Calcium signaling *via* voltage-dependent L-type Ca^{2+} channels

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In excitable cells such as muscles and neurons, the voltage-dependent L-type Ca^{2+} channel is one of the most important pathways for Ca^{2+} influx from extracellular space in response to the cell membrane depolarization. Ca^{2+} ion through this channel works as a Ca^{2+} signal that regulates fast (on time scale of milliseconds; e.g. muscle contraction) and long-term (on a time scale of days or weeks; e.g. gene expression) cellular responses. Many important signal molecules are activated *via* Ca^{2+} signal, such as Ca^{2+}-dependent protease (calpain), calcium/calmodulin dependent kinases (CaMKs), phosphatase (calcineurin), and mitogen-activated protein kinases (MAPKs). They are activated by temporally and spatially restricted Ca^{2+} signals, thus strictly regulated in native tissues. Many kinds of Ca^{2+} channels are involved in the rise of the intracellular Ca^{2+} concentration. They are not mere routes of Ca^{2+} influx but also machineries that decide which specific signal cascade should be activated. Recently, the molecular mechanisms by which Ca^{2+} influx through L-type Ca^{2+} channels can activate specific signal cascades have been clarified. In this review, we describe the role of Ca^{2+} signaling *via* L-type Ca^{2+} channels particularly in cardiac myocytes and neurons with emphasis on the mechanisms underlying the activation of the specific Ca^{2+} signals by the L-type Ca^{2+} channel machinery.

Keywords: Ca^{2+} signaling / L-type Ca^{2+} channel.

Review Article

Introduction

Ca^{2+} is one of the most strictly regulated ions in the cell, because it is involved in many important signal cascades by activating various Ca^{2+}-binding proteins, such as kinases, phosphatases, and proteases [1]. Ca^{2+} signals induce various physiological responses such as muscle contraction, vesicle secretion, cell differentiation, proliferation, and cell death. Whereas some of the responses to the Ca^{2+} signal are cell-type specific, multiple Ca^{2+} signals are used for different responses in the same cell. In cardiac myocytes, for example, Ca^{2+} triggers muscle contraction in response to every action potential, whereas in pathophysiological circumstances (e.g. cardiac hypertrophy), the Ca^{2+} signal induces phenotypic remodeling of the myocytes *via* gene expression by activating transcription factors. Moreover, the prolonged Ca^{2+} signal leads to cell death. It is thus important to clarify how different Ca^{2+} signal systems can be set up to control different cellular functions for the better understanding of the Ca^{2+} signal transduction in native tissues.

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It is known that some Ca^{2+}-dependent signaling proteins are specifically regulated by the strength and the duration of the Ca^{2+} signal [2]. In addition, it has become apparent that the origin and spatial differences of the Ca^{2+} signal are also important for the distinction of cellular responses. Ca^{2+} used for cell signaling is derived either from the extracellular fluid or internal stores. In the former case, many kinds of plasma membrane channels open in response to stimuli including membrane depolarization, extracellular and intracellular ligands, and mechanical stretch. In the latter case, ryanodine receptor (RyR) and inositol-1, 4, 5-triphosphate ($InsP_3$) receptor are responsible for the release of Ca^{2+} from the internal Ca^{2+} store such as the endoplasmic reticulum (ER) or its muscle equivalent, the sarcoplasmic reticulum (SR). The difference in the spatial arrangement of these Ca^{2+} channels and their immediate downstream signaling molecules appear to be responsible for the specificity of the Ca^{2+} signaling. In this review, we focus on the voltage-dependent L-type Ca^{2+} channel and summarize the role of Ca^{2+} signals through this channel in native tissues. The potential mechanisms by which the macro-molecular complex of L-type Ca^{2+} channel evoke and transduce specific Ca^{2+} signals are also discussed.

Voltage-dependent L-type Ca^{2+} channel

Voltage-dependent Ca^{2+} channels open in response to membrane depolarization and control the selective entry of

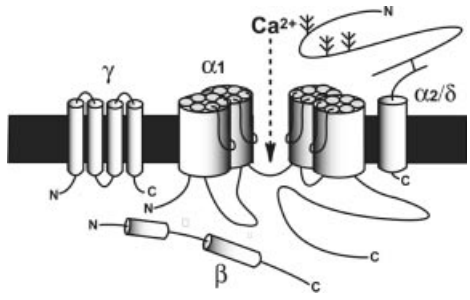

Fig. 1. Schematic structure of the voltage-dependent Ca^{2+} channels. The voltage-dependent Ca^{2+} channels are composed of the pore forming α_1 subunit and the auxiliary β, α_2/δ and γ subunits. The β subunit is entirely cytosolic, whereas α_2/δ and γ subunits have transmembrane regions. Only skeletal muscle L-type Ca^{2+} channels contain γ subunit, while other L-type Ca^{2+} channels consist of α_1, β, and α_2/δ subunits. All three auxiliary subunits interact directly with α_1 subunit independently to each other.

Ca^{2+} ions. The individual channel is composed of multiple subunits (α_1, β, α_2/δ, γ; (Fig. 1). The α_1 subunit is known as the main pore-forming subunit and determines the major functional properties of the channel. Ten genes encoding Ca^{2+} channel α_1 subunits have been cloned and classified systematically according to their characters (Tab. 1) [3]. Among them, Ca_V1 family members ($Ca_V1.1$~$Ca_V1.4$) form L-type Ca^{2+} channels. The other subunits interact with α_1 subunits in various combinations and modulate the channel function, thus are called auxiliary subunits [4].

The L-type Ca^{2+} channel, compared to other voltage-dependent Ca^{2+} channels, has large Ca^{2+} conductance and long opening duration when it is activated by depolarization, thus, called L-type, and is responsible for the entry of large amount of Ca^{2+} into the cytoplasm. This channel was initially purified and cloned as a 1,4-dihydropyridine (DHP) binding protein. In addition to DHPs, small molecular compounds called phenylalkylamines (e.g. verapamil) and benzothiazepines (e.g. diltiazem) can selectively block the L-type Ca^{2+} channel (Tab. 1) and a DHP analogue: Bay k 8644 specifically activates this channel. These pharmacological characteristics distinguish the L-type from other Ca^{2+} channels. These compounds thus afford a great advantage to the study of Ca^{2+} signaling *via* L-type Ca^{2+} channels.

L-type Ca^{2+} channels are expressed in skeletal muscle, cardiac muscle, smooth muscle, neurons, pancreatic β cell, osteoblasts, retina, and lymphocytes (Tab. 1). Studies with the use of L-type specific agonists and blockers have clarified that this channel is involved in various important cellular responses such as muscle contraction, secretion of insulin, gene expression, cell death and survival. The function of L-type Ca^{2+} channels is precisely controlled by protein kinase A (PKA), protein kinase C (PKC), tyrosine kinases, calmodulin kinase II (CaMKII), and G proteins, resulting in the dramatic change of the Ca^{2+} signaling [5]. For example, in cardiac myocytes, the L-type Ca^{2+} channel is phosphorylated and thereby activated by PKA through β-adrenergic receptor pathway, and the influx of Ca^{2+} *via* this channel is increased by 2-5 fold. This enhanced Ca^{2+} signaling results in the inotropic and chronotropic changes of cardiac function. In con-

Tab. 1. Classification of α_1 subunit of voltage dependent calcium channels.

Native channel	Alphabetical classification	Numerical classification	Gene name	Specific blockers	Expressing tissues
L-type	α_{1S}	Cav1.1	CACNA1S	Dihydropyridines Phenylalkylamines Benzothiazepines Calcisseptine	skeletal muscle
	α_{1C}	Cav1.2	CACNA1C	Dihydropyridines Phenylalkylamines Benzothiazepines Calcisseptine	heart, smooth muscle, adrenal, brain, nervous system, pancreas, osteoblast
	α_{1D}	Cav1.3	CACNA1D	Dihydropyridines Phenylalkylamines Benzothiazepines Calcisseptine	heart, brain, nervous system, pancreas, kidney, cochlea
	α_{1F}	Cav1.4	CACNA1F	Dihydropyridines Phenylalkylamines Benzothiazepines Calcisseptine	retina, thymus, spleen, bone marrow
P/Q-type	α_{1A}	Cav2.1	CACNA1A	ω-agatoxin IV A	brain, nervous system
N-type	α_{1B}	Cav2.2	CACNA1B	ω-conotoxin G VI A	brain, nervous system
R-type	α_{1E}	Cav2.3	CACNA1E	SNX-482	brain, nervous system
T-type	α_{1G}	Cav3.1	CACNA1G	Kurtoxin	brain, nervous system
	α_{1H}	Cav3.2	CACNA1H	Kurtoxin	heart, brain, kidney, liver, smooth muscle
	α_{1I}	Cav3.3	CACNA1I	No specific inhibitor	brain

trast, the regulatory role of PKC has been controversial in the heart, since the stimulation of PKC resulted in both an upregulation and an inhibition of L-type Ca^{2+} channels [5].

So far, many studies have elucidated the functional importance and the regulatory mechanisms of L-type Ca^{2+} channels. However, it has been still an unresolved mystery how Ca^{2+} signals recruited by this channel can play unique roles, albeit the presence of many other Ca^{2+} channels in the same tissues. Recently, certain adaptor molecules and macromolecular complexes associated with L-type Ca^{2+} channels are found to be responsible for the strict and specific regulation of Ca^{2+} signal *via* this channel (see below) [6, 7]. These exciting findings have opened up the new direction toward better understanding of the Ca^{2+} signaling through L-type Ca^{2+} channels.

Ca^{2+}-dependent inactivation of L-type Ca^{2+} channel (Regulation by tethering calmodulin)

Voltage-dependent L-type Ca^{2+} channels open in response to the membrane depolarization, then start to inactivate even during the depolarization (Fig. 2A). Ca^{2+} channels are regulated by two different inactivation mechanisms that are called the voltage-dependent inactivation [8] and the Ca^{2+}-dependent inactivation [6]. In response to the prolonged membrane depolarization, the Ca^{2+} channel shifts to a nonconducting inactivated state, which is called voltage-dependent inactivation. The degree of the voltage-dependent inactivation correlates with the membrane potential. The higher the membrane potential is, the more strongly Ca^{2+} channels are inactivated. On the other hand, the Ca^{2+}-dependent inactivation is induced by the elevation of intracellular Ca^{2+}. Other cations permeable thorough the Ca^{2+} channel, Ba^{2+} and Na^{+}, are not able to induce the Ca^{2+}-dependent inactivation (Fig. 2A), indicating that this inactivation mechanism operates only when Ca^{2+} enters through the Ca^{2+} channels. The Ca^{2+}-dependent inactivation is faster than the voltage-dependent inactivation, thus is a powerful negative feedback mechanism to strictly regulate the amount of Ca^{2+} influx *via* the channel. This phenomenon has been shown to be mediated by calmodulin (CaM) that is constitutively bound to L-type Ca^{2+} channels [9].

CaM is a ubiquitous Ca^{2+}-binding protein, which changes its conformation on binding to Ca^{2+}, thus used as a Ca^{2+} sensor in various Ca^{2+} signaling cascades. To induce Ca^{2+}-dependent inactivation, CaM needs to be tethered to Ca^{2+} channel at the basal level of $[Ca^{2+}]_i$ and to work as a Ca^{2+} sensor when the $[Ca^{2+}]_i$ is increased [9]. CaM binds to the carboxyl-terminal of the L-type Ca^{2+} channel α_1 subunits even in the conformational state without Ca^{2+} (apo-CaM). Once Ca^{2+} enters through the Ca^{2+} channel, the Ca^{2+}-bound form of CaM (Ca^{2+}/CaM) interacts with another additional but adjacent site called isoleucine-glutamine (IQ) motif of the Ca^{2+} channel to inactivate the channel (Fig. 2B) [10]. The CaM tethered to the L-type Ca^{2+} channel is necessary to achieve the fast negative feedback regulation, as Ca^{2+} entry occurs in the time scale of milliseconds. When CaM mutants that can not bind to Ca^{2+} are overexpressed in cardiac myocytes, they competitively extrude the endogenous CaM from the Ca^{2+} channels and not only eliminate the Ca^{2+}-dependent inactivation but also prolong the action potential [11]. This indicates that the precise recruitment of CaM to the Ca^{2+} channels is necessary for the Ca^{2+}-dependent inactivation. It also suggests that the Ca^{2+}-dependent inactivation by CaM is necessary for the accurate Ca^{2+} signaling in native tissues, although it must be clarified that the mutant CaMs affect only L-type Ca^{2+} channels.

Interestingly, some of non-L-type Ca^{2+} channels (P/Q-, N- and R-type (Tab. 1)) have a similar Ca^{2+}-dependent inacti-

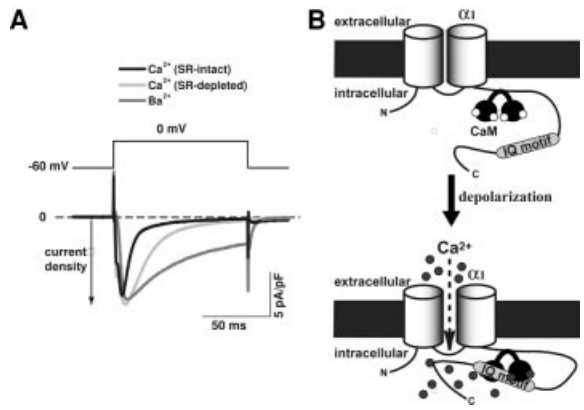

Fig. 2. The mechanism of Ca^{2+}-dependent inactivation. **A.** Ca^{2+} and Ba^{2+} currents through L-type Ca^{2+} channels are recorded by whole cell patch-clamp technique in rat cardiac myocytes. Ca^{2+} channel currents were elicited by a square pulse depolarization from −60 mV to 0 mV. When Ca^{2+} is used as a charge carrier (black line), Ca^{2+} influx decayed more rapidly during the depolarization compared with Ba^{2+} influx (dark gray line), which is defined as the Ca^{2+}-dependent inactivation of the Ca^{2+} channel. Ba^{2+} current shows moderate inactivation that involves the voltage-dependent inactivation. When the intracellular Ca^{2+} store (SR) was depleted by the application of thapsigargin, the Ca^{2+}-dependent inactivation was blunted (light gray line), indicating the participation of Ca^{2+} released from the SR in the Ca^{2+} channel inactivation. **B.** A schematic model of molecular mechanism of Ca^{2+} dependent inactivation is illustrated. In the resting state (upper panel), calmodulin (CaM) without Ca^{2+} is tethered on the carboxyl-terminal of Ca^{2+} channel. Upon depolarization, the channel opens and Ca^{2+} (shown as gray circles) enters through the Ca^{2+} channel. Then, Ca^{2+} is accumulated around the inner mouth of the Ca^{2+} channel and binds to CaM causing the conformational change of CaM (lower panel). The Ca^{2+} bound form of CaM becomes able to interact with IQ motif and induces the inactivation of the Ca^{2+} channel.

vation mechanism induced by CaM tethered to their carboxyl-terminal. However, the spatial range of sensing Ca^{2+} in their system appears to be different from that of the L-type Ca^{2+} channel. In a study in which the intracellular Ca^{2+} buffering was arbitrarily changed by use of Ca^{2+} chelaters, it has been shown that L-type Ca^{2+} channels are inactivated in response to the local Ca^{2+} rise near the channel, which is in contrast to non-L-type Ca^{2+} channels that respond to the global Ca^{2+} rise [12]. It is to say that the Ca^{2+} signal through L-type Ca^{2+} channels is restricted within the privileged space as compared to that through other types of Ca^{2+} channels because of the higher spatial sensitivity to Ca^{2+} in the Ca^{2+}-dependent inactivation mechanism. The Ca^{2+}-depndent inactivation of Ca^{2+} channels is an important mechanism not only for the negative feedback to prevent Ca^{2+} overload but also for the achievement of strictly regulated spatial Ca^{2+} signaling.

It is still unknown how the interaction between CaM and IQ motif induces the conformational change to inactivate the channels. This interaction has been shown to be involved in another important role of the L-type Ca^{2+} channel, that is, the L-type specific Ca^{2+} signals (see Chapter on 'The specific role of the L-type Ca^{2+} channel in CREB activation through ERK pathway'). Thus, the tethered CaM is a pivotal component of the molecular complex associated with the L-type Ca^{2+} channels to understand the molecular feature and the signaling mechanisms of L-type Ca^{2+} channels.

Ca^{2+} signal regulation in cardiac myocyte

Since cardiac myocytes possess a highly organized system to strictly regulate the Ca^{2+} signal *via* L-type Ca^{2+} channels, it has been a good experimental model to understand how the Ca^{2+} signal is regulated in native tissues. Adult cardiac myocytes have a plasma membrane architecture called T-tubular membrane, in which the plasma membrane and sarcoplasmic reticulum (SR) are extremely proximate in the order of nanometers (Fig. 3). When cardiac action potential depolarizes the T-tubular membrane to open the L-type Ca^{2+} channel, the Ca^{2+} entered through this channel diffuses across the junctional space between the plasma membrane and the SR membrane, and triggers the opening of adjacent ryanodine receptors (RyRs) that exist in the SR membrane, resulting in the additional Ca^{2+} release from the internal Ca^{2+} store. This system is called calcium-induced calcium release (CICR), the mechanism to amplify the Ca^{2+} signal from L-type Ca^{2+} channels. Ca^{2+} evolved by the CICR opens neighboring RyRs, and thus the amplified Ca^{2+} signal propagates from the junctional space to the contractile elements to induce muscle contraction by activating sarcomeres that are situated in the cytoplasm. The accumulated Ca^{2+} is then removed from the cytoplasm to the extracellular space or the intracellular store by the Na^+/Ca^{2+} exchanger (NCX) and the Ca^{2+}-pumps (sarco(endo)plasmic reticulum Ca^{2+}-ATPase; SERCA), respectively. By returning the Ca^{2+} concentration to the basal level, cardiac myocytes become ready to respond to the next action potential (Fig. 3). As such, cardiac myocytes use oscillatory Ca^{2+} cycling for the physiological contraction that is initiated by the L-type Ca^{2+} channel, and the cycle is repeated at ~1 Hz in human and ~9 Hz in mouse [1].

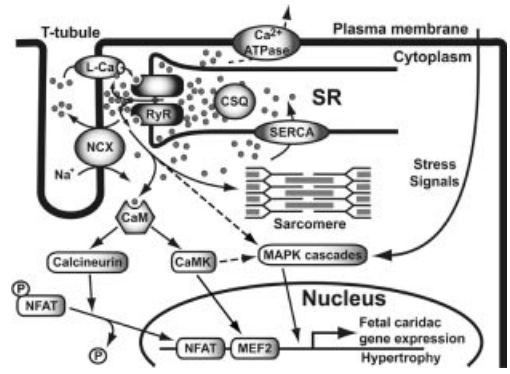

Fig. 3. Calcium signal regulation and transduction in cardiac myocyte. Ca^{2+} signaling in ventricular myocyte is initiated at the T-tubular membrane with the opening of L-type Ca^{2+} channels (L-Ca) in response to action potential (Ca^{2+} is shown as gray filled circles). Ca^{2+} that entered through this channel next binds to the adjacent ryanodine receptor (RyR) and activates the channel to release Ca^{2+} from the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), resulting in the amplification and propagation of Ca^{2+} signaling. Ca^{2+} is accumulated in the SR by Ca^{2+} binding proteins such as calsequestrin (CSQ). Amplified Ca^{2+} signals are used in part to induce Ca^{2+}-dependent inactivation of L-Ca for the negative feedback, whereas they are propagated to reach the sarcomeres to cause the muscle contraction. Cytosolic Ca^{2+} concentration recovers to the basal level as Ca^{2+} is removed to the extracellular space *via* the Na^+/Ca^{2+} exchanger (NCX) and returns to the SR *via* sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA). It is known that the sustained elevation of the intracellular Ca^{2+} level is accompanied with the development of cardiac hypertrophy, that may be because of the collapse of the Ca^{2+} regulatory system induced by physical stress or humoral factors. The morbidly enhanced Ca^{2+} signal functions through Ca^{2+}/CaM-dependent molecules. Calcineurin is activated by Ca^{2+}/CaM, then dephosphorylates the transcriptional factor NFAT, enabling it to enter nucleus to induce the transcription of fetal genes. Calmodulin kinase (CaMK) is also activated by Ca^{2+}/CaM, which induces fetal gene transcription by activating the transcription factor, myocyte enhancer factor 2 (MEF 2). MAPK cascades contribute to cardiac hypertrophy by inducing the fetal gene expression. They are activated by stress signals and presumably by Ca^{2+} signals.

To repeat the accurate Ca^{2+} signal cycling, it is necessary to balance the influx and the efflux of Ca^{2+} in each cycle. In

cardiac myocytes, almost all of the Ca^{2+} influx during the action potential is mediated by L-type Ca^{2+} channels (consisted of $Ca_v1.2$), thus the regulation of L-type Ca^{2+} channel is important to control the Ca^{2+} influx. As described above, Ca^{2+} influx through the L-type Ca^{2+} channel induces a strong Ca^{2+}-dependent inactivation of the channel to prevent excess Ca^{2+} signals. In case of cardiac myocytes, large amount of Ca^{2+} released from the adjacent RyRs accelerates the Ca^{2+}-dependent inactivation of the Ca^{2+} channel (Fig. 3). In fact, when the Ca^{2+} release from RyRs is abolished by the administration of caffeine or thapsigargin that deplete Ca^{2+} in the SR, the inactivation of L-type Ca^{2+} channels is retarded, resulting in the augmentation of Ca^{2+} influx (Fig. 2A) [13]. This means that the lower the Ca^{2+} level of the internal store is, the more Ca^{2+} enters *via* L-type Ca^{2+} channels to recruit Ca^{2+} to the store and vice versa [14]. As such, L-type Ca^{2+} channels, by cross-communicating with proximal RyRs through CICR and the Ca^{2+}-dependent inactivation, function as a sensor of the SR Ca^{2+} content and thereby regulate the whole cell Ca^{2+} level at the optimum range in cardiac myocytes.

L-type Ca^{2+} channels and RyRs are strictly concentrated in the T-tubular membrane region to amplify or restrict the Ca^{2+} signals through their cross-communication. The localization is essential for the physiological contraction of cardiac myocytes; however, the mechanism is still unknown that mediates the precise geometrical arrangements of these Ca^{2+} channels. Under pathological conditions such as heart failure, the loss of T-tubular structure and the impairment of the CICR system have been reported, which have been linked to cardiac arrhythmia and remodeling. Therefore, this is an interesting issue to be elucidated in future to understand how the spatial and temporal regulation of Ca^{2+} signaling is achieved in the cardiac myocytes.

Pathophysiological Ca^{2+} signaling in cardiac myocytes (Cardiac hypertrophy and heart failure)

Physiological oscillatory Ca^{2+} signaling is strictly regulated in cardiac myocytes, as described above. It is well known that abnormal remodeling of the precise regulation of Ca^{2+} signals by artificial manipulation or stresses results in the important disease states such as cardiac hypertrophy and heart failure [15–17]. Thus, Ca^{2+} signaling in cardiac myocytes has been paid much attention from the pathophysiological point of view. When exposed to stress like ischemia or pressure overload, cardiac myocytes adapt themselves to the stresses by increasing in size and by altering the phenotype through the expression of fetal genes, resulting in cardiac hypertrophy [18]. This initial phenotypic change has at first some benefit because the change enhances the cardiac contractility and thus compensates the altered cardiac function. However, if the stresses persist, this compensatory hypertrophic state shifts to the next stage called heart failure in which cardiac myocyte can not perform sufficient contraction. The phenotypic remodeling is controlled by fetal gene expression, in which Ca^{2+} signal plays an important role.

Several transgenic mice lines in which the overexpression of genes encoding components of Ca^{2+} signal pathways, such as $Ca_V1.2$ (α_1 subunit of L-type Ca^{2+} channel) [19], calsequestrin (CSQ) [20], calmodulin kinase IV (CaMKIV) [21], and calcineurin [22] show the hypertrophic phenotype, indicating the importance of the Ca^{2+} signal pathways in the development of cardiac hypertrophy. Some humoral factors, including angiotensin II (Ang II), endothelin-1 (ET-1), and catecholamines are increased under pathophysiological circumstances. These factors and the catecholamine analogs, phenylephrine (PE) and isoproterenol (Iso), share the ability to elevate $[Ca^{2+}]_i$. Administration of these factors to cardiac myocytes also induces hypertrophic responses, although Ca^{2+} signaling is not the sole route leading to cardiac hypertrophy by humoral factors as they activate multiple signaling pathways in cardiac myocytes [18]. It has been shown that the intracellular administration of BAPTA, a Ca^{2+} chelater, prevents fetal gene expression induced by Ang II [23] and that the L-type Ca^{2+} channel blocker and agonist can suppress and facilitate the hypertrophic remodeling induced by PE, respectively, in neonatal cardiac myocytes [15]. These studies suggest that the augmented $[Ca^{2+}]_i$ by these humoral factors may be involved in the signaling cascade(s) that induce hypertrophy in cardiac myocytes.

Ca^{2+}/CaM dependent pathway (calcineurin and CaMK)

Calcineurin/NFAT signaling cascade gives a clue to understand how Ca^{2+} signal induces neonatal gene expression in cardiac hypertrophy. *Molkentin* et al. showed that transgenic mice that express constitutively active form of calcineurin developed cardiac hypertrophy and resulted in heart failure [22]. Calcineurin is a Ca^{2+}/CaM dependent phosphatase which is activated by the increase in $[Ca^{2+}]_i$. Activation of calcineurin dephosphorylates the cytoplasmic transcription factor NFAT that subsequently migrates into the nucleus to upregulate gene expression (Fig. 3). The calcineurin inhibitors (cyclosporine A and FK 506) suppress the NFAT-dependent fetal gene expression and hypertrophic cell remodeling induced by Ang II or PE in cultured neonatal cardiac myocytes [22], indicating that calcineurin is involved in the critical pathway to the induction of cardiac hypertrophy by humoral factors. The important role of calcineurin in the development of cardiac hypertrophy has also been demonstrated *in vivo* as cyclosporine A treatment prevents the pressure overload-induced cardiac hypertrophy [24, 25]. Importantly, however, there are contradictory studies that failed to show the prevention of hypertrophy by the treatment with cyclosporine A in hemodynamic overload [26, 27]. These studies have implicated that various stimuli leading to cardiac hypertrophy

use calcineurin cascade in the downstream of Ca^{2+} signaling, although further studies are necessary to clarify whether the calcineurin activation commonly plays an essential role in the development of cardiac hypertrophy.

CaMK signaling is also known to be involved in the development of the cardiac hypertrophy and heart failure. Activation of CaMKI, II, and IV induces the expression of hypertrophic marker genes in cultured cardiac myocytes, and the CaMK inhibitor KN-62 prevents hypertrophy induced by the administration of ET-1 [28, 29]. The study of transgenic mice overexpressing the active form of CaMKIV in the heart [21] revealed that CaMKIV induces cardiac hypertrophy *in vivo* by activating the transcription factor MEF 2 (Fig. 3). CaMKIV/MEF 2 and calcineurin/NFAT pathways were shown to act synergistically, resulting in a severe hypertrophic phenotype [21, 30]. However, CaMKIV has not been detected in native cardiac myocytes. The CaMKIV overexpressed in the transgenic mouse is considered to mimic the activity of other CaMK isoform(s) that is responsible for native hypertrophic signaling. Recently, the increase in the expression and the phosphorylation of endogenous CaMKIIδ_C has been reported in a rat model of the cardiac hypertrophy induced by pressure overload [31]. Among the CaMK isoforms, the CaMKIIδ_C is thus most likely to be involved in the native hypertrophic signaling in cardiac myocytes. Transgenic mice that express CaMKIIδ_C have been shown to develop a dilated cardiomyopathy, in which phosphorylation of Ca^{2+}-handling proteins (RyRs and phospholamban) are increased [31]. CaMKII is known to phosphorylate other Ca^{2+}-handling proteins such as the L-type Ca^{2+} channel and SERCA to alter their functions. Thus, the activation of CaMK(s) in cardiac myocytes induces not only the fetal gene expression but also the alteration of Ca^{2+} signaling. The abnormal Ca^{2+} signaling deteriorates the pathological remodeling of cardiac myocytes, thus resulting in the impairment of contractile function [31].

Possibilities of relationship of Ca^{2+} signal and MAPK cascades in cardiac myocytes

Besides the Ca^{2+}/CaM signaling pathway, it is well known that mitogen-activated protein kinase (MAPK) cascades are involved in the fetal gene expression in cardiac hypertrophy. MAPKs are serine/threonine kinases that are activated upon tyrosine/threonine phosphorylation by upstream MAPK kinases (MAPKKs), following the activation by upstream kinase MAPKKKs. Activated MAPKs phosphorylate and activate nuclear transcription factors such as c-myc, c-jun and ATF-2, resulting in gene expression. The three best characterized MAPKs cascades are the extracellular-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs cascades. All of these cascades are known to be activated in the cardiac hypertrophy induced by physical stress like pressure overload or the humoral factors described above and are involved in fetal gene expression and induction, or suppression of apoptosis [18, 32–34].

MAPKs are activated by various stimuli in cardiac myocytes [18]. Some of them have been shown to require the Ca^{2+} entry and/or the intracellular Ca^{2+} to activate MAPKs. The activation of ERK by PKA pathway through β-adrenergic receptor stimulation has been shown to be dependent on the extracellular Ca^{2+} in primary culture of neonatal cardiac myocytes [35]. The activation of p38 induced by the administration of daunomycin, an antineoplastic agent which induces apoptosis of cardiac myocytes, was suppressed by the intracellular Ca^{2+} chelating [36]. However, the detailed mechanism by which Ca^{2+} signal activates MAPKs has not been elucidated yet in cardiac myocytes.

Recently, Sagasti et al. identified a novel signaling cascade in that Ca^{2+} signal leads to the activation of p38 in *Caenorhabditis elegans (C. elegans)* [37]. At first, they analyzed a *C. elegans* mutant that lost the asymmetry of neurons: Nsy-1 (Neuronal symmetry-1) and showed that the phenotype could be attributed to the mutation in nsy-1 encoding a homolog of the human ASK1, a MAPKKK that activates JNK and p38 pathways [38]. The expression of Nsy-1 could phosphorylate the MAPKK (MEK 6) and activate p38 [37]. The genetic epistasis analysis revealed that Nsy-1 appears to act in the downstream of CaMKII homolog (Unc-43) and voltage-gated Ca^{2+} channel homologs (Unc-2 and Unc-36, homolog of mammalian N-or P/Q type Ca^{2+} channel α_1 subunits) to determine asymmetric cell fates in AWC olfactory neurons. These results predict that Ca^{2+} signaling through voltage-dependent Ca^{2+} channels and subsequent activation of CaMKII (Unc-43) leads to the activation of the MAPKKK (Nsy-1). Recently, in mammalian neuronal cells, Takeda et al. have confirmed that CaMKII serves as an activator of the ASK1-p38 MAPK pathway in Ca^{2+} signaling [39]. The activation of ASK1 induced by cell depolarization was suppressed by the application of L-type Ca^{2+} channel blocker (nifredipine) and CaMK inhibitor (KN-93). On the other hand, the administration of L-type Ca^{2+} channel agonist (FPL 64176) induced the activation of ASK1. Moreover, the activation of p38 induced by depolarization or constitutively active CaMKII was diminished in neurons deficient in ASK1. These results indicate that the Ca^{2+} channel-CaMKII-ASK1-p38 cascade exists also in mammalian cells, although it has not been clarified yet whether the Ca^{2+} signal through the L-type Ca^{2+} channel is specific to activate the cascade.

It is possible that such cascade exists also in cardiac myocytes and takes part in pathophysiological responses. For example, in cardiac myocytes, L-type Ca^{2+} channel blocker and CaMK inhibitor suppress apoptosis through β-adrenergic receptor pathway [40], which may, at least in part, be controlled by MAPKs [33, 41]. In addition, ASK1-deficient mice have been shown to be resistant against the Ang II-induced cardiac hypertrophy and remodeling [42]. Although

only a little is known about the crosstalk between Ca^{2+} signal and MAPKs in cardiac myocytes, the elucidation of such mechanism will shed light on our understanding of the complex cardiac disorders.

Ca^{2+} signaling in neurons

In neuronal cells, as in the cardiac myocytes, Ca^{2+} enters upon the action potential. Various types of Ca^{2+} channels control the Ca^{2+} influx in neurons, such as the N-methyl-D-aspartate (NMDA) type and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) type glutamate receptors and multiple types of voltage-dependent Ca^{2+} channels (L, P/Q, N, R, T-type) (Tab. 1). Ca^{2+} signals through these channels are differently used in various cell responses such as neurotransmitter release, axonal outgrowth, survival and death, and gene expression, although these channels coexist in a single neuron [43]. Some of the spatial differences in Ca^{2+} signals originate from differences in the subcellular localization of these channels. For example, L-type Ca^{2+} channels that integrate the synaptic activity and control gene expression are mainly expressed in somatic membranes such as the initial segment of axon and the proximal dendrites. In contrast, P/Q, N, and R-type Ca^{2+} channels are found in both soma and synaptic terminals, as they are involved in the secretion of neurotransmitters. However, there are examples that can not be explained by the spatial difference in the localization such that some gene expression is preferentially driven by calcium influx through L-type Ca^{2+} channels, whereas it is poorly induced by calcium entering through NMDA receptors in spite of the fact that there are little differences in their subcellular localization and the duration or magnitude of the somatic Ca^{2+} rise mediated by direct activation of them [44]. Recently, in neurons, some of theses Ca^{2+} signals, specific to the respective Ca^{2+} channels, have been explained by the molecules tethering near the mouths of the channels. In the following chapter, we describe how the entry of Ca^{2+} through L-type Ca^{2+} channels leads to the specific signal in neurons with the accumulating knowledge about the interacting proteins.

The specific role of the L-type Ca^{2+} channel in CREB activation through ERK pathway

A transcription factor; cAMP response element-binding protein (CREB) is a good example to understand how Ca^{2+} signal through the L-type Ca^{2+} channel can specifically control the gene expression. In neurons, CREB is activated upon excitation, which leads to the augmentation of gene expression that control important neural functions such as the activity-dependent survival and the synaptic plasticity of long-term potentiation. The rise of the intracellular Ca^{2+} is known to be a crucial step in these responses because Ca^{2+} activates signal molecules such as CaMK and ERK thus leading to phosphorylation of Ser^{133} of CREB, which is necessary for the activation of CREB [43, 45]. CaMK phosphorylates Ser^{133} rapidly and briefly, while ERK mediates slow and prolonged phosphorylation of the same site (Fig. 4). The prolonged phosphorylation of Ser133 is necessary for CREB to activate gene expression [45]. Interestingly, in hippocampal neurons, not all Ca^{2+} channels that open in response to action potential equally contribute to the activation of CREB. The L-type specific Ca^{2+} channel blocker inhibits the CREB activation almost completely [46] in spite of the fact that the Ca^{2+} influx through L-type Ca^{2+} channels contributes to only 20% of the total Ca^{2+} influx elicited by the action potential [47]. In contrast, the phosphorylation of Ser^{133} in CREB is insensitive to the blockers specific to NMDA receptor or other voltage-dependent Ca^{2+} channels [46]. These results indicate that Ca^{2+} signal through L-type Ca^{2+} channels transmits specific signals for driving CREB to induce gene expression. In some studies, the specific activation of CREB was attributed to the electrophysiological characteristics of the L-type Ca^{2+} channel that allows this channel to preferentially respond to neuronal action potentials [47, 48]. Meanwhile, *Dolmetsch* et al. proposed a new hypothesis that CaM, tethering at the carboxyl-terminus of the L-type Ca^{2+} channel ($Ca_V1.2$), plays a key role in the specific activation of CREB by Ca^{2+}signal *via* L-type Ca^{2+} channel in neurons [46].

As described in chapter 3, CaM is bound to the L-type Ca^{2+} channel at the carboxyl terminal of $Ca_V1.2$. Ca^{2+} is dispensable for the initial binding of CaM. Upon the activation of Ca^{2+} channel, CaM binds to Ca^{2+}, and CaM interacts with another binding site called IQ motif on the carboxyl-terminal of $Ca_v1.2$ (Fig. 2). By the use of pharmacological knock-in method, *Dolmetsch* et al. showed that $Ca_V1.2$ with mutations at IQ motif could not induce either the prolonged activation of ERK or the subsequent activation of CREB in primary culture of cortical neurons, despite that the amount of Ca^{2+} influx induced by depolarization was almost the same compared with that of wild type channels. In addition, the overexpression of CaM mutants that could not bind to Ca^{2+} prevented ERK and CREB activation induced by depolarization of neurons [46]. These results indicate that Ca^{2+} binding to the tethering CaM and the following interaction between Ca^{2+}/CaM and IQ motif of the L-type Ca^{2+} channel is necessary to activate ERK/CREB cascade in response to depolarization. Although the mechanism how the binding between Ca^{2+}/CaM and IQ motif leads to ERK activation is to be elucidated, some clues indicate the importance of carboxyl-terminal region of $Ca_V1.2$. Restricting the mobility of the carboxyl-terminal tail of $Ca_v1.2$ by anchoring it to the plasma membrane caused an inhibition of the depolarization-dependent CREB activation without the change of Ca^{2+} influx [49]. A motif in the carboxyl-terminal tail of $Ca_v1.2$ that interacts with PDZ domain has been shown to be required for the effective activation of CREB-dependent gene expression [7].

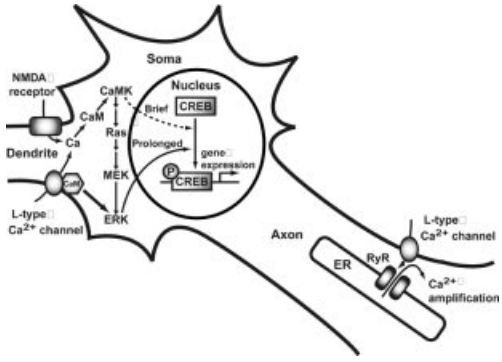

Fig. 4. A schematic model of depolarization-dependent Ca^{2+} signaling in neurons. Ca^{2+} entry, induced by the action potential, activates gene expression in neurons. NMDA receptors and L-type Ca^{2+} channels localized at the proximal dendrite are known to be involved in such Ca^{2+} signaling that phosphorylates a transcription factor, cAMP response element-binding protein (CREB). Ca^{2+} entered through these Ca^{2+} channels binds to cytosolic calmodulin (CaM) followed by the activation of CaMK. CaMK can directly phosphorylate Ser^{133} of CREB, although the phosphorylation is brief thus not enough to induce gene expression. CaMK activation also leads to the activation of the Ras/ERK pathway. The ERK activation induces slow but prolonged phosphorylation of CREB, which is essential for the gene expression. In contrast to NMDA receptors, L-type Ca^{2+} channels have a specific activation pathway leading to the activation of ERK through CaM that is tethered at the carboxyl terminus of α_1 subunit. This explains why L-type Ca^{2+} channels have the priority to efficiently drive the CREB-dependent transcription in neurons. However, the molecular mechanism linking CaM, associated with the L-type Ca^{2+} channel, and Ras/ERK pathway has been unknown.
L-type Ca^{2+} channels are also localized in axon where they colocalize with RyRs on the internal Ca^{2+} store, the ER. As is seen in cardiac myocytes, Ca^{2+} entry *via* the L-type Ca^{2+} channel opens the adjacent RyR, releasing Ca^{2+} from the internal store. The Ca^{2+} signaling amplified by the CICR is involved in neuronal disorders such as spinal cord injury.

These results indicate that the conformational change of carboxyl-terminal region of $Ca_V1.2$ induced by the binding of Ca^{2+}/CaM and IQ motif is important for the activation of ERK/CREB pathway, which may be mediated by the interaction with PDZ domain containing molecules. Since the PDZ interacting motif is not found in $Ca_v1.3$, it is conceivable that the interaction is specific to $Ca_V1.2$, which may account for the different roles among the L-type Ca^{2+} channel family members.

The mechanism by which the L-type Ca^{2+} channel specifically activates CREB implicates that only a localized Ca^{2+} signal near the channels is essential even though the resulting event occurs far from the channels such as gene expression in nucleus. It is expected that the signaling cascade from L-type Ca^{2+} channels to ERK/CREB will be unraveled by the identification of the molecule(s) that interact with L-type Ca^{2+} channel tail at its PDZ domain. Such interactions between Ca^{2+} channels and signal molecules have been found in other channel machineries in neurons such as NMDA receptors [1, 50]. The similar system may operate in specific Ca^{2+} signals through specific Ca^{2+} channels in tissues other than neurons.

The role of L-type Ca^{2+} channel in spinal cord injury

Demyelination seen in multiple sclerosis, some neuronal disorders in spinal cord during stroke, and spinal cord trauma are characterized by abnormal conduction of action potentials along axons and cell death, often resulting in serious clinical disability. The importance of Ca^{2+} influx in the development of neuronal disorders have been suggested by studies of various neuronal diseases including glutamate cytotoxicity [51], hypoxic white matter injury [52], and spinal cord trauma [53]. Elevated intracellular Ca^{2+} could lead to necrosis by the activation of Ca^{2+}-dependent protease (calpain) [54, 55] and the apoptosis accompanied with DNA fragmentation [56]. Neurons have many Ca^{2+} channels, thus it has been extensively discussed which type of Ca^{2+} channel is responsible for such pathological Ca^{2+} signals. Among Ca^{2+} channels, L-type Ca^{2+} channel is well known to be involved in the development of such neuronal disorders induced by physical trauma and ischemia in the spinal cord white matters.

In the study of isolated spinal cord, the pre-treatments, such as the chelating of the extracellular Ca^{2+} or the application of nonspecific Ca^{2+} channel inhibitors (Co^{2+}, Cd^{2+}), have been shown to ameliorate the disorders of neuronal excitability induced by physical trauma. The similar result was obtained by the administration of L-type Ca^{2+} channel blockers (diltiazem and verapamil) [57]. Moreover, the anoxic injury of spinal cord white matter could be inhibited by the prior application of L-type Ca^{2+} channel blockers (DHP, diltiazem, and verapamil) [52]. As co-application of the L-type and N-type Ca^{2+} channel blockers (diltiazem and ω-conotoxin G VI A) suppressed these neuronal disorders cooperatively [57], the L-type Ca^{2+} channel is not the sole route, but plays an important role in such pathological Ca^{2+} signaling.

Besides the Ca^{2+} entry, it is shown recently that Ca^{2+} released from the internal store in response to the L-type Ca^{2+} channel activation could be involved in the neuronal disorders induced by ischemia [58]. In neurons, the Ca^{2+} release from the intracellular store is controlled by RyR and $InsP_3$ receptors. Among theses channels, RyR 1 and RyR 2 were shown to cooperate with L-type Ca^{2+} channels ($Ca_v1.2$ and $Ca_v1.3$ respectively) in neuronal cells [58, 59], suggesting the involvement of such functional interactions between

RyRs and L-type Ca^{2+} channels that amplify the Ca^{2+} signaling as found in cardiac myocytes (see chapter on 'Ca^{2+} signal regulation in cardiac myocyte', Fig. 3). Colocalization of RyRs and L-type Ca^{2+} channels was found in the subcellular region where ER is adjacent to the plasma membrane of axon by electron microscopic inspection. In addition, the Ca^{2+} release from the intracellular store induced by ischemia was suppressed by the administration of L-type Ca^{2+} channel blockers [58]. These results indicate that the amplification mechanism of Ca^{2+} signaling initiated by the interaction between L-type Ca^{2+} channels and RyRs exists also in axons, and that the L-type Ca^{2+} channel may augment toxic Ca^{2+} signals from either extracellular or intracellular origin in neuronal disorders (Fig. 4). However, it is unlikely that such a Ca^{2+} amplification machinery in axons is equipped only for the cytotoxicity. The physiological importance of the mechanism should be elucidated in future studies.

Conclusion

Ca^{2+} signals through L-type Ca^{2+} channels are involved in various physiological and pathophysiological cellular responses by activating signaling molecules such as CaMKs and MAPKs. The Ca^{2+} channel operates not only as a mere route for Ca^{2+} entry from the extracellular space but also as the machinery extensively designed to control Ca^{2+} signals in native tissues. For example, it senses and feeds back the intracellular Ca^{2+} content to the Ca^{2+} entry to maintain the Ca^{2+} homeostasis in cardiac myocytes (see chapter on 'Ca^{2+} signal regulation in cardiac myocyte'). In neurons, the Ca^{2+} channel serves as a specific Ca^{2+} signal transducer to induce MAPK activation and gene expression (see chapter on 'The specific role of the L-type Ca^{2}+ channel in CREB activation through ERK pathway'). The communications with other molecules such as CaM and RyR are necessary to confer these special functions to the L-type Ca^{2+} channels. Especially, the communication with RyR is interesting, as it enables the L-type Ca^{2+} channel to recruit the Ca^{2+} signal not only from the extracellular but also from the intracellular origin.

Besides the roles described above, the L-type Ca^{2+} channel is specifically involved in various cellular functions such as (1) gene expression required for skeletal muscle differentiation ($Ca_v1.1$) [60], (2) secretion of insulin from pancreatic β cells ($Ca_v1.2$ and $Ca_v1.3$) [61-63] and (3) cell proliferation of T-cells by activation of ERK *via* CaMK cascades ($Ca_v1.4$) [64]. In these tissues, it is highly considerable that L-type Ca^{2+} channels have discrete mechanism to control specific Ca^{2+} signals upon cooperation with other Ca^{2+} signaling molecules. Clarification of these mechanisms will be of great help to understand the fine-tuning of Ca^{2+} signals and provide us clues for the treatment of pathophysiological state induced by Ca^{2+} signaling disorders.

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