Functional Expression and Inactivation of L-type Ca^{2+} Currents During Murine Heart Development - Implications for Cardiac Ca^{2+} Homeostasis

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Key Words

Cardiac • Cell differentiation • Ion channels • Ca^{2+} -channel • Inactivation • CICR

Abstract

Background/Aims: The expression and regulation of Ca^{2+} signaling in embryonic cardiomyocytes has been shown to be different from those in adult heart cells, particularly the L-type Ca^{2+} -channel current (I_{CaL}) increases during development. However, little is known about the underlying reasons for this increase of I_{CaL} density and developmental changes in the process of I_{CaL} inactivation, a critical regulator of intracellular Ca^{2+} homeostasis. In the present work, we therefore studied functional differences of I_{CaL} between embryonic and fetal cardiomyocytes and its interaction with intracellular Ca^{2+} homeostasis and Ca^{2+} -induced Ca^{2+} release (CICR). Moreover, we examined the process of voltage- (VDI) and Ca^{2+} -dependent inactivation (CDI) of I_{CaL} during murine embryonic heart development. **Methods:** The electrophysiological characteristics of I_{CaL} inactivation were analyzed in embryonic ventricular cardiomyocytes of early (E9.5-11.5) and late developmental, fetal (LDS,

E16.5-18.5) stages and of adult mice using the whole-cell patch-clamp technique. **Results:** Fast, Ca^{2+} -dependent inactivation kinetics (τ_f) were significantly accelerated in LDS-derived cardiomyocytes (2.53 ± 1.43 ms, $n=9$) as compared to EDS (5.09 ± 2.19 ms, $n=8$, $p=0.009$), whereas slow, voltage-dependent inactivation time constants (τ_s) were unchanged. In cardiomyocytes derived from LDS we observed an increase in the maximal gating charge (Q_{max}), suggesting an increase in the number of L-type Ca^{2+} channels at the sarcolemma, whereas the channel open probability (P_o) was unchanged. Accordingly a significantly higher I_{CaL} density was found in LDS (-14 ± 2.26 pA/pF, $n=14$) versus EDS-derived cardiomyocytes (-10.03 ± 1.43 pA/pF, $n=13$, $p<0.05$). Since ryanodine (10 μM) failed to alter τ_f at both developmental stages, a major contribution of CICR and bulk Ca^{2+} to the acceleration of the fast inactivation kinetics during heart development seems to be unlikely. **Conclusion:** Our data suggest that the increase of local subsarcolemmal Ca^{2+} , evoked by the higher expression of L-type Ca^{2+} channels at the sarcolemma, rather than bulk Ca^{2+} accelerates I_{CaL} inactivation during embryonic heart development.

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Introduction

Even in immature cardiac cells intracellular concentrations of Ca^{2+} [Ca^{2+}]_i have to be controlled very precisely to maintain Ca^{2+} homeostasis. In differentiating cardiomyocytes previous studies showed that the density of cardiac transmembrane charge movement and I_{CaL} increases during maturation [1]. Compensatory mechanisms, e.g. I_{CaL} inactivation, are supposed to contribute to the control of bulk [Ca^{2+}]_i. As detailed information about development-dependent biophysical characteristics of I_{CaL} inactivation is lacking we wanted to discover whether increasing I_{CaL} density during development is paralleled by enhanced I_{CaL} inactivation. Furthermore this study aims to clarify if the increase of I_{CaL} density is evoked by an increasing number of L-type Ca^{2+} channels in the membrane and/or altered open probability, and also whether CICR contributes to inactivation already at the embryonic stage.

In adult cardiomyocytes inactivation of L-type Ca^{2+} channels is of fundamental physiological relevance since it allows to tightly control intracellular Ca^{2+} levels. Ca^{2+} ions and membrane voltage contribute to the inactivation of the L-type Ca^{2+} channel through separate, independent mechanisms, called CDI and VDI [2, 3]. In general, the contribution of CDI and VDI to the decay of I_{CaL} is determined by depolarization of the cell or by phosphorylation of the L-type Ca^{2+} channel protein [4–7]. The close relationship of L-type Ca^{2+} channels with the Ca^{2+} extrusion mechanisms of the sarcoplasmic reticulum (SR), formed by T-tubuli and ryanodine receptors (RyRs), significantly contribute to the CDI process due to a strong increase of local subsarcolemmal [Ca^{2+}]_i during the CICR process [8, 9]. In cells that lack this close association, e.g. embryonic cardiomyocytes [10], CDI is supposed to be more closely related to the overall current density of I_{CaL} [11]. Detailed information is however still lacking.

The slower phase of I_{CaL} inactivation is mainly influenced by VDI [12]. This conclusion was drawn from the observation that I_{CaL} carried by ions that do not provoke CDI, such as Ba^{2+} or Na^{+} , inactivate much slower than currents carried by Ca^{2+} [13, 14]. Also for VDI, the molecular basis of inactivation has been intensively studied. Although earlier studies could not demonstrate a large quantitative impact of VDI on the inactivation process in general, recent studies analyzed this aspect in more detail. VDI strongly increased with depolarisation and was fast at positive membrane potentials [4, 7].

Since all these studies have been exclusively

performed in neonatal and adult cardiomyocytes from different species, our present work aimed to study CDI and VDI at the murine embryonic and fetal stage.

To investigate the mechanisms of inactivation, we have compared biophysical characteristics of I_{CaL} inactivation measured in EDS, LDS and adult cardiomyocytes. We have chosen experimental conditions where pure voltage and Ca^{2+} -dependent mechanisms drive the inactivation process. We found a strong increase of fast inactivation kinetics paralleled by an increasing number of ion channels in the membrane as the underlying reason for the increase of I_{CaL} during heart development reported recently [1]. CICR did not significantly contribute to the alteration of I_{CaL} inactivation.

Materials and Methods

The investigation conforms to the institutional guidelines of the University of Cologne, which are defined by the European Community Guiding Principles in the Care and Use of Animals.

Cell preparation

Embryonic ventricular myocytes

For harvesting murine embryonic ventricular cardiomyocytes mice (strain HIM:OF1) were bred and superovulated as described earlier [15]. Briefly, for isolation of early and late stage cardiomyocytes, E9.5–E11.5 (EDS) and E16.5–E17.5 (LDS) embryos were used, respectively. After embryos were harvested, hearts were dissected and atria separated from ventricles. Single ventricular cardiomyocytes were obtained using enzymatic dissociation procedures as described previously [16, 17]. The isolated, floating cells were plated on sterile gelatin-coated glass coverslips, cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing 20% fetal bovine serum and kept in the incubator for 24–48 hours until attachment was achieved that allowed electrophysiological experiments.

Adult ventricular myocytes

Ventricular cardiomyocytes were isolated from adult mouse hearts (strain HIM:OF1; 4–6 weeks) using a standard Langendorff apparatus as described [18]. In contrast to embryonic cardiomyocytes, adult cells could already be used for experiments directly after enzymatic dissociation.

Electrophysiological recording

Standard voltage clamp recordings were performed using the classic whole-cell patch-clamp technique [19]. Current was recorded using an EPC-9 amplifier (Heka, Lambrecht, Germany) and pipettes (2–4 MΩ resistance when filled with standard intracellular solution) were made from thin walled borosilicate glass capillaries (Harvard Apparatus LTD, Edenbridge, UK) on a DMZ Universal Puller (DMZ, München, Germany). Glass coverslips containing isolated cardiomyocytes were transferred

Fig. 1. Basic characterization of the L-type Ca^{2+} current (I_{CaL}) in embryonic and fetal cardiomyocytes. The current-voltage (I - V) relationship of I_{CaL} was determined by applying test pulses (T, 100 ms) from -60 mV to $+50$ mV in 10 mV increments at a frequency of 0.2 Hz. Pre-pulses from a HP of -80 mV to -40 mV (P, duration: 50 ms) together with Tetrodotoxin (TTX, 10 μM) were applied to inactivate Na^+ currents (I_{Na}). According to previous studies, the prestep to -40 mV is useful to inactivate also T-type Ca^{2+} current (I_{CaT}) (see protocol in A). Representative I - V traces recorded from an early (EDS, B) and late (LDS, C) development stage cardiomyocyte. D) Changes in cellular capacity and (E) I_{CaL} density during heart development. Peak I_{CaL} measured at $+10$ mV was determined as the difference between the peak inward current and baseline current at the end of depolarization. The ordinate shows current density (pA/pF), which was obtained by normalizing currents to membrane capacitance. F) Averaged I - V curves of peak I_{CaL} in EDS- and LDS-derived cardiomyocytes. Asterisks indicate statistically significant differences.

to the recording chamber and placed upon the stage of an Axiovert 135 TV inverted microscope (Zeiss, Germany). The cells were constantly superfused with experimental external solutions using a gravitational perfusion system with a perfusion rate of about 2 ml/min. The temperature of the bath as well as of the perfusion solutions was kept constant at 37°C . If not otherwise stated, voltage-clamp protocols were delivered to the cells from a holding potential (HP) of -80 mV. The current-voltage (I - V) relationship of I_{CaL} was determined by applying a standard I - V protocol for I_{CaL} . According to previous studies, the prestep to -40 mV was useful to inactivate T-type Ca^{2+} current (I_{CaT}) [6, 20].

The recovery from short-term inactivation was evaluated according to previously published methods [21]. Tail currents were measured at -50 mV as described [22]. For measurements of gating currents any contaminating currents were blocked by 2 mM Co^{2+} [23, 24]. Additionally we used TEA and Cs^+ to block potassium channels and TTX to avoid contamination by Na^+ channels [25]. Data were digitized at 10 kHz, filtered at 1 kHz, stored on hard disk and analyzed using Pulse-Fit (Heka, Lambrecht, Germany) analysis software. As reported previously [15, 26], I_{CaL} did not show significant run down (see also Figure 3). Protocols are described in detail in the figure legends.

Experimental solutions

The composition of the different solutions was as follows (in mMol/l): the standard extracellular solutions used to dissociate and to store adult myocytes prior to experiments contained: 135 NaCl, 4 KCl, 1 MgCl_2 , 2.5 Hepes and 5 glucose (pH 7.4 adjusted with NaOH). Extracellular solutions used to superfuse cells during electrophysiological recording contained: 120 NaCl, 5 KCl, 1 MgCl_2 , 20 TEA-Cl and 10 Hepes, adjusted to pH 7.4 with TEA-OH. To this solution 2 mM of either CaCl_2 or BaCl_2 was added. Ca^{2+} free extracellular solution

contained 3 EGTA, 1.5 MgCl_2 and no Ca^{2+} . The standard intracellular solution used to fill the patch pipettes contained: 120 CsCl, 10 EGTA, 3 MgCl_2 , 5 ATP- Mg^{2+} , 5 Hepes, adjusted to pH 7.4 with CsOH, thus making Cs^+ the main intracellular cation. For the measurement of gating currents we used an external solution with 160 TEA-Cl, 2 BaCl_2 , and 10 HEPES (pH adjusted to 7.4 with TEA-OH). To this solution 10 μM TTX and 2 mM Co^{2+} (instead of 2 mM Ba^{2+}) were applied to block I_{Na} and contaminating ionic currents. The internal (pipette) solution contained 56 CsCl, 68 CsF, 2.2 MgCl_2 , 4.5 EGTA, 9 HEPES, 4 MgATP, and 14 creatine phosphate (Tris salt), adjusted to pH 7.4 with CsOH.

Fig. 2. Time constants of inactivation. The inactivation kinetics of representative I_{CaL} traces recorded from EDS (A), LDS (B) and adult (C) cardiomyocytes (test potential +10 mV) were fitted either with mono- (gray lines) or bi-exponential (dotted gray lines) algorithms. The beginning of the depolarization current traces corresponds to time point "0". The bi-exponential fitting traces of adult cardiomyocytes show fast and slow inactivation components. D-F) Statistical analysis of EDS (D), LDS (E) and adult (F) cells in which I_{CaL} was best fitted either with mono and/or bi-exponential algorithms. G) Superposition of representative peak I_{CaL} traces (black lines) and fast and slow inactivation time constants determined with bi-exponential fits to the decaying portion of I_{CaL} (dotted gray lines) at the different developmental stages. Normalized traces are shown as insets.

All substances were, if not otherwise stated, obtained from Sigma-Aldrich Chemie GmbH, Germany.

Data analysis

For the analysis of inactivation, mono- and bi-exponential fits, using a simplex optimization algorithm, were applied to the decaying part of individual I_{CaL} traces. Original traces were fitted using Pulse-Fit (Heka). The maximum of I_{CaL} was taken as time point "0" and fitting performed until the end of the depolarization step. The best fit, judged by visual inspection and by examination of the root mean square (RMS) value, was used [27] as a read out for the deviation between fit and data. To decide whether a mono- or bi-exponential fitting procedure was preferred, all traces were fitted with both fitting algorithms and then compared.

Data were fitted using Origin 7 (OriginLab Corporation, Northampton, MA, USA). Gating currents were analyzed as described recently [22]. Data are expressed as mean $\pm$ SEM for the number of cells indicated (*n*). A Student's *t*-test was used to compare data between different groups. A *p*-value of less than 0.05 was considered statistically significant different.

Results

Basic characterization of I_{CaL} in embryonic ventricular cardiomyocytes

As a basic characterization of I_{CaL} , I_{CaL} densities and *I-V* curves of EDS- and LDS-derived cardiomyocytes

	Inactivation parameters					
	Times constants (ms)			Amplitudes (pA)		
	Fast	slow	mono	fast	slow	mono
EDS (n=8)	5.09±2.19	30.60±1.62	32.80±1.95	-223.91±21.94	-146.30±27.98	-397.66±51.68
LDS (n=9)	2.53±1.43*	28.84±1.97	27.33±2.18	-451.56±29.62	-185.08±21.63	-589.33±41.60
Adult (n=5)	1.97±0.77 [#]	35.50±2.16	25.58±1.33	-579.40±58.12 [#]	-165.80±27.94	-750.20±80.16

Table 1. Mean values of inactivation time constants and amplitudes of mono- and bi-exponential components of I_{CaL} inactivation recorded from EDS, LDS and adult cardiomyocytes. Values are means±SEM. * indicates where LDS cardiomyocytes are significantly different to EDS ($P < 0.05$) and # indicates where adult cardiomyocytes are significantly different compared to EDS ($P < 0.05$). Due to not fully inactivated currents, inconsistencies between current densities and current amplitudes of fast and slow inactivation might occur.

were determined (Fig. 1). The capacitance recorded from individual cells was taken to normalize currents as described previously [15]. It was significantly smaller in cells derived from EDS (42.70 ± 4.50 pF, $n=13$) versus LDS (57.34 ± 5 pF, $n=14$, $p < 0.05$) (Fig. 1D). The I_{CaL} density (Fig. 1E) at 0 mV was significantly higher at LDS (-14 ± 2.26 pA/pF, $n=14$) as compared to EDS (-10.03 ± 1.43 pA/pF, $n=13$, $p < 0.05$). At both stages $I-V$ curves showed activation of I_{CaL} beginning at -30 mV and peak currents between 0 and $+10$ mV (Fig. 1F). The amplitude of I_{CaL} at $+10$ mV was also significantly larger at LDS (-15.12 ± 2.07 pA/pF, $n=12$) compared to EDS (-9.02 ± 1.29 pA/pF, $n=8$, $p < 0.05$) (Fig. 1F).

To exclude that I_{CaL} density varied during the culture process, control experiments were performed 24 hours after dissociation. Compared to standard recordings after 48 hours no significant differences were observed for EDS (-8.95 ± 2.35 pA/pF, $n=6$ versus -10.03 ± 1.43 pA/pF, $n=13$, $p=0.54$) and LDS-derived cardiomyocytes (-14 ± 2.26 pA/pF, $n=14$ versus -13.94 ± 2.26 pA/pF, $n=4$, $p=0.3$).

Inactivation kinetics and time constants

To determine potential differences in inactivation kinetics during development, mono- versus bi-exponential fitting algorithms were tested in EDS, LDS and adult cardiomyocytes. The RMS value and visual inspection helped to decide whether mono- or bi-exponential fittings were preferred.

In adult and LDS-derived cardiomyocytes I_{CaL} inactivation kinetics were significantly faster than at the EDS (Fig. 2A-C). Accordingly, in EDS ventricular cardiomyocytes, most of the I_{CaL} traces showed a mono-exponential decay (Fig. 2D), whereas in LDS only approximately 50% could be fitted with a single exponential function (Fig 2E). However in adult cardiomyocytes,

a bi-exponential decay kinetic was mostly observed (Fig. 2F). The ongoing development may account for that finding. Moreover, we cannot exclude that cells from the same ventricle may be in a different maturation status. Fig. 2G shows a superposition of representative peak I_{CaL} traces (black lines) and their respective bi-exponential fits (dotted gray lines) at the different developmental stages. The analysis of the fast and slow time constants and their amplitudes is summarized in Table 1. Taken together, fast inactivation time constants (τ_f) were significantly accelerated in adult and LDS-derived cardiomyocytes. Although there was a tendency of faster I_{CaL} inactivation in adult versus LDS cells, it was not statistically resolvable. In parallel to the increase of I_{CaL} density during development we also observed significantly increasing amplitudes of τ_f . At all stages no significant differences in slow inactivation time constants (τ_s) and amplitudes of τ_s were observed. The incomplete inactivation of I_{CaL} traces explains inconsistencies between maximal I_{CaL} current densities and current amplitudes of fast and slow inactivation kinetics.

Recovery from inactivation

Inactivation of I_{CaL} is membrane potential dependent. At positive potentials and high stimulation frequencies full channel recovery does not occur, resulting in a decrease in depolarization-induced I_{CaL} amplitude (short term inactivation).

To study the recovery from short-term inactivation with Ca^{2+} as the charge carrier, a standard two-pulse protocol (Fig. 3A) (pulse duration 100 ms, HP -80 mV) was used. Fig. 3B and 3C illustrate superimposed current traces recorded from representative EDS and LDS-derived cardiomyocytes, respectively. The fractional recovery of I_{CaL} from short-term inactivation was calculated as shown in the inset of Fig. 3D. Data fitted

Fig. 3. Recovery of I_{CaL} from short-term inactivation. A) A standard two-pulse protocol (pulse duration 100 ms) was used to characterize recovery from short-term inactivation. The cell membrane was depolarized from a HP of -80 mV to a test potential of +10 mV. A voltage step to +10 mV was used after a variable inter-pulse interval. The pulse interval was augmented from 0 to 400 ms in 40 ms increments. (B) and (C) show representative original traces where apparently no significant run-down was present in EDS and LDS. Note the rapid recovery of I_{CaL} . (D) Mean fractional recovery from short-term inactivation observed in EDS- and LDS-derived cardiomyocytes in the presence of 2 mM Ca^{2+} . The fractional recovery of I_{CaL} from short-term inactivation is defined as the ratio b/a and shown as inset. Curves were fitted by a biexponential function. Time constants were not significantly different (see text).

with a bi-exponential function showed that the time constant for the recovery process during the first 200 ms (more than 90% of recovery) amounted to 32.5 ± 7 ms ($n=9$) in EDS *versus* 30.9 ± 8 ms ($n=11$, $p>0.05$) in LDS cardiomyocytes (Fig. 3D). Although at a step potential of +10 mV the amplitude of I_{CaL} recovered to steady state levels already after a 160 ms interpulse interval, the I_{CaL} amplitude was still decreased pointing to a significant number of L-type Ca^{2+} channels remaining in the inactive state. Using an interpulse interval of 400 ms, but changing the holding potential of the interpulse from -40 to 0 mV, we found that recovery from inactivation was also voltage-dependent (data not shown). Thus recovery from inactivation of I_{CaL} is voltage- and time-dependent, but not depending on the developmental stage.

Steady-state activation and inactivation kinetics of I_{CaL} using Ca^{2+} and Ba^{2+} as charge carriers

To study the voltage dependence of inactivation, we applied the experimental protocol shown in Fig. 4Aa. Ba^{2+}

was used as the charge carrier. As for I_{CaL} , the amplitude of I_{BaL} was larger in cardiomyocytes derived from LDS than EDS (Fig. 4Ab,c). In contrast to experiments using Ca^{2+} as the charge carrier, we observed a left shift of the I - V curve in LDS (Fig 4B), as previously reported for adult cardiomyocytes [28].

The voltage dependence of activation and steady state inactivation of I_{CaL} were studied in the presence of 2 mM Ca^{2+} (Fig. 4C) or 2 mM Ba^{2+} (Fig. 4D). Activation and steady-state inactivation curves of the individual cells were fitted with a Boltzmann equation. The statistical analysis is given in Table 2. The data show that the I_{CaL} activation curves calculated for LDS and EDS were parallel but shifted to more negative potentials in LDS-derived cardiomyocytes (approximately 3 mV for Ca^{2+} and Ba^{2+}). The threshold of inactivation was approximately -50 mV for EDS and LDS. In contrast to the results for activation, inactivation curves of cardiomyocytes derived from LDS shifted to more positive potentials. In the presence of Ca^{2+} the shift was

Fig. 4. I_{CaL} current carried by 2 mM Ba^{2+} and steady-state activation and inactivation curves. A) Typical $I-V$ traces recorded according to the voltage-clamp protocol shown as inset (a) in the presence of 2 mM Ba^{2+} as the charge carrier in a representative EDS- (b) and LDS (c) - derived cardiomyocyte. The voltage-clamp protocol consisted of a depolarizing pre-step (50 ms, -60 mV) followed by test pulses ranging from -60 to +50 mV for 100 ms in 10 mV increments. B) $I-V$ curves of the peak inward currents of EDS- and LDS-derived cardiomyocytes. The $I-V$ curves were measured as described in the

legend of figure 1 but in the presence of 2 mM Ba^{2+} . Activation and steady-state inactivation curves were obtained under conditions in which 2 mM Ca^{2+} (C) or 2 mM Ba^{2+} (D) was used. Steady state inactivation was determined by stepping to various pre-pulse voltages (-60 to +40 mV, 10 mV increments) for 5 s prior to depolarization to a fixed test potential of +10 mV for 100 ms to evoke channel opening. There was a 5 ms interpulse interval to allow for resetting of the activation gate between the end of prepulse and the beginning of the test pulse. Peak currents obtained at all voltages were normalized to the maximal value. Steady state activation was determined by stepping to various prepulse voltage levels (-50 to +40 mV, 10 mV increments) for 100 ms from a HP of -80 mV prior to repolarizing to a fixed test pulse at -50 mV to evoke the tail currents. Peak tail currents were normalized. Both, the steady state activation and inactivation curves, were fitted with a single Boltzmann equation. The activation curves shifted at LDS to the left, whereas the inactivation curves shifted to more positive voltages. Data represent means $\pm$ SEM.

2mM Ca ²⁺					2mM Ba ²⁺				
	<i>k</i>		<i>V</i> _{1/2} (mV)			<i>k</i>		<i>V</i> _{1/2} (mV)	
	Activation	Inactivation	Activation	Inactivation		Activation	Inactivation	Activation	Inactivation
EDS	7.94±0.27	6.22 ±0.49	-12.48±0.14	-31.09 ±0.31	5.51±0.46	6.47 ±0.28	-10.98±0.23	-29.65 ±0.51	
LDS	7.67±0.16	6.17 ±0.36	-14.80±0.72	-25.59 ±0.19*	6.15±0.58	6.13 ±0.24	-17.28±0.19	-26.059±0.58	

Table 2. Steady-state activation and inactivation parameters for I_{CaL} recorded in EDS and LDS-derived cardiomyocytes under condition in which 2 mM Ca^{2+} (EDS, n=5 and LDS, n=8) or 2 mM Ba^{2+} (EDS, n=5 and LDS, n=9) were used. Data (means $\pm$ SEM) were fitted with a Boltzmann function ($y = A2 + (A1-A2)/(1 + \exp((V_{1/2} - x)/k))$) that describes conductance (y) as a function of the potential (x). $V_{1/2}$ is half-activating or half-inactivating potential, and k is the slope factor. * indicates statistical significance.

Fig. 5. Voltage-dependent inactivation of I_{CaL} . Availability-voltage (A - V) relationships during development were obtained with standard double-pulse voltage-clamp protocols (1 s lasting pre-pulses ranging from -50 mV to $+60$ mV in 10 mV increments followed by a 500 ms long depolarizing voltage step to $+60$ mV). The two pulse protocols were separated by a 50 ms lasting interval at -50 mV. The amplitude of the current evoked by the test-pulse voltage steps was normalized to the amplitude of the current evoked during the pre-pulse step to -50 mV. The figures represent the A - V relationship recorded in the presence of extracellular Ca^{2+} (A, Ca^{2+} - and voltage dependency) in EDS, LDS and adult cardiomyocytes as well as in the presence of Ba^{2+} (B, voltage dependency) following the 1 s lasting pre-pulse. C), D) and E) represent the comparison of the A - V relationships recorded in presence (CDI) and absence (VDI) of extracellular Ca^{2+} in EDS, LDS and adult cardiomyocytes, respectively.

significant ($V_{1/2}$: -31.09 ± 0.31 and -25.59 ± 0.19 for EDS and LDS, respectively, $p < 0.05$). As suggested by Cohen and Lederer [29], the shift observed in LDS can be interpreted as a consequence of a local increase of the Ca^{2+} concentration.

Voltage- and Ca^{2+} -dependent inactivation

To evaluate the contribution of Ca^{2+} to the inactivation of embryonic I_{CaL} in more detail, we replaced Ca^{2+} by Ba^{2+} in the extracellular solution eliminating Ca^{2+} -dependent inactivation [30] (see Materials and Methods).

Fig. 6. Effect of voltage on I_{CaL} inactivation time constants. Superimposed, normalized I_{CaL} and I_{BaL} traces recorded from EDS (A, B) and LDS (C, D) -derived ventricular cardiomyocytes; current was evoked by 100 ms lasting depolarizing voltage steps to -20 mV, 0 mV and +20 mV, respectively (HP -80 mV). Recordings were performed in presence of 2 mM Ca^{2+} (A, C) and 2 mM Ba^{2+} (B, D). Note the enhancement and voltage dependence of inactivation during development. Inactivation time constants for Ca^{2+} (E, F) and Ba^{2+} (G), when currents were fitted with mono- (Ba^{2+}) and bi- (Ca^{2+}) (fast and slow) exponential algorithms.

To test whether there might be a development-dependent difference in the amount of current, which is inactivated, we studied the steady-state inactivation at the isochronic state [4, 6] as well as the time- and voltage-dependence of inactivation. Fig. 5 illustrates data recorded in EDS and LDS-derived ventricular cardiomyocytes during a double-pulse voltage clamp protocol (for details see figure legend) that was used to bring the data to be a close approximation to inactivation at a steady-state level. These data cannot be used to report the time scale of the development of inactivation and kinetic differences, but suggest that more current can be inactivated in cardiomyocytes derived from LDS as compared to EDS. The availability-voltage relationship ($A-V$) for recordings in the presence of Ca^{2+} (CDI + VDI, with $V_{1/2}$ values of -25.1 ± 1.4 mV in EDS, -27.5 ± 2 mV in LDS and -32 ± 3.7 mV in adult cells) and in the absence of Ca^{2+} (replacement by Ba^{2+} , VDI, with -26.8 ± 1.7 mV in EDS, -28.6 ± 1.8 mV in LDS and -33 ± 2 mV in adult cells) is shown in Fig. 5A

and 5B. Fig. 5C, 5D and 5E indicate the differences of $A-V$ curves recorded in the presence (○) and absence (●) of extracellular Ca^{2+} in EDS, LDS and adult cardiomyocytes, respectively.

To determine the time- and voltage-dependence of inactivation, currents were normalized to the respective peak value of I_{CaL} (Fig. 6). Measurements in an individual cell were performed first in 2 mM extracellular Ca^{2+} (Fig. 6A and 6C) and then in equimolar extracellular Ba^{2+} (Fig. 6B and 6D). At both developmental stages inactivation of Ca^{2+} - and Ba^{2+} -driven I_{CaL} increased with depolarization. This was confirmed by calculating time constants of inactivation as shown in Fig. 6E-G. Due to its typical mono-exponential inactivation pattern, Ba^{2+} -driven I_{BaL} was fitted only with a single exponential function. As shown in Fig. 6G time constants for current inactivation were slower when Ba^{2+} was applied.

The effect of membrane voltage on decay of I_{CaL} and I_{BaL} is shown in Fig. 7A-D. The inactivation of I_{CaL}

Fig. 7. The voltage dependence of the decay of I_{CaL} . (A, B) VDI and CDI in EDS and LDS-derived cardiomyocytes in the presence of Ca^{2+} or Ba^{2+} (C, D) as the charge carrier. Currents were evoked by 100 ms depolarizing voltage steps from -20 mV to $+30$ mV in 10 mV increments. Inactivation of I_{CaL} and I_{BaL} was quantified by measuring the amplitude of the current at fixed times following activation in relation to the initial peak amplitude of the current. Data show the degree of inactivation at 10, 20, 50 and 100 ms after peak I_{CaL} . (E) Proportion of the inactivation due to Ca^{2+} at 10 ms following activation.

carried by Ca^{2+} (Fig. 7A and 7B) is bell-shaped with a maximum of inactivation at ~ 0 mV suggesting CDI [31]. As shown for different time points (10 ms to 100 ms) inactivation was also time-dependent. As compared to Ca^{2+} -carried currents we observed a reduction of the time-dependent inactivation when Ba^{2+} was used as charge carrier (Fig. 7C and 7D). Moreover, with the exception of the data recorded at -20 mV, decay of I_{BaL} was not voltage-dependent. Fig. 7E shows the proportion of inactivation caused by Ca^{2+} 10 ms after activation of I_{CaL} . These results clearly prove that I_{CaL} inactivation depends on Ca^{2+} and membrane potential.

Effect of the SR Ca^{2+} release inhibitor ryanodine on I_{CaL} inactivation

Several studies showed that in ventricular cardiomyocytes influx of Ca^{2+} through I_{CaL} serves as a trigger of Ca^{2+} release from the SR (CICR). This SR Ca^{2+} release mechanism, which is a consequence of the close morphological relationship between L-type Ca^{2+} channels and RyR2, leads in turn to an acceleration of the fast, Ca^{2+} -dependent I_{CaL} inactivation [32]. To test whether CICR contributes to our observation of acceleration of I_{CaL} inactivation during embryonic heart development, we applied ryanodine (10 μ M), a blocker

Fig. 8. L-type Ca^{2+} channel gating current and open probability (P_o) during murine embryonic heart development. Voltage protocol (upper panel) and representative currents (lower panel) recorded in EDS (A) and LDS-derived ventricular cardiomyocytes (B). Currents were evoked by a 20 ms test pulse to +90 mV (HP -100 mV). (C) Charge movement evoked by voltage steps from -50 mV to +80 mV in EDS and LDS-derived cardiomyocytes. Data were fitted with a single Boltzmann equation ($Q_{\text{ON}} = Q_{\text{max}} / (1 + \exp(-(V - V_{1/2})/k))$, where Q_{max} is the maximum amount of charge, $V_{1/2}$ is the potential of half-maximal charge movement, and k is the slope factor). (D) Original ON-gating currents (upper panel) and ON-gating charge (Q_{ON} , lower panel) at +50 mV pointing to a higher functional expression of L-type Ca^{2+} channels in the sarcolemma at LDS. (E) Tail-activation (G - V) curves calculated from tail current densities (J_{tail}). (F) Tail-current density at the reversal potential (J_{rev}) plotted against Q_{ON} (linear regression), indicating no significant changes of the L-type Ca^{2+} channel open probability P_o . Asterisks indicate statistically significant differences.

of SR Ca^{2+} release. Ryanodine did not substantially change I_{CaL} amplitude at EDS (-8.95 ± 2.35 pA/pF, $n=6$ (controls) versus -7.47 ± 1.25 pA/pF, $n=6$ (ryanodine), $p=0.15$) and LDS (-14.13 ± 1.62 pA/pF, $n=6$ (controls) versus -12.70 ± 0.8 pA/pF, $n=4$ (ryanodine), $p=0.08$). τ_f was not significantly different in EDS (4.72 ± 1.46 ms, $n=6$ (controls) versus 5.61 ± 1.59 ms, $n=6$ (ryanodine), $p=0.61$) and LDS-derived cardiomyocytes (2.24 ± 0.48 ms, $n=6$ (controls) versus 3.01 ± 1.01 ms, $n=6$ (ryanodine), $p=0.12$).

Our data suggest that CICR has no substantial effect

on I_{CaL} inactivation in embryonic and fetal murine cardiomyocytes and that influx of extracellular Ca^{2+} through the L-type Ca^{2+} channels may be responsible for CDI.

L-type Ca^{2+} channel gating current during embryonic murine heart development

To gain more insight into the mechanisms explaining the increase in I_{CaL} density and the acceleration of inactivation in LDS-derived cardiomyocytes, we

investigated gating charge movements and tail currents. This approach aimed at clarifying whether an increase in the number of L-type Ca^{2+} channels expressed on the membrane (N) or the same number of more active channels (increase of the open probability, P_o) or both factors contribute to the observed increase of I_{CaL} density.

For measurements of charge movements Ba^{2+} was replaced by Co^{2+} to block contaminating currents as described [23]. Application of the voltage protocol shown in the inset of Fig. 8A evoked gating currents in EDS- and LDS-derived cardiomyocytes as shown as original traces in Fig. 8A and Fig. 8B, respectively.

The total charge moved during the depolarization steps, Q_{ON} , was calculated according to previously published methods [33, 34]. Briefly, for each individual cell, Q_{ON} was normalized to cell capacitance and plotted against voltage to obtain the $Q_{\text{ON}}-V$ relationship (Fig. 8C). The curves were fitted with a single Boltzmann function for each developmental stage. Q_{MAX} , $V_{1/2}$ and k amounted in EDS ($n=4$) to 3.93 ± 0.76 pC/pF, -9.93 ± 0.82 mV and 12.51 ± 0.69 and in LDS ($n=5$) to 6.56 ± 0.04 pC/pF, -8.42 ± 0.76 mV and 14.55 ± 0.65 , respectively as has similarly been described for other species [33, 35]. These data demonstrate that more charge is moved in LDS-derived cardiomyocytes. To test whether an increase of the number of channels (N) and/or changes of P_o account for our observation, we calculated Q_{ON} at the reversal potential (Fig. 8D, *Lower panel*) (+50 mV) as an indirect read out for N [22, 36]. As shown in Fig. 8D we found a significant increase of N in LDS-derived cardiomyocytes ($p < 0.005$).

When measuring the tail current density (J_{tail}), we observed an increase of J_{tail} in cardiomyocytes derived from LDS as compared to EDS (Fig. 8E). Plotting J_{tail} of individual cells at the reversal potential (+50 mV), J_{rev} , versus Q_{ON} (Fig. 8F) provides an index of the relative opening probability P_o . In contrast to the finding of an increasing number of channels in the sarcolemma contributing to the higher I_{CaL} density at LDS, we could not observe significant differences for P_o in EDS and LDS-derived cardiomyocytes. Taken together, our data imply that the increase of N mainly contributes to the increase of I_{CaL} density during embryonic heart development, whereas P_o remains unchanged.

Discussion

As shown for native cardiac tissue from other species [37], we could recently show that during murine

embryonic stem (ES) cell differentiation, the functional expression of I_{CaL} increased from EDS to LDS-derived cardiomyocytes [38]. A detailed analysis of the underlying reasons, however, was missing so far. Although an increase of I_{CaL} requires compensatory processes preventing cytosolic Ca^{2+} overload, e.g. inactivation of I_{CaL} , information about such mechanisms was also still lacking. Here we provide evidence for (i) an increase of I_{CaL} density from EDS towards LDS in *native murine* cardiomyocytes confirming the data from ES cell-derived cardiomyocytes. This finding is explained by (ii) an increase in the number of L-type Ca^{2+} channels in the plasma membrane but not by a change of the open probability. Moreover, we found (iii) a switch from a monophasic to a biphasic decay of I_{CaL} , (iv) significant contribution of Ca^{2+} to the fast component and membrane voltage to the slow component of I_{CaL} decay and (v) significant increase of the contribution of Ca^{2+} to I_{CaL} decay during maturation at more positive membrane potentials. Most interestingly, (vi) a significant contribution of CICR to the decay of L-type Ca^{2+} channels could be excluded.

The role of I_{CaL} inactivation has previously been studied in adult cardiomyocytes from fish [39], rat [40, 41], rabbit [1] and guinea pig [4-7], as well as in heterologous expression systems [42, 43]. In the present study we have focused in detail on the functional expression of I_{CaL} during embryonic heart development to test whether the increase of I_{CaL} holds also true for native murine tissue and to elaborate whether an increase in the number and/or of the open probability of L-type Ca^{2+} channels may explain the increase of current. Moreover we studied biophysical changes of inactivation kinetics as compensatory mechanisms in detail. This approach could be useful to gain more insight into pathophysiological processes of the failing heart since (a) failing cardiomyocytes lose their well defined microarchitecture leading, as present in embryonic cardiomyocytes, to an increased distance between L-type Ca^{2+} channels and RyRs and (b) a re-expression of an embryonic gene profile and embryonic splice variants of proteins involved in Ca^{2+} handling occurs during the course of heart failure [44]. Earlier studies proved that mammalian ventricular myocytes isolated from neonatal hearts show only little evidence of t-tubule development and less density of L-type Ca^{2+} channels in the surface sarcolemma as compared to adults [10, 45]. Accordingly, Escobar and coworkers [46] demonstrated an acceleration of the activation phase of the ventricular Ca^{2+} transient during development in rat cardiomyocytes suggesting establishment of CICR during postnatal

development. When our data were brought in close approximation to inactivation at a steady-state level [4, 6], we noticed that more current could be inactivated at LDS. This points to a potential stage-dependent gain of importance of I_{CaL} and that an increasing sub-sarcolemmal Ca^{2+} concentration, either evoked by Ca^{2+} ions flowing through L-type Ca^{2+} channels itself and/or gain of CICR, may have accelerated CDI. To test whether the L-type Ca^{2+} channels and/or CICR led to an acceleration of I_{CaL} inactivation, we tested the effect of ryanodine as a blocker of Ca^{2+} release from the SR on τ_f . Although bulk Ca^{2+} might have potentially been altered, ryanodine did not affect L-type Ca^{2+} channel inactivation. This finding points to the particular importance of sarcolemmal Ca^{2+} cycling during embryogenesis. Accordingly, we could recently show that at EDS but also at LDS, CICR is low as compared to the adult stage [47]. As a consequence also CICR-mediated CDI may be low and explain the lacking effect of ryanodine. These data imply that Ca^{2+} flowing through the L-type Ca^{2+} channel but not Ca^{2+} released from the SR accelerated τ_f in LDS-derived cardiomyocytes. As a limitation of our study, we cannot exclude that the patch pipette solution, which requires EGTA, may have also contributed to our findings. It was not possible to conduct measurements without EGTA in the pipette since murine embryonic cardiomyocytes tend to elevate $[Ca^{2+}]_i$ when patched. Although it has previously been shown that the application of EGTA does not interfere with fast and slow inactivation kinetics of I_{CaL} [48], we can not exclude that bulk Ca^{2+} contributes at least in part to CDI of I_{CaL} at the embryonic/fetal stage of murine heart development.

As proof of the presence of CDI [5], the relationship between fast inactivation kinetics of the currents carried by Ca^{2+} and membrane voltage was U-shaped. Besides the acceleration of τ_f in LDS-derived cardiomyocytes the U-shape was even more pronounced when fast inactivation kinetics were studied at LDS and absent when Ba^{2+} was used as charge carrier. In summary, these data imply that the proportion of Ca^{2+} contribution to total inactivation is less at EDS than at LDS. Due to the application of Co^{2+} and the prestep to -40 mV the presence of low-voltage activated T-type Ca^{2+} currents, potentially leading to misinterpretation of our results, is rather unlikely [6, 20].

Similar to recent work in adult guinea pig ventricular myocytes [4, 5, 7], we found also contribution of voltage to the inactivation of I_{CaL} when Ba^{2+} was used as charge carrier. In contrast to these studies though, we could only show clear changes of voltage-dependence between $-$

20 mV and more depolarized potentials in EDS and LDS cardiomyocytes. Hence, at more negative membrane potentials Ca^{2+} drives most of the inactivation at both developmental stages, whereas mainly voltage contributes to inactivation at positive membrane potentials at EDS. When Ba^{2+} was applied, we observed a left shift of the $I-V$ curve at LDS but not at EDS, making development-dependent changes of Ba^{2+} effects most likely. Previous data derived from adult cardiomyocytes evidenced that Ba^{2+} causes inactivation by directly binding to the Ca^{2+} -inactivation site [49]. However, since under Ba^{2+} mainly voltage drives inactivation, I_{BaL} becomes significantly stronger as compared to I_{CaL} . After binding of Ba^{2+} to the Ca^{2+} binding site the ensuing local increase of ions may have led in our experiments to the typical left shift of the $I-V$ curve, which can generally be observed after β -adrenergic stimulation in Ca^{2+} containing solutions. McDonald et al [50] and Byerly et al [51] suggested that the reason for the shift in $I-V$ peak with Ba^{2+} could also be a shift in the voltage dependence of I_{CaL} . Moreover, any variation or modification in the behavior of the Ca^{2+} channel subunits can influence the activation and/or inactivation process of the channel, e.g. the level of γ subunit regulates the amount of Ca^{2+} influx by shifting the steady-state inactivation curve to the left and by increasing L-type Ca^{2+} current amplitude [52].

In general, inactivation time constants were much faster in EDS and LDS-derived cardiomyocytes compared to those described for adult cells [4, 5, 13]. These differences could depend on the developmental stage and on the species used. Inactivation of I_{CaL} leads to a reduction of transsarcolemmal Ca^{2+} influx and hence also determines the duration of the action potential (AP) plateau phase. In fact, because of the higher heart rate and differences in ion channel expression, the plateau phase of APs is very brief and the repolarization is more rapid in rat and mouse than in other species such as dog, rabbit, or guinea pig [53]. Therefore, also I_{CaL} inactivation needs to be faster. Because of the same reasons, i.e. increase of heart rate and ion channel expression during maturation, APs become shorter at the adult than at the embryonic, fetal or neonatal stage. The plateau phase of neonatal rat cardiomyocytes for example becomes very short or even absent in the adult heart [54]. Accordingly, we found that the plateau phase in APs of the early murine embryonic heart is significantly prolonged as compared to late embryonic and particularly adult stages (unpublished observation). Thus, the acceleration of I_{CaL} inactivation may, at least in part, contribute to the shortening of the AP plateau during development.

Developmental changes of diverse Ca^{2+} -dependent signaling pathways and their interaction with L-type Ca^{2+} channels influencing I_{CaL} inactivation [1, 37, 55] can however not be excluded. Also molecular aspects might have contributed to the observed differences. For example a change/switch in channel subunit expression could potentially lead to alterations of inactivation and gating charge. Liu and coworkers [56] recently demonstrated an increase in $\alpha_{1\text{C}}$ -subunit mRNA with a concomitant increase in abundance of $\alpha_{1\text{C}}$ -subunit protein during rat heart development.

The further biological importance of our findings is based on the sustained increase of I_{CaL} and I_{CaL} inactivation, both happening in parallel to maintain intracellular Ca^{2+} homeostasis. However, the open probability of I_{CaL} remains unchanged pointing to an increasing L-type Ca^{2+} channel protein expression in the sarcolemma during development. Interestingly, the expression of NCX, which as a protein does not only underlie regulation by different signaling pathways but also works more 'simple', e.g. electrogenic, is contrarily regulated [57-59]. While we found strong functional and protein expression at the embryonic stage NCX expression declines towards the fetal stage. Thus, low L-type channel expression levels at the embryonic stage may be compensated by strong NCX expression as a

more simple Ca^{2+} providing system for contraction or *vice versa*. Effects of NCX on I_{CaL} inactivation were not studied yet. Since NCX-mediated Ca^{2+} influx may theoretically contribute to the inactivation process of I_{CaL} due to its strong forward and reverse mode at the embryonic stage [57], future studies should elaborate on that important topic.

We conclude that during embryonic heart development inactivation of I_{CaL} is both Ca^{2+} - and voltage-dependent with a higher contribution of CDI to τ_f at the fetal stage. These findings are explained by an increase of sarcolemmal L-type Ca^{2+} channel expression and the local subsarcolemmal Ca^{2+} concentration. Thus, local rather than bulk Ca^{2+} influences inactivation processes in embryonic cardiomyocytes and CICR does not significantly contribute to the acceleration of τ_f .

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References

- Protas L, DiFrancesco D, Robinson RB: L-type but not T-type calcium current changes during postnatal development in rabbit sinoatrial node. *Am J Physiol Heart Circ Physiol* 2001;281:H1252-59.
- Chad JE, Eckert RO: Calcium domains associated with individual channels can account for anomalous voltage relations of Ca-dependent responses. *Biophys J* 1984;45:993-99.
- Hadley RW, Lederer WJ: Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *J Physiol* 1991;444:257-68.
- Mitarai S, Kaibara M, Yano K, Taniyama K: Two distinct inactivation processes related to phosphorylation in cardiac L-type Ca^{2+} channel currents. *Am J Physiol Cell Physiol* 2000;279:C603-10.
- Findlay I: Voltage- and cation-dependent inactivation of L-type Ca^{2+} channel currents in guinea-pig ventricular myocytes. *J Physiol* 2002;541:731-40.
- Findlay I: b-Adrenergic stimulation modulates Ca^{2+} - and voltage-dependent inactivation of L-type Ca^{2+} channel currents in guinea-pig ventricular myocytes. *J Physiol* 2002;541:741-51.
- Findlay I: Voltage-dependent inactivation of L-type Ca^{2+} currents in guinea-pig ventricular myocytes. *J Physiol* 2002;545:389-97.
- Morrill JA, Brown RH Jr, Cannon SC: Gating of the L-type Ca channel in human skeletal myotubes: an activation defect caused by the hypokalemic periodic paralysis mutation R528H. *J Neurosci* 1998;18(24):10320-34.
- Pelzer D, Grant AO, Cavalie A, Pelzer S, Sieber M, Hofmann F, Trautwein W: Calcium channels reconstituted from the skeletal muscle dihydropyridine receptor protein complex and its alpha 1 peptide subunit in lipid bilayers. *Ann NY Acad Sci* 1989;560:138-54.
- Brette F, Orchard C: T-tubule function in mammalian cardiac myocytes. *Circ Res* 2003;92:1182-92.
- Argibay JA, Fischmeister R, Hartzell HC: Inactivation, reactivation and pacing dependence of calcium current in frog cardiomyocytes: correlation with current density. *J Physiol* 1988;401:201-26.
- Budde T, Meuth S, Pape HC: Calcium-dependent inactivation of neuronal calcium channels. *Nat Rev Neurosci* 2002;3(11):873-83.

- 13 Lee KS, Marban E, Tsien RW: Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *J Physiol* 1985;364:395-411.
- 14 Ferreira G, Yi J, Rios E, Shirokov R: Ion-dependent inactivation of barium current through L-type calcium channels. *J Gen Physiol* 1997;109:449-61.
- 15 Reppel M, Sasse P, Piekorz R, Tang M, Roell W, Duan Y, Kletke A, Hescheler J, Nuernberg B, Fleischmann BK: S100A1 enhances the L-type Ca^{2+} current in embryonic mouse and neonatal rat ventricular cardiomyocytes. *J Biol Chem* 2005;280(43):36019-28.
- 16 Hescheler J, Fleischmann BK, Lentini S, Maltsev VA, Rohwedel J, Wobus AM, Addicks K: Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res* 1997;36:149-162.
- 17 Fleischmann BK, Duan Y, Fan Y, Schoneberg T, Ehlich A, Lenka N, Viatchenko-Karpinski S, Pott L, Hescheler J, Fakler B: Differential subunit composition of the G protein-activated inward-rectifier potassium channel during cardiac development. *J Clin Invest* 2004;114(7):994-1001.
- 18 Linz KW, Meyer R: Control of L-type calcium current during the action potential of guinea-pig ventricular myocytes. *J Physiol* 1998;513:425-42.
- 19 Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* 1981;391:85-100.
- 20 Balke CW, Rose WC, Marban E, Wier WG: Macroscopic and unitary properties of physiological ion flux through T-type Ca^{2+} channels in guinea pig heart cells. *J Physiol* 1992;456:247-65.
- 21 Leroy J, Pereverzev A, Vajna R, Qin N, Pfitzer G, Hescheler J, Malécot CO, Schneider T, Klöckner U: Ca^{2+} -sensitive regulation of E-type Ca^{2+} channel activity depends on an arginine-rich region in the cytosolic II-III loop. *Eur J Neurosci* 2003;18(4):841-55.
- 22 Takahashi SX, Miriyala J, Colecraft HM: Membrane-associated guanylate kinase-like properties of β -subunits required for modulation of voltage-dependent calcium channels. *Proc Natl Acad Sci USA* 2004;101:7193-98.
- 23 McDonough SI, Mori Y, Bean BP: FPL 64176 modification of $\text{Ca}_v1.2$ L-type calcium channels: dissociation of effects on ionic current and gating current. *Biophys J* 2005;88:211-23.
- 24 Kawai F, Kurahashi T, Kaneko A: Nonselective suppression of voltage-gated currents by odorants in the newt olfactory receptor cells. *J Gen Physiol* 1997;109:265-73.
- 25 Josephson, IR, Varadi G: Beta subunit increases calcium currents and gating charge movements of human L-type Ca^{2+} channels. *Biophys J* 1996;70:1285-93.
- 26 Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK, Hescheler J: Establishment of beta-adrenergic modulation of L-type Ca^{2+} current in the early stages of cardiomyocyte development. *Circ Res* 1999;84:136-45.
- 27 Yao X, Chang AY, Boulpaep EL, Segal AS, Desir GV: Molecular cloning of a glibenclamide-sensitive, voltage-gated potassium channel expressed in rabbit kidney. *J Clin Invest* 1996;97:2525-33.
- 28 Osaka T, Joyner RW: Developmental changes in calcium currents of rabbit ventricular cells. *Circ Res* 1991;68(3):788-96.
- 29 Cohen NM, Lederer WJ: Changes in the calcium current of rat ventricular myocytes during development. *J Physiol* 1988;406:115-46.
- 30 Shi C, Soldatov NM: Molecular determinants of voltage-dependent slow inactivation of the Ca^{2+} channel. *J Biol Chem* 2002;277(9):6813-21.
- 31 Nakayama S, Brading AF: Inactivation of the voltage-dependent Ca^{2+} channel current in smooth muscle cells isolated from the guinea-pig detrusor. *J Physiol* 1993;471:107-27.
- 32 Adachi-Akahane, Cleemann SL, Morad M: Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *J Gen Physiol* 1996;108:435-54.
- 33 Colecraft HM, Alseikhan B, Takahashi SX, Chaudhuri D, Mittman S, Yegnasubramanian V, Alvania RS, Johns DC, Marban E, Yue DT: Novel functional properties of Ca^{2+} channel β subunits revealed by their expression in adult rat heart cells. *J Physiol* 2002;541:435-52.
- 34 Jones LP, Wei SK, Yue DT: Mechanism of auxiliary subunit modulation of neuronal α_{1E} calcium channels. *J General Physiol* 1998;112:125-43.
- 35 Neely A, Wei X, Olcese R, Birnbaumer L, Stefani E: Potentiation by the beta subunit of the ratio of the ionic current to the charge movement in the cardiac calcium channel. *Science* 1993;262:575-78.
- 36 Wei X, Neely A, Lacerda AE, Olcese R, Stefani E, Perez-Reyes E, Birnbaumer L: Modification of Ca^{2+} channel activity by deletions at the carboxyl terminus of the cardiac alpha 1 subunit. *J Biol Chem* 1994;269:1635-40.
- 37 Namiki T, Joyner RW, Wagner MB: Developmental changes in time course of recovery from inactivation in L-type calcium currents of rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2007;292:295-303.
- 38 Abi-Gerges N, Ji GJ, Lu ZJ, Fischmeister R, Hescheler J, Fleischmann BK: Functional expression and regulation of the hyperpolarization activated non-selective cation current in embryonic stem cell-derived cardiomyocytes. *J Physiol* 2000;523:377-89.
- 39 Shiels HA, Blank JM, Farrell AP, Block BA: Electrophysiological properties of the L-type Ca^{2+} current in cardiomyocytes from bluefin tuna and Pacific mackerel. *Am J Physiol Regul Integr Comp Physiol* 2004;286(4):R659-68.
- 40 Pignier C, Potreau D: Characterization of nifedipine-resistant calcium current in neonatal rat ventricular cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2000;279:H2259-68.
- 41 Branchaw JL, Banks MI, Jackson MB: Ca^{2+} - and voltage-dependent inactivation of Ca^{2+} channels in nerve terminals of the neurohypophysis. *J Neurosci* 1997;17(15):5772-81.
- 42 Adams BA, Tanabe T: Structural regions of the cardiac Ca channel α_{1C} subunit involved in Ca-dependent inactivation. *J Gen Physiol* 1997;110:379-89.
- 43 Peterson BZ, DeMaria CD, Yue DT: Calmodulin is the Ca^{2+} sensor for Ca^{2+} -dependent inactivation of L-type calcium channels. *Neuron* 1999;22:549-58.
- 44 Sack MN, Rader TA, Park S, Bastin J, McCune SA, Kelly DP: Fatty acid oxidation enzyme gene expression is downregulated in the failing heart. *Circulation* 1996;94:2837-42.
- 45 Haddock PS, Coetzee WA, Cho E, Porter L, Katoh H, Bers DM, Jafri MS, Artman M: Subcellular $[\text{Ca}^{2+}]_i$ gradients during excitation-contraction coupling in newborn rabbit ventricular myocytes. *Circ Res* 1999;85:415-27.
- 46 Escobar AL, Ribeiro-Costa R, Villalba-Galea C, Zoghbi ME, Pérez CG, Mejía-Alvarez R: Developmental changes of intracellular Ca^{2+} transients in beating rat hearts. *Am J Physiol Heart Circ Physiol* 2004;286:H971-78.
- 47 Sasse P, Reppel M, Hescheler J, Fleischmann BK: CICR in the embryonic heart. *Biophys J* 2005;88(1):321A-2A.
- 48 Sham JS, Cleemann L, Morad M: Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc Natl Acad Sci USA* 1995;92:121-25.

- 49 Shirokov R, Levis R, Shirokova N, Rios E: Ca-dependent inactivation of cardiac L-type Ca channels does not affect their voltage sensor. *J Gen Physiol* 1993;102:1005-30.
- 50 McDonald TF, Cavalie A, Trautwein W, Pelzer D: Voltage-dependent properties of macroscopic and elementary calcium channel currents in guinea pig ventricular myocytes. *Pflugers Arch* 1986;406(5):437-48.
- 51 Byerly L, Chase PB, Stimers JR: Permeation and interaction of divalent cations in calcium channels of snail neurons. *J Gen Physiol* 1985;85(4):491-518.
- 52 Held B, Freise D, Freichel M, Hoth M, Flockerzi V: Skeletal muscle L-type Ca(2+) current modulation in gamma1-deficient and wildtype murine myotubes by the gamma1 subunit and cAMP. *J Physiol* 2002;539(Pt 2):459-68.
- 53 Binah O, Arieli R, Beck R, Rosen MR, Palti Y: Ventricular electrophysiological properties: is interspecies variability related to thyroid state? *Am J Physiol Heart Circ Physiol* 1987;252(21):H1265-74.
- 54 Wahler GM, Dollinger SJ, Smith JM, Fleman KL: Time course of postnatal changes in rat heart action potential and in transient outward current is different. *Am J Physiol Heart Circ Physiol* 1994;267:H1157-66.
- 55 An RH, Davies MP, Doevendans PA, Kubalak SW, Bangalore R, Chien KR, Kass RS: Developmental changes in beta-adrenergic modulation of L-type Ca²⁺ channels in embryonic mouse heart. *Circ Res* 1996;78:371-78.
- 56 Liu L, O'Hara DS, Cala SE, Poornima I, Hines RN, Marsh JD: Developmental regulation of the L-type calcium channel alpha1C subunit expression in heart. *Mol Cell Biochem* 2000;205(1-2):101-9.
- 57 Reppel M, Sasse P, Malan D, Nguemo F, Reuter H, Bloch W, Hescheler J, Fleischmann BK: Functional expression of the Na⁺/Ca²⁺ exchanger in the embryonic mouse heart. *J Mol Cell Cardiol* 2007;42(1):121-32.
- 58 Reppel M, Reuter H, Sasse P, Hescheler J, Fleischmann BK: NCX current in the murine embryonic heart: development-dependent regulation by Na⁺. *Ann NY Acad Sci* 2007;1099:175-82.
- 59 Reppel M, Fleischmann BK, Reuter H, Sasse P, Schunkert H, Hescheler J: Regulation of the Na⁽⁺⁾/Ca⁽²⁺⁾ exchanger (NCX) in the murine embryonic heart. *Cardiovasc Res* 2007;75(1):99-108.