Effects of Atrial Natriuretic Factor and Vasopressin on Cyclic Nucleotides in Cultured Kidney Cells

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SUMMARY The cellular mechanism of the action of atrial natriuretic factor (ANF) is thought to involve activation of guanylate cyclase. Increasing evidence shows a direct tubular effect of ANF. Part of the ANF-induced diuresis has been suggested to be due to inhibition of the action of arginine vasopressin (AVP) in the cortical collecting tubule. In this study we investigated the effect of ANF on cyclic nucleotide production in primary cultures of cortical collecting tubule cells immunodissected with a monoclonal antibody. ANF caused a dose-dependent stimulation in cyclic guanosine 3',5'-monophosphate (cGMP) production; the half-maximal stimulation was observed at ~1 nM of ANF. ANF (0.01–100 nM) had no effect on cyclic adenosine 3',5'-monophosphate (cAMP) accumulation in cortical collecting tubule cultures. AVP caused a dose-dependent increase in cAMP production, and this effect was not altered by the simultaneous addition of ANF (100 nM). Similarly, ANF-induced cGMP stimulation was not influenced by AVP (10 nM). We conclude that 1) ANF has a direct stimulatory action on cGMP production by cultured cortical collecting tubule cells and 2) any interaction between ANF and AVP is likely to occur at steps distal to cyclic nucleotide formation. (Hypertension 11: 392–396, 1988)

KEY WORDS • atrial natriuretic factor • cyclic adenosine 3',5'-monophosphate • cyclic guanosine 3',5'-monophosphate • immunodissection • cortical collecting tubule

THE mechanism of the diuretic action of atrial natriuretic factor (ANF) is not completely understood. According to the traditional view, the bulk of its action is brought about by hemodynamic changes. This view is challenged by studies in which the diuretic action has been dissociated from hemodynamic changes and by an increasing number of studies showing a direct tubular effect. These tubular actions have been demonstrated mainly in the medullary collecting tubule, and the possible effects of ANF in the cortical collecting tubule (CCT) are unresolved. Since cyclic guanosine 3',5'-monophosphate (cGMP) is an acknowledged second messenger of ANF, in this study we have examined the effects of ANF on cGMP accumulation in primary cultures of immunodissected CCT cells. Furthermore, as ANF has been shown to exert some of its actions by inhibiting adenylate cyclase activation by other hormones, we also tested the possibility that ANF might suppress arginine vasopressin (AVP)–induced cyclic adenosine 3',5'-monophosphate (cAMP) accumulation and consequently reduce its antidiuretic action. Our results indicate that 1) ANF is a potent stimulator of cGMP accumulation in cultured CCT cells and 2) this effect occurs at physiological concentrations of the hormone. However, we found no evidence favoring an ANF-mediated inhibition of the action of AVP.

Materials and Methods

Immunodissection and Culture of Cortical Collecting Tubule Cells

Cells originating from rabbit (New Zealand white) CCT and connecting tubule were isolated by solid-phase immunoadsorption using a monoclonal antibody (MCAB) directed against an ectoantigen shared by these segments. The details of generating MCABs, their purification, and characterization have been de-
scribed elsewhere. For immunodissection, high performance liquid chromatography-purified MCAB ST. 12, which reacts with CCT and connecting tube cells, was immobilized on bacteriological Petri dishes by an overnight incubation at 0 to 4°C, at a protein concentration of 130 μg/ml, at pH 7.8. The dishes were washed four times with phosphate-buffered saline and incubated for 10 minutes with 0.1% bovine serum albumin. Partially digested tubular fragments, obtained by enzymatic digestion, were then applied and were allowed to adhere to the antibody-coated dishes for 5 minutes. This step was followed by five washes with minimum essential medium containing 0.1% bovine serum albumin, and the adherent cells were detached by digestion with 0.1% protease XIV (Sigma Chemical, St. Louis, MO, USA) at 37°C for 30 minutes. CCT cells were washed, resuspended in RPMI 1640 medium with 5% fetal bovine serum; 0.125% serum supplement (SerXtend; New England Nuclear, Boston, MA, USA); 1 mM pyruvate; glucose, 4 g/L; 15 mM HEPES; penicillin, 50 U/ml; streptomycin, 50 μg/ml; tylosin, 12 μg/ml; and 10⁻³ M 2-mercaptoethanol, equilibrated with 5% CO₂ in air. The cells were seeded at a density of 2 to 4 x 10⁶/cm² in tissue culture dishes.

Characterization of Cells
Aliquots of freshly isolated as well as cultured cells were stained with a panel of fluorochrome-conjugated cell-specific MCABs and lectins and analyzed by flow cytometry, as described earlier.

Incubation of Cells with Effectors
After 4 to 5 days of culture, the cells were released from the plates by a 30-minute incubation with Ca²⁺-free medium, followed by a 5- to 8-minute trypsinization (0.25% trypsin, 1 mM EDTA). The cells were washed, resuspended in RPMI 1640 medium (without serum) containing 0.5 mM isobutylmethylxanthine, incubated alone at 37°C for 10 minutes and then with the agonists for an additional 10 minutes, except where indicated otherwise. The reaction was stopped by the addition of 0.35 N HCl/0.25 N acetic acid. The tubes were incubated in boiling water for 2 minutes, cooled on ice, and centrifuged, and the supernatants were brought back to pH 6.2 with NaOH and stored at −20°C until assay for cyclic nucleotides. (The cyclic nucleotide content of these samples represents the sum of intracellular and extracellular contents.)

cAMP and cGMP Assay
Cyclic nucleotides were assayed by radioimmunoassay following acetylation, using reagents obtained from Biomedical Technologies Inc. (Stoughton, MA, USA). Validity of the assays was established by linearity of sample dilutions and by loss of immunoreactivity following incubation with phosphodiesterase.

Statistical Analysis
Each experiment consisted of repeated measures on a batch of cells from the same animal. Thus, for an ANF effect on cGMP, the basal level was subtracted from each subsequent ANF concentration and Hotelling's T² analysis was used to test overall whether these deltas were different from zero. Given a statistically significant Hotelling's result, five individual paired t tests were used to compare each concentration to baseline using 0.01 as the cutoff value to obtain an overall α level of 0.05 in the Bonferroni adjustment (α/tests or 0.05/5). The same approach was used for an effect on cAMP.

Profile analysis was used to compare the effects of synthetic ANF (rat ANF-[126-150]; Peninsula Laboratories, Belmont, CA, USA) and AVP (Sigma) plus ANF on both cAMP and cGMP across concentrations using the observed values after subtracting the basal value. Results are given as means ± SEM.

Results
The purity of the immunodissected cells, based on flow cytometric analysis following staining of the cells with fluorescein isothiocyanate-labeled MCABs (ST.12, ST.46) and Helix pomatia lectin, which react specifically with the collecting tubules including the arcades, was greater than 97%. Staining with other MCABs (ST.1, ST.10, and DT.6) that are specific for the proximal tubule, distal convoluted tubule, and thick ascending limb of the loop of Henle, respectively, indicated less than 1% contamination with these cell types. The purity of the cultured cells was evaluated similarly and was found to be greater than 95%, based on staining with fluorescein isothiocyanate-conjugated MCAB ST.12 and Helix pomatia lectin.

Figure 1 shows phase contrast photomicrographs of cultured CCT cells on Day 7 following seeding. The cells had typical epithelial morphology and appeared homogenous, with no evidence of fibroblast contamination. The cells also showed hemicyst formation, which is characteristic of transporting epithelia.

The time course of changes in cGMP accumulation in response to the addition of 10⁻³ M ANF is shown in Figure 2. These experiments indicated little change in cGMP content between 6 and 12 minutes; therefore, in subsequent experiments the cells were incubated with ANF for 10 minutes.

The relationship between cGMP content and ANF concentration is illustrated in Figure 3. Addition of 10⁻¹¹ M ANF, the lowest concentration tested, was found to increase cGMP content significantly, from a basal value of 2.9 ± 0.3 to 5.9 ± 1.3 fmol/10⁴ cells (p < 0.05), where as 10⁻⁷ M ANF caused an approximately 12-fold increase (p < 0.001).

It is also shown in Figure 3 that ANF, in the concentration range of 10⁻¹¹ to 10⁻⁷ M, did not alter basal cAMP content of cultured CCT cells after a 10-minute exposure. In three experiments we have found that 10⁻⁷ M ANF, during incubations lasting from 1.5 to 12 minutes, was also ineffective in altering basal cAMP production (data not shown). On the other hand, AVP caused a dose-dependent enhancement in cAMP accumulation in these cells. The threshold concentration was 6.4 x 10⁻¹² M, and half-maximal response was at
about $3 \times 10^{-10}$ M of AVP. If the incubations were performed in the presence of $10^{-7}$ M ANF, this dose-response relationship was not significantly altered (Figure 4). This finding is consistent with the profile analysis that detected no ANF-AVP interaction ($p > 0.9$) and no ANF effect ($p > 0.9$).

To test the possible effect of AVP on ANF-induced cGMP accumulation, ANF dose-response experiments were performed in the presence of $10^{-9}$ M AVP. As can be seen in Figure 5, the normalized responses in the presence or absence of AVP were not significantly different at any of the ANF concentrations tested. Similarly, AVP ($10^{-12}-10^{-7}$ M) alone did not alter basal cGMP production by CCT cells significantly (data not shown).

Discussion

Several lines of recent evidence indicate that ANF, besides its effects on the renal vasculature, can also affect tubular function directly. ANF has been shown to inhibit sodium-coupled transport processes in brush border membrane vesicles of the proximal tubule and to reverse the angiotensin II-stimulated sodium and water transport in the proximal tubule. It has also been found to decrease the amiloride-sensitive sodium transport in the LLC-PK$_1$ cell line, which is derived from pig kidney cells and resembles proximal tubule.

In accordance with the presence of high affinity binding sites for ANF in the medullary collecting duct...
revealed by autoradiography, ANF has also been shown to enhance cGMP accumulation in inner medullary collecting duct cells. Furthermore, according to microcatheterization experiments, ANF exerts a specific inhibitory effect on sodium reabsorption by this segment. Similarly, human ANP was found to inhibit oxygen consumption by inner medullary collecting duct cells, probably through inhibition of sodium transport. A similar effect, however, could not be demonstrated in the outer medullary collecting duct. The effect of ANF on cGMP accumulation in the outer medullary and cortical collecting tubules is also unsettled, with no effect seen in some cases and only a modest effect observed with pharmacological doses in other studies.

The results of the present study clearly indicate that ANF is a potent stimulator of cGMP accumulation in primary cultures of rabbit CCT cells. The finding that a significant enhancement in cGMP levels can be achieved by concentrations of ANF comparable to circulating levels suggests that this effect may have physiological relevance.

The mechanisms through which the increase in cGMP levels would translate into a biological response in CCT is a matter for speculation at this stage. As ANF has been shown to inhibit the hormone-induced cAMP accumulation in several systems, it might interfere with the action of AVP and thereby limit water reabsorption in the collecting duct. Such an interaction between ANF and antidiuretic hormone has been shown to occur in isolated perfused CCT of the rabbit as well as in the urinary bladder of the toad, a tissue that shares many similarities with the mammalian collecting duct. In the CCT the effect of ANF on the hydraulic conductivity response induced by chlorophenylthio-cAMP or forskolin was also investigated. The effect of these agents was unaltered by ANF, suggesting that it inhibits the response to AVP by acting proximal to the catalytic subunit of adenylate cyclase.

Our data, however, do not support such an interpretation, as the AVP-induced cAMP response was similar in the presence and in the absence of ANF. These results indicate that if ANF inhibits the action of AVP, it probably does so at steps distal to cAMP formation. A similar conclusion can be reached on the basis of results reported by several other laboratories. However, in primary cultures of papillary collecting ducts, ANF was reported to inhibit AVP-induced cAMP accumulation, even at concentrations that did not affect cGMP formation. Similarly, according to a preliminary report by Werness et al., a small but statistically significant inhibition of AVP-induced cAMP production was observed in microdissected CCT. At present, we have no explanation for these divergent results.

Another type of interaction between ANF and AVP has been shown to exist in vascular smooth muscle cells. Here, AVP has been found to inhibit the ANF-induced cGMP response without altering basal cGMP levels. In cultured CCT cells we have found no evidence for such an interaction between these two hormones (see Figure 5).

Obviously, one has to be cautious when extrapolating results from cell culture systems to the in vivo situation. However, as has been shown by Garcia-Perez and Smith and by Spielman and colleagues, immunodissected CCT cells retain their hormonal responsiveness, and recent studies from our laboratory indicate that differentiated transport functions and cell-specific antigens are also preserved in primary cultures of CCT cells. Therefore, observations made on such differentiated cultures are likely to have in vivo importance.

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