



## Effect of Extracellular Calcium Depletion on Atrial Natriuretic Factor Secretion and Gene Expression

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### Abstract

Extracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_o$ ) may be one of the factors affecting atrial natriuretic factor (ANF) release. In the previous studies, there are opposite reports about the effect of  $[\text{Ca}^{2+}]_o$  on ANF secretion. Therefore, the effect of  $[\text{Ca}^{2+}]_o$  depletion on ANF secretion and gene expression was studied in the present research. Isolated right atria from rats were perfused for 6 and 2 hours in the presence and absence of  $[\text{Ca}^{2+}]_o$ , and ANF secretion were measured using radioimmunoassay method. Cyclohexamide was used in another group as a protein synthesis inhibitor. ANF mRNA level in atria measured by Real time PCR.  $[\text{Ca}^{2+}]_o$  depletion induced a significant increase in basal ANF release. This stimulatory effect peaked at 2 h, then started to decrease and reached around basal levels by 6 h. Protein synthesis inhibition by cyclohexamide did not abolish or significantly decrease the stimulatory effect of  $\text{Ca}^{2+}$ -free media on ANF secretion. The ANF gene expression was not significantly affected by  $\text{Ca}^{2+}$ -free media. These results suggest that the contractile and endocrine function of atrial cardiocytes are differently affected by  $[\text{Ca}^{2+}]_o$  and may represent an adaptation to the dual roles of the atrial myocytes contraction and secretion.

*Keywords:* Atrial natriuretic factor; Extracellular calcium; Gene expression.

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### 1. Introduction

Atrial natriuretic factor (ANF) is a polypeptide hormone continuously produced and released by contractile atrial myocytes of mammals and participates in regulating body fluid and blood pressure. Thus, atrial cardiomyocytes are involved in both mechanical and endocrine functions of the

heart. Appropriate mechanical or neuroendocrine stimuli increase the rate of ANF release with or without a concomitant increase in synthesis [1, 2]. For example, stimulation of ANF release by atrial muscle stretch is well-documented phenomenon demonstrated *in vivo* and *in vitro* experiments and is considered as the principal stimulus for ANF release [3-6]. However, exact mechanism(s) mediating ANF synthesis and secretion from atrial cardiocytes are ill-defined [7]. Extracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_o$ )

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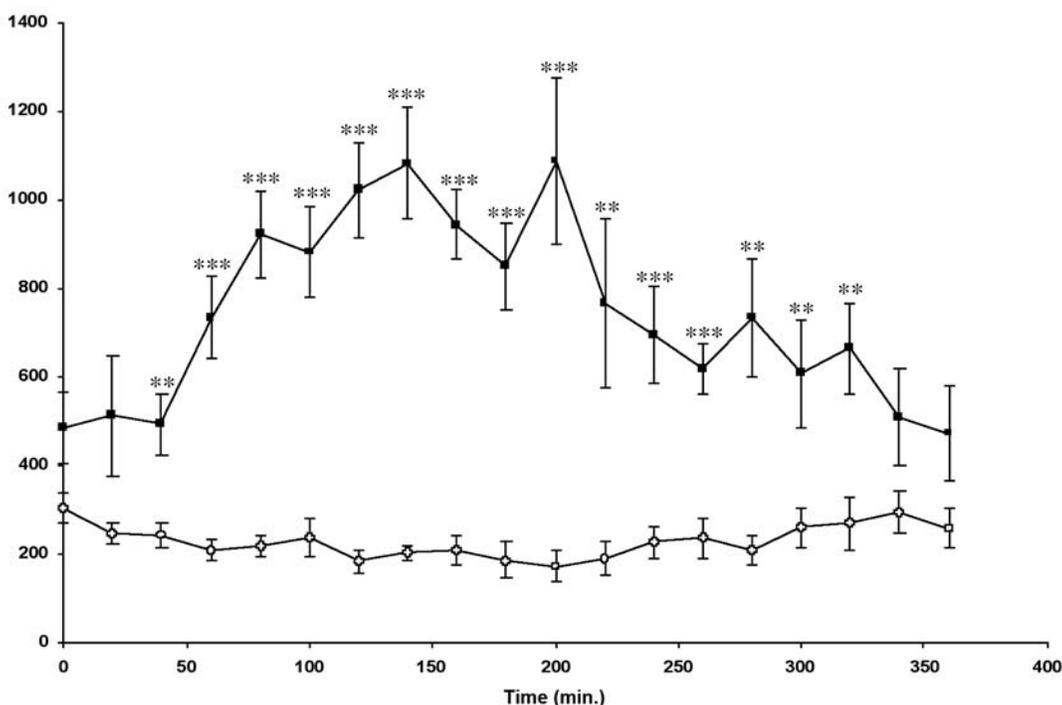
may be one of the factors affecting ANF secretion, as it plays a crucial role in the stimulus-secretion coupling of rennin [8] and parathormone [9] and also it has a key role in the process of muscle excitation-contraction coupling. Reports on the regulatory role of  $[Ca^{2+}]_o$  for ANF secretion have been controversial. Some studies have reported augmented ANF secretion with increase in extracellular  $Ca^{2+}$  [10-12]; Some others have presented evidence indicating inhibition by  $[Ca^{2+}]_o$  [13-15] and also it was suggested that  $[Ca^{2+}]_o$  is not involved in the regulation of ANF secretion [16, 17].

In the present work, the effect of  $[Ca^{2+}]_o$  depletion on ANF secretion was studied using an isolated perfused right atria model. In addition, the levels of ANF gene expression in the presence and absence of  $[Ca^{2+}]_o$  were compared.

## 2. Materials and methods

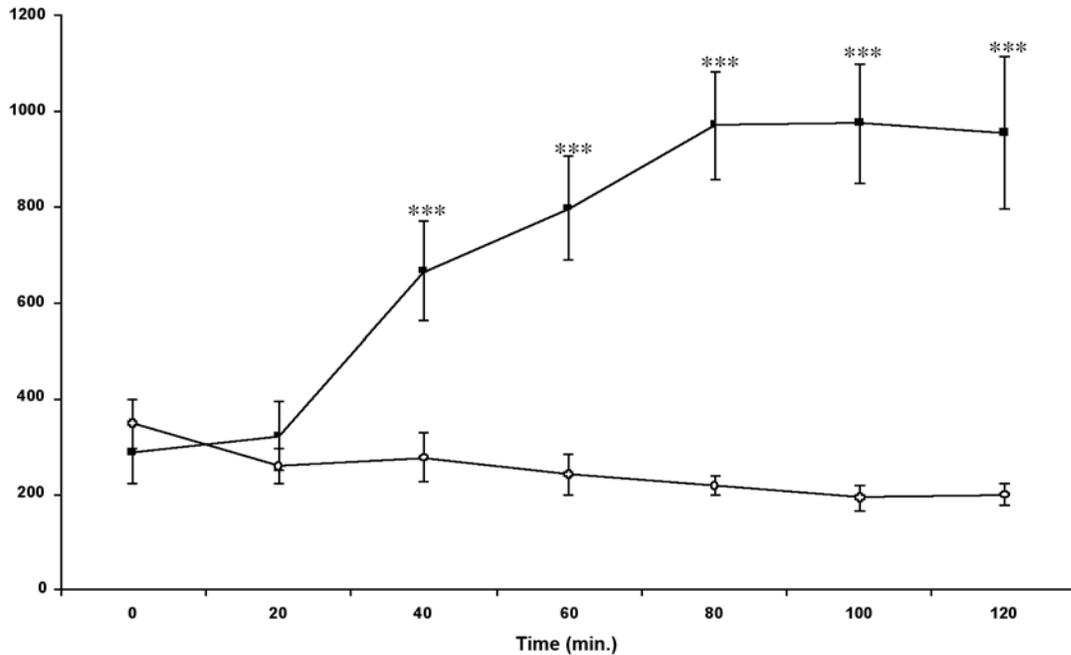
### 2.1. Isolation of right atria and perfusion protocols

The atria were prepared as described previously [18]. Briefly, the hearts were removed from male Sprague-Dawley rats (300-350 g; Charles River Laboratories, Montreal, PQ, Canada) and placed in a supplemented Krebs-Ringer bicarbonate buffer (KRBB) solution. A PE-160 cannula (1.14 mm ID) was inserted into the inferior vena cava. The cannula had an opening midway facing the atrial chamber. The coronary sinus was ligated, the left auricle was removed, the lower five-sixth of the ventricles was removed, and a PE-10 cannula was inserted into the right atrium via the tricuspid valve. The right atrium dissected in this manner was placed in a heated (30 °C) organ chamber. The organ chamber contained 50 ml of KRBB that was replenished at a rate of 2.5 ml/min. and gassed with 95%  $O_2$ -5%  $CO_2$ .



**Figure 1.** Basal atrial natriuretic factor (ANF) release from isolated right atria and effect of  $Ca^{2+}$ -free media on secretion. Atria were perfused with regular Krebs-Ringer bicarbonate buffer (KRBB) (○) or  $Ca^{2+}$  omitted and EGTA (2 mM) added KRBB (■) for 360 min. irANF immunoreactive ANF.

Values are means  $\pm$  SE; n=5, \*\* $p$  < 0.01; \*\*\* $p$  < 0.001 (by unpaired test).



**Figure 2.** ANF release from isolated right atria and effect of  $\text{Ca}^{2+}$ -free media on secretion. Atria were perfused with regular ( $\square$ ) or  $\text{Ca}^{2+}$  omitted and EGTA (2 mM) added KRBB ( $\blacksquare$ ) for 120 min. irANF immunoreactive ANF. Values are means  $\pm$  SE; n=5. \*\*\* $p$ < 0.001 (by unpaired t test).

The superior vena cava was attached to a cannula from which KRBB (preheated to 30 °C and gassed with 95%  $\text{O}_2$ -5%  $\text{CO}_2$ ) was infused at a rate of 3 ml/min. The outflow from the inferior vena cava was connected to a pressure transducer (Narco Bio-System, Houston, TX) that was connected to a physiograph (Narco Bio-System). The intra-atrial pressure was adjusted to 0.5 mmHg. The flow of KRBB was from the superior vena cava, into the right atrium, and out the tricuspid valve. The perfusion medium was collected for a 5 min. period in siliconized glass tube by use of a fraction collector (Retriever II, ISCO, Lincoln, NE).

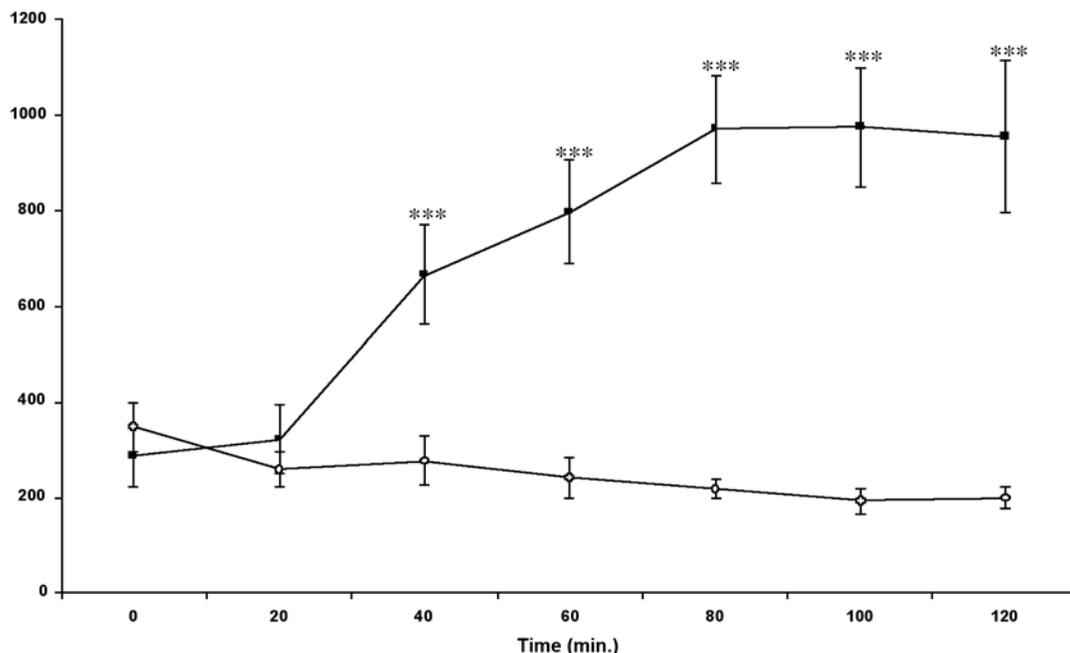
After 60 min. of equilibration, in two groups (n=5), perfusion was continued with KRBB containing  $\text{Ca}^{2+}$  (2.5 mM) for 2 or 6 h. In another two groups (n=5), perfusion were continued using  $\text{Ca}^{2+}$  free KRBB [no  $\text{CaCl}_2$  + EGTA (2 mM)] for 2 or 6 h. In another group (n=5), after 60 min. equilibration, 2 h. perfusion was continued using KRBB with no  $\text{Ca}^{2+}$  added plus 2 mM

EGTA and 10  $\mu\text{g/ml}$  cyclohexamide (as a protein synthesis inhibitor). During all experiments, perfusion medium was collected for 5 min. of each 20 min. period. A 50  $\mu\text{l}$  aliquot was transferred to another tube and kept at -20 °C until for ANF RIA.

At the end of the perfusion period, the tissue was rapidly removed and placed in a dissecting dish containing cold buffer. The cannulas were rapidly removed, and the remaining ventricular tissue, blood vessels, and connective tissue were swiftly cut away. The right atrium was flash-frozen in liquid nitrogen and kept at -80 °C for subsequent RNA extraction.

## 2.2. ANF RIA

RIA was performed using the double-antibody technique as previously described [19]. A 1:2 dilution of perfusate was used for the ANF RIA. Antisera raised against rat ANF<sub>99-126</sub> was obtained from Peninsula Labs. (Belmont, CA).  $^{125}\text{I}$ -labeled ANF<sub>99-126</sub> was made by ANF iodination in our lab. Cross-



**Figure 3.** Effect of cyclohexamide on Ca<sup>2+</sup>-free media stimulated ANF release. Atria were perfused Ca<sup>2+</sup> omitted and EGTA (2 mM) added KRBB (□) or Ca<sup>2+</sup> omitted and EGTA (2 mM) added KRBB in the presence of cyclohexamide (10 µ/ml) (■) for 120 min. irANF immunoreactive ANF.

Values are means ± SE (n=5). There is not significant difference between two groups ( $p > 0.05$ ) (by unpaired t test).

reactivity of ANF antisera with BNP peptide was less than 0.01%.

### 2.3. RNA extraction

Tissue samples were extracted using trizol (GBCO BRL; Burlington, ON, Canada) and RNA was quantified by spectrophotometry.

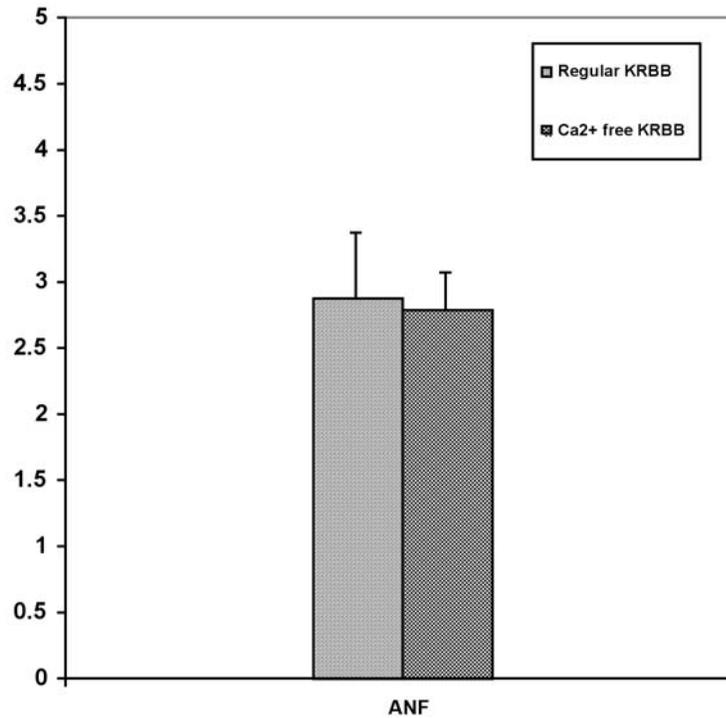
### 2.4. Real time RT-PCR

Total RNA samples (1 µg of each sample) were reverse-transcribed using Super Script II RNase H-Reverse Transcriptase and oligo(dt)12-18 primer of a reverse transcription kit (GIBCO BRL) for 50 min. at 42 °C. Then the reverse transcriptase was inactivated by heating for 15 min. at 70 °C. The polymerase chain reaction (PCR) were conducted using primers specific to ANF (Sense: 5'-GCCGGTAGAAGATGAGGT-CA-3' & Antisense: 5'-GGGCTCCAATCC TGTC AATC-3') that were designed from published sequences [20], and were made by Qiagen. The expected lengths of PCR

products for ANF was 269 bp. PCR carried out using fast start taq DNA polymerase, reaction buffer, dNTP, SYBR Green I, MgCl<sub>2</sub> from a real Time PCR kit (Roche) and light cycler instrument (Roche). The thermal cycle program was 30 cycles that each cycle was set as denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 25 s. As a house keeping gene, β-actin mRNA was simultaneously amplified at 30 cycles under same profile as used for ANF. The specificity of the amplified PCR products were approved using the melting curve analysis by increasing temperature from 68 to 95 and also using gel electrophoresis.

### 2.5. Statistical analysis

All data are reported as mean ± SE. Unpaired student's t-test was used to significance of the difference between means. Significance was accepted at  $p < 0.05$ .



**Figure 4.** Relative abundance of ANF transcripts in rat's right atria after 2 hours perfusion with regular or Ca<sup>2+</sup> omitted and EGTA (2 mM) added KRBB. Relative expression levels of ANF mRNA are normalized against  $\beta$ -actin. Values are means  $\pm$  SE (n=5). There is not significant difference between two groups ( $p>0.05$ ) (by unpaired t-test).

### 3. Results

The beating rate in atriums perfused with regular KRBB averaged  $165 \pm 15$  Beats/min. But in Ca<sup>2+</sup>-free KRBB groups, few minutes after perfusion with Ca<sup>2+</sup>-free media, the atrial beating were abolished.

#### 3.1. Effect of extracellular calcium depletion on ANF secretion

Immunoreactive (ir) ANF secretion rates significantly increased by Ca<sup>2+</sup>-free KRBB in comparison with regular KRBB (Figure 1). This stimulatory effect peaked at 2 h, then started to decrease and reached around basal levels by 6 h.

Regarding morphologic study, light and electron microscopy showed cell death and necrosis in atriums perfused by Ca<sup>2+</sup>-free KRBB for 6 h; it seemed that 6 h is too long for perfusion and so in other set of experiments, 2 h perfusion with Ca<sup>2+</sup>-free KRBB was performed. As expected, ir-ANF secretion rates during 2 h perfusion with

regular and Ca<sup>2+</sup>-free KRBB were similar to first 2 h in 6 h perfusion experiments (Figure 2).

Two hours perfusion with Ca<sup>2+</sup>-free KRBB didn't cause tissue death, because at the end of 2 h perfusion, switching to regular KRBB restarted atrial beating.

#### 3.2. Effect of cyclohexamide on ANF secretion

To determine the effect of protein synthesis inhibition on Ca<sup>2+</sup>-free media stimulated release of ANF, perfusion was performed in the presence of cyclohexamide as a protein synthesis inhibitor. As showed in Figure 3, cyclohexamide couldn't abolish or significantly decrease the stimulatory effect of Ca<sup>2+</sup>-free media on ANF secretion.

#### 3.3. Atrial ANF gene expression

Figure 4 shows atrial ANF mRNA levels in regulated and Ca<sup>2+</sup>-free KRBB groups after 2 h of perfusion. The ANF gene expression was not significantly affected by Ca<sup>2+</sup>-free

media ( $p > 0.05$ ). Also in the 6 h perfusion groups, the amount of ANF mRNA in regular and  $\text{Ca}^{2+}$ -free KRBB were not significantly different (data not shown). But the data wasn't considered because too long perfusion that causes cell death may affect the amount of ANF mRNA.

#### 4. Discussion

I examined the effect of  $[\text{Ca}^{2+}]_o$  depletion on perfused right atria and showed that  $\text{Ca}^{2+}$ -free medium stimulates ANF secretion without significant effect on ANF gene expression. I also showed that protein synthesis inhibition cannot prevent this stimulatory effect of  $[\text{Ca}^{2+}]_o$  depletion on ANF secretion.

These results are in agreement with some studies [13-15, 21] and in disagreement with some other [10, 12, 22]. The discrepancy between these two lines of evidence cannot be easily reconciled. But, most plausible reason may reside in the differences in atrial preparations.

The cellular mechanism involved in releasing ANF in response to  $[\text{Ca}^{2+}]_o$  depletion has not so far been explored. The secretory mechanisms of parathormone and rennin which have also been shown to be negatively regulated by  $[\text{Ca}^{2+}]_o$  may have common features with the ANF mechanism. Although  $\text{Ca}^{2+}$ -free medium abolished beating of atria because of cellular depolarization, but this stimulatory effect on ANF secretion cannot refer to atrium depolarization caused by  $[\text{Ca}^{2+}]_o$  depletion; because perfusion with high- $\text{K}^+$  media that causes atrial depolarization and abolishes atrial beating, has not changed ANF release [5]. This dissociation of ANF secretion from depolarization-induced changes in  $\text{Ca}^{2+}$  flux is unusual if not unique among cells that secrete peptides or neurotransmitters and probably represent an adaptation to the dual roles of the atrial myocytes, contraction and

secretion. The stimulatory effect of  $[\text{Ca}^{2+}]_o$  depletion also is not related to changes in osmolarity, because replacement of  $\text{Ca}^{2+}$  with  $\text{Sr}^{2+}$  cannot prevent increase in secretion [5].

No significant effect of cyclohexamide as a protein synthesis inhibitor on  $[\text{Ca}^{2+}]_o$  depletion-stimulated ANF secretion is in agreement with the previous studies showed no inhibitory effect of cyclohexamide on stimulated ANF secretion [23, 24]. Therefore, most of response to  $\text{Ca}^{2+}$ -free stimulated ANF secretion is based on previously synthesized, stored hormone and newly synthesized ANF is the minor component of this response. Decline in  $\text{Ca}^{2+}$ -free stimulated ANF secretion after two hours of perfusion is probably due to exhaustion of these pools [25]. In a pilot study we fractionated ANF granules using ultracentrifuge and OptiPrep™ (as a density gradient media) and examined the effect of 2 h  $\text{Ca}^{2+}$ -free medium on the pattern of granule size. The result (not shown) indicated no effect on the size distribution pattern of granules. It suggests that  $\text{Ca}^{2+}$ -free medium does not deplete a specific type of ANF granules.

The gene expression experiment more confirmed that  $\text{Ca}^{2+}$ -free medium has no effect on ANF synthesis. Most of the previous works that evaluated the effect of  $[\text{Ca}^{2+}]_o$  depletion on ANF secretion, have not determined ANF mRNA level. But in two studies that mRNA has been determined, they have reported opposite results. One study has used an *in situ* method and showed that continuous perfusion of  $\text{Ca}^{2+}$ -free solution to rat for 48 h, increases the amount of ANF mRNA in the heart [26]. The cause of this discrepancy between our result and the result of this study can be probably related to duration of experiment (48 h vs. 2 h) and the difference in method (*in vivo* vs. *in vitro*). On the other hand, in another study using cardiocytes cell culture,  $[\text{Ca}^{2+}]_o$  depletion for 24 h has caused 3 times decrease in ANF

mRNA. But in that study ANF secretion is also decreased [22].

In summary, in this study, it was confirmed that  $[Ca^{2+}]_o$  depletion increases ANF secretion without significant effect on mRNA expression and ANF synthesis. These results suggest that the contractile and endocrine function of atrial cardiocytes are differently affected by  $[Ca^{2+}]_o$  atrial beating, a  $Ca^{2+}$  dependent process was abolished after perfusion with  $Ca^{2+}$ -free media. In contrast, ANF release was stimulated under the above conditions.

Although no physiological condition is expected to entail a very low or none- $Ca^{2+}$  environment, our finding should only be taken as indicative differences between atrial cardiocytes and most other endocrine cells.

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