Changes in extracellular pH mediate the chronotropic responses to L-arginine

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Abstract

We have recently shown that exogenous nitric oxide (NO) elicits a positive chronotropic response by stimulating the hyperpolarization-activated current, I h. Objective: To examine whether L-arginine (L-Arg) can mimic the chronotropic effect of NO by enhancing its endogenous production. Methods: In spontaneously beating guinea pig atria we evaluated the heart rate (HR) response to increasing concentrations of L-Arg (1 μmol/l to 10 mmol/l), and compared it with that for d-Arg or L-lysin (L-Lys) (all in free base (FB) or hydrochloride (HCl) formulation). Results: L-ArgFB > 100 μmol/l caused a reversible dose-dependent increase in HR (peak effect 164 ± 7 bpm at 10 mmol/l, P < 0.05, n = 8). However, a similar HR response occurred with d-ArgFB (n = 7) or L-LysFB (n = 6). All FB formulations increased the perfusate pH (peak [pH] = 8.61 ± 0.03). Although alkalinization can stimulate NO release from the endothelium, this is unlikely to have contributed to HR changes in our preparation, since neither N-methyl-L-arginine, (100–500 μmol/l, which per se reduced HR by 8 ± 1%, P < 0.05, n = 9) nor NO scavenging (fresh 5% red blood cells, n = 9) caused a rightward shift of the concentration–response curve to L-ArgFB. Furthermore, as opposed to FB formulations, L-ArgHCl, d-ArgHCl or L-LysHCl > 1 mmol/l significantly decreased HR and [pH] (n = 17). The chronotropic effects of L-ArgFB or L-ArgHCl were reproduced by changing [pH] with NaOH (n = 8) or HCl (n = 7), whereas the HR increase with L-ArgFB was prevented by clamping [pH] at 7.42 ± 0.07 (n = 10). Conclusions: In vitro, L-Arg can markedly affect HR through a pH-mediated, NO-independent mechanism. Our data show that the opposing changes in [pH] induced by different formulations of L-Arg can importantly confound the assessment of the biological effects of this amino acid. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

We have recently shown that exogenous nitric oxide (NO) can increase heart rate (HR) in vitro, through the activation of an intracellular pathway involving cGMP and the hyperpolarization-activated current I h [1]. Since NO is tonically released in the heart [2], this mechanism may be involved in the regulation of intrinsic HR. Some circumstantial evidence supports this hypothesis; for instance, conscious endothelial NO synthase (eNOS)-deficient mice, which lack the major source of NO synthesis in the cardiovascular system, have significantly lower HR than wild type mice [3,4]. Conversely, administration of 1 mmol of the NOS substrate L-arginine (L-Arg) has been shown to elicit a positive chronotropic response in vitro [5]. It is debatable, however, whether in the absence of prolonged L-Arg depletion or inducible NO synthase (iNOS) stimulation, extracellular supply of L-Arg would result in increased NO production [6,7]. Indeed, since the intracellular concentration of this amino acid far exceeds the half-maximal saturating concentration for eNOS [6,7], extracellular provision of L-Arg should not affect NO synthesis. Recent findings, however, suggest that cytoplasmic stores of L-Arg may not be directly accessible to NOS and delivery of extracellular L-Arg to the caveolae

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may be required for NO synthesis [8]. Thus, the aim of this study was twofold. First, does endogenous NO production regulate intrinsic HR? Secondly, does l-Arg supplementation exert a chronotropic effect by increasing the synthesis of NO? We found that inhibition of NOS has a small but significant negative chronotropic effect in isolated guinea pig atria. Although extracellular supply of l-Arg produced significant changes in HR, these were not mediated by an increased synthesis of NO. Indeed, we demonstrate that arginine-induced changes in extracellular pH are responsible for the chronotropic effect of this amino acid.

2. Methods

Experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the United Kingdom “Animals (Scientific Procedures) Act 1986”.

The spontaneously beating guinea pig atrial preparation has been described in detail previously [1]. Briefly, the atria were mounted vertically in a preheated (37±0.1°C), continuously oxygenated, water jacketed bath containing 60 ml of Tyrode solution (Section 2.1). Sutures connected the right atrium to a stainless steel hook and the left atrium to an isometric force transducer (Harvard Apparatus). Data were acquired on a Power Macintosh 8500 computer using a Biopac MP100 data acquisition system and AcqKnowledge 3.5 software. Beating rate was triggered from contraction and the signals were displayed in real time. Data were stored on a compact disk for off-line analysis.

2.1. Solution and drugs

The Tyrode contained (mmol/l): NaCl 120, KCl 4, MgCl 2, NaHCO3 25, CaCl2 1.8, Na2HPO4 0.1, glucose 11. The perfusate was aerated with 95% O2 and 5% CO2 (pH 7.4) and its temperature was continuously monitored (Digitron 1408-K gauge) and kept at 37±0.1°C. Water was of reagent grade from an Elga water purification system.

Two distinct formulations of l-Arg, l-Arg free base (l-ArgFB) and l-Arg hydrochloride (l-ArgHCl), were used. Concentration–response relationships were constructed by adding either l-ArgFB or l-ArgHCl cumulatively (in half-logarithmic increments every 4 to 5 min) to the perfusate in a concentration range from 1 μmol/l to 10 mmol/l.

To determine whether the effects of l-Arg are NO-mediated, we evaluated the chronotropic response to the inactive enantiomer d-Arg [9,10] both in the FB and HCl formulation. Moreover, we examined the rate effect of l-ArgFB after preincubation of the preparation with Nω-methyl-l-arginine (l-NMMA) to inhibit NOS (100–500 μmol/l for 30–40 min) [9] or in the presence of 5% suspension of fresh human red blood cells (RBC, prepared as described by Mendes Ribeiro et al. [11]) to scavenge NO [12–14]. Finally, we tested the chronotropic response to similar concentrations of l-Lysine (l-LysFB and l-LysHCl). This amino acid has properties similar to l-Arg, but it cannot serve as a substrate for NOS [10].

All drugs were obtained from Sigma Chemical Co.

2.2. pH Of the Tyrode perfusate

The pH of the perfusate was monitored throughout the experiments (Corning 220 pH-meter). Since millimolar concentrations of ArgFB or ArgHCl caused alkalinization or acidification of the perfusate respectively (see Results), we tested whether similar changes in pH, obtained by adding sodium hydroxide (NaOH, stock concentration 0.01 to 0.1 mol/l) or hydrochloride (HCl, stock 0.01 to 0.1 mol/l), could mimic the rate response to the two formulations of arginine. In addition, we evaluated the chronotropic response to l-ArgFB while clamping pH by adding HCl to the perfusate.

2.3. Statistical analysis

Data are presented as mean±SEM (±SD for pH measurements). One-way analysis of variance followed by Fisher’s posthoc test was used to evaluate intra- and intergroup differences. Statistical significance was accepted at P<0.05.

3. Results

After 120–200 min of stabilization, the spontaneous beating rate of atrial preparations reached a stable value which averaged at 179±3 bpm (n=54). The effect of time on the beating rate was tested in seven preparations. As shown in Fig. 1A (top trace), no significant changes in beating rate occurred over the time-course of the experiments reported below (−1±1 bpm after 30 min, −2±1 bpm after 60 min, both nonsignificant [NS]).

3.1. Chronotropic response to l- and d-ArgFB and l-LysFB

Fig. 1A (middle trace) shows a typical chronotropic response to increasing concentrations of l-ArgFB in the isolated guinea pig atria with mean data for eight preparations plotted in Fig. 1B. Whereas 1 to 100 μmol/l of l-ArgFB had no effect on the beating rate, higher concentrations (≥500 μmol/l) elicited a reversible dose-dependent positive chronotropic response, which peaked at the maximal concentration used (1±4±7 bpm at 10 mmol/l, P<0.05 vs. B/L [B/L indicates the basal beating rate after stabilization]). At these concentrations, however, l-
ArgFB also caused a progressive increase in the pH of the perfusate (from 7.40±0.003 to 8.61±0.036, n=3 measurements, data not shown).

Identical results were obtained when similar concentrations of either the inactive d-isomer of ArgFB (n=7, see Fig. 1a bottom trace, and Fig. 1b) or l-LysFB (n=6) were tested; i.e., concentrations of d-ArgFB or l-LysFB ≥500 μmol/l elicited a dose-dependent positive chronotropic response with a peak increase in rate at 10 mmol/l (+68±6 bpm for d-ArgFB and +71±7 bpm for l-LysFB, P<0.05 vs. B/L for both). Likewise, d-ArgFB [15] and l-LysFB caused a progressive increase in the pH of the Tyrode perfusate (from 7.40±0.001 to 8.60±0.044) in the concentration range from 0.5 to 10 mmol/l.

3.2. Effect of NOS inhibition or NO scavenging on the chronotropic effect of ArgFB

An increase in extracellular pH has been previously shown to be the mechanism by which both l- and d-ArgFB can stimulate NO release in cultured endothelial cells [16]. To test whether a pH-mediated release of NO contributed to the increase in beating rate with the FB formulation of arginine, we examined the chronotropic response to l-ArgFB in the presence of l-NMMA (100–500 μmol/l, n=9), or after adding a NO scavenger to the perfusate (5% human RBC, n=9 [12–14]).

l-NMMA decreased the spontaneous beating rate by 8±1% (P<0.05, for a raw data trace see Fig. 2a trace) whereas the inactive d-isomer of NMMA (d-NMMA 100–500 μmol/l, n=6) [9] was ineffective (−0.4±0.5%, NS). l-NMMA or NO scavenging did not affect the concentration–response curve to l-ArgFB nor the concomitant changes in the perfusate pH (Fig. 2b and c). In both cases there was a dose-dependent increase in beating rate in a concentration range from 0.5 to 10 mmol/l, which was virtually identical to that seen with l-ArgFB alone (peak effect of +66±6 bpm with l-NMMA, and +68±6 bpm with RBC, P<0.05 vs. B/L, Fig. 2b cf. Fig. 1).
3.3. Chronotropic response to the L- or D-isomer of ArgHCl or to L-LysHCl

The concentration–response to L-ArgHCl was tested in six preparations. Fig. 3a (top trace) and Fig. 3b show that L-ArgHCl had no effect on HR at concentrations from 1 μmol/l to 1 mmol/l whereas higher concentrations elicited a negative chronotropic effect (n=6, peak response of −16±3 bpm at 10 mmol/l, P<0.05) and decreased the pH of the perfusate (pH=7.28±0.026 at 10 mmol/l L-ArgHCl, see Fig. 3c). The D-isomer of ArgHCl (n=5, see Fig. 3a bottom trace, and Fig. 3b) or L-LysHCl (n=6) had a similar effect on rate (peak response at 10 mmol/l of −15±2 bpm and −12±2 bpm respectively), and produced virtually identical changes in the pH of the Tyrode solution (Fig. 3c).

3.4. Chronotropic effect of changes in the pH of the perfusate

To assess the importance of extracellular pH in determining the chronotropic effect of L-ArgFB or L-ArgHCl, we tested the HR response (1) to L-ArgFB while clamping the perfusate pH with HCl; (2) to pH changes obtained by adding NaOH or HCl to the Tyrode to mimic the pH effect of increasing concentrations of the FB or HCl formulation of arginine.

Fig. 4 shows that when the increase in pH associated with increasing concentrations of L-ArgFB was prevented by simultaneous application of HCl (n=10), the beating rate did not change (−1±1 bpm at 0.1 or 1 mmol/l, ±1±2 bpm at 5 mmol/l, and ±3±2 bpm at 10 mmol/l of L-ArgFB, pH range from 7.40±0.030 to 7.42±0.072). Fig. 5 (raw data in Fig. 5a top trace, mean data for eight experiments in Fig. 5b), on the other hand, shows the chronotropic effect of adding NaOH to the perfusate to mimic the pH changes seen with ArgFB. Each increase in pH (to 7.47±0.014→7.55±0.047→8.38±0.180→8.62±0.053, see Fig. 5c) was accompanied by an increase in HR of a magnitude similar to that seen with ArgFB (+7±1 bpm→+11±1 bpm→+43±5 bpm→+69±6 bpm, P<0.05, Fig. 5b, cf. Fig. 1 and Fig. 2). Conversely, when HCl was used to mimic the pH changes seen with ArgHCl,
we observed a decrease in HR of 7±1 bpm at pH = 7.33±0.020 and of 17±1 bpm at pH = 7.28±0.027 (n = 7, P < 0.05, Fig. 5a bottom trace and Fig. 5b, cf. Fig. 3). Thus, the chronotropic effect of L-ArgFB or L-ArgHCl was fully mimicked by modifying pH of the Tyrode perfusate.

4. Discussion

The present study demonstrates that the NO precursor L-Arg can modulate mammalian HR in vitro at concentrations greater than 100 μmol/l. However, the chronot-
The chronotropic effect of L-Arg is not mediated by a substrate-dependent increase in NO production since (1) it also occurs with D-Arg and with another basic amino acid, L-Lys, and (2) it is not attenuated by inhibition of NOS or NO scavenging. Moreover, the chronotropic response to L-Arg varies with the formulation of this amino acid and with the associated changes in the perfusate pH; i.e., ArgFB causes an increase in beating rate and alkalinization of the perfusate, whereas ArgHCl elicits a negative chronotropic response and a decrease in pH. A casual relationship between extracellular pH and the chronotropic effect of L-Arg was confirmed by showing that the HR changes with the two formulations of L-Arg are fully mimicked by modifying extracellular pH with NaOH or HCl (in the absence of L-Arg), whereas preventing alkalinization of the perfusate with HCl abolishes the rate response to increasing concentrations of L-ArgFB.

4.1. Negative chronotropic effect of NOS inhibition

Constitutive NOS is expressed in the coronary endothelium, in atrial and ventricular myocytes, and in pacemaking tissue [17]. Recent evidence indicates that NO is tonically released in the mammalian heart where it reaches concentrations of 1–3 μmol/l [2]. Whereas endogenous NO is known to modulate cardiac contractility and relaxation [17,18] and to affect the chronotropic response to autonomic stimuli [19–21], its effect on basal HR has not been established.

We have recently shown that micromolar concentrations of NO donors elicit a NO–cGMP-dependent positive chronotropic response in vitro [1]. Here, we found that L-NMMA, a blocker of NOS, causes a reduction in the beating rate in guinea pig atria by 8±1% (P<0.05, see Fig. 2a), whereas the D-isomer of NMMA is ineffective. Similar
reductions in HR with NOS inhibition have been incidentally reported by others in the rat in vitro [5,22,23] and following intracoronary infusion of 1-NMMA in vivo in the dog [24,25], though in two other studies in the same species the HR reduction with NOS inhibition failed to reach statistical significance [19,26]. In addition, conscious
eNOS-deficient mice have a significantly lower HR than the wild type mice [3,4]. Taken together these data indicate that endogenousely released NO exerts a relatively small but significant tonic positive chronotropic effect.

4.2. Mechanism of the chronotropic effect of $l$-Arg

In addition to inhibiting NOS, enhancing the endogenous production of NO might provide another means of testing the importance of this molecule in regulating sinoatrial node activity. This could be achieved either by stimulation of NOS with "agonists" (such as bradykinin or substance P [18]) or, possibly, by increasing the extracellular provision of $l$-Arg. However, both bradykinin and substance P have been shown to exert a NO-independent negative chronotropic effect by stimulating the release of acetylcholine from local cholinergic neurons [27,28].

Circumstantial evidence suggests that $l$-Arg might exert a positive chronotropic effect through NO. For instance, an increase in HR in response to 1 mmol/l $l$-Arg was noted by Kojda et al. [5] in Langendorff-perfused rat hearts whereas NOS inhibition with N-nitro-$l$-arginine decreased the beating rate of this preparation. Although it is well established that $l$-Arg is the substrate for NO synthesis [9,10,29], the mechanism by which supplying high concentrations of this amino acid should boost NO production remains controversial. The intracellular concentration of $l$-Arg (800 to 1000 $\mu$mol/l in endothelial cells) is known to be tenfold higher than the plasma level, exceeding by far the half-maximal saturating concentration for the constitutive NOS ($K_m \sim 3$ $\mu$mol/l) [6,7]. This suggests that, in the absence of $l$-Arg depletion or iNOS activation [30], the extracellular supply of $l$-Arg should not be a limiting factor for NO production. Nonetheless, recent findings in cultured endothelial cells indicate that not all $l$-Arg stores may be available to NOS and direct delivery of extracellular $l$-Arg to the caveolae may be required for NO synthesis [8].

We found that concentrations of $l$-ArgFB greater than 100 $\mu$mol/l significantly increase the spontaneous beating rate. To assess whether an enhanced production of NO was responsible for the positive chronotropic response to $l$-ArgFB, we tested the effect of the $d$-isomer, $d$-ArgFB, and that of another basic amino acid, $l$-LysFB (neither of which can serve as a substrate for NOS). The lack of stereospecificity of the HR response to ArgFB (Fig. 1), and the fact that $l$-LysFB was equally effective, indicate that the chronotropic effect of $l$-ArgFB cannot be secondary to a substrate-related enhanced production of NO. Although both $l$- and $d$-ArgFB (and in fact any alkaline buffer) have been shown to stimulate NO release from cultured endothelial cells via an increase in $[\text{pH}]_o$ [16], this mechanism is unlikely to have contributed to HR changes in our preparation, since there was no rightward shift in the concentration–response curve to $l$-ArgFB in the presence of $l$-NMMA or RBC (Fig. 2, cf. Fig. 1). We then considered two other mechanisms that could potentially affect pacemaking: i.e., changes in the osmolarity and $pH$ of the perfusate with $l$-Arg. Since only marked hyperosmolarity ($\geq 150$ mmol/l sucrose) affects HR in vitro [31,32], this mechanism could not play any significant part in the chronotropic effect of concentrations of $l$-Arg (500 $\mu$mol/l to 10 mmol/l) used in our experiments. However, we demonstrated that $l$-ArgFB and $l$-ArgHCl cause opposing changes in extracellular $pH$ which play a key role in eliciting the chronotropic effect of this amino acid (Fig. 4 and 5). This is consistent with previous findings in isolated atrial preparations, where alkalosis was shown to elicit tachycardia whereas acidosis decreased HR, e.g., Ref. [33]. The cellular mechanism responsible for the $pH$-dependent modulation of the pacemaker activity has been elucidated by Satoh and Seyama [34] in isolated patch-clamped sinoatrial node cells. They showed that changing the extracellular $pH$ from 7.4 to 8.5 caused an increase in spontaneous beating rate which was accompanied by a greater amplitude of the $I_C$ current, and by an increase in the maximal conductance of the $l$-type calcium current and of the steady-state outward current (without affecting their gating properties). In contrast, decreasing $pH$ from 7.4 to 6.5 elicited opposite effects on these ionic currents and on the beating rate.

5. Summary

In freshly isolated guinea pig atria we showed that inhibition of NOS has a small but significant negative chronotropic effect. Although we found that extracellular supply of $l$-Arg markedly affects the spontaneous beating rate, we provide evidence that this does not occur through an increased synthesis of NO. This finding is consistent with the idea that exogenous $l$-Arg cannot stimulate NO synthesis by the constitutive NOS in the absence of $l$-Arg depletion [6,30,35,36], but it does not exclude the potential therapeutic role of this amino acid in pathological conditions associated with the impairment of the $l$-Arg/NO pathway [37,38]. Our study is the first to show that different formulations of $l$-Arg exert opposing, $pH$-dependent, chronotropic responses in vitro. This highlights the importance of considering NO-independent effects when $l$-Arg is used to enhance NO synthesis or reverse NOS inhibition.

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