Brain natriuretic peptide secretion in adult rat heart muscle cells: The role of calcium channels

La sécrétion du peptide natriurétique de type B dans les cardiomyocytes de rat adulte : rôle des canaux calciques

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KEYWORDS
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Summary
Background. — The cellular mechanisms that regulate B-type natriuretic secretion are not well elicited. Intracellular fluctuation of calcium ions rate seems to be implicated.
Aims. — In this study, we evaluate the role of ventricular transmembrane calcium channels in the secretion of BNP in normal adult rats (group N) and pressure overload hypertrophied ones (group H).
Methods. — We measured plasma BNP concentration and BNP concentration in culture media of cardiomyocytes from N and H group in the presence and absence of calcium channels antagonists.
Results. — Plasma BNP concentration was increased in H group in comparison to N group (0.630 ± 0.008 ng/ml versus 0.106 ± 0.004 ng/ml; \(p < 0.01\)). This increase in BNP level was also obtained in culture media of H group in comparison to N group (3.45 ± 0.7 ng/ml versus 0.53 ± 0.22 ng/ml). However, the presence of calcium channels antagonists in the culture media of cardiomyocytes had decreased BNP concentration in both N (nifedipine: 0.22 ± 0.04 ng/ml;
Introduction

Brain natriuretic peptide (BNP) belongs to the family of hormones which also includes Atrial natriuretic peptide (ANP) [1], Type-C natriuretic peptide (CNP) [2] and Type-D natriuretic peptide (DNP) [3].

BNP was first isolated from porcine brain tissue [4]. In humans, it is mainly produced by the muscle cells of the ventricles of the heart, in response to stretching, and it seems to be a reliable indicator of stress [5]. BNP is relevant in diagnosis and treatment as well as in predicting prognosis [6]. Its physiologic effects antagonise those of angiotensin II and, since there is a strong relationship between plasma BNP concentration and impaired ventricular function [7], BNP assays are currently used to monitor cardiac decompensation.

The cellular mechanisms which control secretion of this peptide are as yet poorly understood, although it seems that changes in intracellular calcium concentration affect BNP gene expression in ventricular hypertrophy [8].

Comparing normal animals with a group of model rats in which ventricular hypertrophy had been induced by pressure overload, we measured BNP concentrations in both the blood, and in the culture supernatants of ventricular muscle cells co-cultured with and without calcium-channel blockers (nifedipine, verapamil and diltiazem) in order to enhance our understanding of the mechanisms which regulate BNP secretion, in both the normal physiologic situation and the pathogenesis of heart disease.

Materials and methods

The experiments were carried out on 2- to 3-month-old adult Wistar rats weighing 250 ± 50 grams. The rats were split into two groups of eight rats each: a control group (N); and another group (H), in which ventricular hypertrophy had been induced by pressure overload generated by clipping the abdominal aorta for 12 weeks [9]. The position of the clips in the group H rats was radiologically checked a week before sacrifice. The degree of hypertrophy was estimated using the equation: ([weight of the hypertrophied heart/weight of the control heart] × 100. Morphological changes were investigated in histological sections of heart tissue prepared from animals in both groups.

After sacrifice, all the hearts were recovered. Ventricular muscle cells were isolated by enzymatic digestion (the Langendorff method) and used to establish "rapid-attachment" primary cultures [10]. In brief, the rats were injected with heparin and anaesthetised with a combination of ketamine and xylazine. The heart was then quickly extracted by thoracotomy and transferred into a cold Tyrode solution. The aorta was cannulated and the heart mounted on a Langendorff column for successive perfusions at 37 °C of:

- Tyrode solution for 3 min;
- calcium-depleted Tyrode solution for 4 min;
- the same solution supplemented with 0.05% collagenase for 20 min (Worthington, type II), 0.06 mM CaCl₂ and 0.1% bovine serum albumin (BSA).

Once the ventricle had become flaccid, it was rinsed through with Kraft Brüh (KB) solution "power soup" for
2 min. The isolated cells were filtered and kept for 1 h in the KB solution.

For the primary cultures, isolated N and H cells were washed in M199 medium (Gibco, Life Technologies) supplemented with $10^{-7}$ mol/dm$^3$ insulin, 0.2% BSA and 1% antibiotic. These cells were then plated out (10$^4$ cells/ml) in 35-mm culture dishes that had been pre-treated with laminin (20 $\mu$g/ml, Sigma) and pre-incubated at 37 $^\circ$C for 30 min. All culture media were replaced 2 h later with the same medium supplemented with creatine (5 mM, Sigma), l-carnitine (2 mM, Sigma) and taurine (5 mM, Sigma); L-type calcium-channel blockers (nifedipine, verapamil and diltiazem) were added to some of the cultures to a final concentration of $2 \times 10^{-6}$ mol/dm$^3$ [11].

**BNP assays**

BNP concentrations in the blood and in the culture supernatants of ventricular muscle cells (from both normal and hypertrophied rats) were measured by ELISA (IBL, Hamburg).

**Solutions**

The parent nifedipine, diltiazem and verapamil solutions (Sigma) were dissolved in DMSO to make up 1 mM stock solutions.

**Histology**

Thin sections (5 $\mu$m) were prepared from the hearts and left ventricles of N and H rats, and stained with Haematoxylin-Eosin (HE) and Masson trichrome (MT) for light microscopy ($\times$ 10). The thickening of the myocardium was estimated on the HE-stained heart sections, and the presence of collagen was analysed on the MT-stained ventricle sections.

**Statistical analysis**

Repeated measurements were tested by analysis of variance using SPSS software. Intra-group and inter-group factors were analysed using the Mauchly test with Greenhouse-Geisser correction. A value of $p < 0.05$ was used for statistical significance.

**Results**

**Ventricular hypertrophy**

The degree of ventricular hypertrophy (as estimated using the equation given in the Materials and methods section) was $35 \pm 1\%$ ($n=8$, $p < 0.01$). Analysis of micrographs of HE-stained sections showed that hearts from the H group were hypertrophied compared with those from the N group (thick lines, Fig. 1B). Hypertrophy was accompanied by higher collagen levels (Fig. 1D) than seen in normal myocardial tissue (Fig. 1C), as shown by the green staining in the MT sections.

**BNP concentration**

The blood BNP concentration was significantly higher in the H group (0.630 $\pm$ 0.008 ng/ml versus 0.106 $\pm$ 0.004 ng/ml; $n=8$; $p < 0.01$) (Fig. 2). Similarly, a higher concentration was observed in the H culture supernatant compared with the N group (3.45 $\pm$ 0.7 ng/ml versus 0.53 $\pm$ 0.22 ng/ml; $n=8$) (Fig. 3). Co-culture of the ventricular muscle cells...
with calcium-channel blockers at a concentration of $2 \times 10^{-6}$ mol/dm$^3$ significantly reduced the concentration of BNP in the culture supernatant ($p < 0.05$) — by about 63% in the cultures of normal cells, and by 93% in the hypertrophied cells. The BNP concentrations in the N group were:

- nifedipine: $0.22 \pm 0.04$ ng/ml;
- verapamil: $0.19 \pm 0.05$ ng/ml;
- diltiazem: $0.17 \pm 0.03$ ng/ml) (Fig. 4).

In the H group, they were:

- nifedipine: $0.18 \pm 0.05$ ng/ml;
- verapamil: $0.23 \pm 0.04$ ng/ml;
- diltiazem: $0.28 \pm 0.1$ ng/ml ($p < 0.05$) (Fig. 5).

Discussion

BNP is a circulating hormone produced in the heart, mainly by ventricular cells in normal haemodynamic conditions (whereas ANP is mainly secreted by atrial cells). When haemodynamic stress induces hypertrophy, the BNP concentration in the blood rises as the ventricular cavity expands

[12]; in parallel, the concentration of ANP also rises [13]. In our pressure overload experimental hypertrophy model, the blood BNP concentration was significantly elevated, in line with published data. Moreover, BNP secretion was sustained in primary cultures of ventricular muscle cells isolated from both groups, and higher concentrations were observed with the H cells.

Nifedipine, verapamil and diltiazem are the best-characterised L-type blockers of calcium flux, and are in routine use in cardiovascular clinical practice. Blocking membrane calcium channels significantly reduced BNP concentrations in the culture supernatants of both normal and hypertrophied ventricular cells.

Calcium ions not only regulate heart contraction but also a whole series of other parameters such as gene transcription and cell metabolism. Two different types of voltage-dependent calcium channels are found on heart cells, leading to two different types of calcium flux with distinct biophysical and pharmacological properties, namely T flux (which is involved in cardiac automatism) and L
flux (which is involved in the coupling of stimulation and contraction) [14,15]. Heidrich et al. 2008 [16], studying neonatal and adult cardiomyocytes, showed that a calcium-dependent protein, chromogranin B (CGB), may modulate calcium release via action at the inositol 1,4,5-triphosphate receptor (InsP3R), thereby regulating BNP expression and secretion by these cells. They also showed that increased ventricular expression of CGB is associated with increased BNP secretion. We suggest that calcium-channel blocker-induced inhibition of the calcium ion influx could be partly responsible for variations in BNP secretion mediated by the CGB/InsP3R signal.

Similarly, Kudoh et al., 2003 [8], studying a muscle cell hypertrophy model based on mechanical stretching in vitro, showed that calcium-chelating agents, stretch-sensitive cation-channel blockers and/or calcium-channel blockers can modulate expression of the BNP gene as well as that of others (including ANP), via secondary, calcium-dependent mediators.

In the conditions of our experiment, blocking L-type calcium channels for 24 h seemed to reduce calcium ion influx into ventricular muscle cells thereby inhibiting the activity of calcium-dependent protein kinases like calcineurin and calmodulin II, and reducing synthesis and expression of the BNP gene and secretion of its product.

Thus, it is likely that reducing the calcium ion influx by blocking calcium channels inhibited BNP gene expression in both N and H ventricular cells, thereby inhibiting secretion of the peptide.

**Conclusion**

In the light of all the previously published data, our findings suggest that L-type calcium-channels are involved in the regulation of BNP secretion in both the normal physiological situation and ventricular hypertrophy. Further investigation of the effects of calcium on expression of the BNP gene (as well as those of other effectors in the calcium homeostasis system) is warranted.

**References**


