of enhancement was smaller than that by ATP (table 2).

The effect of ATP or ATP analogues to enhance guanylate cyclase activity might possibly be due to

inhibition of the decomposition of GTP, thereby supplying the enzyme with higher concentrations of its substrate. This possibility was studied by the use of [$\alpha$-$^{32}$P]GTP as the substrate. The concentration of GTP after incubation was determined based on radioactivities in the spot of GTP after separation on a thin-layer plate of polyethyleneimine-cellulose. In the presence of GTP-regenerating agents, roughly 80% of added GTP remained as such after 5-min incubation of the cyclase assay, regardless of whether the reaction mixture was further supplemented with ATP (or ATP analogues) or not. In the absence of the regenerating agents, 1 mM GTP decreased to 0.41 mM without addition of adenine nucleotides, to 0.55 mM with 0.5 mM ATP and to 0.75 mM with 0.5 mM AppNHp during 5-min incubation. The increase in GTP concentration from 0.4 to 0.8 mM caused 30–40% increases in guanylate cyclase activity in the presence of ANF. However, 400–600% increases in the cyclase activity were in fact induced by ATP or AppNHp under these conditions. Thus, the enhancing effect of ATP or ATP analogues on guanylate cyclase was not accounted for by the inhibition of the breakdown of the enzyme substrate.

The ANF-induced activation was enhanced by ADP as well. ADP might be converted to ATP as a result of the phosphate transfer from GTP in the medium. But, ADP$\beta$S, which is not capable of being phosphorylated, was still effective. Other nucleotide triphosphates, UTP and CTP, as well as adenosine and AMP did not increase guanylate cyclase activity in either the presence or absence of ANF. Thus, the effect of ATP to enhance ANF-induced activation of membrane guanylate cyclase is explainable by its allosteric effect on the enzyme activity rather than by the phosphorylation of membrane proteins. The allosteric site(s) is (are) rather selective to ATP but may be occupied by ADP or ATP- or ADP-analogues as well which produce smaller effects than ATP itself. A similar non-phosphorylating mechanism has been proposed for ATP-induced modulation of EGF receptor functions such as tyrosine kinase activity [11,12].

In conclusion, the Mg-ATP complex is a strong potentiator of ANF in activation of membrane guanylate cyclase of various membrane tissues. Conceivably, intracellular ATP supports the generation of intracellular signals upon stimulation of membrane ANF receptors. A likely possibility is the existence of an ATP-binding site on the ANF receptor-guanylate cyclase complex. The occupancy of this site may lead to a conformational change of the complex, thereby facilitating ANF-induced activation of the catalytic activity.

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