Increased Na\(^+\) concentration and altered Na/K pump activity in hypertrophied canine ventricular cells

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Abstract

Objective: To investigate whether hypertrophy in the dog with chronic atrioventricular block (CAVB) alters [Na\(^-\)] and Na/K-pump function of ventricular myocytes. Methods: We measured the [Na\(^-\)] dependence of the Na/K pump current, \(I_p\). This relation was used as a calibration curve for [Na\(^-\)], based on \(I_p\). We measured \(I_p\) at the time of access and extrapolated [Na\(^-\)] at the pump sites, i.e. subsarcolemmal [Na\(^-\)], [Na\(^-\)]\(_{sub}\), from the calibration curve. Results: The extrapolated [Na\(^-\)]\(_{sub}\) was significantly higher in CAVB (7.9 mM vs. 3.2 mM in control). The [Na\(^-\)] dependence of \(I_p\) in CAVB myocytes was shifted to the right (range of [Na\(^-\)]: 0–20 mM). In resting cells, the \(I_p\), i.e. steady state Na efflux, which matches Na influx, was higher in CAVB (0.25 ± 0.02 vs. 0.47 ± 0.06 pA/pF, \(P<0.05\)). Maximal \(I_p\) density was not different, and DHO sensitivity was not altered. Conclusions: Hypertrophy in CAVB cells is associated with increased [Na\(^-\)]\(_{sub}\). This results from an increase in Na\(^+\) influx, and a decreased sensitivity of \(I_p\) for Na in the range of [Na\(^-\)] studied. There is no evidence for a decrease in total pump capacity or for a functional Na/K-ATPase isoform shift. The rise in Na\(^+\) contributes to the contractile adaptation and preservation of sarcoplasmic reticulum Ca\(^2+\) content at the low heart rates of the dog with CAVB.

Keywords: Calcium (cellular); Hypertrophy; Na/K-pump

1. Introduction

During cardiac hypertrophy and heart failure, a rise in [Na\(^-\)], could potentially contribute to increasing or maintaining contractility, as it would increase Ca\(^2+\) influx via the Na/Ca exchanger [1,2]. With cellular remodeling during these pathophysiological states, expression and/or function of several of the Na\(^+\) transporters are affected, which can lead to an increase in [Na\(^-\)]. Several studies have reported a decrease in activity of the Na/K-ATPase, due to downregulation of the number of pumps and/or due to a shift in isoform expression (e.g. in rat [3–6], in dog [7,8], and in human [9–11]). The Na/H exchanger appears to be upregulated and this would also result in an increase in [Na\(^-\)]\(_i\) (reviewed in Refs. [12,13]). For the Na\(^+\) channel, an increase in the non-inactivating window cur-
frequencies of stimulation [29]. This contributes to maintaining function at low heart rates, but leads to a negative force–frequency behaviour. In addition, we found an increase in Ca\(^{2+}\) influx via the Na/Ca exchanger [30]. These dogs are also more sensitive to the pro-arrhythmic effects of ouabain [29]. All of these findings suggest an increase in [Na\(^+\)], in the present study we have therefore measured [Na\(^+\)]\(_{\text{subs}}\), and investigated potential underlying mechanisms. The membrane current generated by the Na/K-ATPase, \(I_p\), was used as a probe for subsarcolemmal [Na\(^+\)], [Na\(^+\)]\(_{\text{subs}}\), because several studies have shown that the Na\(^+\) concentration near the membrane in dialyzed cells may be different from that in the bulk cytosol [31–35]. In addition this approach allowed us to simultaneously characterize one of the major Na\(^+\) flux pathways.

2. Methods

2.1. Animal model and cell isolation

A complete atrioventricular block was induced in adult mongrel dogs by chemical ablation of the AV node. This procedure has been described in detail before [36]. Animal experiments and treatment were in accordance with the European directive for the protection of vertebrate animals used for scientific purposes. A total of 18 dogs was studied after 8±1 weeks of complete AVB. A group of 15 dogs in sinus rhythm, with comparable body weight (BW) and age, served as control. The procedure for cell isolation has been described in detail before [37,38]. The heart was removed under deep anaesthesia. Heart weight (HW) was immediately determined, and for all CA VB dogs, hypertrophy was confirmed by a HW/BW of 11.4±0.6 versus 8.0±0.2 for control dogs \((P<0.001)\). For the present study, myocytes were obtained from the same animals for which the Ca\(^{2+}\) homeostasis has been described [30]; only cells isolated from the midmyocardial layer of the left ventricular free wall were used. Cells were kept at room temperature.

2.2. Solutions

The experiments on isolated cells were carried out in plastic Petri dishes placed on the stage of an inverted microscope. The bath medium was a Tyrode solution containing (in mM): 144 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 10 Hepes, 5 glucose and 1.8 CaCl\(_2\); the pH was 7.35 (adjusted with NaOH). Individual cells were superfused with different test solutions applied to the cell under study via a multibarreled and valve-controlled pipette. Solution changes at the surface of the cell were complete within 100–200 ms and the temperature drop near the cells when test solutions were changed was 1–1.5 °C maximally. The bath was kept constant at 36 °C. The standard extracellular superfusion medium contained (in mM): 144 NaCl, 0 or 5.4 KCl, 0.5 MgCl\(_2\), 1.8 CaCl\(_2\), 10 Hepes, 5 glucose (pH 7.35 with NaOH). When the Na\(^+\) concentration in the superfusion solution was varied, Na\(^+\) was replaced by N-methyl-d-glucamine. In order to abolish K\(^+\)-sensitive conductances and Na/Ca exchange, 2 mM BaCl\(_2\) and 5 mM NiCl\(_2\) were added to the test solutions. The Na/K pump was suppressed by omitting K\(^+\) from the superfusion solution, and activated by rapidly superfusing with a 5.4 mM K\(^+\) solution. Dihydro-ouabain (DHO; Sigma) was used to identify the sensitivity of the Na/K pump to cardiac glycosides. The patch pipette solution contained (in mM): 120 K-aspartate, 3 MgCl\(_2\), 0.15 CaCl\(_2\), 20 tetraethylammonium chloride (TEA-Cl), 5 EGTA, 10 Hepes, 5 MgATP, 5 glucose, pH 7.30 (with KOH); Na\(^+\) was added (2, 5, 10, 20 mM) by replacing K-aspartate by equimolar amounts of Na-aspartate. To activate the \(I_p\) maximally an internal solution with 100 mM Na\(^+\) and 0 K\(^+\) was used. K\(^+\) was omitted from the solution since intracellular K\(^+\) competes with the binding of Na\(^+\). K-aspartate was replaced by 100 mM Na-aspartate and 20 mM TEA-Cl.

2.3. Electrical recording

Membrane currents were measured by means of the single electrode, whole cell patch technique using an Axoclamp 2A voltage clamp amplifier. Resistance of the patch electrodes was about 2 MΩ. The cell surface area was calculated from the capacitive charge flowing during small hyperpolarizing voltage pulses. Cell capacity of control and AVB ventricular myocytes used for \(I_p\) measurements was 165±5 pF (91 cells; five hearts) in control hearts and 185±4 pF (122 cells; nine hearts), respectively \((P<0.05)\). The Na/K pump current, \(I_p\), was measured as the K\(^+\)-activated, DHO-sensitive outward current. To avoid interference with activation of \(I_{Ks}\) and \(I_{Kc}\), and to obtain maximal activation of the voltage-dependent Na/K pump, \(I_p\) was measured at −20 mV. \(I_p\) measurements were carried out 6–18 h after cell isolation; only rod-shaped cells with clear striations were used.

2.4. Statistics

The data are presented as means±S.E.M. Differences between means were tested by the Student’s t-test. A two-way ANOVA analysis was used to detect significance between \(I_p\) versus [Na\(^+\)]\(_{\text{pip}}\) of control and CA VB dogs. Differences were considered significant if \(P<0.05\).

3. Results

3.1. A calibration curve for [Na\(^+\)]\(_{\text{subs}}\)

Because of the uncertainty about [Na\(^+\)]\(_{\text{subs}}\) with cell dialysis we used the sensitivity of Na/K pump for internal Na\(^+\) as a probe to estimate Na\(^+\) near its intracellular binding sites facing the subsarcolemmal space. Na/K
3.2. Measuring $[\text{Na}^+]_{\text{subs}}$ in myocytes

Immediately after establishing the whole-cell configuration, the cell was superfused with a 0 mM K$^+$ solution and briefly switched to a solution with 5.4 mM K$^+$. Fig. 2A illustrates the time course of $I_p$ from the moment access to the cell was made. $[\text{Na}^+]_{\text{pip}}$ in this experiment was 10 mM Na$^+$. Initially $I_p$ at $-20$ mV was 0.24 pA/pF, 2 min later 0.68 pA/pF. The initial $I_p$ value was considered to be determined by the basal $[\text{Na}^+]$ near the binding sites. When the cell was slowly dialedyzed by the higher $[\text{Na}^+]$ solution $I_p$ increased to attain a steady-state value after about 2–3 min.

The mean values for the initial $I_p$ in resting myocytes estimated at the moment at which access was made were 0.25±0.02 pA/pF ($n$=83 cells of seven hearts) and 0.47±0.06 pA/pF ($n$=81 cells from eight hearts), for control and CA VB dogs, respectively ($P<0.05$). Fig. 2B shows the values of $[\text{Na}^+]_{\text{subs}}$ obtained by extrapolation of the $I_p$ densities (mean±2 S.E.M.) to $[\text{Na}^+]_{\text{pip}}$. Horizontal arrows indicate the initial resting $I_p$ with the upper arrow related to CA VB cells; the curves are similar as in Fig. 1C. The shaded areas indicate the range of the mean values (arrows)±2 S.E.M. for $I_p$ at rest and for the extrapolated range of $[\text{Na}^+]_{\text{subs}}$ (projection onto the x-axis). From this, we can extrapolate that $[\text{Na}^+]_{\text{subs}}$ was 3.2 mM (range 2.8–3.5 mM) in control versus 7.9 mM (range 5.8–11.4 mM) in CA VB dog myocytes. The higher extrapolated $[\text{Na}^+]_{\text{subs}}$ in quiescent cells is determined by the higher $I_p$ density and by the rightward shift of the CA VB calibration curve. Such a shift can result from a downregulation of the overall Na/K pump activity, or from a reduction in $[\text{Na}^+]$, sensitivity of the Na/K-ATPase.

3.3. Is the Na/K-ATPase downregulated?

To examine whether the shift of the $I_p/[\text{Na}^+]_{\text{pip}}$ curve was caused by a downregulation of functional Na/K pump molecules, we measured the maximally activated $I_p$. Maximal activation was obtained by dialyzing the cells with a pipette solution containing 100 mM $[\text{Na}^+]$ and 0 mM K$^+$. Maximal $I_p$ densities in control and CA VB cells are illustrated in Fig. 3A and amounted to 3.4±0.2 pA/pF ($n$=26 cells from five hearts) and 3.5±0.2 pA/pF ($n$=27 from five hearts), respectively. The almost identical maximal $I_p$ densities indicate that the maximal capacity of the Na/K pump to extrude Na$^+$ was not different in control and CA VB cells.

3.4. Is there a shift in functional isoform composition?

In the dog, two isoforms of the Na/K-ATPase have been described, $\alpha$1 and $\alpha$3 [4,7,8,40]. The $\alpha$1 is the dominant isoform, and represents around 85% of all $\alpha$ protein in the normal dog heart. The $\alpha$3 isoform has a higher sensitivity for cardiac glycosides, but a lower affinity towards internal Na$^+$. The presence of two populations of functional Na/K pumps with different sensitivities to cardiac glycosides can be detected by studying the inhibition of $I_p$ as a function of cardiac glycoside concentration [41]. If two different
Fig. 1. $I_{o}$ activation by intracellular $[\text{Na}^{+}]$. (A) Activation of $I_{o}$ in a control cell, $[\text{Na}^{+}]_{\text{pip}}$ 10 mM. Upper trace, solution switch between 0 and 5.4 mM $K^{+}$. Lower trace, membrane current; zero current level is indicated by the horizontal line at the bottom of the calibration mark. Holding potential: $-20$ mV; cell capacity: 137 pF. (B) Experimental procedure for equalizing $[\text{Na}^{+}]_{\text{pip}}$ to $[\text{Na}^{+}]_{\text{pip}}$ (10 mM). For equilibrating $[\text{Na}^{+}]_{\text{pip}}$ with $[\text{Na}^{+}]_{\text{pip}}$, the cell was clamped at 0 mV and the cell was superfused with 10 mM $Na^{+}$ for 3 min. $I_{o}$ was measured at $-20$ mV with 5.4 mM $K^{+}$ in the presence of 150 mM $Na^{+}$. Cell capacity 154 pF. (C) $I_{o}$ density (pA/pF) of control (open circles) and CAVB (solid circles) cardiomyocytes as a function of $[\text{Na}^{+}]_{\text{pip}}$, which equals $[\text{Na}^{+}]_{\text{sub}}$. Data were fitted according to the Hill equation.
Fig. 2. Estimation of $[\text{Na}^+]_{\text{obs}}$ from the $I_p$ versus $[\text{Na}^+]_{\text{pip}}$ curve. (A) Protocol to determine resting $I_p$ in a control cardiomyocyte. Upper trace: solution switch between 0 and 5.4 mM K$^+$. Lower trace: membrane current; the first activation of $I_p$ was carried out immediately after access to the cell was made. Subsequent activations were made 1, 1.5 and 2 min after rupture of the cell membrane. Cell capacity: 140 pF, $[\text{Na}^+]_{\text{pip}}$: 10 mM. (B) $I_p$ versus $[\text{Na}^+]_{\text{pip}}$ calibration curves as in Fig. 1C; open circles: control cells, solid circles: CAVB cells. The mean resting $I_p$ density is indicated by the horizontal arrows, shaded areas indicate the range ±2 S.E.M. The $I_p$ values are projected on the x-axis as indicated by the vertical arrows and shaded areas, yielding extrapolated $[\text{Na}^+]_{\text{obs}}$ values.

isoforms contribute to $I_p$, the concentration dependence of the inhibition of $I_p$ is biphasic and the response can be fitted to a two-binding site model with two $K_D$ values. We used this property to investigate an eventual shift in the sensitivity towards dihydro-ouabain (DHO). Fig. 3B shows a full range concentration–$I_p$ inhibition curve by DHO for CAVB myocytes. The pipette Na$^+$ concentration was 100 mM with 0 mM K$^+$; the DHO solution was superfused
after $I_p$ attained a steady-state value in 5.4 mM $K^+$. This curve could be fitted according to a two-binding-site model [41] indicating the presence of Na/K pump binding sites with different affinities towards DHO. From this fit, we could deduce that the high affinity sites, or the $\alpha_3$ isoform, represented $17\%$ of the total $I_p$-generating sites. If the fraction of high-affinity pump molecules contributing to $I_p$ had been changed, the percentage of $I_p$ that would be blocked by the low concentration of DHO ($10^{-5}$ M) would be different. However, the fraction of $I_p$ that could be inhibited by $10^{-5}$ M DHO in control cells ($0.21 \pm 0.04$, $n=8$ cells, three hearts) was not statistically different from the value for CAVB ($0.17 \pm 0.02$, 13 cells, six hearts). This indicates that the relative contribution of the $\alpha_3$ isoform to $I_p$ was the same in control and CAVB. At $10^{-6}$ M DHO, the concentration that inhibits half of the low-affinity pumps, we did not observe a difference either, again consistent with the absence of an alteration in isoform composition in CAVB.

4. Discussion

In this study, we investigated the subsarcolemmal Na$^+$ concentration in compensated cardiac hypertrophy in dogs with chronic atrioventricular block. To estimate [Na$^+$]$_{sub}$ the Na/K pump current was used as a local sensor of Na$^+$ in the subsarcolemmal space. The major findings are that in ventricular myocytes from CAVB dogs versus control cells: (1) the Na$^+$ activation curve of $I_p$ for CAVB was shifted to the right, (2) $I_p$ in quiescent cells was greater, (3) the extrapolated [Na$^+$]$_{sub}$ was higher, (4) the functional maximal Na/K pump capacity was unchanged, (5) the fraction of $I_p$ generated by high-affinity (for cardiac glycosides) pump molecules was the same.

4.1. The Na/K pump current as a measure for [Na$^+$] in the subsarcolemmal space

Large discrepancies have been reported to exist between bulk [Na$^+$] in the cytosol and local [Na$^+$] near the inner side of the membrane of dialyzed cells by measuring membrane currents which are sensitive to intracellular Na$^+$, i.e. the Na/K pump current [31–33], the Na/Ca exchange current [31,32,34,35,42] and the [Na$^+$]-activated K$^+$ current [43,44]. Even in the subsarcolemmal space microheterogeneity of [Na$^+$], has been shown by micro-electron probe analysis [44]. Fig. 1A is consistent with these reports and it illustrates that, even at a holding potential of $-20$ mV, and a small driving force for Na$^+$, $I_p$ transients occur when the Na/K pump is switched on after a short period of Na/K inhibition. Such transients indicate that [Na$^+$] is not controlled in the subsarcolemmal space and continuous activation of the pump results in a much smaller [Na$^+$]$_{sub}$, than [Na$^+$]$_{pip}$. Conversely, after long periods in 0 mM K$^+$ and 150 mM Na$^+$, [Na$^+$]$_{sub}$ tended to be higher than [Na$^+$]$_{pip}$ indicating that the background Na$^+$ influx markedly influences local [Na$^+$]. Therefore, local [Na$^+$] seen by the binding sites of the Na/K pump and the Na/Ca exchanger may markedly deviate from concentrations found in the cytosol of dialyzed cells, and can be higher as well as lower. To make the calibration curve $I_p/[Na^+]_{pip}$ suitable to estimate [Na$^+$]$_{sub}$ from $I_p$, an experimental procedure was designed to equalize [Na$^+$]$_{pip}$ with [Na$^+$]$_{sub}$. With this procedure, we could thus extrapolate the [Na$^+$]$_{sub}$ from the resting $I_p$ measured at the moment of access, before [Na$^+$]$_{sub}$ was influenced by cell dialysis.

It is important to point out that Na$^+$ gradients are much
less likely to occur in non-dialyzed cells, at least in the steady state. Indeed, in physiological conditions, gradients are expected to occur only transiently upon abrupt changes in the Na\(^+\) influx or Na\(^+\) efflux. One could thus argue that [Na\(^+\)] should be measured in undialyzed cells. However, this approach also has its inherent shortcomings, such as the possibility that uncontrolled membrane potential changes will affect Na\(^+\) fluxes and [Na\(^+\)].

4.2. Intracellular Na\(^+\) in hypertrophic cells

The [Na\(^+\)] estimated from the steady-state \(I_p\) density was about 4 mM higher in hypertrophic compared to control cells. Several studies have reported an increase in [Na\(^+\)] in cardiomyocytes from failing and hypertrophic hearts [17,19,20,45–47]. The mean rise in [Na\(^+\)] in these reports was about 5 mM similar to our findings. A few studies reported no change in [Na\(^+\)], (e.g. Ref. [21]; for review, see Ref. [48]). Although no direct explanation can be found to explain these controversial findings, results may be influenced by differences in the models of hypertrophy, the developmental stage of the hypertrophic process and techniques used to measure [Na\(^+\)].

\(I_p\) in resting cells was twice as large in CAVB than in control cells. Since the Na/K pump is the main transporter extruding Na\(^+\) in a quiescent myocyte, the total Na\(^+\) influx has to equal Na/K pump-dependent Na\(^+\) efflux. For a similar stoichiometry, a doubling of \(I_p\) signifies a twofold rise of the Na\(^+\) efflux, and thus, in the steady state, a doubling of the Na\(^+\) influx. A similar rise in Na\(^+\) influx was recently found in ventricular myocytes of the failing rabbit heart [19].

A second factor that might contribute to an increased [Na\(^+\)] in resting cells is an altered Na/K pump activity, i.e. a decrease in the maximal pump capacity and/or a decrease in sensitivity for [Na\(^+\)]. For a constant Na\(^+\) influx, both alterations will be accompanied by a rise in [Na\(^+\)]. Although an extensive literature exists on Na/KATPase activity and ouabain binding site density in tissue homogenates of hypertrophic and failing hearts, few reports have actually measured the Na/K pump function in intact cells. Most tissue studies report a decrease in Na/K-ATPase activity and/or ouabain binding sites (e.g. Ref. [9]).

Our functional measurements indicate that maximal Na/K pump activity in CAVB dogs is unaltered, as was also recently reported for the failing rabbit heart [19]. Although the maximal Na/K pump activity was unchanged in CAVB, we observed a rightward shift of the [Na\(^+\)]-dependent \(I_p\) activation in the range of 0–20 mM [Na\(^+\)]. This could indicate the presence of a functional isoform shift with a higher proportion of \(a3\) with a lower [Na\(^+\)] affinity. Contradictory results have been reported on \(a3\) isoform expression in the dog. A decrease (in pressure overload hypertrophy [8] and pacing-induced failure [49]) as well as a rise (pacing-induced failure [7]) have been reported. Our functional test of DHO sensitivity provides no evidence for an altered contribution of the high-affinity α isoform in our CAVB hypertrophy model. The rightward shift of the curve therefore needs another explanation. Recently, a novel mechanism regulating Na\(^+\) via the Na/K pump has been proposed to explain the reduction of [Na\(^+\)], in ventricular preparations and cells of rabbits treated with angiotensin-converting enzyme (ACE) inhibitors [50–52]. Treatment of rabbits with captopril decreased the Na\(^+\) activity by about 4 mM. The fall in [Na\(^+\)] could largely be explained by an increase in the apparent affinity of the main pump isoform for Na\(^+\) in favour of K\(^+\) binding. These findings indicate that the competitive inhibition of Na\(^+\) binding by K\(^+\) might be regulated by an angiotensin II-induced, protein-kinase C-dependent phosphorylation of the pump molecules.

Angiotensin II has been implicated as an important factor in different models of hypertrophic remodelling (e.g. Ref. [53]). If it plays a role in the CAVB model, part of the effect on the selectivity of cation binding may be lost, or at least be underestimated, when cells are bathed in an angiotensin II-deficient medium after isolation. However, in the study by Rasmussen et al. [54], the effects of ACE inhibition became only evident when animals were treated for at least 24 h, indicating that endogenous angiotensin II had long-lasting effects.

4.3. Functional implications of an increased [Na\(^+\)]

Cardiac tissues which have an internal Na\(^+\) concentration that is a few mM higher at rest than other cells typically display a negative force–frequency relationship (for review, see Ref. [24]). The higher contractility at low frequencies of stimulation has been related to suppression of rest-decay of contractile parameters, of SR Ca\(^{2+}\) content and Ca\(^{2+}\) transients in preparations with a higher [Na\(^+\)], [24,55,56]. In this developmental stage of hypertrophy in CAVB dogs, SR function is still intact but the Na/Ca exchanger is upregulated [30], a change that potentially could compete with SR loading. Together with the rise in [Na\(^+\)], however, the myocyte is able to keep its SR Ca\(^{2+}\) content high at the low frequencies of the CAVB dog (30 vs. 105 beats/min in control). Although a rise in [Na\(^+\)], contributes to maintain contractile function as part of the compensatory adaptation in the dog with CAVB, the increased loading of SR may trigger spontaneous release of Ca\(^{2+}\) and contribute to arrhythmias. This correlates with the higher incidence of arrhythmias in this model of hypertrophy [36].

In conclusion, the basal Na\(^+\) concentration in the subsarcolemmal space of hypertrophied ventricular myocytes of CAVB dogs is higher than in cardiomyocytes from control dogs. The increase in [Na\(^+\)] is determined by a higher Na\(^+\) influx and a shift of the Na/K pump activation curve to the right. The higher [Na\(^+\)] is an important factor determining contractile compensation by lessening Ca\(^{2+}\) extrusion via the Na/Ca exchanger during
inter-beat intervals and keeping SR Ca$^{2+}$ content high at the low intrinsic heart rate in AVB dogs.

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References


