Inhibition of spontaneous activity of rabbit atrioventricular node cells by KB-R7943 and inhibitors of sarcoplasmic reticulum Ca2+ ATPase

Hongwei Cheng, Godfrey L. Smith, Jules C. Hancox, Clive H. Orchard

1. Introduction

The cardiac atrioventricular node (AVN) conducts electrical excitation from the atria to the ventricles. Its slow rate of conduction co-ordinates atrial and ventricular contraction and can protect against some types of arrhythmia [1,2]. The AVN also possesses intrinsic pacemaker activity, although the mechanisms underlying this activity are not fully understood. It has been suggested that in the sinoatrial node (SAN) spontaneous Ca2+ release from the sarcoplasmic reticulum (SR) contributes to pacemaking: early work showed that inhibition of SR Ca2+ release decreases the spontaneous frequency of the SAN [3–5], and more recent work has provided evidence that sodium–calcium exchange (NCX) activity is necessary for SAN pacemaker activity (e.g. [6–8]), with Ca2+ release from the SR contributing to pacemaking by activating inward NCX current (iNCX) [3–10].

Less is known about the role of Ca2+ in AVN pacemaking. Rabbit AVN cells exhibit [Ca2+]i transients during spontaneous activity and possess functional NCX, with an iNCX density similar to that in ventricular myocytes [11–13]. Recent evidence from experiments with the SR inhibitor ryanodine implicates Ca2+ release from the SR in pacemaking in intact AVN preparations [14], and indicates that the rate of spontaneous action potentials (APs) and [Ca2+]i transients in rabbit AVN cells [13] is sensitive to SR inhibition, consistent with a link between Ca2+ handling and spontaneous activity in these cells.

The present study was designed to investigate further the role of Ca2+ in the spontaneous activity of the AVN, in particular the contribution of SR Ca2+ release to AVN pacemaking and whether this involves iNCX. The NCX inhibitor KB-R7943, which has been used extensively in studies of atrial, ventricular and SAN cells (e.g. [8,15–18]), and the SR Ca2+ ATPase (SERCA) inhibitors thapsigargin [19] and cyclopiazonic acid (CPA) [20], were used to investigate the role of NCX and SR Ca2+ release in the generation of spontaneous APs and [Ca2+]i transients in cells isolated from the rabbit AVN.

2. Methods

2.1. AVN myocyte isolation

Male New Zealand White rabbits (2–3 kg) were killed humanely in accordance with UK Home Office legislation, and AVN cells isolated from the heart using a combination of enzymatic and mechanical dispersion, as described previously [21]. In brief, hearts
were Langendorff-perfused at 37 °C with Ca^{2+}-containing solution, then Ca^{2+}-free solution containing EGTA (100 μM), and then enzyme-containing solution (1 mg/ml collagenase, type I, Worthington, USA and 0.1 mg/ml protease, type XIV, Sigma, USA), followed by removal of the AVN, identified by its relation to anatomical landmarks [22]. AVN cells were dispersed from the AVN and re-suspended and stored in refrigerated (4 °C) Kraftbrühe ‘KB’ solution [23] until use.

2.2. Solutions and chemicals

All chemicals were purchased from Sigma–Aldrich (UK), and all solutions were made with deionised Milli-Q water (Millipore Systems, USA), unless stated otherwise. The cell isolation and ‘KB’ solutions have been described previously [21,24]. The normal Tyrode solution used to superfuse cells contained (in mM) [25]: 140 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 5 HEPES (pH 7.4 with NaOH). For spontaneous action potential recording, the K\(^+\)-based pipette solution contained (in mM) [26]: 110 KCl, 10 NaCl, 0.4 MgCl\(_2\), 10 HEPES, 5 glucose, 5 K\(_2\)ATP, 0.5 GTP-Tris (pH 7.1 with KOH). For L-type calcium current (\(I_{\text{Ca,L}}\)) recording, the internal solution was similar, except that KCl was replaced with CsCl, and 5 mM BAPTA was added [27]. For \(I_{\text{NCX}}\) recording, the internal solution was also Cs\(^+\)-based, and contained (in mM) [12]: 110 CsCl, 10 NaCl, 0.4 MgCl\(_2\), 1 CaCl\(_2\), 5 EGTA, 10 HEPES, 5 glucose, 20 TEACl (pH 7.2 with CsOH), and the external solution was potassium-free Tyrode containing 10 μM nitrindipine (to inhibit L-type calcium current) and 10 μM strophantidin (to inhibit the Na\(^+\)/K\(^+\) pump).

The low-sodium Tyrode solution used to inhibit forward mode NCX contained 40 mM NaCl (LiCl replacement). KB-R7943 (Tocris, USA), thapsigargin and CPA were dissolved in dimethyl sulfoxide (DMSO) made up as 5 M stock solution which was kept at 4 °C. Nickel (Ni\(^{2+}\)) chloride, used to block \(I_{\text{NCX}}\), was made up at 5 M stock solution which was kept at 4 °C. Although KB-R7943 has been used as a selective inhibitor of NCX [8,15–17], it is not entirely selective [30]. Moreover, whilst many of the above effects of KB-R7943 are consistent with the hypothesis that NCX is involved in pacemaker activity (see Section 4), a reduction in AP upstroke velocity and overshoot potential are difficult to explain solely on the basis of an effect of KB-R7943 on NCX.
Fig. 1. Effects of KB-R7943 on spontaneous action potentials and \([\text{Ca}^{2+}]_{i}\) transients. (A) KB-R7943 inhibits spontaneous action potentials. The top trace shows a representative slow time-base recording of membrane potential from an AVN myocyte before, during and after exposure to 5 μM KB-R7943, as indicated by the horizontal bars above the trace. The lower traces show sections of the top panel from the periods indicated, in the absence (left) and presence (middle and right) of KB-R7943, displayed at a faster time-base. The time scale bar in the lower left panel applies to all three lower panels. (B) KB-R7943 inhibits spontaneous \([\text{Ca}^{2+}]_{i}\) transients. The top trace shows a slow time-base averaged fluorescence plot of confocal line-scan image from an AVN myocyte (different cell from A) before, during and after application of 5 μM KB-R7943, as indicated above the trace. The lower traces show sections of the top panel from the periods indicated, in the absence (left) and presence (middle and right) of KB-R7943, displayed at a faster time-base. The time scale bar in the lower left panel applies to all three lower panels.
quently, to clarify the effects of KB-R7943 on AVN cell activity we investigated its effects on AVN \( I_{\text{NCX}} \) and \( I_{\text{Ca,L}} \).

### 3.2. Inhibition by KB-R7943 of AVN \( I_{\text{NCX}} \)

\( I_{\text{NCX}} \) was elicited using voltage ramps from +60 to −80 mV with major interfering currents blocked (see Section 2 and [12]). Fig. 2Ai shows mean net current density–voltage relations under control conditions and in the presence of 5 \( \mu \)M KB-R7943 or 5 mM Ni\(^{2+}\). In control solution the voltage ramp elicited an outwardly rectifying current which was markedly reduced in amplitude in the presence of 5 \( \mu \)M KB-R7943 or 5 mM Ni\(^{2+}\) \((P<0.001)\). From these net current recordings, \( I_{\text{NCX}} \) was obtained as the Ni\(^{2+}\)-sensitive current (Fig. 2Aii, control – nickel). The KB-R7943-sensitive current (Fig. 2Aii, control – KB-R7943) was similar to the Ni\(^{2+}\)-sensitive current at potentials negative to the observed reversal potential \( (E_{\text{rev}}) \) over the voltage range studied, although the drug-sensitive current was slightly smaller than the Ni\(^{2+}\)-sensitive current at positive voltages (between +20 and +60 mV; \( P<0.05, \) two-way ANOVA with Bonferroni post-hoc comparison). Examination of the residual KB-R7943 insensitive component of \( I_{\text{NCX}} \) (control – nickel) – (control – KB-R7943) in Aii showed no current at negative voltages (indicating that 5 \( \mu \)M KB-R7943 was as effective as Ni\(^{2+}\) at negative voltages), whilst at potentials positive to \( E_{\text{rev}} \) there was some residual \( I_{\text{NCX}} \) in the presence of KB-R7943.

### 3.3. KB-R7943 inhibition of \( I_{\text{Ca,L}} \)

Fig. 2B illustrates the time-course of effect of KB-R7943. Fig. 2Bi shows a representative plot of outward current (measured at +60 mV) against time, whilst Fig. 2Bii shows inward current (measured at −80 mV) from the same cell. Rapid application of either Ni\(^{2+}\) or KB-R7943 led to rapid changes of both inward and outward currents. However, although both agents caused almost complete inhibition of peak inward current, only Ni\(^{2+}\) completely inhibited peak outward current; some residual outward current remained at +60 mV in the presence of KB-R7943. These results show that 5 \( \mu \)M KB-R7943 was able to inhibit AVN \( I_{\text{NCX}} \) rapidly and completely at voltages relevant to the diastolic depolarization of AVN cells.

### Table 1

Effect of 5 \( \mu \)M KB-R7943 on spontaneous action potentials (APs) in rabbit atrioventricular node cells \((n=14)\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>At 15 s after KB-R7943 exposure</th>
<th>At 40 s after KB-R7943 exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous AP rate (beats/s)</td>
<td>3.99 ± 0.47</td>
<td>3.64 ± 0.46</td>
<td>1.87 ± 0.48</td>
</tr>
<tr>
<td>[% change, compared with control]</td>
<td>[−3.2 ± 13.9%]</td>
<td>[−40.4 ± 17.0%]</td>
<td></td>
</tr>
<tr>
<td>Slope of pacemaker diastolic depolarization (mV s(^{-1}))</td>
<td>113.9 ± 13.3</td>
<td>64.5 ± 8.2</td>
<td>32.4 ± 8.8</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−34.2 ± 10.0%]</td>
<td>[−67.5 ± 9.8%]</td>
<td></td>
</tr>
<tr>
<td>Maximal upstroke velocity ( (V_{\text{max}}, \text{V s}^{-1}) )</td>
<td>5.76 ± 0.99</td>
<td>3.03 ± 0.81</td>
<td>1.24 ± 0.48</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−50.0 ± 6.0%]</td>
<td>[−81.5 ± 5.2%]</td>
<td></td>
</tr>
<tr>
<td>Maximal repolarization velocity ( (V_{\text{rep}}, \text{V s}^{-1}) )</td>
<td>−1.53 ± 0.16</td>
<td>−0.92 ± 0.09</td>
<td>−0.45 ± 0.12</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−37.1 ± 6.4%]</td>
<td>[−68.5 ± 8.1%]</td>
<td></td>
</tr>
<tr>
<td>AP duration at 50% repolarization ( (\text{APD}_{50}, \text{ms}) )</td>
<td>63.24 ± 6.09</td>
<td>67.23 ± 6.83</td>
<td>50.29 ± 12.81</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−12.7 ± 13.2%]</td>
<td>[−19.2 ± 20.4%]</td>
<td></td>
</tr>
<tr>
<td>Maximal diastolic potential ( (\text{MDP}, \text{mV}) )</td>
<td>−55.08 ± 1.85</td>
<td>−44.69 ± 2.21</td>
<td>−35.40 ± 2.90</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−18.7 ± 3.3%]</td>
<td>[−35.8 ± 4.7%]</td>
<td></td>
</tr>
<tr>
<td>Overshoot ( (\text{peak of AP}, \text{mV}) )</td>
<td>18.59 ± 2.05</td>
<td>4.18 ± 4.38</td>
<td>−9.92 ± 5.11</td>
</tr>
<tr>
<td>[AP amplitude (mV)]</td>
<td>73.68 ± 2.89</td>
<td>48.87 ± 5.90</td>
<td>25.48 ± 6.95</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−34.2 ± 6.9%]</td>
<td>[−65.9 ± 8.8%]</td>
<td></td>
</tr>
</tbody>
</table>

When the data were analysed for this table, if spontaneous \([\text{Ca}^{2+}]\) transients had stopped in the presence of KB-R7943, the values for maximal diastolic potential and peak of AP were taken as the ‘resting’ potential, and the values for AP rate and other parameters were taken as 0.

\* \( P<0.05 \) versus control.

\** \( P<0.01 \) versus control.

\# \( P<0.01 \) compared with 0.

### Table 2

Effect of 5 \( \mu \)M KB-R7943 on spontaneous \([\text{Ca}^{2+}]\) transients in rabbit atrioventricular node cells \((n=12)\).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>At 15 s after KB-R7943 exposure</th>
<th>At 40 s after KB-R7943 exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous ([\text{Ca}^{2+}]), transient rate (beats/s)</td>
<td>3.05 ± 0.24</td>
<td>1.35 ± 0.24</td>
<td>0.58 ± 0.21</td>
</tr>
<tr>
<td>[% change, compared with control]</td>
<td>[−53.0 ± 8.0%]</td>
<td>[−79.1 ± 7.4%]</td>
<td></td>
</tr>
<tr>
<td>Percentage increase of diastolic ([\text{Ca}^{2+}]) baseline compared with control (%)</td>
<td>−9.3 ± 3.24</td>
<td>−16.9 ± 2.89</td>
<td></td>
</tr>
<tr>
<td>[% change]</td>
<td>[−13.1 ± 3.5%]</td>
<td>[−29.2 ± 4.8%]</td>
<td></td>
</tr>
<tr>
<td>([\text{Ca}^{2+}]), transient peak ((F_{\text{p}})); ( F_{\text{p}} ): the peak fluorescence intensity, ( F_{\text{c}} ): the control diastolic ([\text{Ca}^{2+}]) baseline ((\mu \text{M KB-R7943 vs control}) )</td>
<td>1.64 ± 0.08</td>
<td>1.44 ± 0.12</td>
<td>1.16 ± 0.12</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−27.6 ± 10.4%]</td>
<td>[−60.6 ± 12.7%]</td>
<td></td>
</tr>
</tbody>
</table>

When the data were analysed for this table, if spontaneous \([\text{Ca}^{2+}]\) transients had stopped in the presence of KB-R7943, diastolic \([\text{Ca}^{2+}]\) and \([\text{Ca}^{2+}]\), transient peak had the same values, and spontaneous \([\text{Ca}^{2+}]\), transient rate and \([\text{Ca}^{2+}]\), transient amplitude were taken as 0.

\* \( P<0.01 \) versus control.

\** \( P<0.01 \) compared with 0.
Fig. 2. KB-R7943 inhibits the nickel-sensitive $I_{\text{NCX}}$ in rabbit AVN cells. (Ai) Mean net current densities recorded by a ramp protocol from +60 to −80 mV (duration = 250 ms, holding potential = −40 mV, frequency = 0.33 Hz) from AVN myocytes ($n=5$) during superfusion with the control solution and exposure to 5 μM KB-R7943 or 5 mM nickel chloride. (Aii) Control – nickel represents the nickel-sensitive $I_{\text{NCX}}$ density by subtracting the current density during exposure to nickel from that in control; control – KB-R7943 represents the KB-R7943-sensitive current; and (control – nickel) − (control – KB-R7943) represents the residual nickel-sensitive $I_{\text{NCX}}$ after KB-R7943 inhibition. *$P<0.05$, **$P<0.01$: control – nickel versus control – KB-R7943. (Bi and Bii) Representative time-courses of the net current densities obtained at +60 mV (Bi) and −80 mV (Bii) from an AVN cell during superfusion with the control solution and exposure to 5 μM KB-R7943 and 5 mM nickel as indicated.

Fig. 3. KB-R7943 inhibits $I_{\text{Ca,L}}$ in rabbit AVN cells. (A) Representative $I_{\text{Ca,L}}$ traces from an AVN myocyte in the absence (control) and presence of 5 μM KB-R7943 and on washout, as indicated. The corresponding protocol used to elicit $I_{\text{Ca,L}}$ and the time scale bar are shown underneath. (B) The time-course of mean $I_{\text{Ca,L}}$ densities (at 0 mV, protocol shown as in A) from AVN myocytes ($n=12$) in absence (control) and presence of 5 μM KB-R7943 (and following washout), as indicated.
3.4. Low extracellular sodium ([Na\(^+\)]\(_e\)) inhibits spontaneous activity. After 15, 30 and 60 s exposure, respectively, and significantly (P < 0.01) decreased action potential and diastolic potential and did not cause cessation of spontaneous APs; nifedipine (0.2 μM) had little effect on maximum diastolic potential. However, spontaneous APs and [Ca\(^{2+}\)]\(_i\); transient rates were reduced (by 41 ± 18% (P < 0.05, n = 9), and 70 ± 16% (P < 0.01, n = 10), respectively), both I\(_{\text{NCX}}\) and I\(_{\text{Ca,L}}\) were still affected: Ni\(^{2+}\)-sensitive inward I\(_{\text{NCX}}\) at −80 mV was inhibited 84 ± 10% (P < 0.01, n = 3), whilst peak I\(_{\text{Ca,L}}\) was decreased by 23 ± 5% (P < 0.01, n = 7).

To investigate the possible contribution of decreased I\(_{\text{Ca,L}}\) to the effect of KB-R7943 on spontaneous activity, we empirically determined a concentration of the Ca\(^{2+}\) channel blocker nifedipine (0.2 μM) that produced approximately the same fractional decrease of I\(_{\text{Ca,L}}\) as that observed in response to KB-R7943. This concentration of nifedipine significantly (P < 0.01; n = 6) decreased I\(_{\text{Ca,L}}\) from −17.72 ± 2.38 pA/pF to −12.46 ± 1.84 pA/pF (i.e. by 29.9 ± 4.1%) after 15 s, −13.09 ± 1.51 (by 25.2 ± 2.2%) after 30 s, and −12.77 ± 1.40 (by 26.7 ± 2.2%) after 60 s exposure. This concentration of nifedipine decreased spontaneous AP rate by 11.8 ± 4.6% (P < 0.05 versus control) after 15 s, by 12.1 ± 3.1% after 30 s (P < 0.01 versus control; not significantly different from effect at 15 s), and by 10.7 ± 4.4% after 60 s (P < 0.05 versus control; not significantly different from effect at 15 or 30 s). Interestingly, these percentage decreases in rate are similar to the 13% decrease observed in cells which did not stop beating in response to KB-R7943 (above). However, in contrast to the response to KB-R7943, 90 s exposure to nifedipine had little effect on maximum diastolic potential and did not cause cessation of spontaneous APs; nifedipine did, however, significantly (P < 0.05) decrease upstroke velocity, by 33.7 ± 4.2%, 44.4 ± 6.2%, and 44.6 ± 6.8% after 15, 30 and 60 s exposure, respectively, and significantly (P < 0.01) decreased action potential overshoot by 47.4 ± 8.5%, 46.4 ± 9.0%, and 50.3 ± 10.3% after 15, 30 and 60 s exposure, respectively.

3.4. Low extracellular sodium ([Na\(^+\)]\(_i\)) inhibits spontaneous action potentials

Application of low [Na\(^+\)]\(_i\) has been shown previously to abolish spontaneous Ca\(^{2+}\) transients from AVN cells at ambient temperature [13]. In light of this and the observed effects of KB-R7943 on I\(_{\text{Ca,L}}\) (Fig. 3), [Na\(^+\)]\(_i\) reduction (to 40 mM; see Section 2) was used to reduce forward mode NCX activity. In 9 out of 11 cells, reduction of [Na\(^+\)]\(_i\) caused spontaneous APs to stop rapidly within ~5 s. In the remaining 2 cells, although spontaneous APs did not stop with brief (~10 s) exposure to low [Na\(^+\)]\(_i\), the AP rate decreased by 27% (P < 0.05; versus control solution, n = 2), and then recovered following return to control solution. Sustained exposure to low [Na\(^+\)]\(_i\) was not possible in these experiments as this led to cell contracture under our conditions.

3.5. SERCA blockade inhibits spontaneous activity

Thapsigargin and CPA were used to investigate the effect of depleting the SR by inhibiting SR Ca\(^{2+}\) uptake. Fig. 4A shows the effect of rapid application of 2.5 μM thapsigargin [31] on spontaneous APs. After exposure to thapsigargin, spontaneous AP rate initially increased and then decreased before stopping. Spontaneous APs stopped within 30 s in 3 out of 10 cells, and in all cells after ~90 s. Spontaneous APs (i.e. reverse mode NCX) transients showed a similar response: Fig. 4B shows a representative recording of the effect of thapsigargin on spontaneous Ca\(^{2+}\) transients. On initial exposure to thapsigargin, diastolic Ca\(^{2+}\) and spontaneous rate increased; this was followed by a decrease in diastolic Ca\(^{2+}\) and a progressive decrease in rate and cessation of spontaneous [Ca\(^{2+}\)]\(_i\); transients. Thapsigargin caused cessation of spontaneous Ca\(^{2+}\) transients within 30 s in 5 out of 7 AVN cells, and after 60 s in all 7 cells. Table 3 shows mean data illustrating the time-course of the effect of thapsigargin on AP and [Ca\(^{2+}\)]\(_i\); transient parameters (at 5, 15 and 30 s exposure to thapsigargin, incorporating measurements from all cells studied. By 60 s all cells were quiescent). The effects of thapsigargin were time-dependent (Table 3 and Fig. 4) and irreversible. Spontaneous AP frequency and slope of diastolic depolarization initially increased slightly (albeit not significant) at 5 s, and then decreased following 15 and 30 s exposure to thapsigargin. Maximum diastolic potential and AP amplitude decreased progressively in the presence of thapsigargin. The Ca\(^{2+}\); transient rate initially increased at 5 s and then decreased following 15 and 30 s exposure to thapsigargin; diastolic calcium initially increased slightly (albeit not significant) and subsequently decreased during exposure to the compound.

CPA (30 μM) was also used to inhibit SERCA. Its effects on spontaneous activity were similar to those of thapsigargin. In brief, after 30 s exposure to CPA, spontaneous APs had ceased in 3 out of 9 cells, and [Ca\(^{2+}\); transients had ceased in 6 out of 10 cells; spontaneous action potentials and [Ca\(^{2+}\); transients had ceased in all cells after 90 s exposure to CPA. Incorporating data from all cells studied (n = 9 for APs, n = 10 for [Ca\(^{2+}\); transients), spontaneous AP frequency initially increased by 6.9 ± 3.0% after 5 s (P < 0.05 versus control) and then decreased by 58.4 ± 11.8% (P < 0.01 versus control) after 30 s exposure to CPA. Similarly, [Ca\(^{2+}\); transient frequency initially increased, by 64.1 ± 17.6% after 5 s (P < 0.01 versus control) and then decreased by 68.9 ± 16.1% (P < 0.05 versus control) after 30 s exposure to CPA.

4. Discussion

4.1. Actions of the NCX inhibitor KB-R7943

KB-R7943 has been used widely in experiments on intact hearts, isolated cardiac tissues and cells [e.g.8,15–18]. When KB-R7943 was first tested on guinea-pig ventricular I\(_{\text{NCX}}\), it was reported to exhibit preferential inhibition of outward I\(_{\text{NCX}}\) (i.e. reverse mode NCX) [32], however it was found subsequently to inhibit both modes of NCX function under bi-directional I\(_{\text{NCX}}\) recording conditions [15,33]. This compound has been used in experiments that implicated NCX in canine SAN activity [34] and, at the same concentration as used here, has also been reported to inhibit spontaneous activity of guinea-pig isolated SAN cells [8]. However, to our knowledge the present study is the first in which this agent has been used in experiments on an AVN preparation. Under our conditions, 5 μM KB-R7943 produced bi-directional block of AVN I\(_{\text{NCX}}\), though with an apparent preference for inward I\(_{\text{NCX}}\) (i.e. forward mode NCX). 5 mM Ni\(^{2+}\) is an effective inhibitor of I\(_{\text{NCX}}\) when used under NCX-selective conditions [35,36]. Thus, the similarity of inward KB-R7943-sensitive current and Ni\(^{2+}\)-sensitive inward current under I\(_{\text{NCX}}\)-selective conditions suggests a maximal or near maximal inhibition of forward mode I\(_{\text{NCX}}\) at this concentration of KB-R7943. NCX can be anticipated to be operating in the forward mode (inward I\(_{\text{NCX}}\)) over the diastolic potential range of AVN cells. Thus, the marked effect of KB-R7943 on AVN cell spontaneous rate (for both APs and [Ca\(^{2+}\); transients) is consistent with a significant role for NCX in AVN pacemaking. This notion is supported by the inhibitory effect of rapid application of a low [Na\(^+\)]\(_i\) solution at physiological (this study) and ambient (reported previously [13]) temperatures.

However, KB-R7943 is not entirely selective for NCX: Kimura and colleagues found that the compound inhibited I\(_{\text{K}}\), I\(_{\text{Ca,L}}\), and the inward rectifier K\(^+\) current I\(_{\text{K1}}\) with IC\(_{50}\) values of 14, 8 and 7 μM respectively [32]. Rabbit AVN cells lack I\(_{\text{K1}}\) [21,26] and Na chan-
**Fig. 4.** Effects of thapsigargin on spontaneous action potentials and [Ca\(^{2+}\)]\(_i\) transients. (A) Thapsigargin inhibits spontaneous action potentials. The top trace shows a slow time-base recording of membrane potential from a representative AVN myocyte before, during and after application of 2.5 μM thapsigargin, as indicated above the trace. The lower traces show sections of the top panel from the periods indicated, in the absence (left) and presence (middle and right) of thapsigargin, displayed at a faster time-base. The time scale bar in the lower left trace applies to all three lower traces. In the first few seconds after thapsigargin exposure, initially the spontaneous AP rate increased and then decreased, with cessation of activity at ∼60 s after thapsigargin exposure. (B) Thapsigargin inhibits spontaneous [Ca\(^{2+}\)]\(_i\) transients. The top trace shows a slow time-base averaged fluorescence plot of a confocal line-scan image from a representative AVN myocyte (different cell from A) before, during and after application of 2.5 μM thapsigargin as indicated above the trace. The lower panels show sections of the top trace from the periods indicated, in the absence (left) and presence (middle and right) of thapsigargin, displayed at a faster time-base. The time scale bar in the lower left trace applies to all three lower traces. In the first few seconds after thapsigargin exposure, initially the [Ca\(^{2+}\)]\(_i\) transient rate increased, and the diastolic calcium baseline was elevated; the spontaneous [Ca\(^{2+}\)]\(_i\) transient rate then decreased and stopped at ∼20 s following thapsigargin exposure.
4.2. Influence of SERCA inhibition on AVN spontaneous activity

In the previous sections, we have established the role of SR 
Ca2+ handling in AVN spontaneous activity and AP transients. 
In the present study, we investigated the effect of SERCA 
inhibition on AVN spontaneous activity and AP characteristics. 

**Spontaneous AP (n = 10)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>At 5 s after thapsigargin exposure</th>
<th>At 15 s after thapsigargin exposure</th>
<th>At 30 s after thapsigargin exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate (beats/s)</td>
<td>3.22 ± 0.25</td>
<td>3.40 ± 0.33</td>
<td>2.44 ± 0.36*</td>
<td>1.48 ± 0.44**</td>
</tr>
<tr>
<td>[% change, compared with control]</td>
<td>[5.6 ± 4.3%]</td>
<td>[−25.1 ± 6.9%]</td>
<td>[−57.8 ± 10.9%]</td>
<td></td>
</tr>
<tr>
<td>Slope of pacemaker diastolic depolarization (mV/s−1)</td>
<td>97.7 ± 18.9</td>
<td>102.2 ± 17.6</td>
<td>68.1 ± 18.5</td>
<td>33.6 ± 10.3</td>
</tr>
<tr>
<td>[% change]</td>
<td>[12.6 ± 12.1%]</td>
<td>[−31.9 ± 7.4%]</td>
<td>[−65.1 ± 11.7%]</td>
<td></td>
</tr>
<tr>
<td>Maximal diastolic potential (MDP, mV)</td>
<td>−52.74 ± 3.09</td>
<td>−50.55 ± 3.02</td>
<td>−50.68 ± 2.71</td>
<td>−46.31 ± 2.47</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−4.1 ± 1.4%]</td>
<td>[−3.6 ± 1.6%]</td>
<td>[−11.5 ± 3.5%]</td>
<td></td>
</tr>
<tr>
<td>AP amplitude (mV)</td>
<td>63.96 ± 3.99</td>
<td>59.65 ± 3.75</td>
<td>59.68 ± 4.46</td>
<td>43.20 ± 9.82</td>
</tr>
<tr>
<td>[% change]</td>
<td>[−6.5 ± 1.9%]</td>
<td>[−7.2 ± 3.7%]</td>
<td>[−35.0 ± 14.4%]</td>
<td></td>
</tr>
</tbody>
</table>

**Spontaneous [Ca2+], transient (n = 7)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>At 5 s after thapsigargin exposure</th>
<th>At 15 s after thapsigargin exposure</th>
<th>At 30 s after thapsigargin exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate (beats/s)</td>
<td>3.27 ± 0.41</td>
<td>4.36 ± 0.36*</td>
<td>1.95 ± 0.56*</td>
<td>0.35 ± 0.34*</td>
</tr>
<tr>
<td>[% change, compared with control]</td>
<td>[38.7 ± 13.1%]</td>
<td>[−43.9 ± 13.1%]</td>
<td>[−87.5 ± 12.5%]</td>
<td></td>
</tr>
<tr>
<td>Percentage increase of diastolic Ca2+ baseline compared with control (%)</td>
<td>–</td>
<td>4.52 ± 2.495</td>
<td>−7.05 ± 4.89</td>
<td>−27.47 ± 7.21</td>
</tr>
</tbody>
</table>

When the data were analysed for this table, if spontaneous activity had stopped in the presence of thapsigargin, the values for maximal diastolic potential were taken as the ‘resting’ potential, and the values for AP rate and other AP parameters were taken as 0; and spontaneous [Ca2+] transient rate was taken as 0.

### Notes

- *P < 0.05, versus control.
- **P < 0.01, versus control.
- ***P < 0.001, versus control.

Nelsons are sparse or absent from the compact node [37]; moreover, the maximal upstroke velocity of AVN control APs in our KB-R7943 experiments (<6 V s−1) is inconsistent with a major role for Ica in driving the AP upstroke in the cells studied here. Thus, effects of KB-R7943 on Ica or IKr are unlikely to have contributed significantly to the effects of this agent observed in the present study. On the other hand, IcL plays a major role in AP genesis [38,39].

Under our conditions 5 μM KB-R7943 inhibited IcL by ~31%, which is compatible with earlier ventricular cell data [30,32,33]. This secondary effect of KB-R7943 is likely to contribute significantly to changes in AP upstroke velocity and overshoot seen here, whilst progressive depolarization in MDP may also have contributed via facilitating IcL inactivation. When the role of IcL was tested by investigating the effect of a nifedipine concentration that produced a similar decrease of IcL to KB-R7943, this compound produced a marked effect on AP upstroke velocity and overshoot, consistent with the established role of IcL in AVN APs [38,39]. However, in no cell studied did nifedipine induce quiescence (although spontaneous AP rate decreased by ~12%). Thus, any secondary effect of KB-R7943 on IcL is likely only to have had a comparatively minor effect on spontaneous AP rate, and the observed cessation of spontaneous activity in KB-R7943-treated cells is unlikely to be attributable to an effect on IcL. The ‘resting’ potential at which cells become quiescent in KB-R7943 (~26 mV) is more positive than the known zero current potential of AVN cells (~40 mV) [21,26]. This may suggest an additional action of KB-R7943, potentially on an outward resting conductance. Although this avenue was not pursued in the present study, an inhibitory effect on delayed rectifier K+ current has been reported [30] and the rapid delayed rectifier (IKr) is active during AVN AP repolarization and the diastolic depolarization [14,40,41].

4.2. Influence of SERCA inhibition on AVN spontaneous activity

Recent studies have shown that inhibition of ryanodine receptors (RyRs) in AVN cells has a marked inhibitory effect on spontaneous AP and calcium transient rate [13,14]. If SR Ca2+ release is involved in AVN cell pacemaking, then inhibition of SR Ca2+ reuptake by inhibition of SERCA would also be predicted to reduce spontaneous rate. Indeed, SERCA inhibition has been observed to slow the spontaneous rate of guinea-pig [3,8], canine [42] and rabbit [43] SAN preparations. The immediate effect of SERCA inhibition by both thapsigargin and CPA in the present study was an initial increase in diastolic [Ca2+]; and in spontaneous AP and [Ca2+] transient rate. This is consistent with a scheme in which transiently raised [Ca2+], due to reduced SR reuptake of Ca2+, leads to greater activation of a sarclemmal electrogenic process (most likely involving the NCX, as discussed above). The subsequent decline in spontaneous AP/[Ca2+] transient rate would be anticipated to follow this transient increase, once the SR were depleted of Ca2+ and this had been removed from the cytosol. In time, the loss of SR Ca2+ would in turn remove the influence of cyclic SR Ca2+ release on sarclemmal electrogenic. CPA was found to produce qualitatively similar results to thapsigargin; thus data with both agents implicate SR Ca2+ cycling in spontaneous activity.

In the SAN, a calcium “clock” has been proposed along with the traditional voltage clock (i.e. membrane currents generated by voltage-dependent ion channels) to bring about spontaneous activity [44,45]. It has been proposed that after a calcium transient of the SAN cell, as SR calcium content increases and ryanode receptors (RyRs) recover from inactivation, high [Ca2+] in the SR lumens activates RyRs, causing a localized subsarcolemmal calcium release (i.e. a calcium spark) [46]. This causes a local increase of cytoplasmic [Ca2+], stimulating inward INCX. This brief inward current causes a small diastolic depolarization, with summation of such individual events bringing the membrane potential to threshold for an action potential. This rhythmic spontaneous SR Ca2+ release/spark shows inherent rhythmicity, giving rise to the term “calcium clock” [44,46]. In our previous study of AVN cells at ambient temperature calcium sparks occurred relatively infrequently [13] and in our present experiments calcium sparks were not detected in spontaneously beating AVN cells. It seems unlikely, therefore, that the calcium clock mechanism identified for the rabbit SAN [46] underpins the role of SR Ca2+ release in AVN cell pacemaking; our data are more consistent with a scheme in which Ca2+ transients initiated by the AP upstroke in turn activate sarclemmal NCX to extrude Ca2+ generating an inward current that persists into diastole.

4.3. Strengths, limitations and conclusions

In comparison to the SAN [3,4,6–10,42–46], comparatively few data exist regarding Ca2+ handling by AVN cells [13,14]. The present results not only add new information about Ca2+ handling, but also further implicate SR Ca2+ cycling in electrogenic of spontaneously active AVN cells. Caveats in the use of KB-R7943 are highlighted earlier in the ‘Discussion’. The use of this compound is nevertheless warranted because it is arguably the most effective commercially available NCX inhibitor and because it has been used in prior cardiac studies [8,15–18,32–34]. The AVN is known to be a heterogeneous structure [1,2,47] and cells were isolated from the entire AVN region, so that it is not possible to attribute...
with certainty AVN regional sub-types to individual cells studied. However, this uncertainty is shared with numerous previous AVN studies (e.g. [13,21,26]), and the relatively slow AP upstroke velocities observed here are most compatible with ‘N-like’ or ‘NH-like’ APs [47]. Although concurrent Ca\(^{2+}\) transient and AP recordings would permit changes to both events to be viewed in parallel, we found that a combination of Fluoro-4 dye loading and whole-cell recording rapidly led to quiescence in nearly all cells studied in this way. Thus, in order to minimize cell dialysis and buffering of Ca\(^{2+}\) transients, Ca\(^{2+}\) transients and APs were therefore recorded in separate experiments. Nevertheless, the limited data that we have obtained recording both parameters simultaneously (not shown) showed no cyclic changes of [Ca\(^{2+}\)]\(_i\) in the absence of APs; this is consistent with a scheme in which the AP upstroke initiates the SR Ca\(^{2+}\) release process which itself ultimately then influences diastolic depolarization. Our thapsigargin and CPA data demonstrate that inhibition of SERCA prevents effective Ca\(^{2+}\) cycling and thereby influences spontaneous activity. Inhibition of spontaneous activity by two KB-R7943 concentrations differing >20 fold, together with the inhibitory effect of low [Na\(^+\)] strongly implicate the NCX as a major electrogenic transport process that couples SR Ca\(^{2+}\) release to spontaneous AP genesis. Further work is required to determine whether NCX is the only such process or whether, as has been suggested for the SAN, other mechanisms such as [Ca\(^{2+}\)]\(_i\) sensitive cation channels [48] may also be involved.

Acknowledgments

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References


