Atrial natriuretic peptide modulates the hyperpolarization-activated current (I_f) in human atrial myocytes

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Abstract

Objectives: The relationship between atrial stretching and changes in cell excitability is well documented. Once stretched, human atrial myocytes (HuAM) release atrial natriuretic peptide (hANP). Receptors for hANP (NPR) are coupled to a guanylyl cyclase (GC) activity, and are present on HuAM, but the electrophysiological effects of hANP are largely unknown. We investigated the effect of hANP on I_f, the hyperpolarization-activated current present in HuAM, and the underlying intracellular pathway.

Methods: HuAM were isolated from atrial appendages and utilized for patch-clamp recording.

Results: hANP caused a significant and concentration dependent shift of the midpoint activation potential (D V_h) toward less negative potentials of 6.9 ± 1.0 mV at 0.1 nM; 13.0 ± 2.6 mV at 1 nM and 15.3 ± 2.2 mV at 10 nM (p < 0.001 for all); a parallel increase of I_f rate of activation occurred. The effect of hANP was completely blocked by isatin, a potent antagonist of NPR (p < 0.01 vs. hANP). In the presence of the inhibitors of guanylyl cyclase (ODQ and LY83583), hANP caused a significantly smaller D V_h (p < 0.01 vs. hANP for both). 8Br-cGMP mimicked the effect of hANP, both in the presence and absence of KT5823, a selective inhibitor of Protein kinase G. Pretreatment with pertussis toxin (PTX) did not change the effect of hANP, thus excluding a major role for the coupling of NPR with the Gi-Proteins system. Pretreating cells with cyclopentyladenosine (CPA), an A1-adenosine receptor agonist, completely blocked hANP effect. Adding hANP to maximal serotonin concentration produced an additive response.

Conclusions: Our data demonstrate for the first time that ANP is able to increase I_f, likely through a modulation of intracellular cGMP and cAMP levels. This effect could have implications in the relationship between stretch and arrhythmogenesis in the human atrium.

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Keywords: Atrial natriuretic peptide; Human atrial myocytes; Hyperpolarization-activated current; Patch-clamp; Signal transduction

1. Introduction

Electrical and mechanical activities are closely integrated in cardiomyocytes [1]. Action potentials trigger cell twitches; on the other hand, mechanical changes (i.e. cell stretch) can modulate electrical activity, either by activation of stretch activated channels (SAC) [2], and/or by increasing intracellular calcium [1]. Thus mechanical disturbance might result in the occurrence of electrical arrhythmogenic changes and increased risk of arrhythmias [1]. In fact, the relationship between atrial stretching and arrhythmogenesis is well documented [3], but the underlying mechanisms are not fully understood.

Atrial distension, by stretching atrial myocytes, is the main stimulus for releasing atrial natriuretic peptide (ANP) both in animal models [4] and in humans [5]. Human ANP (hANP) is synthesized and stored as pro-hormone in granules localized in human atrial myocytes (HuAM) [6] and, when released, interacts with specific receptors, thus exerting its autocrine, paracrine and endocrine actions. To date three types of natriuretic peptides receptors (NPR) have been identified: NPR-A and NPR-B are membrane-bound receptors having a guanylyl cyclase (GC) activity [6]; NPR-C is
the so-called “clearance receptor” [6] because its major role is to remove hANP, but it seems to be also coupled with G-proteins [7]. Both NPR-A and NPR-C are present on atrial myocytes of primates [8] but, to our knowledge, no clear-cut data are available in humans. Previous data demonstrate the expression of NPR-A in human atrial tissue [9] and the modulation of both L-type calcium current ($I_{Ca,L}$) and transient outward current ($I_{to}$) by hANP [10]. These currents play a key role in human atrial repolarization both in physiological and pathological conditions, being profoundly affected in atrial fibrillation [11,12] and in dilated atria [13]. Thus a direct electrophysiological action of ANP released upon atrial stretching might represent a further link between changes in cell length and electrical response of HuAM.

The constitutively expressed hyperpolarization-activated current ($I_f$) is an inward Na/K current, which modulates the diastolic membrane potential, thus contributing to the phase 4 of the action potential [14,15]. Under physiological conditions $I_f$ conductance in human atrium is not large enough to depolarize cell membrane and to initiate spontaneous activity [16]. However, pathological conditions [17] and/or activation of specific intracellular pathways by neurohumoral signals [14,15], likely involved in atrial arrhythmias, are able to cause a functional increase of $I_f$. Under these conditions $I_f$ could influence cell excitability and predispose to the occurrence of atrial arrhythmias [16,18].

Therefore, the aim of the present study was to investigate the effect of hANP on native $I_f$ in HuAM and related intracellular pathways. Preliminary results appeared in abstract form [19].

2. Methods

2.1. Patients

Tissue specimens of human right atrial appendages were obtained from 27 patients of both sexes (21 male, 6 female), aged from 43 to 82 years (63.8 ± 1.8) undergoing cardiac surgical procedure, for valve replacement ($n = 7$) and/or coronary artery bypass graft ($n = 22$). All patients gave their informed consent for the use of their tissue samples. The investigation conforms with the principles outlined in the “Declaration of Helsinki” [20] and was approved by the local ethical committee. All patients were in sinus rhythm before surgery. Twenty-two patients were under pharmacological treatment with several combinations of Ca²⁺-antagonists, nitrates, β-adrenergic blockers, acetylsalicylic acid, ACE-inhibitors, and others. Our previous data suggested that the properties of $I_f$ were not significantly influenced by these treatments [14,15].

2.2. Cell isolation

HuAM were enzymatically isolated from specimens of human atrial appendages. The detailed procedure and solutions for transport of atrial tissue and cell isolation have been previously published [14].

2.3. Electrophysiological recordings

Cells were transferred to a recording chamber mounted on the stage of an inverted microscope (Nikon Diaphot) and superfused by gravity with a six-line microperfusor system placed near the cell at a flow rate of 1 ml/min (temperature, 37 °C). The experimental set-up used for patch-clamp recording in the whole-cell configuration was similar to that described previously [14]. Cells were perfused with normal Tyrode’s solution to measure membrane capacitance ($C_m$), and with modified Tyrode’s solution for $I_f$ measurements. $C_m$ was measured by ± 10 mV voltage steps applied from a holding potential of −70 mV.

Fig. 1. Determination of activation curve for $I_f$. Typical recording of $I_f$ in a single HuAM. Traces were obtained by hyperpolarizing steps (−10 mV) from −60 to −130 mV, applied from a holding potential of −30 mV (A). The related activation curve for $I_f$ was obtained by fitting the following experimental data points with a Boltzmann function (B).
and calculated as described previously [14]. $I_f$ was elicited by hyperpolarizing steps ($-10$ mV) ranging from $-60$ to $-130$ mV applied from a holding potential of $-30$ mV; such a holding potential was chosen in order to maintain the $f$-channels deactivated before applying hyperpolarizing steps, especially in the presence of agonists which tend to shift the threshold for $I_f$ activation toward less negative voltages. Current amplitudes at different membrane potentials were calculated as the difference between the less negative value or “peak” at the beginning of the test pulse and the value at steady state. Steady-state values of the hyperpolarization-activated current were extrapolated by fitting the entire trace from the initial “peak” to the end of the step to a single exponential decay, which gave the time constant ($\tau$) for $I_f$ activation.

2.4. Solutions

Cells were superfused with Tyrode’s solution containing (in mM): D-(+)-glucose 10, Hepes 5.0, NaCl 140, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1.2; pH adjusted to 7.3 with NaOH. To measure $I_f$, Tyrode’s solution was modified by adding 1 mM BaCl$_2$ (to block inward rectifier-like channels), 2 mM MnCl$_2$ (to block $I_{Ca,L}$) and 0.5 mM 4-aminopyridine (to block transient outward potassium current, $I_{to}$); KCl was increased to 25 mM in order to amplify $I_f$. This extracellular solution is conventionally used to record $I_f$ because it allows to isolate and magnify $I_f$ without altering its voltage-dependence [21]. Internal pipette solution contained (in mM): K-L-aspartic acid 130, Hepes 10, Na$_2$-ATP 5, Na$_2$-GTP 0.1,

![Fig. 2](https://academic.oup.com/cardiovascres/article-abstract/63/3/528/346534/fig2.jpg)

**Fig. 2.** Effect of 10 nM hANP on $I_f$. Traces show the typical effect of 10 nM hANP on the $I_f$ evoked by a double-pulse voltage protocol (see inset) (A). Activation curves of the $I_f$ in the absence (○) and in the presence of 10 nM hANP (●) (B).

![Fig. 3](https://academic.oup.com/cardiovascres/article-abstract/63/3/528/346534/fig3.jpg)

**Fig. 3.** hANP shifts $V_h$ in a concentration-dependent manner. The average activation curves show the effect of hANP at 0.1 nM (A), 1 nM (B) and 10 nM (C). The bar graph (D) summarizes the results for each hANP concentration: $\Delta V_h[0.1 \text{ nM}] = 6.9 \pm 1.0$ mV ($n = 9$), $\Delta V_h[1 \text{ nM}] = 13.0 \pm 2.6$ mV ($n = 10$), $\Delta V_h[10 \text{ nM}] = 15.3 \pm 2.2$ mV ($n = 12$). Each column represents mean ± S.E.M. of the $V_h$ shift; *$p<0.001$ vs. corresponding controls, *$p<0.05$ vs. ANP 0.1 nM.
EGTA 11, MgCl₂ 2.0, CaCl₂ 5.0, pH adjusted to 7.2 with KOH (pCa=7.0).

### 2.5. Drugs

Drugs were dissolved, according to their solubility, either in distilled water, EtOH, dimethyl sulfoxide or medium (SIGMA, M199). These diluents did not exert electrophysiological effects at the final concentrations used in our experimental conditions. Stock solutions of ODQ and PTX were directly added to cell suspension to perform incubation before experiments. Neither ODQ nor PTX seemed to influence the basic properties of $I_C$ during the incubation; in fact, $V_h$ was $-86.6 \pm 2.6$ mV for cells pre-incubated in ODQ ($n=12$) and $-92.3 \pm 3.7$ mV for those pre-incubated in PTX ($n=18$), not statistically different from cells stored in control conditions ($-87.7 \pm 1.4$ mV). Carbachol (2 μM, SIGMA) was used to assess the effectiveness of G₁-protein inhibition by PTX.

$hANP$ (SIGMA) was used at the following concentrations: 0.1, 1 and 10 nM. If the concentration is not differently indicated, the highest concentration was used. Cyclopentyladenosine (CPA), a selective A₁-adenosine receptor agonist, was dissolved in 100% ethanol to prepare a stock solution of 10 mM, which was then diluted with Tyrode’s solution to the final concentration [14]. 5-Hydroxy-tryptamine (5-HT) was dissolved in distilled water and stored solution of 10 mM, which was then diluted with Tyrode’s solution to get the final 5-HT concentration [15].

Isatin (Aldrich), a potent antagonist of NPR [22], 8Br-cGMP (SIGMA), and LY83583 (Calbiochem), an inhibitor of GC [23], were used at 100 μM. ODQ (Calbiochem), an inhibitor of GC [24], was used at 10 μM and cells were pre-incubated for at least 30 min at 37 °C. KT5823 (Calbiochem), a selective inhibitor of the PkG [25], was used at 0.1 μM.

Pertussis toxin (PTX) (SIGMA) was used at 0.5 μg/ml and cells were pre-incubated with PTX for at least 3 h at 37 °C. The boltzmann function:

$$g_I = g_{\text{max}} \cdot \frac{1 + \exp\left(-\frac{V_m - V_h - \tau}{k}\right)}{1 + \exp\left(-\frac{V_m - V_h}{k}\right)}$$

2.6. Data analysis and statistics

The amplitude of $I_f$ was normalized to $C_m$. From the $I-V$ relations, specific conductance of $I_f$ was determined for each cell according to the equation $g_f = I/(V_m - V_{rev})$, where $g_f$ is the conductance calculated at membrane potential $V_m$, $I$ the current amplitude, and $V_{rev}$ is calculated from the analysis of tail currents [14]. $g_{\text{max}}$ was obtained by fitting values with the Boltzmann function: $g_f = g_{\text{max}} \cdot \left[1 + \exp\left((V_m - V_h)/k\right)\right]$, where $k$ is the slope factor describing the slope of the activation curve. All data are presented as mean ± S.E.M.

The Instat program (Graph Pad, vers. 3.05) was used for statistical analysis; $p < 0.05$ was considered significant. Student’s $t$-test on paired and unpaired data were used for statistical comparisons. We used one way analysis of variance (ANOVA) followed by Dunnett multiple comparison (to compare $hANP$ 10 nM alone with the several tested drugs) or repeated measures ANOVA followed by the Student–Neuman–Keuls test (to compare data on $hANP$ and 5-HT or CPA). To evaluate the relationship between midpoint of $I_C$ activation and the effect of agonists (that is, $hANP$, in the absence or presence of PTX, and 8Br-cGMP), a linear regression was used, according to the equation $Y=a+bX$, where $X$ and $Y$ are, respectively, $V_h$ in the absence and presence of agonists.

![Fig. 4](https://academic.oup.com/cardiovascres/article-abstract/63/3/528/346534)

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**Fig. 4.** The intracellular pathway of $hANP$ stimulation. (A) Summary of the effect of $hANP$ in the absence and presence of 100 μM isatin, a potent antagonist of NPR, 10 μM ODQ and 100 μM LY83583, two inhibitors of GC. Effect of $hANP$ was mimicked by 10 μM 8Br-cGMP in the absence and presence of 0.1 μM KT5823, a selective inhibitor of PkG. Each column represents mean ± S.E.M. of the shift in $V_h$; numbers indicate the number of tested cells. *p<0.01 vs. hANP alone. (B) Midpoint ($V_h$) of $I_C$ activation curve recorded in control conditions is plotted against $V_{-h}$ measured after agonist application. Each point refers to an individual cell, challenged with 0.1, 1 or 10 nM $hANP$ (crossed, open or closed circles, respectively), 0.1 or 10 nM $hANP$ in PTX pretreated cells (open or closed triangles, respectively), 8Br-cGMP in the absence and presence of KT5823 (open and closed squares, respectively). Points were fitted by the linear relationship $Y=a+bX$ with $a=11.5$ and $b=0.995$ ($p<0.0001$, $n=63$).
3. Results

3.1. $I_f$ properties

Single HuAM, isolated from right atrial appendages, had an average membrane capacitance ($C_m$) of $51.3 \pm 1.6 \, \text{pF}$ ($n=278$).

A hyperpolarization-activated current was present in almost all HuAM (113 out of 127 cells, 89%) in agreement with our previous data [14,15]; since we have previously demonstrated that this current has the electrophysiological properties of the $f$-current [14,15], we refer to it as $I_f$.

Fig. 1 shows a typical recording of $I_f$ obtained from an HuAM bathed in modified Tyrode’s solution (A) and its activation curve obtained from the trace analysis (B). Statistical analysis of the individual activation curves gave an average value of ionic specific conductance ($g_f$) of $31.6 \pm 2.4 \, \text{pS/pF}$ and an average value of midpoint activation potential ($V_h$) of $-87.7 \pm 1.4 \, \text{mV}$ ($n=113$). These data are consistent with previous values reported in literature [14,15,26].

3.2. Effect of hANP on $I_f$

Fig. 2A shows the typical effect of 10 nM hANP on $I_f$ evoked from a single HuAM by a two-pulse protocol: from a holding potential of $-30 \, \text{mV}$ the cell was hyperpolarized to $-70 \, \text{mV}$ (a voltage relatively close to the physiological resting potential of HuAM) and then to $-110 \, \text{mV}$ (a potential close to the voltage of $I_f$ maximal activation). Perfusion with 10 nM hANP increases the $I_f$ amplitude during the first pulse, while the residual current activated during the second, more negative step, is slightly decreased; this suggests that a shift of the activation curve rather than an increase of the maximal amplitude occurs. The occurrence of such a shift was confirmed by the analysis of the activation curves measured before and after exposure to 10 nM hANP, obtained by plotting the normalized $I_f$ conductance vs. membrane potential (Fig. 2B).

Fig. 3 reports the average activation curves obtained at three concentrations of hANP: 0.1 nM (A), 1 nM (B) and 10 nM (C). The bar graph (D) summarizes the shift of $V_h$ ($\Delta V_h$) showing a clear-cut concentration-dependent effect; corresponding maximal specific conductance of $I_f$ was $25.5 \pm 1.2$, $20.3 \pm 0.8$ and $27.6 \pm 1.1 \, \text{pS/pF}$ in the presence of 0.1, 1 and 10 nM hANP, respectively. In parallel with the effect on $V_h$, $I_f$ kinetics was also modified by hANP. In particular, 10 nM hANP significantly increased the rate of current activation ($1/\tau$, in s$^{-1}$) from 0.9 $\pm$ 0.2 to $1.7 \pm 0.2$ at $-90 \, \text{mV}$ ($n=8$, $p<0.05$); from 1.2 $\pm$ 0.2 to $1.8 \pm 0.2$ at $-100 \, \text{mV}$ ($n=7$, $p<0.05$); from 1.4 $\pm$ 0.2 to $2.8 \pm 0.5$ at $-110 \, \text{mV}$ ($n=8$, $p<0.001$); from 1.5 $\pm$ 0.2 to $2.5 \pm 0.2$ at $-120 \, \text{mV}$ ($n=7$, $p<0.001$); and from 1.9 $\pm$ 0.2 to $3.3 \pm 0.2$ at $-130 \, \text{mV}$ ($n=4$, $p=0.002$).

3.3. Signalling pathway of hANP stimulation

Previous data, particularly the concentration-dependent effect of hANP, suggested the involvement of ANP receptors. This was further supported by the observation that the effect of hANP was completely blocked by isatin: in the presence of isatin, from an average value of $-80.2 \, \text{mV}$ in control conditions, $V_h$ was only shifted by $0.1 \pm 1.5 \, \text{mV}$. 

![Fig. 5. Effect of hANP in the presence and absence of PTX pretreatment. Traces show the typical effect, obtained in a single PTX pretreated HuAM, on $I_f$ elicited before (○) and after (●) the perfusion with 10 nM hANP (A). The average activation curves show the effect of 0.1 nM hANP (B) and 10 nM hANP (C) in HuAM pre-incubated with PTX.](https://academic.oup.com/cardiovascres/article-abstract/63/3/528/346534)
Isatin, an endogenous indole, is a potent antagonist of NPR, with no subtype selectivity; moreover isatin has been reported to inhibit GC activity [22,27].

Since NPR-A and NPR-B are receptor subtypes coupled with GC activity, two inhibitors of this enzyme, ODQ and LY83583, were tested. In the presence of ODQ, hANP caused a shift of $V_h$ significantly smaller that in its absence ($\Delta V_h = 3.6 \pm 2.1$ mV, $n = 12$, $p < 0.01$ vs. hANP alone). Similar results were obtained with LY83583, being $\Delta V_h = 0.3 \pm 2.6$ mV, $n = 5$ ($p < 0.01$ vs. hANP alone). These data suggest that hANP acts on f-channel by increasing intracellular cGMP levels.

To confirm this hypothesis, we perfused HuAM with 8Br-cGMP, a stable analogue of cGMP. 8Br-cGMP was able to shift the activation curve of $I_f$ by $13.6 \pm 3.1$ mV (from $-95.2$ to $-81.6$ mV, $n = 8$) ($p = 0.003$ vs. corresponding controls, NS vs. hANP). Interestingly, the effect of 8Br-cGMP was not significantly reduced in the presence of KT5823, a potent and selective inhibitor of PKG: in the presence of KT5823, direct application of 8Br-cGMP shifted $V_h$ by $10.2 \pm 1.5$ mV, $n = 10$ ($p < 0.0001$ vs. corresponding controls, NS vs. hANP and 8Br-cGMP alone). All these data are summarized in Fig. 4A.

To evaluate the relevance of the coupling between the NPR-C and the G$_i$-Protein system, we pre-incubated HuAM with pertussis toxin (PTX) to inactivate G$_i$-Proteins. The effectiveness of the G$_i$-Protein blockade was tested by assessing the effect of 2 $\mu$M carbachol on a voltage ramp protocol from $-120$ to $+60$ mV from a holding potential of $-70$ mV. Carbachol increased both inward and outward current in HuAM not pretreated with PTX and it was completely ineffective in PTX pretreated cells (PTX) ($n = 3$ for both). Specific f-current conductance ($g_f$) and $V_h$ for cells pre-incubated with PTX were, respectively, $45.5 \pm 9.2$ pS/pF ($n = 18$) and $92.3 \pm 3.7$ mV, not statistically different from control cells. Traces in Fig. 5A were recorded in a PTX pretreated HuAM and show the typical effect before (open circles) and after (closed circles) the perfusion with 10 nM hANP. PTX pretreatment did not alter the effect of 0.1 nM hANP, being $\Delta V_h$ (PTX) ($9.9 \pm 3.3$ mV, $n = 7$, $p < 0.03$ vs. corresponding controls) not different from

![Fig. 6. Influence of inhibition or stimulation of adenylyl cyclase on the effect of hANP. (A–C) Example of the effect of 10 nM hANP in a single HuAM in the presence of 5 $\mu$M CPA, a selective agonist of type 1 adenosine receptors. CPA caused a leftward shift of $I_f$ activation curve (A) and reduction of current measured at 80 mV; furthermore, it prevented the effect of subsequent superfusion with hANP, which did not modify $I_f$ (B). Panel C summarizes the results obtained in four different cells (**$p < 0.01$ vs. control); each column represents mean ± S.E.M. of the shift in $V_h$. (D–F) Example of the effect of 10 nM hANP in a single HuAM in the presence of 1 $\mu$M 5-HT. 5-HT shifted $I_f$ activation curve toward less negative potentials (A) and increased current measured at $-80$ mV; following superfusion with hANP caused a further rightward shift of the activation curve (B). Panel C summarizes the results obtained in four different cells (*$p < 0.05$; **$p < 0.01$ vs. control; †$p < 0.05$ vs. 5-HT alone); each column represents mean ± S.E.M. of the shift in $V_h$.](https://academic.oup.com/cardiovascres/article-abstract/63/3/528/346534)
that observed in the absence of PTX (6.9 ± 1.0 mV). Similar data were obtained for the highest hANP concentration (10 nM), being ΔVₙₕ[PTX] (10.4 ± 1.9 mV, n = 7, p = 0.001 vs. corresponding controls) not significantly different from ΔVₙₕ measured in not-pretreated cells (15.3 ± 2.2 mV). The average activation curves for PTX pretreated cells are shown in Fig. 5B (hANP 0.1 nM) and Fig. 5C (hANP 10 nM). Since Vₙₕ showed a large variability, we tested whether the effect of different agonists (hANP in the absence or presence of PTX, 8Br-cGMP in the absence or presence of KT5823) may depend on the effect of different agonists (hANP in the absence or presence of KT5823) may depend on Iₙ activation voltage of basal conditions. However, Fig. 4B suggests that this was not the case. Points obtained by plotting Vₙₕ measured in control vs. Vₙₕ measured after agonist application, gave a distribution which was fitted (p < 0.0001, n = 63) by a linear relationship. The line intercepted y-axis at 11.5 mV and its slope was 0.995 with a 95% confidence interval from 0.976 to 1.015 (i.e. not statistically different from unity). This suggests that Iₙ was shifted similarly by all agonists in cells independently from the starting value of Vₙₕ. Moreover, ANOVA analysis showed that Vₙₕ measured before drug (hANP or 8Br-cGMP) application was not statistically different among experimental groups, being −91.7 ± 1.4 mV for control cells used for hANP experiments and −87.1 ± 3.0 for control cells used for 8Br-cGMP experiments, −92.3 ± 3.7 mV for HuAM pre-incubated in PTX, and −81.8 ± 2.9 mV for those pre-incubated in KT5823 (p = 0.1173). Similarly, Vₙₕ did not differ significantly among the previous experimental groups and those in which cells were incubated with NPR antagonist (ISA) or GC inhibitors (ODQ and LY83583) (p = 0.07).

Finally, the effect of basal activity of adenylyl cyclase on hANP effect was evaluated. In Fig. 6, cells were superfused with maximal concentrations of either CPA, a selective A1 agonist, or 5-HT. As expected [14], CPA (5 μM), by inhibiting adenylyl cyclase activity, shifted Vₙₕ toward more negative values by 4.3 ± 0.9 mV (p < 0.01 vs. corresponding controls); subsequent application of hANP (10 nM) was completely ineffective (Fig. 6A–C). An opposite result was obtained by enhancing adenylyl cyclase activity through 5-HT₄ activation [15] (Fig. 6D–F). In this case, 5-HT caused the expected rightward shift of Vₙₕ (8.6 ± 2.0 mV, n = 4, p < 0.05 vs. corresponding controls); adding hANP (10 nM) caused a further significant shift of Vₙₕ (19.4 ± 4.3 mV, p < 0.01 vs. control; p < 0.05 vs. 5-HT alone). This result suggests that basal adenylyl cyclase activity influences the effect of hANP.

4. Discussion

Our data demonstrate for the first time that hANP is able to positively modulate the hyperpolarization-activated current (Iₙ) in HuAM. The principal findings are: (1) hANP increases Iₙ at physiologically relevant potentials in a concentration-dependent manner, by interacting with NPR.

(2) The effect of hANP on the f-channel depends on an increase of the intracellular cGMP levels, but not on phosphorylation by PKG. (3) The coupling between NPR and G-proteins does not play a relevant role in the effect of hANP on f-channel.

4.1. The effect of hANP on Iₙ is receptor-mediated

In our experiments, Iₙ was increased by superfusion of the HuAM with hANP at low concentrations (from 0.1 to 10 nM), the maximal effect being reached at concentrations beyond 1 nM, comparable with the plasma concentration of hANP reported in patients with chronic atrial fibrillation [28]. Since hANP is released by HuAM [5], it should be expected that the plasma concentration is lower compared to that reached in the proximity of the release site, especially if one considers the “clearance action” of NPR-C [6].

The effect of hANP on Iₙ appears to depend on the stimulation of NPR present on HuAM, since it was completely blocked by isatin, a potent and specific, although not selective, antagonist of NPR [22]. ANP mainly interacts with NPR-A and NPR-C [6], which have been identified in atrial tissue of primates, while NPR-B is absent [8]. Molecular evidence confirms the expression of NPR-A in the human right atrium [9]. Specific antagonists for all NPR have been synthesized: in particular, NPR-A and NPR-B are selectively blocked by HS-142-1 [29] while NPR-C is inhibited by C-ANP-23 [6]. Since a precise characterization of NPR was beyond the aim of this study, we used isatin as a non-selective antagonist of NPR. Isatin acts fundamentally in two ways: (1) by antagonising the binding sites for hANP; (2) by inhibiting the guanylyl cyclase activity of the NPR. Recent data suggest that isatin also inhibits soluble GC in human platelets [27]. This allowed us to by-pass the missing information about the subtypes of NPR in HuAM. A previous paper reporting the direct electrophysiological effect of hANP in HuAM showed that hANP decreases Iₙ_a,-l and Iₙ_o in HuAM [10], but no definitive evidence of a receptor-mediated effect was provided. Due to the non-selectivity of isatin on NPR subtypes and its inhibitory action on GC, at present we cannot precisely attribute the effect of hANP on Iₙ to a specific receptor subtype, in particular, type A or B. However, the inhibition of the effect of hANP by isatin together with the observation that this effect was concentration-dependent suggests the involvement of a receptor-mediated mechanism.

4.2. Intracellular pathway of hANP stimulation

The observed effect appears to be dependent on cGMP production by NPR, since it was prevented by two GC inhibitors (LY83583 and ODQ). LY83583 is an inhibitor of both soluble and particulate GC [23], while ODQ is a selective inhibitor of soluble guanylyl cyclase [24]. As for the action of LY83583, our data are consistent with previous findings, where the effect of ANP was completely blocked
by this GC-inhibitor in guinea pig cardiomyocytes [23]. As for the action of ODQ, our data are in contrast with others previously reported in literature, showing the ineffectiveness of ODQ on ANP mediated effect [23,30]. Several reasons could account for these divergent observations. First, in different experimental models (HuAM vs. guinea pig tracheal rings and ventricular myocytes), different coupling mechanisms might be involved. Second, previous studies evaluated the effects of ANP (inhibition of L-type calcium current [23], and relaxant effect [30]) probably involving a phosphorylating action by PkG. In HuAM, we assessed an electrophysiological effect of hANP, which certainly does not involve PkG. Third, a novel, not well identified link between particulate and soluble guanylyl cyclase seems to exist, as suggested by recent evidence [31]. Finally, our results should be interpreted in the light of recent observations showing that some of the effect of ANP are partially mediated by soluble guanylyl cyclase [31].

8Br-cGMP was able to mimic the effect of hANP on native f-channel in HuAM, also in the presence of KT5823, a selective inhibitor of PkG, thus excluding a major role for phosphorylation of the f-channel by PkG, and suggesting a direct modulation of the f-channel by cGMP. These results agree with previous data by Yoo et al. [32] showing a cGMP mediated action of NO on sinoatrial cells. To date, four isoforms of f-channels have been identified (HCN1–4), all of them showing a Cyclic Nucleotide Binding Domain (CNBD) in their molecular sequence [33]. However, the isoforms differ in their biophysical and pharmacological properties, such as sensitivity to intracellular cyclic nucleotides. Indeed, HCN1 is almost insensitive to intracellular cyclic nucleotides [34]. In contrast, HCN2 and HCN4 are largely modulated by cAMP and cGMP [33]. Present knowledge provides evidence for HCN2 and HCN4 in the human atrium [33]. Our functional findings support this view: the great magnitude of $V_h$ shift induced by hANP in HuAM is consistent with the expression of HCN isoforms highly sensitive to cyclic nucleotides. Actually, Musialek et al. [35] demonstrated that, in guinea pig isolated atria, cGMP could act both directly, binding to f-channel, and indirectly, by increasing cAMP levels, due to the inhibition of the cGMP-inhibited phosphodiesterase (PDE-3). Since a previous study suggested a very high basal adenyl cyclase activity in HuAM [36], we cannot exclude that the observed effect of hANP on $I_f$ might be partially mediated by an increase of intracellular cAMP level, due to the inhibition of PDE-3. This mechanism is also supported by the following considerations. Results obtained by pretreating cells with CPA (which stimulates A1-receptors and G1 Proteins) indicate that inhibition of basal adenyl cyclase activity prevents the effect of hANP. On the other side, adding maximal hANP concentration (10 nM) to maximal 5-HT concentration (1 μM) [15] produced an additive response, which is apparently difficult to be explained when considering the hypothesis that both 5-HT and hANP exert their effects through cAMP. However, as recently demonstrated by Zaccolo and Pozzan [37], PDE activity prevents diffusion and equilibration of cAMP concentration within the cytosol. As a consequence, PDE inhibition generated a rise in cAMP higher than that induced by maximal stimulation of Gs via β-AR [37]. Being the effect of maximal β-AR or 5-HT stimulation quantitatively comparable [14,15], one can hypothesize that hANP, due to a cGMP-mediated inhibition of PDE activity, exert an additive effect to that caused by 5-HT. The concomitant inhibition of PDE activity and stimulation of adenyl cyclase activity would allow to reach intracellular cAMP levels (and a functional effect) higher than that induced by maximal 5-HT concentration.

The third type of NPR (NPR-C) is known as a “clearance receptor” [6], but it can also decrease cAMP level by interacting with G1-Proteins [7]. We tested the relevance of this receptor subtype by pre-incubating HuAM with PTX, which inactivates G1 Proteins. Since the effect of hANP in untreated and treated cells was similar, our data suggest that coupling between NPR and G1-Proteins does not play a major role in modulating hANP action on the f-channel. Thus, it can be suggested that the hANP-induced increase in intracellular cGMP level is more important for f-channel modulation than a decrease of basal intracellular cAMP levels due to the activation of the G1-Protein system by the NPR-C subtype.

4.3. Conclusion and limitations

In conclusion, our results for the first time provide data for a potentially new mechanism involved in the complex relationship between stretch and arrhythmogenesis: they suggest a link between the auto- and paracrine actions of hANP and a direct effect on the hyperpolarization-activated current which is constitutively expressed in HuAM. Previous studies on HuAM showed a direct effect of hANP on other ion currents, namely $I_{Ca-L}$ and $I_{Na}$ [10]. Thus, on the basis of those and present results, the total effect of hANP on the human atrial action potential cannot be easily predicted and requires further investigation. However, the depolarizing shift in the activation curve of $I_f$ produced by hANP, increases the amplitude of this depolarizing current and its influence on the diastolic membrane potential, which might be translated into an increased propensity to spontaneous activity of HuAM [16]. This is a potentially novel mechanism for stretch-related atrial arrhythmogenesis.

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References


