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Functional roles of L-type calcium channels in murine embryonic hearts

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Abbreviations and units

Ab, antibody	kb, kilo base pairs
ATP, adenosine-5'-triphosphate	kDa, kilo Dalton
bp, base pair	kV, kilo volt
BSA, bovine serum albumin	LB, luria Betrani medium
cAMP, cyclic AMP	MEM, minimal essential medium
cDNA, complementary DNA	min, minute
cfu, colony forming units	ml, millilitre
CIP, calf intestinal phosphatase	mM, millimolar
CMV, cytomegalovirus	M.O.I, multiply of infection
Da, Dalton	ng, nanogram
DHP, dihydropyridine	PAGE, polyacrylamide gel electrophoresis
DMEM, Dulbecco's MEM	PCR, polymerase chain reaction
DMSO, dimethylsulfoxide	PKC, protein kinase C
DNA, deoxyribonucleic acid	RNA, ribonucleic acid
DNTP, 2'deoxynucleoside-5'-triphosphate	RNase, ribonuclease
DTT, dithiothreitol	rpm, revolutions per minute
E.coli, Escherichia coli	RT-PCR, reverse transcriptase polymerase chain reaction
EDTA, ethylenediamine tetra-acetic acid	SDS, sodium dodecyl sulphate
EGTA, ethylene-bis (oxyethylenenitrilo)-tetra-acetic acid	s, second
EGFP, enhanced green fluorescence protein	S.E., standard error of the mean
FCS, fetal calf serum	sDNA, single strand DNA
g, gram (weight) respective gravity (for centrifugation)	Tris, Tris-(hydroxymethyl)-aminomethane
GTP, guanosine-5'-triphosphate	U, unit
HEK 293, human embryonic kidney cells	UV, ultraviolet
hr, hour	

1. Introduction

Rise in intracellular calcium triggers a variety of processes such as muscle contraction, secretion, neurotransmission, chemotaxis and gene expression (Berridge et al. 2000). Although there are many channels, exchangers and pumps involved in controlling intracellular Ca^{2+} levels, voltage-dependent Ca^{2+} channels (VDCCs) play a key role in this process (Bootman et al. 2001). VDCCs are a large family of integral membrane proteins that control the selective flow of Ca^{2+} ions down their electrochemical gradient in response to changes in membrane potential. Thus, VDCCs are molecular switches coupling electrical signals at the cell surface to physiological events in the cell.

1.1 Voltage–dependent calcium channels

Calcium channels were first purified and biochemically characterized from the transverse tubule membranes of skeletal muscle (Curtis and Catterall 1984; Flockerzi et al. 1986). Related proteins have been identified in heart, brain and a number of excitable cell types and tissues (for review see Hofmann et al. 1999). The channels are complex proteins composed of four or five distinct subunits, which are encoded by multiple genes (Figure 1). The α_1 subunit of 190-250 kDa is the largest subunit of the complex and is organized in four homologous domains (I-IV) with six transmembrane segments (S1-S6) in each. It incorporates the ion conducting pore, the voltage sensor and gating apparatus, as well as the known sites of channel regulation by second messengers, drugs, and toxins. The α_1 subunit is associated with the transmembrane, disulfide-linked $\alpha_2\delta$ subunit complex and the β subunit that is attached to the cytosolic surface of the α_1 subunit. Some calcium channel complexes e.g. the skeletal muscle channel contain an additional transmembrane γ subunit. $\alpha_2\delta$, β and γ subunits usually are designated as auxiliary subunits since they are not required for principal channel function but rather modulate certain channel properties such as voltage-dependence of activation and inactivation or serve as targets for channel regulation by protein kinases and other factors. The β subunit is also involved in the dynamic cell surface trafficking of the channel complex (Weisgerber et al. 2003). Although auxiliary subunits modulate a number of properties of the calcium channel complex, the pharmacological and electrophysiological diversity of calcium channels arises primarily from the existence of multiple α_1 subunits.

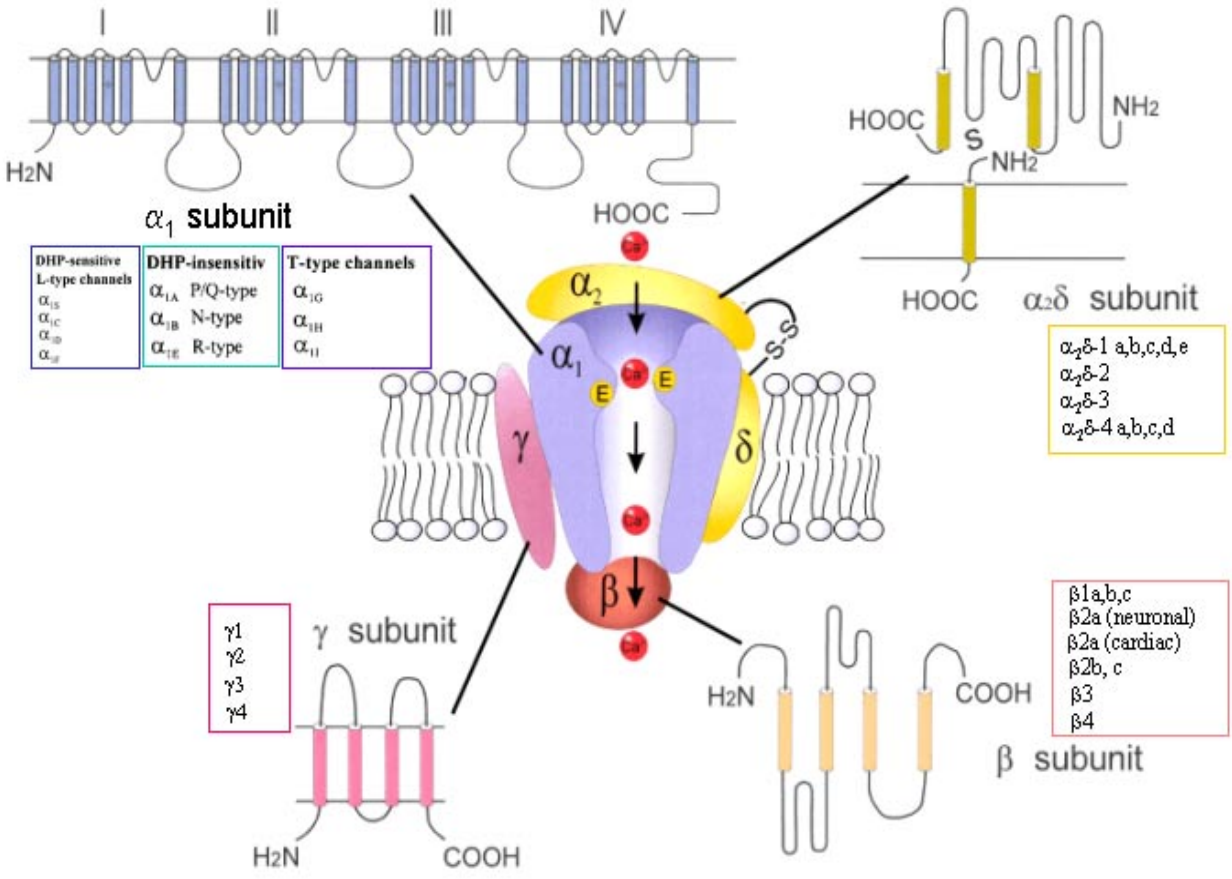


Figure 1. Subunit composition of voltage-dependent calcium channels. The putative folding structures of the individual subunits are illustrated. Several genes and/or splice variants exist for each of the calcium channel subunits (see boxes). (Adapted from Klugbauer et al. 2002a and Hofmann et al. 1999).

Mammalian α_1 subunits are encoded by ten distinct genes. Phylogenetic analysis indicates that the common ancestor of modern voltage-dependent calcium channel genes has evolved in a very early evolutionary event into the high-voltage-activated (HVA) and low-voltage-activated (LVA) calcium channel families (Figure 2). The designations "HVA" and "LVA" refer to the activation voltage of both channel classes (Figure 3). HVA channels require strong membrane depolarization to activate and their activation threshold is high. Therefore, the channels are also called "high-threshold" channels. In contrast, LVA channels have a lower activation threshold and are activated at more negative membrane potentials. As a consequence, if a neuron or heart cell is depolarized from resting potential, LVA calcium channels will activate first followed by the HVA channels. The primary sequences of HVA and LVA channel are distantly related to each other sharing less than 30% sequence identity.

While the HVA channel family was quite conserved during evolution a duplication of the ancestral HVA gene gave rise to two distinct subfamilies with to date four and three homologous genes respectively. The principal evolution of calcium channels into three major branches must have occurred well over 500 million years ago, since the nematode *Caenorhabditis elegans* contains one member of each subclass. Historically, various names had been given to the corresponding gene products, giving rise to distinct and sometimes confusing nomenclatures. In 2000 a phylogenetically-based nomenclature was adopted (Ertel et al. 2000; Figures 2; Table 1). According to this nomenclature, α1 subunits are classified into three principal subfamilies, namely Ca_v1, Ca_v2 and Ca_v3. The structural differences among these three classes of α_1 subunits are reflected in marked differences in their biophysical properties, their regulation and their sensitivity to blockers. In the following section the hallmark features of the calcium channel α_1 subunit families will be briefly summarized. The structure and functional properties of the various auxiliary calcium channel subunits have been described in detail in a recent review by Arikkath and Campbell (Arikkath and Campbell 2003) and will not be discussed here.

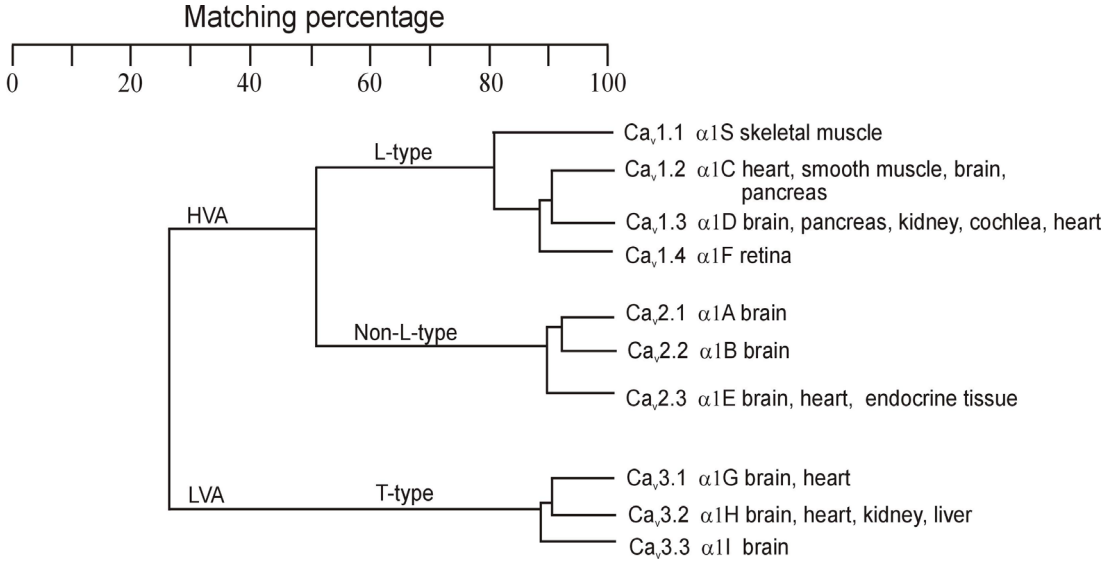


Figure 2. Sequence similarity of voltage-gated calcium channel α_1 subunits. Phylogenetic representation of the primary sequences of the calcium channels. Only the membrane-spanning segments and the pore loops are compared. First, all sequence pairs were compared, which clearly defines three families with intra-family sequence identities above 80% (Ca_v1, Ca_v2, Ca_v3). Then, a consensus sequence was defined for each family and these three sequences were compared to one another, with inter-family sequence identities of about 50% (Ca_v1 versus Ca_v2) and 28% (Ca_v3 versus Ca_v1 or Ca_v2) (Adapted from Klugbauer et al. 2002a and Ertel et al. 2000).

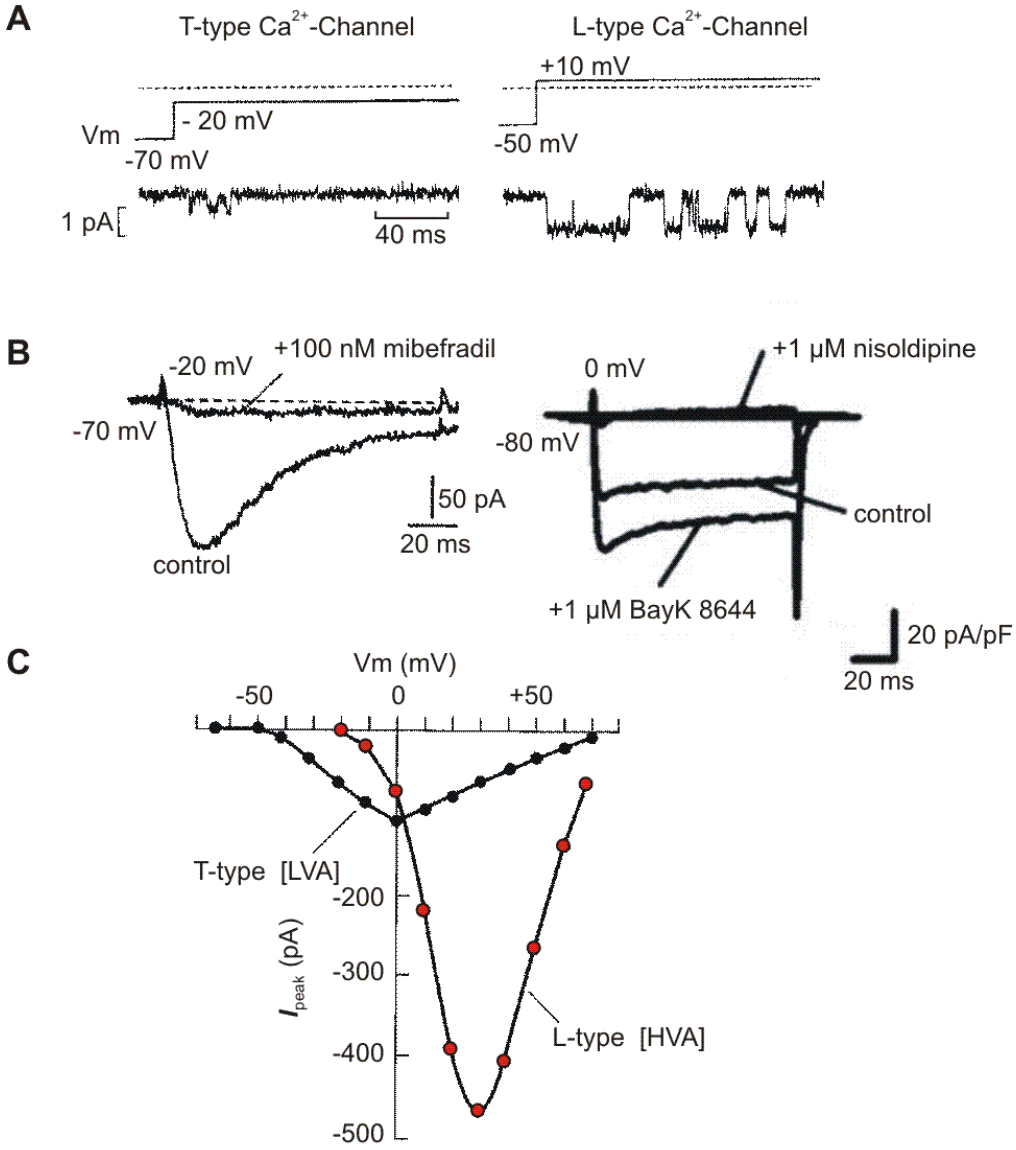


Figure 3. **L-type and T-type Ca^{2+} currents**. **A**, single-channel currents flowing through L-type (HVA) and T-type (LVA) Ca^{2+}-channels recorded from a cell-attached patch on a guinea pig cardiac ventricular myocyte. Ba^{2+} has been used instead of Ca^{2+} as the permeant ion to prevent inactivation of L-type current. Currents were activated by shifting the membrane potential (Vm) from –70 mV or –50 mV to test potentials of either –20 mV or +10 mV depending on the current type. T-type channel openings are characterized by their tiny conductance and transient time course. In contrast, L-type channel openings are long-lasting and reveal higher single-channel conductance. **B**, Modulation of T-type and L-type currents by agonists and antagonists. Whole cell current traces were evoked from a rat cerebellar Purkinje neuron (left panel) and a mouse cardiomyocyte (right panel) by stepping from Vm of –70 mV or –80 mV to test potentials of either –20 mV or 0 mV. The T-type current is blocked

by the addition of 100 nM mibefradil (left panel). The L-type current is blocked by the antagonistic 1,4-dihydropyridine (DHP) nisoldipine and is potentiated by the agonistic DHP BayK8644. **C**, Peak current-voltage relation of T- and L-type currents measured in an isolated canine cardiac atrial cell. The I-current-voltage relationship of the T-type component is at least 30 mV more negative than that of the L-type component. (Panel A adapted from Nilius et al. (1985); panel B *left* from Bean and McDonough 1998; Panel B *right* from Seisenberger et al. 2000; Panel C from Bean 1985)

1.1.1 The Ca_v1 calcium channel subfamily

This family comprises four members that have been designated as $Ca_v1.1$ (α1S), $Ca_v1.2$ (α1C), $Ca_v1.3$ (α1D) and $Ca_v1.4$ (α1F). The proteins share about 75 % sequence identity between each other. The highest sequence homology is seen in the transmembrane core region whereas the N- and C-termini diverge more strongly. Ca_v1 channels are the molecular correlate with the L-type calcium current (Figure 3). This current has been first detected in cardiac myocytes (Reuter 1967) and since than was found in many other cell types. The L-type Ca^{2+} current is characterized by its slow inactivation kinetics (L= long-lasting) when Ba^{2+} is used as carrier. It is important to mention that slow inactivation kinetics are only observed at artificial ionic conditions. In a physiological ionic environment i.e. if Ca^{2+} instead of Ba^{2+} is the conducting ion L-type currents do inactivate rapidly. This so-called Ca^{2+}-dependent inactivation (CDI) represents a crucial feedback mechanism preventing excessive influx of Ca^{2+}, which would be potentially toxic for the cell. The mechanisms that underlie this feedback inhibition have been uncovered only recently. It was found that permeating Ca^{2+} inhibits L-type channels by interacting with calmodulin that is tightly bound to specific regions in the C-terminus of the channel (for a detailed review of CDI see Budde et al. 2002).

L-type channels are the molecular targets of the organic calcium channel blockers, including 1,4-dihydropyridines (e.g. nifedipine), phenylalkylamines (e.g. verapamil), and benzothiazepines (e.g. diltiazem) that are used in the therapy of cardiovascular diseases. These drugs are thought to act at three distinct but partially overlapping receptor sites in the α1 subunit (Striessnig et al.1998; Hofmann et al.1999). Phenylalkylamines are intracellular pore blockers, which are thought to enter the pore from the cytoplasmic side of the channel and block it. Their receptor site is formed by amino acid residues in the S6 segments in domains III and IV, in close analogy to the local anaesthetic receptor site on sodium channels

(Hockerman et al. 1997; Schuster et al. 1996). 1,4-dihydropyridines can be channel activators or inhibitors, and therefore are thought to act allosterically to shift the channel toward the open or closed state, rather than by occluding the pore. Their receptor site includes amino acid residues in the S6 segments of domains III and IV and the S5 segment of domain III. The dihydropyridine receptor site is closely apposed to the phenylalkylamine receptor site and shares some common amino acid residues. Diltiazem and related benzothiazepines are thought to bind to a third receptor site but the amino acid residues that are required for their binding overlap extensively with those required for phenylalkylamine binding.

The four Ca_v1 subunits reveal distinct tissue distributions. $Ca_v1.1$ is almost exclusively expressed in skeletal muscle where it is a key determinant of the excitation-contraction coupling (Tanabe et al. 1987; Adams and Beam 1990). $Ca_v1.2$ is the most widespread distributed member of the Ca_v1 family. The primary transcript of this gene is extensively spliced giving rise to a variety of mature mRNAs. For example, the $Ca_v1.2a$ isoform is expressed in cardiomyocytes (Mikami et al. 1989) whereas the $Ca_v1.2b$ isoform is predominantly found in smooth muscle (Biel et al. 1990). $Ca_v1.2$ transcripts are also expressed in many types of neurons as well as in endocrine cells. $Ca_v1.2$ is involved in the control of important physiological functions including smooth muscle tone (Davie and Hill 1999; Moosmang et al. 2003), heart contractility (Reuter 1979), secretion of hormones (Milani et al. 1990) and integration of synaptic inputs (Bean 1989). The $Ca_v1.3$ channel was originally cloned from neuronal and endocrine tissue and may be involved in the control of hormone secretion (Williams et al. 1992; Seino et al. 1992). The recent studies indicate that the channel is also expressed in heart conduction tissue (Platzer et al. 2000). Inactivation of the channel resulted in deafness and cardiac rhythmicity (Platzer et al. 2000). The $Ca_v1.4$ gene is the only calcium channel gene localized on the X-chromosome (Xp11.23). The channel is specifically expressed in retinal photoreceptors and bipolar cells. Loss-of-function mutations in the gene have been identified in patients suffering from congenital stationary night blindness (Strom et al. 1998; Bech-Hansen et al. 1998).

1.1.2 The Ca_v2 calcium channel subfamily

The Ca_v2 subfamily includes three channels ($Ca_v2.1$ (α_{1A}), $Ca_v2.2$ (α_{1B}) and $Ca_v2.3$ (α_{1E})) that are mainly expressed in neurons. Ca_v2 channels conduct dihydropyridine-insensitive currents that play a key role in the initiation of rapid synaptic transmission (Südhof 1995). The

channels are differentiated on the basis of their biophysical properties and their sensitivity to specific peptide toxins from spiders and marine snails (Table 1; Miljanich et al. 1995). These peptide toxins are potent blockers of synaptic transmission because of their specific effects on the Ca_v2 family of calcium channels. Both P-type and Q-type Ca^{2+} channels are encoded by $Ca_v2.1$ gene. The P-type and Q-type Ca^{2+} currents, first recorded in Purkinje (Llinas and Sasaki 1989) and cerebellar granule neurons (Randall and Tsien 1995), are specifically blocked by the ω-agatoxin IVA (Nowycky et al. 1985). The N-type current carried by $Ca_v2.2$ channel is distinguished from the L-type current by its more negative activation voltage and its faster activation kinetics. The $Ca_v2.2$ channels are blocked by low to moderate concentrations of ω-conotoxin GVIA and related cone snail toxins (McCleskey et al. 1987).. Cerebellar granule neurons contain R-type calcium current that is resistant to peptides inhibiting N- and P/Q type channels (Randall and Tsien 1995). Expression studies in heterologous systems indicate that the R-type current is produced by the $Ca_v2.3$ channel (Schneider et al. 1994). The $Ca_v2.3$ channels are blocked specifically by the synthetic peptide toxin SNX-482 derived from tarantula venom (Miljanich et al. 1995).

1.1.3 The Ca_v3 calcium channel subfamily

The three members of the Ca_v3 subfamily ($Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H) and $Ca_v3.3$ (α1I)) differ from the channels of the Cav1 and Cav2 subfamily by their significantly more negative activation range (LVA channels; Figure 3). In comparison to L-type currents, Ca^{2+} currents carried by Ca_v3 channels inactivate rapidly, deactivate slowly and have small single-channel conductance (Figure 3). With respect to their ***t***ransient kinetics and ***t***iny unitary conductance the currents are designated T-type currents. T-type currents have been characterized in muscle cells and in a variety of neurons where they play a key role in shaping the action potential (Bean 1985; Carbone and Lux 1984). Recent analysis of mice deficient for $Ca_v3.1$ (Kim et al. 2001) and $Ca_v3.2$ (Chen et al. 2003) indicate that these channels are major determinants of thalamic pacemaking and relaxation of coronary arteries, respectively. So far, there are no drugs or peptide toxins (Heady et al. 2001; Sidach and Mintz 2002) that selectively block Ca_v3 channels with high affinity. The Ca_v3 family of calcium channels is insensitive to both the classical calcium channel blockers that block Ca_v1 channels and the spider and cone snail toxins that block the Ca_v2 channels. Low micromolar concentrations of mibefradil, formerly Ro 40-5967, are widely used to block T-type calcium currents (Mishra and Hermsmeyer 1994). However 10 to 30 fold higher concentrations of this substance also block

cardiovascular L-type channels. Ethosuximide, a first-line drug in the treatment of absence epilepsy, was found to block T-type currents in thalamic relay neurons (Coulter et al. 1989). However, electrophysiological studies in rat and cat thalamic neurons suggest that block of other ionic channels may be also relevant for the pharmacological effect of this drug (Leresche et al. 1998). Development of more specific and high affinity blockers of the Ca_v3 family of calcium channels would be useful for therapy and for more detailed analysis of the physiological roles of these channels.

Table 1. Physiological functions and pharmacology of calcium channels.

Channel	Current	Specific antagonists	Cellular functions	Refs
$Ca_v1.1$	L-type	Dihydropyridines Phenylalkylamines Benzothiazepines,	Excitation-contraction coupling	a, b
$Ca_v1.2$	L-type	Dihydropyridines Phenylalkylamines Benzothiazepines,	excitation-contraction coupling, hormone release, regulation of transcription,	
$Ca_v1.3$	L-type	Dihydropyridines Phenylalkylamines Benzothiazepines,	synaptic integration regulation of transcription, synaptic integration	
$Ca_v1.4$	L-type	Dihydropyridines Phenylalkylamines, Benzothiazepines	neurotransmitter release from rods and bipolar cells	
$Ca_v2.1$	P/Q-type	ω-agatoxin IVA	Neurotransmitter release, dendritic Ca^{2+} transients	c,d
$Ca_v2.2$	N-type	ω-conotoxin-GVIA	neurotransmitter release, dendritic Ca^{2+} transients	
$Ca_v2.3$	R-type	SNX-482	repetitive firing	
$Ca_v3.1$ $Ca_v3.2$ $Ca_v3.3$	T-type T-type T-type	Mibefradil, Ethosuximide,	pacemaking, repetitive firing	e, f, g

(Refs: a) Striessnig et al.1998; b) Hofmann et al.1999; c) Südhof 1995; d) Miljanich et al. 1995; e) Mishra and Hermsmeyer 1994; f) Coulter et al. 1989; g) Leresche et al. 1998).

1.2 Cardiac calcium channels

1.2.1 Physiological roles of calcium channels in heart

Voltage-gated calcium channels control two key processes required for normal heart function. First, Ca^{2+}-influx through calcium channels is a prerequisite for excitation-contraction coupling in cardiomyocytes, and hence for heart contraction. Second, Ca^{2+} influx contributes to the generation of pacemaker potentials in cardiac conduction tissue, and hence is involved in the regulation of heart rhythmicity (for recent review see Hofmann et al. 1999; Stieber et al. 2003). A typical heart cell contains both L- and T-type currents (Figure 3). Electrophysiological and biochemical studies have unveiled the specific roles of both current types and the molecular identity of the underlying proteins (Larsen et al. 2002).

Two T-type Ca^{2+} channels have been detected in vertebrate heart: $Ca_v3.1$ and $Ca_v3.2$ (Bohn et al. 2000; Ferron et al. 2002). The $Ca_v3.1$ is highly enriched in sino-atrial pacemaker cells (Bohn et al. 2000) and may act together with HCN channels (Biel et al. 2002) and L-type Ca^{2+} channels in the generation of pacemaker potentials. $Ca_v3.2$ is mainly expressed in coronary arteries and plays an important role in the determination of the tone of these vessels (Chen et al. 2003). There is evidence that in heart the T-type current also contributes to pathological processes such as ventricular hypertrophy, post-myocardial infarction and arrhythmogenesis that occurs during atrial fibrillation (Martinez et al. 1997; Huang et al. 2000; Nattel et al. 1999).

Cardiac myocytes express three different L-type calcium channel genes: $Ca_v1.1$, $Ca_v1.2$, and $Ca_v1.3$. Among these channels, $Ca_v1.2$ is the predominantly expressed isoform and is essential for excitation-contraction coupling (Mikami et al. 1989; Welling et al. 1997; Lipscombe 2002). The major role of the $Ca_v1.3$ subunit is likely in the control of cardiac rhythmogenesis since mice lacking this subunit reveal sinoatrial dysfunction (Platzer et al. 2000; Mangoni et al. 2003; Takimoto et al. 1997; Wyatt et al. 1997). So far, no specific function has been reported for the only faintly expressed $Ca_v1.1$ subunit.

1.2.2 Modulation of cardiac L-type calcium channels

In the heart, the positive inotropic effect of catecholamines is conferred by the upregulation of L-type calcium channel activity. L-type calcium currents are modulated by a wide variety of hormones and neurotransmitters. The underlying signalling cascade of this important physiological process has been investigated by many laboratories over the last two decades but is still not completely understood (for review see Trautwein and Utz 1996). It is well established that binding of catecholamines to β_1 adrenergic receptors in the plasmalemma of cardiomyocytes leads to the stimulation of the G_s protein, the activation of the adenylyl cyclase and the production of cyclic adenosine monophosphate (cAMP). Cyclic AMP activates the cAMP-dependent protein kinase A (PKA) which in turn phosphorylates its cellular substrates leading to a 3- to 7- fold increase in the amplitude of the L-type calcium current (Kameyama et al. 1985; Hartzell and Fischmeister 1992). The resulting increase in the Ca^{2+} influx triggers an enhanced calcium release from sarcoplasmatic ryanodine receptors. Consequently, the cytosolic Ca^{2+} concentration rises allowing a stronger interaction between actin and myosin filaments. Electrophysiological analysis indicates that PKA increases the amplitude of the L-type current because it increases the open probability of this channel. As a consequence the same membrane depolarization will activate a much larger L-type current in the presence of PKA than in the absence of PKA (Yue et al 1990).

Although the fact that cAMP kinase-dependent phosphorylation significantly affects the function of the cardiac calcium channels is undisputed, the molecular target of PKA and the mechanism causing the cardiac calcium channel modulation is still controversially discussed. As pointed out in the previous chapters the cardiac L-type calcium channel is a multi-subunit complex consisting of the principal $Ca_v1.2a$ α_1 subunit and the auxiliary $\alpha_2\delta$ and β subunits. Early biochemical studies suggested that the cardiac α_1 subunit was not a substrate for phosphorylation by cAMP dependent kinase (Schneider and Hofmann 1988). However, more recent results have revealed that the originally purified 160 to 195 kD protein represented a truncated version of full length channel (De Jongh et al. 1996). The full length 240 kD form of the α_1 subunit was shown to be phosphorylated by cAMP kinase on a single serine residue (Ser1928) in the C-terminal domain (De Jongh et al. 1996, Mitterdorfer et al 1996). This site is missing in the originally purified form of the α_1 subunit that is truncated at residue 1870 in the C-terminal sequence. Because the α_1 subunit is phosphorylated only on Ser1928 in transfected cells, regulation may involve phosphorylation of this site. In support of this

notion, mutation of Ser1928 to alanine reduced the regulation of calcium channel activity caused by cAMP in transfected HEK 293 cells from a 35% increase in calcium channel current to a 7% increase (Gao et al 1997). Although the observed extent of regulation of calcium channel activity falls well short of the magnitude recorded in native cardiac cells (3- to 7- fold increase) these results indicate that at least part of the regulation of cardiac calcium channel activity is caused by phosphorylation of the $Ca_v1.2$ α_1 subunit. However, in contradiction to the proposed model, Zong and his coworkers observed no cAMP-induced increase of the calcium current in CHO or HEK293 cells coexpressing all subunits of the calcium channel complex (Zong et al 1995). Taken together, it seems likely that the additional regulatory influences not yet reproduced and characterized in transfected cells are important for modulation of the activity of cardiac calcium channels *in vivo*. For example, emerging evidence indicates that specific PKA anchoring proteins (so-called AKAPs) are required for regulation of cardiac Ca^{2+} channels through the PKA pathway (Fraser 1998; Marx 2003). The absence of heart-specific AKAPs or an insufficient expression level of these proteins may explain why calcium channel regulation could not be reconstituted reliably in heterologous expression systems.

Another prime candidate for mediating PKA effects is the calcium channel β subunit. Both β1 and β2 isoforms are expressed in cardiac tissue (Biel et al. 1991, Hullin 1992) and likely are both associated in $Ca_v1.2$ channels. The β subunits of cardiac calcium channels are phosphorylated by PKA in intact hearts treated with β-adrenergic agonists (Haase et al 1996). Biochemical experiments indicate that PKA phosphorylates three sites of cardiac β_{2a} (Ser459, Ser478 and Ser479) in vitro (Gerhardstein et al 1999). To test the functional relevance of these phosphorylation sites, cardiac β_{2a} was coexpressed with the C-terminal truncated form of $Ca_v1.2$ α_1 subunit lacking Ser1928 in HEK293 cells. The channel activity could be substantially (greater than two fold) activated by PKA, indicating that the phosphorylation of β subunit contributes to the urpregulation of calcium channel (Bunemann et al 1999). Mutation of Ser478/Ser479 to alanines but not of Ser459 on β_{2a} prevented the upregulation of channel activity (Bunemann et al 1999). These results suggest that the phosphorylation of either Ser478, Ser479 or both contributes to calcium channel regulation by PKA at least in the presence of C-terminally truncated α_1 subunit.

1.3 Mouse knockout models to study the function of L-type calcium channels

Since the cloning of L-type calcium channel and their auxiliary subunits, heterologous expression studies have revealed a large body of knowledge about function and regulation of these channels. In recent years, a strategy to study calcium channels by knocking out genes encoding these channels in transgenic mice has proven tremendously valuable in clarifying and confirming heterologous expression studies and defining *in vivo* functions of the calcium channels. In attempts to identify the native role of the various L-type calcium channel subunits, a variety of knockout mice have been produced (Table 2).

Table 2. Deletion of L-type Ca^{2+} channel subunits in mice

Deleted subunit gene	Tissue distribution	Phenotype	Refs
$Ca_v1.2$	Heart, smooth muscle, brain	Embryonic lethal<14.5	a
$Ca_v1.3$	Endocrine, smooth muscle, heart, brain	Deaf, arrhythmia, bradycardia	b
β1	Skeletal muscle, heart, brain	Decreased L-type Ca^{2+} current, immobile, death at birth (asphyxia)	c
β2	Heart, smooth muscle, brain	Embryonic lethal<9.5 , decreased cell surface expression of calcium channel	d
β3	Brain, heart, aorta	Decreased L- and N- type Ca^{2+} current	e, f
γ1	Skeletal muscle	Increased L-type Ca^{2+} current amplitude	g

(Refs: a) Seisenberger et al. 2000; b) Platzer et al. 2000; c) Gregg et al. 1996; d) Weisgerber et al. 2003; e) Scott et al. 1996; f) Namkung et al. 1998; g) Freise et al. 2000)

$Ca_v1.2$ knockout mice

Mice lacking the $Ca_v1.2$ L-type Ca^{2+} channel die *in utero* before day 15 postcoitum (p.c.) indicating that this channel is required for normal embryonic development (Seisenberger et al.

2000). Interestingly, contraction of mutant and wild type embryonic hearts is indistinguishable until day 12.5 p.c. This finding indicates that up to this time the $Ca_v1.2$ channel is dispensable for excitation-contraction coupling. Electrophysiological measurements in $Ca_v1.2$-deficient cardiomyocytes uncovered the presence of a novel, L-type-like Ca^{2+} channel. It was speculated that this channel may functionally compensate for the loss of $Ca_v1.2$ during early embryognesis but is not sufficient to maintain heart function in later stages of development. The molecular identity of this channel was not known so far and was investigated in this study.

$Ca_v1.3$ knockout mice

Mice deficient for $Ca_v1.3$ are viable and have a normal life span (Platzer et al. 2000). Although the deletion of $Ca_v1.3$ has no obvious effect on embryonic development, $Ca_v1.3$ knockout mice reveal sinoatrial node dysfunction resulting in bradycardia and arrhythmia. Thus, $Ca_v1.3$ is likely to be involved in the generation of pacemaker potentials in the sinus node region.

Further analysis of the phenotype of $Ca_v1.3$ null mice revealed that these mice are deaf. Previous studies had suggested that Ca^{2+} entry through L-type calcium channels is required for neurotransmitter release in auditory hair cells of chicken (Martinez-Dunst et al. 1997) and mice (Moser et al. 2000). Given the development of deafness in the knockout mice, it is very likely that the $Ca_v1.3$ channel is the molecular basis for the L-type current in hair cells.

β1 subunit knockout mice

The β1 subunit is expressed in a wide variety of tissues, predominantly in skeletal muscle and brain with lower expression levels in the spleen and heart (Ruth et al. 1989; Varadi et al. 1991; Pragnell et al. 1991). β1 knockout mice are unable to move and die at birth from asphyxiation (Gregg et al. 1996). The L-type currents were decreased and the amounts of $Ca_v1.1$ were greatly reduced, suggesting the trafficking role β1-subunit (Gregg et al. 1996). The β1 knockout mice show a reduction in muscle mass with disorganization of thick and thin filaments of skeletal muscle. The early death of the homozygous animals has not permitted a close examination of the role of the β1-subunit in brain and heart.

β2 subunit knockout mice

The inactivation of the cardiac β2 subunit of voltage-dependent calcium channels results in low cardiac calcium current densities and in embryonic death at embryonic day 9.5 (Weisgerber et al. 2003). It is likely that the early death of the β2 subunit knockout mice is caused by retention of both the $Ca_v1.2$ and the $Ca_v1.3$ subunit in the endoplasmatic reticulum (ER) because it was shown that the β2 subunit suppresses an ER-retention signal present in the α1 subunits (Bichet et al. 2000).

β3 subunit knockout mice

In β3 knockout mice electrophysiological analyses indicated a 30% reduction in Ca^{2+} channel current density, a slower inactivation rate, and a decreased dihydropyridine-sensitive current. Moreover a significant reduction in dihydropyridine responsiveness was observed in these mice (Namkung et al. 1998). Despite the reduction in L-type calcium channel density, β3 null mice showed normal blood pressure. A high salt diet significantly elevated blood pressure only in the β3-null mice and resulted in hypertrophic changes in the aortic smooth muscle layer and cardiac enlargement (Murakami et al. 2002; Murakami et al. 2003).

γ1 knockout mice

The γ1 knockout mice are viable and show no distinguished phenotype from wild type. The features of γ1 knockout mice are the increased L-type current amplitude, the deceleration of the inactivation and shifts in the steady state inactivation to more positive potentials (Freise et al. 2000). However, despite these effects on the L-type channels in skeletal muscle, no effect of γ1 is observed on skeletal EC coupling (Ursu et al. 2001).

2. Aim of the study

The structure, function and physiological roles of voltage-gated Ca^{2+} channels have been extensively investigated by molecular biology, biochemical and electrophysiological methods in heart cells (An et al.1996; Davies et al. 1996; Liu et al. 1999). In contrast, there is only limited knowledge on the transcriptional regulation of these channels during heart development. One major goal of this study was to address this important issue by systematically determining the expression levels of the six known calcium channel α_1 subunits during mouse embryogenesis. To this end semi-quantitative RT-PCR should be used to measure the amount of calcium channel mRNAs in cardiomyocytes isolated at different time points of heart development. For selected subunits these studies should be supported by Western blot analysis.

It was previously shown that the $Ca_v1.2$ channel is required for normal embryonic development (Seisenberger et al.2000). Mice deficient for this subunit die in utero before day E15.5. Surprisingly, the analysis of $Ca_v1.2$ knockout cardiomyocytes derived from day E12.5 revealed that these cells are able to contract. Further studies indicated that at this time point rhythmic contractility depends on a Ca^{2+} current with L-type characteristics but with an usual low affinity for dihydropyridines. It was thus suggested that this calcium current could functionally compensate for the lacking $Ca_v1.2$ current during early embryogenesis. Since the same current may also be physiologically relevant during normal heart development it would be very important to know by which calcium channel gene it is encoded. Therefore, a major goal of the present study was to elucidate the molecular identity of the L-type calcium current in $Ca_v1.2$ deficient cardiomyocytes. The experimental approach used to achieve this goal was to compare the expression level of all six calcium channel α_1 subunits in murine cardiomyocytes derived from $Ca_v1.2$ deficient embryos with that in wild type cardiomyocytes. This approach was expected to allow the identification of channels that were differentially expressed. The identity of isolated candidate channels with the current of $Ca_v1.2$ deficient cardiomyocytes should be verified by expression of the respective cDNAs in heterologous systems followed by electrophysiological characterization.

Finally, an adenoviral gene delivery system should be established allowing efficient gene transfer into native cardiomyocytes and the cardiac-like HL-1 cell line. Such a high-efficient expression system is urgently needed as a tool for functional studies on calcium channel

regulation in the context of native cardiac cells. In one set of experiments antisense constructs should be used to substantially decrease the synthesis of endogenous $Ca_v1.3$ and $Ca_v1.1$ in the cardiac HL-1 cell line. These studies should be complemented by adenoviral-mediated overexpression of cardiac α_1 subunit (full length and C-terminally truncated forms) and cardiac $\beta 2$ subunit (wild type and mutated forms).

3. Materials and Methods

The molecular and cell biological methods applied are described in this section.

3.1 Materials

3.1.1 Reagents

All reagents used were of molecular biology grade. Reagents were from Sigma (Taufkirchen, Germany) and Merck (Darmstadt, Germany) unless stated otherwise. Biochemicals, biomaterials and solutions for molecular and cell biology experiments were mainly from Invitrogen (Karlsruhe, Germany), New England Biolabs (Frankfurt, Germany), GibcoBRL (Invitrogen; Karlsruhe, Germany) and Stratagene (Heidelberg, Germany).

Reagent	Source
Zero blunt TOPO PCR cloning kit	Invitrogen
Superscript II reverse transcriptase	Invitrogen
Rnase H	Invitrogen
Trizol LS reagent	Invitrogen
Fetal calf serum	Invitrogen
Protein standard	Invitrogen
Restriction enzymes	New England Biolabs
T4 ligase	New England Biolabs
DNA standard	GibcoBRL
MEM	GibcoBRL
DMEM	GibcoBRL
Trypsin / EDTA solution (10×)	GibcoBRL
penicillin / streptomycin	GibcoBRL
pBluescript	Stratagene
E.coli XL1 blue MRF’	Stratagene
Taq DNA polymerase	Promega (Mannheim, Germany)
DNA sequencing kit	PE Biosystems (Weiterstadt, Germany)

FuGen	Roche (Mannheim, Germany)
Adeno-X expression system	Clontech (BD Biosciences; Heidelberg, Germany)
Primers	MWG Biotech (Ebersberg, Germany)
goat anti-rabbit IgG	Jackson ImmunoResearch (Hamburg, Germany)
goat anti-mouse IgG	Jackson ImmunoResearch (Hamburg, Germany)
Cav1.2 antibody	Alamone (Munich, Germany)
Cav1.3 antibody	Chemicon (Hofheim / TS, Germany)
β-actin antibody	Abcam (Cambridge, UK).
ECL system	PerkinElmer Life Sciences (Rodgau–Jügesheim, Germany)

3.1.2 Preparation of general solutions

All solutions were prepared with ultra-pure water (Millipore, Molsheim, France) and were autoclaved unless otherwise specified. The composition of specific solutions is given in the relevant methods. The preparation of general solutions is presented below.

1 M DTT
1.5 g Dithiothreitol
33µl 3 M NaAcetat, pH 5.2 (final 0.01 M)
H_2O ad 10 ml

0.5 M EDTA, pH 8.0
93.05g $Na_2EDTA \cdot 2H_2O$
300 ml H_2O
H_2O ad 500 ml, pH 8.0,

1 M $MgCl_2$
10.17 g $MgCl_2 \cdot 6H_2O$
H_2O ad 50 ml

5 M NaCl
292g NaCl
H_2O ad 1000ml

3M Natriumacetat
40.8g NaAcetat $\cdot 3H_2O$
H_2O ad 100 ml, pH 5.2A

Proteinase K (20mg/ ml)
0.08g Proteinase K
H_2O ad 4 ml

RNase A (10mg/ ml) 100 mg RNaseA
100 µl 1 M Tris, pH 7.5 (final 10 mM)
30 µl 5 M NaCl (final 15 mM)
H_2O ad 10 ml

10 % SDS 100g SDS
H_2O ad 1 000 ml

10x TBE 540g Tris
275g boric acid
200 ml 0.5 M EDTA, pH 8.0 or 37.2g EDTA·$2H_2O$
H_2O ad 5 000 ml

10x TBS 12.1g Tris (final 10 mM)
87.6g NaCl (final 150 mM)
700 ml H_2O
H_2O ad 1 000 ml, pH 8.2

1M Tris, pH 8.0 60.5g Tris-base
300 ml H_2O
H_2O ad 500 ml, pH 8.0

3.2 Cell culture procedures

3.2.1 Cell culture solutions

Unless purchased sterile, all solutions and supplements used in cell culture work were sterilized with a 0.2-µm syringe filter. They were stored at 4°C and pre-warmed to 37°C prior to use.

1x PBS 40g NaCl
1g KCl
3.9 g $Na_2HPO_4 \cdot 2H_2O$
1g KH_2PO_4
300 ml H_2O
H_2O ad 5000 ml. pH 7.4,

Trypsin/EDTA	10 ml trypsin solution stock (0.5% trypsin/ 0.2% EDTA) PBS ad 90 ml, pH 7.4

3.2.2 Culturing of HEK293 and HL-1 cell lines

Human embryonic kidney cells (HEK293) were maintained at 37°C, 5% CO_2 in minimal essential medium (MEM) with 1.8 mM L-glutamine, 0.2% $NaHCO_3$, 10% fetal calf serum (FCS), 100 U penicillin and 100 µg/ml streptomycin. HEK293 cells were used for transfection and for adenovirus amplification.

Murine cardiomyocyte HL-1 cells at passage 71 were obtained from Dr. W.C. Claycomb (Louisiana State University Health Science Center, New Orleans, LA, USA), who first established and characterized the cell line (Claycomb et al. 1998). Cells were grown in T25, gelatin-fibronectin coated flasks, as previously described (Claycomb et al. 1998). Cells were maintained in 'Claycomb medium' (JRH Biosciences, Lenexa, KS, USA), supplemented with 10% fetal bovine serum, 10 µM noradrenaline (Sigma), 100 U penicillin and 100 µg/ml streptomycin. The cultures were grown at 37°C in an atmosphere of 5% CO_2 and 95% air at a relative humidity of approximately 95%. The medium was changed every 24 hr.

3.2.3 Isolation and cell culturing of murine embryonic cardiomyocytes

Individual embryos were obtained after breeding of wild type or heterozygous $Ca_v1.2$ (+/-) mice. The embryonic hearts were dissected and then cardiomyocytes were isolated as described (Davies et al. 1996) at day E9.5, E12.5 or E15.5. Embryonic cardiomyocytes were cultured at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U penicillin and 100 µg/ml streptomycin. The dispersed cells were plated on 60-mm culture plates (Corning, NY, USA) for 48 hr and then harvested for RNA isolation.

3.2.4 Cell culture techniques

3.2.4.1 Passaging of Cells

The cells were passaged when they approached confluence. Growth medium was removed and cells were washed two times with PBS. Afterwards, cells were incubated with Trypsin/EDTA (2 ml per 75-cm^2 flask) for 2 min at 37°C to allow detachment of the cells. The digestion of trypsin was stopped with 8 ml culture medium and the sedimented cells were divided in a 1:3 ratio and cultured in culture flasks (TPP, Trasadingen, Switzerland).

If the cell density was to be determined, 50 µl of the cell suspension was dropped on a Neubauer hemacytometer and the cell numbers were counted in four 0.1-mm^3 chambers under a binocular microscope. The required cell concentration was adjusted by diluting the cell suspension with fresh culture medium. Cells were seeded in appropriate density in culture flasks or plates.

3.2.4.2 Freezing, storage and thawing of cells

For long time storage confluent cells from one 75-cm^2 flask were trypsinized and the detached cells were centrifuged at 500 g for 5 min. The pellet was resuspended in 3 ml freezing medium containing FCS + 10% [V/V] DMSO. 1-ml cell aliquots were first frozen in cryovials at –70°C overnight and then transferred to liquid nitrogen for long time storage. For thawing, cryopreserved cells were rapidly warmed in 37°C water bath and immediately dissolved in pre-warmed culture medium.

3.3 Maintenance and transformation of *E.coli*

3.3.1 Solutions and media

LB-Medium

10g	Select Peptone 140 (GibcoBRL)
5g	Select Yeast Extract (GibcoBRL)
5g	NaCl
1g	D (+) Glucose
H_2O ad 1 000 ml	

Agar plates	20g	Select Peptone 140
	10g	Select Yeast Extract
	10g	NaCl
	30g	Select Agar
	2g	D(+) Glucose
	H_2O ad 2 000 ml	

3.3.2 *E.coli* culture and storage

The strain used for amplification of plasmid DNA was *E.coli* XL1 blue MRF' [genotype: *Δ(mrcA)183 Δ(mrcCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 lac [F' proAB lacI^{q}ZΔM15 Tn10*(Tet^r)]. Bacteria strains were long-term stored at -80°C in LB medium supplemented with 10% glycerol.

Culturing of *E.coli* was done in LB medium supplemented with selective antibiotics (ampicillin or kanamycin at 100 µg/ml). For small liquid cultures 3-7 ml LB medium were inoculated with a single bacterial colony and the bacteria were grown in glass or vessels overnight at 37°C on a shaker set at 225 rpm. Bacteria were harvested by centrifugation.

Agar plates were prepared using LB medium and agar (15 g/l). Dry LB agar plates were inoculated with a single bacterial colony or with a bacterial suspension by spreading with a sterile inoculation loop or a glass spreader respectively to achieve single colony growth. The plates were incubated inverted overnight at 37°C. Plates containing 50 µg/ml ampicillin were used for adenoviral vectors. Plates containing 50 µg/ml kanamycin were used for pShuttle vectors. For short-term storage agar plates were kept at 4°C.

3.3.3 Transformation of *E.coli* with plasmids

3.3.3.1 Preparation of transformation competent bacteria

100 ml LB medium were inoculated with a pre-culture of *E.coli* XL1 blue MRF' and cultured at 37°C overnight. The culture was placed on ice when the OD_{600} reached 0.35- 0.38. The culture was centrifuged at 4000 g, 4°C for 15 min. The cell pellet was resuspended in 15 ml

1xTSS (10% polyethyleneglycol 3350, 50 mM $MgCl_2$, 5% DMSO in LB medium). The cells were shock frozen in a dry-ice/ethanol bath and stored in 400-µl aliquots at -80°C.

3.3.3.2 Transformation of *E.coli*

Plasmid DNA at an appropriate concentration was mixed with 400 µl thawed competent bacteria and incubated on ice for 30 min. This mixture was added to 2.6 ml LB medium and incubated at 37°C, 150 rpm for 1 hr. The bacteria were plated onto agar plates containing the corresponding selective antibiotic and incubated overnight at 37°C. To extract plasmid DNA from the transformed colonies, Mini-prep (3.4.6) was applied and the DNA was analyzed with restriction digestion enzymes (3.4.1).

3.3.3.3 Preparation of electrocompetent bacteria

100 ml of LB medium were inoculated with *E.coli* XL1 blue MRF' and cultured at 37°C, 225 rpm overnight. The culture was placed on ice when the OD_{600} reached 0.5- 0.7 and chilled on ice for 20 min. The culture was centrifuged at 4 000 g, 4°C for 20 min. The cell pellet was washed two times in 10 ml ice-cold water and one time in 10 ml cold 10% glycerol with centrifugation at 4 000 g, 4°C for 15 min. The bacteria cells were centrifuged and resuspended in 2 ml cold 10% glycerol. The cells were stored in 50 -µl aliquots at -80°C.

3.3.3.4 Electroporation of *E.coli*

Electroporation with high voltage is the most efficient method for transforming bacteria. 40-µl thawed electrocompetent cells were mixed with up to 500 ng plasmid DNA and incubated on ice for 1 min. The mixture was transferred into an ice-cold electroporation cuvette (EquiBio, Angleur, Belgium). The cuvette was placed into an electroporator (Gene Pulser II; Bio-Rad, Munich, Germany) and pulsed with 2.5 kV, 25 µF and a pulse control of 200 Ω. Thereafter, 1 ml LB medium was added and the culture was grown at 37°C, 145 rpm for 1 hr. An aliquot of 100-µl transformed bacteria was spread on an antibiotic selective LB agar plate and grown overnight at 37°C.

3.3.4 Zero blunt TOPO cloning

A rapid and selective method was used to clone PCR products. The Zero blunt TOPO PCR cloning kit was purchased from Invitrogen. A 5-µl reaction contained 0.5 to 4 µl PCR product and 0.5 µl PCR-Blunt II-TOPO vector. The mix was incubated at room temperature for 5 min and the reaction was stopped with1 µl stop solution. The mix was then added to a vial of One-Shot competent cells (Invitrogen) and incubated on ice for 30 min. After a heat shock for 30 s at 42°C, the tube was incubated on ice for 2 min. 250 µl of SOC medium (Invitrogen) was added to the tube and the tube was shaken horizontally at 37°C, 125 rpm for 1 hr. Two volumes (10 µl and 50 µl) of bacteria were plated onto agar plates containing 50 µg/ml kanamycin and incubated at 37°C overnight.

3.4 DNA processing and manipulation

3.4.1 Restriction endonuclease digestion of DNA

Restriction endonucleases were used to digest double-stranded DNA for analytical or preparative purposes. These restriction enzymes were purchased from New England Biolabs and used according to the supplier's instructions. A typical reaction consisted of a 20 µl volume containing 1-3 µg of DNA, 1× reaction buffer (from supplier), and 100 µg/ml BSA. The reaction was incubated at the temperature recommended (usually 37°C) for times from 30 min to overnight. Complete digestion was confirmed by agarose gel electrophoresis.

3.4.2 Phenol-chloroform extraction

To inactivate and remove contaminant proteins, DNA solutions were extracted with a mixture of phenol-chloroform. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1) (Roth, Karlsruhe, Germany) was added to an aqueous DNA solution. After vigorous vortex the emulsion was centrifuged at 13000 g, 4°C for 5 min. The upper aqueous layer containing the purified DNA was carefully transferred to a fresh tube and was subsequently precipitated by absolute ethanol.

3.4.3 Ethanol precipitation of DNA

DNA was precipitated from aqueous solutions using salt and alcohol. A 1/10 volume of 3.3 M sodium acetate, pH5.2 and a 2.5 volume of ice-cold absolute ethanol were added to the

solution. After 15 min at -80°C, the solution was centrifuged at 13 000 g, 4°C for 15 min. The pellet was washed with 70% ethanol, dried and dissolved in water. All DNA solutions were stored at -20°C.

3.4.4 Dephosphorylation of linearized plasmid DNA by CIP

To prevent the re-ligation of linearized vector and to promote the insertion of the desired DNA fragment, calf intestinal phosphatase (CIP) was used to dephosphorylate the 5' ends of the vector.

Dephosphorylation was carried out directly following plasmid linearization. 1U CIP (Boehringer, Mannheim, Germany) was added to the digestion mixture. After 20 min incubation at 37°C, the mixture was loaded to agarose gel in which the CIP and restriction enzymes were inactivated. The dephosphorylated plasmid was electroeluted from the agarose gel (3.4.10) and precipitated with ethanol (3.4.3).

3.4.5 Ligation of DNA fragments

DNA fragments bearing either sticky ends (both vector and insert have compatible overhangs) or blunt ends (the ends of vector and insert have no overhangs) can be ligated *in vitro* with T4 DNA ligase.

Linearized, dephosphorylated vector (approximately 50 fmol) was mixed with an excess of insert DNA (100 fmol) in a volume of 15 µl containing 1× ligase buffer and 1U T4 DNA ligase. The ligation mixture was incubated at 16°C for 6-16 hr and was subsequently used to transform bacteria.

3.4.6 Mini-prep: small-scale preparation of plasmid DNA

Following amplification of plasmid DNA in *E.coli*, DNA was extracted using a rapid method ('Mini-Prep') to screen for clones with the correct insert. Single colonies were picked from agar plates, inoculated into 3 ml of LB medium containing the suitable selective antibiotic and cultured overnight at 37°C, 225 rpm. The culture was centrifuged at 5.000 g for 1 min. The bacterial pellet was resuspended in 125 µl buffer MP1 (50 mM Tris-Cl, pH8.0, 10 mM

EDTA, 100 µg/ml RNase A) and transferred to a 1.5 ml tube. 125 µl buffer MP2 (200 mM NaOH, 1% SDS) was added to the tube and the lysate was incubated at room temperature for 5 min. 125 µl buffer MP3 (3 M potassium acetate, pH5.5) was added. After gentle vortex, the mix was incubated on ice for 15 min and subsequently centrifuged at 13000 g, 4°C for 15 min. The supernatant containing the plasmid DNA was transferred to a fresh 1.5-ml tube and precipitated by 270 µl isopropanol. The DNA pellet was washed with 70% ethanol and dissolved in 50 µl water.

3.4.7 Maxi-prep: large-scale preparation of plasmid DNA

For preparation of mg amounts of plasmid DNA, the Qiagen Plasmid Purification kit (Qiagen, Hilden Germany) was used. A bacterial colony known to contain the desired insert was pre-cultured for 6 hr in 3 ml LB medium containing a selective antibiotic. 100 ml LB medium was inoculated with the pre-culture and grown overnight. The bacteria were centrifuged at 6000 g, 4°C for 10min and the pellet was resuspended in 10 ml cold buffer P1. 10 ml lysis buffer P2 was added and the lysate were incubated for 5 min at room temperature. The mix solution was neutralized with 10 ml buffer P3 and incubated on ice for 20 min. This mix was ultra-centrifuged at 70000 g, 4°C for 30 min and the supernatant was decanted onto an anion exchange column equilibrated with buffer QBT. The column was washed twice with 30 ml buffer QC and the DNA was eluted with 15 ml buffer QF. The DNA was precipitated with 10.5 ml isopropanol and centrifuged to DNA pellet at 10000 g, 4°C for 30 min. The DNA pellet was washed with 70% ethanol, dried under vacuum and dissolved in water to an appropriate concentration.

3.4.8 Quantification of DNA solutions

The concentration of DNA was determined by spectrophotometry (Shimadzu, Manchester, England). The ultraviolet (UV) absorption was measured at a wavelength of 260nm (OD_{260}) using a quartz cuvette. For double-stranded DNA one OD_{260} corresponds to approximately 50 µg/ml DNA. In addition, the OD_{280} was measured to estimate the purity of the nucleic acid sample. A ration A_{260}/A_{280} significantly less than 1.8-2.0 would indicate phenol or protein contamination.

3.4.9 Agarsoe gel electrophoresis

DNA was separated by agarose gel electrophoresis. 0.7-2% (w/v) agarose was melted in 1×TBE electrophoresis buffer and supplemented with ethidium bromide (0.5 µg/ml). The agarose gel was placed in an electrophoresis tank (Bio-Rad, Munich, Germany) and submerged in 1×TBE buffer. The DNA samples were mixed with 6×DNA loading dye (18% Ficoll typ 400, 0.5 M EDTA, 50 mg/ml bromphenolblue, 200 mM Tris) and loaded into the agarose gel wells. In addtion 10-µl DNA standard was loaded in parallel with the samples. Horizontal electrophoresis was carried out at approximately 110 V. The stained gel was photographed under UV light. The length of a DNA fragment was determined by comparison of its mobility to that of DNA standards. The DNA bands of the correct size were excised and subsequently electroeluted.

3.4.10 Electroelution : recovery of DNA from agarose gels

DNA in agarose gel slice was electroeluted with 300 µl 1×TBE buffer in dialysis plastic membranes (Roche, Mannheim, Germany). The electroelution chamber (Bio-Rad, Munich, Germany) was cooled on ice and a constant current of 145 mA was applied for 1.5 hr. After elution, the TBE in the membrane was transferred to a 1.5-ml tube and the DNA was precipitated as 3.4.3.

3.4.11 Sequencing of DNA

DNA was sequenced using Bigdye Terminator Cycle DNA Sequencing kit (PE applied biosystems, Weiterstadt, Germany) and the ABI prism 310 Genetic Analyzer (PE applied biosystems). A sequencing reaction consisted of 0.5 µg plasmid DNA or 90 ng purified PCR product, 3.2 pmol primer and 5 µl of reaction Ready Mix in a 20 µl volume. To incorporate the fluorescent nucleotides into the template, the reaction was cycled 25 times as follows:

Denaturation: 95°C, 30 s
Annealing: 50°C, 50 s
Elongation: 60°C, 4 min

Separation of template from unincorporated nucleotides was achieved by centrifugation through a Centrisep size-exclusion column (PE applied biosystems). The eluted template was

dried, resuspended in Template Suppression Reagent (PE applied biosystems) and denatured at 95°C for 3 min. The sample was then loaded onto the Genetic Analyzer. The obtained raw data were processed and analyzed with the supplied software.

3.4.12 DNA amplification by polymerase chain reaction (PCR)

Specific DNA segments can be amplified by PCR. Primers were commercially synthesized (MWG Biotech, Ebersberg, Germany). They were designed corresponding to the DNA segment to be amplified.

Reaction parameters for DNA amplification were as follows:

Template DNA	200 ng (except in negative control PCR)
Forward-primer	25 pmol
Reverse-primer	25 pmol
Taq DNA polymerase	1-2 U
PCR reaction buffer	1x
dNTPs	10 pmol each dATP,dGTP,dCTP,dTTP
Final reaction volume	50 µl

Thermal cycle paramenters for PCR were as follows:

Initial denaturation	94°C, 1 min
30-40 cycles of	
Denaturation:	94°C, 1 min
Primer annealing:	45-65°C, 1 min
Elongation:	72°C, 30 s-2 min

The annelaing temperature was varied according to the melting temperature of the primers. Elongation time depended on the length of the desired product, with 1 min used for amplifaction of 1 kb.

3.5 RNA applications

3.5.1 RNA isolation and quantification

Frozen heart tissue or cultured cardiomyocytes were homogenized in Trizol LS Reagent (Invitrogen), and total RNA was isolated according to the manufacturer's recommendations. Total RNA was then quantified by spectrophotometer. To verify the absence of genomic DNA in the prepared RNA samples, control PCR reactions were performed in the absence of reverse transcriptase. The concentration of RNA was photometrically measured at 260 nm and one OD_{260} corresponded to 40 µg/ml for single stranded RNA.

3.5.2 First strand cDNA synthesis

First strand cDNA synthesis was carried out using Superscript II reverse transcriptase (Invitrogen). A reaction mixture contained 4 µg of total template RNA, 100 pmol of random hexamer primers, 1.25 mM dNTPs, 10 mM DTT, 2 units of Rnase H, 1× first strand buffer and 50 units of Superscript II reverse transcriptase. Template RNA and random hexamer primers were mixed with water to a final volume of 11µl and incubated for 10 min at 70°C. The buffer, DTT and dNTPs were added and the solution was incubated for 2 min at 42°C. The reverse transcriptase was then supplemented and the mixture was incubated for 1 hr at 42°C. The reverse transcriptase was heat-inactivated for 5 min at 90°C. Rnase H was finally added and the total solution was incubated for 20 min at 37°C. The first strand cDNA samples were stored at -20°C.

3.5.3 Gene expression analysis at the mRNA level using RT-PCR

For gene expression analysis, first strand cDNA was subjected to PCR in order to amplify cDNA fragments derived from expressed target gene-specific mRNA transcripts. The cDNAs were first analyzed with a GAPDH specific RT-PCR as an internal control. Specific primer pairs were used to detect $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ $Ca_v2.3$, $Ca_v3.1$ and $Ca_v3.2$ transcripts (Table 3). Each RT-PCR reaction contained 10% of first strand cDNA reaction mix, 1xPCR buffer, 25 pmol of each primer, and 2.5 units of *Taq* DNA polymerase (Promega) in a total volume of 50 µl. The PCR cycles were denaturing at 94°C for 1 min and annealing at 1 min during 35-40 cycles. The PCR products were separated on 2% agarose gels and stained with ethidium bromide. The gels were recorded and analyzed by imaging software (Bio-Rad Gel 2000, Quantity One Software version 4). The relative amount of the amplicons was determined and

normalized to that of the GAPDH fragment. The identity of the PCR products was verified by DNA sequencing.

The relative expression levels of two $Ca_v1.3$ splice variants ($Ca_v1.3$ (1a) and $Ca_v1.3$ (1b)) were analyzed by using a mixture of two forward primers (Dutr01 and Race20, 1:1 ration) and the reverse primer Race12 (Table 3). Detection of the two splice variants by this reaction mixture was possible because this PCR reaction yielded different sized fragments.

Table 3 Primers used to amplify the calcium channel cDNAs and the predicted product size.

Name	Primer	5'Location [bp]	Sequence [5'-3']	Size [bp]
$Ca_v1.1$	F	1235	GCGAGGTCATGGACGTGGACGA	200
(L06234)	R	1434	GATCACCAGCCAATAGAAGAC	
$Ca_v1.2$	F	3527	CAGGAGGTGATGGAGAAGCCA	316
(L01776)	R	3842	CTGCAGGCGGAACCTGTTGTT	
$Ca_v1.3$	F	3217	GGGGTCCAGCTGTTCAAGGGGGAA	555
(AJ437292)	R	3771	GCATGATGAGGACGAACATCATG	
$Ca_v3.1$	F	2998	GTGGAGGGTTTCCAGGCAGAGG	279
(AJ012569)	R	3276	GTGCTGGAGCTCTTGGG	
$Ca_v3.2$	F	3105	AGAGGAAGATTTCGATAAGCT	328
(AF051946)	R	3432	GCTGTTCCAGCTGGAGCGCC	
$Ca_v1.3$(1a)	Dutr01	-45	AGCAYAGTGCCCTGCACACAGTA	590
(AJ437291)	RACE12	545	CAGTCCATACGCTATAATCTTCAGAAATGT	
$Ca_v1.3$(1b)	RACE20	-31	GCTCGGTGGCTGTATTTTCAAC	649
(AJ437292)	RACE12	618	CAGTCCATACGCTATAATCTTCAGAAATGT	
GAPDH	F		CGGCAAATTCAACGGCACAGTCA	597
	R		GGTTTCTCCAGGCGGCACGTCA	

F, forward primer. R, reverse primer. The accession numbers of the calcium channel cDNA sequences are indicated below its names.

3.6 Protein analysis

3.6.1 Whole cell lysates

Cardiomyocytes or cultured HL-1 cells were harvested, washed with PBS and centrifuged at 5000 g for 5 min. The embryonic hearts or harvested cells were exposed to one freeze-thaw cycle and homogenized in lysis buffer (20 mM KH_2PO_4, pH 7.2, 1 mM EDTA) containing protease inhibitors (1 mM benzamidine, 0.1 mM PMSF and protease inhibitor cocktail [1:500, Sigma]). The lysates were heated for 5 min at 95°C and subjected to protein concentration measurement.

3.6.2 Measurement of protein concentrations

The protein content was estimated using the Bradford protein determination method. Bovine serum albumin (BSA) (GibcoBRL) standards were prepared from concentrations of 25 to 100 ng/µl in water. 1 ml of Bradford solution was added to 100 µl of BSA standard or diluted protein sample. The mix solution was left at room temperature for 5 min and the absorbance was measured at 595 nm. A calibration curve of the protein concentration versus absorbance was obtained and protein sample concentration was calculated.

Bradford solution

250 mg Coomassie Brillant Blue G 250
100 ml Ethanol
200 ml 84.5% H_3PO_4
H_2O ad 2 000 ml

3.6.3 SDS-PAGE

Whole cell lysates were resolved for analytical purposes by polyacrylamide gel electrophoresis (PAGE). The use of anionic detergent sodium dodecyl sulfate (SDS) enabled separation according to the molecular weight of the proteins. Resolving gel solution was prepared and poured between two glass plates. After polymerization, the top of the gel was washed with water. Freshly prepared stacking gel solution was filled. Subsequently the gel was placed in a vertical electrophoresis apparatus (Mini-PROTEAN II Cell; Bio-Rad) filled with 1×running buffer. The protein lysates were denatured in Lamemmli sample buffer (0.25 M Tris-Cl pH 6.8, 1.6% SDS, 5% glycerol, bromophenol blue, with 0.1 M DTT) and boiled for 10 min. The protein samples and a protein standard were loaded onto the SDS-PAGE gel. The gel was run at 100-200 V. The resolving gel was subjected to western blot analysis.

Resolving gel (11%)	1.875 ml 1.5 M Tris, pH 8.8
	2.875 ml H_2O
	2.45 ml acrylamide/bis-acrylamid solution
	25 µl APS
	10 µl TEMED
Stacking gel	1.25 ml 0.5 M Tris, pH6.8
	3.05 ml H_2O
	0.65 ml acrylamide/bis-acrylamid solution 12.5 µl APS
	5 µl TEMED
Running buffer (10×)	30 g Tris-base
	142 g glycine
	50 ml 20% SDS
	H_2O ad 1 000 ml

3.6.4 Western-blot analysis

After electrophoresis proteins were transferred to a PVDF membrane (Millipore, Eschborn, Germany). A PVDF membrane was placed onto the polyacrylamide gel and both were sandwiched between electrode papers, pads and a plastic support. The proteins were transferred to the membrane by electrophoresis towards the anode at 200 mA for 1hr in ice-cold transfer buffer. None-specific binding sites on the blotted membrane were blocked in 1×TBST buffer (1×TBS, 0.1% Tween 20) containing 3% BSA for 1 hr at room temperature. The membrane was washed two times in 1×TBST for 15 min before incubation with the primary antibodies. To detect $Ca_v1.2$ and $Ca_v1.3$, the upper part of the membrane was incubated overnight at 4°C with the respective primary antibodies (1:200). For β-actin, the lower part of the membrane was incubated overnight at 4°C with the β-actin primary antibody (1:200). After incubation with the primary antibodies the immublots were washed three times with 1×TBST for 10 min. For $Ca_v1.2$ and $Ca_v1.3$, immunoblots were then incubated with goat anti-rabbit secondary antibody (1:10000 diluted in 1×TBST) for 1 hr at room temperature. For β-actin, immublots were incubated with goat anti-mouse secondary antibody (1:10000 diluted in 1×TBST) for 1 hr at room temperature. The immunoblots were visualized by the ECL system (NEN Life Science Products, Boston, MA) and the films were developed in an automated machine (Kodak, Stuttgart, Germany).

Transfer buffer	3.03 g Tris
	14.4 g glycin
	200 ml methanol
	H_2O ad 1 000 ml

3.7 Adeno-X Expression System

In order to introduce genes into mammalian cells, the Adeno-X expression system (Clontech; BD Biosciences, Heidelberg, Germany) was applied. The components of the system included pShuttle vector, Adeno-X viral DNA (PI-Sce I/I-Ceu I digested), adeno-X forward primer, Adeno-X reverse primer, I-Ceu I, PI-Sce I and 10× double digestion buffer. The entire process consisted of three steps (Figure 4 taken from the manual of Adeno-X expression system, Clontech). First, an expression cassette was constructed by cloning the target gene into pShuttle. Second, the expression cassette was transferred to Adeno-X viral DNA by ligation. Finally, the recombinant adenoviral plasmid was packaged by transfecting HEK 293 cells. Adenovirus was harvested by collecting the viral supernatant from the transfected HEK 293 cells. The viral titer was then determined with the Adeno-X Rapid Titer kit (Clontech; BD Biosciences) and the virus was used to infect cells.

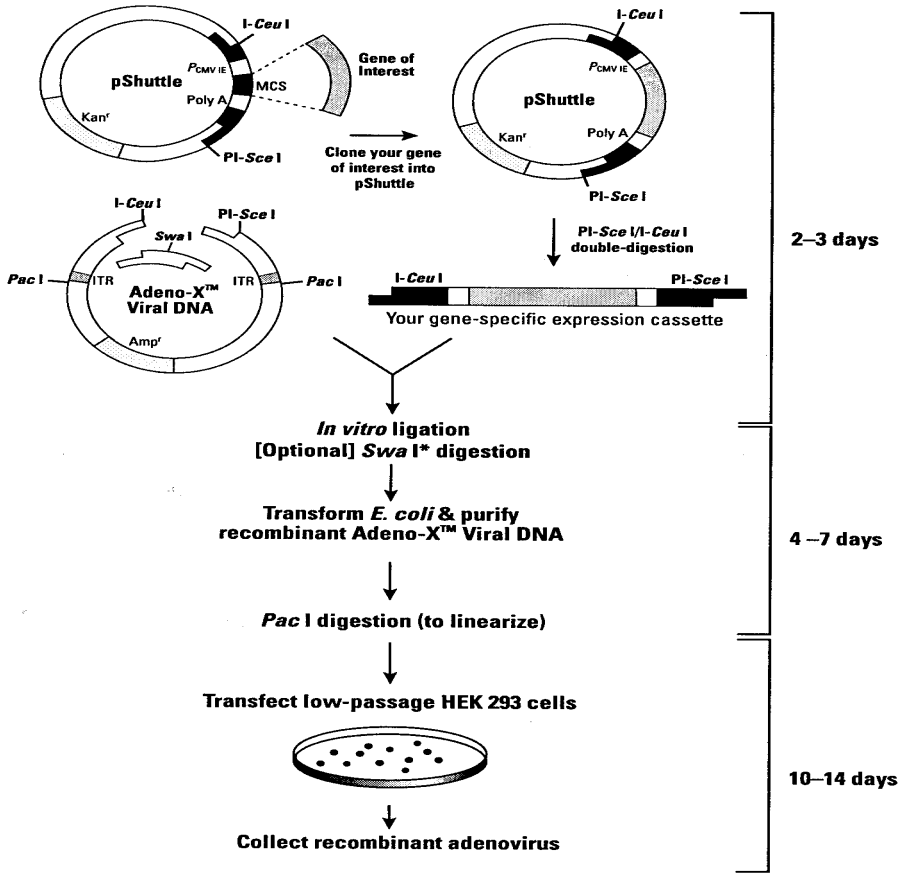


Figure 4. Protocol overview of constructing recombinant adenovirus with the Adeno-X Expression System (taken from the manual of Adeno-X expression system, Clontech).

3.7.1 Cloning the insert into pShuttle

3.7.1.1 Anti-sense constructs

The anti-sense or sense control fragments were obtained by PCR with primer pairs specific for the IIS6 and III S1 segments of the calcium channel α_1 subunits (Table 4) The recognition sites of Not I (GCGGCCGC) and Kpn I (GGTACC) were introduced at the 5' and 3' end of the fragments.

Table 4 Primers for obtaining sense or anti-sense fragments

$Ca_v1.1$	Sense	forward reverse	AGAAAGCGCAGGAAGATGTCG CTGCAGCTCGGCCAGAGGGCG	255bp
	anti-sense	forward reverse	CTGCAGCTCGGCCAGAGGGCG AGAAAGCGCAGGAAGATGTCG	255bp
$Ca_v1.2$	Sense	forward reverse	CAGGAGGTGATGGAGAAGCCA CTGCAGGCGGAACCTGTTGTT	318bp
	anti-sense	forward reverse	CTGCAGGCGGAACCT CAGGAGGTGATGGAGAAGCCA	318bp
$Ca_v1.3$	Sense	forward reverse	AAACCAGAAGTCAACCAGATAG ACCCACACGTATCGGGTTGGT	267bp
	Antisense	forward reverse	ACCCACACGTATCGGGTTGGT AAACCAGAAGTCAACCAGATAG	267bp

The PCR products were sequenced and then ligated into the Not I and Kpn I sites of the pEGFP vector (Clontech). Subsequently, the EGFP-sense or antisense constructs were cloned into pShuttle at the sites of Apa I and Kpn I.

3.7.1.2 Overexpression constructs of calcium channel subunits

In order to enable coexpression of EGFP (enhanced green fluorescent protein) and calcium channel subunits (cardiac α_{1C} full length, cardiac α_{1C} truncated, β2 and β2 mutation subunits), the cDNAs of calcium channel subunits (provided by Dr. N. Klugbauer) were cloned into the Not I and Kpn I. sites of a modified pShuttle-EGFP vector. This vector contains two separate CMV promoters, one for expression of EGFP and one for expression of the resepctive target gene. The correctness of all constructs was confirmed by sequencing.

3.7.2 Subcloning the pShuttle cassette into the Adeno-X Genome

To obtain recombinant adenoviral DNA, pShuttle (or modified pShuttle-EGFP) vectors were digested with the restriction enzymes PI-Sce I and I-Ceu I. The obtained expression cassettes containing the promotor regions, the target genes and the poly A-signal region were purified

by gelelectrophoresis and ligated into Adeno-X viral DNA that was digested with the same enzymes (Figure 3). The products obtained were circular recombinant E1/E13-deleted adenoviral genomes. The putative recombinant Adeno-X DNA was analyzed by PCR (primers included in the Adeno-X expression system) and by restriction digestion analysis with PI-Sce I and I-Ceu I.

3.7.3 Producing recombinant adenovirus

The recombinant Adeno-X viral plasmids were linearized with Pac I and subsequently transfected into HEK 293 cells with FuGen reagent (Roche). One week later, the cells were collected and lysed with three consecutive freeze-thaw cycles (the cells were frozen in a dry ice/ethanol bath and thawed by placing the tube in a 37°C water bath). After the third cycle, the cells were briefly centrifuged to pellet debris and the supernatant was directly added to a fresh HEK 293 culture dish. When approximately 50% of the cells detached from the dish, the cells were collected and the lysate was prepared by three consecutive freeze-thaw cycles. The virus supernatant was the primary viral stock and used for determining viral titer. The viral titer was determined by the plaque assay or by the Adeno-X Rapid Titer kit (Clontech; BD Biosciences).

3.7.4 Adenoviral infection of cardiomyocytes

Adenoviral infection was performed on cells that had been cultured overnight in 60-mm culture dishes (Corning, NY, USA) or in 24-well plates. The infection procedure was similar to that described (Eizema et al. 2000). The cells were changed into low serum medium containing 5% FCS and infected with adenovirus at a multiplicity of infection (MOI) of 10-50 particles/cell for 12 hr. The cells were then cultured in normal growing medium with 10% FCS for an additional 48-72 hr. The efficiency of adenoviral infection was observed by the GFP expression. The expression of target genes was analyzed by RT-PCR or western-blot analysis.

4. Results

4.1 Enhanced expression of L-type $Ca_V1.3$ calcium channels in murine embryonic hearts from $Ca_V1.2$ deficient mice

In this part, the developmental expression of six potential calcium channel α_1 subunits was systematically evaluated by RT-PCR at embryonic days 9.5, 12.5 and 15.5 p.c. in fetal mouse heart tissue and in isolated fetal cardiomyocytes. In an attempt to investigate the developmental consequences of calcium channel dysfunction, the expression of calcium channels in cardiomyocytes derived from $Ca_v1.2$ deficient embryos was compared with that in wild type cardiomyocytes. Major differences in the expression level of other calcium channel genes with a predominant up-regulation of a $Ca_v1.3$ splice form were found in $Ca_v1.2$ (-/-) embryos.

4.1.1 Expression of calcium channel α_1 subunit mRNAs in murine fetal heart and cardiomyocytes

Due to the very low amount of embryonic heart tissue, we used a RT-PCR approach to investigate the expression of Ca^{2+} channel α_1 subunits in the developing heart. In agreement with other publications, we obtained amplicons for $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$, $Ca_v3.1$ and $Ca_v3.2$ channels (Hofmann et al. 1999; Ménard et al. 1999; Takimoto et al. 1997). No specific DNA fragments were detected for the R type $Ca_v2.3$ and $Ca_v1.4$ calcium channels (not shown and Seisenberger et al. 2000). The relative mRNA expression was determined at embryonic day 9.5 p.c., a time point when the heart starts regular beating, at embryonic day 12.5 p.c., a time point when $Ca_v1.2$-deficient embryos still develop and at embryonic day 15.5 p.c., a time point when the heart is fully developed (Passier et al. 2000) and requires the presence of the $Ca_v1.2$ channel (Seisenberger et al. 2000). In order to compare expression levels obtained from independent experiments the data was normalized by using GAPDH as internal standard. Figure 5A shows representative gels displaying the results from RT-PCR with total RNA isolated from intact embryonic hearts. Transcripts of the L-type channels $Ca_v1.1$, $Ca_v1.2$ and $Ca_v1.3$ are present throughout the fetal heart development. However, while at day 9.5 p.c. the expression levels of the three channels are about at the same levels, the $Ca_v1.2$ subunit is up-regulated about 3 fold when the heart development proceeds (Figure 4B). In contrast, expression levels of $Ca_v1.3$ and $Ca_v1.1$ are only slightly increasing during embryogenesis. As

a result, $Ca_v1.2$ becomes the predominant L-type channel isoform in late heart developmental stages. In isolated fetal cardiomyocytes, up-regulation of $Ca_v1.2$ was even slightly more pronounced than in total heart (Figure 6). Again, the increase of the other two L-type channel isoforms is much smaller than that of $Ca_v1.2$. In general, the changes in mRNA levels are more pronounced in cardiomyocytes than in total heart tissue, which may be caused by various expression levels in multiple cell types in heart tissue. While in total heart the $Ca_v3.1$ T-type Ca^{2+} channel subunit could be consistently found at all three time points investigated, $Ca_v3.2$ is barely detectable at all developmental stages (Figure 5 and 6). At day 15.5, $Ca_v3.2$ could be detected neither in total heart nor in isolated cardiomyocytes indicating that $Ca_v3.1$ is the major cardiac T-type channel isoform (Bohn et al. 2000; Ménard et al.1999). Like $Ca_v1.2$**,** the $Ca_v3.1$ subunit is also up-regulated during early fetal development. However, in contrast to $Ca_v1.2$, the $Ca_v3.1$ subunit levels do not further increase between 12.5 p.c. and 15.5 p.c. indicating that gene expression of the two isoforms is differentially regulated. Interestingly, unlike in total heart, expression of $Ca_v3.1$ was not observed in isolated cardiomyocytes at day 9.5 (Figure 6). This finding indicates that at this time point $Ca_v3.1$ could be expressed in cell types different from cardiomyocytes.

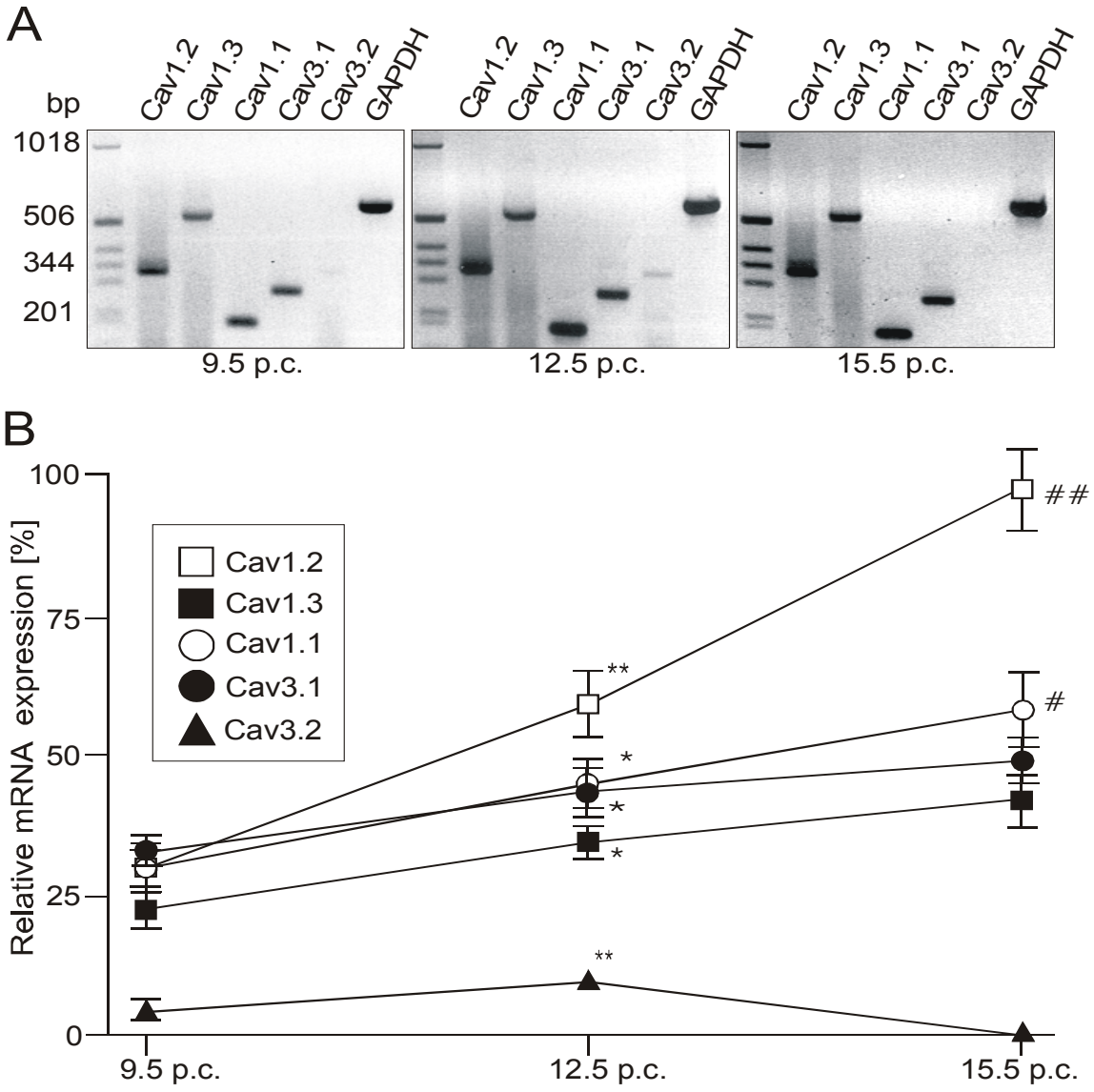


Figure 5. Expression profiles of α_1 subunit genes during murine fetal heart development. **(A)** RT-PCR-based analysis of calcium channel α_1 subunit mRNA from murine heart at day 9.5 p.c., 12.5 p.c. and 15.5 p.c. For each lane, 0.4µg of total RNA was used to perform RT-PCR with primer pairs shown in Table 1. GAPDH was used as an internal control. **(B)** Relative expression levels of α_1 subunit genes. The intensities of the respective ethidium bromide-stained amplicons were measured and normalized against the GAPDH amplicon as internal control. Data are presented as means ± S.E. (n=3). *P<0.05, **P<0.01 vs the group of day 9.5 p.c. #P<0.05, ##P<0.01 vs the group of day 12.5 p.c.

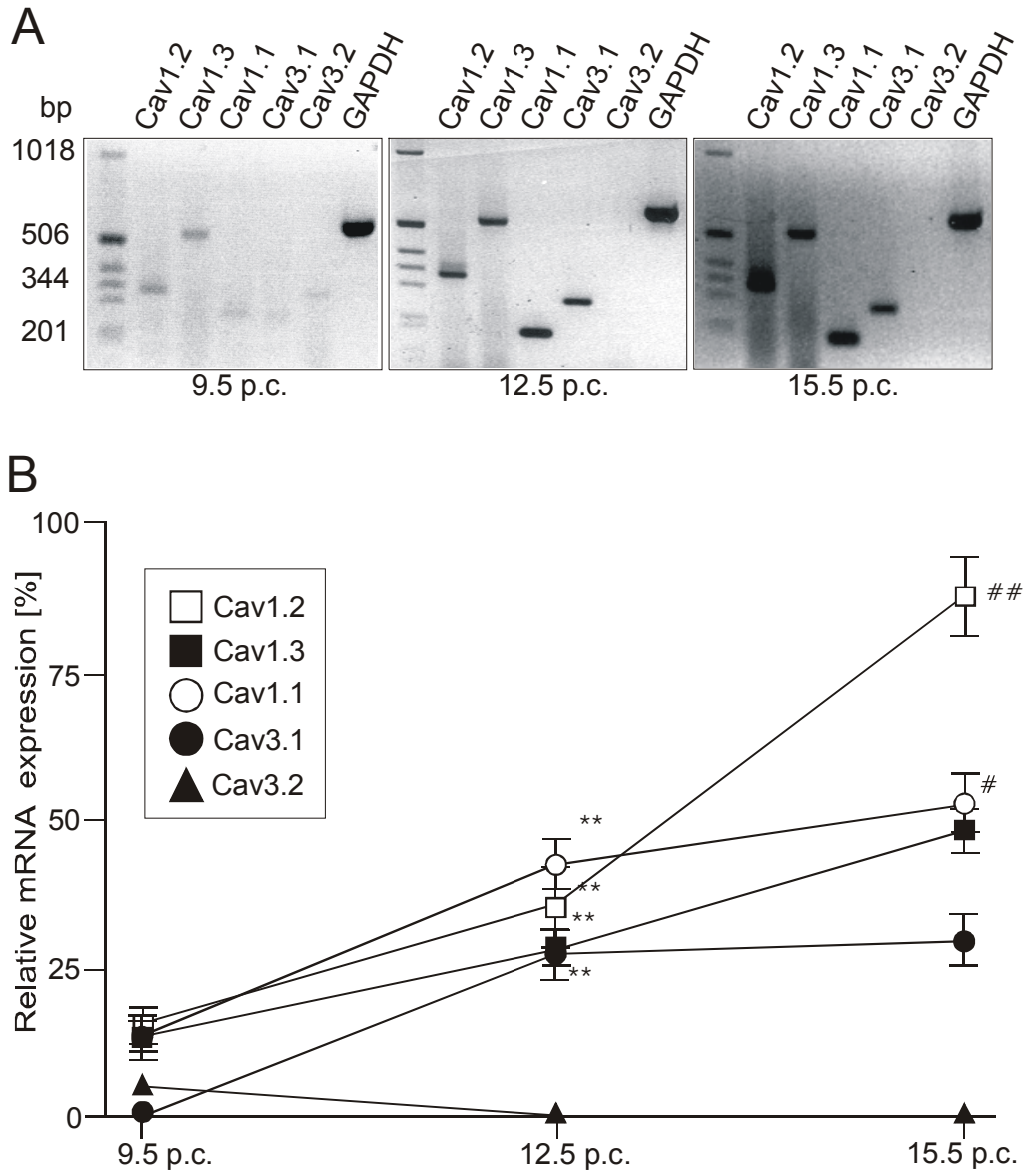


Figure 6. Expression profiles of $\alpha_1$subunit genes in isolated embryonic cardiomyocytes. **(A)** RT-PCR-based analysis of calcium channel α_1 subunit mRNA from cardiomyocytes isolated from murine embryos at day 9.5 p.c., 12.5 p.c. and 15.5 p.c. For each lane, 0.4 μg of total RNA was used to perform RT-PCR with primer pairs shown in Table 1. GAPDH was used as an internal control. **(B)** Relative expression levels of α_1 subunit mRNA as compared to GAPDH expression. Data are presented as means ± S.E. (n=3). n.d. indicates that expression was below the level of detection. *P<0.05, **P<0.01 vs the group of day 9.5 p.c. #P<0.05, ##P<0.01 vs the group of day 12.5 p.c.

4.1.2 Different expression levels of calcium channel α_1 subunit mRNAs in $Ca_v1.2$-deficient mice

Embryos deficient for the $Ca_v1.2$ gene die before day 14.5 p.c. In contrast, heterozygous mice ($Ca_v1.2$ (+/-)) are viable revealing no overt cardiac phenotype (Seisenberger et al 2000). Our previous study showed that hearts of $Ca_v1.2$ (+/+) and $Ca_v1.2$ (-/-) embryos contract with the same frequency at day 12.5 p.c., indicating that up to this time point $Ca_v1.2$ is either not required for rhythmic heart contraction or is functionally compensated by up-regulation of other calcium channels. Therefore, we investigated the expression of calcium channel α_1 subunit mRNAs in cardiomyocytes isolated from day 9.5 p.c. and 12.5 p.c. of $Ca_v1.2$ (-/-) embryos. Figure 7 shows that the $Ca_v1.2$ gene deletion influences expression of three other channels, $Ca_v1.1$, $Ca_v1.3$ and $Ca_v3.1$. At day 9.5 p.c. $Ca_v1.1$ is up-regulated in $Ca_v1.2$ (-/-) cardiomyoctes. However, this up-regulation does not further proceed during development. At embryonic day 12.5 p.c. expression levels of $Ca_v1.1$ are in the same range for both $Ca_v1.2$ (+/+) and $Ca_v1.2$ (-/-) cardiomyocytes. The most obvious compensatory effect is seen in the expression of $Ca_v1.3$. At day 9.5 p.c. this subunit is up-regulated about 4 fold in $Ca_v1.2$ (-/-) cardiomyocytes (Figure 7B). Unlike with $Ca_v1.1$, a 2 to 4- fold up-regulation of $Ca_v1.3$ is also present at day 12.5 p.c. Transcripts of the $Ca_v1.2$ subunit could also be detected in (-/-) cardiomyocytes (Figure 7A). However, these transcripts do not give rise to a functional calcium channel protein because exon 3 was disrupted by the introduction of a premature stop codon and a neomycin resistance cassette (Seisenberger et al. 2000). Interestingly, the presence of the neomycin cassette has no major influence on $Ca_v1.2$ mRNA expression at day 9.5 p.c. when $Ca_v1.2$ expression levels are low. At day 12.5 p.c. expression levels of the mutated $Ca_v1.2$ mRNA are about 40 % lower than that of the wild-type mRNA (Figure 7B). Finally, deletion of $Ca_v1.2$ affects expression levels of $Ca_v3.1$. In wild-type cardiomyocytes this channel is not substantially expressed at day 9.5 p.c., but (-/-) cells express this subunit in amounts that roughly correspond to expression levels of wild type cells seen at day 12.5 p.c. (Figure 7B).

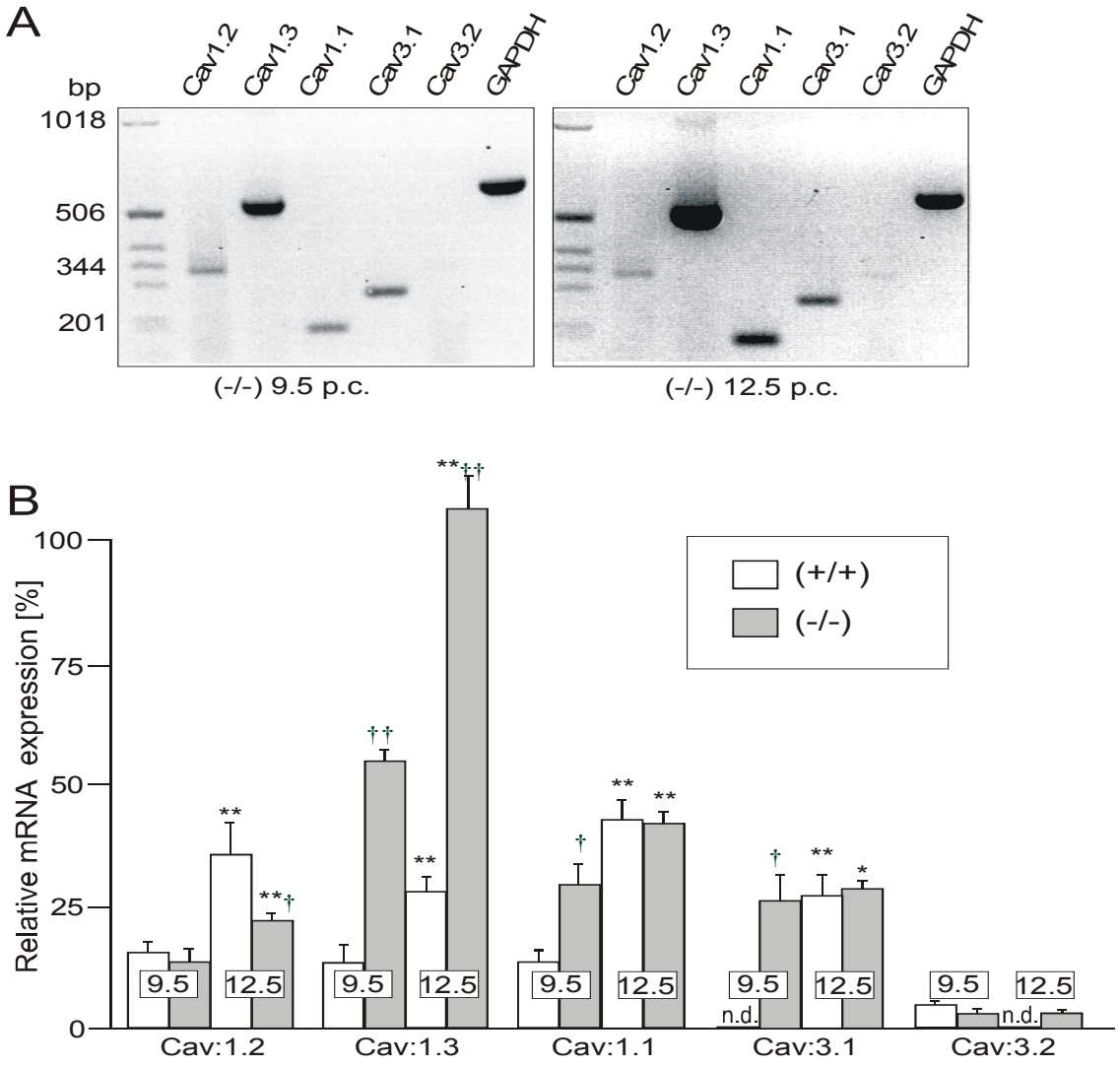


Figure 7. Expression of calcium channel α_1 subunit mRNAs in $Ca_v1.2$ (-/-) and $Ca_v1.2$ (+/+) embryos. (A) RT-PCR-based analysis of calcium channel α_1 subunit mRNA in cardiomyocytes isolated from murine $Ca_v1.2$ (-/-) embryos on day 9.5 p.c. and 12.5 p.c. For each lane, 0.4 µg of total RNA was used to perform RT-PCR. GAPDH was used as an internal control. **(B)** Relative mRNA expression levels of α_1 subunits as compared to GAPDH expression. Data are presented as means ± S.E. (n=3). n.d. indicates expression below the level of detection (values for $Ca_v1.2$ (+/+) cardiomyocytes are from fig 2). *P<0.05, **P<0.01, the group of day 12.5 p.c. vs the group of day 9.5 p.c., †P<0.05, ††P<0.01, the group of $Ca_v1.2$ (-/-) vs the group of $Ca_v1.2$ (+/+) at the same day.

4.1.3 Expression of $Ca_v1.3$ splice variants in $Ca_v1.2$-deficient murine embryonic hearts

To further analyze the expression of $Ca_v1.3$ in $Ca_v1.2$-deficient hearts, we investigated the expression levels of two $Ca_v1.3$ N-terminal splice variants, $Ca_v1.3$ (1a) and the recently identified novel isoform $Ca_v1.3$ (1b) (Klugbauer et al. 2002b) (Figure 8A). RT-PCR indicated that both splice variants of $Ca_v1.3$ were slightly increased at day E9.5 in $Ca_v1.2$ (-/-) hearts. Notably, the up-regulation of $Ca_v1.3$ observed at day E12.5 was even more pronounced. At this time, $Ca_v1.3$ (1b) was clearly the dominant splice variant (Figure 8B).

These PCR results were confirmed by Western blots with a $Ca_v1.3$ specific antibody (Figure 8C). The amount of $Ca_v1.3$ protein was substantially increased in the heart of $Ca_v1.2$ (-/-) embryos. As expected, $Ca_v1.2$ was absent in knockout embryos whereas the expression levels of β-actin were not affected by the knockout.

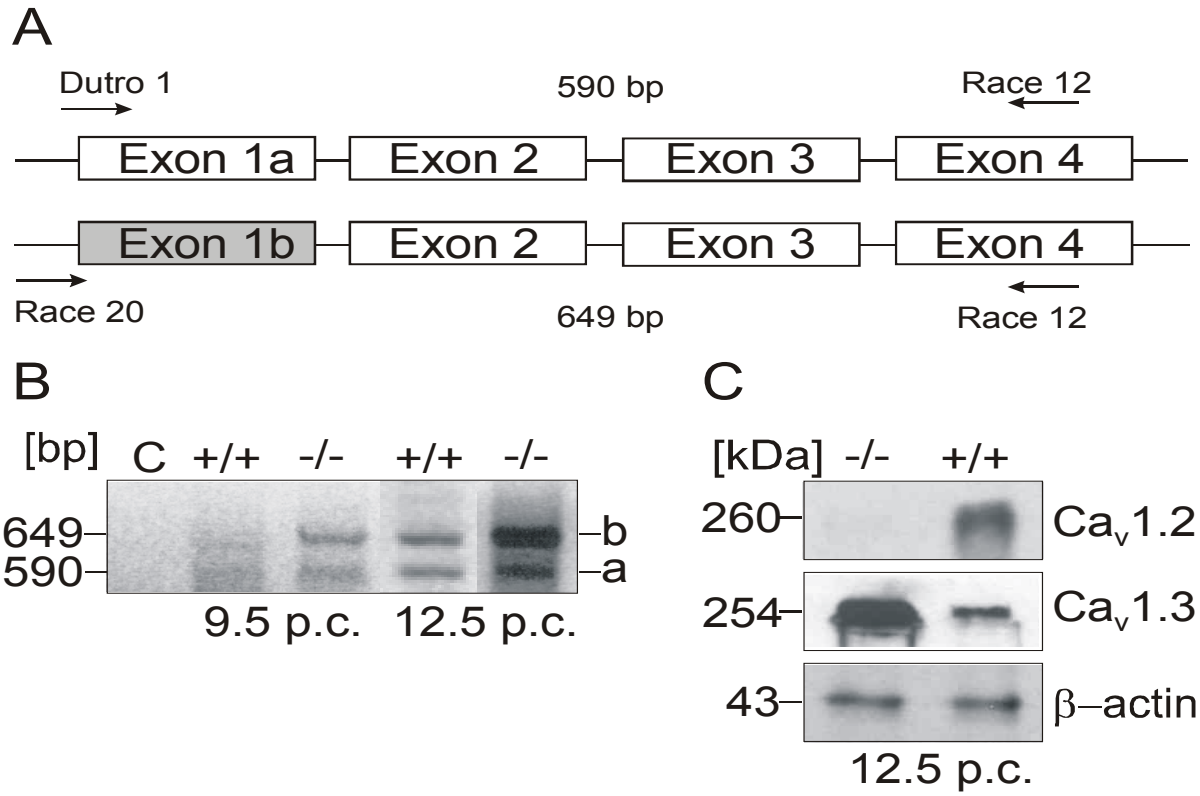


Figure 8. Expression of $Ca_v1.3$ in $Ca_v1.2$ (-/-) and $Ca_v1.2$ (+/+) embryos. (A) Scheme of exones 1-4 of $Ca_v1.3$ (1a) and $Ca_v1.3$ (1b). Dutro1 and Race 20 are primers specific for the N-terminus of $Ca_v1.3$ (1a) and $Ca_v1.3$ (1b), respectively. The forward primers are: Dutr01 (5'-AGC AYA GTG CCC TGC ACA CAG TA-3') and RACE 20 (5'-GCT CGG TGG CTG TAT TTT CAA C -3') with the common reverse primer RACE12 (5'-CAG TCC ATA CGC TAT AAT CTT CAG AAA TGT-3'). **(B)** RT-PCR based analysis of $Ca_v1.3$ splice variants in embryonic hearts at day 9.5 p.c. and day 12.5 p.c.. **(C)** Western blotting of $Ca_v1.2$, $Ca_v1.3$ and β-actin in murine embryonic hearts at day 12.5 p.c..

4.2 Antisense depletion and overexpression of L-type calcium channel subunits by recombinant adenovirus-mediated gene transfer in cardiac HL-1 cell line

The expression of most calcium channel cDNAs in heterologous systems such as *Xenopus* oocytes or HEK293 cells induces robust currents with principal biophysical properties of native calcium currents. However, heterologously expressed channels lack normal physiological regulation. For example, the enhancement of L-type calcium currents by β-adrenergic stimulation, a process of fundamental physiological importance, could not be reliably mimicked in heterologous systems so far. Very likely, physiological channel regulation requires a cellular environment that is not represented in oocytes or the usual tumour cell-derived expression systems. A possible strategy to circumvent this problem consists in expression of cDNAs in primary cells or in permanent cell lines that are closely related to primary cells. I made use of this approach to study the regulation of cardiac L-type channels. As cell type to express cardiac channels the "cardiac-type" HL-1 cell line was chosen. Since these cells can not be efficiently transfected with standard transfection protocols an adenovirus-based gene delivery system was established. Both antisense and sense constructs of calcium channel cDNAs were used in order to deplete or over-express distinct calcium channel subunits. In the following sections I will demonstrate that adenovial-mediated gene transfer yields high levels of transgene expression in cardiac HL-1 cells making this systems ideally suited for future physiological studies in heart cells.

4.2.1 Delivery of foreign genes to cultured cardiac myocytes by an adenovirus-mediated expression system

As mentioned above, delivery of plasmid DNA to cardiac myocytes by conventional means, such as Ca^{2+} phosphate precipitation and lipofection, has proven challenging. By contrast, recombinant adenoviral constructs can transfect cardiac myocytes with nearly 100% efficiency (Martin et al. 1997; Eizema et al. 2000). Here, we adapted the adenoviral-mediated gene expression method to the cultured cardiac myocyte HL-1 cell line. Figure 9 shows a scheme of the adenoviral vectors that we constructed. To selectively deplete mRNA of $Ca_v1.1$, $Ca_v1.2$ and $Ca_v1.3$ a set of fusion vectors containing the EGFP coding region fused to a portion of the calcium channel sequence in antisense orientation were constructed (Figure 9A). To obtain high selectivity for a given channel isoform antisense regions were obtained from channel regions where $Ca_v1.1$, $Ca_v1.2$ and $Ca_v1.3$ are most divergent. Sense constructs

containing the same calcium channel fragments sense orientation were used as the technical control. Expression of EGFP detected by green fluorescence was used as a marker for successful gene delivery and transcription.

For overexpression of channel subunits tandem vectors containing two independent CMV promotor-driven transcription units, one for EGFP (marker gene) and one for the respective transcripts were designed (Figure 9B).

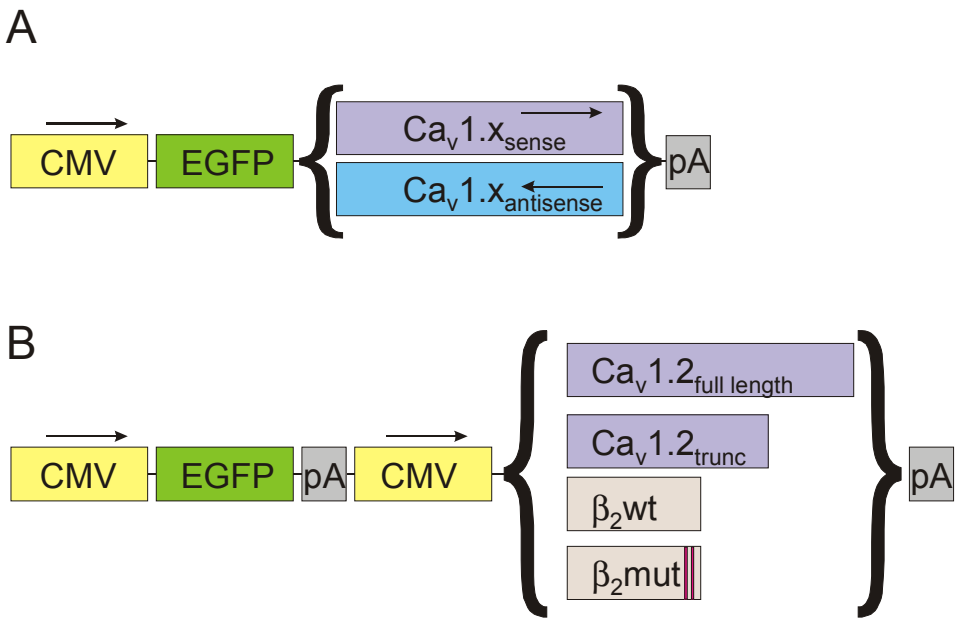


Figure 9. Schematic representation of adenoviral vectors used for sense and antisense overexpression. (A) Fusion constructs for antisense depletion. Selected portions of the coding region of $Ca_v1.1$, $Ca_v1.2$ or $Ca_v1.3$ (see Materials and Methods) were fused in sense or antisense orientation to the 3'-end of the EGFP cDNA. Apoly-A-tract (pA) was added to increase mRNA stability. Antisense and sense for a given $Ca_v1.x$ encode always the same region while in opposite orientation as indicated in the figure. The CMV promotor was used for high level expression. **(B)** Tandem promotor constructs for calcium channels overexpression. The vector contains two CMV promotor-driven transcription units, one for EGFP and one for the indicated calcium channel subunits. The $Ca_v1.2_{trunc}$ channel lacks part of the cytosolic C-terminus of the full length channel including the putative PKA phosphorylation sites. In the mutant β_2 subunit (β_2 mut) the serine residues (Ser-459, Ser-478) that are phosphorylated by PKA are mutated to alanines.

To examine the efficiency of gene transfer by the adenovirus vectors we used EGFP as a reporter gene. The percentage of cells expressing EGFP was determined by comparing white light phase and fluoresent microscopic views of the transfected cardiac HL-1 cells in culture. In agreement with the other study (Eizema et al. 2000), I found that the transfection efficiency concurred well with an increase in M.O.I (multiplicity of infection) (Figure 10A). It can be safely concluded that nearly 100% of cardiac HL-1 cells express EGFP 72h after exposure to M.O.I 30 pfu/cell. Figure 10B illustrates the time course of EGFP expression after transfection of HL-1 cells with M.O.I 30 of the EGFP-$Ca_v1.2$ antisense fusion vector. Transfected cells are bright green, indicating successful transfection of EGFP. Overall, the percentage of striated cells showing green fluorescence increased from 20% to nearly 100% when the time of viral exposure was increased from 24 hrs to 72 hrs. Similar results were obtained with all adenoviral constructs shown in Figure 9.

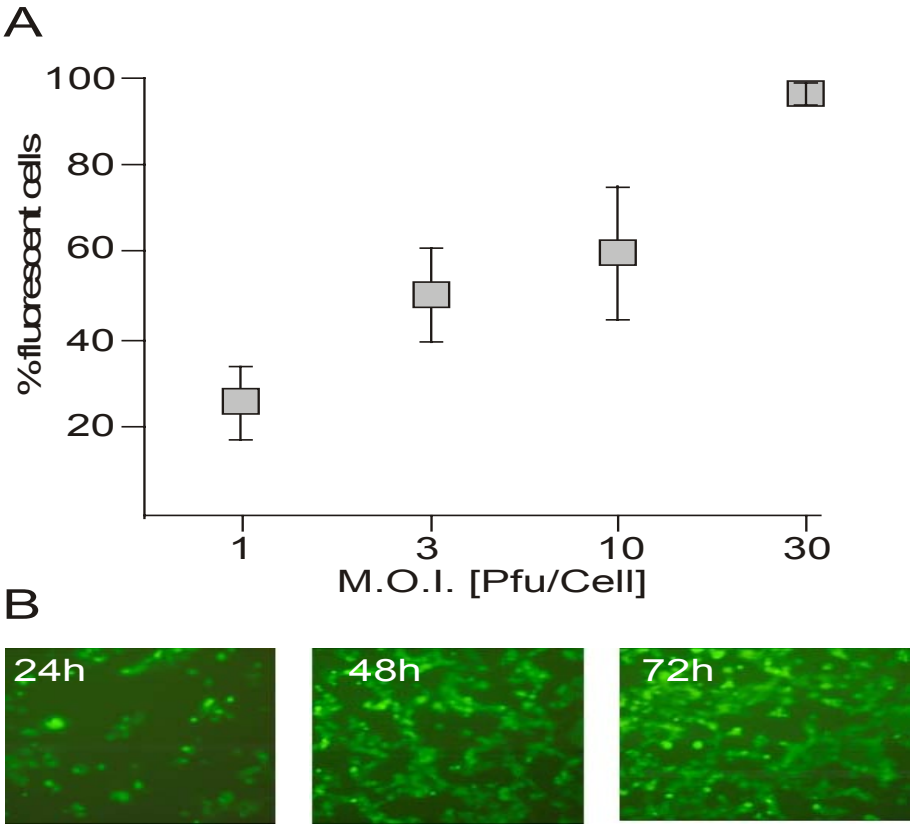


Figure 10. Detection of green fluoresence in cardiac HL-1 cells transfected with EGFP-Ca_v1.2 antisense fusion vector. (A) Percentage of HL-1 cell expressing EGFP after infection with increasing M.O.I. levels. Cells were infected with a virus titre of M.O.I of 1, 3, 10, and 30, respectively, as described in Materials and Methods. Seventy two hours after infection 10 different fields of each culture plate were examined by comparative phase and fluorescence microscopy to determine percentage of cells expressing EGFP. Data are presented as means ± S.E. (n=3) . **(B)** Fluorescent photomicrographs of HL-1 infected with recombinant adenovirus vectors at M.O.I 30. The cells are shown at time points of 24 h, 48 h and 72 h after transfection, respectively.

4.2.2 Depletion of Ca_v1.x calcium channel expression in HL-1 by adenovirus-mediated antisense expression

Cardiac HL-1 cells were infected at a M.O.I of 30 with antisense fusion vectors specific for Ca_v1.1, Ca_v1.2 or Ca_v1.3, respectively. The adenovirus-mediated sense vectors were always used as virus control while non-infected cells were used as normal control. The antisense target regions for Ca_v1.1, Ca_v1.2 and Ca_v1.3 were selected from regions where the three Ca_v1.x calcium channels are most divergent from each other. Previous studies have shown that antisense RNAs inhibit gene expression by specifically binding to their respective target

mRNAs (Crooke et al. 1999; Baker et al. 1999). Specific interaction of antisense RNA with the target mRNAs induces a cellular process that results in the degradation of the mRNAs. Therefore, we first examined the effects of the adenovirus-mediated antisense strategy by RT-PCR. Cardiac HL-1 cells were harvested 72 h after infection with adenovirus. The cellular mRNA was isolated and the amount of $Ca_v1.x$ mRNA was determined. Profound decreases of $Ca_v1.x$ mRNA levels were found with all three antisense vectors (Figure 11A). However, the most significant decrease was observed after expression with the $Ca_v1.1$ antisense vector. The quantitative differences in the reduction efficiency of antisense vectors point out that several factors interfere with mRNA degradation. Moreover, this result indicates that the sequence revealing highest antisense activity varies among different genes. The incubation with the adenovirus-mediated sense vectors had no statistically significant effects on the mRNA expression of $Ca_v1.x$ mRNA.

To demonstrate that the decrease of mRNA levels was followed by a decrease in the protein levels we performed Western blot analysis (Figure 11B). Interestingly, protein levels were roughly like in control cells three days after infection, a time point when the mRNA levels were still significantly decreased (Figure 11A). However, at day four after infection protein levels turned out to be much lower in transfected than in control cells. The delay of protein turn-down with respect to the time point of mRNA degradation possibly is caused by the relatively long half-live time of calcium channel α_1 subunit proteins in the cell.

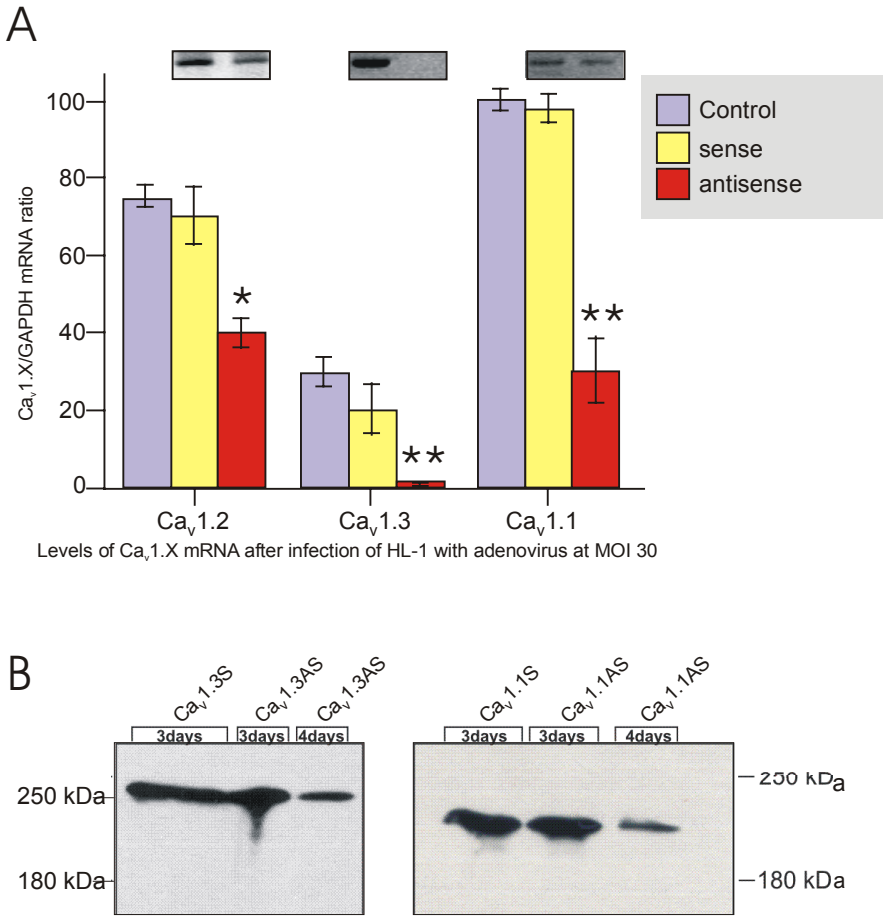


Figure 11. Depletion Ca$_v$1.x calcium channel subunits after infection of cardiac HL-1 with adenovirus-mediated Ca$_v$1 antisense vectors. (A) Graphical representation of mean Ca$_v$1.x/GAPDH mRNA rations. The antisense effects on Ca$_v$1.2, Ca$_v$1.3 and Ca$_v$1.1 mRNA were examined by RT-PCR 72 h after infection of HL-1 with adenovirus at M.O.I. 30. Data are presented as means ± S.E. (n=4). Representative ethidium bromide stained gels are shown on top of the related graphic columns. *P<0.05, **P<0.01 vs control group. **(B)** Western blot of a representative experiment at M.O.I 30 indicates the protein expression levels of Ca$_v$1.3 and Ca$_v$1.1 after infection with adenovirus-mediated sense or antisense vectors. (S, sense; AS, antisense).

4.2.3 Overexpression of calcium channel subunits in cardiac HL-1 cells

The enhancement of calcium channel activity by phosphorylation plays a key role in the autonomous control of the heart beat. Despite extensive efforts of many laboratories the exact mechanism underlying this crucial process is not yet understood. It has been speculated that the C-terminus of the α_1 subunit and/or the cardiac β subunit may serve as target for PKA-mediated phosphorylation. As a basis to study this issue we constructed recombinant Ca$_v$1.2

adenoviruses to overexpress the full length $Ca_v1.2$ ($Ca_v1.2_{fl}$), a C-terminally truncated ($Ca_v1.2_{trunc}$), the wild type β_2 subunit or a $\beta_2$mutant lacking the two putative PKA phosphorylation sites. Figure 12A shows a Western blot with cells that have been transfected with increasing doses of the $Ca_v1.2_{fl}$-virus. The anti $Ca_v1.2$ antibody detects a specific signal of about 210 kDa in cells transfected with the virus. In lane 2 representing a M.O.I. of 30 the signal is significantly stronger that in lane 1 representing a M.O.I. of 10. In cells transfected with a control virus (lane 3) a faint band with a slightly higher molecular mass (about 250 kDa) is visible. This band probably represents the full length $Ca_v1.2$ channel that is endogenously present in HL-1 cells. Taken together the experiments show that the adenoviral vector induces the production of the $Ca_v1.2_{trunc}$ in a dose–dependent manner.

The overexpression of full length $Ca_v1.2$ via adenovirus-mediated overexpression vectors was examined on either 7% or 8.5% SDS-PAGE gels (Figure 12B). The same amount protein was loaded on the two types of gels. Clearly cells transfected with the $Ca_v1.2$-virus revealed a much more intensive signal with the anti-$Ca_v1.2$ antibody (lane 1 and 3) than control cells (lane 2 and 4) demonstrating overexpression of $Ca_v1.2_{fl}$. On the 7% SDS-PAGE, the $Ca_v1.2_{fl}$ band was quite diffuse. Increasing the concentration of the gel to 8.5 % resulted in a significant sharpening of the signal (compare lanes 1 and 3 or 2 and 4). The overexpression of wild type β_2 subunit in HL-1 is shown in Figure 12C. Similar overexpression was found for the β_2-mutant (not shown). Taken together, the experiments convincingly demonstrate the usefulness of adenoviral vectors for gene transfer in HL-1 cells.

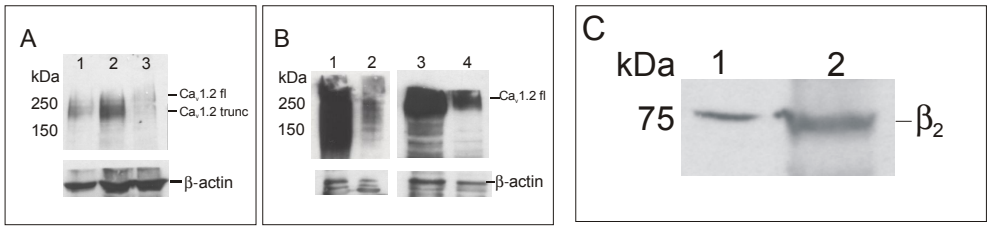


Figure 12. Overexpression of calcium channel subunits in HL-1 cells 72 h after infection with adenoviral tandem CMV expression vectors. (A) Overexpression of truncated $Ca_v1.2$ ($Ca_v1.2_{trunc}$). Proteins were separated on a 7% SDS-PAGE, blotted and probed with an anti $Ca_v1.2$ antibody. Lane 1: M.O.I 10 $Ca_v1.2$ truncated, Lane 2: M.O.I 30 $Ca_v1.2$ truncated, Lane 3: control vector. The antibody detects the overexpressed $Ca_v1.2$ trunc and the endogenous full length (fl) $Ca_v1.2$. In the lower panel β-actin has been visualized by a specific antibody to

demonstrate that equal amounts of protein have been loaded in lanes 1-3. **(B)** Overexpression of full lengh $Ca_v1.2$. Western blot was done either on 7% SDS-PAGE (1:M.O.I 30 $Ca_v1.2$ full length and 2: control lane) or 8.5% SDS-PAGE (3: M.O.I 30 $Ca_v1.2$ full length and 4: control lane). **(C)** Overexpression of β_2 subunit. 1: control, 2: M.O.I 30 $\beta_2$wt.

5. Discussion

5.1 Functional roles of L-type calcium channels in murine embryonic hearts

In this study, I investigated for the first time the developmental expression levels of all cardiac calcium channel α_1 subunits in murine fetal hearts. In agreement with previous electrophysiological data (Protas et al. 2001), I observed an up-regulation of three subunits underlying L-type calcium currents ($Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$). Up-regulation of the $Ca_v1.2$ subunit was much more pronounced than that of the other two subunits. This finding emphasizes the particular importance of $Ca_v1.2$ for heart function, especially during late fetal stages and in the adult heart. In contrast, in early cardiac developmental stages when the expression levels of $Ca_v1.2$ are low, mechanisms that do not necessarily include the $Ca_v1.2$ channel may control heart contraction (Yasui et al. 2001; Viatchenko-Karpinski et al. 1999). Like $Ca_v1.2$, the $Ca_v1.1$ and $Ca_v1.3$ subunits are also significantly up-regulated between days E9.5 and E12.5. In contrast to $Ca_v1.2$, the expression levels of the two latter subunits increase only slightly after day E12.5. Taken together, these findings imply that $Ca_v1.1$ and $Ca_v1.3$ probably fulfil an unknown role in early heart development that may change at later stages of cardiac development. A function for $Ca_v1.1$ has not been reported, but for $Ca_v1.3$ it has been shown that this channel is important for the generation of spontaneous action potentials in sino-atrial node cells of the adult heart (Zhang et al. 2002; Mangoni et al. 2003).

The expression levels of the two cardiac T-type calcium channels differ profoundly from each other during heart development. $Ca_v3.2$ expression levels were very low. In isolated cardiomyocytes and in heart tissue this subunit could not be detected at day E15.5 suggesting that $Ca_v3.2$ does not play a substantial role in adult heart muscle. However, the low but clearly measurable expression of $Ca_v3.2$ at day E9.5 and E12.5 points to a possible role of this subunit in early heart development. The low expression levels of $Ca_v3.2$ may also reflect that this channel is only present in a subset of heart cells (e.g. cells of the conduction tissue) or in smooth muscle cells of heart vessels, and therefore was not detected in whole heart preparations. This notion is supported by a recent study reporting the deletion of $Ca_v3.2$ in mice (Chen et al. 2003). Consistent with our findings mice lacking this channel have no primary defect in myocardial function but reveal constitutively constricted coronary arterioles. As a consequence the heart of knockout mice is not sufficiently supplied with oxygen and develops focal myocardial fibrosis. In conclusion, $Ca_v3.2$ is essential for normal relaxation of

coronary arteries in the adult heart and may have an additional role during the early stages of heart formation *in utero*.

In agreement with two previous studies (Bohn et al. 2000; Cribbs et al. 2001), the $Ca_v3.1$ mRNA was consistently detected in the developing heart indicating that $Ca_v3.1$ is the major cardiac T-type calcium channel isoform. Like $Ca_v1.2$, the $Ca_v3.1$ subunit was up-regulated during early fetal development. However, in contrast to $Ca_v1.2$, the $Ca_v3.1$ expression level did not further increase after day 12.5 p.c. At day 15.5 p.c., a time point when the heart is fully developed the expression level of $Ca_v3.1$ was about one fourth of that of the $Ca_v1.2$ channel. This ratio is in good agreement with previous electrophysiological measurements that revealed a 1:4 ratio of the T- and L-type currents densities (see Figure 3). Surprisingly, a cardiac phenotype has not been reported for $Ca_v3.1$ knockout mice (Kim et al. 2001). At present, one can only speculate upon mechanisms explaining this finding. First, it is possible that the T-type current is not required for normal function of cardiomyocytes but is needed under specific (pathological) physiological circumstances (e.g. at hyperpolarized membrane potentials during hypoxia) that have not been uncovered so far. Alternatively, the loss of $Ca_v3.1$ may be functionally compensated for by up-regulation of one of the two other T-type calcium channel genes. Clearly, an in-depth functional examination of the heart function of $Ca_v3.1$ mice accompanied with the determination of expression levels of the calcium channel subunits will be required to clarify this important issue.

As pointed out, mice deficient for $Ca_v1.2$ die *in utero* before day E14.5. The results of this study indicate that the loss of this key subunit increases the mRNA levels of $Ca_v1.3$, $Ca_v1.1$ and $Ca_v3.1$. While the increase of the latter two subunits is only moderate, the mRNA and the protein concentration of the $Ca_v1.3$ subunit are profoundly up-regulated. RT-PCR analysis indicated that the increase in $Ca_v1.3$ expression is mainly due to the up-regulation of the $Ca_v1.3(1b)$ isoform of this channel. The electrophysiological and pharmacological properties of the $Ca_v1.3(1b)$ subunit heterologously expressed in HEK293 cells are in good agreement with that of the residual L-type calcium current found in $Ca_v1.2$ (-/-) cardiomyocytes. These results strongly suggest that Ca^{2+} influx through $Ca_v1.3(1b)$ is a major entry pathway in $Ca_v1.2$ (-/-) cardiomyocytes.

The functional relevance of $Ca_v1.3$ up-regulation is evident. Most likely, the up-regulation of $Ca_v1.3$ constitutes a compensatory mechanism of the cell to rescue the lost $Ca_v1.2$ function.

This hypothesis is supported by a report (Weisgerber et al. 2003) that inactivation of the cardiac β_2 subunit of voltage-dependent calcium channels results in low cardiac calcium current densities and early death at E10.5. It is likely, that the early death of the β_2 subunit (-/-) mice is caused by ER-retention of the $Ca_v1.2$ and the $Ca_v1.3$ subunit because it was shown that the β_2 subunit suppresses an ER-retention signal present in the α_1 subunits (Bichet et al. 2000). However, the compensatory increase in the $Ca_v1.3$ subunit is not sufficient to allow development of the embryo beyond day 14 p.c.. It remains an unsolved issue why the $Ca_v1.2$ channel cannot be functionally replaced by $Ca_v1.3$ in the adult heart. As shown in this report, in wild type heart the $Ca_v1.2$ mRNA concentration increases after day 12.5 p.c.. Thus, one may speculate that only this channel can provide the necessary calcium ions for contraction of the adult heart. There is an increasing evidence that ion channels can fulfil their various physiological roles in the cell only if they are integrated into specific networks of regulating cytosolic proteins (Pawson and Scott 1997). For example, specific anchoring proteins are required to attach ion channels to distinct subcellular compartments such as the triads of skeletal muscle (Flucher et al. 2000) or the synapse of neurons (Sheng and Wyszynski 1997). Contraction of cardiomyocytes is triggered by a Ca^{2+}-induced Ca^{2+} release mechanism (CICR; Ferrier and Howlett 2001). CICR is only possible if the L-type calcium channel of the sarcolemmal membrane is localized in close proximity to the ryanodine receptor of the sarcoplasmatic reticulum. The cellular proteins required for forming this structural complex are not known so far. However one may speculate that anchoring proteins that are specifically designed to bind $Ca_v1.2$ cannot efficiently interact with $Ca_v1.3$ or other calcium channels. In conclusion, in the absence of $Ca_v1.2$ the subcellular structures that are needed for efficient CICR may not be formed.

Another mechanism explaining the cardiac dysfunction of $Ca_v1.2$ deficient mice refers to the intrinsic current properties of this channel. While $Ca_v1.2$ and $Ca_v1.3$ share the principal hallmark features of L-type channels they do differ from each other in some electrophysiological details. For example, $Ca_v1.3$ activates at somewhat more negative membrane potentials and more rapidly than $Ca_v1.2$ (Koschak et al. 2001). It is possible that contraction of adult cardiomyocytes can be only accomplished with a calcium channel that has exactly the electrophysiological profile of $Ca_v1.2$. Given the above-mentioned functional differences between $Ca_v1.2$ and $Ca_v1.3$ one may ask what the physiological role of $Ca_v1.3$ in adult cardiomyocytes may be. $Ca_v1.3$ deficient mice display sinus arrhythmia (Platzer et la. 2000) indicating that this channel is involved in the generation of pacemaker potentials. It is

not known whether or not $Ca_v1.3$ mice have altered heart contractility. Side by side measurement of CICR in wild-type and $Ca_v1.3$ cardiomyocytes will be necessary to identify a possible role of this channel in cardiac contraction.

The results of this study add to the growing evidence that the different α_1 subunits of the high voltage-activated calcium channels can substitute for each other in certain but not in all functions. Our results indicate that there is a transcriptional cross-talk between the $Ca_v1.2$ and $Ca_v1.3$ calcium channel genes. A cross-talk between these two calcium channels has also been described in pancreatic ß cells of $Ca_v1.3$-deficient mice (Namkung et al. 2001). Furthermore, a compensatory increase in the expression of calcium channel $Ca_v1.2$ subunit mRNA in Purkinje cells of the cerebellum was described in tottering mice (Campbell et al. 1999). Tottering mice inherit a recessive mutation of the P/Q-type $Ca_v2.1$ subunit. In wild type mice, $Ca_v1.2$ mRNA is expressed at extremely low levels in Purkinje cells, but, in tottering mice, $Ca_v1.2$ mRNA is significantly increased in these cells. The results of these studies support the notion that a compensatory up-regulation of the calcium channel mRNA levels occurs only in specific cell types. Clearly, future studies will be necessary to verify the above hypothesis and to pinpoint the mechanism by which the mutual control of $Ca_v1.2$, $Ca_v1.3$ and other Ca_v genes is organized.

5.2 Adenovirus-mediated expression system as a feasible gene-transfer approach in cardiomyocytes

To study the physiological roles of calcium channels, development of genetically modified (knockout or transgenic) animal models is very useful inasmuch as functional consequences of calcium channel manipulations can be studied at the organ or whole animal model. On the other hand, to elucidate the molecular details of channel function and to identify signaling cascades regulating channel activity, cell lines transfected with expression vectors encoding the protein of interest are required. In the past extensive studies on calcium channel function have been undertaken in tumour-derived cell lines such as HEK293 or CHO cells. While these cells were very useful for performing site-directed mutagenesis-based structure-function analysis they turned out to be insufficient to pinpoint regulatory pathways of calcium channel regulation. In particular, the molecular pathway underlying β-adrenergic regulation of L-typ calcium current has remained elusive so far. To overcome this problem, cell lines that are more closely related to "normal" cardiomyocytes were proposed as model system. HL-1 cells,

an immortalized cardiac cell line has been shown to contain cAMP-regualted calcium and pacemaker currents (Sartiani et al. 2002). Thus, this cell probably is well suited as model system to study the molecular basis of cardiac-type calcium channel regulation. Unfortunately, gene transfer by traditional transfection methods [e.g. calcium phosphate] is rather insufficient in these cells. Thus, a more efficient gene delivery method is required as a basis for further studies in these cells.

In this study I have addressed this issue by developing recombinant adenoviral gene transfer. Adenoviral vectors provide most efficient and convenient method for delivery of DNA constructs to a variety of cells, including cardiomyocytes (Eizema et al. 2000; Martin et al. 1997). Because the recombinant virus is replication defective, its titer can be kept constant and adjusted to introduce a predetermined number of heterologous gene copies in cultured cells, thereby producing controlled expression of recombinant protein. In fact, we demonstrated with the GFP reporter gene in our experiments that increase in the multiplicity of infection produces a higher level of expression and it is possible to infect nearly all cardiac HL-1 cells in culture with M.O.I 30.

Infection of cardiac HL-1 cells with adenovirus-mediated antisense resulted in a dramatic decrease in endogenous $Ca_v1.x$ mRNA expression after 72 h, presumably by an increased degradation of the respective channel transcripts. Control vectors generating sense mRNAs had slight but statistically not significant effects on endogenous $Ca_v1.x$ mRNA indicating the specificity of the antisense effect. Down regulation of the transcripts was consistently followed by a decrease of the respective calcium channel proteins. Interestingly, the decrease of calcium channel protein was observed with a delay of about 24 h after the breakdown of the mRNA. This delay probably reflects relatively long half-life times of calcium channel subunits in the cells. Nevertheless, the antisense RNA-induced down regulation of calcium channel proteins clearly indicates that reductions of mRNA amounts cannot be compensated by the cell [e.g. by increase in efficiency of protein translation].

Interestingly, we also found out that the inhibitory efficiency of the antisense vectors is variable among three $Ca_v1.x$ antisense vectors. The reasons for this diversity are unclear. Probably, sequence-specific differences in mRNA secondary structures, mRNA-bound proteins or accessibility of hybridized mRNA to Rnase H are involved in this effect. Thus,

target sequences for antisense strategies must be carefully selected and optimized for any given mRNA.

Consistent with previous studies in cardiomyocytes (Eizema et al. 2000; Martin et al. 1997) the CMV promoter used in our vectors induced high rates of transcription in HL-1 cells. Moreover, the tandem CMV promoter vectors allowed to express simultaneously high levels of GFP and the gene of interest. Thus, transfected cells could be easily identified at their green fluorescence. Indeed, our biochemical and preliminary electrophysiologcal data confirm that the vast majority of green fluorescent cells contained the overexpressed calcium channel subunits.

In conclusion, the adenovirus vectors designed in this study proved to be very efficient as system for high-efficient overexpression of calcium channel subunits as well as for antisense inhibition of calcium expression. Undoubtedly, our adenoviral vectors will serve as powerful tool for future studies on the function and regulation of calcium channel subunits in cardiomyocytes and HL-1 cells.

6. Summary

Voltage-gated calcium (Ca^{2+}) channels play a crucial role in the control of heart contraction and are essential for normal heart development. In the past, most studies have focused on the subunit composition, function and regulation of these channels in the adult body. In contrast, there was only little information on the developmental regulation of the channels during embryogenesis. In the first part of this study this important issue was addressed. By semi-quantitative RT-PCR the expression levels of the six cardiac calcium channel α_1 subunits was determined at different time points during mouse heart development. Furthermore, the expression levels of these subunits were compared in side by side experiments with the expression levels of mouse embryonic hearts deficient for the $Ca_v1.2$ subunit. In adult cardiomyocytes the $Ca_v1.2$ L-type calcium channel is the predominant channel type and is essential for excitation-contraction coupling. Although the inactivation of the $Ca_v1.2$ gene caused embryonic lethality before embryonic day E14.5, hearts were contracting before E14 depending on a dihydropyridine-sensitive calcium influx. We analyzed the consequences of the deletion of the $Ca_v1.2$ channel on the expression level of other voltage-gated calcium channels in the embryonic mouse heart and in isolated cardiomyocytes. A strong compensatory up-regulation of the $Ca_v1.3$ calcium channel was observed on the mRNA as well as on the protein level. RT-PCR indicated that the recently identified new $Ca_v1.3$ (1b) isoform was strongly up-regulated whereas a more moderate increase was found for the $Ca_v1.3$ (1a) variant. Heterologous expression of $Ca_v1.3$ (1b) in HEK293 cells induced Ba^{2+} currents with properties similar to those found in $Ca_v1.2$-/- cardiomyocytes suggesting that this isoform constitutes a major component of the residual L-type calcium current in $Ca_v1.2$-/- cardiomyocytes. In summary, these results imply that calcium channel expression is dynamically regulated during heart development and that the $Ca_v1.3$ channel may substitute for $Ca_v1.2$ during early embryogenesis.

In addition to the developmental investigations an adenoviral expression system was established to overexpress or deplete calcium channel subunits in the cardiac HL-1 cell line. Such a system is urgently required since non-viral transfection works inefficient in the above mentioned cardiac cell lines. The first set of vectors induces the transcription of antisense mRNA to deplete calcium channel subunits. Transcription is driven by the high-efficiency CMV promoter. The cDNA of GFP was fused to the antisense mRNA to detect transfected cells by their green fluorescence. The effectiveness and specificity of the vectors was

demonstrated for the $Ca_v1.1$ and $Ca_v1.3$ subunit by RT-PCR and Western blot analysis. The second set of vectors allows CMV promoter-driven overexpression of calcium channel subunits. The vectors contain a second transcription unit (tandem vectors) to express GFP as a marker protein. The system was successfully tested for full length and truncated $Ca_v1.2$ as well as for the wild type β_2 subunit. Taken together, the developed vectors will provide a valuable tool for further investigations into the physiological regulation of calcium channels.

7. Appendix

Electrophysiological experiments performed by Dr. Andrea Welling are described in this section.

7.1 Method for electrophysiological analyses

Electrophysiological experiments were done as described earlier (Seisenberger et al. 2000; Klugbauer et al. 2002b). HEK293 cells were transiently transfected with the expression vector for the $Ca_v1.3$ (1a) or (1b) subunit (0.2 µg per well) and the expression vectors for the β3 and the $\alpha_2\delta$-1 subunits (0.15 µg each per well) using lipofectamine according to the manufacturer (Invitrogen). The holding potential (HP) was –80 mV. Current-voltage relations (I-V) were recorded from –60 to +50 mV with 10 mV increments and a frequency of 0.2 Hz. Cumulative dose-response curves were measured using 2-3 different nisoldipine concentrations per cell. Trains of test pulses were to 0 or +10 mV for 40 ms with 0.1 Hz for I_{Ba} of native cells or to -10 or 0 mV for 100 ms with 0.2 Hz for I_{Ba} of expressed channels. IC_{50} values were calculated by fitting the averaged dose-response curves to the Hill equation:

$$I/Imax = 1/(1 + ([nisoldipine]/IC_{50})^H),$$

where [nisoldipine] is the concentration of nisoldipine, IC_{50} is the half-blocking concentration, H is the Hill coefficient, I is the current measured at any concentration of nisoldipine, and Imax is the current measured in the absence of drug. The data for the I_{Ba} of wild type cardiomyocytes were fitted with a two component Hill equation.

The intracellular solution contained (in mM): 60 CsCl, 50 aspartic acid, 68 CsOH, 1 $MgCl_2$, 5 K-ATP, 1 $CaCl_2$, 10 HEPES, 11 EGTA with pH = 7.4. Seals were formed in Barium-tyrode of the following composition (in mM): 130 NaCl, 4.8 KCl, 5 $BaCl_2$, 1 $MgCl_2$, 5 glucose, 5 HEPES; pH 7.4. The bath solution contained (in mM): 130 N-methyl-D-glucamine (NMDG), 4.8 CsCl, 5 $BaCl_2$, 5 glucose, 5 HEPES at pH = 7.4 (NMDG-tyrode). Nisoldipine stock solution was 10 mM in ethanol. On each experimental day, nisoldipine was diluted from the stock solution into the NMDG-tyrode to the indicated concentrations.

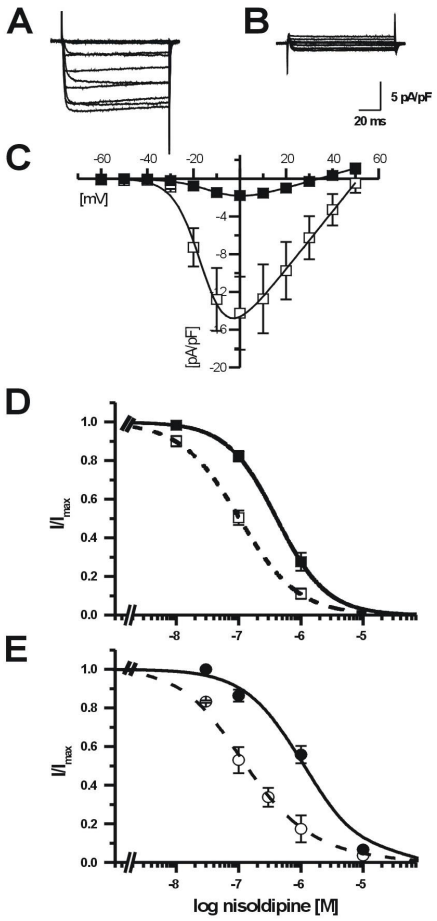
7.2 Electrophysiological and pharmacological properties of the $Ca_v1.3$ splice variants

It was tempting to speculate that the previously described (Seisenberger et al. 2000) L-type like dihydropyridine-sensitive calcium current of $Ca_v1.2$ (-/-) cardiomyoctes could be caused by the upregulated $Ca_v1.3$ channel. To test this hypothesis, $Ca_v1.3$ (1a) and $Ca_V1.3$ (1b) were expressed in HEK 293 cells and compared the expressed currents with currents from $Ca_v1.2$ (-/-) cardiomyocytes. The expression of both the β2 and β3 subunits has been reported in cardiac muscle (Hullin et al. 2003). In the initial experiments $Ca_v1.3$ (1b) was coexpressed with the β2a or β3 subunit, but in the presence of the β2a subunit only current densities below 1.0 pA/pF were obtained. Therefore we investigated $Ca_v1.3$ subunit in further experiments in the presence of the β3 subunit. The coexpression of β3 with $Ca_v1.3$ will also facilitate comparability of our results with those of others (Koschak et al. 2001).

Both isoforms induced L-type barium currents with slow inactivation kinetics in 55% ($Ca_v1.3$(1a)) and 16% ($Ca_v1.3$(1b)) of transfected HEK 293 cells (Appendix FigureA, B). Some of the untransfected HEK 293 cells ("control cells") showed a small rapid inactivating inward current with a current density at 0 mV of 0.64 ± 0.08 pA/pF (n=3). A similar current could be detected in pcDNA3 vector transfected cells. The current was not sensitive to the dyhydropyridine nisoldipine and disappeared at an HP of –40 mV, suggesting that this endogenous current was not an L-type current. To safely separate cells current, only cells showing a slowly inactivating current and a current density above 1.0 pA/pF were considered for analysis. With 5 mM Ba^{2+} as charge carrier, the current density of $Ca_v1.3$(1a)- and $Ca_v1.3$(1b)-transfected cells was 11.8± 2.0 pA/pF (n=30) and 1.9± 0.24 pA/pF (n=25), respectively. The *I-V* curves indicated that the current through the $Ca_v1.3$ splice forms activated negative from –40 mV and were maximal between –10 mV and 0 mV (Appendix Figure C). This result is in good agreement with the L-type current described in $Ca_v1.2$(-/-) cardiomyocytes (Seisenberger et al. 2000).

I_{Ba} of wild type cardiomyocytes was blocked by nisoldipine with IC_{50} values of 0.1 and 3.9 µM. In contrast, I_{Ba} currents of $Ca_v1.2$ knock-out cells were blocked with an IC_{50} of 1.1 µM (Figure 8E). The IC_{50} values for the I_{Ba} nisoldipine block induced by the heterologously expressed $Ca_v1.3$ splice variants differed from each other, being 0.1 µM ($Ca_v1.3$(1a)) and 0.41 µM ($Ca_v1.3$(1b)) (Appendix Figure D). The latter value is close to that found in $Ca_v1.2$(-/-)

cardiomyocytes, suggesting that $Ca_v1.3(1b)$ channels could be responsible for the L-type like Ca^{2+} current in $Ca_v1.2$ knockout cells.



Appendix Figure. $Ca_V1.3$ splice variants express functional calcium channels in HEK 293 cells with electrophysiological and pharmacological characteristics similar to I_{Ba} of embryonic $Ca_V1.2(-/-)$ cardiomyocytes. A, B, C and D show results obtained with $Ca_V1.3(1a)$ and $Ca_V1.3(1b)$ channels transiently expressed in HEK 293 cells together with β_3 and $\alpha_2\delta$-1 subunits. (A, B) Representative current traces for $Ca_V1.3(1a)$ (A) and $Ca_V1.3(1b)$ (B) to potentials from –60 to +60 mV (10-mV increments) from an HP of –80 mV. (C) Averaged current voltage (I-V) realtions (mean ± S.E.; □, $Ca_V1.3(1a)$, n = 13; ■, $Ca_V1.3(1b)$, n = 18). The HP was –80 mV. (D, E) Nisoldipin block of I_{Ba} from expressed (D) and native (E) L-type calcium channels. Native channels were measured in primary embryonic cardiomyocytes cultured at day 12.5 p.c. Trains of test pulses were from –80 mV to the maximum current at 0.1 or 0.2 Hz. Data points are the mean ± S.E.M. The data for $Ca_V1.2+/+$ cardiomyocytes were fitted with a two-exponential Hill-equation, whereas data for $Ca_V1.2-/-$ cardiomyocytes, $Ca_V1.3(1a)$ and $Ca_V1.3(1b)$ were fitted with a simple Hill-equation. IC_{50} values are 0.1 µM for $Ca_V1.3(1a)$ (□, n = 12); 0.41 µM for $Ca_V1.3(1b)$ (■, n = 8); 1.1 µM for $Ca_V1.2-/-$ (●, n = 3-8 per point); 0.1 and 3.9 µM for $Ca_V1.2+/+$ (○, n = 3-6 per point).

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8.1 Own publications and abstract for this study

1. **Man Xu**, Andrea Welling, Susanne Paparisto, Franz Hofmann, Norbert Klugbauer. Enhanced expression of L-type Cav1.3 calcium channels in murine embryonic hearts from Cav1.2 deficient mice. *J. Biol. Chem*. 2003 Oct; 278(42):40837-41

2. **Man Xu**, Andrea Welling, Susanne Paparisto, Franz Hofmann, Norbert Klugbauer. Enhanced expression of L-type Ca1.3 calcium channels does not functionally compensate the deletion of $Ca_v1.2$ in murine embryonic hearts. (abstract). Joint Cold Spring Harbor Laboratory / Wellcome Trust meeting "Pharmacogenomics", Cambridge UK, 2003 September 24-28.

3. **Man Xu**, Andrea Welling, Carmen Baumann, Franz Hofmann, Norbert Klugbauer. Use of adenovirus-mediated gene transfer to study the regulation of calcium channel subunits by phosphorylation (in preparation)

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1. **Man Xu**, Andrea Welling, Susanne Paparisto, Franz Hofmann, Norbert Klugbauer. Enhanced expression of L-type Cav1.3 calcium channels in murine embryonic hearts from $Ca_v1.2$ deficient mice. *J. Biol. Chem*. 2003 Oct; 278(42):40837-41

2. **Man Xu**, Andrea Welling, Susanne Paparisto, Franz Hofmann, Norbert Klugbauer. Enhanced expression of L-type Ca1.3 calcium channels does not functionally compensate the deletion of $Ca_v1.2$ in murine embryonic hearts. (abstract). Joint Cold Spring Harbor Laboratory / Wellcome Trust meeting "Pharmacogenomics", Cambridge UK, 2003 September 24-28.

3. **Man Xu**, Yi-ping Wang, Wei-bo Luo, Ya-Ming Xu, Li-Jiang Xuan. Salvialonate suppress the proliferation of mouse mesangial cells by inhibition of endothelin release. *Acta Pharmacol. Sin*. 2001 July; 22(7): 634-638

4. **Man Xu**, Yi-ping Wang, Wei-Kang Sun, Ya-Ming Xu, Li-Jiang Xuan

Effect of salvianolate on renal function and endogenous endothelin release in chronic renal failure rats, *Chinese Journal of Pharmacology and Toxicology,* 2001 Feb; 15(1): 32-35

5. **Man Xu**, Andrea Welling, Carmen Baumann, Franz Hofmann, Norbert Klugbauer. Use of adenovirus-mediated gene transfer to study the regulation of calcium channel subunit by phosphorylation (in preparation)

Conferences attended

- Joint Cold Spring Harbor Laboratory / Wellcome Trust meeting "Pharmacogenomics". September 24-28, 2003, Welcome Trust Genome Campus, Cambridge, UK. (Poster)

- 8th Chinese National Congress of Pharmacology, November 24-27, 2002, Shanghai, China. (15 min oral presentation)

- XIVth World Congress of Pharmacology, July 7-12, 2002, San Francisco, CA (Poster)

- 4th VW-Symposium on conditional mutagenesis, April 14-16, 2002, Herrsching am Ammersee, Germany

Prizes

- DAAD Prise für hervorragende Leistungen ausländischer studierender (Annual Prize for the Best Performances of Foreign Students) (2002)

- Award for Chinese Young Pharmacologists Symposium (2002)

- Award for Excellent Graduate Student, Chinese Academy of Sciences (2000)

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