Effect of Atrial Natriuretic Factor on $\text{Na}^+\cdot\text{K}^+\cdot\text{Cl}^-$ Cotransport of Vascular Smooth Muscle Cells

Nancy E. Owen, Eugene N. Bush, William Holleman, and Martha E. O'Donnell

SUMMARY We previously demonstrated that vascular smooth muscle cells possess a prominent $\text{Na}^+\cdot\text{K}^+\cdot\text{Cl}^-$ cotransport system that can be markedly stimulated by elevations in levels of intracellular cyclic guanosine 3',5'-monophosphate (cGMP). Since others have shown that atrial natriuretic factor (ANF) can bind to specific membrane receptors and can enhance cGMP levels in vascular smooth muscle cells, we asked whether ANF could also stimulate $\text{Na}^+\cdot\text{K}^+\cdot\text{Cl}^-$ cotransport in vascular smooth muscle cells. It was discovered that rat atriopeptin III stimulated Na$^+$-K$^+$-Cl$^-$ cotransport of vascular smooth muscle cells in a concentration-dependent manner. In contrast, rat atriopeptin III had no effect on two other sodium transport systems known to be present in vascular smooth muscle cells (i.e., Na$^+$-H$^+$ exchange and Na$^+$-K$^+$-adenosine triphosphatase (ATPase). These studies indicated that ANF selectively stimulates Na$^+$-K$^+$-Cl$^-$ cotransport of vascular smooth muscle cells. We then asked whether ANF-stimulated Na$^+$-K$^+$-Cl$^-$ cotransport was dependent upon the ability of ANF to enhance intracellular cGMP levels. When rat atriopeptin III-stimulated increases in cGMP were inhibited with the quinolinedione LY 83583, rat atriopeptin III could no longer stimulate Na$^+$-K$^+$-Cl$^-$ cotransport of vascular smooth muscle cells. Thus it appeared that the effects of ANF were dependent upon the ability of ANF to elevate intracellular cGMP levels. Finally, we asked whether ANF effects on Na$^+$-K$^+$-Cl$^-$ cotransport were related to the biological activity of ANF. Using vasorelaxation of histamine-constricted aortic strips as an index of biological activity, we observed a tight correlation between the biological activity of six ANF analogues and their ability to stimulate Na$^+$-K$^+$-Cl$^-$ cotransport and to elevate intracellular cGMP levels of vascular smooth muscle cells. It appears that ANF interacts with a specific membrane receptor to elevate intracellular cGMP, which then leads to stimulation of Na$^+$-K$^+$-Cl$^-$ cotransport. (Hypertension 10 [Suppl I]: M28-M30, 1987)

KEY WORDS • atrial natriuretic factor • Na$^+$-K$^+$-Cl$^-$ cotransport • cyclic GMP • vascular smooth muscle cells

THE Na$^+$-K$^+$-Cl$^-$ cotransport system is a bidirectional symport system that is physiologically important for vectorial transport of salt and water, and for regulation of cellular volume.\textsuperscript{1,2} This transport system has been shown to be present in a wide variety of cells and tissues, including both secretory and absorptive epithelial tissue,\textsuperscript{3} erythrocytes (avian\textsuperscript{4} and human\textsuperscript{5}), and tissue culture cells (HeLa,\textsuperscript{5} MDCK\textsubscript{12,4,7} and human fibroblasts\textsuperscript{8}). Common characteristics exhibited by Na$^+$-K$^+$-Cl$^-$ cotransport in each of these cell types include an absolute requirement for all three ions,\textsuperscript{9} inhibition by loop diuretics such as furosemide or bumetanide,\textsuperscript{9} and dependence upon the energy state of the cell.\textsuperscript{7} In addition, Na$^+$-K$^+$-Cl$^-$ cotransport has been shown to be regulated by cyclic nucleotides in most of the cells in which it has been demonstrated to be present.\textsuperscript{3,4,8} The nature of this regulation, however, is very cell- and tissue-specific.

Our laboratory was the first to demonstrate the presence of Na$^+$-K$^+$-Cl$^-$ cotransport in vascular smooth muscle (VSM) cells.\textsuperscript{10} We found that Na$^+$-K$^+$-Cl$^-$ in VSM cells displayed the above-mentioned common characteristics and that the system could be inhibited by increases in levels of intracellular cyclic adenosine 3',5'-monophosphate (cAMP)\textsuperscript{10} and stimulated by increases in levels of intracellular cyclic guanosine 3',5'-monophosphate (cGMP).\textsuperscript{10,11} It was discovered that when intracellular cAMP levels were enhanced by treatment with the permeable cAMP analogue, 8-bromo-cAMP (8-Br-cAMP), or with the $\beta$-adrenergic agonist isoproterenol, Na$^+$-K$^+$-Cl$^-$ cotransport was inhibited. We also found that the permeable cGMP analogue 8-bromo-cGMP (8-Br-cGMP) could stimulate Na$^+$-K$^+$-Cl$^-$ cotransport (Table 1).
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Results and Discussion

When VSM cells were challenged with increasing concentrations of rat AP III, rat AP III stimulated Na⁺-K⁺-Cl⁻ cotransport in a concentration-dependent manner. In six separate quadruplicate determinations, the concentration yielding half-maximal stimulation of cotransport ($K_\text{m}$) was 9 nM. This value was in close agreement with reported $K_\text{m}$ values for ANF binding to specific membrane receptors of VSM cells \(16\) and with reported $K_\text{m}$ values for increasing intracellular cGMP in VSM cells.\(12,13\) The observation that ANF influences Na⁺-K⁺-Cl⁻ cotransport is in agreement with the findings of O'Grady et al. \(25\) and of Solomon et al. \(24\) These workers showed that ANF influences Na⁺-K⁺-Cl⁻ cotransport of teleost intestine \(23\) and stimulates chloride secretion in shark rectal gland.\(24\)

Subsequently, the effects of rat AP III on two other sodium transport systems was evaluated. It was found that rat AP III did not affect either Na⁺-H⁻ exchange or the Na⁺,K⁺-ATPase of VSM cells. This finding agrees with the work of others who have shown that ANF does not affect Na⁺,K⁺-ATPase activity of human erythrocytes \(25\) or rat kidney.\(26\) However, it disagrees with a published report demonstrating that ANF can inhibit Na⁺-H⁻ exchange in porcine kidney epithelial cells (LLC-PK,) \(27\). It is noteworthy that while the effects of ANF have been tested on three sodium transport systems, such measurements had not previously been carried out using a single cell type (e.g., VSM cells). Our findings suggested that rat AP III specifically stimulated Na⁺-K⁺-Cl⁻ cotransport of VSM cells, and probably did so through interaction with a specific membrane receptor.

These experiments established that ANF could stimulate Na⁺-K⁺-Cl⁻ cotransport and others had shown that ANF could stimulate elevations in intracellular cGMP levels; however, it was not known if these events occurred in series or in parallel. Because of this uncertainty, it was of interest to examine the relationship between ANF effects on Na⁺-K⁺-Cl⁻ cotransport and on cGMP levels in VSM cells. Thus we determined the effect of rat AP III on Na⁺-K⁺-Cl⁻ cotransport in the presence and absence of the quinolinedione, LY 83583; LY 83583 has been shown to block cGMP formation in a variety of tissues.\(28\) It was found that Na⁺-K⁺-Cl⁻ cotransport occurring in the presence of 100 nM rat AP III was markedly reduced by 10 μM LY 83583 (see Table 1). Maximal inhibition was obtained with 10 μM LY 83583 and half maximal inhibition was seen at 0.3 μM LY 83583. It was found that the effects of LY 83583 could be reversed by addition of 8-Br-cGMP in the presence of a cGMP phosphodiesterase inhibitor. These findings demonstrated that when the ability of ANF to enhance intracellular cGMP levels was blocked, the ability of ANF to stimulate Na⁺-K⁺-Cl⁻ cotransport was inhibited. On this basis it was concluded that ANF stimulates Na⁺-K⁺-Cl⁻ cotransport in VSM cells through elevations in intracellular cGMP.

Although we provided strong evidence to suggest that ANF acted through cGMP to activate Na⁺-K⁺-Cl⁻ cotransport of VSM cells selectively, the biologi-

| Table 1. Effect of ANF on Na⁺-K⁺-Cl⁻ Cotransport of Vascular Smooth Muscle Cells |
|--------------------------|--------------------------|
| Treatment               | Na⁺-K⁺-Cl⁻ cotransport |
|                         | (mol K⁺ influx/g protein/min) |
| Control                 | 4.23 ± 0.50             |
| 8-Br-cGMP               | 6.91 ± 0.28             |
| Rat AP III              | 6.95 ± 0.49             |
| LY 83583                | 2.19 ± 0.51             |
| Rat AP III + LY 83583   | 2.27 ± 0.37             |
| 8-Br-cGMP + LY 83583 + M&B 22,948 | 5.91 ± 0.92         |

Data are expressed as Na⁺-K⁺-Cl⁻ cotransport (bumetanide-sensitive K⁺ influx) and values are means ± SEM of triplicate determinations from four separate experiments. AP = atriopeptin. Vascular smooth muscle cells were obtained from rat thoracic aorta as described in Materials and Methods. Cells were preincubated for 5 minutes in HEPES-buffered minimal essential medium containing 10 mM LY 83583 or vehicle. The medium was then replaced with identical fresh medium containing the agents to be tested. Concentrations were 8-Br-cGMP, 50 μM; rat AP III, 100 nM; LY 83583, 10 μM; and M&B, 100 μM.

Few physiologically occurring agents have been shown to elevate intracellular cGMP levels. One agent that does have this activity is atrial natriuretic factor (ANF).\(12-15\) ANF is a collective term for a group of bioactive peptides that interact at specific receptors to activate particulate guanylate cyclase.\(12,13\) It has been demonstrated to cause natriuresis, diuresis, and vasorelaxation.\(19\)

Although the complementary DNA sequence has been determined and it is well established that ANF is derived from a preprohormone,\(20\) little is known about its mechanism of action. Because VSM cells possess specific high affinity ANF receptors and because ANF activates guanylate cyclase and causes an increase in cGMP in these cells, our laboratory investigated the effect of ANF on Na⁺-K⁺-Cl⁻ cotransport of VSM cells. This report summarizes our studies and relates our work to other published reports concerning the effects of ANF on ion transport processes.

Materials and Methods

Rat atriopeptin III (AP III) and other ANF analogues were generously provided by Dr. William Holleman (Abbott Laboratories, N., Chicago, IL, USA). 6-Anilino-5,8-quinolinedione (LY 83583) was provided by Lilly Research Laboratories (Indianapolis, IN, USA). M & B 22,948 (2-0-propoxyphenyl-8-aza-purin-6-one) was kindly provided by May and Baker Ltd. (Dagenham, Essex, United Kingdom). Bumetanide was the gift of Hoffmann-La Roche (Nutley, NJ, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

VSM cells were derived from explants of rat thoracic aorta as previously described.\(10,11\) Measurements of Na⁺-K⁺-Cl⁻ cotransport,\(9\) Na⁺-H⁺ exchange,\(21\) Na⁺,K⁺-adenosine triphosphatase (ATPase) activity,\(22\) intracellular cGMP levels,\(10,11\) and vasorelaxation\(22\) were carried out as described in previous publications.

![Image](http://hyper.ahajournals.org/DownloadedFrom)
The biological relevance of ANF effects on sodium transport had not been determined. We assessed the biological activity of six ANF analogues by determining their ability to induce vasorelaxation in histamine-constricted aortic strips (Table 2). We then assessed the ability of these agents to stimulate Na⁺-K⁺-Cl⁻ cotransport of VSM cells. A very tight correlation was discovered between the biological activity (ability to induce vasorelaxation of aortic rings) and stimulation of Na⁺-K⁺-Cl⁻ cotransport of VSM cells. Furthermore, the same potency order was noted for effects on intracellular cGMP levels (data not shown).

On the basis of these studies, we concluded that ANF stimulation of Na⁺-K⁺-Cl⁻ cotransport parallels biological activity. At present, the nature of this relationship is undefined. The only two known physiological roles of this cotransport are vectorial transport of salt and water in epithelia and volume regulation in single cells. It is possible that some of the natriuretic and diuretic effects of ANF could be mediated through direct effects on the Na⁺-K⁺-Cl⁻ transporter. The identity of the relationship between ANF effects on Na⁺-K⁺-Cl⁻ cotransport and ANF-mediated vasorelaxation remains to be demonstrated.

### Table 2. Biological Activity of ANF Analogues and Their Effect on Na⁺-K⁺-Cl⁻ Cotransport

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(K_v) for vasorelaxation (nM)</th>
<th>(K_v) for Na⁺-K⁺-Cl⁻ cotransport (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANF (104–126)</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>ANF (103–126)</td>
<td>1.3</td>
<td>7.6</td>
</tr>
<tr>
<td>ANF (105–126)</td>
<td>5.1</td>
<td>75.9</td>
</tr>
<tr>
<td>[Asn[11]]ANF</td>
<td>63.1</td>
<td>26.9</td>
</tr>
<tr>
<td>ANF (103–123)</td>
<td>81.3</td>
<td>63.1</td>
</tr>
<tr>
<td>[d-Ala⁴]ANF</td>
<td>1.7</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Values are means ± SEM from four separate experiments. Vasorelaxation and Na⁺-K⁺-Cl⁻ cotransport were measured as described in Materials and Methods. \(K_v\) is the concentration that produces 50% of the maximum response.

### References

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