Differential distribution and regulation of mouse cardiac Na\(^+\)/K\(^+\)-ATPase α\(_1\) and α\(_2\) subunits in T-tubule and surface sarcolemmal membranes

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Abstract

Objectives: Two Na+/K+-ATPase (NKA) α-subunit isoforms, α\(_1\) and α\(_2\), are expressed in the adult mouse heart. The subcellular distribution of these isoforms in T-tubule and surface sarcolemmal (SSL) membranes and their regulation by cAMP-dependent protein kinase (PKA) is unclear.

Methods: We used formamide-induced detubulation of mouse ventricular myocytes to investigate differential functional distribution and regulation by PKA of α\(_1\) and α\(_2\) in T-tubule versus SSL membranes by measuring NKA current (\(I_{\text{pump}}\)) and NKA-mediated Na\(^+\) efflux (−d[Na]/dt).

Results: \(I_{\text{pump}}\) is composed of 88% α\(_1\)-mediated \(I_{\text{pump}}\) (\(I_{\alpha_1}\)) and 12% α\(_2\)-mediated \(I_{\text{pump}}\) (\(I_{\alpha_2}\)). α\(_1\) and α\(_2\) subunits demonstrate distinct ouabain affinities (105±6 and 0.3±0.1 μmol/L respectively) but similar affinity for intracellular Na\(^+\) (K\(_{1/2}\)Na\(^+\) of 16.6±0.8 and 16.7±2.6 mmol/L respectively). Detubulation reduced (i) \(I_{\text{pump}}\) density (1.42 ±0.1 to 1.20±0.04 pA/pF), (ii) cell capacitance (181 ± 12 to 127± 17 pF), and (iii) \(I_{\alpha_2}\) contribution (12 to 6%). Total \(I_{\text{pump}}\) density was ∼60% higher in T-tubule (1.94 pA/pF, derived) vs. SSL membranes. Although T-tubule membranes represent only 30% of total surface area, they generate ∼70% of \(I_{\alpha_2}\) and ∼37% of \(I_{\alpha_1}\). \(I_{\alpha_1}\) density was substantially higher than \(I_{\alpha_2}\) in SSL (\(I_{\alpha_1}/I_{\alpha_2}=16:1\)) but this was markedly reduced in T-tubules (4:1). In addition to differential localisation, isoprenaline (ISO, 1 μmol/L) significantly increased α\(_1\)-mediated NKA Na\(^+\) affinity (from 16.6±0.8 to 13.3±1.4 mmol/L) and caused a small increase in maximal NKA Na\(^+\) efflux rate. ISO had no effect on α\(_2\)-mediated NKA activity.

Conclusion: These data suggest that NKA α\(_1\) and α\(_2\) subunits are differentially localised and regulated by PKA in T-tubule and SSL membranes and may have distinct regulatory roles in cardiac excitation–contraction coupling.

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1. Introduction

The cardiac Na\(^+\)/K\(^+\)-ATPase (NKA) is the primary mechanism by which intracellular sodium ([Na\(^+\)], and hence intracellular calcium [Ca\(^{2+}\)], is regulated in the heart. The NKA establishes and maintains the physiological transmembrane [Na\(^+\)] gradient which is essential for a plethora of cellular functions [17,20,26,45] and indirectly controls myocardial contractility by influencing Na\(^+\)/Ca\(^{2+}\) exchange (NCX) activity [30,34] and indirectly setting sarcoplasmic reticulum (SR) Ca\(^{2+}\) load and contractility.

The NKA is a heteromeric enzyme composed of an α subunit (112 kDa) and a glycosylated β subunit (53 kDa) [27]. The catalytic α-subunit contains binding sites for Na\(^+\), K\(^+\), ATP and cardiac glycosides. Four isoforms of the α-subunit have been identified and demonstrate tissue-specific distribution [28,38,41,44]. It is widely reported that the existence of multiple α-subunit isoforms with tissue-specific...
distribution, is coupled to specialised and specific physiological roles [19,31,32,40,46]. Cardiac α isoform expression varies depending on species. α1 and α2 are expressed in rat, guinea pig and mouse heart [15,22], while three isoforms (α1, α2, and α3) are present in human heart [23,39]. Experimentally, NKA α1 and α2 activity can be distinguished based on their differing sensitivity to cardiac glycosides. In voltage-clamped guinea-pig ventricular myocytes, Gao et al. [14] demonstrated a clear biphasic relationship between increasing concentration of dihydro-ouabain (DHO) and inhibition of whole-cell Na+/K+ pump current (Ipump). This biphasic relationship was due to the presence of both high DHO affinity α2 pumps and low affinity α1 pumps.

In physiological terms, it has been proposed that the Na+/K+-ATPase may be specifically tailored for a tissue by differential expression of a mix of functionally different pump isoforms [14]. Studies by Lingrel and colleagues investigated the possibility that α-subunit isoforms are functionally and spatially distinct in the mouse heart. Measurement of cardiac contractility in Langendorff perfused mouse hearts with genetically reduced levels (∼50%) of cardiac Na+/K+-ATPase α1 or α2 isoforms lead to the proposal of a compartmentalisation model whereby α2 regulates [Ca2+]i, and cardiac contractility within membrane regions (T-tubules) in close proximity to the Ca2+ regulatory machinery (e.g. L-type Ca2+ channels, sarcoplasmic reticulum Ca stores, NCX) and α1 localises to the surface sarcolemma and plays a general housekeeping role by regulating bulk [Na+]i [22]. In agreement with this concept, selective inhibition of α2 activity in mouse astrocytes with genetically modified levels of α2 subunit expression, increases [Na+]i, and [Ca2+]i, (via NCX) in the cytosolic environment between plasma (PM) and endoplasmic reticulum (ER) membranes [16].

Although the validity of the compartmentalisation model has recently been contested [11,37], immunofluorescence studies in guinea-pig ventricular cardiac myocytes suggest that α1 subunits are predominantly located in the peripheral sarcolemma whilst α2 are mainly distributed in the T-tubules [42]. A similar pattern has been reported in primary cultured rat astrocytes, neurons and arterial myocytes [24], but the opposite pattern has been reported in rat ventricular myocytes [29]. Further studies are required to clarify this situation.

Myocyte detubulation enables direct functional measurements of ion channel and transporter function in surface sarcolemma (SSL) vs. T-tubule membranes [3,25]. Detubulation is achieved by subjecting myocytes to osmotic shock which seals off the T-tubules leaving them functionally intact but isolated from the SSL. In detubulated myocytes only currents carried by SSL channels and transporters are accessible. By this method it has been demonstrated that L-type Ca2+ current (ICa) [25], NCX activity, and Na+/K+-ATPase activity [8,47] are preferentially concentrated in the T-tubules of rat ventricular myocytes. This evidence is in favour of a model whereby all the components required for efficient excitation–contraction coupling are localised in the T-tubules and in close proximity to the SR Ca2+ store. However, detubulation has yet to be used to investigate the distribution of Na+/K+-ATPase α1 and α2-subunit function in T-tubule and SSL membranes. This may shed light on different physiological roles of α1 and α2 in the heart.

In the present study we have assessed the functional distribution of α1 and α2 subunits in T-tubule versus SSL membranes by formamide-induced detubulation of mouse ventricular myocytes and measurement of Na+/K+ pump current (Ipump) and Na+/K+-ATPase-mediated Na+ efflux rate (∼d[Na+]i/dt). We have estimated, (i) the composition of plasma membrane surface area in terms of T-tubule and SSL membranes, (ii) Ipump amplitude and density in T-tubule and SSL membranes, (iii) the contribution of Ion1 and Ion2 to total Ipump, (iv) the Ion1/Ion2 ratio in T-tubule and SSL membrane compartments, and (v) the effect of ISO stimulation on α1 and α2 Na+/K+-ATPase activity.

2. Materials and methods

2.1. Animals

All animals used in this study received humane care in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996). This study was also subjected to local ethical review by the Ethical Review Process Committee of King’s College London and Loyola University Chicago.

2.2. Myocyte isolation

C57Bl/6 mice were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg) and heparin (100 IU). Hearts were excised, perfused in a retrograde fashion through Langendorff apparatus. Ventricular myocytes were isolated by Langendorff perfusion and enzymatic dispersion (0.3 mg/mL Type-2 collagenase, Worthington) following a modified version of the AfCS procedure protocol (# PP00000125) [21].

2.3. Myocyte detubulation

Detubulation was induced by osmotic shock as described previously [25]. Briefly, 1.5 mol/L formamide was added to the cell suspension for 15–20 min, then withdrawn. Myocytes were plated on laminin-coated cover-slips for 20 min and incubated with 10 μmol/L di-8-aminophylythylpyridinium (di-8-ANEPPS) in Ca2+-free Tyrode solution (in mmol/L, NaCl 137, KCl 5.4, MgCl2 0.5, glucose 10, HEPES 10, pH 7.4) in the dark for 20 min, imaged by confocal microscopy (excitation 488 nm, peak emission 515+/- 15 nm) and analysed with Image J software (NIH). All confocal images were captured midway through the myocyte z-axis.
2.4. Electrophysiological recording of Na+/K+ pump current (£pump)

Mouse ventricular cardiac myocytes were voltage-clamped and whole-cell £pump recorded at 35 °C using the perforated-patch technique. Electrodes were made from thin-walled (1.5 mm outer diameter, 1.17 mm inner diameter) borosilicate glass capillaries (Harvard Apparatus Ltd, UK) and fire-polished using a three-stage electrode puller (DMG Universal Puller, Zeitz-Instrumente Vetries GmbH, Germany). Electrode resistance was 1–2 MΩ when filled with the standard pipette solution. Following gigahm seal formation, series resistance was monitored with a repetitve 5 mV pulse (~80 mV holding potential). During membrane permeabilization, series resistance typically fell to 10–15 MΩ within 10 min. Membrane capacitance was recorded after permeabilization by standard techniques [4] by imposing a 25 ms square step from ~80 to ~75 mV and integrating the area under the capacitance transient. £pump was recorded continuously at 10 Hz sampling frequency at 0 mV. Pipette and extracellular solutions were designed to inhibit all voltage-gated channels and the Na/Ca exchanger. Standard pipette solution contained (in mmol/L) NaCl 140, KCl 5, MgCl2 1, NiCl2 2, BaCl2 1, procaine 0.5, glucose 10, extracellular solution (5K) contained (in mmol/L) NaCl 140, pipette solution on the day of use. Standard K-containing myocytes and the Na/Ca exchanger. Standard pipette cellular solutions were designed to inhibit all voltage-gated 10 Hz sampling frequency at 0 mV. Pipette and extracellular solutions were designed to inhibit all voltage-gated channels and the Na/Ca exchanger. Standard pipette solution contained (in mmol/L) CsCH3O2S 90, NaCH3O2S 35, NaCl 15, CsCl 5, MgCl2 1, HEPES 10, pH 7.2 at 35 °C with CsOH. Amphotericin B (225 μg/mL) (from Streptomyces, Sigma, UK) in DMSO (0.74% v/v) was added to the pipette solution on the day of use. Standard K-containing extracellular solution (5K) contained (in mmol/L) NaCl 140, KC1 5, MgCl2 1, NiCl2 2, BaCl2 1, procaine 0.5, glucose 10, HEPES 10, pH 7.4 at 35 °C. K-free solution (0K) was prepared by removing KC1 with no correction for osmoregulation. In all experiments £pump was defined as that sensitive to the removal of extracellular K and was calculated as the product of steady-state 5K minus 0K current. Ouabain was added to 5K solution on the day of use (from a 10 mmol/L stock) and protected from light.

2.5. Measurement of Na+ efflux through the Na+/K+ pump

Na+K-pump flux was determined as the rate of pump-mediated [Na+]i decline and dual excitation fluorescence measurements (at 340 and 380 nm; F340 and F380) were performed as previously described [9]. Myocytes were Na+-loaded by inhibiting the Na+/K+ pump in a K+-free solution containing (mmol/L): 145 NaCl, 2 EGTA, 10 HEPES, and 10 glucose (pH =7.4). [Na+]i, decline was measured on pump reactivation in solution containing (mmol/L): NaCl 140, TEA-Cl 4 KCl, 2 EGTA, 1 MgCl2, 10 HEPES, and 10 glucose (pH =7.4). Because cell volume does not change with this protocol [10], [Na+]i, decline reflects Na+ efflux. The rate of [Na+]i, decline (~d[Na+]i/dt) was plotted versus [Na+]i, and fitted with: ~d[Na+]i/dt= Vmax (1 + (Km/[Na+]i)N+1. In separate experiments, ~d[Na+]i/dt was measured in the presence of 10 mmol/L ouabain to determine Na+ pump independent Na+ efflux. This was subtracted from Na+/K+ pump-mediated efflux. In some experiments, cells were treated with 1 μmol/L ISO during the latter part of pump inhibition and throughout reactivation. Ouabain (10 μmol/L) was used in some experiments to preferentially inhibit high ouabain affinity Na+/K+-ATPase α2 subunits. All experiments measuring [Na+]i, with SBFI were carried out at room temperature (25 °C).

2.6. Statistical analysis

Quantitative data are shown as mean±standard error of the mean (SEM). n values for electrophysiological experiments are given as the number of cells from number of animals. Student t test was used for statistical discriminations, with P<0.05 considered significant and non-significance indicated (ns).

3. Results

3.1. Formamide treatment induces detubulation of mouse ventricular myocytes

Fig. 1A shows an x–y confocal image of a control myocyte stained with di-8-ANEPPS (representative of the staining pattern in 30 cells from 7 hearts): the T-tubule membrane network is clearly visible. The dotted line running along the longitudinal axis of the cell marks the area of fluorescence intensity (FI) analysis and shows repetitive fluorescence peaks at ~2 μm intervals and two larger peaks representing SSL membrane staining. Fast Fourier transformation (FFT) of FI profiles allows determination of T-tubule interval. FFT of FI profiles from control myocytes (13 cells from 6 hearts) gave a prominent peak at 0.54±0.01 μm and i.e., a mean T-tubule interval of 1.84±0.02 μm. Fig. 1B is a representative (54 cells from 7 hearts) x–y confocal image of a di-8-ANEPPS stained detubulated myocyte. FFT of FI profiles from detubulated myocytes reveals no periodicity. These results are indicative of successful detubulation and limited dye access from the SSL to the T-tubule membrane network in detubulated myocytes.

3.2. Cell capacitance and I_pump distribution in surface sarcolemmal and T-tubular membrane compartments

In cardiac myocytes the membrane system is composed of surface sarcolemmal (SSL) and T-tubular compartments. T-tubule cell capacitance and localised Na+/K+ pump current were calculated by subtracting control values from those recorded in detubulated myocytes (T-tubule=Total−SSL). Membrane capacitance was reduced from 181±12 pF in control to 126±17 pF in detubulated myocytes. These data demonstrate that membrane surface area is composed of 30% T-tubule (54 pF) and 70% SSL (126±17 pF) membranes. £pump amplitude recorded at 0 mV in 5 mmol/L KCl, and 50 mmol/L [Na+]i, was reduced from 257±22 pA in control myocytes (1.42±0.1 pA/pF) to 151±17 pA in
detubulated myocytes (1.20 ± 0.04 pA/pF). Therefore, although T-tubules represent only 30% of the membrane surface area, Na⁺ pumps residing there generate ~41% (106 pA) of total Iₚump (the remaining 59% is generated in the SSL). These data are summarised in Fig. 2. Normalising Iₚump amplitude data to cell capacitance demonstrates that functional Iₚump density in T-tubular membranes (1.94 pA/pF) is 60% higher than in SSL membranes (1.20 pA/pF) (T-tubule:SSL Iₚump density ratio = 1.6:1).

We also measured the rate of Na⁺/K⁺-ATPase-mediated Na⁺ efflux (−d[Na⁺]/dt) in control and detubulated myocytes (Fig. 3). Maximal Na⁺ efflux rate (V_max) was 10.7 ± 1.9 mmol/min in control myocytes and 7.5 ± 0.9 mmol/min following detubulation. Despite this not achieving the level of statistical significance, this ~30% difference in the mean V_max values is not incompatible with
the suggestion from the voltage-clamp data that T-tubular Na+/K+-ATPase activity accounts for about ∼40% of the total cellular Na+ efflux.

3.3. *,I,*pump composition in control myocytes (SSL and T-tubule membranes)

The contribution of $\alpha_1$ and $\alpha_2$ to total $I_{\text{pump}}$ was defined by investigating the dose-dependent inhibition of $I_{\text{pump}}$ with ouabain. $\alpha_1$ (low-affinity) and $\alpha_2$ (high-affinity) were defined by their differing sensitivity to ouabain. Maximal $I_{\text{pump}}$ inhibition was achieved by exposure to 10 mmol/L ouabain or 0K solution. Recovery from inhibition was rapid and complete with a return to the pre-inhibition level within 3 min. Fig. 4A is a current recording demonstrating dose-dependent inhibition of $I_{\text{pump}}$ with ouabain. Average data representing the percentage inhibition of $I_{\text{pump}}$ by ouabain was fit with a two-site binding hyperbolic function and $I_{\text{pump}}$ determined by curve stripping (Fig. 4B). In control myocytes $\alpha_1$ contributed 88% to the total recordable current with a $K_d$ of 10.4 μmol/L and $\alpha_2$ contributed the remaining 12% with a $K_d$ of 0.3 μmol/L.

To support the above $I_{\text{pump}}$ data, Na+/K+-ATPase-mediated Na+ efflux was recorded in the presence of low dose ouabain (10 μmol/L) to preferentially inhibit Na+/K+-ATPase $\alpha_2$ subunits. Curve stripping analysis of our $I_{\text{pump}}$ data (Fig. 4B) suggests that 10 μmol/L ouabain, will inhibit 97% of $\alpha_2$-mediated Na+/K+-ATPase activity and only 9% $\alpha_1$ activity. Under these conditions $\alpha_1$-mediated Na+ efflux predominates due to 11-fold specificity for $\alpha_2$ inhibition. $\alpha_2$-mediated Na+/K+-ATPase activity was calculated as the difference between total and $\alpha_1$-mediated activity. Fig. 5 represents total Na+/K+-ATPase-mediated Na+ efflux, and that mediated via $\alpha_1$ and $\alpha_2$ subunits. Curve fitting with the Hill equation demonstrated that maximal Na+ efflux rate ($V_{\text{max}}$) was 10.7±1.9 mmol/min in control myocytes. 80% of this Na+ efflux capacity was due to $\alpha_1$ subunits (8.6±1.6 mmol/min) and the remaining 20% via $\alpha_2$. These data correlate well with estimates of $I_{\text{pump}}$ composition in terms of $\alpha_1$ and $\alpha_2$. Additionally, these data suggest that $\alpha_1$ and $\alpha_2$ subunits have very similar affinity for Na+ ions, with $K_m$ values of 16.6±0.8 and 16.7±2.6 mmol/L respectively.
3.4. Iα1 and Iα2 amplitude and density in SSL and T-tubular membranes

Having determined the contribution of Iα1 and Iα2 subunits to Ipump in control myocytes we constructed a ouabain dose–response curve in detubulated myocytes. Under these conditions, Iα1 contributes 94% and Iα2 only 6% to total recordable Ipump (Kd for ouabain of 170 and 0.2 μmol/L respectively) (Fig. 6). These data define the percentage composition of total Ipump in terms of Iα1 and Iα2 in the SSL membrane compartment.

In control myocytes cell capacitance was 181 pF and total Ipump amplitude (257 pA) was composed of 88% Iα1 (226 pA) and 12% Iα2 (31.1 pA). Therefore, Iα1 density was 1.25 pA/pF, and Iα2 density was 0.17 pA/pF. In detubulated myocytes (in which only SSL membranes contribute to cell capacitance and only SSL pumps contribute to whole-cell Ipump), total Ipump amplitude (151 pA) was composed of 94% Iα1 (142 pA) and 6% Iα2 (9.4 pA). After normalising for SSL cell capacitance (127 pF), SSL Iα1 density was 1.12 pA/pF and Iα2 density was 0.07 pA/pF. With the above information, T-tubule Iα1 and Iα2 amplitude (84 pA and 22 pA respectively) and density (1.54 pA/pF and 0.39 pA/pF respectively) can be determined mathematically. These data are summarised in Table 1, panel A.

3.5. β-adrenergic regulation of Na+/K+/ATPase α1 and α2 subunits

In addition to investigating whether Na+/K+/ATPase α-subunit isoforms demonstrate differential subcellular localisation, we also determined whether this is associated with differential regulation by PKA. 1 μmol/L ISO was used to maximally activate the protein kinase A (PKA) signalling cascade and mimic the effect of β-adrenergic stimulation. Under control conditions, ISO induced a significantly (P= 0.035) ~26% increase in Na+ sensitivity, shifting the Km for Na+ (K1,2 Na+) from 16.6±0.8 mmol/L to 12.3±1.3 mmol/L but had no effect on Vmax (from 10.7±1.9 to 11.2±1.7 mmol/min) (Fig. 7A). In the presence of 10 μmol/L ouabain, ISO induced a similarly significant (P=0.048) 20% stimulation of α1-mediated Na+/K+/ATPase Na+ affinity (Km decreasing from 16.6±0.8 to 13.3±1.4 mmol/L) again with no effect on Vmax (from 8.6±1.6 to 9.3±0.7 mmol/min) (Fig. 7B). α2-mediated Na+/K+/ATPase activity was calculated mathematically as previously described (Fig. 7C). ISO had no effect on α2-mediated Vmax (from 2.1±3.1 to 1.8±2.9 mmol/min).

| Table 1A | α1 and Iα2 in SSL and T-tubule membrane compartments |
|----------|---------------------------------------------------|
| Ipump (pA)  | Iα1 density (pA/pF)  | Iα2 density (pA/pF)  |
| SSL+/T-tubule (control) | 226 | 1.25 | 31 | 0.17 |
| SSL alone (detubulated) | 142 | 1.12 | 9 | 0.07 |
| T-tubule (calculated) | 84 | 1.54 | 22 | 0.39 |

(A) Iα1 and Iα2 amplitude in control (SSL and T-tubule) and detubulated (SSL) myocytes. Iα1 and Iα2 amplitudes in T-tubule were calculated mathematically as described in the text. Iα1 and Iα2 densities were defined by normalising to cell capacitance. Since the individual current amplitudes for α1 and α2 shown are derived from a single curve strip of the mean data, these numbers have no associated errors.
4. Discussion

In the present study we have investigated the distribution and function of Na⁺/K⁺-ATPase α₁ and α₂-subunits in SSL and T-tubule membranes in mouse ventricular cardiac myocytes using the technique of myocyte detubulation.

The accuracy of this experimental approach relies on successful and efficient detubulation. The close correlation between the reduction in cell surface area reported here (~30%) and direct electron microscopic measurements of total sarcolemma in T-tubules in mouse ventricular myocytes (36%) [33] suggests that this method is quantitatively reliable. Furthermore, staining of control myocytes with di-8-ANEPPS revealed a mean T-tubule interval of ~1.84 µm, which is very similar to that observed in the rat (1.86 µm) [3]. Repetitive T-tubular staining was completely lost following detubulation.

Although α₁ and α₂ isoforms are present in mouse ventricular myocytes, α₁ is the predominant Na⁺/K⁺-ATPase isoform. α₁-mediated $I_{\text{pump}}$ ($I_{\alpha_1}$) contributes 88% to total recordable $I_{\text{pump}}$ and α₂-mediated $I_{\text{pump}}$ ($I_{\alpha_2}$) contributes the remaining 12%. The overall subcellular localisation of Na⁺/K⁺-ATPase activity indicates that $I_{\text{pump}}$ density is 60% higher in T-tubule vs. SSL membranes (although T-tubule membranes represent only 30% of total membrane area) and that Na⁺ pumps residing there generate ~41% of total $I_{\text{pump}}$, ~37% of $I_{\alpha_1}$ and ~70% of $I_{\alpha_2}$. Moreover, this study provides the first quantitative determination of the relative distribution of α₁ and α₂-mediated Na⁺/K⁺-ATPase activity in mouse ventricular myocytes. We have shown that $I_{\alpha_1}$ density predominates over $I_{\alpha_2}$ in both SSL and T-tubule membrane compartments. However, the relative ratio of $I_{\alpha_1}$: $I_{\alpha_2}$ is markedly different in T-tubule versus SSL membranes. $I_{\alpha_1}$ density is substantially higher than $I_{\alpha_2}$ in SSL membranes ($I_{\alpha_1}$/$I_{\alpha_2}$ density ratio of 16:1), but in T-tubule, the dominance of $I_{\alpha_1}$ over $I_{\alpha_2}$ is markedly reduced (4:1). Furthermore, the T-tubule:SSL $I_{\alpha_1}$ ratio (4.1:1) suggests that $I_{\alpha_1}$ is relatively uniformly distributed between T-tubule and SSL membranes whereas $I_{\alpha_2}$ is ~5 times more concentrated in the T-tubules (T-tubule:SSL ratio of 5.3:1). These data are summarised in Table 1B. A similar pattern has been reported in rat ventricular myocytes in a recent abstract, with ~4.5 times higher functional density of $I_{\alpha_2}$ in the T-tubules and uniform $I_{\alpha_1}$ distribution between T-tubule and SSL membranes [6].

Recent studies in detubulated rat myocytes have reported that $I_{\text{Ca}_{\text{a}}}$, NCX and Na⁺/K⁺-ATPase activity are concentrated

### Table 1B

| Relative ratios | $I_{\text{pump}}$ density (pA/pF) | $I_{\alpha_1}$ density (pA/pF) | $I_{\alpha_2}$ density (pA/pF) | $I_{\alpha_1}$: $I_{\alpha_2}$ |
|-----------------|----------------------------------|---------------------------------|---------------------------------|-----------------------------|
| SSL             | 16:1                             | 0.07:1                          |                                 |                             |
| T-tubule        | 4:1                              | 0.26:1                          |                                 |                             |
| T-tubule:SSL ratio | 1.6:1                           | 1.38:1                          | 5.33:1                          |                             |

(B) Relative ratios of $I_{\text{pump}}$ composition in SSL and T-tubule membranes derived from the data in panel A.
in the T-tubules [8,25,47]. Hence, co-localisation of a specific Na+/K+-ATPase α subunit isoform with NCX and the L-type Ca channel in T-tubules would form a structural basis of cardiac excitation–contraction coupling and local control of contractility as described by the compartmentalisation model of James et al. [22]. Recently, the validity of this compartmentalisation model has been contested [11,37], and the authors have now concluded that both α1 and α2 isoforms can indirectly regulate cardiac contractility through modulation of forward mode Na/Ca exchange. Our data suggests that α1 and α2 subunits are differentially localised. IA1 is greater than IA2 in both T-tubule and SSL membrane compartments but the predominance of IA1 over IA2 is lower in T-tubule. Therefore, it is possible that by altering the extent to which α1 predominates over α2 (i.e. altering the IA1/IA2 balance), previously hidden differential physiological roles for the two isoforms may be revealed.

In addition to differential subcellular localisation we have also demonstrated that ISO significantly stimulates α1-mediated Na+/K+-ATPase activity (predominantly via an increase in Na+/K+-ATPase Na+ affinity). In agreement, recent data from our laboratory have demonstrated isoform-specific stimulation of IA1 in guinea-pig myocytes following PKA stimulation with forskolin [42]. Conversely, in the present study we have shown that ISO has no significant effect on α2-mediated Na+/K+-ATPase activity, but due to the small contribution of α2 to total Na+/K+-ATPase activity coupled with its mathematical derivation, this conclusion should be viewed with caution. Previously published studies from ourselves [42] and others [15] have also suggested that β-stimulation activates α1 but not α2. However, on the basis of the data presented in this present study, it is possible that ISO does influence α2-mediated pump function but this is below the limit of detection of this method.

In terms of β-adrenergic stimulation in the heart, many proteins involved in excitation–contraction coupling are targets for PKA phosphorylation (e.g. L-type Ca2+ channel, phospholamban, ryanodine receptor, troponin I). Until recently the exact mechanism of Na+/K+-ATPase pump regulation by PKA has remained elusive. It is now clear that this role is played by phospholemman (PLM) [1,7,42], a member of the FXYD family of proteins that are tissue specific regulators of the Na+/K+ pump. PLM is the primary sarcolemmal substrate for PKA [35] and PKC [36] and regulates the cardiac Na+/K+ pump by applying a tonic inhibition that is relieved by genetic PLM knockout and PLM phosphorylation [1,7]. Previously, we have demonstrated both functional and physiological association between PLM and the Na+/K+-ATPase α1 isoform [42] (but not with α2), and more recently we have demonstrated that the stimulatory effect of ISO on Ipump in PLM wildtype voltage-clamped mouse ventricular myocytes is preferentially mediated via stimulation of α1-mediated current [1]. With regards to biochemical evidence, co-immunoprecipitation studies have demonstrated association of PLM with the α2 subunit in rabbit ventricular myocytes and bovine sarclemmal microsomes [2,5]. However no association has been reported in guinea-pig ventricular myocytes and rat cardiac homogenates [13,42].

The evidence presented here supports a model whereby α1 and α2 subunits demonstrate differential localisation and potentially differential regulation by PKA/β-adrenergic stimulation in mouse ventricular T-tubule and SSL membranes. As β-stimulation leads to elevated [Na+]i, as a direct consequence of positive chronotropy [12,18,43], it may be that the α1 subunit, located in SSL membranes and regulated by PLM, is primarily involved in controlling bulk [Na+]i, (as suggested by James et al. [22]) and controlling the delicate balance of [Na+]i, by allowing it to rise sufficiently to contribute to the positive inotropic effect of β-stimulation while protecting against the deleterious effects of [Na+]i, and [Ca2+]/ overload which may lead to cardiac arrhythmias and diastolic dysfunction.

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