Cheng, H., Li, J., James, A. F., Inada, S., Choisy, S. C. M., Orchard, C. H., Zhang, H., Boyett, M. R., & Hancox, J. C. (2016). Characterization and influence of cardiac background sodium current in the atrioventricular node. *Journal of Molecular and Cellular Cardiology, 97*, 114-124. https://doi.org/10.1016/j.yjmcc.2016.04.014
Characterization and influence of cardiac background sodium current in the atioventricular node

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A R T I C L E   I N F O
Article history:
Received 8 December 2015
Received in revised form 1 April 2016
Accepted 25 April 2016
Available online 27 April 2016

Keywords:
Atioventricular node
AVN
Background current
IB Na
Pacemaking

A B S T R A C T
Background inward sodium current (IB Na) that influences cardiac pacemaking has been comparatively under-investigated. The aim of this study was to determine for the first time the properties and role of IB Na in cells from the heart’s secondary pacemaker, the atioventricular node (AVN). Myocytes were isolated from the AVN of adult male rabbits and mice using mechanical and enzymatic dispersion. Background current was measured using whole-cell patch clamp and monovalent ion substitution with major voltage- and time-dependent conductances inhibited. In the absence of a selective pharmacological inhibitor of IB Na, computer modelling was used to assess the physiological contribution of IB Na. Net background current during voltage ramps was linear, reversing close to 0 mV. Switching between Tris- and Na+-containing extracellular solution in rabbit and mouse AVN cells revealed an inward IB Na, with an increase in slope conductance in rabbit cells at ~ 50 mV from 0.54 ± 0.03 to 0.91 ± 0.05 nS (mean ± SEM; n = 61 cells). IB Na magnitude varied in proportion to [Na+] o. Other monovalent cations could substitute for Na+ (Rb+ > K+ > Cs+ > Na+ > Li+). The single-channel conductance with Na+ as charge carrier estimated from noise-analysis was 3.2 ± 1.2 pS (n = 6). Ni2+ (10 mM), Gd3+ (100 μM), ruthenium red (100 μM), or amiloride (1 mM) produced modest reductions in IB Na. Flufenamic acid was without significant effect, whilst La3+ (100 μM) or extracellular acidosis (pH 6.3) inhibited the current by > 60%. Under the conditions of our AVN cell simulations, removal of IB Na arrested spontaneous activity and in a simulated 1D-strand, reduced conduction velocity by ~ 20%. IB Na is carried by distinct low conductance monovalent non-selective cation channels and can influence AVN spontaneous activity and conduction.

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1. Introduction

The atioventricular node (AVN) is normally the only site through which electrical activity can pass from atria to ventricles; slow conduction through the AVN facilitates completion of atrial contraction prior to that of the ventricles [1–3]. The filtering properties of the AVN can also serve a protective function during some supraventricular tachyarrhythmias [2,3]. The AVN possesses pacemaking properties and should the sinoatrial node (SAN) fail or normal conduction become impaired, the AVN can take over pacemaking of the ventricles [2,3]. In the heart’s primary pacemaker, the sinoatrial node, the cellular basis of pacemaking is established to involve both calcium and membrane ‘clocks’, with spontaneous rate influenced by cellular Ca2+ dynamics and by multiple sarcoplasmic reticulum Ca2+ release mechanisms [4,5]. In contrast, the cellular electrophysiological basis of AVN pacemaking is incompletely understood, though it is clear that this is also likely to involve multiple ionic conductances [6–8]. For example, in the rabbit intact AVN inhibition of the hyperpolarization activated pacemaker current ‘If’ slows but does not stop AVN junctional rhythm [9,10]; this is consistent with an important though not obligatory role for If in AVN pacemaking. There is also evidence from both rabbit and dog preparations that intracellular Ca2+ cycling influences AVN pacemaking rate [11–14], whilst Cav1.3 and 3.1 have been implicated in mouse AVN pacemaking [8].

The potential importance of a background inward conductance in SAN pacemaking has long been recognised and such a conductance was incorporated even in early models of SAN pacemaking (e.g. [15–17]). In 1990, Denyer and Brown provided strong, though indirect, evidence for a role for a background inward current in rabbit isolated SAN cell pacemaking during If inhibition with Cs+ [18]. A subsequent study by Hagiwara and colleagues provided direct evidence for a Na+-dependent background inward current (IB Na) in SAN cells from the same species [19]. Much less is known in this regard for the AVN. Strong, albeit indirect,
evidence that AVN cells possess a marked 'resting' permeability to one or more types of inwardly moving cation comes from the fact that voltage-clamped small AVN tissue preparations and AVN cells exhibit a 'zero current' potential of ~ −40 mV (e.g. [6,20–22]), which is some distance from the K⁺ equilibrium potential. Spontaneously active AVN cells arrested by Ca²⁺ channel blockade also exhibit a 'resting' potential of near ~ −40 mV [23]. However, no experimental data are available that address directly the nature of background inward current in the AVN. Moreover, uncertainties remain regarding the underlying basis for cardiac background inward current, with suggestions including that it might result from 'leak' forms of activity of the Na-K pump or Na-Ca exchange [24,25]. Consequently, the present study was undertaken to determine whether or not AVN cells possess a cation current analogous to SAN IB,Na and, if present, forms of activity of the Na-K pump or Na-Ca exchange [24,25].

2. Methods

2.1. AVN cell isolation

Male New Zealand White rabbits (2–3 kg) were killed humanely in accordance with UK Home Office legislation. AVN cells were isolated by enzymatic and mechanical dispersion as described previously [21,26]. The AVN region within the Triangle of Koch was identified in relation to anatomical landmarks and removed for cell dispersion [21,26]. AVN cells from male C57BL/6 mice (19–31 g) were isolated using a similar method, which is described in detail in [27]. Murine AVN cells were used to determine the presence of IB,Na in AVN cells from an additional species to rabbit (Fig. 2). All experiments shown in other figures were performed on rabbit AVN cells. Cells were stored in refrigerated (4 °C) Kraftbrühe ‘KR’ solution [28] until use.

2.2. Electrophysiological recording

Cells were placed in an experimental chamber mounted on the stage of an inverted microscope (Eclipse TE2000-U, Nikon, Japan) and superfused with a Tyrode’s solution containing (in mM) NaCl 136.9, KCl 5.4, NaH2PO4 0.33, CaCl2 1.8, MgCl2 0.5, HEPES 5 and Glucose 5 (pH 7.4 with NaOH). Whole-cell patch-clamp recordings were made using an Axopatch-1D amplifier (Axon Instruments, USA). Patch-pipettes (A-M Systems, USA) were pulled and heat-polished to a final resistance of 2–3 MΩ (Narishige PP-83 and Narishige MF-83, Japan). Protocols were generated and data recorded on-line with pClamp 10.0 software (Axon instruments, USA) via an analog-to-digital converter Digidata 1322 (Axon Instruments/Molecular Devices, USA). Membrane currents were recorded in whole cell voltage-clamp mode, with a digitization frequency of 10 kHz. For cation current recordings, the currents were recorded in whole cell voltage-clamp mode, with a Digidata 1322 (Axon Instruments/Molecular Devices, USA). Membrane resistance of 2

2.3. Estimation of single channel conductance through “noise analysis”

Single channel currents were estimated from the variance of the Na⁺-dependent background current, calculated from the integral of the power spectral density. The single channel conductance was calculated as the slope of the single channel current-voltage relation between −110 and −80 mV, where the current-voltage relation approached the asymptote predicted by the Goldman-Hodgkin-Katz (GKH) flux equation (see below). Further details are provided in the online supplementary information and have been described elsewhere [30].

2.4. Data analysis

Whole cell current analysis was performed using Clampfit from the pClamp 10.0 software suite. Statistical analysis was performed using Microsoft Office Excel (Microsoft Corporation), Origin (OriginLab Corporation) and Prism (Graphpad Software, Inc.). Graphs were drawn using Graphpad Prism or Igor Pro (Wavements Inc.). All data are expressed as mean ± SEM.

2.5. Computer modelling of AVN activity

The most biophysically detailed available cell and tissue models of the AVN are those for rabbit AVN by Inada et al [7]. Only the ‘N’ cell model exhibits automaticity [7] and this was therefore used to investigate the influence of IB,Na on rabbit AVN cell spontaneous and driven action potentials. The Goldman-Hodgkin-Katz (GKH) flux equation was chosen to simulate IB,Na:

\[
I_{\text{IB,Na}} = P_{\text{Na}}V_m \left( \frac{R^2}{F^2} \right) \left( e^{-\frac{[\text{Na}^+]_o}{R} - \frac{[\text{Na}^+]_i}{R}} \exp\left( \frac{-V_m F}{RT} \right) \right)
\]

where \(P_{\text{Na}}\) is the Na⁺ permeability, \(V_m\) is the membrane potential, \(F\) is Faraday’s constant, \(R\) is the gas constant, \(T\) is the absolute temperature, and \([\text{Na}^+]_o\) and \([\text{Na}^+]_i\) are the intracellular and extracellular Na⁺ concentrations. \(P_{\text{Na}}\) was determined by fitting \(I_{\text{IB,Na}}\) from Fig. 1Biv by the GKH flux equation \((P_{\text{Na}} = 7.308 \times 10^{-1} \text{ L/s; cell capacitance, } C_m = 29 \text{ pf})\). To eliminate \(I_{\text{IB,Na}}\) from the AV node, \(I_{\text{IB,Na}}\) was calculated as above (but for physiological \([\text{Na}^+]_o\) and \([\text{Na}^+]_i\) was subtracted from the N cell model. The conduction velocity was determined using a 1D string model. The string model consisted of 100 elements (myocytes). The length of each element was 100 μm. Conduction was calculated using the reaction-diffusion equation:

\[
\frac{\partial V_m}{\partial t} = \nabla \cdot (D \nabla V_m) - I_{\text{stim}} + I_{\text{stim}}
\]
where $D$ is the diffusion coefficient, $I_{ion}$ is the ionic current and $I_{stim}$ is the stimulation current. $D$ was taken to be 0.003 mSmm$^2$ (equivalent to a coupling conductance of 0.3 mS). The stimulus was applied at the first three elements. The conduction velocity was determined as the average conduction velocity calculated from the 30th element to the 70th element.

Fig. 1. Background currents elicited by voltage step (Ai–Aiv) and descending voltage ramp (Bi–Biv) protocols in rabbit AVN cells. Ai: Representative families of currents in Tris Na$^+$-free and 150 mM-Na$^+$ solutions. For clarity of display, only selected current traces are shown (protocol is shown underneath). Aii: Representative Na$^+$-dependent inward background currents obtained by subtracting the currents in Tris Na$^+$-free from those in 150 mM-Na$^+$ solution (see Ai). Aiii: Mean current-voltage relations for currents (end pulse) in Tris Na$^+$-free and 150 mM-Na$^+$ solutions (mean ± SEM, $n = 8$ cells). Aiv: Mean current-voltage relation for the subtracted Na$^+$-dependent inward background current, $I_{b,Na}$ (mean ± SEM, $n = 8$ cells). Bi: Representative currents respectively in Tris Na$^+$-free and 150 mmol/L-Na$^+$ solutions (protocol is shown underneath). Bii: Representative Na$^+$-dependent inward background current obtained by subtracting the current in Tris Na$^+$-free from that in 150 mM-Na$^+$ solution (see Bi); grey line denotes a fit to the data with a Goldman-Hodgkin-Katz (GHK) current equation for diffusion of permeant ions. Biii: Mean current-voltage relations for currents in Tris Na$^+$-free and 150 mM-Na$^+$ solutions (mean ± SEM (dotted lines), $n = 61$ cells). Biv: Mean current-voltage relation for the subtracted Na$^+$-dependent inward background current, $I_{b,Na}$ (mean ± SEM, $n = 61$ cells).
3. Results

3.1. Background current during voltage steps and ramps

Net background current and Na–Tris difference current were studied using voltage step and ramp protocols (lower panels in Fig. 1Ai and Bi). In the presence of 150 mM extracellular Na+, voltage steps to potentials between −120 and +50 mV (in 10 mV increments, pulse frequency 0.2 Hz) elicited currents that showed little time-dependence during the applied voltage command. Holding current at −40 mV was inward under these conditions (Fig. 1Ai panel b). When the superfusate was Tris-free, both outward and inward current components were smaller (Fig. 1Ai panel a) and the holding current became markedly less inward. Representative Na+-Tris difference currents are shown in Fig. 1Aii and were time-independent and inwardly directed over the full range of membrane potentials tested. Mean current-voltage (I–V) relations for net current in Na+- and Tris-containing solutions are shown in Fig. 1Aiii, whilst the mean I–V relation for Na+-sensitive (Na+-Tris difference) current is shown in Fig. 1Aiv, and was inwardly directed across the entire range of test potentials. The time-independence of the currents observed during voltage steps enables the use of a voltage-ramp protocol to survey background current rapidly across a wide range of potentials. Thus, we also examined currents elicited by a descending ramp protocol (between +40 and −100 mV over 150 ms; frequency 0.2 Hz). Representative currents in Na+-containing and Tris-containing solutions are shown in Fig. 1Bi, with the corresponding Na+-Tris difference current shown in Fig. 1Bii. The net current in Na+-containing solution was linear, reversing close to 0 mV (Fig. 1Bi), whilst the Na+-dependent (Na+-Tris difference) current was inwardly directed across the entire potential range of the voltage ramp. Mean I–V relations for net current in Na+ and Tris-containing solutions are shown in Fig. 1Biii, whilst mean Na+-sensitive difference current is shown in Fig. 1Biv. The mean I–V relation for Na+-sensitive difference current during voltage-ramps was similar to that for currents elicited by voltage steps (compare Fig. 1Aiv and Biv); consequently the voltage ramp protocol was employed for most subsequent experiments. The presence of a Na+-sensitive inward background current was not exclusive to rabbit AVN, as we also recorded a similar current from murine AVN cells (Fig. 2). Fig. 2A shows representative mouse AVN cell currents in Na- and Tris-containing solutions, elicited by the same voltage ramp protocol as used to record rabbit AVN cell IB_Na. Fig. 2B shows the Na+-Tris difference current, representing IB_Na, whilst Fig. 2C shows mean murine AVN cell IB_Na from 6 experiments. Fig. 2D shows the mean current-voltage (I–V) relation for IB_Na from murine AVN cells, with the mean current from rabbit cells superimposed in red. The I–V relations for IB_Na for the two species were similar, indicating both that the current is not restricted to rabbit AVN and that it was remarkably similar in amplitude in mouse and rabbit AVN cells.
3.2. Na⁺ dependence and effects of ionic substitution

The effects of altering [Na⁺]₀ between 0 and 200 mM on net current magnitude and profile are shown in Fig. 3Ai: as [Na⁺]₀ was progressively reduced from 150 mM, the net inward current component became smaller and the current reversed at progressively more negative voltages. Fig. 3Aii shows (for the same cell as Fig. 3Ai) Na⁺-sensitive difference currents in 30, 75, 150 and 200 mM [Na⁺]₀. Fig. 3B shows the concentration-dependence of the [Na⁺]₀-dependent current at two selected voltages (−50 and −100 mV), showing a linear dependence of current density on log [Na⁺]₀, whilst Fig. 3C shows the [Na⁺]₀-dependence of the slope conductance of the Na⁺-sensitive current. The linear dependence of current magnitude on log [Na⁺]₀ is similar to that reported for SAN IB,Na [19]. The mean slope conductance at −50 mV in Tris-containing [0 Na⁺] solution was 0.54 ± 0.03 nS (n = 61; compared to 0.45 ± 0.18 nS previously reported for SAN cells under similar conditions [19]) whilst in 150 mM mean slope conductance increased to 0.91 ± 0.05 nS (compared to 0.87 ± 0.33 nS for SAN cells [19]). Considered collectively, the data in Fig. 3 demonstrate a strong dependence of current magnitude on [Na⁺]₀ and throughout the rest of this report this current component is denoted IB,Na.

Fig. 4 shows the effects of monovalent cation substitution on the profile and magnitude of the background current. Fig. 4Ai shows records from a single cell in solutions containing 150 mM of Tris, Li⁺, Na⁺, Cs⁺, K⁺ and Rb⁺, whilst Fig. 4Aii shows Tris-difference currents for each metal cation. Fig. 4B shows mean current density plots for current at −50 and −100 mV for IB,Na and the equivalent current with the other cations. The current was monovalent non-selective, with its amplitude largest in Rb⁺ and smallest in Li⁺ (Rb⁺ > K⁺ > Cs⁺ > Na⁺ > Li⁺). The estimated relative slope conductance ratios at −50 mV for these ions compared with Na⁺ were, respectively, 5.96, 3.00, 2.33, 1.00 and 0.58. For the SAN, Hagiwara et al used the relative slope conductance ratio for K⁺ compared to Na⁺ to estimate the reversal potential (E_CV) for the net monovalent NSCC, assuming physiological [Na⁺]₀ and [K⁺]₀ concentrations; this yielded a value of around −21 mV [19]. Using a P_C/P_K ratio of 3.0 from the present study, together with [Na⁺]₀ of 140 mmol/L, [Na⁺]₀ of 8 mM and [K⁺]₀ of 5.4 mM, [K⁺]₀ of 140 mM, we obtained an E_CV of −26.9 mV, which is close to the value estimated by Hagiwara et al for SAN cells [19]. As an additional check, we used the above ion concentrations together with a P_C/P_K value for SAN cells of 2.27 from [19], and closely matched the previously estimated E_CV for SAN cells, with a derived value of −20.3 mV.

3.3. Sensitivity of IB,Na to pharmacological inhibition

Taken together, the data in Figs. 1–4 suggest that IB,Na is the [Na⁺]₀-sensitive component of a background monovalent non-selective cation channel (NSCC) current. Gd³⁺ ions block a number of NSCCs [32] and consequently we tested the effects of 100 μM Gd³⁺ on IB,Na. Fig. 5Ai and Aii show mean currents in Na⁺-containing and Tris-containing solution, whilst Fig. 5Bi and Bii show comparable data for the same sample of cells, when the superfusate contained 100 μM Gd³⁺. As shown in Fig. 5Aii and Bii, Gd³⁺ led to a reduction in IB,Na amplitude across the tested range. In 9 cells, at −100 mV IB,Na amplitude was decreased by 49.1 ± 4.3% by this concentration of Gd³⁺ (Fig. 5C). In a further 9 cells, 1 mM La³⁺ inhibited IB,Na by 52.4 ± 9.2%. A second lanthanide, lanthanum (La³⁺) also inhibited IB,Na, with 100 μM La³⁺ blocking the current by 68.8 ± 5.5% (n = 8; Fig. 5C). 1 mM La³⁺ inhibited IB,Na by 71.6 ± 3.9% (n = 8). Ruthenium red (100 μM), which inhibits multiple cation channels [32,33], inhibited IB,Na by 50.9 ± 6.5% (n = 6; Fig. 5C). By contrast, increasing the [Ni²⁺] in the superfusate from 2 to 10 mM (a concentration sufficient to inhibit maximally cardiac Na–Ca exchange [34]) reduced IB,Na by only ~20% (Fig. 5C). Amiloride has been suggested to inhibit partially IB,Na [19] in the SAN and we found it to inhibit AVN IB,Na by −40% (Fig. 5C). Flufenamic acid (FFA) has been shown to inhibit TRMP4-related NSCCs in SAN cells [35]; however it was without significant

![Fig. 3](image-url)
In principle, the difference in power spectra of the current “noise” between Na⁺-containing and Tris-containing (Na⁺-free) external solutions can be used to estimate single channel conductance, because the whole-cell current variance is a function of the current amplitudes through single open channels, and consequently the power spectra at any voltage provide a measure of the unitary current amplitude at that voltage [36]. We used currents generated by voltage step commands between −110 and +20 mV to obtain the Na⁺-dependent (Na⁺-Tris difference) current, deriving from their current-voltage relation the asymptote shown in Fig. 6A. Over the voltage range at which the asymptote was achieved (−110 to −80 mV inclusive), the DC component of current in both Na⁺-containing and Tris-containing solutions was removed (Fig. 6Bi and Bii), and the power spectra calculated. The power spectrum of the Na⁺-dependent current, calculated as the difference between the power spectrum in Na⁺-containing and Na⁺-free solutions, was fitted with equation S1 (Fig. 6C). The power spectra at each voltage were integrated to obtain the variance, from which the unitary current amplitudes were estimated (Fig. 6D). The slope conductance of the mean unitary current voltage–relation was 3.2 ± 1.2 pS.

3.5. Investigating the potential physiological role of \( I_{\text{IB,Na}} \)

None of the agents tested in the experiments described in Fig. 5 produced complete inhibition of \( I_{\text{IB,Na}} \), nor would they be expected to be \( I_{\text{IB,Na}} \)-selective under action potential (AP) recording conditions. Therefore we reasoned that, in the absence of a specific blocker, the potential role of \( I_{\text{IB,Na}} \) in electrical activity of the AVN may best be investigated using computer modelling. The “N” cell model from the Inada et al. AVN electrophysiology model, which exhibits spontaneous activity in the absence of external stimulation [7] was therefore chosen to study the influence of \( I_{\text{IB,Na}} \). Fig. 7A shows spontaneous APs produced by this cellular model. It contains background current (which can be interpreted as the sum of all background currents) and the effect of block of \( I_{\text{IB,Na}} \) was simulated by subtracting \( I_{\text{IB,Na}} \) calculated using the GHK equation (Eq. (1)) fitted to experimental data (Fig. 1Biv). After block of \( I_{\text{IB,Na}} \), pacemaking ceased, because (consistent with the block of an inward current) during the pacemaker potential the membrane potential now failed to reach the threshold potential, attaining quiescence at a value of −53 mV. Experimental data indicate that AVN cells exhibit “zero current” potentials of −40 mV (e.g. [6, 20–22]) and additional simulations were performed (online Supplement Fig. S1) in which L-type Ca current was abolished to induce quiescence in the presence of \( I_{\text{IB,Na}} \). This intervention induced quiescence at −40 mV; thus, the effect of \( I_{\text{IB,Na}} \) removal in Fig. 7A was to produce a hyperpolarization of ‘resting’ potential. Under normal conditions with SAN dominance, the AVN is driven and does not show pacemaking. Fig. 7B shows the effect of block of \( I_{\text{IB,Na}} \) on the driven AP. The AP shape and duration were not affected, but the resting membrane was hyperpolarized (again consistent with the block of an inward current; Fig. 7B).

Hyperpolarization of the resting membrane may affect excitability and, therefore, conduction velocity; this was examined using a 1D string model (see Methods). The conduction velocity obtained under control conditions (\( I_{\text{IB,Na}} \) present; 16.7 cm s\(^{-1}\)) is typical of the rabbit AVN [7]. Block of \( I_{\text{IB,Na}} \) decreased the conduction velocity by −20% (to 13.3 cm s\(^{-1}\)). Fig. 7C shows that experimental data for \( I_{\text{IB,Na}} \) were well fitted by Eq. (1). Additionally, the predicted I–V relation for \( I_{\text{IB,Na}} \) under ‘physiological’ conditions (\([\text{Na}^+]_o\) set to 140 mM; \([\text{Na}^+]_i\) set to 8 mM) was inward across the entire range of physiologically relevant membrane potentials (Fig. 7C).

4. Discussion

This study provides the first evidence for the presence and role of \( I_{\text{IB,Na}} \) in the AVN and, to our knowledge, the first experimental estimate...
for the single channel conductance of the channels that underlie \( I_{B,Na} \) for any cardiac cell type.

### 4.1. On the nature of \( I_{B,Na} \)

The current-voltage relation for net background current under bivalent conditions (Na\(^+\) outside/Cs\(^+\) inside) in this study was linear, reversing close to 0 mV, consistent with a dominant identity of total background current under our recording conditions as a NSCC. The estimated \( E_{rev} \) for this monovalent NSCC, with physiological Na\(^+\) and K\(^+\) values, of \(-26.9\) mV indicates that, as previously suggested for the SAN [19], it would carry inward current over the diastolic potential range in AVN cells. \( I_{B,Na} \) was measured as the external Na\(^+\)-sensitive component of this NSCC, under the same conditions as used previously to study an analogous conductance in SAN cells [19]. Our results indicate that \( I_{B,Na} \) is both present in the AVN and also of similar magnitude to that reported for the SAN [19]. The strong similarity between \( I_{B,Na} \) in rabbit and murine AVN cells seen here suggests conservation of the current

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**Fig. 5.** Effects of gadolinium (Gd\(^{3+}\)), lanthanum (La\(^{3+}\)), ruthenium red, nickel (Ni\(^{2+}\)), amiloride, flufenamic acid (FFA) and acidic pH on Na\(^+\)-dependent inward background current (\( I_{B,Na} \)). Ai and Aii: in control condition, mean I–V relations for currents in Tris Na\(^+\)-free and 150 mM-Na\(^+\) solutions (Ai), and mean \( I_{B,Na} \) (Aii) (mean ± SEM (dotted lines), \( n = 9 \)). Bi and Bii: with application of 100 \( \mu \)M Gd\(^{3+}\), mean I–V relations for currents in Tris Na\(^+\)-free and 150 mM-Na\(^+\) solutions (Bi), and mean \( I_{B,Na} \) (Bii) (mean ± SEM (dotted lines), \( n = 9 \)).

C: A summary of the effects of 100 \( \mu \)M Gd\(^{3+}\), 100 \( \mu \)M La\(^{3+}\), 100 \( \mu \)M ruthenium red, 10 mM Ni\(^{2+}\), 1 mM amiloride, 100 \( \mu \)M flufenamic acid (FFA), and acidosis of pH 6.3 on \( I_{B,Na} \) at \(-100\) mV. *\( p < 0.05 \), **\( p < 0.01 \); the numbers of cells for each experiment are given in parentheses.
in cells from this region across species. There is prior evidence for the presence of an I_{b,Na} in non-pacemaker cells, but one that it is of substantially smaller magnitude [19,37].

The GHK voltage dependence and Eisenmann III permeability sequence for I_{b,Na} distinguish this current from voltage-dependent ‘persistent’ or ‘late’ Na current [38]. Additionally, it has been suggested that minor transport modes of Na–Ca exchange might account for cardiac background inward current [25,39] and AVN cells exhibit a robust Na–Ca exchange current [26,40], the persistence in our experiments of I_{b,Na} in the presence of a maximally effective Na–Ca exchange blocker [19,37]. Similarly, the presence of I_{b,Na} in the absence of external Ca^{2+}, together with its low single channel conductance and insensitivity to FFA distinguish this current from the TRPM4-mediated FFA-sensitive, Ca-activated NSCC observed for SAN cells [35]. Furthermore, despite some sensitivity to lanthanides and other manoeuvres that inhibit NSCCs, the permeability sequence and low single channel conductance for I_{b,Na} seem difficult to reconcile with properties of other members of the transient receptor potential (TRP) family of NSCCs [32]. For example, whilst TRPV4 has an Eisenmann IV permeability sequence for monovalent cations, close to the sequence for I_{b,Na}, and is also sensitive to ruthenium red [41], its single channel conductance at negative voltages lies between 30 and 60 pS [42], 10-fold or more our estimate for channels mediating I_{b,Na}. On the other hand, the single channel conductance estimated here is close to that for the epithelial Na channel (ENaC; 4–5 pS), but ENaC has a higher sensitivity to inhibition by amiloride and in contrast with I_{b,Na} has a high Na/K relative permeability [43]. Low conductance NSCC behaviour has been induced in the Na/K pump by exposure to the marine toxin, palytoxin (PTX), with a PTX induced single channel conductance of ~7 pS [24]. The P_{Ca}/P_{Na}, P_{Ca}/P_{Na}, and P_{Ro}/P_{Na} ratios for this toxin-induced NSCC were reported to be 1.13, 1.01 and 1.11 [24], unlike the relative conductance ratios for AVN I_{b,Na}, respectively of 2.33, 3.00 and 5.96. Although the molecular architecture of a number of ion channel transcripts in the AVN has been mapped, at present this information does not extend to NSCC candidates [44,45].

4.2. Physiological role of I_{b,Na}

The lack of an identified molecular correlate for channels carrying I_{b,Na} precludes elucidation of its physiological role(s) through genetic modification and no selective pharmacological inhibitor of the current has yet been discovered. Additionally, Na substitution cannot be used under physiological recording conditions to discriminate I_{b,Na} from other conductances as this intervention would also affect I_{f} and Na–Ca exchange current. Computational modelling thus affords the only available means of assessing the physiological contribution of I_{b,Na}. One study has suggested that I_{f} and I_{b,Na} may play ‘reciprocal’ roles in pacemaking of SAN cells, in which membrane hyperpolarisation following a reduction in either current leads to augmentation of the other, thereby stabilising pacemaker rate [46]. In another modelling study
membrane potential failed to reach the threshold for AP initiation, leading to an arrest of spontaneous activity. When APs were triggered, removal of ICa,L did not alter AP shape, but hyperpolarized membrane potential, associated with a decreased excitability manifested as a (20%) slowed conduction velocity. In the absence of ICa,L and IKr reduction this reduction was increased to ~53% (see Supplemental Fig. S2). These results are consistent with partial ICa,L and IKr reduction being able to contribute to the (patho)physiological modulation of AVN cell spontaneous rate.

The smaller amplitude of ICa,L in non-pacemaker cell types [19,37] together with the concomitant presence of current generated by channels of inward rectifying K⁺ current, IK1, likely limits the impact of this current on electrogenesis in those cells, though it is possible that the current may still influence Na homeostasis [52].

4.3. Limitations

Although to our knowledge this is the first study to provide an estimate of single channel conductance for channels mediating cardiac ICa,L, "noise analysis" is an indirect rather than direct method of observing single channel activity. Its application in the present study is predicated on the assumption that Na⁺ removal affected only background current. This is a reasonable assumption given that the experimental solutions utilized in this study (and the earlier report of SAN ICa,L [19]) were designed to inhibit major overlapping ion channel and transporter currents. For example, activation of cardiac Na-dependent K⁺ channels requires ~20 mM intracellular [Na⁺] [53,54] and, our pipette solution was both largely Cs⁺-based and Na⁺-free, which precludes IK1 current activation in our experiments. Additionally, the presence of strophantidin and nickel in the external solution makes significant contamination by Na-K-ATPase or Na/Ca exchange currents unlikely; moreover, the properties of the power spectrum obtained indicate that the currents were predominantly produced by channels showing gating behaviour, ruling out transporter-currents. Thus, it is reasonable to conclude that the channels identified through power-spectral analysis of Na⁺-Tris difference currents are distinct. Estimation of single channel conductance through this method required measurements in the voltage range –80 to –110 mV, rather than at diastolic potentials, in order to obtain currents of adequate size for power spectral analysis. This range of voltages was chosen as it avoided the underestimate of conductance through the effects of GHK rectification (i.e. the current voltage relation of the Na-dependent current achieved the asymptote in this voltage range). Future work to obtain direct measurements of single ICa,L channels would provide valuable independent validation of the single channel conductance estimate obtained from our analysis. However, the low single channel conductance may make such measurements somewhat challenging to make.

The AVN is electrically and structurally heterogeneous [2,55]. Whilst isolated AVN cell populations are also heterogeneous [31,56,57], it is not possible to attribute a precise origin from within the AVN to cells studied; thus the present study does not address directly issues of potential regional differences in the distribution within the AVN of ICa,L. The fact that the underlying genetic basis for the channel (in any cardiac region) remains to be determined also precludes mapping ICa,L Channel transcript or protein levels within AVN sub-regions. In principle, this

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**Fig. 7.** Predicted role of ICa,L in the AV node action potential. (A) predicted role of ICa,L in AV node pacemaking. The traces show electrical activity calculated using the N cell model from Inada et al. [7] before and after the elimination of ICa,L from the N cell model. In the presence of ICa,L, the model shows robust pacemaking, but after elimination of ICa,L pacemaking is abolished. (B) Predicted role of ICa,L in the driven AV node action potential. The 10th action potential during 2.5 Hz stimulation is shown. Action potentials before and after the elimination of ICa,L are shown. After the elimination of ICa,L, the resting membrane is hyperpolarized. (C) Current-voltage relationships for ICa,L. Solid black line, experimental ICa,L from Fig. 1Biv. Solid grey line, GHK equation under physiological conditions (for all simulations [Na⁺]o = 150 mM [Na⁺]i = 5.4 mM). As shown in panel C (dashed line), the GHK simulated current under physiological conditions was slightly smaller than that recorded experimentally (with 150 mM [Na⁺]o and 0 [Na⁺]i). It is the smaller current under physiological conditions that was incorporated into action potential simulations.
Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmcc.2016.04.014.

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