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Capturing single L-type Ca^{2+} channel function with optics

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Abstract

Advances in imaging technology have allowed optical analysis of Ca^{2+}-permeable ion channel activity. Here, we briefly review novel developments in optical recording of L-type voltage-dependent Ca^{2+} channel (LTCC) function with high spatial and temporal resolution. Underlying principles supporting the use of total internal reflection fluorescence (TIRF) microscopy for optical measurement of channel activity and new functional characteristics of LTCCs revealed by application of this approach are discussed. Visualization of Ca^{2+} influx through single LTCCs ("LTCC sparklets") has demonstrated that channel activity is regionally heterogeneous and that clustered channels are capable of operating in a cooperative, or "coupled" manner. In light of these findings, we describe a current molecular model for the local control of LTCC activity and coupled gating in physiological and pathological contexts.

1. Introduction

Spatial confinement of intracellular second messenger activity has been increasingly recognized as a fundamental means of controlling specificity in divergent signaling processes. As an elemental second messenger that is neither synthesized nor degraded, calcium is a fitting example of this concept. Ca^{2+} ions are continually moved between extracellular and intracellular environments and among subcellular compartments in an intricate and dynamic interchange that is essential for cellular communication. The complex nature of transient subcellular Ca^{2+} signals has only recently come into view [1, 2], with pioneering discoveries owing much to key advances in imaging technology. The progression of our ability to visualize micro and nanodomain Ca^{2+} signaling events in space and time has thus proven essential for fully understanding how cells function and how errant Ca^{2+} signals can result in disease.

In excitable cells, influx of Ca^{2+} ions via L-type voltage-dependent Ca^{2+} channels (LTCCs) controls numerous physiological processes such as excitability, contraction, secretion, neurotransmitter release and synaptic plasticity. LTCCs are composed of pore-forming $\alpha 1$ subunits associated with a combination of accessory, β, γ, and $\alpha_2\delta$ subunits which influence channel trafficking and gating [3-5]. Alternative splicing of mRNA encoding LTCC subunits confer tissue-selective channels with distinct pharmacological and electrophysiological profiles [6-8]. Channel function is further influenced by interaction with a variety of regulatory and structural proteins, and is subject to modification by several ubiquitous kinases and phosphatases, which serve to finely tune channel function to cellular

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demand [4, 9, 10]. Importantly, alteration of LTCC expression or function has been linked to multiple human pathological conditions including cardiac arrhythmia, hypertension, immune deficiency and autism [11].

To a large extent, our current understanding of LTCC biophysical properties is the result of experiments employing the patch-clamp technique to isolate and record transmembrane currents. However, patch-clamp applied alone is insufficient in providing information regarding localization and organization of multiple active channels, which is critical for understanding how Ca^{2+} influx through LTCCs mediates a diversity of signaling pathways and cellular functions (e.g. contraction and transcription). Recent work employing optical methods in combination with patch-clamp electrophysiology have overcome this issue and provided novel information about LTCC organization. These efforts have revealed unique features of channel regulation that may have profound therapeutic implications given the physiological importance of these channels. In this review, we discuss the use of advanced microscopy in combination with Ca^{2+}-sensitive fluorescent indicators to optically record LTCC activity and new aspects of Ca^{2+} signaling revealed by this approach.

2. Optical recording of Ca^{2+} channel activity

The physiological importance of Ca^{2+} ions first became known during the early 1880's when Sidney Ringer reported the observation that frog hearts would not beat unless a calcium salt was added to the extracellular solution [12]. It is now apparent that signaling in all excitable cells involves control of not only global cytosolic Ca^{2+}, but also fine coordination of short-lived Ca^{2+} gradients confined to various subcellular compartments. Nonetheless, the study of small amplitude Ca^{2+} transients in live cells has been hindered by limited microscope and dye technology, until recently. Accordingly, with the emergence of improved fluorescence-based Ca^{2+}-sensitive indicators and advanced microscopy equipment, optical techniques have rapidly evolved as a means to study subcellular Ca^{2+}-signals with unsurpassed spatiotemporal resolution.

The first optical demonstration of dynamic changes in intracellular Ca^{2+} revealed Ca^{2+} "waves" in sperm-activated Medaka fish eggs [13]. This early investigation detected emitted light from aequorin, a bioluminescent photoprotein with Ca^{2+}-binding EF-hand motifs isolated from the jellyfish *Aequoria victoria*. Later, microinjected aequorin was used to simultaneously measure pulsatile Ca^{2+} increases and corresponding contractions in frog atrial myocytes [14]. However, irreversible consumption of this Ca^{2+} indicator upon light emission and limited signal amplitudes hindered use of aequorin for analysis of intracellular Ca^{2+}. The subsequent generation of several Ca^{2+}-sensitive fluorescent indicators (e.g. fura-2, fluo-4) triggered a mass of studies to view and characterize subcellular Ca^{2+} gradients in living cells [15].

The first two-dimensional analysis of local Ca^{2+} influx through single plasmalemmal Ca^{2+}-permeable channels was achieved using wide-field microscopy in combination with whole-cell patch-clamp in toad stomach smooth muscle cells (Figure 1A) [16]. In this study, thapsigargin was applied to fluo-3 loaded cells to eliminate Ca^{2+} release from intracellular stores. Subsequent application of 20 mM caffeine evoked submembrane transient changes in fluo-3 fluorescence with seemingly random distribution. These transients correlated with single channel openings in whole-cell current traces (Figure 1A). With the use of a wide field microscope, fluorescence was detected from a relatively large cytosolic volume, due to collection of light from within and outside the focal plane. Accordingly, changes in fluorescence with this approach reflect total accumulation of regional cytosolic Ca^{2+} rather than submembrane gradients near the mouth of active channels, which decreases the rate of signal rise and decay with distance from the focal plane. However, the relationship between

the total change in fluorescence and degree of Ca^{2+} influx was later shown to be linear, regardless of whether the transient is in focus, thus allowing accurate optical determination of single Ca^{2+} channel current from total fluorescence increase or "signal mass" [17].

The development of laser scanning confocal microscopy first permitted "optical sectioning" to examine transient fluorescent signals within close proximity to the point source of Ca^{2+} influx in excitable cells. Line-scanning confocal imaging of fluo-4 fluorescence led to the detection of spontaneous localized intracellular Ca^{2+} release (i.e. "Ca^{2+} sparks") in quiescent cardiomyocytes resulting from short-lived activation of a cluster of ryanodine receptors residing in the sarcoplasmic reticulum [18]. Subsequent investigation of the elementary plasmalemmal Ca^{2+} signals evoking these intracellular Ca^{2+} release events used line-scanning confocal microscopy in combination with the cell-attached patch configuration to demonstrate, for the first time, transient intracellular Ca^{2+} signals at the intracellular mouth of a single LTCC located within the voltage-clamped patch membrane [19]. In this study, signal amplitudes were enhanced by raising extracellular [Ca^{2+}] in the presence of the LTCC agonist FPL64176, effectively increasing the driving force for Ca^{2+} and prolonging the duration of channel openings, respectively. In cardiomyocytes, each fluorescent transient produced by opening of a single LTCC, referred to as "LTCC sparklet", was found to promote Ca^{2+} spark ignition by triggering activation of four to six ryanodine receptors in closely apposed sarcoplasmic reticulum.

Although confocal microscopy offers the ability to record from a restricted volume (~0.8 μm axial section, Figure. 1B), this point scanning method is associated with limited temporal resolution, unless one-dimensional line scan mode is used. However, significant disadvantages exist with line scanning in that the total number of active sites per cell cannot be obtained and event amplitude and duration may be misrepresented due to outlying Ca^{2+} signals arising from regions near the scan line. A more suitable approach for recording from large areas of membrane is total internal reflection fluorescence (TIRF) microscopy. In particular, the use of TIRF in combination with ultra-fast high sensitivity electron multiplying charge coupled device (EMCCD) cameras has allowed the direct observation of changes in Ca^{2+} within a thin (< ~100 nm, Figure. 1C) optical section of the plasmalemmal surface. Indeed, this approach was first successfully implemented in the optical recording of single N-type Ca^{2+} channel activity in a heterologous expression system [20, 21]. Considering that TIRF allows selective illumination of fluorophore near the surface membrane, this technique resolves Ca^{2+} influx in regions surrounding Ca^{2+}-permeable channels while avoiding detection of bulk cytoplasmic indicator and therefore has emerged as an advantageous method for optical measurement of Ca^{2+} channel activity.

3. Use of total internal reflection fluorescence (TIRF) for imaging Ca^{2+} sparklets

Total internal reflection fluorescence (TIRF) microscopy relies on the physical principle that incident light encountering a boundary with a lower refractive index at the proper angle is completely reflected with the generation of a highly restricted electromagnetic field (evanescent waveform) in the lower-index medium [22]. Whereas the evanescent field carries identical properties as that of incident light (i.e. wavelength, frequency), its intensity decays exponentially with distance, such that illumination extends tens of nanometers, typically one third of the incident wavelength, from the coverslip-cell interface. This precisely restricted illumination limits the harmful effects of laser exposure on live cells and produces a very thin excitation field, thus offering exceptional resolution for imaging targets embedded in or directly adjacent to the plasmalemma. In this section, we briefly describe a general protocol for the use of TIRF in combination with whole-cell patch-clamp electrophysiology to image LTCC sparklets.

First, isolated native or cultured cells are allowed to adhere to a glass coverslip mounted in a recording chamber in physiological saline solution at room temperature. After allowing cell adhesion (~20 min), cells are washed by continuous superfusion. Thapsigargin or cyclopiazonic acid is used to inhibit sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity and prevent SR Ca^{2+} loading. This helps to prevent interference of LTCC-associated fluorescent signals by Ca^{2+}-release events from intracellular stores. Cells are then patch clamped in the whole-cell configuration to allow manipulation of membrane potential as well as introduction of Ca^{2+} indicator and an excess of EGTA (10 mM). Cytosolic Ca^{2+} indicator and EGTA act as fluorescent and non-fluorescent buffers that compete for Ca^{2+} ions. As a result, Ca^{2+} ions crossing the plasmalemma via Ca^{2+}-permeable channels bind to the lower affinity, but fast binding Ca^{2+} dye (e.g. fluo 5). Upon release, this Ca^{2+} is taken up by the more abundant, high-affinity, and non-fluorescent EGTA, thus restricting fluorescence signals to areas near the source of entry. This approach in combination with the thin optical section in TIRF mode increases signal-to-noise and permits the detection of low intensity fluorescent signals. Because the time course of the fluorescence signals closely parallels the time course of current recordings (Figure 1C), analytical methods to quantify Ca^{2+} sparklet activity are alike that of open probability (P_O) analysis for single channel currents. Thus, Ca^{2+} sparklet activity can be expressed as nP_S, which is analogous to nP_O, and where n refers to the number of quantal levels and P_S is the probability of Ca^{2+} sparklet occurrence (for details on this analysis see [23]).

Although larger optical sections produced by the two-dimensional (i.e. swept field, spinning-disk) confocal microscope makes observation of small amplitude sparklet events challenging, it should be noted that this method has been used to detect LTCC sparklets and is particularly useful for recording sparklets in situ. For example, loading EGTA-AM to buffer Ca^{2+} and fluo-4 as a Ca^{2+} indicator, our laboratory has recently observed localized LTCC-mediated Ca^{2+} influx events in intact pressurized resistance arteries (Nystoriak and Navedo, unpublished observations). These findings lay the groundwork for future experiments aiming to investigate mechanisms of LTCC sparklet regulation by physiological modulators of vascular tone such as intravascular pressure or endothelial-derived vasoactive factors.

4. Cardiovascular LTCC sparklets

Optical investigation of local Ca^{2+} influx has revealed unique features regarding regulation of LTCC function in the vasculature, which may apply to channels expressed elsewhere. Considering the strong voltage-dependence of LTCC activation and broad expression of functional LTCCs throughout the plasmalemma of arterial smooth muscle cells, it was long assumed that channel activation is random and that all LTCCs have a similar open probability at a given membrane potential. Yet these assumptions were challenged when the Santana laboratory reported that subpopulations of LTCCs in isolated arterial myocytes operate in a continuously high open probability mode, producing distinct sites of Ca^{2+} influx [24]. Based on pharmacological, biophysical and molecular properties, the molecular entity underlying these Ca^{2+} influx events was identified as $Ca_V1.2$ channels. For instance, fluorescent Ca^{2+} influx events produced by opening of single LTCCs (i.e. LTCC sparklets) are concomitant with inward Ca^{2+} conductance, enhanced by dihydropyridine agonists (i.e. Bay-K 8644), and abolished by dihydropyridine antagonists (i.e. nifedipine) [25]. Consistent with this, heterologous expression of the pore-forming $Ca_V1.2$ (i.e. α_{1C}) subunit with accessory $Ca_V\beta3$ and $Ca_V\alpha2\delta1$ subunits in tsA-201 cells recapitulates LTCC sparklets with identical pharmacological and biophysical properties as Ca^{2+} sparklets in native smooth muscle cells [23-25]. Furthermore, smooth muscle cells expressing dihydropyridine-insensitive $Ca_V1.2$ channels produced sparklets that are resistant to nifedipine [25]. Surprisingly, LTCC sparklets are observed even at hyperpolarized membrane potentials

(−70 mV) and display several activity modes; namely silent, low and high (i.e. persistent) nP_S sparklets. A silent LTCC sparklet refers to a typically dormant site, which can be triggered by LTCC agonists, for instance. Low activity sparklets show intermittent, transient openings, whereas high activity ("persistent") sparklet sites are characterized by prolonged openings and nearly continuous Ca^{2+} influx. Finally, an amplitude histogram for these events can be fit with a multi-component Gaussian function such that the quantal nature of Ca^{2+} influx through LTCCs is revealed, with a single quantal unit of Ca^{2+} influx typically elevating $[Ca^{2+}]_i$ in the vicinity of the channel by ~38 nM in the presence of 20 mM external Ca^{2+} [24].

The spatial distribution of LTCC sparklet sites in arterial myocytes, although varied between cells, differentiated from a Poisson distribution, indicating non-stochastic LTCC activation occurring at defined sites within the myocyte surface membrane [26]. This finding raises a fundamental question: If functional LTCCs are located broadly throughout the plasmalemma of arterial myocytes, what are the mechanistic bases underlying regional heterogeneity of LTCC activity? This conundrum is not yet fully resolved, but considerable mounting evidence suggests that a subpopulation of LTCCs can be differentially regulated by a host of targeted regulatory factors. For example, LTCC sparklet activity is markedly enhanced by protein kinase C (PKC) [24, 27]. Activation of this serine/threonine kinase with phorbol esters (i.e. phorbol 12,13-dibutyrate) activates previously silent LTCC sparklet sites and further increases activity at low nP_S sites. Further analysis of this effect demonstrates that spatial heterogeneity of sparklet activity is not random, but results from functional coupling of specific clusters of LTCCs to PKCα by means of the multivalent scaffolding protein, AKAP150 (*A-kinase anchoring protein 150;* ortholog of human AKAP79) [23, 26, 27]. In this model, AKAP150 binds and targets PKC to specific regions of the surface membrane within close proximity to substrate LTCCs. Consistent with this, genetic ablation of PKCα or AKAP150 abolished persistent Ca^{2+} sparklet activity [27]. In addition to its role in the targeting of PKC, AKAP150 also binds and targets the cAMP-dependent protein kinase (PKA), and the Ca^{2+}/calmodulin-dependent phosphatase calcineurin to LTCCs. In this model, PKA upregulates LTCC sparklets in a similar fashion as PKC, and calcineurin opposes PKC and PKA activity and thereby inhibits LTCC sparklet activity [28]. Hence, localization of specific kinase and phosphatase activity by the scaffolding protein AKAP150 may represent a mechanism for regional heterogeneity of enhanced Ca^{2+} influx by LTCCs. Thus far, the molecular determinants of spatially heterogeneous AKAP150 expression in arterial myocyte submembrane compartments are unclear. However, previous findings in neurons suggest that this may involve targeting to membrane regions rich in the acidic phospholipid phosphatidylinositol 4,5 bisphosphate, F-actin or cadherin adhesion proteins via the N-terminal basic domain of AKAP150 [29-31].

In arterial myocytes, localized LTCC-mediated Ca^{2+} signals influence a variety of physiological phenomena. Persistent LTCC sparklets contribute ~50% of the steady-state Ca^{2+} entry [32] and directly regulate contractility and vascular tone under physiological conditions. Furthermore, LTCC sparklets can indirectly influence SR function by contributing Ca^{2+} influx to a cytosolic Ca^{2+} pool from which the SR can draw to accelerate reloading of intracellular Ca^{2+} stores [33]. Therefore, by way of elevating SR Ca^{2+} load, sparklets can presumably increase the frequency of RyR-mediated Ca^{2+} sparks and activation of nearby large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, ultimately promoting hyperpolarization and decreased LTCC activation. It is conceivable that submembrane Ca^{2+} gradients produced by LTCC sparklets may also directly influence nearby BK_{Ca} channel activity, as this could represent additional feedback regulation to prevent uncontrolled Ca^{2+} entry in vascular smooth muscle leading to excessive vasoconstriction.

Cardiac and smooth muscle exhibit several similarities and differences with respect to local LTCC signals. In the heart, LTCC expression is concentrated in T-tubules where they are well situated to raise Ca^{2+} within a cytoplasmic cleft such that Ca^{2+}-induced Ca^{2+} release occurs via activation of proximal RyRs. These functional "couplons" between individual LTCCs and RyRs form the elementary basis for generation of cell-wide spikes in cytosolic $[Ca^{2+}]_i$, action potential prolongation and contraction [19, 34]. Accordingly, coupling strength between sparklets and sparks, and therefore cardiac contractility is proportional to the amount of Ca^{2+} flux via LTCCs and local $[Ca^{2+}]_i$ [35]. Conversely, a separate subpopulation of LTCCs in cardiomyocytes, restricted to caveolae rather than T-tubules, do not appear to be involved in SR Ca^{2+} release, but instead participate in NFAT-dependent excitation-transcription events. It is proposed that Ca^{2+} microdomains generated by caveolae-associated LTCCs contribute to pro-hypertrophic signaling in cardiomyocytes (see below) [36]. The specific elements involved in segregation of LTCC-mediated signals within these specialized microdomains in the heart are yet to be examined.

5. Coupled gating of L-type Ca^{2+} channels

Examination of the amplitude distribution produced by LTCC sparklets led to a striking discovery: the frequency of multi-channel openings far exceeded the probability of observing simultaneous openings of independently gating channels, suggesting that a group of several LTCCs may activate and inactivate in a synchronous manner (e.g. coupled gating) (Figure 2A). Importantly, synchronized opening and closing of multiple channels can be observed by both electrophysiological and optical analytical methods (Figures 2B and C). To explore this further, a Markov chain model has been applied to these recordings to yield the coupling coefficient or strength of "coupled" events (κ) [37]. In this model, values of κ range from 0 for total independent channel gating to 1 for channels exhibiting only synchronized openings. In experiments performed in arterial smooth muscle, neonatal ventricular myocytes and tsA-201 cells expressing $Ca_V1.2$ channels, values of κ ranged from 0.1 to 1, with a median κ of 0.2. Under normal conditions, most Ca^{2+} influx events were found to be the result of independent gating and coupled LTCC gating was relatively weak. However, it remains plausible that physiological stimuli (e.g. increase in intravascular pressure, β-adrenergic agonists) can evoke increases in $[Ca^{2+}]_i$ via induction of LTCC coupling.

Although the precise molecular details of coupled gating in LTCC clusters are not fully understood, spatial proximity between channels is thought to be necessary for coupled gating to occur. Recent work by Dixon et al supports this hypothesis [38]. In this study the authors used a light-activated fusion system consisting of flavin-binding, kelch repeat F box1 (FKF1) and gigantea (GI) proteins of *Arabidosis thaliana* was used to fuse $Ca_V1.2$ channels and determine whether protein-protein interaction among LTCCs enable coupled gating. The C-termini of $Ca_V1.2$ channels were tagged with either FKF1 or GI and expressed in tsA-201 cells. Illumination with blue light (473 or 488 nm) induced channel fusion via structural modulation of FKF1, promoting high-affinity binding of GI. Remarkably, fusion of neighboring channels after light stimulation substantially increased the likelihood of coupled events and resulted in a leftward shift in voltage-dependence of activation leading the authors to suggest that physical interaction between C-termini may underlie coupled gating. Yet, how this interaction evokes synchronous gating of several channels is unclear. Notably, light-induced fusion of channels resulted in a shift in voltage-dependent activation towards more hyperpolarized membrane potentials. Thus, one could speculate that interaction between neighboring C-termini may couple pore openings between adjacent channels by equivalent exertion of torque on the voltage-sensors of multiple channels via upstream S6 transmembrane helices, which are thought to impact pore opening in voltage-

gated channels [39]. Nonetheless, further experiments are required to fully address the mechanistic basis of coupled gating.

In Dixon et al, physical interactions were induced artificially, being driven by light-sensitive fusion proteins [38]. Hence, another important issue raised by findings of coupled gating of LTCCs is what native factors promote coupling between channels in vivo. One possibility is that a scaffolding protein, such as AKAP150, simultaneously anchors C-termini of multiple channels. Existing evidence suggests that displacement of the ubiquitous Ca^{2+} sensor calmodulin from the C-terminus of AKAP150-associated LTCC α1 subunits by PKC activators, CaM inhibitors or conformational changes produced by Timothy syndrome is a key step in the induction of LTCC coupling [37] (Figure 2D). Therefore, a potential role for AKAP150 in coupled gating of LTCCs can have broad implications for Ca^{2+} signaling across multiple organ systems, as this scaffolding protein is ubiquitously expressed and influences LTCC function in a variety of cell types [27, 40-42]. Thus, coupled gating may represent a common mechanism by which LTCCs can enhance local Ca^{2+} concentrations and potentially influence downstream signaling pathways. For example, one can speculate that the coupling strength between sparklet and sparks in the heart may be substantially increased in couplons with AKAP150-coupled LTCCs than in areas with independently gating channels, as the coordinated opening of multiple channels is more likely to generate the amplification of Ca^{2+} influx necessary to maximally activate RyRs during EC coupling [43]. In support of this, action potential-evoked $[Ca^{2+}]_i$ transients are slightly decreased in cardiac cells from an AKAP150 knockout ($AKAP150^{-/-}$) mouse [42]. In addition, AKAP150 can behave as an accessory subunit of LTCCs, regulating $Ca_V1.2$ kinetics in ventricular myocytes, and is responsible for prolonged cardiomyocytes action potentials during Timothy syndrome. However, considering that not all AKAP150-associated LTCCs consistently display coupling behavior, it is likely that additional factors are also required for cooperative LTCC gating to occur.

6. LTCC-mediated mechanisms of disease

Increased Ca^{2+} influx via upregulated LTCC expression and/or activity [44-46] in resistance arteries is a major contributor of enhanced arterial tone during several forms of hypertension [47-50]. Consistent with this, LTCC sparklet activity and the frequency of coupled gating events were found to be significantly higher in vascular smooth muscle in response to the potent endogenous vasoconstrictor angiotensin II [45, 46], which is elevated during hypertension. Multiple pathological alterations may collectively contribute to increased LTCC activity in hypertensive animals, including increased expression of $Ca_V1.2$ [44], activation of PKCα [46] and possibly PKC α-dependent reactive oxygen species (ROS) signaling [51, 52]. Similar to that observed during hypertensive conditions, persistent LTCC sparklet activity in arterial myocytes is also increased by acute exposure of isolated myocytes to supraphysiological levels of extracellular D-glucose as well as in genetically engineered diabetic (*db/db*) mice [45]. These recent findings suggest that impaired regulation of LTCC sparklet activity may be a common mechanism of elevated intracellular Ca^{2+} during vascular dysfunction. However, during acute hyperglycemia and type-II diabetes, increased LTCC sparklet activity and coupled gating is dependent on local activation of PKA, rather than PKC [45]. Interestingly, this study found that anchoring of PKA by an AKAP protein was also critical, as a specific peptide inhibitor of PKA/AKAP interactions (i.e. Ht-31) eliminated the glucose-induced increase in LTCC sparklet activity. Although more experiments are needed to address this issue, selective activation of AKAP-bound PKA may explain this kinase dependent facilitation of LTCC activity, exclusive from parallel potassium channel activation and membrane potential hyperpolarization.

How AKAP-targeted PKA is activated by elevation of extracellular glucose is still unclear. One intriguing possibility involves glucose-induced activation of NADPH oxidase [53] producing discrete sites of ROS generation. Consistent with this hypothesis, localized and direct oxidative kinase activation upon stimulation with hydrogen peroxide has recently been linked to enhanced Ca^{2+} sparklet activity in arterial myocytes [52]. Whether localized stimulation of adenylyl cyclase and subcellular cAMP production accompanies this discrete PKA-dependent LTCC activation upon stimulation with elevated extracellular glucose has not yet been tested. Additionally, hyperglycemic conditions are known to stimulate extracellular release of uridine triphosphate and adenosine triphosphate in a variety of cell types, including arterial myocytes [54-56]. Binding of these nucleotides to vascular $P2Y_2$ and $P2Y_6$ receptors evokes an increase in cytosolic Ca^{2+}, which is dependent on both Ca^{2+} influx via LTCCs and intracellular Ca^{2+} release [57], and ultimately decreases arterial diameter. Further studies are required to address these issues.

A current model for the control of excitation-transcription coupling by LTCCs suggests that a rise in cytosolic Ca^{2+} triggers activation of the Ca^{2+}/CaM-dependent phosphatase calcineurin, which in turns dephosphorylates members of the NFAT transcription factor family [26, 58, 59]. Removal of phosphate groups at several N-terminal serine and threonine residues within NFAT unmasks nuclear import signals [58, 60], leading to nuclear translocation and activation or deactivation of NFAT-target genes. Importantly, Ca^{2+}-induced NFAT activation is evoked by a variety of stimuli and this signaling pathway is linked to induction of specific genetic programs during the progression of multiple disease states including Alzheimer's disease, myocardial infarction, heart failure, and hypertension [53, 54, 61, 62]. In arterial myocytes, nuclear translocation of NFATc3, the isoform predominantly expressed in smooth muscle, is a key pathophysiological event responsible for downregulating the expression of voltage-dependent and Ca^{2+}-activated potassium channels [61, 63, 64]. Accordingly, downregulation of these channels may underlie membrane potential depolarization, elevated cytosolic $[Ca^{2+}]_i$ and higher arterial tone during hypertension and diabetes. Activation of NFAT in the vasculature during hyperglycemic conditions and diabetes has also been linked to increased production of the matrix cytokine osteopontin [57], a key mediator of vascular inflammation, which is active in the development of atherosclerotic lesions [65]. Thus, specific factors contributing to NFATc3 nuclear translocation represent potentially therapeutic targets for future treatments against vascular disease.

As in the vasculature, compartmentalized Ca^{2+} signals in cardiomyocytes may also be essential for signaling during pathological stresses contributing to hypertrophic growth and remodeling [66]. For example, calcineurin and NFAT activation during cardiac hypertrophy may be independent of global Ca^{2+} transients, but rather more linked to local Ca^{2+} influx via a subgroup of channels residing in membrane subdomains [36, 67]. Nonetheless, the source of "hypertrophic" Ca^{2+} contributing to calcineurin-NFAT activation in the heart remains controversial. A recent study identified a subpopulation of LTCCs that are not localized to the T-tubules, but are associated with caveolae [36]. Selective blockade of caveolae-associated LTCCs using caveolin-3-targeted Rem proteins nearly abolished Ca^{2+} influx-induced NFAT nuclear translocation, but did not significantly alter contractility. Additionally, evidence both supports and refutes signaling microdomains housing T-type voltage-dependent Ca^{2+} channels as important mediators of hypertrophic Ca^{2+} influx [68, 69]. Finally, inhibition of canonical transient receptor potential (TRP) channels also significantly reduces calcineurin-NFAT activity and was protective in transgenic mice against loss of cardiac functional performance following pressure-overload stimulation [70]. Interestingly, it was recently shown that nuclear translocation of NFATc3 upon overexpression of TRPC3 was completely abolished by the LTCC inhibitor nifedipine [71],

suggesting that TRP channels may modulate expression or activity of LTCCs to induce hypertrophic signaling in the heart.

7. The future of fluorescence-based investigation of Ca^{2+}-permeable channels

Ongoing endeavors to investigate functional coupling between Ca^{2+} transients and signaling pathways at molecular resolution will without question rely on further advances in imaging and indicator technology to isolate fluorescent signals with fewer cytotoxic effects and improved signal-to-noise. A major goal of future analyses of Ca^{2} signaling pathways will be to visualize localized transient events in more physiologically relevant contexts than that of former studies, which mostly imaged isolated cells loaded with acetoxymethyl ester indicators. Indeed, conventional dyes are severely limited in situ or in vivo due to interfering fluorescence arising from high-intensity signals in adjacent cell types, which may also readily take up indicator. To circumvent this issue, transgenic mice have recently been developed to express genetically encoded Ca^{2+} indicators, offering the possibility of long-term in situ and in vivo imaging with cell-specific and even microdomain-specific expression profiles [72, 73]. Several variants of GCamP, a circularly permutated enhanced green fluorescent protein (GFP) attached to calmodulin and an N-terminal fragment of myosin light chain kinase (M13) [74, 75], have now been expressed to record Ca^{2+} signals in neural networks, heart, and vascular endothelial cells [72, 76, 77]. The GFP fluorescent yield of GCamP proteins increases with cooperative binding of calcium ions by calmodulin and M13 fragments over a range of physiological Ca^{2+} concentrations ($K_D \approx 150$ nM), making it useful to study intracellular events occurring on millisecond timescales. This indicator can also be selectively targeted to various subcellular compartments. For example, tagging of GCaMP2 with Lck, a Src tyrosine kinase containing tandem myristoylation and palmitoylation domains, increased the local expression of GCamP2 near the membrane in astrocytes of rat hippocampal astrocyte-neuron co-cultures by as much as fourteen fold compared with unmodified GCaMP2 [78]. This strategy lead to the discovery of Ca^{2+} “microdomains” in fine astrocytic processes that were the result of transmembrane Ca^{2+} flux, Ca^{2+} signals that previously went undetected using organic Ca^{2+} dyes. Although further experiments are required to determine the nature of these astrocytic microdomain signals, this study suggests that Lck-tagged GCaMP provides a powerful strategy to monitor near-membrane signals in small volume cellular compartments.

A new height of target specificity in Ca^{2+} imaging was reached in a recent study in which a genetically encoded troponin-based Ca^{2+} indicator, TN-XL, was tethered to the carboxy terminus of $Ca_V2.2$ channels heterologously expressed in HEK293 cells [79]. In combination with TIRF microscopy, this approach allows detection of nanodomain changes in fluorescence solely at the pore of the Ca^{2+}-permeable channel of interest, without interfering fluorescence from regions outside the vicinity of the Ca^{2+} channel. In fact, FRET-based estimates approximate a distance of 55 Å between the sensor and the cytoplasmic mouth of the channel. With stronger buffering of 10 mM EGTA, Ca^{2+} nanodomains were estimated from TN-XL fluorescence to be just 40 nm in diameter, roughly two-fold larger than estimates of the diameter of a single Ca^{2+} channel. Importantly, it was shown that fused Cav2.2/TN-XL is resistant to endogenous proteolysis and addition of this sensor to the channel structure did not substantially interfere with channel gating kinetics or pore conductance, although resulting currents were actually slightly enhanced. These innovations provide new insights for the future use of progressively improved genetically encoded indicators fused to Ca^{2+}-permeable channels to isolate nanodomain Ca^{2+} signals at the pore of single channels.

8. Conclusions

Subcellular compartmentalization of intracellular Ca^{2+} is essential to the versatility of this ionic second messenger. Optical analysis of local Ca^{2+} signals arising through single or clusters of Ca^{2+}-permeable channels represents a powerful new approach with the potential for revealing important new information regarding the regulation of Ca^{2+} channels in excitable cells during health and disease. Used as an adjunct technique to patch clamp electrophysiology, TIRF microscopy has already lead to major improvements in our understanding of functional regulation and spatial characteristics of LTCC-mediated Ca^{2+} influx in excitable cells. One can anticipate that further development of novel tools to aid in optical investigation of LTCC function will ultimately facilitate a more complete understanding of channel regulation relating to the role these channels play in numerous cellular processes.

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Highlights

- Advances in imaging technology (TIRF and confocal) have allowed optical analysis of L-type Ca^{2+} channel activity with high spatiotemporal resolution in excitable cells.
- L-type Ca^{2+} channel activity varies regionally throughout the plasmalemma of excitable cells.
- A subpopulation of L-type Ca^{2+} channels can operate in a cooperative or coupled manner with profound implications in health and disease.
- Emerging approaches such as chemical or genetically encoded Ca^{2+} indicators are promising developments in our pursuit of understanding nano- and microdomain Ca^{2+} signals.

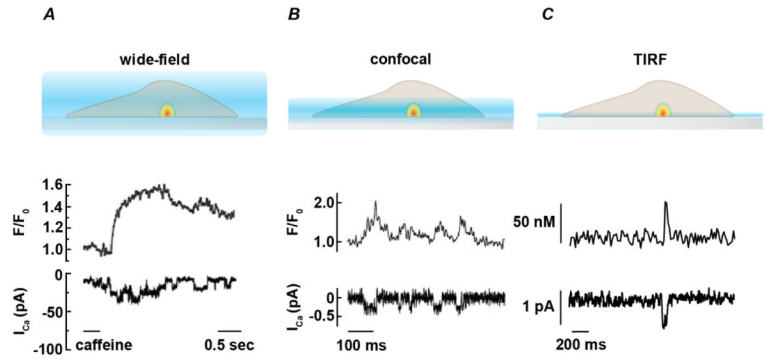

Figure 1. Relationship between excitation field and temporal resolution of fluorescent signals

A. Schematic representation (*top*) showing excitation pattern (*blue*) for conventional wide-field fluorescent microscope. Fluorescent signal from cytosolic Ca^{2+} indicator is collected from within and outside of focal plane. Images reflect Ca^{2+} signals near point source (Ca^{2+}-permeable channel) as well as Ca^{2+} accumulation in the cytosol. Therefore, rise and decay of the observed fluorescent signal is slower than corresponding single channel current measurements (*bottom*, redrawn from [16]). ***B***. Cartoon representing excitation profile for confocal microscope with representative single channel current (I_{Ca}) and $[Ca^{2+}]_i$ (F/F_O) traces using line scan confocal microscopy (redrawn from [19]). Excitation field and Ca^{2+} influx is represented as in *A*. ***C***. Representation of excitation profile for total internal reflection fluorescence (TIRF) microscope with representative single channel current (I_{Ca}) and $[Ca^{2+}]_i$ (F/F_O) traces (redrawn from [24]). Excitation field and Ca^{2+} influx is represented as in *A*.

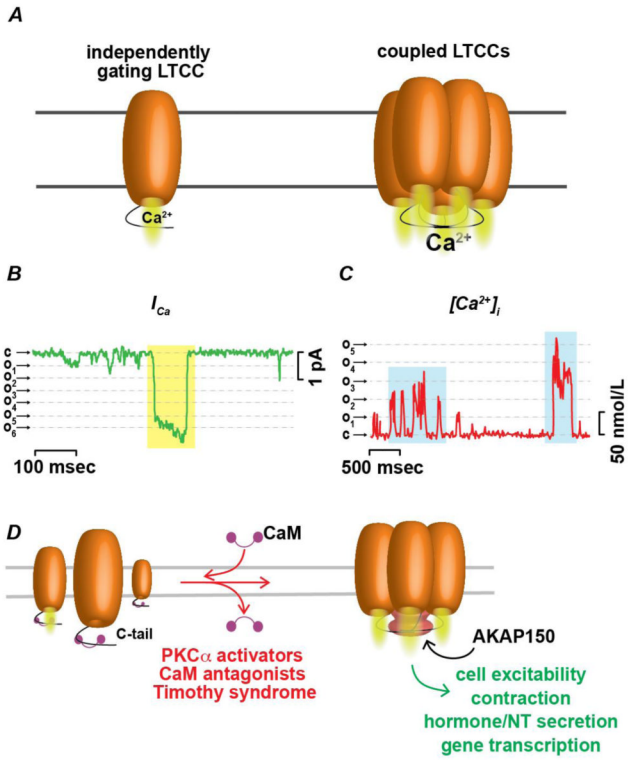

Figure 2. Mechanisms leading to coupled gating between physically interacting LTCCs

A Cartoon showing Ca^{2+} influx via independently gating LTCC (*left*) and Ca^{2+} influx via multiple physically coupled channels opening and closing in synchrony. ***B*** Representative single and coupled (*yellow highlight*) LTCC current record during step depolarization from −80mV to −30 mV. Dashed lines show amplitude of quantal levels. ***C*** Representative $[Ca^{2+}]_i$ trace showing single and coupled (*blue highlights*) Ca^{2+} influx events. (*B* and *C* redrawn from [37]) ***D*** Proposed mechanism of coupled gating between LTCCs. In this model, calmodulin (CaM) binding of LTCC carboxy (COOH) termini prevents interaction between neighboring channels. Dissociation of CaM from LTCCs facilitates physical interaction between C-termini via association with the scaffolding protein AKAP150. Coupling is promoted pharmacologically by CaM antagonists, activators of PKCα, and during pathological conditions of timothy syndrome and hypertension. Coupled gating of LTCCs may represent a ubiquitous physiological and pathological mechanism mediating local amplification of Ca^{2+} influx across excitable cells potentially contributing to fine control of excitability, contractility, secretory and transcriptional pathways.