Atrial Natriuretic Factor in Purkinje Fibers of Rabbit Heart

Madhu Bala Anand-Srivastava, Gaétan Thibault, Christophe Sola, Edward Fon, Marie Ballak, Christian Charbonneau, Helena Haile-Meskel, Raul Garcia, Jacques Genest, and Marc Cantin

The Purkinje fibers of the rabbit false tendons (chordae tendineae spuriae) are endocrine cells containing immunoreactive atrial natriuretic factor (ANF) and ANF messenger RNA (mRNA). These cells, as visualized by immunocytoultramicrotomy, contain immunoreactive ANF in their secretary granules and their Golgi complex and exhibit ANF mRNA, as visualized by in situ hybridization with an ANF complementary RNA probe. The content of immunoreactive ANF and ANF mRNA of the Purkinje fibers is midway between that of atrial and ventricular working cardiocytes. High-pressure liquid chromatography analysis of immunoreactive ANF using antibodies against the C-terminal and N-terminal moieties of the molecule indicates that part of immunoreactive ANF contained in Purkinje fibers is the propeptide [Asn',Tyr126]ANF whereas part was nonspecifically cleaved into C-terminal and N-terminal ANF. The chordae tendineae spuriae exhibit binding sites for ANF (K_a:~1.0 nM; B_max:~2.3 fmol/mg). ANF profoundly decreases basal and stimulated (epinephrine, dopamine, isoproterenol, and forskolin) adenylate cyclase activity and cyclic adenosine monophosphate (AMP) levels. ANF has little effect on norepinephrine-stimulated adenylate cyclase activity or on norepinephrine-stimulated cyclic AMP levels. ANF produces only a slight increase in guanylate cyclase activity and cyclic guanosine monophosphate levels at high (10^(-7)-10^(-6) M) concentrations. These results suggest an autocrine function for ANF in the modulation of the impulse in the peripheral conduction cells (Purkinje fibers) of the rabbit through changes in second messenger levels. (Hypertension 1989;13:789-798)

Since the discovery of cablelike structures in the heart ventricular cavities by Purkinje,1 the cells forming the peripheral, extramural conduction network have been extensively investigated in rabbit,2,3 cat,2 dog,4 sheep,5 and cow.6 In these species, and to a varying degree, the Purkinje fibers have been found to be different from working ventricular cardiocytes because of the presence of intermediate filaments (skeletin or desmin),8 the peculiar organization of the contractile apparatus,2,3,6,9 myofilament polyribosome complexes,2 variations in Z-band ultrastructure,6,9 the absence of a T-system,4,10,11 and simplification of the intercalated disk.4,10,11 The aim of the present study was to find out whether immunoreactive atrial natriuretic factor (irANF) and ANF messenger RNA (mRNA) are present in these cells as in atrial12 and working ventricular cardiocytes13 and in the conduction system of the rat14,15 and to assess the role of ANF on second messengers in these cells in both normal and stimulated conditions.

Materials and Methods

Animals

Female New Zealand White rabbits (2.5 kg body wt) were used throughout.

Peptides

The propeptide of ANF [Asn',Tyr126]ANF was purified from isolated rat atrial granules.16 [Asn',Arg28] ANF was produced by incubating 150 μg [Asn',Tyr126] ANF with 50 milliunits IRCM-serine protease #1,17 pH 7.4, at room temperature in a 0.1 M BES buffer containing 1 mM Na2 EDTA and by subsequent purification on a C18-Vycor column with 0.13% heptfluorobutyric acid and acetonitrile as the mobile phase. The purity and concentration of the peptide were verified by amino acid analysis.18 Synthetic [Ser8,Tyr126]ANF and [Asp11,Ala27]ANF were obtained from Bio-Mega Inc. (Laval, Québec, Canada).

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Measurement of Immunoreactive Atrial Natriuretic Factor in Chordae Tendinea Spuriae, Atria, and Ventricular Septum

Ten rabbits were anesthetized with sodium pentobarbital. irANF was measured in chordae tendinea spuriae (CTS), both atrial appendages, and the middle portion of the interventricular septum as already described.13-15,19 Proteins were measured according to the technique of Lowry et al.20

High-Performance Liquid Chromatography Pattern of Immunoreactive Atrial Natriuretic Factor in Chordae Tendinea Spuriae

The CTS from 10 rabbits were harvested and treated as above. The extracts were centrifuged at 15,000 rpm for 10 minutes, and the supernatants were kept frozen at -70°C until used. The ANF peptides were extracted on Sep-Pak cartridges (Water Associates, Milford, Massachusetts) and analyzed by reverse phase high-performance liquid chromatography (HPLC) on a C18 µ-Bondapak (0.4x30 cm) column as already described.14,15,19 Two-milliliter fractions were collected. The radioimmunoassay (RIA) for C-terminal21 and N-terminal18 ANF and a two-site immunoradiometric assay for the whole propeptide [Asn1, Tyr126]ANF22 were performed on lyophilized aliquots as described.

Antibodies

Three different antibodies were used throughout. A monoclonal antibody (2H2) against rat synthetic [Arg101, Tyr126]ANF, which is directed against region 101-103 of rat [Ser99, Tyr108]ANF, was used. Polyclonal antibodies were produced in rabbits by the injection of synthetic rat [Arg101, Tyr126]ANF coupled with bovine thyroglobulin. The cross-reactivity of these antibodies to various ANF fragments has already been described.24 Polyclonal antibodies against the synthetic N-terminal portion of [Asp11, Ala37]ANF that were coupled to bovine serum albumin were also produced in the same manner.18 With this antibody and [125I] [Asn1, Tyr126]ANF as tracer, similar cross-reactivities were obtained with various N-terminal moieties of rat [Asn1, Arg98]ANF, rat [Asn1, Tyr126]ANF, and rat [Asp11, Ala37]ANF. No cross-reactivity could be detected with synthetic rat [Ser99, Tyr126]ANF, rat [Ser103, Tyr126]ANF, and rat [Glu11, Arg98]ANF. This finding indicates that the epitope responsible for immunization is probably located between positions 11 and 30.18 All these antibodies were purified by affinity chromatography, and monospecificity was determined by Western blot. The results of Western blot analysis14,15 revealed the monospecificity of each antibody and confirmed several studies in which the cross-reactivity of each antibody was thoroughly assessed by other techniques for RIA or immunocytochemical purposes.21,24

Immunohistochemistry

The hearts of five rabbits were perfused and prepared for immunohistochemistry. The avidin-biotin-peroxidase method13-15 was used for each of the three antibodies.

Electron Microscopy

Ten rabbits were perfused through both cardiac ventricles with 20 ml Ringer-Locke fluid and then with 2% glutaraldehyde buffered with cacodylate HCl (0.1 M, pH 7.4). Portions of atrial appendages, of CTS, and of interventricular septum were harvested and processed for electron microscopy as already described.13-15 For immunocryoultramicroscopy, five rabbits were perfused as above but with 4% formaldehyde (prepared with paraformal) containing 0.1% glutaraldehyde and buffered with 0.1 M phosphate (pH 7.2). Fragments of atria and CTS were then prepared for immunocryoultramicroscopy as already described,25-27 and each of the antibodies was used. The polyclonal antibodies against C-terminal [Arg101, Tyr126]ANF were used at a concentration of 0.03 µg/ml; the polyclonal antibodies against the N-terminal portion of [Asp11, Ala37]ANF were used at a concentration of 1.0 µg/ml. The monoclonal antibody against C-terminal ANF was used at a concentration of 1.0 µg/ml. The specificity of immunostaining was assessed as already described.13-15,25-27

In Situ Hybridization

Plasmid pKS+ANF-3-A was constructed by ligation of Pst I digested and dephosphorylated pKS (Stratagene Inc., La Jolla, California) with a 650 bp Pst I insert isolated from pANF3-A28 by standard procedures.29

Probe Preparation

Cesium chloride–banded plasmid pKS+ANF3-A was linearized by Ava II digestion, twice phenol-extracted, precipitated, and resuspended in RNase-free water (1 mg/ml). The probes were synthesized with an RNA transcription kit (Stratagene Inc.). [35S]Uridine 5'-triphosphate (New England Nuclear, Dupont Canada, Mississauga, Ontario, Canada; 1,350 Ci/mmol) was used as the radionucleotide. T7 polymerase was used to synthesize the complementary RNA (cRNA) probe, and T3 polymerase was used to synthesize the control (mRNA) probe. Unincorporated labeled nucleotides were eliminated by chromatography on Sephadex G-50 (Phar- macia, Uppsala, Sweden). The specific activity of probes was 2-4×10⁶ cpm/µg.

Hybridization

Under pentobarbital anesthesia, the hearts of five rabbits were first perfused through both ventricles with autoclaved Ringer-Locke fluid containing 0.05% diethyl pyrocarbonate (DEPC) (Sigma Chemical Co., St. Louis, Missouri) for 1 minute and then with formaldehyde (4% paraformal in phosphate-buffered saline; pH 7.4). Portions of the atrial appendages and of the free walls of the right and left ventricles with attached CTS were dissected and
Adenylate Cyclase Activity Determination

Determination of Cyclic AMP and Cyclic GMP Levels

cAMP and cGMP levels were determined in the false tendons by RIA as described previously with kits from New England Nuclear and Amersham, respectively.

Atrial Natriuretic Factor–Receptor Binding Determination

[^125I]ANF binding was determined as described at 25°C by incubating 20 µl crude homogenates of false tendons (~50 µg) for 60 minutes with 10 pM[^125I]ANF-(99-126) in 200 µl of a reaction mixture consisting of 50 mM Tris HCl, pH 7.5, 5 mM MgCl₂, 1 µM aprotinin, 1 mg/ml bacitracin, 4 mg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and various amounts of unlabeled ANF. Binding reactions were initiated by the addition of crude homogenates. The receptor[^125I]ANF complex was separated from free[^125I]ANF by filtration through GF/C filters pretreated with polyethyleneimine. The filters were washed three times with 4 ml ice-cold Tris-HCl buffer, pH 7.5, and radioactivity was counted in a γ-counter (LKB, Uppsala, Sweden).

Results

Biochemistry

As determined by RIA (Table 1), the CTS contained approximately 40 times less irANF than the

control. As determined by RIA (Table 1), the CTS contained approximately 40 times less irANF than the
TABLE 1. Immunoreactive Atrial Natriuretic Factor Concentration in Rabbit Heart

<table>
<thead>
<tr>
<th></th>
<th>irANF (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Both atria</td>
<td>192.8±17.9</td>
</tr>
<tr>
<td>Chordae tendineae spuriae</td>
<td>4.9±1.0*</td>
</tr>
<tr>
<td>Ventricular septum</td>
<td>0.07±0.01**</td>
</tr>
</tbody>
</table>

Statistical analysis of data was performed by one-way analysis of variance and the Dunnett test. irANF, immunoreactive atrial natriuretic factor.

* p<0.05 as compared with irANF values in 1 and 3.
** p<0.05 as compared with irANF values in 1 and 2.

Immunohistochemistry

All the CTS showed a much higher reactivity (Figure 2A) with the three antibodies used than that encountered in the middle ventricular wall. The reaction was lesser, however, than that encountered in atrial appendages.

Ultrastructure

As previously described,3 the CTS of the rabbit are made up of Purkinje fibers separated and surrounded by connective tissue sheaths whose exterior surface is covered by endothelium. Fibroblasts, nerve terminals, Schwann cells, and a few mast cells are present in the matrix of collagen and elastic fibers. The Purkinje fibers (Figure 2B), which make up the vast majority of cellular elements in the CTS, contain a central nucleus with large spaces at either pole. The periphery of the cell contains bands of myofilaments generally similar to those encountered in working ventricular cardiocytes except for their loose arrangement and the frequent absence and poor organization of Z-bands. Myofilament polyribosome complexes were present along with bundles of intermediate filaments (skeletin or desmin).8 The paranuclear Golgi complexes were often extensive and made up of saccules with peripheral vesicles (Figure 2C). Rough endoplasmic reticulum cisternae were scarce and sometimes located close to the Golgi complex. Structures resembling secretory granules were scarce and often located in the central perinuclear Golgi area of the cell. No T system was present, and intercalated disks were simplified in comparison with those visualized in working ventricular cardiocytes.

In Situ Hybridization

Hybridization between labeled ANF cRNA probes and cell mRNA encoding pro-ANF was demonstrated by specific and discrete deposits of silver grains in the emulsion overlying rabbit atrial cardiocytes (Figure 3A). The density of silver grains, as already described in the rat40,41 was much lower in rabbit working ventricular cardiocytes (Figure 3B). No labeling occurred over other cell types in the myocardium, endocardium, or epicardium. Only a background level of silver grains was detected in atrial appendages whereas the ventricular septum contained approximately 2,700 times less ANF than the atrial appendages. HPLC analysis of the acid extract of CTS (Figure 1) showed that three immunoreactive peaks, which were detected by three specific RIAS, eluted at corresponding positions of ANF(99-126), ANF(1-98), and ANF(1-126). However, other immunoreactive peaks were also observed indicating some nonspecific proteolytic degradation of ANF.

**Figure 1. Analysis of extracts of chordae tendineae spuriae by reverse phase high-performance liquid chromatography (HPLC). Elution was done with linear gradient of CH₃CH in 0.1% trifluoroacetic acid. Elution position of known peptides, ANF-(99-126), ANF-(1-98), and ANF-(1-126) are indicated by brackets. Immunoreactive atrial natriuretic factor (ir-ANF) peptides were detected by specific radioimmunoassays for N-terminal portion (O---O), C-terminal portion (O---O), and pro-ANF (O---O).**
sections that were hybridized with the probe identical to the coding sequence of rat ANF mRNA. In addition, the radioautographic labeling was significantly reduced by RNase pretreatment of sections before in situ hybridization. These controls emphasize the specificity of the cRNA probe. In mixed primary cultures of rat atrial cardiocytes, the cardiocytes displayed an intense selective labeling after exposure of the cells to ANF cRNA, and only a few background grains were found after exposure to the ANF mRNA probe (data not shown). In the rabbit CTS (Figure 3C and 3D), the number of silver grains present on Purkinje fibers was midway between those found over atrial and working ventricular cardiocytes. Here again, only a background level of silver grains was detected in sections hybridized with the probe identical to rat ANF mRNA, and the intensity of the reaction was markedly reduced by RNase pretreatment of the sections.
Binding Studies

To investigate if the from rabbit heart. The binding was competitively inhibited by increasing concentrations of unlabeled ANF with an apparent dissociation constant ($K_d$) of about 1.0 nM. The maximal binding capacity observed was about 2–2.5 fmol/mg protein.

Second Messenger Studies

In order to investigate whether these ANF receptors were functional, the effects of ANF on an adenylate cyclase–cAMP system were studied. ANF inhibited adenylate cyclase activity in a concentration-dependent manner with an apparent $K_i$ of about 1 nM (Figure 5). The maximal inhibition observed was approximately 60%. ANF also decreased the cAMP levels in the CTS in a concentration-dependent manner with an apparent $K_i$ of approximately 1 nM (Figure 6). Table 2 shows that isoproterenol, epinephrine, norepinephrine, and dopamine increased the adenylate cyclase activity and cAMP levels to various degrees. ANF inhibited the stimulatory effect of isoproterenol, epinephrine, and dopamine on cAMP production and adenylate cyclase activity whereas the norepinephrine-
stimulated adenylate cyclase activity was decreased only slightly, and cAMP levels were not affected by ANF. In addition, forskolin, a positive inotropic agent, which has been reported to activate the adenylate-cyclase-cAMP system in a variety of tissues, also increased cAMP levels in these false tendons by approximately eightfold. ANF was also able to partially inhibit the forskolin-stimulated cAMP production. In addition, ANF also stimulated guanylate cyclase activity (Figure 7) and increased cGMP levels (Figure 8) at high concentrations (10^{-7} to 10^{-6} M).

Discussion

The present results indicate that the Purkinje fibers of rabbit CTS, like those of the rat, are endocrine cells containing relatively large amounts of both irANF and ANF mRNA. In the rat, the intracavitary Purkinje fibers are of two types: one that resembles atrial cardiocytes (Purkinje type I) and another that is more akin to ventricular cardiocytes (Purkinje type II). Both types contain a much greater number of secretory granules harboring irANF and a much larger Golgi complex. The results of the present study indicate that, in the rabbit, the Purkinje fibers are of only one type, as already described, the number of secretory granules containing irANF is much smaller, and the Golgi complex is less elaborate. Thus, the rat model may apply to small mammals whereas the rabbit model, because of its general resemblance to Purkinje fibers in other species such as cat, dog, sheep, and cow, may apply to larger mammals. The amount of irANF, as demonstrated by RIA, is greater in rabbit Purkinje fibers than in rabbit working ventricular cardiocytes. Likewise, the amount of ANF mRNA, as demonstrated by hybridization in situ, is midway between that of atrial and working ventricu-
Table 2. Effect of Atrial Natriuretic Factor on Basal and Agonist-Stimulated Cyclic Adenosine Monophosphate Levels and Adenylate Cyclase Activity in Rabbit False Tendons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cAMP levels (pmol/mg protein)</th>
<th>Adenylate cyclase activity (pmol cAMP/mg protein/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Change (%)</td>
</tr>
<tr>
<td>Baseline</td>
<td>531</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine (50 μM)</td>
<td>1,331</td>
<td>150 ↑</td>
</tr>
<tr>
<td>Epinephrine (50 μM)</td>
<td>1,356</td>
<td>150 ↑</td>
</tr>
<tr>
<td>Dopamine (100 μM)</td>
<td>1,067</td>
<td>101 ↑</td>
</tr>
<tr>
<td>Isoproterenol (50 μM)</td>
<td>787</td>
<td>48 ↑</td>
</tr>
<tr>
<td>Forskolin (50 μM)</td>
<td>5,859</td>
<td>739 ↑</td>
</tr>
</tbody>
</table>

Cyclic adenosine monophosphate (cAMP) levels in false tendons were determined as described in Materials and Methods. The false tendons were incubated at 37°C in the presence of 3-isobutyl-1-methylxanthine (1.0 mM) and various agonists, alone or in combination with atrial natriuretic factor (ANF) for 10 minutes. The reaction was stopped by trichloroacetic acid. cAMP was extracted as in Materials and Methods, and cAMP levels were determined by using a radioimmunoassay kit. Values are the means of duplicate determinations from one of three separate experiments.

Adenylate cyclase activity was determined as under Materials and Methods. Values are mean±SEM of triplicate determinations from one of three separate experiments.

ular cardiocytes. The irANF contained in these secretory granules and in the Golgi complex is recognized by both antibodies against the C-terminal [Ser^{9},Tyr^{126}]ANF and the N-terminal [Asn^{9},Arg^{98}]ANF moieties of the molecule. It is thus evident that, as confirmed by HPLC analysis, the secretory granules of this cell type, as in atrial cardiocytes, contain at least 30% of the whole ANF propeptide. The presence of other immunoreactive peaks may indicate a partial nonspecific degradation of pro-ANF during processing of tissues. The immunocytochemical results also indicate that Golgi saccules and vesicles in Purkinje fibers also contain the ANF propeptide.

Since rabbit Purkinje fibers are endocrine cells containing secretory granules, their regulated secretion is by definition secretagogue-dependent, and the effects of ANF are possibly autocrine. The results of the present study suggest the presence of receptors for the C-terminal portion of ANF on these cells, which appear to be linked to second messengers. C-terminal [Ser^{9},Tyr^{126}]ANF at physiological concentrations produces a pronounced dose-dependent decrease in adenylate cyclase activity and cAMP content. The presence of ANF receptors coupled to an adenylate cyclase system has also been shown in rat heart sarcotendinmma and cultured atrial and ventricular cardiocytes. The results on cAMP are compatible with the studies on adenylate cyclase and suggest that the decrease of cAMP levels by ANF is the result of its inhibitory effect on adenylate cyclase. Since ANF has also been reported to stimulate the guanylate cyclase-cGMP system, we were also interested in investigating whether ANF receptors present in CTS are also coupled to this signal transduction system. We have found that the effects of ANF on (presumably) particulate guanylate cyclase and cGMP are far less

![Diagram](image-url)
pronounced and occur at much higher concentrations of the peptide.

Catecholamines have been shown to play an important role in the regulation of impulse conduction. Since adenylate cyclase and cAMP are known to be mediators of catecholamine action, we were interested in examining the effects of ANF on catecholamine-stimulated adenylate cyclase activity and cAMP levels in false tendons. We found that isoproterenol, epinephrine, norepinephrine, and dopamine increased the adenylate cyclase activity and cAMP levels to various degrees. ANF inhibited the stimulatory effects of isoproterenol, epinephrine, and dopamine on cAMP production and adenylate cyclase activity, and identical results were obtained with forskolin. On the other hand, norepinephrine-stimulated adenylate cyclase activity was only slightly decreased, and cAMP levels were not affected by ANF.

Since, in the central nervous system, ANF-containing neurons and ANF binding sites are present and inhibitory effects of the peptide on their firing rate have been described, the situation may be similar in Purkinje fibers. In these cells, cAMP is able to produce a positive inotropic effect and to induce arrhythmias whereas cGMP depresses the slow response action potential and prevents isoproterenol-induced arrhythmias. The powerful effect of ANF on inhibition of adenylate cyclase and production of cAMP in these cells may indeed indicate that the autocrine secretion of ANF may modulate the conduction impulse of these cells while ANF-induced production of cAMP may have effects only at high concentrations. Because the amount of ANF released locally may be high, the system may be implicated as well. The slight inhibition of norepinephrine-stimulated adenylate cyclase activity and the lack of inhibitory effect of ANF on cAMP levels may indicate that its effects on the adenylate cyclase-cAMP system are selective.

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