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ORIGINAL PAPER

Late Na^+ current produced by human cardiac Na^+ channel isoform $Na_v1.5$ is modulated by its β_1 subunit

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Abstract Experimental data accumulated over the past decade show the emerging importance of the late sodium current (I_{NaL}) for the function of both normal and, especially, failing myocardium, in which I_{NaL} is reportedly increased. While recent molecular studies identified the cardiac Na^+ channel (NaCh) α subunit isoform ($Na_v1.5$) as a major contributor to I_{NaL}, the molecular mechanisms underlying alterations of I_{NaL} in heart failure (HF) are still unknown. Here we tested the hypothesis that I_{NaL} is modulated by the NaCh auxiliary β subunits. tsA201 cells were transfected simultaneously with human $Na_v1.5$ (former hH1a) and cardiac β_1 or β_2 subunits, and whole-cell patch-clamp experiments were performed. We found that I_{NaL} decay kinetics were significantly slower in cells expressing $\alpha + \beta_1$ (time constant $\tau = 0.73 \pm 0.16$ s, $n = 14$, mean $\pm$ SEM, $P < 0.05$) but remained unchanged in cells expressing $\alpha + \beta_2$ ($\tau = 0.52 \pm 0.09$ s, $n = 5$), compared with cells expressing $Na_v1.5$ alone ($\tau = 0.54 \pm 0.09$ s, $n = 20$). Also, β_1, but not β_2, dramatically increased I_{NaL} relative to the maximum peak current, I_{NaT} ($2.3 \pm 0.48\%$, $n = 14$ vs. $0.48 \pm 0.07\%$, $n = 6$, $P < 0.05$, respectively) and produced a rightward shift of the steady-state availability curve. We conclude that the auxiliary β_1 subunit modulates I_{NaL}, produced by the human cardiac Na^+ channel $Na_v1.5$ by slowing its decay and increasing I_{NaL} amplitude relative to I_{NaT}. Because expression of $Na_v1.5$ reportedly decreases but β_1 remains unchanged in chronic HF, the relatively higher expression of β_1 may contribute to the known I_{NaL} increase in HF via the modulation mechanism found in this study.

Keywords Whole-cell sodium current · Heterologous expression · Human sodium channel subunits

Introduction

Experimental data accumulated over the past decade show the emerging importance of the late sodium current (I_{NaL}) for the function of both normal and, especially, failing myocardium, in which I_{NaL} is reportedly increased [1–3] The importance of the contribution of I_{NaL} to heart failure (HF) mechanisms has been demonstrated in experiments in which "correction" of I_{NaL} in failing cardiomyocytes resulted in:

1. rescue of normal repolarization;
2. reduced beat-to-beat action potential duration (APD) variability; and
3. improvement of Ca^{2+} handling and contractility [1, 3–5].

Accordingly I_{NaL} has recently emerged as a novel possible target for cardioprotection to treat the failing heart [6, 7].

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Voltage-clamp studies have identified several types of single Na^+ channel activity and whole-cell Na^+ currents that could contribute to APD in cardiomyocytes. The variety of Na^+ channel activities identified so far has been classified (for review see Ref. [6]) in terms of the late (or persistent) Na^+ current i.e. I_{NaL} (or I_{pNa}), and background Na^+ currents. In contrast with I_{NaL}, background Na^+ currents have been poorly characterized and have no clear molecular identity.

Major biophysical and pharmacological characteristics of the whole-cell I_{NaL} have been studied in great detail in human cardiomyocytes by our research group [3, 8, 9] and can be summarized as follows:

1. potential-independent slow inactivation and re-activation ($\sim$0.5 s);
2. steady-state activation and inactivation similar to that for I_{NaT}; and
3. low sensitivity to the specific toxins TTX and STX similar to the cardiac Na^+ channel isoform $Na_v1.5$.

A slowly inactivating I_{NaL} with aforementioned biophysical characteristics has been identified in ventricular cardiomyocytes and cardiac Purkinje cells of dogs [1, 3, 5, 10–12], guinea pigs [13–15], rabbits [16], rats [17] and mice [18]. I_{NaL} is also produced by the heterologously expressed cardiac Na^+ channel isoform main α-subunit $Na_v1.5$ [7, 19].

Despite explosive interest in this new component of the Na^+ current (for recent reviews see Refs. [6, 7, 14, 20]) the mechanisms of I_{NaL} regulation in normal heart and its alterations in HF are not yet understood and are likely to need further collective efforts based on different approaches including detailed biophysical and molecular biology examinations in addition to traditional pharmacological studies. Utilizing antisense inhibition and siRNA technologies our most recent studies explored the molecular identity of I_{NaL} in ventricular cardiomyocytes [7, 21]. These studies suggested the cardiac Na^+ channel α-subunit isoform ($Na_v1.5$) was a major contributor to I_{NaL}.

Although most recent studies have shown that I_{NaL} is strongly and differently modulated by intracellular Ca^{2+} in the cardiomyocytes of normal and failing hearts [18, 22], Na^+ channels operate not in isolation but within macromolecular complexes [23, 24], which are critical attributes of Na^+ channel function (in addition to membrane voltage and ion concentrations). The macromolecular complexes include auxiliary β-subunits, phospholipids and elements of the cytoskeleton, each of which can modulate Na^+ channel function including I_{NaL} (for review see Ref. [7]). The β-subunit gene family has four members—β_1 (SCN1B), β_2 (SCN2B), β_3 (SCN3B), and β_4 (SCN4B) (for review see Ref. [24]). Despite high homology between β_1 and β_3, and β_2 and β_4 the different functional role of these newly discovered isoforms (β_3 and β_4) could not be ruled out. In addition there is a splice variant β_1A of SCN1B that is expressed in embryonic brain and adult heart in rat [25]. All five β-subunits are expressed in rodent heart and are differently localized to specific sub-cellular domains and cell types. The β_1 subunit is non-covalently attached to the α subunit, and the β_2 subunit is covalently linked to the α subunit by a disulfide bond [26]. Numerous studies indicate a possible role of β auxiliary subunits in modulating Na^+ channel expression and function (for review see Ref. [24]), but the possible implication of β-subunits in I_{NaL} modulation has not been studied in detail, especially in HF. Our previous studies using the canine chronic HF model showed that in the state of HF the protein level of $Na_v1.5$ is reduced but remains unchanged for β_1 and β_2 subunits, making these β subunits relatively upregulated [27].

Thus, an intriguing possibility could be that differential expression of α- and β-subunits in normal and failing hearts can contribute, at least in part, to I_{NaL} alterations observed in HF. Accordingly, in this study, using a heterologous expression system, we specifically tested whether I_{NaL} is modulated by β_1 or β_2 co-expression with $Na_v1.5$. Our experiments show that β_1 substantially and significantly affects I_{NaL}, whereas the effects of β_2 were insignificant. More specifically, the β_1 subunit modulates I_{NaL} by two mechanisms one of which slows I_{NaL} decay and the other of which increases I_{NaL} amplitude relative to the peak transient (I_{NaT}) current.

Methods

Human kidney epithelial cells tsA201 were transiently transfected by $Na_v1.5$ alone and/or simultaneously with human cardiac β_1 or β_2 subunits (tagged by GFP or fivefold excess of β-encoding cDNA, as previously suggested [28]). We chose tsA201 cells because there was no expression of endogenous α or β_1 and β_2 subunits compared with that of some HEK293 cell lines (our unpublished data). Whole-cell recordings were made using the conventional patch-clamp technique. The heterologous expression system provides an unique possibility of measuring the peak transient current (I_{NaT}) and I_{NaL} at the same physiological $[Na^+]_o$, = 140 mM. This is impossible in cardiomyocytes because of voltage-control problems. The I_{NaL} amplitude was measured as averaged current within 200–220 ms after the onset of depolarization (2 s duration) and was normalized to the peak I_{NaT} (see examples in Fig. 3a, b). The I_{NaL} decay was evaluated by the single-exponential fit starting 200 ms after the onset of depolarization, as previously suggested [8]. The data points of the peak current in the current–voltage relationships were fitted to the function [29]:

$$I_{Na}(V_t) = G_{max} * (V_r - V_t)/\left(1 + \exp\left(\left(V_{1/2G} - V_t\right)/k_G\right)\right) \quad (1)$$

where G_{max} is a normalized maximum Na^+ conductance, V_r is a reversal potential, V_t is the testing voltage, and $V_{1/2G}$ and k_G are the midpoint and slope of the respective Boltzmann functions underlying the NaCh steady-state activation (SSA). The data points were fitted to model equations by use of nonlinear regression (Origin 7.0 software, Microcal Software, MA, USA).

The steady-state availability (or inactivation, SSI) terms ($V_{1/2A}$, the midpoint and k_A, the slope of the relationship) were measured by a standard double-pulse procedure with the 2-s-duration pre-pulse (V_p) ranging from −130 to −40 mV. The data points of I_{NaT} normalized to I_{NaT} measured at −30 and −130 mV prepulse were fitted to a Boltzmann function A (V_p):

$$A\left(V_p\right) = 1/\left(1 + \exp\left(\left(V_p - V_{1/2A}\right)/k_A\right)\right). \quad (2)$$

Full details can be found in an extended "Materials and methods" section in the online supplement linked with the online version of this article.

Results

The reporter GFP gene expression monitored the transient transfection of the β-subunits (Fig. 1). Evidently both β_1 (Fig. 1a) and β_2 (Fig. 1b) can be detected both perinuclear and, importantly, in membrane compartments of the cells in the optical slices under the confocal microscope. Our confocal imaging thus validated the transfection procedure, ensuring that β subunits are located in the cell membrane together with functional Na channel α subunits. It is also important to note that we did not make any attempt to quantify the β-subunits expression level based on these results because our objective was to study β subunit effects on I_{NaL} in all-or-none fashion.

To carefully evaluate I_{NaL}, we averaged 20–50 original current traces, and the "zero" current (obtained after TTX 25 μM application) was subtracted from the current traces (typical examples are shown in Fig. 2). We determined effects of β subunits on I_{NaL} kinetics and I_{NaL} relative amplitude compared with that of I_{NaT} (Fig. 3). We found that co-expression of α with the β_1 subunit but not the β_2 subunit significantly slows I_{NaL} decay (Fig. 3a) and substantially increases the relative amplitude of I_{NaL} (Fig. 3b). Accordingly $\alpha + \beta_2$ can be interpreted as a mock control for these studies. The effect of the β_1 subunit on these I_{NaL} values was independent of the reporter GFP gene. This is evident from comparison of the β_1-GFP construct with β_1 alone (Fig 3b, c). Furthermore, the β_1 subunit caused a significant rightward shift of the SSI curves for both peak

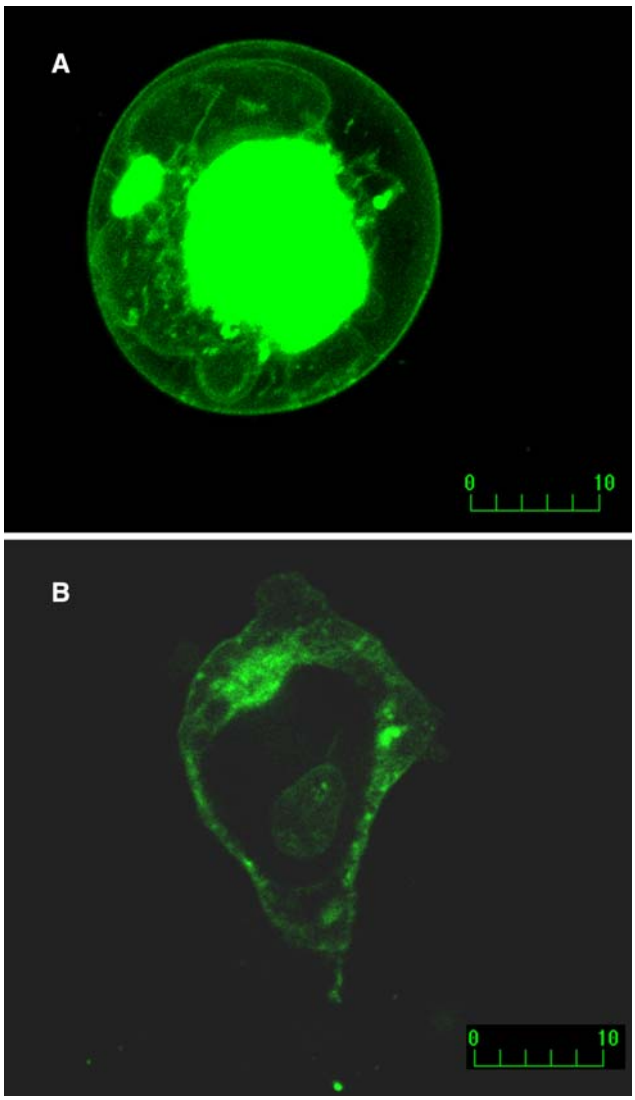

Fig. 1 Confocal microscopy of the live tsA201 cells transfected with $Na_v1.5 + \beta_1$ (**a**) or $Na_v1.5 + \beta_2$ (**b**). Fluorescence of GFP linked to β subunit C-terminus at the cell membrane is evident for both β subunits. Optical slices were 0.5 μm (Zeiss Axiovert 100, Bio-Rad MRC 1024, excitation/emission wavelength 488/522 nm, laser power 10%)

I_{NaT} and I_{NaL} (measured at 200 ms; not shown). Representative examples of I_{NaT} are shown in Fig. 4, statistics for SSI data are given in Table 1. The current–voltage relationship and the steady-sate activation values remained unchanged (Fig. 5, Table 1).

Discussion

For the first time we demonstrate that the β_1 subunit can significantly modulate I_{NaL} produced by the heterologously expressed $Na_v1.5$. The modulation includes slowed

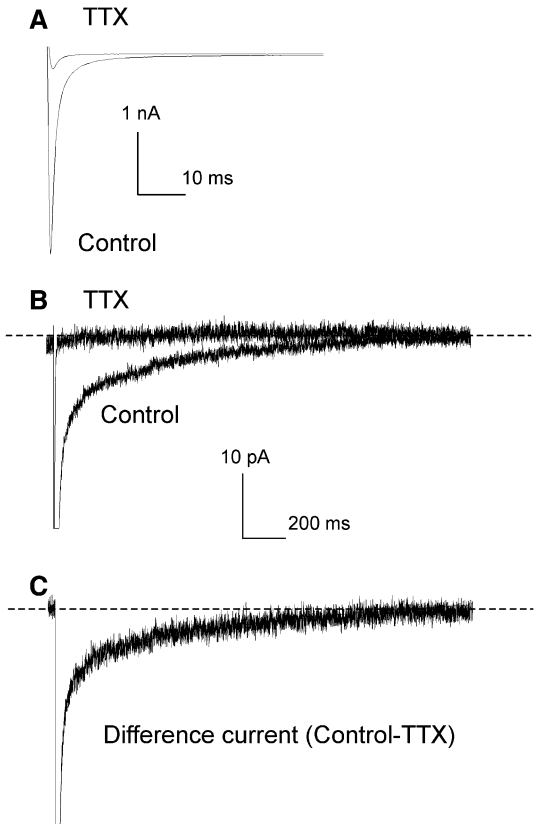

Fig. 2 Experimental approach used to elucidate I_{NaL} of heterologously expressed $Na_v1.5$. **a**, **b** Typical examples of the I_{Na} currents recorded in tsA201 cells transiently transfected by $\alpha + \beta_1$-GFP. Shown are averaged traces from 50 sweeps before and after TTX (25 μM) application to assess "zero" current. **c** Difference I_{NaL} current obtained by subtraction of "zero" current. Note different currents and time scales in **a**, **b**, and **c** to demonstrate peak (I_{NaT}) and I_{NaL}. $V_h = -120$ mV, $V_m = -30$ mV, 23°C

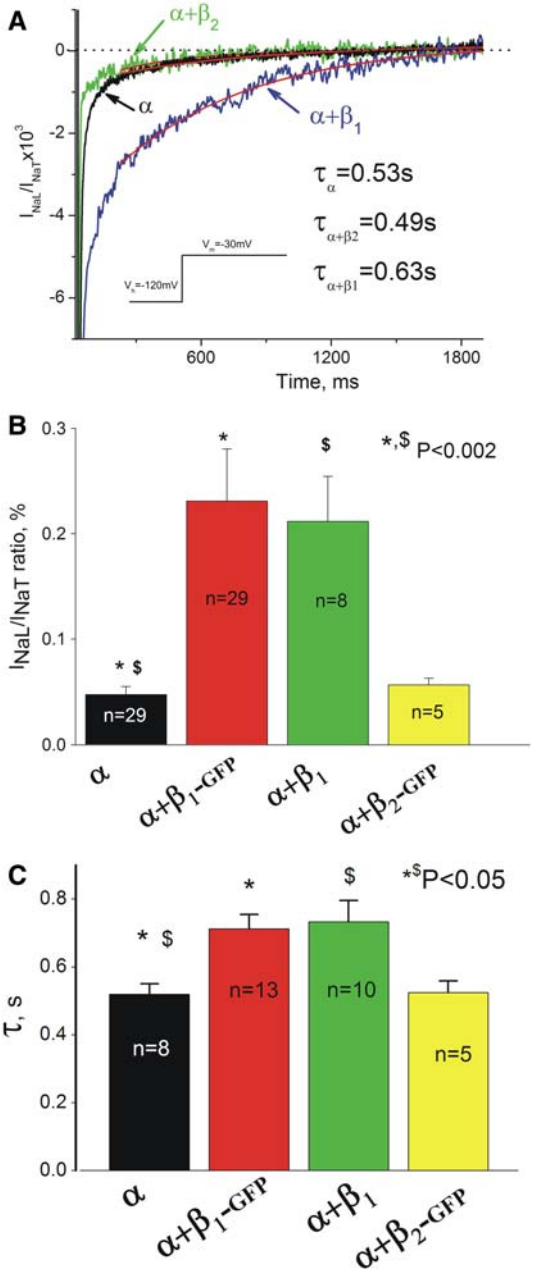

Fig. 3 Effects of the β subunits on the I_{NaL} produced by the heterologously expressed human cardiac NaCh isoform α subunit ($Na_v1.5$). **a** Representative examples of superimposed current traces recorded in tsA201 cells transfected with cDNA encoding human cardiac NaCh α-subunit alone or together with β_1 ($\alpha + \beta_1$) or β_2 ($\alpha + \beta_2$). Shown are averaged (10–20) currents with a single-exponential fit to I_{NaL} decay (*solid lines*) starting 200 ms after the onset of depolarization. The time constant (τ) values are given in the panel. The current amplitudes are relative to the peak I_{NaT}. The voltage-clamp procedure given in the *inset*. **b**, **c** Statistical analysis of the I_{NaL}/I_{NaT} ratio (**b**) and the decay time course (**c**) changes in response to coexpression of α with β subunits. The statistically significant difference (*P*) in panels **b** and **c** was evaluated by ANOVA followed by the Bonferroni's post hoc test. *Bars* in panels **b** and **c** represent means ± SE, *n* number of cells. There was no significant difference between $\alpha + \beta_1$-GFP compared with $\alpha + \beta_1$ (1:5 cDNA ratio) or for α alone compared with $\alpha + \beta_2$-GFP for both **b** and **c** panels

inactivation, augmented amplitude relative to I_{NaT}, and rightward SSI shift.

Although β subunits do not form ion-conducting pores, they are important modulators of Na_v function, expression levels at the plasma membrane (trafficking), and cell adhesion [23, 24]. Recent studies support the emerging significance of the β_1 auxiliary subunit in modulation of $Na_v1.5$ function. It has been shown that the β_1-subunit:

1. is involved in abnormal NaCh activity associated with the LQT3 mutation [30];
2. aggravates NaCh dysfunction in Brugada syndrome [31];
3. modifies block of NaCh by fatty acids [32] and lidocaine [33]; and
4. modulates trafficking of $Na_v1.5$ [34].

As to modulation of the late Na^+ channel activity by the β subunits, there are only a few controversial reports. Both β_1 and β_3 subunits exhibit dual and opposite effects on the

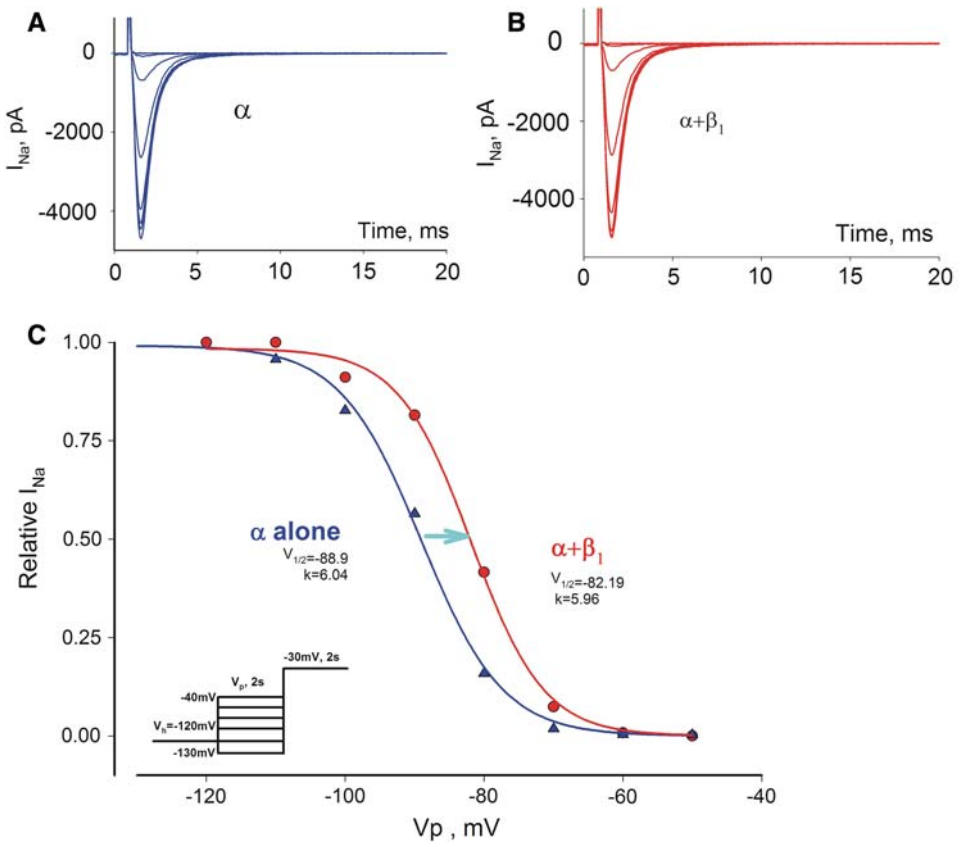

Fig. 4 Effect of β_1 subunit on the steady-state inactivation (SSI) voltage-dependency of the heterologous expressed $Na_v1.5$. **a**, **b** Representative raw current recordings at −30 mV in response to a different 2-s-long prepulse potential (voltage procedure is shown in **c**). **c** Data points of the relative (normalized to maximum) peak I_{NaT} measured at −30 mV and plotted against prepulse voltage (V_p). *Solid lines* represent the Bolzmann fit (Eq. 2 in "Methods"), and values are given on the plots. Statistics for values evaluated in numerous cells are given in Table 1. $V_h = -140$ mV, $[Na]_0 = 140$ mM, 24°C

Table 1 Steady-state activation (SSA) and inactivation (SSI) data for I_{Na} in the heterologously expressed cardiac $Na_v1.5$ (α) without or with its auxiliary β subunits

Conditions	SSI data			SSA data		
	$V_{1/2A}$ (mV)	K_A (mV)	*n*	$V_{1/2G}$ (mV)	K_G (mV)	*n*
α alone	−88.2 ± 0.9	−6.2 ± 0.2	11	−35.4 ± 1.2	5.5 ± 0.2	11
α + β_1 + GFP	−85.1 ± 1.1*	−5.7 ± 0.4	13	−36.1 ± 2.1	6.4 ± 0.4	11
α + β_1 (1:5)	−81.8 ± 0.9*	−5.8 ± 0.4	12	−34.7 ± 1.2	5.9 ± 0.2	7
α + β_2 + GFP	−88.3 ± 1.8	−6.1 ± 0.4	9	−36.8 ± 1.2	5.9 ± 0.3	5

SSA and SSI data were obtained from I_{Na} data fit to Eqs. 1 and 2 ("Methods"), respectively. Data represent means ± SE, *n* stands for the cell number

*$P < 0.05$ versus α alone. Statistically significant differences between results within the experimental groups were evaluated by ANOVA followed by Bonferroni's post hoc test and were considered significant at $P < 0.05$

decay time course of the Na^+ current produced by heterologously expressed rat brain αIIA ($Na_v1.2$) [35]. The current decay was assessed by use of two exponential models and the fast time constant, τ_1, was accelerated whereas the slow time constant, τ_2, was increased more than twofold (10.9 vs. 25 ms) by β_1 and β_3, respectively. In line with this finding it has been shown that expression of the β_1 subunit increased slowly, inactivating current (called persistent current by the authors) produced by the new epilepsy-related $Na_v1.1$ mutant D1866Y [36]. These data are in line with our findings reported here.

Transient expression of $Na_v1.5$ into the HEK293 cell line stably expressing β_1 subunit reduced a non-inactivating current component measured 750 ms after depolarization onset [37]. A similar non-inactivating Na^+ current has been also reported in rat cardiomyocytes; it was highly TTX-sensitive and could be augmented by hypoxia or cyanides [38, 39]. In both cases, when the non-inactivated current was measured, the authors used intracellular solutions deprived of ATP and containing artificial (non-physiological) anion fluoride. It has long been known that fluoride can retard Na^+ channel inactivation significantly,

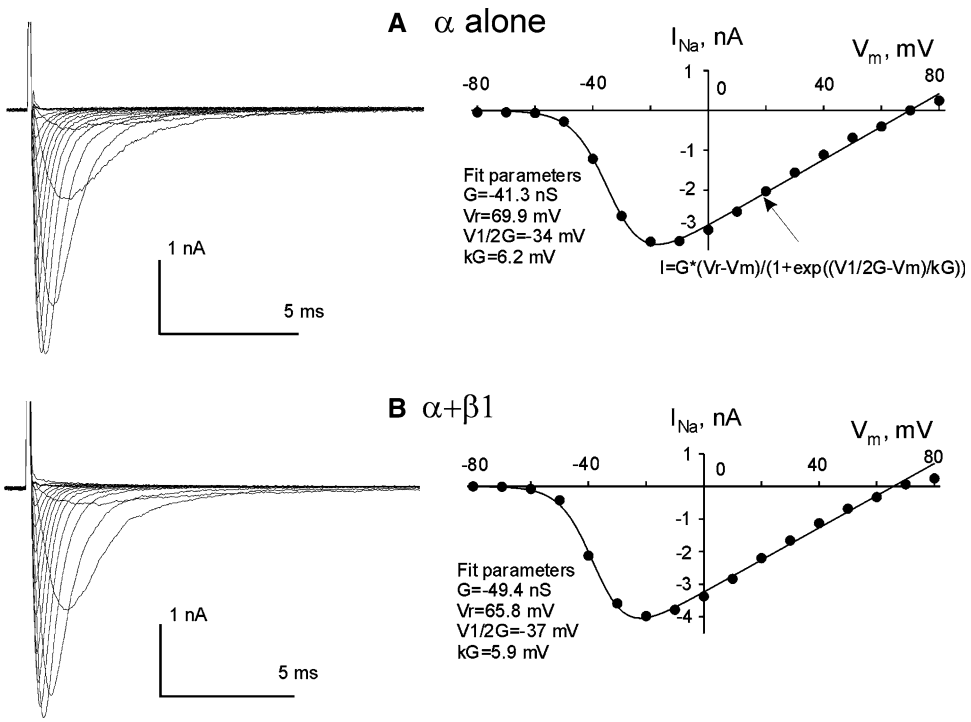

Fig 5 Voltage–current relationship for heterologous expressed $Na_v1.5$ (α alone **a**, or with the β_1 subunit **b**). **a**, **b** *Left panels* raw current recordings at different membrane potentials, *right panels* data point plots, with the fits (*solid lines*) to Eq. 1 ("Methods"). The equation is given in **a**, and fit values for the steady-state activation are indicated on the plots in **a** and **b**. The statistical data for the steady-state activation are given in Table 1

as was shown in internally perfused axons [40], and in cardiac cells [41]. Metabolic regulation of Na^+ channel inactivation and its rundown was demonstrated in neonatal cultured rat cardiomyocytes [42]. Therefore, this non-inactivating I_{Na} component is likely to be related to these non-physiological experimental conditions. In contrast, our current recordings were performed in close-to-physiological conditions (physiological [Na^+] and with no fluoride) and they did not reveal any non-inactivating current either in heterologously expressed $Na_v1.5$ clone (see Fig. 2, and Ref. [19]) or in cardiomyocytes from human hearts [3, 8]. In addition to the non-inactivating current discussed above, a background Na^+ current that could be recorded at very negative membrane potentials (−120 mV) has been reported in rabbit cardiac Purkinje cells and ventricular cardiomyocytes [43]. In contrast, recent studies [11, 12] show that canine cardiac Purkinje cells exhibit slowly inactivating I_{NaL}, similar to that described in ventricular cardiomyocytes of humans and dogs [3, 8]. Unlike background Na^+ currents, this I_{NaL} in Purkinje cells possesses steady state inactivation and is not activated at resting membrane potentials. Furthermore, non-inactivating Na^+ current was not present in human cardiomyocytes [3, 8]. Our single-channel data, in fact, excluded the presence of the non-inactivating component under close-to-physiological conditions for both $Na_v1.5$ clone and for human cardiomyocytes [2, 19]. Thus, the non-inactivating or background Na^+ currents are more likely to be species-dependent and/or were recorded in the presence of artificial anion fluoride and absence of ATP in the intracellular milieu. The molecular and genetic origins of background currents in cardiac cells remain unknown, but Denis Noble in his recent review [6] suggested that they could result from a leak form of Na^+–K^+ ATPase [44] or from NCX [45]. Accordingly, it is important to emphasize that we report here for the first time the modulatory effect of the β_1 subunit on cardiac-type late Na^+ current I_{NaL} (also known as persistent Na^+ current I_{pNa}) rather than on background non-inactivated currents of yet unknown nature reported in some previous studies.

The potency of the β_1 subunit to modulate I_{NaL} shown herein has been confirmed in the native cell environment by our preliminary study in normal dog cardiomyocytes in which antisense inhibition of SCN1B significantly accelerated I_{NaL} decay ([46], see Fig. 8A in Ref. [7]). It has been also shown that at the protein level $Na_v1.5$ is down-regulated whereas β_1 remained unchanged in HF, pointing toward relative higher membrane content of β_1 [27], but the SSI shift was not found in HF [2, 3, 29]. The SSI shift is dependent on variety of factors, including intracellular [Ca^{2+}], cytoskeleton, and membrane lipid content, that may affect SSI in different ways negating the β_1-related effect ([22], see review [7]). These data together with the findings of our study suggest a potential mechanism for the contribution of β_1 to HF-related I_{NaL} alterations [1, 3]. Furthermore, in addition to the I_{NaL} decay slowing, β_1 can also change I_{NaL} via its well-known effect of SSI shift. This β_1-induced SSI shift under physiological conditions (i.e. at a resting potential of ∼−80 mV) may have profound effect on I_{NaL} enhancement during action potentials, as

more Na^+ channels operating in late modes become available.

The next important question is how, specifically, the β_1 subunit interacts with Na^+ channel to produce the observed I_{NaL} changes. One possibility, however, could be related to the C-terminus (CT). The role of the CT in regulating $Na_v1.5$ inactivation via the Ca^{2+}-calmodulin-dependent interaction with the III–IV linker, responsible for the initial fast inactivation, has recently been suggested [47, 48]. Direct interaction between the cytoplasmic CT domain of $Na_v1.1$ with β_1 and β_3 has recently been demonstrated [36] and thus provides a possible molecular mechanism for the I_{NaL} modulation found here.

Single-channel studies in heterologously expressed $Na_v1.5$ and human cardiomyocytes show that late NaCh activity is arranged in two major gating modes—late scattered mode (LSM) and 'burst" mode (BM) [19]. Numerical evaluation based on the Markovian chain model revealed that BM + LSM is responsible for the intermediate phase (40–300 ms) whereas LSM is responsible for the ultra-late (>300 ms) phase on I_{NaL} inactivation [2]. In this study we analyzed the amplitude and decay of I_{NaL} after 200 ms, thus presumably the function of LSM mode. Therefore, the β_1 subunit may increase the probability of occurrence of these modes (i.e. make modal switch more probable), thus increasing relative I_{NaL}/I_{NaT}. Also β_1 affects the gating kinetics (inactivation) of LSM. The idea of β_1 subunit involvement in the modal switch has previously been suggested for muscular isoform $Na_v1.4$ [49].

We did not find β_2-related effects on I_{NaL} in our experimental setting. The role of this subunit in physiological function of NaCh is not clear. Initially the β_2 subunit was implicated in intercellular adhesion and recruitment of the cytoskeletal protein ankyrin to the plasma membrane at sites of cell-to-cell contact [50]. Accordingly the direct role of the β_2 subunit on NaCh gating was not suggested, because NaCh protein has direct attachment to the sub-membrane cytoskeleton via ankyrin (for a review see Ref. [48]) and may directly be related to the cytoskeleton-dependent effects on Na^+ channel gating [51, 52]. It has been recently demonstrated that β_1/β_2 chimeras may cause the additional closed state for $Na_v1.5$ that is accessible at hyperpolarized potentials, although this effect was not produced by the wild type β_2 subunit [53]. Given the multi-modal origin of $Na_v1.5$-related I_{NaL} it is not obvious how this subunit can play a role in the kinetic transition constants assuming the new closed state [2]. Although our experiments do not reveal apparent effects on the I_{NaL} properties studied, we cannot exclude the possibility that the β_2 subunit can still affect late Na^+ channel openings taking into account a possible role of neuronal isoforms (for a review see Ref. [7]).

We conclude that the auxiliary subunit β_1 modulates I_{NaL}, produced by the human cardiac Na^+ channel $Na_v1.5$ by slowing its decay and increasing I_{NaL} amplitude relative to I_{NaT}. Because expression of $Na_v1.5$ reportedly decreases but β_1 remains unchanged in chronic HF, the relatively higher expression of β_1 may contribute to known I_{NaL} increase in HF via the modulation mechanism found in this study.

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