Analogue-Specific Action In Vitro of Atrial Natriuretic Factor on Human Red Blood Cell Ca\(^{2+}\)-ATPase Activity

FAITH B. DAVIS, SUSAN D. BLAS, MATTHEW M. DAVIS, AND PAUL J. DAVIS

SUMMARY Specific atrial natriuretic factor (ANF) analogues have been found to have inhibitory activity in vitro in a calmodulin-dependent, human red blood cell membrane Ca\(^{2+}\)-adenosine triphosphatase (ATPase) model. Studied at 10\(^{-4}\) to 10\(^{-8}\) M concentrations, atriopeptin I (residues 127-147 of rat prepro-ANF sequence) and atriopeptin III (residues 127-150) progressively inhibited Ca\(^{2+}\)-ATPase activity by up to 20% (p < 0.001). This degree of inhibition was consistent with activities of other (calmodulin-independent) enzyme inhibitors in this model. Therefore, the C-terminal Phe-Arg-Tyr sequence (residues 148-150) is unnecessary for atriopeptin action on Ca\(^{2+}\)-ATPase. Human and rat atrial peptides with amino acids 123-150 were inactive, indicating that the 123-126 sequence (Ser-Leu-Arg-Arg) must be cleaved to activate atriopeptins in this system. Human ANF fragment 129-150 also had no effect on Ca\(^{2+}\)-ATPase, defining the importance of residues 127-128 (Ser-Ser) proximal to the disulfide bridge (joining 129 to 145). The addition of purified calmodulin to red blood cell membranes in the presence of inhibitory ANF did not restore Ca\(^{2+}\)-ATPase activity to normal levels, indicating that the ANF effect on this enzyme is calmodulin-independent. Atriopeptin I and atriopeptin III had no effect on red blood cell Na\(^{+}\),K\(^{+}\)-ATPase activity in vitro. Thus, the structure-activity relationships of ANF analogues in this novel human cell membrane model are highly specific. Although the inhibitory action of ANF analogues on Ca\(^{2+}\)-ATPase, a calcium pump-associated enzyme, may be unique to the red blood cell, the calcium dependence of the gluconeogenic effects of ANF in the kidney would be supported by inhibition of this ATPase. (Hypertension 12: 428-433, 1988)

KEY WORDS • atrial natriuretic factor • adenosine triphosphatase • calcium • human red blood cells

Atrial natriuretic factor (ANF) has natriuretic and blood pressure-lowering actions in intact animals and has also been shown to inhibit the release of renin and aldosterone. The diverse effects of ANF also include stimulation of arginine vasopressin secretion and of renal gluconeogenesis. The mechanisms of ANF actions are incompletely understood, but they are thought to involve enhanced guanylate cyclase activity (e.g., in the kidney) and, perhaps, lowering of intracellular Ca\(^{2+}\) concentrations. On the other hand, the renal tubular gluconeogenic action of ANF is Ca\(^{2+}\)-dependent, and gluconeogenesis in the kidney is regulated in part by changes in intracellular Ca\(^{2+}\) content.

We have recently used the red blood cell (RBC) membrane Ca\(^{2+}\)-adenosine triphosphatase (ATPase) model to study structure-activity relationships in vitro of thyroid hormone analogues and sex steroid analogues. These relationships have demonstrated highly specific structural requirements, and activities have been limited to biologically active analogues. The possibility that ANF may inhibit membrane Ca\(^{2+}\)-ATPase activity was explored in the current human RBC studies. The ANF analogues employed are indicated in Figure 1.

Materials and Methods

Reagents

Na\(_{2}\)-adenosine 5'-triphosphate (ATP) and ouabain were obtained from Sigma (St. Louis, MO, USA). The ANF analogues rat atriopeptin I (AP I; residues 127-147 of the rat prepro-ANF sequence),...
rat atriopeptin III (AP III; residues 127-150), rat ANF fragment 135-150, human ANF fragment 129-150, AP III analogue with p-methylbenzyl-3-thioproionic acid (Mpr) at Position 129 and d-alanine at Position 131 (PL-058), and human cardiodilatin (1-16) were purchased from Peninsula Laboratories (Belmont, CA, USA). Peptides 123-150 (Met 134, human) were obtained from U.S. Biochemical (Cleveland, OH, USA). Beef brain calmodulin was purified in our laboratory by phenothiazine-Sepharose affinity chromatography.12

**RBC Membranes**

RBCs were obtained from healthy human volunteers, after informed consent, and membranes were prepared by hypotonic lysis in 5 mM Tris, 0.1 mM EDTA at pH 7.6, as previously described.13 These calmodulin-depleted and Ca²⁺-depleted membranes were washed twice in 0.9% NaCl, twice in 10 mM Tris buffer, pH 7.45, and were stored in 10 mM Tris buffer at -70 °C until use within 24 to 72 hours of preparation. Each membrane preparation was used for two to three assays, and each assay was performed with membranes from a single donor. Selected experiments used RBC membranes, prepared in the same way, from mature female Wistar rats.

**Ca²⁺-ATPase Activity**

RBC membrane enzyme activity was measured by ATP hydrolysis at 37 °C.13 The assay medium contained 25 mM Tris buffer, pH 7.45, 75 mM NaCl, 25 mM KCl, 1 mM MgCl₂, 0.1 mM EGTA, and, in selected samples, 0.15 mM CaCl₂, leading to a free Ca²⁺ concentration of 20 μM, as determined by ion-specific electrode and by a computer program kindly provided by Dr. John T. Penniston (Mayo Clinic, Rochester, MN, USA). The membrane protein concentration was 1 mg/ml, and under these conditions, enzyme activity was linear to 120 minutes; therefore, assay time was routinely 90 minutes. Concentrations of Ca²⁺, Mg²⁺, ATP, and protein were optimal for conditions of time and temperature. Ca²⁺-ATPase activity was the difference in ATP hydrolysis (generation of inorganic phosphate) in the presence and absence of 20 μM free Ca²⁺ and was expressed as μmoles of Pₗ per milligram of membrane protein per 90 minutes. Blanks were included for nonenzymatic ATP hydrolysis and membrane content of endogenous Pₗ. Phosphate was measured by the Fiske-Subbarow method,14 and protein was measured by the technique of Lowry et al.,15 with bovine serum albumin as the standard. All assays were performed on duplicate samples, and results are expressed as the means ± SE from three experiments. Intra-assay coefficient of variation in Ca²⁺-ATPase activity was 1%, and interassay variation was 10%. In 12 experiments included in this report, 49% of the total ATPase activity was Ca²⁺-stimulable.

In ANF studies, analogues were solubilized in 10 mM Tris buffer and incubated with membranes for 60 minutes at 37 °C before enzyme assay. Control samples contained ANF diluent (10 mM Tris buffer), without peptide. In selected studies, purified bovine brain calmodulin (10⁻⁸-10⁻⁶ M) was added to AP I samples (10⁻⁸-10⁻⁶ M) to determine whether AP I effects on Ca²⁺-ATPase were subject to reversal by calmodulin.

**Na⁺,K⁺-ATPase Activity**

Na⁺,K⁺-ATPase activity was measured by our previously reported method.16 The assay medium was the same as for Ca²⁺-ATPase assay, except that Ca²⁺ was absent, and enzyme activity was measured as the difference in ATP hydrolysis in the presence and absence of 1 mM ouabain.

**Statistical Analysis**

Comparison of enzyme activities in the presence and absence of various ANF analogues was by paired t test.

**Results**

**Ca²⁺-ATPase Inhibition by ANF Analogues**

Rat AP I and AP III (10⁻⁸-10⁻⁶ M) significantly inhibited Ca²⁺-ATPase activity in human erythrocyte membranes (Figure 2). In the studies presented in this figure, the control Ca²⁺-ATPase activity was 0.379 ± 0.022 μmol Pₗ/mg/90 min and the activities in the presence of 10⁻⁶ M AP I and AP III were 0.307 ± 0.017 and 0.303 ± 0.022 μmol Pₗ/mg/90 min, respectively. In other assays, inhibition of enzyme activity by 10⁻⁶ M AP I has ranged from 27 to 65%, probably as a function of RBC donor variability. These concentrations are similar to those effective in studies of ANF in other subcellular fraction models.9, 17, 18 In this Ca²⁺-ATPase model,
residues 127–147 (AP I) of the rat prepro-ANF sequence were required for inhibition of the enzyme. AP III (127–150) also inhibited the enzyme, thereby indicating that the C-terminal Phe-Arg-Tyr sequence (absent in AP I) was unnecessary for this effect.

Preincubation of membranes at 37 °C was performed with AP I added at various time points. Samples with 15-, 30-, and 60-minute preincubation exposure of membranes to $10^{-6}$ M AP I, as well as samples with AP I added at the end of preincubation, just before enzyme assay (0-minute preincubation) were compared with respect to Ca$^{2+}$-ATPase activity. Control samples with Tris buffer added at the same time points showed no change in Ca$^{2+}$-ATPase activity, while samples with AP I added showed a direct correlation between inhibition of enzyme activity and duration of AP I preincubation (Figure 3). For this reason, all studies were conducted with a 60-minute preincubation. In additional studies, we noted that if membranes are preincubated with AP I for 60 minutes, then centrifuged and resuspended in assay buffer without AP I, some irreversible inhibition of enzyme activity remains, suggesting that AP I may bind irreversibly to the membranes (results not shown).

Exogenous purified calmodulin, $10^{-9}$ and $10^{-6}$ M, did not affect the action of $10^{-8}$ or $10^{-6}$ M AP I on Ca$^{2+}$-ATPase (Table 1). This finding suggests that AP I inhibition of this enzyme is not mediated by peptide binding to calmodulin.

### Critical Sequences Defined by Non-Inhibitory ANF Analogue Activity

Human ANF-(123–150) did not inhibit Ca$^{2+}$-ATPase (Figure 4), indicating that the sequence Ser-Leu-Arg-Arg (123-126) must be cleaved to yield a peptide (AP III) that inhibits the enzyme. Rat ANF-(123–150), with isoleucine at position 134 rather than methionine, was also ineffective in altering Ca$^{2+}$-ATPase activity (results not shown). A human ANF fragment containing residues 129–150, which resembles AP III except for the Ser-Ser (127–128) deletion, was also ineffective against Ca$^{2+}$-ATPase (see Figure 4), defining the critical nature of the serine sequence in this model. ANF fragments 135–150 and 129–150 containing Mpr at Position 129 and D-alanine at Position 131 (PL-058) were also ineffective. The presence of either of these two fragments in a concentration of $10^{-6}$ M did not alter the inhibitory

### Table 1. Effect of ANF Analogues on Basal and Calmodulin-Stimulated Human RBC Ca$^{2+}$-ATPase Activity

<table>
<thead>
<tr>
<th>ANF analogue</th>
<th>Basal (μmol P$_i$/mg/90 min)</th>
<th>$10^{-8}$ M calmodulin</th>
<th>$10^{-6}$ M calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.608 ± 0.055</td>
<td>0.698 ± 0.054*</td>
<td>0.608 ± 0.056</td>
</tr>
<tr>
<td>Atriopeptin I</td>
<td>$10^{-8}$ M</td>
<td>0.567 ± 0.059†</td>
<td>0.589 ± 0.061†</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$ M</td>
<td>0.419 ± 0.070†</td>
<td>0.407 ± 0.072†</td>
</tr>
<tr>
<td>Atriopeptin III</td>
<td>$10^{-8}$ M</td>
<td>0.581 ± 0.058†</td>
<td>0.597 ± 0.061†</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$ M</td>
<td>0.429 ± 0.065†</td>
<td>0.427 ± 0.068†</td>
</tr>
</tbody>
</table>

Results are means ± SE of three experiments performed in duplicate. Basal activity is that without calmodulin. Control activity is that without atriopeptin. $P_i$ = inorganic phosphate.

* $p < 0.001$, comparing basal and calmodulin values.

† $p < 0.001$, †$p < 0.01$, comparing control and atriopeptin values.
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**Peptide Sequence Recognized by Human RBC Ca\textsuperscript{2+}-ATPase**

From the observations just summarized, we conclude that a specific, 21-residue sequence is recognized by this human erythrocyte Ca\textsuperscript{2+}-ATPase model (Figure 5, hatched circles). Residues 123-126 (stippled circles) suppress the inhibitory effects of residues 127-147, thus allowing full activity of the enzyme, while residues 148-150 (open circles) do not contribute to the polypeptide inhibitory effect. In studies of rat RBC membrane Ca\textsuperscript{2+}-ATPase activity, 10\textsuperscript{-8} M AP I and AP III inhibited the enzyme by 56 and 39%, respectively, demonstrating a response similar to that of the human RBC enzyme.

**Lack of Na\textsuperscript{+},K\textsuperscript{+}-ATPase Effect by ANF Analogues**

Rat AP I did not inhibit Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in this preparation (control, 0.244 ± 0.027 μmol Pi/mg/90 min vs AP I, 0.256 ± 0.020 μmol Pi/mg/90 min). Findings with AP III were similar. These results indicate that the inhibitory effect of peptide residues 127-147 is specific for Ca\textsuperscript{2+}-ATPase, rather than a more generalized toxic effect on membranes. ATPase activity measured in the absence of Ca\textsuperscript{2+} and ouabain (but in the presence of Na\textsuperscript{+},K\textsuperscript{+}, and Mg\textsuperscript{2+}) was also unaffected by AP I or AP III.

**Discussion**

In the human RBC Ca\textsuperscript{2+}-ATPase model, an ANF sequence of residues 127-147 of rat prepro-ANF (AP I) significantly inhibited enzyme activity in vitro at concentrations of 10\textsuperscript{-8} to 10\textsuperscript{-6} M. Structure-activity studies showed that the inclusion of residues 123-126 (Ser-Leu-Arg-Arg) excluded action in the Ca\textsuperscript{2+}-ATPase system and that the Ser-Ser sequence at 127-128, immediately proximal to the disulfide bridge, is critical to the inhibitory activity of ANF analogues (see Figure 4). Residues 148-150 (Phe-Arg-Tyr) at the C-terminus of AP III (127-150) are not required for activity in this model; that is, AP I and AP III are equally active. In contrast, these two analogues, at concentrations up to 10\textsuperscript{-6} M, had no effect in vitro on erythrocyte Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity. Thus, in this human enzyme model, the requirements for a specific ANF structure have been defined as shown schematically in Figure 5. These requirements are distinct from those reported for the natriuretic and renal blood flow effects of ANF,\textsuperscript{19} in which, for example, AP I is more active than AP III and the inclusion of one or more residues (123-126) proximal to 127 enhances activity.\textsuperscript{20}

These differences may reflect the nature of the cell used in the present studies or an action of ANF that is independent of its effects on sodium excretion and renal hemodynamics. Since ANF has been reported to decrease intracellular Ca\textsuperscript{2+} concentration in isolated renal glomerular mesangial cells,\textsuperscript{8} it appears unlikely that an ANF effect on membrane Ca\textsuperscript{2+}-
ATPase ("calcium pump") activity in that cell model would be inhibitory. On the other hand, ANF has gluconeogenic actions on kidney cells that are Ca2+-dependent and are reproduced by the use of Ca2+ ionophore.9 How the gluconeogenic signal of ANF is distinguished by target cells from that relating to sodium handling is unclear, although we speculate that the structure-activity relationships for ANF we describe here do relate to carbohydrate metabolism. Renal tubular gluconeogenesis in vitro is maximally stimulated at 10−7 M ANF,6 consistent with the concentration dependence we have reported for Ca2+-ATPase. We have reported a growth factor (erythropoietin) response in erythroid precursors that involves inhibition of membrane Ca2+-ATPase21 and that is thought to contribute to erythropoietin-induced changes in RBC precursor Ca2+ content. On the other hand, it is possible that ANF action on RBC is limited to the erythroid series and wholly unrelated to its previously described actions in the kidney or blood vessels.

The range of concentrations of ANF required in the present studies (10−8-10−6 M) is consistent with that required to develop cyclic guanosine 3′,5′-monophosphate or particulate guanylate cyclase responses to ANF in a variety of cultured cells,22 including bovine adrenocortical and aortic smooth muscle cells, human lung fibroblasts, and canine kidney epithelia. As pointed out, these concentrations of ANF also stimulate renal gluconeogenesis.6 Although the affinities (Kd) of ANF receptor sites usually are described to be in the nanomolar range, Leitman et al.22 have recently pointed out that there is a poor correlation between certain in vitro biological effects of ANF and ANF receptor site affinity and number. Whether this lack of correlation represents a quality of cells maintained and studied in vitro or reflects other factors, such as problems in receptor measurement or metabolism by specific tissues of ANF analogues, is unclear.

The RBC Ca2+-ATPase model we have employed in these studies has previously been shown in endocrine and pharmacological experiments to exhibit biologically relevant structure-activity requirements. Examples of the latter are studies of thyroid hormone analogues10 and sex steroids.11 A potent inotropic agent—milrinone23—that shares structural homologies with thyroid hormone stimulates cardiac membrane Ca2+-ATPase, but not the RBC enzyme.24 Calcium channel blockers known to have anticalmodulin actions25 inhibit the calmodulin-dependent Ca2+-ATPase in RBCs and block the calmodulin-dependent stimulation of RBC Ca2+-ATPase by thyroid hormone.26

Because a number of peptides have been reported to bind to calmodulin,27 we conducted studies of the ability of purified eukaryote calmodulin to reverse the effect of ANF on RBC Ca2+-ATPase. Calmodulin did not antagonize the action of AP 1 on RBC Ca2+-ATPase, indicating that this effect is calmodulin-independent. The magnitude of the ANF effect on Ca2+-ATPase (20% reduction) is comparable to that obtained in our hands with other calmodulin-independent inhibitors of Ca2+-ATPase, such as erythropoietin21 and N-[4-azido-2-nitrophenyl]-2-aminoethylsulfonate28 (F. B. Davis, P. J. Davis, and S. D. Blas, unpublished observations, 1986).

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