ABSTRACT: Natriuretic peptides seem to be a potent regulator of cell Ca\(^{2+}\) signalling in their action on the cardiovascular system. It was therefore the aim of this study to investigate the effect(s) of B-type natriuretic peptide (BNP) on the action potential and the L-type calcium current \((I_{CaL})\) in the rat left ventricular myocytes. Perforated and whole cell patch clamp technique was used to record action potential (AP) and \(I_{CaL}\) in current and voltage clamp mode, respectively. At the concentration tested of 10\(^{-7}\) M, BNP significantly increased the action potential duration at 50% and at 90% of repolarization by 16.85% and 16.39% respectively, and the phase II slope of the AP by 52.5%; reduced the \(I_{CaL}\) amplitude with a 16.17% decrease in the peak amplitude; reduced (16.51%) the inactivation time course of current decay; increased the \(V_{0.5}\) activation of the L-type calcium channel by 32.84% and decreased \(V_{0.5}\) inactivation by 34.39%. These data suggest that BNP modulates cardiomocyte function by reducing \(I_{CaL}\) and modifying the AP. This study may show a novel facet to evaluate the paracrine/autocrine effect of BNP on the normal heart function.

INTRODUCTION

The cardiac L-type calcium channel current \((I_{CaL})\) plays an important role in cardiac excitation-contraction coupling [1-2]. A number of neurohumoral agents including hormones and neurotransmitters modulate the \(I_{CaL}\) to control myocardial contractility in order to meet the body’s demands. One such hormone family are the natriuretic peptides (NPs). NPs consist of a group of genetically distinct peptides with a 17-amino acid disulfide ring structure that affect the cardiovascular and endocrine systems through their actions in diuresis, natriuretic, vasorelaxation and aldosterone and renin inhibition [3]. There are three members: atrial natriuretic peptide (ANP) also known atrial natriuretic factor (ANF), brain or B-type natriuretic peptide (BNP) and C-type (CNP).

In the heart, ANP expression is confined to the atria, BNP is expressed in both the atria and ventricles and the extent of CNP expression in the heart is unclear. ANP and BNP secretion is triggered by transmural pressure and myocyte stretch [4]. NPs bind two classes of cell surface receptors, the guanylyl cyclase-linked A and B receptors (NPR-A and NPR-B) and the C receptor (NPR-C) [5]. NP signalling is terminated either by binding to NPR-C which internalizes and degrades NPs thus removing them from the circulation or through cleavage by neutral endoperoxidase [6-7]. BNP seems to be a reliable indicator of cardiac stress [8]. BNP is relevant in diagnosis and treatment as well as in predicting prognosis [9]. Its physiologic effects antagonize those of angiotensin II and, since there is a strong relationship between plasma BNP concentration and impaired ventricular function [10], Recombinant BNP (nesiritide)
assays are currently used to monitor cardiac decompensation [11].

At the cellular level, natriuretic peptides act through cGMP-dependent mechanisms [12] and seem to modulate intracellular calcium concentrations in cardiac growth [13]; however, there is currently no direct and published evidence to show the effects of the bioactive fragment BNP-32 per se on the ionic channels conductance’s in heart muscle cells. Herein we specifically evaluate the autocrine effects of BNP-32 on the action potential (AP) and the density of I_{Ca,} on freshly isolated adult rat ventricular myocytes. We give evidence that BNP-32 affect the AP and decreases \( I_{Ca} \) of normal cardiomyocytes.

METHODS

Cell dissociation

Male Wistar rats weighing 250-300 g were sacrificed in accordance with the United Kingdom Home Office Guidelines on the operation of Animals (Schedule 1, Scientific Procedures Act 1986) and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Ventricular myocytes were enzymatically dissociated by retrograde perfusion of rat hearts. Briefly, animals were injected with heparin and anesthetized (i.p. ketamine hydrochloride 10 mg/kg; Troy laboratories PTY limited, Australia); the heart was quickly removed via thoracotomy and transversely cut. It was rinsed with KB (Kraft Brüh) solution for 2 min. The isolated cells were filtered when the heart was flaccid, it was rinsed with KB solution; 4 min with a nominally Ca2+-free Tyrode solution; 5 min with the Tyrode solution; 4 min with a nominally Ca2+-free Tyrode solution; and centrifuged at 250 rpm to remove KB solution. Brüh) solution for 2 min. The isolated cells were filtered when the heart was flaccid, it was rinsed with KB solution; 4 min with a nominally Ca2+-free Tyrode solution; 5 min with the Tyrode solution; 4 min with a nominally Ca2+-free Tyrode solution; and centrifuged at 250 rpm to remove KB solution.

Solutions and reagents

The composition of the Tyrode solution was (in mM): NaCl, 140; KCl, 5; CaCl2, 1.8; MgCl2, 1.8; HEPES, 10; MgSO4, 5; CaCl2, 0.08; EGTA, 0.5; creatine, 5; Na2ATP, 5; taurine, 20; HEPES, 10; MgCl2 8; D-Glucose, 10; pH 7.4 (with Tris). The KB solution contained (in mM): KCl 30, K2SO4 50, HEPES 10, MgCl2 8; D-Glucose, 11; MgSO4, 5; CaCl2, 0.08; EGTA, 0.5; creatine, 5; Na2ATP, 5; taurine, 20; HEPES, 10; D-Glucose, 10; pH 7.2 (with KOH).

BNP-32 was purchased from Sigma-Aldrich (Gillingham, Dorset, U.K). The amino acid sequence is Asn-Ser-Lys-Met-Ala-His-Ser-Ser-Ser-Cys-Phe-Gly-GLn-Lys-Ile-Asp-Arg-Ile-Gly-Ala-Val-Ser-Arg-Leu-Gly-Cys-Asp-Gly-Leu-Arg-Leu-Phe. BNP-32 was dissolved in distilled H2O to obtain a 1.0 mM stock solution, which was stored frozen in 10 µL aliquots and added to ECS at the required final concentration, 10−M.

I_{Ca} were recorded in Na+ and K+-free solution to suppress Na+ and K+ currents prior to and after superfusion with 10−M BNP-32. The external control solution (ECS) contained (in mM): N-methyl D-Glucamine (NMG), 130; CsCl, 5.4; CaCl2, 1.8; MgCl2, 1.8; D-Glucose, 11; HEPES, 5; Tetrodotoxin (TTX) 0.01; pH 7.4 adjusted with HCl. The pipette solution (PS) was (mM): TEACl, 20; CsCl, 130; MgATP, 5; GTPNa2, 0.3; EGTA, 10; HEPES, 5; adjusted to pH 7.20 with tris.

To record action potentials, pipettes tips were filled with an antibiotic (Amphotericin-B)-free solution containing (in mM): KCl 30, K2SO4 50, HEPES 10, MgCl2 8; D-Glucose, 11; MgSO4, 5; CaCl2, 0.08; EGTA, 0.5; creatine, 5; Na2ATP, 5; taurine, 20; HEPES, 10; D-Glucose, 10; pH 7.25 adjusted with KOH. It should be noted that chloride ions were partly substituted by sulphate to minimize junction potential [16]. The rest of the pipette was backfilled with the same solution containing Amphotericin-B (150 µM; Sigma).

Electrophysiological measurements

Voltage clamp experiments were performed at room temperature in the whole cell configuration of the patch clamp technique [17]. The electrical signal was recorded by a patch amplifier (Axopatch 200B, Axon instruments) interfaced to a personal computer using the pCLAMP software (version 9.2, Axon instruments) for data acquisition and analysis. Patch electrodes were fabricated with a two-stage patch pipette puller (model PP-830, Narishige Scientific Instrument, Narishige, Japan) to give tip resistances of 3-5 MΩ. The micropipettes were filled with the relevant intracellular solution as described above. Membrane capacitance (Cm) was estimated from the capacitance transient elicited by a 10 mV depolarizing step from a holding potential (HP) value of −90 mV and calculated according to the equation: 
\[
C_m = \frac{I_0 - I_s}{\Delta V_m}, \quad \text{where } I_0 \text{ is the time constant of the membrane capacitance, } I_s \text{ is the initial current value, } \Delta V_m \text{ the amplitude of voltage steps. No capacitance correction was used, } I_{Ca}. \quad \text{were elicited using a double pulse protocol to obtain current-to-voltage (I/V) curves and steady-state activation and inactivation curves.}
\]

From a holding potential of −60 mV, 500 ms depolarizing pulses to different membrane potentials (10 mV increments, i.e., the conditioning pulse) were followed by a 5 ms return to −60 mV, and then by a 500 ms test pulse to 0 mV at which the maximum I_{Ca} was obtained. Peak current amplitudes (normalized to cell capacitance) for I/V curves and steady-state activation curves were measured from the conditioning pulses. Steady-state inactivation curves were plotted from the normalized current recorded during test pulses. Data for activation and inactivation curves were fitted with a simple Boltzmann function: 
\[
I/V_{max} = \frac{1 + \exp[-(V_{m} - V_{1/2})/k]}{1 + \exp[V_{m} - V_{1/2}]/k]^{1}, \quad \text{where } I/V_{max} \text{ is the relative current, } V_{m} \text{ is the membrane potential, } V_{1/2} \text{ is the voltage of half-maximal activation or inactivation and } k \text{ is the slope factor. The time course of decay of calcium currents was analyzed by using a monoexponential or biexponential fit of current traces obtained at 0 mV. APs were recorded in current clamp mode and the myocytes were stimulated.}
with 2 ms suprathreshold current pulses at 0.5 Hz with a sampling rate of 25 kHz. The parameters recorded in ECS and after achieving steady state with ECS containing 10^{-7} M BNP-32 included: resting membrane potential (RMP), time taken to reach half the AP amplitude (APD_{50}) and slope of phase II of the AP, which was fitted using a linear function. For each cell, 4 APs before and after exposure to BNP-32 were averaged for data analysis.

Statistical analysis
Results were expressed as mean ± standard error (SEM). Statistical analysis was performed using analysis of variance, followed by the Newman-Keuls multiple range test for multiple comparison. Statistical significance was considered when \( p < 0.05 \). All statistical analyses were carried out using SPSS software (London, United Kingdom) and figures were prepared using Origin version 5.0 (Microcal Origin Software Inc, Northampton, MA, USA).

RESULTS

Effect of BNP on the action potential
As shown in figure 1, 10^{-7} M BNP prolonged the AP and significantly increased the phase II slope by 52.5% from \(-6.5 ± 0.6 \text{ mV/ms}\) to \(-10.0 ± 0.5 \text{ mV/ms}\); \( p < 0.001 \); (Table I). The mean APD_{50} and APD_{90} were increased by 16.85% and 16.39% respectively in the presence of BNP (from \(5.9 ± 0.3 \text{ ms}\) in control to \(6.6 ± 0.4 \text{ ms}\) for APD_{50} and from \(30.5 ± 1.2 \text{ ms}\) in control to \(35.5 ± 1.3 \text{ ms}\); \( p < 0.01 \)) (Table I). However, there was no change in RMP with a mean of \(-70.1 ± 0.4 \text{ mV}\) for control recording and \(-68.1 ± 1.5 \text{ mV}\) in the presence of BNP-32 (Table I). Based on these findings, we investigated the effects of BNP on ICaL next.

Effect of BNP on ICaL
We tested the effect of 10^{-7} M BNP on the ICaL using the whole cell patch clamp technique.

The mean (± SEM) membrane capacitance of the cells used in the recordings was \(120 ± 10 \text{ pF}\). Superimposed traces of ICaL current at 0 mV under control and in the presence of BNP is shown in figure 2A. We observed a decrease in the current amplitude upon the application of BNP over all potentials tested (Fig. 2B). The peak

| Table I: Comparison of Phase II Slopes, Time Taken to Reach Half the AP Amplitude (APD_{50}), and Resting Membrane Potential (RMP) in the Absence and Presence of 10^{-7} M BNP |
|-----------------|-----------------|-----------------|
| **Control**     | **+ BNP 10^{-7} M** |
| **Slope** (mV/ms) | \(-6.5 ± 0.6\) | \(-10.0 ± 0.5**\) |
| **APD_{50}** (ms) | \(5.9 ± 0.3\)  | \(6.6 ± 0.4*\)   |
| **APD_{90}** (ms) | \(30.5 ± 1.2\) | \(35.5 ± 1.3*\)  |
| **RMP** (mV)    | \(-70.1 ± 0.4\) | \(-68.1 ± 1.5\)  |

\({}^*p < 0.01\) denotes statistical significance. \(**p < 0.001\)
amplitude at 0 mV was significantly reduced by 16.17 % (control = –6.8 ± 0.50; +BNP-32 = –5.7 ± 0.36 pA/pF; \( p < 0.01 \)). The inactivation time course of the current decay was also significantly decreased by 16.51 % (\( p < 0.001 \); Table II) suggesting reduced Ca\(^{2+}\)-dependent inactivation in the presence of BNP. Thus, our findings show that BNP attenuates both the amplitude and time course of IC\(_{\text{aL}}\).

Effect of BNP on the voltage dependence of availability of IC\(_{\text{aL}}\).

Figure 3A shows example recordings of current generated with the conventional double-pulse protocol used to ascertain the voltage dependence of IC\(_{\text{aL}}\) activation and inactivation (see methods). We found that there was a significant shift in the activation curve to more positive potentials (Fig. 3B & Table II) with corresponding \( V_{0.5} \) values of -10.2 ± 0.3 mV for control recordings and -6.8 ± 0.6 mV for recordings in the presence of 10\(^{-7}\) M BNP (32.84% increase, \( p < 0.01 \)). The respective slopes of the curves in ECS and in the presence of BNP were 5.3 ± 0.2 and 5.5 ± 0.2 and were not significantly different. In addition, there was also a significant decrease in inactivation with \( V_{0.5} \) values of -18.9 ± 0.2 mV for control recordings and -34.4 ± 0.5 mV for recordings in the presence of BNP (34.39% decrease, Fig. 3B and Table II, \( p < 0.01 \)). The respective slope values were 6.4 ± 0.4 and 6.7 ± 0.3 and were not significantly different. These data demonstrate that BNP affects the voltage-dependent activation and inactivation of IC\(_{\text{aL}}\). Overall, there was a decrease in the window current in the presence of BNP (Fig. 3B).

DISCUSSION

In the present study we provide evidence that BNP modulated the AP, decreased the density L-type calcium current and the time course of the current decay. BNP also modified the channel’s sensitivity for activation and inactivation. We should note that we had high yields of rod shaped clearly striated cardiomyocytes (≥ 85%) isolated from the left ventricles where BNP is usually expressed and released in pathophysiological states [16]. Furthermore, the concentration of BNP tested in this study could be considered supra-physiological; however, our started experiments were undertaken to determine an optimal range of concentrations to test. Moreover, the level tested might indeed be supra-physiological in circulating plasma but similar to concentrations in local interstitia surround

**TABLE II**

VOLTAGE-DEPENDANT AVAILABILITY AND INACTIVATION TIME CONSTANTS OF IC\(_{\text{aL}}\) IN CONTROL CONDITIONS AND WITH 10\(^{-7}\) M BNP

Data for activation and inactivation curves were fitted with a simple Boltzmann function. Half-activation or inactivation values are given in millivolts. \( \tau_f \) & \( \tau_s \) = fast and slow component of inactivation, respectively, obtained by fitting current traces elicited by a depolarizing pulse to 0 mV from a holding potential value of -60 mV. Values are given as mean ± SEM, and number of experiments (n) is given in parenthesis.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>+ BNP 10(^{-7}) M (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACTIVATION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} )</td>
<td>-10.2 ± 0.3</td>
<td>-6.8 ± 0.6</td>
</tr>
<tr>
<td>( K )</td>
<td>5.3 ± 0.2</td>
<td>5.5 ± 0.2</td>
</tr>
<tr>
<td><strong>INACTIVATION</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{0.5} )</td>
<td>-18.9 ± 0.2</td>
<td>-34.4 ± 0.5*</td>
</tr>
<tr>
<td>( K )</td>
<td>6.4 ± 0.4</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td><strong>INACTIVATION DECAY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \tau_f )</td>
<td>10.1 ± 0.5</td>
<td>10.3 ± 0.3</td>
</tr>
<tr>
<td>( \tau_s )</td>
<td>87.8 ± 0.4</td>
<td>102.3 ± 2.0**</td>
</tr>
</tbody>
</table>

\( *p < 0.01 \) denotes statistical significance. \( **p < 0.001 \)
ing the ventricular myocyte. To the best of our knowledge, there is no information available on local concentrations of BNP released into the myocardial interstitium.

Based on our findings, we propose that BNP modulates Ca\textsuperscript{2+} handling by the left ventricular myocytes. By binding to its receptors (NPRA), BNP activates the particulate guanylyl cyclase system (pGC) causing the generation of cGMP [12, 17], which has been shown to inhibit L-type calcium channel via phosphorylation [18]. The ICaL serves three functions: first, the amplitude and time course of its decay determines the shape of the plateau of the action potential; second, it acts as a trigger to release Ca\textsuperscript{2+} from the sarcoplasmic reticulum; and third, it plays a role as a Ca\textsuperscript{2+} loading mechanism. Thus, the decreased activation of the Ca\textsuperscript{2+} channel by BNP may cause decreased influx of Ca\textsuperscript{2+}, attenuating the Ca\textsuperscript{2+}-induced-Ca\textsuperscript{2+} release mechanism causing the decreased release of Ca\textsuperscript{2+} via the Ryanodine-2 receptors [19]. This would result in decreased intracellular Ca\textsuperscript{2+} available for myofilament contractility suggesting a negative inotropic effect of BNP on left ventricular myocytes. We also found that the time course of the ICaL decay was significantly decreased suggesting reduced calcium-dependent inactivation in the presence of BNP. This could speculate the involvement of additional mechanisms not cGMP-dependent that need to be investigated.

The attenuation of the ICaL by BNP would explain the modification of the phase II slope of the AP. The prolongation of the APD\textsubscript{90} with the decrease of ICaL may appear paradoxical; however, previous studies have shown that Ca\textsuperscript{2+}-dependent inactivation of ICaL is a major determinant of AP duration [20-21]. As shown here, reduced Ca\textsuperscript{2+}-dependent inactivation of ICaL would therefore lead to more Ca\textsuperscript{2+} entry and hence a prolongation of the AP duration. Alternatively these changes may also be due to the involvement of K\textsuperscript{+} channels such as IK\textsubscript{1} seeing here by the prolongation of APD\textsubscript{90} but this was beyond the scope of the present study.

The decrease in the window current demonstrates the modulators role of BNP on the AP. The window current represents the amount of Ca\textsuperscript{2+} influx through the Ca\textsuperscript{2+} channels, which has been shown to be associated with the maintenance of the plateau phase of the AP and is linearly linked to the amplitude of the Ca\textsuperscript{2+} transient [19]. Therefore, the data in this study showing a decrease in the window current is consistent with the decrease in the amplitude of the ICaL. Together with the decrease in the Ca\textsuperscript{2+}-dependent inactivation of ICaL these data suggest that BNP modulates the duration and shape of the plateau phase (phase II) of the AP. Interestingly, previous studies have shown that the AP duration is prolonged in hypertrophy [22], a clinical investigation where BNP is usually elevated [4, 23-25].

The clinical implications of this study can be exposed as follow: It is generally considered that BNP expression is a beneficial compensatory mechanism via its vasodilating, natriuretic, diuretic and anti hypertrophic effects. Although, one of the pathophysiological hallmarks of heart failure is impaired Ca\textsuperscript{2+} homeostasis that results in contractile dysfunction, arrhythmogenesis [26] and the expression and release of BNP by the ventricular myocardium [23, 27]. The role of altered ICaL in disease states remains unclear [26]. In this work we showed that the application of BNP attenuated the ICaL. Thus, it is possible to project that in heart failure where the level of circulating BNP is markedly elevated there is a similar inhibition of the ICaL and consequently changes in the AP. Moreover, intravenous BNP (nesiritide) is used in patients with acute decompensated heart failure due to its vasodilatory effects [11]. However, recent meta-analyses of clinical trials evaluating the effects of recombinant BNP in the treatment of acute decompensated heart failure have shown increased mortality in patients [28-30]. It is possible that in a subset of patients, the infusion of BNP is causing harm rather than benefit by suppressing the expression and/or activity of SERCA2a [31-32] as well as other Ca\textsuperscript{2+} regulatory proteins and, altering the AP. This leads to important questions: is heart failure a state of BNP deficiency or BNP resistance and when does BNP contribute to the transition from beneficial hypertrophy to overt heart failure? And what is the role of BNP on Ca\textsuperscript{2+} signalling in this context?

In conclusion, we give evidence that BNP alters the AP and ICaL of heart muscle cells. This work can reveal a novel constitutive mechanism for the autocrine action of BNP on the electrophysiology of ventricular cardiomyocytes.

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