PLoS one

Increased Expression of the Auxiliary β2-subunit of Ventricular L-type Ca2+ Channels Leads to Single-Channel Activity Characteristic of Heart Failure

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Background. Increased activity of single ventricular L-type Ca2+-channels (L-VDCC) is a hallmark in human heart failure. Recent findings suggest differential modulation by several auxiliary β-subunits as a possible explanation. *Methods and Results*. By molecular and functional analyses of human and murine ventricles, we find that enhanced L-VDCC activity is accompanied by altered expression pattern of auxiliary L-VDCC β-subunit gene products. In HEK293-cells we show differential modulation of single L-VDCC activity by coexpression of several human cardiac β-subunits: Unlike β1 or β3 isoforms, β2a and β2b induce a high-activity channel behavior typical of failing myocytes. In accordance, β2-subunit mRNA and protein are up-regulated in failing human myocardium. In a model of heart failure we find that mice overexpressing the human cardiac CaV1.2 also reveal increased single-channel activity and sarcolemmal β2 expression when entering into the maladaptive stage of heart failure. Interestingly, these animals, when still young and non-failing ("Adaptive Phase"), reveal the opposite phenotype, *viz*: reduced single-channel activity accompanied by lowered β2 expression. Additional evidence for the cause-effect relationship between β2-subunit expression and single L-VDCC activity is provided by newly engineered, double-transgenic mice bearing both constitutive CaV1.2 and inducible β2 cardiac overexpression. Here in non-failing hearts induction of β2-subunit overexpression mimicked the increase of single L-VDCC activity observed in murine and human chronic heart failure. *Conclusions*. Our study presents evidence of the pathobiochemical relevance of β2-subunits for the electrophysiological phenotype of cardiac L-VDCC and thus provides an explanation for the single L-VDCC gating observed in human and murine heart failure.

INTRODUCTION

Homeostasis of intracellular Ca2+ concentration $[Ca^{2+}]_i$ is essential for cardiac function and integrity; its dysregulation is a hallmark of advanced heart failure [1,2]. Voltage-dependent L-type Ca2+-channels (L-VDCCs) are the source of trigger Ca2+ entering cardiomyocytes [3]. Data derived from numerous studies support an involvement of L-VDCC in pathological changes of $[Ca^{2+}]_i$ in heart failure. Although still controversial, L-VDCC current density appears unchanged in failing cardiomyocytes [1,4,5]. Whole-cell currents are determined by a number of parameters, including number of channels, single-channel current amplitude and time spent in the open state. Therefore, altered number of active channels or activity of individual L-VDCC is not necessarily reflected by calcium current density. In fact, despite no change in whole-cell L-VDCC density (I_{Ca}), single-channel activity was significantly increased in ventricular myocytes from human end-stage failing hearts [6]. Chen et al. [7] showed attenuated I_{Ca} increase by (S)-BayK8644 in human failing myocardium whereas basal whole-cell currents were unchanged, indicating that single-channel activity is enhanced while channel density is lowered. These findings confirm the idea of an "electrophysiological heart-failure phenotype" of single L-VDCCs. The biochemical nature of this change in phenotype has not been delineated, although phosphorylation [8,9] and dephosphorylation [10,11] have been implicated. Activities of kinases and phosphatases not only change channel function but interfere with neurohumoral modulation of the L-VDCC; *e.g.* β-adrenergic regulation is blunted in heart failure possibly due to hyperphosphorylation of L-VDCCs [6,7]. Using heterologous recombination we have shown that distinct subunit compositions of L-VDCC induce single-channel characteristics similar to the biophysical phenotype of "hyperphosphorylated" L-VDCC [12]. The latter suggests that changes in gene expression of L-VDCC subunits may form the basis of a heart-failure phenotype of L-VDCC. In mammalian hearts L-VDCCs are composed of an ion conducting pore (CaV1.2 or α1C), and two auxiliary subunits, an α2δ and a β-subunit. Most

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investigators agree that β-subunit diversity is of physiological and pathophysiological importance [13–18]. In fact, some studies have revealed altered β-subunit patterns in human heart failure [19,20], suggesting that an altered β-subunit expression pattern is of functional relevance. Delineation of pathophysiological mechanisms in human heart is difficult because of wide inter-individual variance, including age, medication, state of disease *etc.* Human tissue also offers a limited choice of truly independent variables, such as time, disease stage and treatment options. Animal models offer control of any relevant factor to test pathophysiological concepts. We analyzed β-subunit gene expression in both human non-failing and failing hearts as well as in transgenic mice overexpressing the human $Ca_V1.2$ (α_{1C}) subunit (tg $Ca_V1.2$). The latter was chosen because of phenotypical characteristics common with human heart failure, *e.g.* early blunting of β-adrenergic signaling, slow progression towards hypertrophy and calcium overload in failing myocytes [21,22]. Most importantly, in young (non-failing; "Adaptive State") tg $Ca_V1.2$ mice we previously found concordance of lowered β2-subunit expression and decreased activity of single L-VDCC [16]. In the present study we find an increase of single L-VDCC activity accompanied by enhanced expression of β2-subunits when these mice have entered the failing state ("Maladaptive State" ≥9 months of age). By examination of a new, double-transgenic mouse bearing both constitutive $Ca_V1.2$ and inducible β2-subunit overexpression in the heart we show a relationship between subunit expression and channel function.

RESULTS

Gating parameters of single L-VDCC in failing human and transgenic myocardium

Single-channel activity of old (≥9 months, and in heart failure) tg $Ca_V1.2$ was significantly increased compared to young (4 months, no hypertrophy or heart failure) tg $Ca_V1.2$ [16] (Table 1). Henceforth, these animals are referred to as "young" and "old" tg $Ca_V1.2$, respectively. Peak ensemble average current (I_{peak}) in old tg $Ca_V1.2$ mice was enhanced (-56 ± 14 fA vs. -23 ± 7 fA, $p<0.05$) due to an increased fraction of active sweeps, mean open time, and mean open probability, and a decrease of mean closed time (t_{closed}). Of further interest, the changes of peak current, fraction of active sweeps and open probability mirror findings obtained from single L-VDCC measurements in human cardiomyocytes from non-failing or failing idiopathic dilated cardiomy-

opathy (DCM) hearts, respectively [6] (Table 1).

L-VDCC subunit expression in non-failing and failing human hearts

Protein expression of $Ca_V1.2$, $\alpha_2\delta$, low molecular weight β1, and β3 was similar in non-failing and failing human myocardium, but we found a significant up-regulation of β2 (Figure 1a,b). There was no difference in gene expression of the $Ca_V1.2$, and the $\alpha_2\delta$ at the protein level (mRNA data not shown). At least two β1-subunit isoforms (β1a,c), four β2-subunit isoforms (β2a–d), and two β3-subunit isoforms (β3a,trunc) are expressed at relevant levels in human myocardium [12]. β1a (GenBank No NM_199247) and β1c (GenBank_199248) are sequence-identical except for replacement of exon 7a by exon 7b in β1c, consistent with previous work [23]. β2a–d isoforms differ only with respect to the N-terminal region (D1 domain). Quantitation by real-time PCR revealed an increased expression of β1c and all β2 isoforms in heart failure, in line with the protein data (Figure 1c).

L-VDCC subunit protein expression in old wild type and old tg $Ca_V1.2$ hearts

β1, β2 and β3 isoforms are expressed at the protein level in old $Ca_V1.2$ mouse heart, although expression of β1-subunit isoforms was faint. Compared to old wild type mice (≥9 months), the old tg $Ca_V1.2$ showed a significant up-regulation of β3-, and β2-subunits (Figure 2), in striking contrast to the down-regulation of β2-subunits we previously observed in young tg $Ca_V1.2$ [16]. In the old tg $Ca_V1.2$ mouse myocytes, up-regulated β2-subunits and overexpressed $Ca_V1.2$ both localize to the surface sarcolemma and the T-tubules (Figure 3), suggesting the functional relevance of altered expression levels.

β-subunit-dependent modulation of $Ca_V1.2$ expressed in HEK293

The diversity of β-subunit expression patterns found in cardiomyocytes necessitated the functional characterization of each β-subunit isoform. Using HEK293 cells constitutively expressing the human $Ca_V1.2$ as a homologous recombination system we show that the gene as well as alternative splicing determines calcium channel gating, extending and elaborating on previous work [12]. This is highlighted by significant differences in peak current and

Table 1. Single L-VDCC gating of young and old tg $Ca_V1.2$ resembles data obtained from human non-failing and failing ventricle

gating parameter	tg $Ca_V1.2$	tg $Ca_V1.2$	human LV	human LV
	4 months	≥9 months	non-failing	failing (DCM)
peak current I_{peak} [fA]	-23 ± 7	-56 ± 14*	-13 ± 5	-28 ± 5*
fraction of active sweeps f_{active} [%]	53.7 ± 5.3	$71.7\pm8.2^{(*)}$	26.4 ± 5.3	56.7 ± 8.0*
open probability P_{open} [%]	4.4 ± 1.2	$6.4\pm0.9^{†}$	3.2 ± 1.3	$6.1\pm1.6^{†}$
mean open time t_{open} [ms]	0.34 ± 0.03	0.45 ± 0.04*	0.54 ± 0.05	0.65 ± 0.04
mean closed time t_{closed} [ms]	6.6 ± 1.0	3.1 ± 0.7*	9.3 ± 1.6	7.5 ± 1.7
number of experiments	13 (13)	11 (5)	16 (12)	9 (6)

In a previous study [6] we found single-channel activity to be significantly increased in ventricular myocytes from human hearts failing due to idiopathic dilated cardiomyopathy compared to non-failing ventricles. In excellent agreement the present study reveals activity of single L-VDCC from ≥9 months old, i.e. failing murine hearts overexpressing the human $Ca_V1.2$ to be significantly increased compared to single-channel activity in 4 months old, *i.e.* non-failing young transgenics obtained in a previous study [16]. Charge carrier: 70 mM Ba^{2+}; holding potential: -100 mV; test potential: $+20$ mV. Note that Schroder et al. [6] did not use a depolarizing bath solution, thus potentials are approximate values.
*$p<0.05$ and $^{(*)}p = 0.07$ in a Student's t-test; $^{†}p<0.05$ in a Mann-Whitney test (performed when data failed normality test). Numbers of experiments given in parentheses indicate number of experiments with only one channel in the patch.
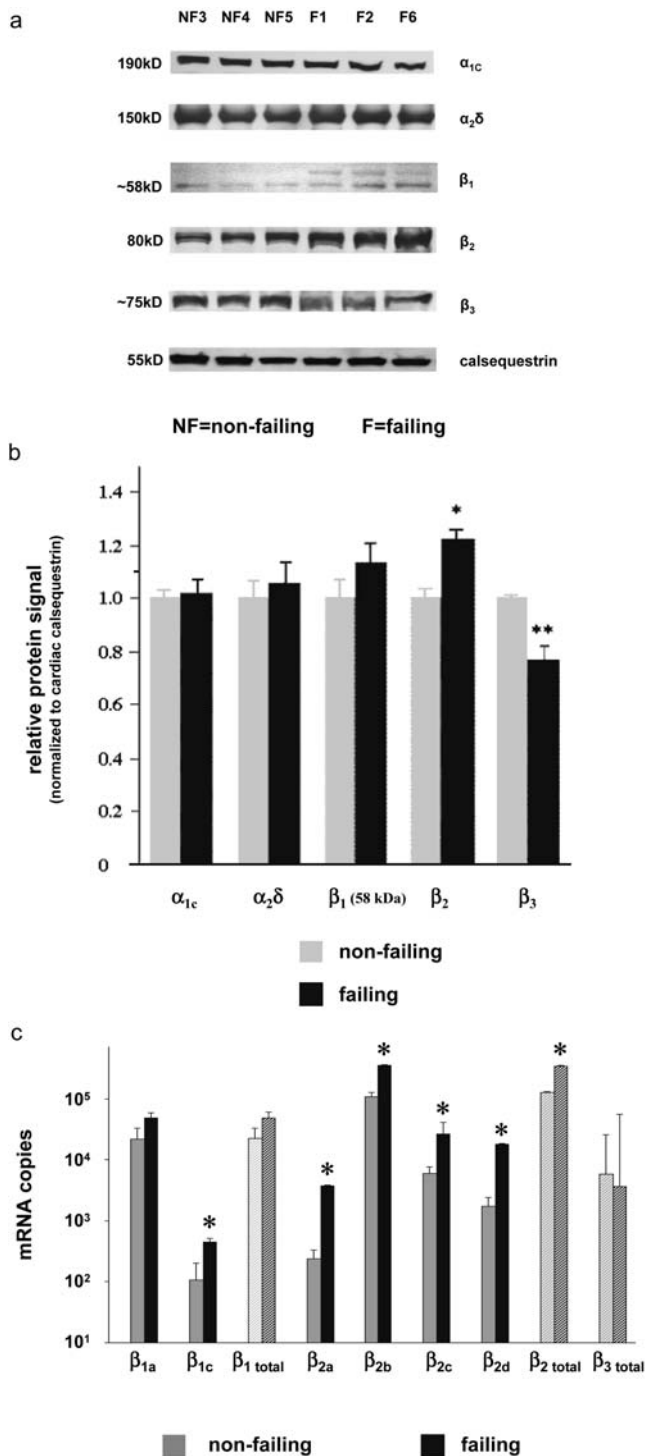doi:10.1371/journal.pone.0000292.t001

Figure 1. Subunit expression of cardiac L-VDCC subunits in human myocardial specimens
(a) Human specimens from non-failing (NF) and failing (F) myocardium (n = 4–5) were analyzed in immunoblots using specific polyclonal antibodies directed against the particular L-VDCC subunits. (b) L-VDCC subunit expression was normalized to cardiac calsequestrin protein expression in the same sample (number of NF/F specimens was always identical for each subunit; n = 5–8). Quantitative analysis of subunit protein expression is depicted as ratio of F vs. NF. * p<0.001; ** p<0.0001. (c) mRNA expression of β-subunit isoforms (NF: n = 5; F: n = 9–13) was measured by real time PCR, and always normalized to cardiac calsequestrin mRNA expression. * p<0.05.
doi:10.1371/journal.pone.0000292.g001

open probability between β-subunits (Table 2), with β_{2a} and β_{2b} exerting the strongest effects. β_{2c} or β_{2d} as well as β_{1a}, β_{1c}, and β_{3a} induced a minor to moderate increase in single-channel activity with no significant effects detected for closed times. Taken together, the data support the view that the single-channel phenotype of failing cardiomyocytes is caused by channel complexes containing β_{2a} or β_{2b}.

Generation of an inducible, heart-specific β_{2a}-subunit overexpression mouse (tg$_{ind}$ β_{2a})

Our functional analyses support the idea of pathophysiological relevance of β_2-subunit up-regulation, but the parallel biophysical and biochemical changes in cardiomyocytes may still be co-incidental. Rather than following the natural course of gene expression changes, transgene-controlled β_2-subunit overexpression should prove its causative role in native tissue. A hybrid drosophila-bombyx ecdysone receptor (VgBmEcR) when coupled to an αMHC promoter should combine strictly drug-controlled, transgene-specific, and cardiac tissue-specific gene induction. We generated this double transgenic model of αMHC VgBmEcR and the (rat) β_{2a} gene, hence referred to as tg$_{ind}$ β_{2a}, under control of the ecdysone response element (Figure 4a). Mice carrying both transgenic constructs developed normally and did not show any signs of developmental or cardiac dysfunction. *In vivo* induction with tebufenozide clearly increased cardiac β_2-subunit expression in tg$_{ind}$ β_{2a} mice at the protein level (Figure 4b) proofing functionality of drug-controlled gene expression. However, the single L-VDCC phenotype after induction was not altered in this mouse when compared with either tebufenozide treated wild-type mice or sham-induced tg$_{ind}$ β_{2a} (Figure 4c, Figure 5a–e).

Characterization of L-VDCC activity in double transgenic (tg Ca$_V$1.2×tg$_{ind}$ β_{2a}) mice

Adaptive down-regulation of β_2-subunit expression in the young tg Ca$_V$1.2 [16] sets the stage for analysis of a β_2-subunit increase in native tissue. For this we crossbred tg Ca$_V$1.2 mice and tg$_{ind}$ β_{2a} mice. Consistent with the hypothesis of a functional predominance of β_1-subunits in the young tg Ca$_V$1.2 mice, (**N.B.**: These animals are "Adaptive", and in fact demonstrate hypercontractility, without failure) activity of single-channels was dramatically increased by β_2-subunit induction in tg Ca$_V$1.2×tg$_{ind}$ β_{2a} 48 hours after induction (Figure 5) (**e.g.** peak current: −72±10 fA vs. −13±2 fA in tg Ca$_V$1.2 likewise treated with tebufenozide, p<0.05). This is evidence for our proposed relationship of structure and function of L-VDCC, at the single channel level, in ventricular myocytes ***ex vivo***.

DISCUSSION

The major, novel result of our study is the concomitant increase in β_2-subunit expression and L-VDCC activity in three independent models: human dilated cardiomyopathy, old tg Ca$_V$1.2 mice spontaneously progressing into heart failure and young ("Adaptive State") tg Ca$_V$1.2 mice with additional tissue-specific inducible overexpression of β_2-subunits. We explain our results by the differential effects of the β-subunits, namely β_{2a} and β_{2b} as the most important modulators in recombinant assays.

The three β-subunits (β_{1-3}) thus far known to be expressed in mammalian hearts have vastly different effects on current density [17,24], kinetics [12,24], and single-channel properties [12,14,17]. Beyond that, some β_2-subunit isoforms have been implicated in mediating membrane targeting [3,25], cardiomyocyte apoptosis [26], cell death [17], adrenergic regulation [27,28] and modula-
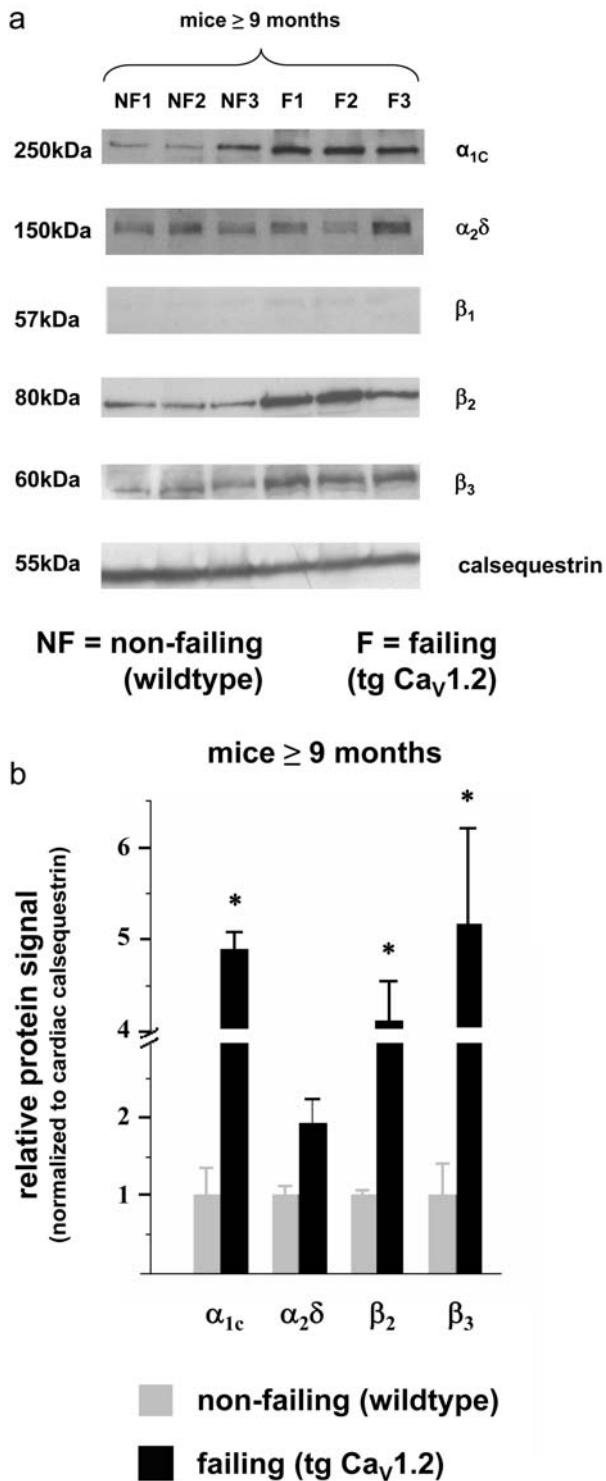
Figure 2. Protein expression of cardiac L-VDCC subunits in old wild-type and tg $Ca_V1.2$ mice

(a) Specimens from old wild-type mice and tg $Ca_V1.2$ in heart failure were analyzed in immunoblots using specific polyclonal antibodies directed against the particular L-VDCC subunits.

(b) Protein expression of L-VDCC subunits was always normalized to cardiac calsequestrin protein expression in the same sample. Quantitative analysis of subunit protein expression is depicted as ratio of 10 months old tg $Ca_V1.2$ vs. age-matched wild-type. β_1 protein bands were faint, and thus not analyzed quantitatively (number of WT/old tg $Ca_V1.2$ specimens was always identical for each subunit; n = 4). * p<0.05.
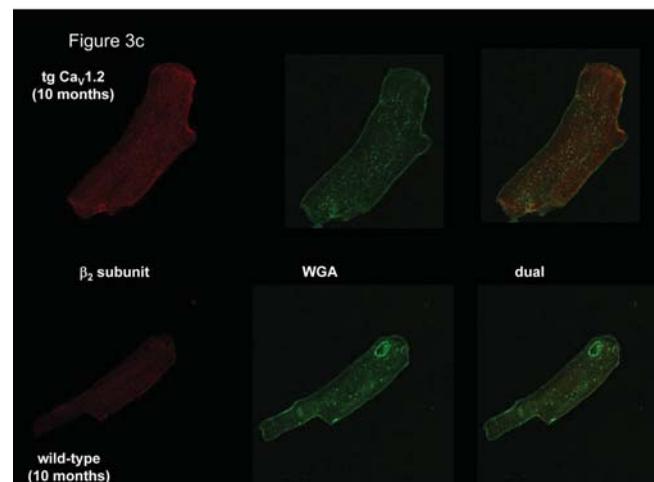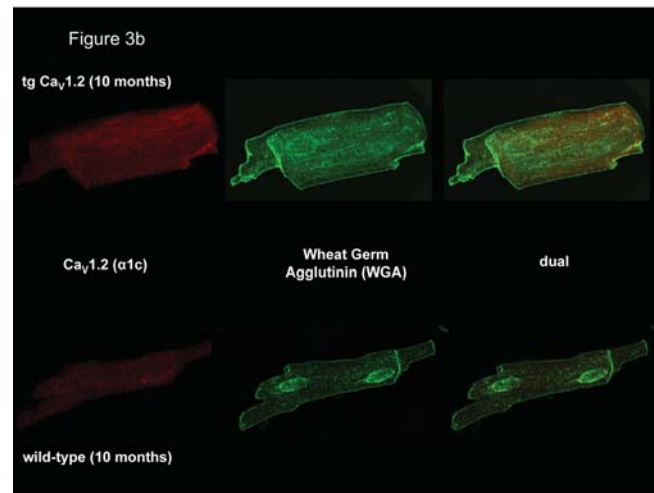doi:10.1371/journal.pone.0000292.g002
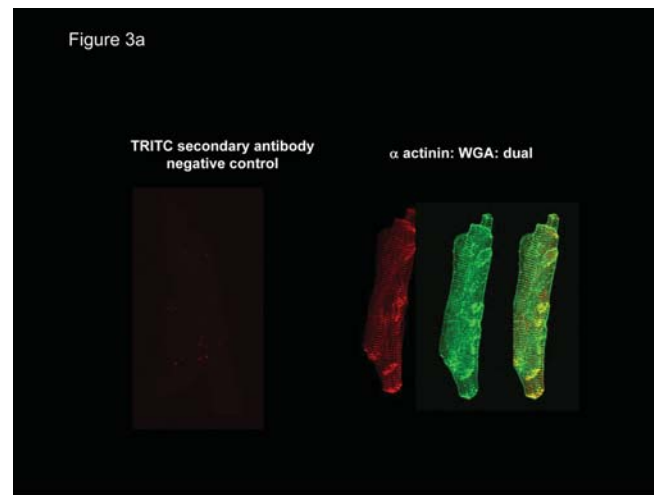
Figure 3. Immunocytochemistry shows increased sarcolemmal expression of $Ca_V1.2$ and β_2-subunits in ventricular myocytes from old tg $Ca_V1.2$ mice

(a) Immunocytochemistry of cardiomyocytes isolated from 10 months old wild-type or tg $Ca_V1.2$ mice. Controls show no specific interaction for TRITC labeled secondary antibody and labeling of sarcolemma, T-tubules and intercalated discs by Oregon Green 488-conjugated wheat germ agglutinin (WGA).

(b,c) Primary antibodies directed against $Ca_V1.2$ and β_2-subunits, respectively, demonstrate predominant t-tubular localization of the respective antigens in old wild-type and tg $Ca_V1.2$.
doi:10.1371/journal.pone.0000292.g003

Table 2. Gating of single recombinant human $Ca_V1.2$ in HEK293 cells is differentially modulated by several coexpressed human cardiac auxiliary β-subunits

gating parameter	β_{1a}	β_{1c}	β_{2a}	β_{2b}	β_{2c}	β_{2d}	β_{3a}	control
peak current I_{peak} [fA]	-36 ± 12	-22 ± 5	$-109\pm25^*$	$-123\pm28^*$	-24 ± 7	-37 ± 16	$-46\pm14^*$	-12 ± 2
fraction of active sweeps f_{active} [%]	53.3 ± 10.5	32.5 ± 5.2	57.6 ± 6.6	58.7 ± 9.1	45.5 ± 14.6	64.1 ± 8.2	48.9 ± 6.4	46.9 ± 6.3
open probability P_{open} [%]	2.1 ± 0.6	$2.4\pm0.5^*$	$6.9\pm1.4^*$	$8.3\pm1.9^*$	1.8 ± 0.6	3.2 ± 1.3	$2.8\pm0.8^{(*)}$	1.1 ± 0.2
mean open time t_{open} [ms]	0.34 ± 0.09	$0.28\pm0.02^*$	$0.31\pm0.02^*$	$0.34\pm0.03^*$	0.23 ± 0.03	$0.26\pm0.01^*$	$0.30\pm0.03^*$	0.22 ± 0.01
mean closed time t_{closed} [ms]	11.5 ± 1.7	15.8 ± 5.3	$7.0\pm0.5^*$	$4.7\pm1.0^*$	10.8 ± 2.6	11.4 ± 1.7	12.1 ± 2.9	12.4 ± 1.3
number of experiments	10 (6)	11 (4)	14 (6)	16 (8)	5 (4)	9 (6)	10 (8)	8 (5)

Single-channel gating parameters of human cardiac $Ca_V1.2$ stably expressed in HEK293 cells transiently cotransfected with several auxiliary β-subunits and a human cardiac $\alpha_2\delta$-2-subunit. Coexpression with β_{2a} and β_{2b} reveals strongest stimulation of single channels compared to control cells without transfection of any β-subunit (ctr). Data were obtained by patch-clamp recordings using cell-attached configuration (charge carrier: 110mM Ba^{2+}; holding potential: -100 mV; test potential: $+10$ mV for 150 ms). $^*p<0.05$ and $^{(*)}p=0.07$ in Bonferroni-corrected post-hoc tests versus control, following one-way ANOVA. Numbers of experiments given in parentheses indicate number of experiments with only one channel in the patch.

doi:10.1371/journal.pone.0000292.t002

tion of CaM Kinase II [29,30]. It is of interest that the pattern of auxiliary subunits [3], including the prevalence of β-subunit isoforms varies among species, with β_2-subunits predominating in small rodents like rats [17], and mice [31], and additional relevant expression of β_1- and β_3-subunits in humans [13,19,23].

In our study the homologous recombination of human L-VDCC subunits indicates the biophysical relevance of β_{2a} and β_{2b} isoforms for up-regulation of single L-VDCC activity. The increase of β_2-subunit gene expression in human heart failure suggests that these L-VDCC subunits form the basis of the "heart-failure phenotype" of single L-VDCC found in human hearts [6]. Young tg $Ca_V1.2$ animals were chosen as a known example of dynamic adaptation of β_2-subunit expression in heart. The functional relevance of this adaptation is illustrated by whole-cell current density, that is increased by only 50% [22,32], while $Ca_V1.2$ protein expression and density of single channels show a 3-fold increment in these transgenic hearts [16,32]. This apparent discrepancy is explained by an up-regulation of β_1- but substantial down-regulation of the β_2-subunit expression [16]. This gene expression is characteristic for the "Adaptive phase" of the model [3,22] putatively limiting calcium overload at the younger ages. We now demonstrate that when this tg $Ca_V1.2$ mouse enters into the "Maladaptive state" with overt heart failure at ≥ 9 months of age, both single L-VDCC activity and β_2-subunit expression increase, mimicking alterations of channel structure and biophysics in terminal human heart failure. Thus, the old tg $Ca_V1.2$ mouse may be regarded as a heart-failure model in which a primary calcium (current) overload can no longer be effectively counterbalanced by adaptive mechanisms, *i.e.* β-subunit expression. The transcriptional mechanisms underlying this bidirectional control of β_2-subunit expression, however, remain to be elucidated in the context of changes in β_1 and β_3 subunit expression in human and old tg $Ca_V1.2$ mouse heart failure.

As a novel and first approach to induce an increased β_2-subunit overexpression in intact animals, rather than in isolated cells [17,24,26] we generated a mouse model of cardiospecific inducible β_{2a}-subunit expression (tg$_{ind}$ β_{2a}). Induction of β_2-overexpression in this mouse model did not affect overall single L-VDCC gating significantly. As a more recent study indicates that an 1:1-stoichiometry of pore-forming α_1- and auxiliary β-subunit may be sufficient for modulation of channel gating [33], we assume that most calcium channel pores are saturated with native β-subunits in the induced tg$_{ind}$ β_{2a}. However, mean closed time was lower in induced tg$_{ind}$ β_{2a} suggesting that a portion of overexpressed β_{2a}

exerts functional action similar to the recombinant channel data presented. To prove our concept that β_2-subunit expression underlies the activity of single L-VDCC of the heart-failure phenotype we crossbred tg$_{ind}$ β_{2a} with tg $Ca_V1.2$ mice [21,22]. Induction of β_{2a}-subunit gene expression in the young double tg mice (tg $Ca_V1.2 \times$ tg$_{ind}$ β_{2a}) led to a premature increase of single L-VDCC activity. This confirms our theory, derived from recombinant channel data, in the relevant tissue *ex vivo*.

Such deliberate overexpression of β_2-subunit *in vivo*, when carried forward in a chronic manner, hopefully will pave the way for understanding the progression of heart failure if these alterations in single L-VDCC gating lead to decompensation at an earlier age of the animal. This knowledge will have direct implications because pharmacological agents which modulate L-VDCC function are in everyday clinical practice and have been shown to be beneficial in various clinical trials targeting different populations [34,35]. We wish to emphasize that, at this point in our studies, we show a relationship between electrophysiological parameters that is consistent with heart failure. In order to prove this, it is necessary to chronically imbue the young animals with a heart-specific increase in the β_2 subunit and follow their transition to heart failure at specific age points as they mature. These experiments are ongoing but will require considerable time.

MATERIALS AND METHODS

Materials
Non-failing and failing human left ventricular specimens were obtained from explanted hearts not transplanted for technical reasons (n = 5), or from orthotopic heart transplantation recipients (n = 8). Heart failure patients were in NYHA class III–IV (17–63 y, 3 females) mean duration of symptomatic heart failure ranged from 9–60 months, peak oxygen exercise capacity was 13.3–15.5 ml kg^{-1} min^{-1} at time of listing. Heart transplant recipients were ambulatory at time of operation and received treatment with inhibitors of the angiotensin-system (100%), β-blockers (75%), aldosterone antagonist (50%), diuretics (62.5%).

Animals
Mice with cardiac-specific heterozygous overexpression of the human $Ca_v1.2$ [21] were bred with non transgenic littermates. These animals were bred with the tg$_{ind}$ β_{2a} as described below. Non transgenic littermates served as WT controls in this study.

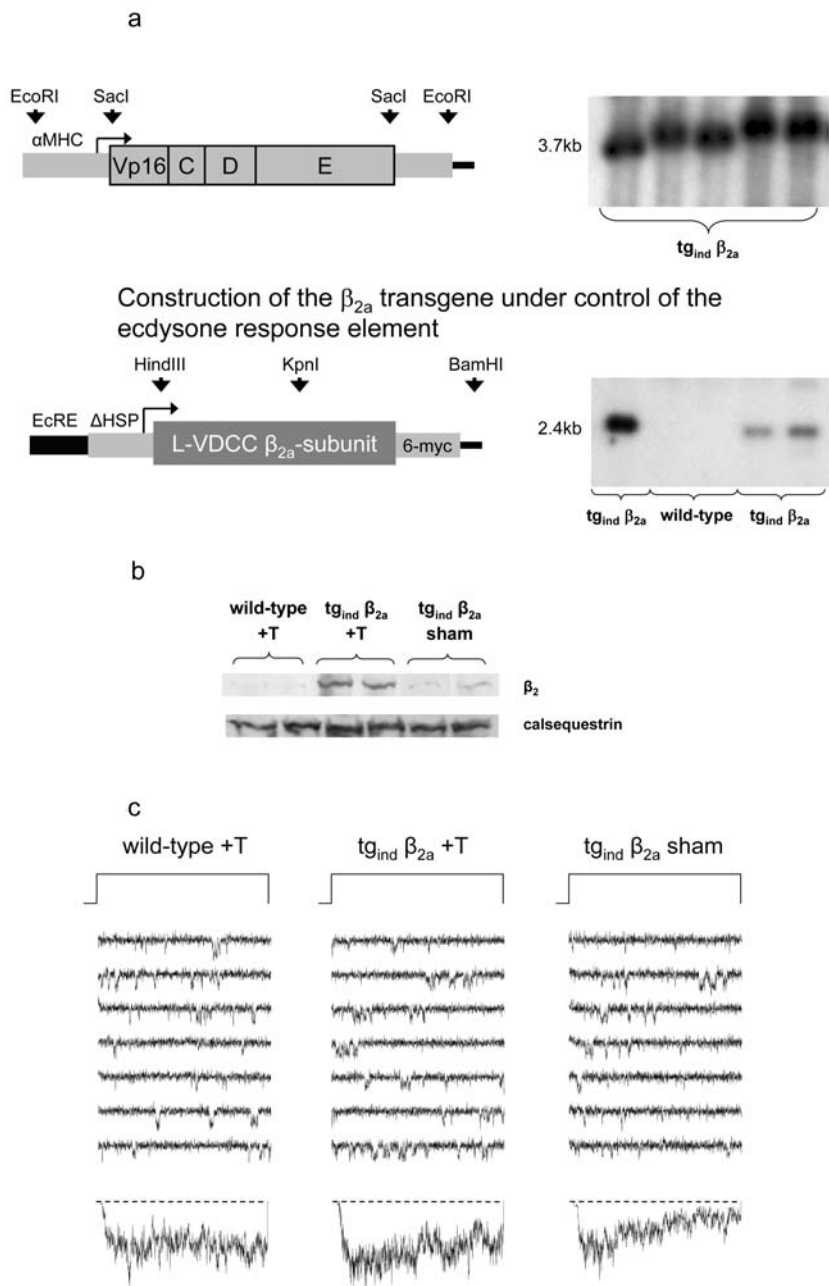The study was approved by the local institutional committees.

Figure 4. tg mouse model with an inducible cardiac overexpression of the β_{2a} under control of a hybrid bombyx-ecdysone receptor
(a) For cardiac-specific expression the hybrid bombyx-ecdysone receptor (VgBmEcR) was placed under the control of αMHC promoter (for details see Materials and Methods). Transgenic mice (tg$_{ind}$ β_{2a}) positive for the hybrid bombyx-ecdysone receptor and the construct of the ecdysone response element (EcRE) and the β_{2a}, respectively, are identified in Southern blots. The radiolabeled probe specific for the coding sequence of VgBmEcR was generated by SacI digestion. It hybridized to a 3.7 kb band in transgene mouse genomic DNA digested by EcoRI digest; the radiolabeled DNA probe specific for the coding sequence of β_{2a} was generated by HindIII/KpnI digest. It hybridized to a 2.4 kb band of genomic DNA digested with HindIII/BamHI in tg$_{ind}$ β_{2a} but not in WT.
(b) 48 h after treatment with the inducing drug tebufenozide (+T) Western-blot analysis with ventricular tissue from 4–5 month old mice reveals increased expression of β_2-protein in tg$_{ind}$ β_{2a} compared to treated wild-type or sham-induced transgenics.
(c) Exemplary traces of single-channel recordings from murine ventricular myocytes. Induction of cardiac overexpression of the β_{2a} (+T) does not alter single-channel behavior compared to either wild-type mice after treatment with the inducing drug or i.p-application of only the vehicle (water/oil-emulsion) to β_{2a}-transgenic mice ("sham"). Data were obtained by patch-clamp recordings using cell-attached configuration (charge carrier: 70 mM Ba²⁺; holding potential: −100 mV; test potential: +20 mV for 150 ms). Bottom traces show ensemble average currents from the respective experiment.
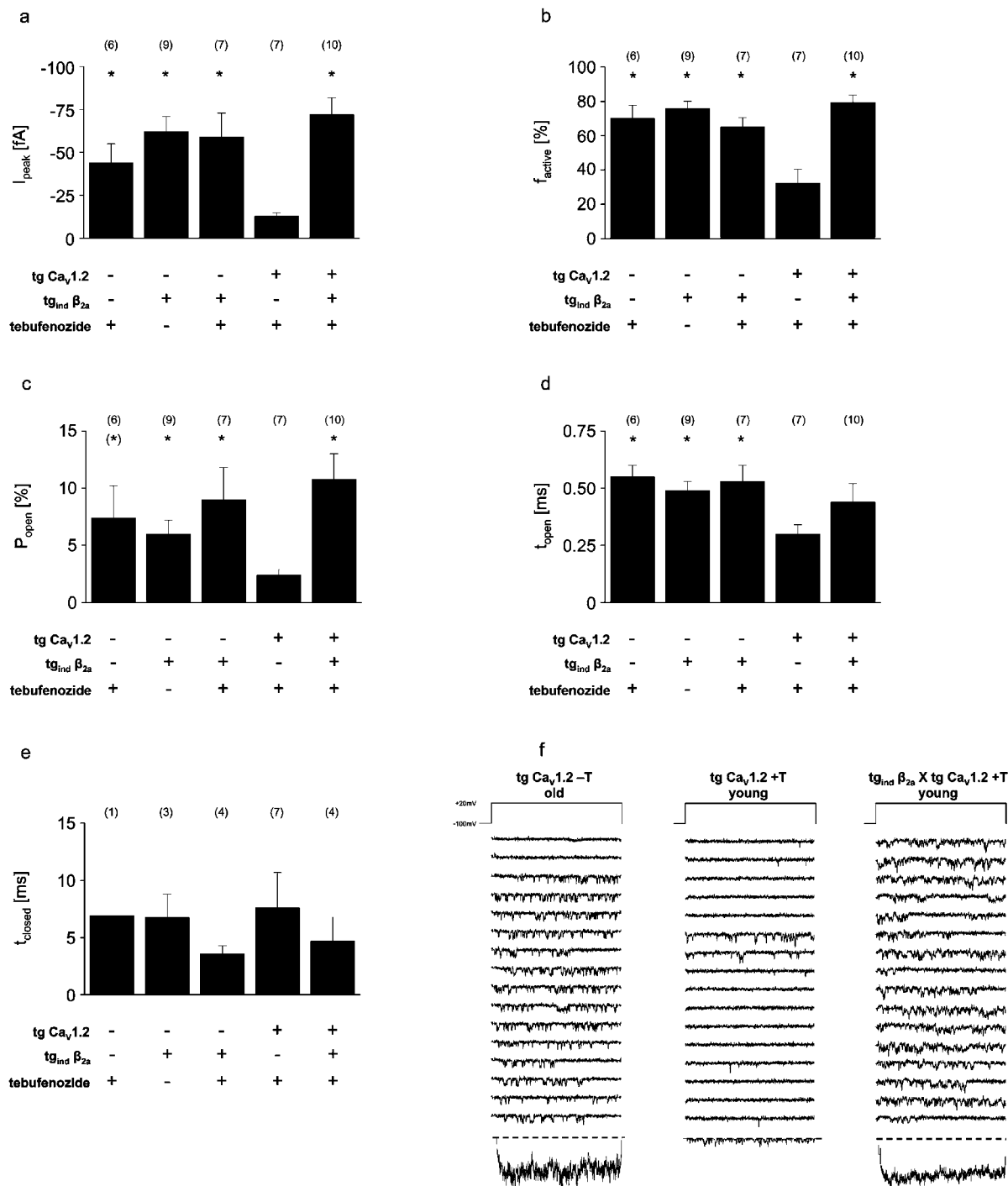doi:10.1371/journal.pone.0000292.g004

Figure 5. Gating of single L-VDCC in ventricular myocytes from mice showing cardiac overexpression of Ca²⁺-channel subunits

(a–e) Single-channel gating parameters of ventricular L-VDCC from murine hearts. Compared to 4–5 months old mice showing a cardiac overexpression of the human $Ca_V1.2$ (tg $Ca_V1.2$), the inducing compound tebufenozide (T) significantly increased single L-VDCC activity in ventricular myocytes from age-matched double-transgenics (tg $Ca_V1.2 \times tg_{ind}$ $β_{2a}$, showing an additional inducible $β_{2a}$-overexpression) 48 h after drug administration. Overexpression of the $β_{2a}$-subunit without overexpression of the human $Ca_V1.2$ does not alter single-channel gating (cp. Figure 4c). Tebufenozide treatment does not affect single-channel gating in ventricular myocytes from wild-type mice. Data were obtained by patch-clamp recordings using cell-attached configuration (charge carrier: 70 mM Ba^{2+}; holding potential: −100 mV; test potential: +20 mV for 150 ms). *$p < 0.05$ and (*)$p < 0.09$ compared to tg $Ca_V1.2$ in Student's t-test. Number of underlying experiments is given in parentheses.

(f) Exemplary traces of single-channel recordings from murine ventricular myocytes. Activity of single L-VDCC is clearly higher in old (≥9 months, failing) tg $Ca_V1.2$ compared to channels from young (4–5 months, non-failing) tg $Ca_V1.2$. Induction of $β_2$-overexpression in hearts of young tg_{ind} $β_{2a} \times tg$ $Ca_V1.2$ by tebufenozide mimicks the heart-failure phenotype of L-VDCC gating otherwise not observed until tg $Ca_V1.2$ enter the "Maladaptive State" at an age ≥9 months. T = tebufenozide. Data were obtained by patch-clamp recordings using the cell-attached configuration (charge carrier: 70 mM Ba^{2+}; holding potential: −100 mV; test potential: +20 mV for 150 ms). Bottom traces show average currents from the respective experiment.
doi:10.1371/journal.pone.0000292.g005

Quantitative analysis of L-VDCC subunit mRNA expression in human myocardium

Real-time PCR: Quantitation was performed with the iCycler iQ real-time PCR detection system using primer/fluorescent probe concentrations of 200 nmol/L either in 1× iQ Supermix (β_{2a-d}) or 1× iQ SyBr Green when no fluorescent probes was used ($\beta_{1a,b}$, β_3, cardiac calsequestrin) (all Bio-Rad®). Both iQ Supermix and SyBr Green based real-time PCR were followed by gel electrophoresis confirming amplification of singular products of expected size. In addition, SyBr Green based real RT-PCR was followed always by melt-curve analysis (70°C–94°C at 0.5°C steps) verifying the similarity of standard and specimen amplification product melting point. Quantitation was performed using intra-assay standard curves of the specific templates. Templates were cloned from human non-failing left ventricle mRNA reversely transcribed by iScript (Biorad®), identity to published sequences was confirmed at both strands.

Human β_1 template: PCR-cloning by sense primer 5′-CTCAAGGGCTACGAGGTTAC-3′ and antisense primer 5′-GTGTTTGGACTGAGACTTTCC-3′ (GenBank NM_000723; resp. positions: 864-883, and 1247-1227), with 94°C (3 min), and 40 cycles of 51°C (30 sec), 72°C (20 sec), and 94°C (20 sec). Real-time PCR was set up with the cloning primers and run at 94°C (3 min), and 40 cycles of 50°C (30 sec), 72°C (20 sec), and 94°C (20 sec). Correlation: ≥0.992, efficiency: ≥93.4%.

Human β_1 isoform templates: PCR cloning of the β_{1a} by sense primer 5′-GCCTCGGCTCCAGCAAA-3′ and antisense primer 5′-CTCACCAAGCTCAGCCTCTTC-3′ (GenBank NM_199247; resp. positions 692-708; 855-835), of the β_{1c} by the same sense but different antisense primer 5′-CTCTGTCGACTTC-TGCTTCTGTTT-3′ (GenBank NM_000723; position 807-784), with 94°C 3 min, 40 cycles 56°C (30 sec), 72°C (20 sec), 94°C (20 sec). Real-time PCR was set up with the respective cloning sense primer and antisense primers, and the fluorescent probe 5′-6FAM-CTCCAGTTCCAGTCTGGGAGATGTGGT XT p (GenBank NM_000723; 720-746) and run at 94°C (3 min), and 40 cycles of 53°C, and 94°C (each 30 sec). Correlation: ≥0.990, efficiency: ≥90.6%.

Human β_{2a-d} isoform templates: PCR cloning of β_{2a-d} isoforms was by isoform specific sense primers:

β_{2a}: 5′-GCATCGCCGGCGAGTA-3′, (GenBank423189, position: 21-36);

β_{2b}: 5′-GACAGACGCCTTATAGCTCCTCAA-3′ (GenBank AF285239; position: 7-30);

β_{2c}: 5′-AGTGGACTGGACCTGCTGAA-3′ (GenBank AF423190; position: 13-32);

β_{2d}: 5′-GCCGCCGCACAGTCATAT-3′ (GenBank AF423191; position: 109-126);

always with the same antisense primer 5′-CGGTCCTCCT-CCAGAGATACAT-3′ (GenBank AF423189; position: 109-89). Real-time PCR was set up with the respective cloning primers, and the fluorescent probe 5′-6FAM-ATGGACGGCTAGTGTAG-GAGTCTGCCGA XT p (GenBank NM_000723; position: 79-52) and run at 94°C (3 min), and 40 cycles of 56.5°C, and 94°C (each 30 sec). Correlation was ≥0.995, efficiency ≥90.4%.

Template of human β_3 and human cardiac calsequestrin: see [12].

Cloning of human β₁-, β₂-, β₃-splice variants and insertion into bicistronic eukaryotic expression vectors

β₁-subunits: Full length β_1-subunit isoform sequences were cloned using two pairs of sequence specific primers: 1st sense primer 5′-CCTCTCCATGGTCCAGAAGACCAGCA-3′ and 1st antisense primer 5′-CAAATAAAGCTTTCTGCATCATG-TCTGTAA-3′, and 2nd sense primer 5-TTACAGACATGA-TGCAGAAAGCTTTATTTG-3′ and 2nd anti-sense primer 5′-GCGCCCACTACATGGCATGTTCCT-3′ (GenBank NM_199247; resp. positions: 147-173; 1048-1019; 1019-1048; 1732-1709). PCR with the 1st primer pair yielded two amplification products due to alternative splicing of exon 7a and exon 7b. Full length message of β_{1A} with either exon 7a or 7b was reassembled in pIRES2-EGFP opened with BglII/SmaI site using the internal HindIII restriction site.

β₂-subunits: Full length β_2-subunit isoform sequences were cloned using two pairs of sequence specific primers derived from GenBank sequences. N-terminal coding sequences for β_2-subunit splice variants were generated using isoform specific primer pairs. β_{2a}: sense primer 5′-CTCTTCATGCAGTGCTGCGGGCTG-GT-3′ and antisense primer 5′- ACTTCCGCTAAGCTTGACC-TTGTG-3′ (GenBank U95019; resp. positions: 496-521, 1397-1374); β_{2b}: sense primer 5′-ATGCTTGACAGACGCCTTAT-AGCT-3′ and same antisense primer (GenBank AF285239; resp. positions: 1-24, 899-876); β_{2c}: sense primer 5′-ATGAATCAGG-GGAGTGGACTGGAC-3′ and same antisense primer (GenBank AF423190; resp. positions: 1-24, 977-894); β_{2d}: sense primer 5-ATGGTCCAAAGGGACATGTCCAAG-3′ and same antisense primer (GenBank AF423191; resp. positions: 1-24, 1061-1038). The C-terminal fragment was amplified by sense primer 5′-CACAAGGTCAAGCTTAGCGGAAGT-3′ and antisense primer 5′-GGCAAAACTCATTGGGGGAT-3′ (GenBank U95019; resp. positions: 1374-1397, 2327-2308). Amplification was performed in cDNA reverse transcribed (Revert Aid Kit, MBI Fermentas®) from mRNA isolated from non-failing human left-ventricular myocardium using Trizol (Invitrogen®) and the poly(A)tract kit (Promega®). PCR conditions always were 40 cycles of 94°C, 58°C, 72°C, (each 1 min); and 5 min 72°C. Amplification products were visualized by UV protected 0.8% agarose gel-electrophoresis, extracted (Perfect Gel Clean-up, Eppendorf-Vaudaux®), and subcloned into pCR2.1-TOPO (Invitrogen®). Sequences of cloned fragments were determined on both strands (MWG-Biotech®). For eukaryotic expression full length β_2-subunit isoforms were reassembled in the pcDNA3 polylinker region (Invitrogen®) opened by BamHI/NotI using the internal HindIII restriction site. Full length coding sequences were inserted by T4 DNA ligation of N-terminal β_2-subunit isoform fragments cut by BamHI (pCR2.1 restriction site) and HindIII (internal restriction site contained in all β_2-subunit isoforms) and of the C-terminal fragment cut by HindIII and NotI (pCR2.1 restriction site). Full-length coding sequences of β_2 isoforms were inserted by T4 DNA ligation into pIRES2-EGFP opened by EcoRI restriction.

β₃-subunits: Full length coding sequence was excised by EcoRI/XhoI and inserted into pIRES2-dsRed2 opened with EcoRI/SmaI.

human $\alpha_2\delta$-2: Full length coding sequence was obtained from Klugbauer et al. [36] excised by restriction with HindIII/XhoI and inserted into pIRES2-dsRed2 opened with NheI blunt/XhoI.

Western-blot analysis of Ca²⁺-channel subunits

Protein expression levels of the L-VDCC subunits were assayed by Western-blot analysis of human and mouse cardiac ventricular protein samples. Briefly, protein extracts were obtained by homogenizing frozen heart tissue in buffer (5% SDS, 50 mM TRIS-HCl, pH 7.4, 250 mM sucrose, 75 mM urea, and 10 mM DTT containing complete protease inhibitor cocktail tablet from Roche) using a Teflon homogenizer. The homogenate was

denatured by incubation at 95°C (2 min) followed by centrifugation at 16,000g (5 min); supernatants (containing membrane fractions and cytosolic proteins) were collected for analysis. Protein was quantified using Bicinchoninic acid (BCA) Protein Assay (Pierce®). For $Ca_V1.2$ and $\alpha_2\delta$-1 Western blots, 60 μg, and for β_2 and β_3 Western blots, 150 μg of total protein were separated on a 8% and 12% SDS-PAGE gel (BioRad®). Gels were transferred to nitrocellulose membranes (Amersham®) according to standard wet transfer procedure. L-VDCC subunits were detected using the following antibodies: anti-human $Ca_V1.2$ against the II-III loop (generous gift from Dr. Hannelore Haase, Max-Delbrück Center, Berlin, Germany; [37,38]); anti-β_2 (generous gift from Dr. Adolfo Cuadra (Dr. M. Hosey), Northwestern University, Chicago, USA; [39]); anti-β_3 and anti-$\alpha_2\delta-1$ (Alomone;[40,41]), and anti-calsequestrin (Santa Cruz;[42]). The anti-β_1 (Swant; cp. [16]) stains a band at ~57 kDa in the membrane of human skeletal muscle (data not shown) where β_{1a} is pre-dominant [43] suggesting that the antibody detects a β_{1a} in human and mouse myocardium. In the present study this antibody detected an additional band of ~65 kDa in murine myocardium and ~70 kDa in human heart. Though our present mRNA data indicate that there are two β_1-isoforms in cardiac tissue we cannot exclude a cross-reaction of the antibody with β_3-subunits since the second band is quite close to the band detected by the β_3-specific antibody from Alomone (see above). Thus we decided to avoid any quantitation of this "high molecular band" detected in murine and human cardiac tissue, respectively.

Immunofluorescence analysis of Ca²⁺-channel subunits

Ventricular myocytes were freshly isolated from 10 month old tg $Ca_V1.2$ and controls as previously described, stored in Kraft-Bruehe solution and plated on laminin-coated with poly-L-lysine and 50 μg/ml mouse laminin (Invitrogen®) coverslips for 1 h at 37° C, 5% CO_2. After incubation myocytes were washed with relaxation buffer (mM: 100 KCl, 5 EGTA, 5 $MgCl_2$, 0.25 dithiothreitol (DTT) in PBS, pH 6.8). Myocytes were then fixed in pre-cooled (−20°C) methanol/acetone (1:1) for 5–10 min at 4°C. To prevent non-specific binding, myocytes were blocked with 10% normal donkey serum (Sigma®) in PBS overnight (labeling buffer) [44]. Primary antibodies were diluted in labeling buffer and incubated with myocytes overnight at 4°C. Primary antibody dilutions for different subunits of the L-VDCC studied were: 1:200 for α_{1C} (Alomone®) and 1:500 for β_2. In the case of Wheat Germ Agglutinin (WGA) labeling, myocytes were incubated overnight at 4°C with Oregon Green 488-conjugated WGA (Molecular Probes®) at a concentration of 1 μg/ml. WGA selectively binding to N-acetyl-d-glucosamine in glycoproteins was used to label the peripheral sarcolemma, the T-tubules and the intercalated disks. After overnight incubation, myocytes were washed with PBS and incubated with secondary antibody in PBS-0.1% BSA for 1 h at room temperature. Secondary antibody for the study of the L-VDCC subunits was tetramethylrhodamine-isothiocyanate (TRITC)-conjugated donkey anti rabbit antibody at 1:400 dilution (Jackson ImmunoResearch®, USA). For negative control experiments, myocytes were kept in labeling buffer overnight without primary antibody and only incubated with secondary antibody at the same concentration. After washing the cells with PBS, coverslips were mounted on slides using Gel/Mount aqueous mounting media (Biomeda®) and images were acquired on a Nikon PCM 2000 laser confocal scanning microscope as 0.5 mm "optical sections" of the stained cells, keeping gain and background values constant through the different samples.

Generation of transgenic mice with inducible cardiac overexpression of β_{2a}

Recent modifications in the Drosophila ecdysone receptor revealed better regulation of gene expression in mammalian cells, however, the dependence on steroidal ligand activation (i.e. ponasteron) with its potential additional effects on gene expression remains. The ecdysone receptor from Bombyx mori is activated by the non-steroidal ligand tebufenozide (effective drug in MIMIC, Dow AgroSciences®, Munich, Germany) without known specific interaction in mammalian cells. This construct regulated β-galactosidase expression in HEK293 cells at concentrations of 1 μM tebufenozide as effectively as the Drosophila ecdysone receptor (data not shown). For our experiments we intraperitoneally injected 9 mg tebufenozide (i.e. twice the dose leading to maximum serum concentration of the drug) 48h before isolation of cardiac myocytes. The hybrid drosophila-bombyx ecdysone receptor (VgBmEcR) was constructed by fusion of the binding and transactivation domain of the modified drosophila system (pVgRXR, Invitrogen®) to the ligand binding domain of the bombyx ecdysone receptor (BmEcR in pBSII KS+BmB1 = ecdysone receptor type B1 of Bombyx mori, obtained from Fujiwara H., Tokyo, Japan) using the restriction enzyme *Bsr*GI and *Not*I. The coding sequence of VgBmEcR was set under control of the promoter of αMHC for cardio-specific expression. The αMHC promoter was excised from pBlue-MHCβ₁ARSV40polyA (obtained from Stefan Engelhardt, Wuerzburg, Germany) using *Dra*I and *Pvu*II and inserted into pVgBmEcR opened with the same enzymes. The ecdysone-regulated plasmid pInd-β₂ₐ was constructed by excision of the β-galactosidase coding sequence from pInd-LacZ (Invitrogen®) using *Hind*III and *Xba*I and insertion of the coding sequence for rat β₂ₐ excised from pCR3-β₂ₐ-6myc (obtained from A.J. Chien, Chicago, USA) with the same restriction enzymes. The linearized coding sequence of constructs were injected simultaneously into embryonic stem cells, and mice transgenic for the VgBmEcR and the β₂ₐ-subunit were identified by PCR using construct-specific primers (not depicted), and by Southern blot using αMHC-VgBmEcR and β₂ₐ specific probes labeled radioactively. Mouse DNA was obtained from mice 3 weeks post delivery and digested with *Eco*RI for proof of αMHC-VgBmEcR-, and *Hind*III/*Bam*HI for Ind-β₂ₐ -genomic integration. Probes specific for αMHC-VgBmEcR and Ind-β₂ₐ were obtained by *Sac*I and *Kpn*I/*Hind*III restriction of the respective coding sequences. Probes were radio-labeled with α-^{32}P-CTP using the Klenow fragment. Animals positive for integrated coding sequences were identified by 3.7 kb hybridization signal for αMHC-VgBmEcR and a 2.4 kb signal for Ind-β₂ₐ (Figure 4a).

Cell culture and co-transfection

Cell culture and transient co-transfection were done as described [12,45]. In brief, HEK293 cells were stably transfected with the full-length $Ca_V1.2$-subunit (GenBank NM_000719) cloned from human heart [46]. Cells were seeded in polystyrene Petri dishes (9.6 cm², Falcon®, Heidelberg, Germany) at a density of 1–2·10⁴ cells cm⁻² and transiently co-transfected with the cDNA plasmids encoding the different human cardiac β₂ splice variants together with human cardiac α₂δ-2-subunit [36]. Lipofection was carried out 24–36 h after plating by incubating (3–6 h) with SuperFect (Qiagen®) and the respective plasmids at a DNA mass ratio of 3:3:1 [12,45]. Transfected cells were grown on Petri dishes in Dulbecco′s modified Eagle′s medium (DMEM, Biochrom KG®, Berlin, Germany) supplemented with 10% fetal bovine serum (Sigma®, Deisenhofen, Germany), penicillin (10 units ml⁻¹) and streptomycin (10 μg ml⁻¹, both from Biochrom®). Electro-

physiological recordings in GFP-positive cells were obtained 48–72 h after transfection.

Isolation of ventricular myocytes

Single ventricular myocytes were isolated from murine hearts by enzymatic dissociation using the method described earlier [47]. In brief, hearts were perfused with a collagenase solution (Worthington type I and II, 75 U l⁻¹) in a Langendorff setup and subsequently cut into small chunks. Myocytes were harvested by pouring the suspension through cheesecloth.

Single-channel recording

Single-channel recordings were performed by using the cell-attached configuration of the patch-clamp method as described earlier [47]. Cells were placed in disposable Petri dishes containing 3 ml of a high-potassium depolarizing solution (mM: 25 KCl, 120 K-glutamate, 2 MgCl₂, 10 Hepes, 2 EGTA, 10 dextrose, 1 CaCl₂, 1 Na₂-ATP; pH 7.3 with KOH). Patch pipettes (borosilicate glass, 6–8M) were filled with pipette solution for myocytes (mM: 70 BaCl₂, 110 sucrose and 10 Hepes; for HEK cells (mM): 110 BaCl₂, 10 Hepes; pH 7.4 with TEA-OH. Ba²⁺ currents were elicited by voltage steps (150 ms at 1.66 Hz) from −100 mV to +20 mV (native channels) or +10 mV (recombinant channels) (≥180 sweeps per experiment). Data were sampled at 10 kHz and filtered at 2 kHz (3 dB, four-pole Bessel) by using an Axopatch 200A amplifier (Axon Instruments®, Foster City, USA). PCLAMP software (CLAMPEX 5.5.1, FETCHAN, and PSTAT 6) was used for data acquisition and analysis (Axon Instruments®, Foster City, USA). Signal-noise ratio and adequate resolution of openings were

similar to previous work [12,16,47], as confirmed by comparison of, *e.g.* data from wild-type mice.

Data analysis and statistics of single-channel recordings

Linear leak and capacity currents (averaged non-active sweeps) were digitally subtracted. Openings and closures were identified by the half-height criterion. The fraction of active sweeps within a patch (f_active), the open probability within active sweeps (P_open), and the peak value of single-channel ensemble average currents (I_peak) were determined as described [16]. Where necessary, these parameters were corrected for the number of channels in a patch, as described [6]. For comparisons unpaired Student's two-tailed t-test or Mann-Whitney test was used where appropriate. Throughout, a level of p<0.05 was considered significant. Values are given as mean±SEM.

Author Contributions

Conceived and designed the experiments: RH JM IB MR ES RG LH AS SH. Performed the experiments: RH JM Sv IB MR KD DR ES RG LH AS SH IK. Analyzed the data: RH JM Sv IB MR KD UH PM LH AS SH IK. Contributed reagents/materials/analysis tools: RH JM IB MB LH AS SH. Wrote the paper: RH JM IB AS SH.

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