

Calcium Signaling

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Calcium ions (Ca²⁺) impact nearly every aspect of cellular life. This review examines the principles of Ca²⁺ signaling, from changes in protein conformations driven by Ca²⁺ to the mechanisms that control Ca²⁺ levels in the cytoplasm and organelles. Also discussed is the highly localized nature of Ca²⁺-mediated signal transduction and its specific roles in excitability, exocytosis, motility, apoptosis, and transcription.

In the furnaces of the stars the elements evolved from hydrogen. When oxygen and neon captured successive α particles, the element calcium was born. Roughly 10 billion years later, cell membranes began to parse the world by charge, temporarily and locally defying relentless entropy. To adapt to changing environments, cells must signal, and signaling requires messengers whose concentration varies with time. Filling this role, calcium ions (Ca²⁺) and phosphate ions have come to rule cell signaling. Here, I describe our current understanding of Ca²⁺-mediated signaling (complementing several excellent reviews [Berridge, 2005; Burgoyne, 2007; Carafoli, 2004; Petersen, 2005; Rizzuto and Pozzan, 2006]) and place particular emphasis on emerging themes related to Ca²⁺ binding proteins, Ca²⁺ entry across the plasma membrane, and the localized nature of Ca²⁺ signals.

Ca²⁺: One Ion, Two Charges

Protein function is governed by shape and charge. Ca²⁺ binding triggers changes in protein shape and charge. Similarly, phosphorylation imparts a negative charge, altering protein conformations and their interactions (Westheimer, 1987). Protein kinases, comprising ~2% of eukaryotic genomes, remove phosphate from ATP and covalently attach it to the free hydroxyl groups of serine, threonine, or tyrosine residues. The abilities of Ca²⁺ and phosphate ions to alter local electrostatic fields and protein conformations are the two universal tools of signal transduction.

Cells invest much of their energy to effect changes in Ca²⁺ concentration ([Ca²⁺]). Underlying the speed and effectiveness of Ca²⁺ is the 20,000-fold gradient maintained by cells between their intracellular (~100 nM free) and extracellular (mM) concentrations. In contrast, the concentration of Ca²⁺'s cousin, Mg²⁺, barely differs across the plasma membrane. Why is Ca²⁺ so avidly excluded from the cytosol? One reason is that Ca²⁺ binds water much less tightly than Mg²⁺ and precipitates phosphate. Hence, cells have evolved ways to sequester this dangerous divalent, perhaps at first to simply reduce its cytosolic levels but later to use its binding energy for signal transduction. Unlike complex molecules, Ca²⁺ cannot be

chemically altered. Thus, to exert control over Ca²⁺, cells must chelate, compartmentalize, or extrude it. Hundreds of cellular proteins have been adapted to bind Ca²⁺ over a million-fold range of affinities (nM to mM), in some cases simply to buffer or lower Ca²⁺ levels, and in others to trigger cellular processes. The local nature of Ca²⁺ signaling is intimately tied to this large range of affinities.

The calcium ion can accommodate 4–12 oxygen atoms in its primary coordination sphere, with 6–8 being most common. The chelating compounds EDTA and EGTA cage Ca²⁺ via a doublet of two amine and four carboxylate groups. In comparison, nature's specialized Ca²⁺ binding proteins, the oxygen atoms of carboxyl and carbonyl groups (and sometimes water) coordinate binding to Ca²⁺. Typically, six to seven oxygen atoms surround Ca²⁺ at ~2.5 Å in a pentagonal bipyramid (Figure 1A) (Strynadka and James, 1989). The professional protein chelator of Ca²⁺ is the EF hand domain (named after the E and F regions of parvalbumin) (Nakayama and Kretsinger, 1994), which is present in hundreds of proteins. Helix-turn-helix motifs are common in proteins ranging from channel voltage sensor "paddles" to DNA-binding proteins. In EF hand helix-turn-helix motifs, negatively charged oxygen atoms cradle Ca²⁺ within a ~12 amino acid loop between two orthogonal α helices (Figure 1B). The affinities of EF hand domains for Ca²⁺ vary ~100,000-fold depending on a variety of factors ranging from critical amino acids in the Ca²⁺ binding loop to side-chain packing in the protein core.

Ca²⁺ Sensor and Adaptor Proteins *Calmodulin, the Archetypal Sensor/Adaptor Protein*

Calmodulin (CaM1-4) is a small, ubiquitous adaptor protein that amplifies Ca²⁺'s diminutive size to the scale of proteins. No other molecule more dramatically emphasizes the evolutionary importance of Ca²⁺ signaling. Having changed only slightly over 1.5 billion years of evolution and being transcribed from three separate chromosomes in humans, expression levels of this protein shaped the beaks of Darwin's finches (Abzhanov et al., 2006).

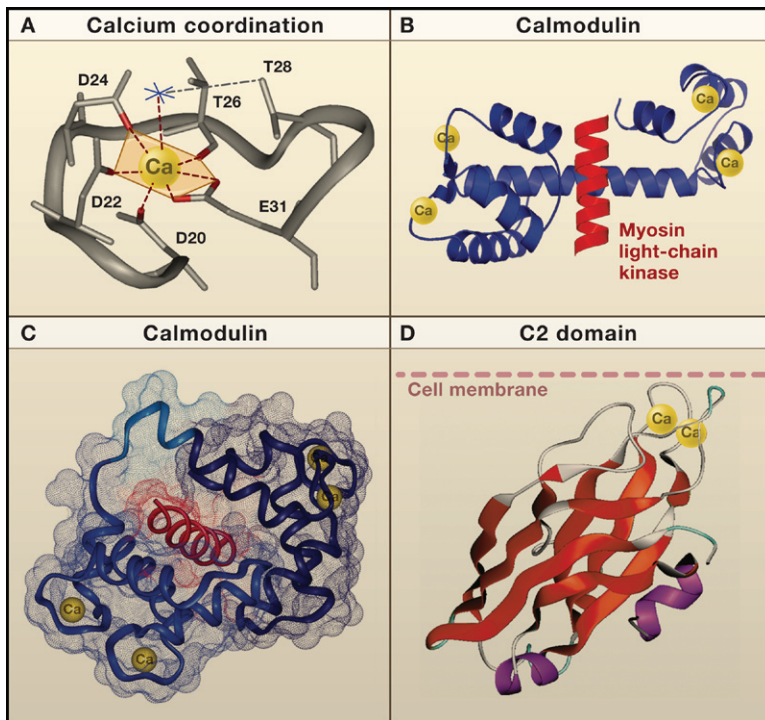


Figure 1. Oxygen Atoms of Amino Acids Chelate Ca^{2+}

(A) Ca^{2+} is coordinated by seven oxygen atoms (five in the plane of the orange pentagon and two perpendicular to the plane, thus forming a bipyramidal pentagon). Adapted from the EF hand calcium-binding proteins data library (http://structbio.vanderbilt.edu/chazin/cabp_database).

(B) Calmodulin has four EF-hand motifs, each with distinct affinities for Ca^{2+} , two at each end of a long jointed α helix. Ca^{2+} binding to calmodulin exposes hydrophobic surfaces. A helical peptide from myosin light chain kinase is colored red. This extended conformation is not stable in solution, but illustrates calmodulin's domains (constructed with Pymol using PDB 3CLN). The red helix represents a hypothetical target peptide.

(C) Ca^{2+} -bound calmodulin wraps around basic amphipathic helices of proteins. Adapted from the EF-Hand Calcium-Binding Proteins Data Library (http://structbio.vanderbilt.edu/cabp_database/cabp.html).

(D) C2 domains bind Ca^{2+} in variable loops containing negatively charged amino acids. The scaffold is an antiparallel β sandwich with an exposed protein-interaction surface. The dashed blue line is the hypothetical plasma membrane (C2 domain of protein kinase C β . PDB 1a25 from the OPM database; <http://opm.phar.umich.edu>).

When Ca^{2+} binds, the shape of the calmodulin domains change, triggering their ability to relieve protein autoinhibition, remodel active sites, and dimerize proteins (Hoeftlich and Ikura, 2002). Hundreds of proteins contain calmodulin recruitment sites characterized by interspersed basic and bulky hydrophobic amino acids bracketed by aromatic residues. Calmodulin is shaped like a dumbbell, but with a flexible joint in its middle (Meador et al., 1992, 1993). The EF hands of calmodulin have distinct affinities for Ca^{2+} , and their binding affinities are often increased by interaction with target proteins. Binding of Ca^{2+} is associated with a large change in conformation and exposure of hydrophobic surfaces within each domain, which triggers calmodulin's Ca^{2+} sensor activity (binding to its targets). Hydrophobic residues, usually containing methionine, wrap around amphipathic regions of target proteins, such as the α helices in myosin light chain kinase (MLCK; Figures 1B and 1C) and calmodulin dependent kinase II (CaMKII). In many cases, both domains wrap around the target, compacting the structure into a globular shape (for movies, see <http://www.molmovdb.org/cgi-bin/morph.cgi?ID=180968-23252>). This Ca^{2+} switch has also been cleverly adapted to a fluorescence resonance energy transfer-based Ca^{2+} sensor (Palmer and Tsien, 2006) and, mimicking nature, will likely be engineered to do much more. Calmodulin also extends the reach of Ca^{2+} by activating phosphorylation pathways. Ca^{2+} /calmodulin binding relieves autoinhibition of the catalytic domain of calmodulin kinase (CaMK) family enzymes. CaMKs multimerize, leading to auto- and interphosphorylations that prolong kinase activity.

S100 Ca^{2+} -sensing proteins are the largest family of EF hand proteins (>25 human genes), putatively targeting more than 90 proteins. Like calmodulin, Ca^{2+} binding in S100 proteins triggers exposure of hydrophobic surfaces to target proteins. Some S100 proteins, as homo- and heterodimers of two 2-EF-hand subunits, assemble/disassemble protein complexes (such as those containing tubulin or p53), although much remains to be discovered (Santamaria-Kisiel et al., 2006). Neuronal Ca^{2+} sensor (NCS) proteins have four EF hands, binding three (frequency, neurocalcin- δ , GCAP) or two (KChIP) Ca^{2+} ions (Burgoyne, 2007). Their structures are also compact and globular, relying on surface charge and membrane association for specificity. Several NCS proteins (recoverin, hippocalcin, VILIP1-3) are Ca^{2+} -triggered switchblades in which a myristoyl group is unsheathed, enabling attachment to membranes.

C2 Domains

Lipid bilayers are like a workbench, holding proteins on a surface to organize their function and increase speed by reducing diffusion from three dimensions to two. Not surprisingly, many proteins have evolved domains that place, or remove, them from the lipid bilayer. Ca^{2+} , with its positive charge, is often used to change a protein's location in the cell from the cytoplasm to a membrane surface (translocation).

A C2 domain is an ~ 120 amino acid segment with a common fold, an 8-stranded antiparallel β sandwich connected by variable loops (Cho and Stahelin, 2005). In many C2 domains, binding of two or three Ca^{2+} ions in the three variable loops creates a substantial electrostatic potential that accelerates a protein's association

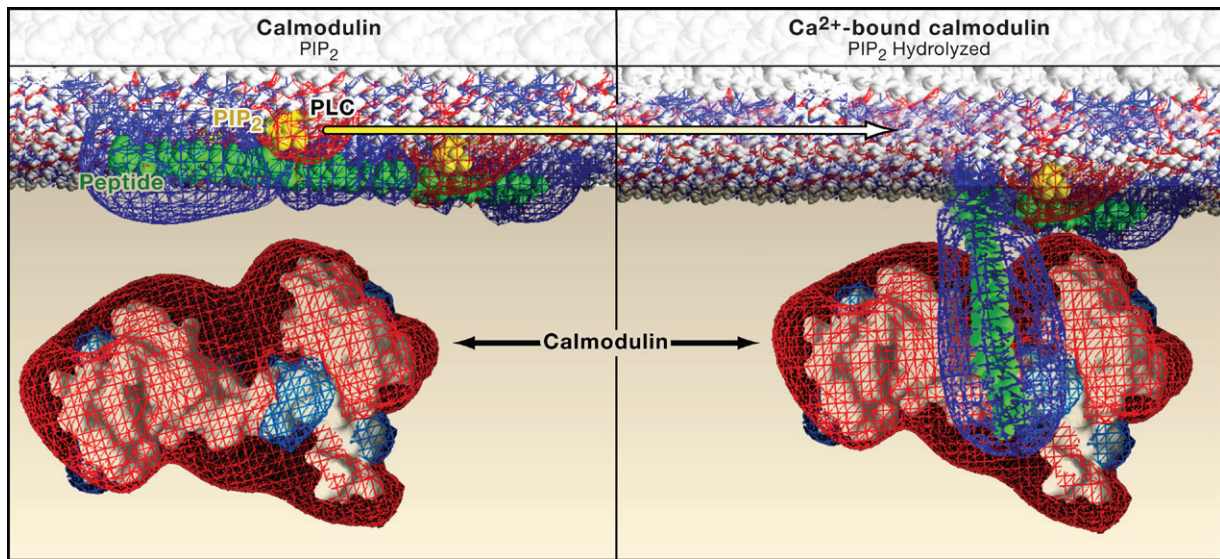


Figure 2. An Electrostatic Switch Mechanism

Membrane-bound phosphatidylinositol 4, 5 bisphosphate (PIP_2) and cytosolic calmodulin are both highly negatively charged (red field lines). PIP_2 accumulates near a positively charged (blue field lines), amphipathic region of a protein, or corresponding peptide, and pins it to the inner leaflet. An increase in local $[\text{Ca}^{2+}]$ activates calmodulin, which “pulls” the basic region off from the membrane. Activation of phospholipase C (PLC) hydrolyzes PIP_2 , which also can reduce the basic cluster’s interaction with the membrane. The best evidence for this mechanism comes from peripheral proteins, such as K-Ras, and MARCKS (McLaughlin and Murray, 2005), but similar mechanisms may also affect the Ca^{2+} -permeant, PIP_2 - and Ca/CaM -gated TRP channels. This is modified from original figures provided by Murray and McLaughlin (McLaughlin et al., 2005; McLaughlin et al., 2002). Ca/CaM in the cytoplasm is shown in its open conformation. PIP_2 -charged head groups are yellow; the hydrophobic peptide with interspersed basic amino acids is green. Blue lines, +25 mV; Red lines, -25 mV electrostatic potentials. Calmodulin image reproduced from *The Journal of General Physiology* (2005) 126, 41–53. Copyright 2005 The Rockefeller University Press.

with anionic membrane leaflets, such as the cytoplasmic surface of eukaryotic plasma membranes and the outer leaflet of outer mitochondrial membranes (Figure 1D). Neutralization of charge by Ca^{2+} binding in the variable loops of some proteins may allow penetration of hydrophobic and aromatic amino acids into the bilayer. The C2 domain is common in signal-transduction proteins; there are approximately 650 human proteins listed in the protein family (Pfam) database with C2 architectures. These include well-known signaling proteins such as phospholipases, protein-kinase C (PKC), phosphoinositide 3-kinase (PI3K), synaptotagmins, rabphilin, and Munc. Additional specificity is conferred upon this group of proteins by a second domain. For example, C1 domains bind diacylglycerol, while pleckstrin homology (PH) and PX domains bind phosphatidylinositol lipids with specificity determined by phosphate positions in the inositol ring. Thus, increases in $[\text{Ca}^{2+}]$ initiate translocation of proteins with C2 and other domains (e.g., protein kinase C family proteins) to specific regions of membranes containing their substrate. Another Ca^{2+} -dependent membrane targeting scheme is employed by annexins, where phosphoryl moieties of the membrane replace charge from carbonyl oxygens and water in a unique Ca^{2+} -binding fold (Gerke et al., 2005).

PIP₂ and Calmodulin Switching

Both phosphatidylinositol 4, 5 bisphosphate (PIP_2) and calmodulin (either Ca^{2+} bound or free) are highly negatively charged, but PIP_2 is bound to inner leaflets of

plasma membranes by its acyl chains, whereas calmodulin is soluble and cytosolic. Both are ubiquitous and abundant. Clusters of positively charged residues on many peripheral (e.g., K-Ras, MARCKS) and integral (e.g., the juxtamembrane regions of ion channels and the EGFR) proteins produce a local positive potential that acts as a basin of attraction for PIP_2 , both enhancing the local concentration of PIP_2 and pulling the cluster close to the membrane. Negatively charged calmodulin competes with PIP_2 and may pull the positively charged protein segment off the membrane (Figure 2) (McLaughlin and Murray, 2005). Further elaboration of this mechanism of competitive switching is enabled by PIP_2 generation/hydrolysis, PKC phosphorylation of the basic cluster, and the degree of Ca^{2+} binding to calmodulin.

Ca²⁺ Pumps Set the Stage for Signaling

Like Sisyphus, ATPase pumps are condemned to push Ca^{2+} uphill for eternity into the endoplasmic reticulum (ER) (via sarcoendoplasmic reticular Ca^{2+} ATPases; SERCA pumps) or out of the cell (via plasma membrane Ca^{2+} ATPases; PMCA pumps) (Figure 3A). To maintain low cytoplasmic $[\text{Ca}^{2+}]$, ATPases exchange protons for two (SERCA) or one (PMCA) Ca^{2+} per ATP hydrolyzed. SERCA (ATP2A1-3) and PMCA (ATP2B1-4) Ca^{2+} pumps are P type ATPases, defined by an obligatory aspartyl phosphate intermediate in the pump cycle (Strehler and Treiman, 2004). A second mechanism, the $\text{Na}^+/\text{Ca}^{2+}$ exchangers (NCX, or SLC8A1-3), and the $\text{Na}^+/\text{Ca}^{2+}$ - K^+

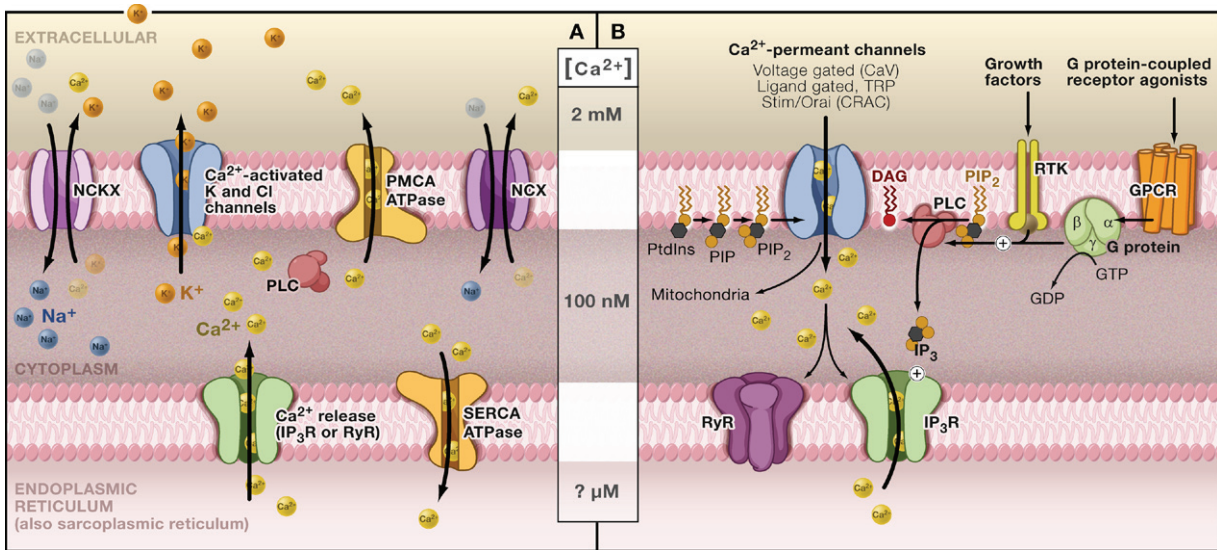


Figure 3. Maintaining and Using Ca²⁺ Gradients for Signaling

(A) The cytoplasmic Ca²⁺ level is low in resting cells. Cytoplasmic [Ca²⁺]_i is maintained at ~100 nM by extrusion via plasma membrane Ca²⁺ ATPase (PMCA) and smooth endoplasmic reticulum Ca²⁺ ATPase (SERCA) transporters. The Na/Ca exchanger (NCX), a major secondary regulator of [Ca²⁺]_i is electrogenic, exchanging three Na ions for one Ca²⁺. Intracellular Ca²⁺ hyperpolarizes many cells by activating K⁺ channels, and in some cells, Cl⁻ channels. This decreases CaV channel activity but increases the driving force across active Ca²⁺-permeant channels.

(B) The core of the Ca²⁺ signaling network. In excitatory Ca²⁺ signaling, plasma membrane ion channels are triggered to open by changes in voltage, or extra- or intracellular ligand binding. When open, ~1 million Ca²⁺ ions/s/channel flow down the 20,000 fold [Ca²⁺]_o gradient (E_{Ca} ~ +150 mV), maintained by elements shown in (A). Initial increases in [Ca²⁺]_i trigger more release, primarily from ER via Ca²⁺-sensitive ryanodine receptors (RyR). G protein-coupled receptor (GPCR) or receptor tyrosine kinase-mediated activation of PLC cleaves PIP₂ into inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ is a ligand for the intracellular IP₃R channel spanning the membrane of the ER. GPCRs catalyze the exchange of guanosine diphosphate (GDP) for GTP on Gα subunits, releasing active Gα and Gβγ subunits that in turn activate PLCβ. RTKs dimerize upon ligand binding, autophosphorylate, and interact with other signaling proteins to activate PLCβ.

exchangers (NCKX; SLC24A1-5) exchange one Ca²⁺ ion for three Na⁺ ions (NCX) or cotransport one K⁺ ion with one Ca²⁺ ion in exchange for four Na⁺ ions (NCKX). Running in their “forward” modes, inward (depolarizing) Na⁺ current drives Ca²⁺ extrusion. The high-affinity, low-capacity PMCAs and the low-affinity, high-capacity Na⁺/Ca²⁺ (-K⁺) exchangers complement each other. The PMCAs are effective at maintaining low internal [Ca²⁺]_i over long durations, whereas NCX and NCKX can make the rapid adjustments needed during generation of action potentials in neurons (Hilgemann et al., 2006). Calmodulin can substantially increase both PMCA Ca²⁺ affinity and ATPase pump rate.

Opening the Ca²⁺ Floodgate

Voltage-gated Ca²⁺-selective channels (CaVs) are the fastest Ca²⁺ signaling proteins and initiate dramatic changes within a single cell (Figure 3B). Each channel conducts roughly a million Ca²⁺ ions per second down the 20,000-fold gradient; a few thousand channels/cell can effect >10-fold changes in intracellular levels within milliseconds. Like transistors, the triggering messengers are the photons of the electromagnetic field. The channel’s antenna is a paddle-shaped helix-turn-helix loop containing positively charged residues (usually arginines) (Long et al., 2005). A change in voltage moves the paddle that in turn pulls the channel “gate” open. Hodgkin and

Huxley first documented that, unlike transistors, conductances in biological membranes can be highly selective. Calcium selectivity is a consequence of moderately high-affinity Ca²⁺ binding in the pore of the channel. Based on mutagenesis of Ca²⁺ channels and inferences from other Ca²⁺-binding proteins such as Ca²⁺-ATPases, seven oxygens contributed by aspartate and glutamate side chains are likely to form the Ca²⁺ cage (Gouaux and Mackinnon, 2005). In the presence of normal extracellular [Ca²⁺]_o, Ca²⁺ block is essential for selectivity as only Ca²⁺ can bind and then enter the pore. When external Ca²⁺ is removed, Ca²⁺ channels become nonselective and allow Na⁺ and K⁺ to transit the membrane.

Cell-to-Cell Signaling

The simplest type of Ca²⁺ compartmentalization is established by the cell’s plasma membrane. Ca²⁺ signaling occurs between cells in two ways. First, cellular Ca²⁺ autonomy can be circumvented by gap junction (connexin) channels, as often occurs in epithelia and always in cardiomyocytes. More commonly, cell-to-cell signaling is effected by transmitter-gated, usually Ca²⁺ permeant, ion channels (e.g., NMDA, nicotinic, purinergic ionotropic). Voltage-gated Ca²⁺ channels (CaV) rapidly increase periplasmic [Ca²⁺]_o that in turn trigger protein-fusion machines (e.g., synaptotagmins and SNARE complexes), enabling vesicles containing transmitter

molecules to fuse to the plasma membrane. Small molecules (ATP, acetylcholine) and single amino acids (e.g., glutamate) released outside the cell gate Ca^{2+} -permeant channels (P2X, nicotinic receptors, NMDA receptors) on adjacent cell membranes. These Ca^{2+} -mediated events dominate much of neuroscience, and corollaries are now appreciated in extracellular communication between almost all cell types. Ca^{2+} control of synaptic release and Ca^{2+} entry via neurotransmitter channels is reviewed elsewhere (Jahn and Scheller, 2006; Sudhof, 2004).

TRP Channels

As these were recently reviewed (Ramsey et al., 2006), only a few points about transient receptor potential (TRP) ion channels are summarized here. TRP ion channels are formed by tetrameric assembly around a pore. Most are weakly voltage-sensitive, nonselective ion channels. The sole yeast, and some mammalian, TRP channels span only intracellular membranes. The majority of the 28 mammalian TRPs comprise plasma membrane channels that depolarize cells and increase intracellular Na^+ and Ca^{2+} . Many TRP channels are greatly potentiated by phospholipase C (PLC) activation by G protein-coupled or tyrosine-kinase receptors (TKR). Intracellular Ca^{2+} gates some mammalian TRP channels but modulates practically all TRP channels. Their physiological roles are most clearly established in sensory systems, and indeed many are activated by environmental signals such as temperature change, pH, volatile chemicals, and plant compounds, but their functions are probably much broader. The central unanswered question in this field is how TRP channels are normally activated *in vivo*.

TRP channels are frequently, but incorrectly, called store-operated channels. In a strict definition of a store-operated channel, the channel is activated by store depletion even when cytoplasmic $[\text{Ca}^{2+}]$ levels are buffered to low levels (otherwise, all Ca^{2+} -activated, and modulated, Ca^{2+} -permeant channels would be store operated channels). Thus far, TRP channels by themselves do not satisfy these conditions.

Store-Operated Ca^{2+} Entry; the Stim/Orai Channel (SOC) Mechanism

Ca^{2+} is constantly seeping out of the ER into the cytoplasm. SERCAs tirelessly pump it back into the ER. If these pumps are blocked, ER $[\text{Ca}^{2+}]$ runs down. Similarly, prolonged incubation of many cells in low Ca^{2+} media allows the PMCAs to extrude the leaked Ca^{2+} , depleting ER Ca^{2+} . In many nonexcitable cells, Ca^{2+} entry across the plasma membrane is infrequent (blood cells for instance are exceptionally "tight"). IP_3 receptor (IP_3R)-mediated release of Ca^{2+} from the ER in response receptor activation empties the ER as PMCAs pump Ca^{2+} out of the cell faster than it can be repleted. Slowly over seconds after such store depletion, a Ca^{2+} entry mechanism is activated. This mechanism is called store-operated Ca^{2+} entry (Putney, 2005). Researchers have paid special attention to a slow, tiny, but highly selective Ca^{2+} conductance that is

activated when ER $[\text{Ca}^{2+}]$ drops (CRAC or Ca^{2+} -release activated current; I_{CRAC}) (Parekh and Penner, 1997). Most importantly, CRAC is activated by a decline in ER $[\text{Ca}^{2+}]$ but not by a rise in cytoplasmic $[\text{Ca}^{2+}]$. Although TRPs and other channels were widely proposed for this role, only CRAC, so far, demonstrates activation that is independent of cytoplasmic Ca^{2+} , consistent with it being critical for store-operated Ca^{2+} entry.

The single-channel conductance of I_{CRAC} is ~ 15 femto-siemens (fS), approximately 1000-fold lower than that of other ion channels (Prakriya and Lewis, 2006) (total CRAC current from an entire cell is only ~ 5 – 10 pA). T cells from two brothers suffering from immunodeficiency had long ago been shown to lack I_{CRAC} (Partiseti et al., 1994). In T cells, the primary Ca^{2+} entry pathway is CRAC; Ca^{2+} -dependent dephosphorylation of the nuclear factor of activated T cells (NFAT) by calcineurin initiates its translocation to the nucleus for regulation of chemokine genes (Feske, 2007). The first clue as to the molecular component of store-operated Ca^{2+} entry came when STIM1, a single transmembrane-spanning domain protein primarily residing in the ER, was found to be essential for I_{CRAC} activation (Roos et al., 2005). STIM1's N terminus sterile α motif may seed multimerization, whereas its Ca^{2+} -binding EF hand is presumably the sensor of ER $[\text{Ca}^{2+}]$ (Lewis, 2007). Subsequently, a four-transmembrane domain plasma-membrane protein, Orai1 (Feske et al., 2006; Zhang et al., 2006), was shown to be required for CRAC activity, and mutagenesis of its pore proved that it was the channel-forming subunit (Prakriya et al., 2006; Yeromin et al., 2006).

Upon ER Ca^{2+} depletion, STIM1 aggregates in the ER just below Orai1 in the plasma membrane (Figure 4A). Although apposition of the ERM domain-containing C terminus of STIM1 is within 25 nm of Orai1, a direct link has not been established (Wu et al., 2006). What causes STIM1 to cluster at plasma-membrane regions adjacent to ER membranes, and what gates Orai? Related to these details is a more important question. Like the interaction between the CaV-Ryanodine receptor Ca^{2+} calcium channels in muscle (see section below), the Stim/Orai channel complex brings Ca^{2+} into a narrow region between the plasma membrane and the ER. What are the targets of this localized release? Interestingly, sub-membranous mitochondria receive (and buffer) much of this localized Ca^{2+} increase. Surely there is much cell biology remaining to be uncovered, and it may have little to do with ER Ca^{2+} repletion.

Ca^{2+} Acts Locally

Like an aged homeowner, evolution has crowded every nook and cranny of the cell with its handiwork of lipids and proteins. Thus, nonuniformity, cooperativity, and compartmentalization are essential features of cell biology, even on the nanomolar scale. Hydrated Ca^{2+} can diffuse $40 \mu\text{m}$ in 1 s (40 nm/ms) in simple saline solution (Einstein, 1905). This mobility is never realized in the crowded, charged cytosolic world where the freedom

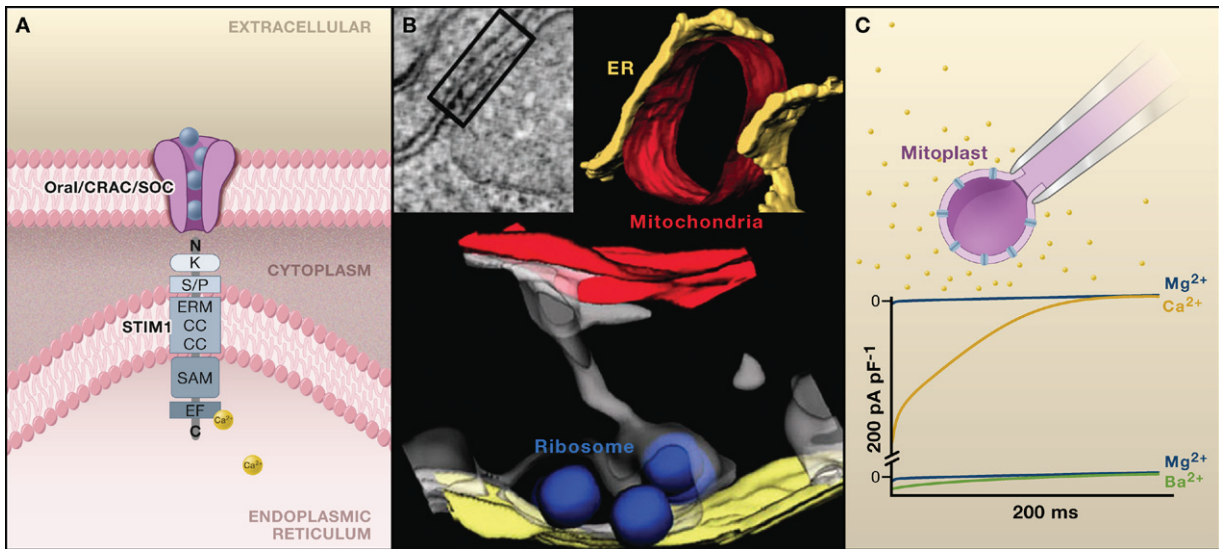


Figure 4. Emerging Ca^{2+} Pathways

(A) Depletion of Ca^{2+} stores activates the Stim/Orai complex. The Ca^{2+} release-activated current (I_{CRAC}) is activated by depletion of Ca^{2+} in the ER. The EF-hand protein, STIM1, presumably senses low $[\text{Ca}^{2+}]$ levels in the ER and migrates within the ER membrane to within 25 nm of the plasma membrane. STIM1 multimerizes, and the CRAC channel is activated. The SAM (sterile- α motif), ERM (ezrin-radixin-moesin), CC (coiled-coil), and S/P (serine-proline-rich) and K (lysine-rich) domains presumably mediate multimerization and other protein interactions. The CRAC channel is a plasma membrane channel comprising four transmembrane subunits (Orai1).

(B) Ca^{2+} and mitochondria. Mitochondria preferentially take up Ca^{2+} from the closely apposed ER. Tomogram (left) of ER flanking mitochondria in a chicken B cell line (DT40 cells) lacking all IP₃R_s (cells are derived from a triple knockout). Scale bar, 250 nm. The mitochondrial outer membrane (red) is tethered (gray, enlarged below) to the ER (yellow); this is modified from (Csordas et al., 2006).

(C) Ca^{2+} current (MiCa) measured from an individual mitoplast under voltage control. Ca^{2+} is driven across the inner mitochondrial membrane by its -150 to -200 mV voltage gradient. MiCa is highly selective, and poorly permeant to even Ba^{2+} and Mg^{2+} divalent ions. Ca^{2+} entering via the MiCa channel (mitochondrial uniporter) stimulates Ca^{2+} -sensitive enzymes of the tricarboxylic acid structure, increasing ATP production. Images reproduced from *The Journal of Cell Biology* (2006) 174, 915–921. Copyright 2006 The Rockefeller University Press.

of Ca^{2+} is measured in nanometers and microseconds. Ca^{2+} exits single ion-channel pores at rates >1 per μs , but fixed and mobile endogenous buffers limit Ca_v -mediated changes in $[\text{Ca}^{2+}]$ to $10 \mu\text{M}$ levels within 20 nm (Naraghi and Neher, 1997), a volume that can accommodate several 40 Å calmodulin molecules (Figure 5). The steep Ca^{2+} gradient around entry sites can give rise to nonhomogeneous activation of Ca^{2+} binding proteins with similar Ca^{2+} affinities. Countering these steep gradients are mobile buffers and mobile Ca^{2+} -trigger proteins, which prolong the Ca^{2+} signal and increase its effective length constant.

The fine reticular ER and mitochondria spread like a vast three-dimensional spider web within cells, actively sequestering Ca^{2+} . The distributed nature of these Ca^{2+} compartments insures that Ca^{2+} is only briefly free before encountering an extrusion (PMCA, NCX) or uptake (SERCA/MiCa) mechanism. Intracellular Ca^{2+} compartments are also Ca^{2+} distribution systems. The pinnacle of evolutionary success in this regard is skeletal muscle, where the sarcoplasmic reticulum is configured to provide Ca^{2+} to the adjacent muscle proteins in a manner that is as efficient and rapid as possible. Rapidity is ensured by two means; first, the light-speed messenger, voltage, activates Ca_v channels. Normally confined to the surfaces of cells, skeletal muscle Ca_v s are distributed throughout the T tubule network, which are deep

invaginations of the plasma membrane. Charged residues in Ca_v s move in response to a change in the transmembrane voltage; the ensuing conformational changes are translated directly to the RyR channels of the sarcoplasmic reticulum. Combining the surface invaginations of the T tubule network, the distributed sarcoplasmic reticulum, and direct coupling between depolarization and RyR channel opening, insures rapid and near simultaneous release of Ca^{2+} to bind adjacent troponin and enable myosin-actin contraction. Skeletal muscle fibers are syncytia, fused from multiple single-muscle cells. In heart, where speed is less important than fidelity, single cells remain distinct, but gap junctions enable them to function as syncytia. In the event of rogue firing by groups of heart cells, gap junctions between metabolically or electrically disparate cardiac cells close to isolate the arrhythmia.

In most cells, the ER/sarcoplasmic reticulum and mitochondrial Ca^{2+} stores are not homogeneously distributed. Mitochondria can divide or fuse and are moved about via microtubule and actin networks (Rice and Gelfand, 2006). Miro, an intrinsic mitochondrial protein with 2 EF hand and GTPase domains, binds to Milton, an adaptor to kinesin (Glater et al., 2006; Stowers et al., 2002). In a kind of ER-mitochondrial Ca^{2+} synapse, mitochondria and ER share sites of close apposition where Ca^{2+} release from ER is preferentially taken up by mitochon-

dria (Rizzuto et al., 1998). Electron microscopic tomographic studies show that smooth ER and mitochondria are joined by 10 nm tethers; experimental shortening of the tethers induces mitochondrial Ca^{2+} overload (Csordas et al., 2006) (Figure 4B). In neurons, they are often found clustered at sites of high channel activity. In skeletal and cardiac muscle, mitochondria closely associate with Ca^{2+} release sites of the sarcoplasmic reticulum but do not appear to form direct connections with RyR channels (Franzini-Armstrong, 2007). Mitochondria also accumulate at immunological synapses, buffer local Ca^{2+} , regulate CRAC current, and influence T cell activation (Hoth et al., 2000). Presumably, the high Ca^{2+} at these active sites drives mitochondrial ATP production (via the Ca^{2+} -sensitive pyruvate-, α -ketoglutarate-, and isocitrate dehydrogenases), but they are also in position to act as high-capacity Ca^{2+} buffers and sentinels for Ca^{2+} overload in order to trigger apoptosis.

Although little is known of free $[\text{Ca}^{2+}]$ in ER (net $[\text{Ca}^{2+}] \sim 1 \text{ mM}$), $[\text{Ca}^{2+}]$ is undoubtedly heterogeneous due to the uneven distribution of ER Ca^{2+} -binding proteins such as calsequestrin, calreticulin, calnexin, and immobile Ca^{2+} buffers. The heterogeneities of $[\text{Ca}^{2+}]$ within the ER may affect protein processing (e.g., via Ca^{2+} -sensitive chaperones such as the Hsp70 protein, BiP). Given the local nature of Ca^{2+} signaling, the heterogeneity of Ca^{2+} release from different parts of the ER must affect Ca^{2+} -dependent processes, but little is known in this submicroscopic, time-varying domain.

Neurons expand the opportunities for localized Ca^{2+} signaling within a single cell by orders of magnitude. Extremely long processes enable localized signaling to be integrated and modified. Even translation and RNA editing might be decentralized into far-off regions of the cell. The extreme example of Ca^{2+} compartmentalization, however, is the dendritic spine, where postsynaptic densities house Ca^{2+} sensors regulating synaptic plasticity. The spine head volume is $0.01\text{--}1 \mu\text{m}^3$ ($10^{-17}\text{--}10^{-15} \text{ l}$), chemically isolated from the main dendrite by the $\sim 100\text{-nm}$ -wide spine neck. When an action potential propagates to the spine, free Ca^{2+} rises to $\sim 1 \mu\text{M}$ within milliseconds (Sabatini et al., 2002), corresponding to $100\text{--}1000 \text{ Ca}^{2+}$ ions in the entire spine head. The Na/Ca exchanger and CaATPases rapidly reduce Ca^{2+} levels ($\tau = 15 \text{ ms}$) (Sabatini et al., 2002). NMDA receptor (NMDAR) channel activation is slower and even more effective in sequentially filling Ca^{2+} -binding sites in calmodulin. Calmodulin thus captures transient Ca^{2+} signals and translates them into more prolonged signals (e.g., via CaM kinase II phosphorylation of substrates). As a crucial feature of signal integrity, the dendritic spine head and its accessibility through the spine neck appear to be actively regulated (Alvarez and Sabatini, 2007).

As compactly laid out as an integrated circuit, synaptic densities are themselves marvels of spatial organization. In the hippocampus, where some types of memory are encoded, glutamate activates NMDAR receptor/channels on the postsynaptic membrane of pyramidal

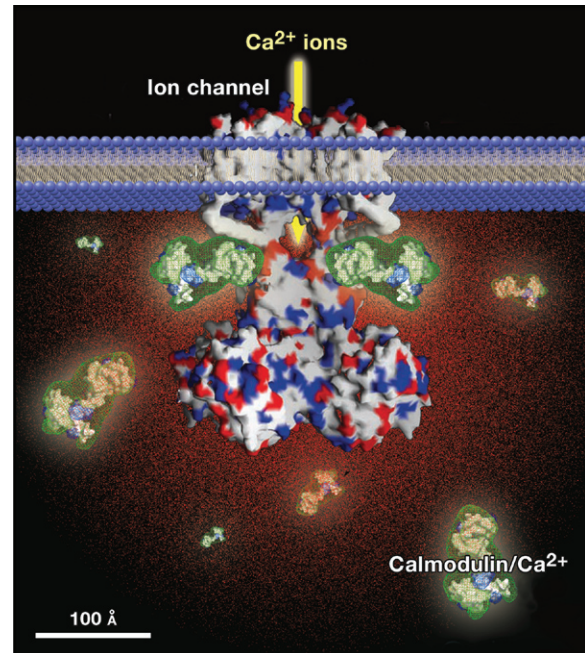


Figure 5. Ca^{2+} Acts Locally

Ca^{2+} enters cells via ion channels at rates of $\sim 10^6/\text{s}$, resulting in a steep gradient of $[\text{Ca}^{2+}]$ (red) lasting less than 1 ms. Intracellular $[\text{Ca}^{2+}]$ falls from $\sim 10 \mu\text{M}$ to $\sim 100 \text{ nM}$ over a few hundred \AA , a volume containing >10 calmodulin molecules at normal cytosolic concentrations. Hypothetical Ca^{2+} channel based on dimensions of tetrameric Kv1.2 (Long et al., 2005).

neurons. The many proteins of the postsynaptic density are organized by scaffolding proteins like PSD95, Homer, and MUPP1 (Kim and Sheng, 2004). Remarkably, MUPP1 has 13 discrete protein interaction domains, bringing together the Ca^{2+} -conducting NMDA receptor/channel, the Ca^{2+} -triggered small G protein activator, SynGAP- α , and Ca^{2+} -triggered CaMKII. Through a cascade of proteins, AMPA-type glutamate receptors are increased at the synapse to result in long-term potentiation of neuronal firing, thus reinforcing and prolonging the signal (Krapivinsky et al., 2004).

Intracellular Ca^{2+} Signaling Networks

A universal mechanism for Ca^{2+} signaling is release from intracellular compartments (Figure 3B). G protein-coupled receptors (GPCRs; primarily Gq/11 subtypes) activating phospholipase C β (PLC β) and TKR activating PLC γ cleave phosphatidylinositol 4, 5 bisphosphate (PIP_2) into 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binding to the IP_3 receptor (IP_3R) ER channel allows diffusion of Ca^{2+} from the ER to increase intracellular $[\text{Ca}^{2+}]$ from $\sim 100 \text{ nM}$ to $\sim 1 \mu\text{M}$ for many seconds. Ca^{2+} binding to the C2 domain of PKC α , $\beta 1$, $\beta 2$, and γ subtypes initiates translocation to the membrane, where coincident DAG binding activates it. Ca^{2+} -sensitive DAG kinase phosphorylates DAG to produce phosphatidic acid, while DAG lipase converts DAG to

arachidonic acid, thus to generate a host of bioreactive molecules. Most commonly associated with the phrase “Ca²⁺ signaling,” this network dominates much of receptor biology (Figure 3B).

Although frequently called “Ca²⁺ channels,” IP₃Rs are actually nonselective cationic channels that conduct Ca²⁺. As with its sister intracellular channel, the RyR, the IP₃ receptor complex is massive: its pore is formed by a homotetramer of ~3000 amino acids. The six transmembrane-spanning domain is at the very C-terminal end of each subunit, leaving room for a large number of cytoplasmic regulatory sites and protein-binding domains. Regulated by an intrinsic suppressor domain, a hinged clamshell-like structure clasps IP₃. Many proteins are proposed to interact with the IP₃R. Of these, IRBIT (IP₃R-binding protein released with IP₃) regulates its IP₃ sensitivity, whereas ERp44 confers redox sensing (Mikoshiba, 2007). The spatial distribution of IP₃Rs (and RyRs) within structurally diverse cells such as neurons is poorly understood and is a relatively overlooked area of research. For reasons that are unclear the highest density of IP₃Rs by far is in cerebellar Purkinje neurons.

Like the IP₃R, RyRs are massive tetrameric (2.2 mDa) channels permeant to Ca²⁺ that span the ER or sarcoplasmic reticulum. The primary natural agonist of RyR is Ca²⁺; low μM [Ca²⁺] opens the channel to allow Ca²⁺ to flow out of the ER/sarcoplasmic reticulum. Like most Ca²⁺-permeant channels, higher cytoplasmic [Ca²⁺] near the mouth of the channel inhibits gating and prevents [Ca²⁺] overload. Eighty percent of the RyR's mass is cytoplasmic, where it interacts with Ca/CaM/CaMKII, FK-506-binding proteins, mAKAP/PKA, PR130/calci-neurin, spinophilin, and sorcin. Other regulators such as triadin, junctin, and calsequestrin appear to regulate ER Ca²⁺ availability to the pore (Bers, 2004). A major area of interest is RyR incontinence, a defect that can induce cardiac arrhythmias or accelerate metabolism to produce lethal hyperthermia. IP₃Rs and/or RyRs are sensitive to the redox status of cells, to nitric oxide/S-nitrosylation, and to quinones/reactive oxygen species (ROS; see below) (Waring, 2005).

Mitochondria, Innate Immunity, and Apoptosis

Ca²⁺ regulates mitochondrial function, movement, and viability. Like the ER, mitochondria can also store mM Ca²⁺ but in mitochondria Ca²⁺ is regulated by fundamentally distinct mechanisms compared to those used in the ER. Whereas Ca²⁺ readily diffuses through large pores in the mitochondrial outer membrane, it crosses the inner mitochondrial membrane via ion channels and transporters. The resting electrical gradient from cytoplasm to the interior of mitochondria is -150 to -200 mV. This gradient is created by active transport of protons (released from oxidized NADH) across the inner mitochondrial membrane. One eukaryotic Ca²⁺ uptake mechanism across inner mitochondrial membranes has been directly measured and identified as a highly Ca²⁺-selective ion conductance channel (Figure 4C) (Kirichok et al., 2004).

This channel, named MiCa (mitochondrial Ca²⁺ channel), binds Ca²⁺ with high affinity ($K_d \leq 2\text{nM}$), enabling high Ca²⁺ selectivity despite relatively low cytoplasmic [Ca²⁺]. MiCa is impermeant to the abundant cytoplasmic Mg²⁺ and K⁺ ions, insuring that only Ca²⁺ dissipates mitochondrial potential.

The Ca²⁺-sensitive dehydrogenases of the Krebs' cycle (McCormack et al., 1990) are stimulated as increased mitochondrial Ca²⁺ boosts ATP production. Increasing ATP production means that more oxygen is reduced to water, but there is also more leakage of free electrons, which results in the formation of superoxides. The resulting oxygen ions, free radicals, and peroxides are collectively called ROS, which are effective but nonselective killers; they oxidize polydesaturated fatty acids in lipids, amino acids, and damage DNA.

Innate immunity is actually a continuum of defense mechanisms from bacteria to humans, many of which involve generation of ROS. Prokaryotes adapted to earth's increasing cyanobacteria-generated oxygen environment by evolving oxidative phosphorylation, an efficient means of ATP production. A benefit of this was the capacity to shed ROS as defensive toxins. If mitochondria originated as an engulfed prokaryote as appears likely, conflicts must have arisen during coevolution of the mitochondria and its host eukaryote; aerobic respiration benefited the organism at the cost of mitochondrial release of ROS into the cytoplasm. When a eukaryotic cell is invaded by a bacterium, the innate immunity program generates antimicrobial molecules (e.g., cationic peptides, histone-derived compounds) and proteases to kill the prokaryote. Thus, it seems likely that apoptosis arose as an adaptation of innate immunity, in which eukaryotes generated proteins targeting the outer mitochondrial membrane to release the offending cytochrome c, perhaps at first simply to control mitochondrial number or the cell's metabolic requirements. The complex translocalization of the BCL-2 proteins, Ca²⁺-dependent annexins, and activation of caspases may illustrate evolutionary refinement of innate immunity pathways with a bacterial symbiont. With high oxidative stress or runaway Ca²⁺-driven ATP generation, a host of proteases (caspases, calpains, cathepsins) and other molecules choreograph cell death. Ca²⁺-dependent annexin 1 association with the membrane, followed by “flipping” to the extracellular face, marks some apoptotic cells for removal (Gerke et al., 2005). These measures preserve the organism at the expense of infected or damaged cells.

Emerging Ca²⁺ Release Sites

Endosomes, Golgi vesicles (and acrosomes), lysosomes, secretory granules, and melanosomes are single membrane-bound compartments that could release Ca²⁺ (Rizzuto and Pozzan, 2006). To be candidates for fast Ca²⁺ release, these compartments must have a Ca²⁺-permeant channel and a favorable electrochemical gradient from compartment to cytoplasm. In addition, func-

tional Ca^{2+} compartments can be formed in restricted spaces, such as cilia (also cilia-like structures such as rods, cones, dendrites, and spines) and various cellular protrusions such as lamellipodia and villi. It is too early to say which of these can release Ca^{2+} and, given the local nature of Ca^{2+} 's actions, what processes they might control. Some evidence supports localized Ca^{2+} release and control of nuclear pores, intervesicular fusion, and protrusions such as growth cones. The leading candidates for release channels in these compartments are members of the TRP ion-channel families, but as the recent discovery of Orai attests, there are Ca^{2+} -permeant channels yet to be discovered. A second major question for any candidate channel is its activation mechanism.

NAD⁺ Derivatives and Ca²⁺ Release

Nicotinamide adenine dinucleotide (NAD⁺) is synthesized by joining nicotinamide (from niacin) with ribose and ADP; addition of a 2' phosphate forms NADP⁺. Additionally, Ca^{2+} /calmodulin regulates some NAD kinases to produce NADP⁺, as occurs following Ca^{2+} entry in neutrophils during the respiratory burst or during fertilization. At acid pH, ADP-ribosyl cyclases exchange the nicotinamide moiety of NADP⁺ with nicotinic acid to produce NAADP⁺. NAADP⁺ is a potent (~100 nM) intracellular Ca^{2+} release messenger identified in sea urchin lysates (Lee, 2005). Proposed to release Ca^{2+} from ER, or lysosomes (Yamasaki et al., 2005), the target receptor for NAADP⁺ is not known.

ADP-ribosyl cyclases (e.g., CD38, CD157) cyclize NAD or NGD to produce cADP ribose (cADPr) or cGDPr (Lee, 2001). TRPM2, a Ca^{2+} -permeant ion-channel activated by nucleotide binding to its C-terminal nudix domain, is one potential target of cADPr or cGDPr. Alternatively, the redox status of cells encoded by the nucleotides NADP⁺/NADPH may modulate IP₃Rs via ERp44 or somehow activate proposed oxidative stress-induced apoptotic intracellular channels such as Bax inhibitor 1. It is curious that ADP-ribosyl cyclase CD38 is an ectoenzyme given that the known nucleotide-binding sites are intracellular. Whether cells take up these extracellular nucleotides or receptors will require more investigation. Sirtuins are generally known as NAD⁺-dependent histone deacetylases involved in gene silencing, but Sir2 can cleave nicotinamide from target proteins and transfer the protein's acetyl group to ADP ribose to generate 2'- or 3'-O-acetyl-ADP ribose (OADPr), a regulator of sirtuin complexes (Liou et al., 2005). Surprisingly, some sirtuins are also ADP ribosyltransferases, creating OAADPr, another potential Ca^{2+} mobilizing agent.

Motility

Ever since Sidney Ringer realized that it was the Ca^{2+} in London's "hard" tap water that was required for heart contraction, Ca^{2+} has been associated with motility (Ringer, 1883). In muscle, Ca^{2+} binds troponin to relieve tropomyosin's block of myosin/actin binding. Motor proteins, such as myosin, dynein, and kinesin,

are ATPases that are often modulated by Ca^{2+} , especially via adaptors such as calmodulin and the kinases they activate. Sperm swim without extracellular Ca^{2+} , although Ca^{2+} clearly regulates flagellar shape (Qi et al., 2007). In cells of the immune system, significant increases in cytoplasmic $[\text{Ca}^{2+}]$ usually reduce or halt cell motility (Gallo et al., 2006). The clustering of mitochondria at sites of Ca^{2+} release may require the Miro family of mitochondrial RhoGTPases but may also depend on Ca^{2+} -dependent regulation of several cytoskeletal elements or motor proteins.

Ca^{2+} /calmodulin regulates cell shape through control of myosin's interaction with cytoskeletal actin. Vaso-regulation by the control of smooth muscle contraction exemplifies such pathways. Smooth muscle contracts when CaV channels open; Ca/CaM activates myosin light-chain kinase (MLCK, a CamK) to phosphorylate the myosin head light chain and enable myosin ATPase activity (Ledoux et al., 2006). Smooth-muscle relaxation is intrinsically and extrinsically modified. As smooth muscle $[\text{Ca}^{2+}]$ declines due to normal homeostatic mechanisms, MLCK phosphatases initiate smooth-muscle relaxation. In addition, receptor-mediated $[\text{Ca}^{2+}]$ increases in vascular endothelium lying adjacent to smooth muscle can initiate relaxation via release of vasoactive agents. Ca/CaM binding in endothelial cells activates nitric-oxide synthases (NOS3) (Dudzinski et al., 2006). Dimeric nitric-oxide synthases are a kind of miniature electron-transport chain in which electrons flow from NADPH at the C-terminal reductase of the enzyme to the oxygen at its N-terminal oxygenase domain, oxidizing L-Arg to L-citrulline and releasing nitric oxide. A reactive gas and molecule of innate immunity and signal transduction nitric oxide dramatically relaxes smooth muscle. Nitric oxide diffuses readily into adjacent smooth muscle, stimulates soluble guanylyl cyclase, and in turn protein kinase G, to phosphorylate exchangers and channels that lower $[\text{Ca}^{2+}]$. NO also converts thiol groups (e.g., cysteines) to form S-nitrosothiols, a common posttranslational modification of proteins.

A simple way to detach cells in tissue culture is simply to lower extracellular $[\text{Ca}^{2+}]$. Cadherins, a family encoded by >100 genes, are critical to cell adhesion, and by coupling to catenins, vinculin, and actin, to development and morphology (Bamji, 2005). A short stretch of extracellular negatively charged amino acids binds up to three Ca^{2+} ions; removal of Ca^{2+} bends the cadherin structure and exposes it to proteases. Although Ca^{2+} does not appear to affect the canonical Wnt/ β -catenin/planar cell-polarity pathway, it has been proposed to regulate Wnt signaling indirectly. Like cadherins, integrins also bind extracellular divalent cations (α subunit; Ca^{2+} or Mg^{2+}), but the number of ions bound can be altered by ligands, such as components of the extracellular matrix. On the intracellular surface, talin, paxillin, and α -actinin regulate focal adhesion kinases. Many of these pathways intersect with other Ca^{2+} -signaling pathways.

Propagation Extends the Spatial Reach of Ca^{2+}

Action potentials are a well-known mechanism to propagate increases in Ca^{2+} , up to a meter in humans. But in all cells, Ca^{2+} release from the ER is a nonlinear, cooperative process; IP_3 binds to four receptor sites on the IP_3R (ITPR1-3), one on each subunit of the tetramer (Mikoshiba, 2007). In both ER and sarcoplasmic reticulum, IP_3Rs and RyRs are at first potentiated, then inhibited by Ca^{2+} . Small perturbations in conditions, such as ambient $[\text{Ca}^{2+}]$, $[\text{IP}_3]$, and various regulators, result in uncoordinated bursts of local release across a cell (called “sparks” for their appearance in Ca^{2+} imaging fluorescence microscopy [Guatimosim et al., 2002]). Increasing spark frequency can cascade and become regenerative. This regenerative release is seen as two- or three-dimensional waves of changes in $[\text{Ca}^{2+}]$ that propagate within cells. Such patterns are visually arresting; waves can interact, create spirals, and annihilate. Incorporation of spatial and temporal components of Ca^{2+} release, inhibition, buffering, and diffusion into sets of coupled nonlinear differential equations can reproduce these phenomenon in silico.

Timely and spatially variant Ca^{2+} oscillations control easily observable events such as muscle contraction, fertilization, and secretion. On the molecular scale, the consequences of Ca^{2+} oscillations (waves) require a wealth of measurements not yet realizable. A reactant's (Ca^{2+} , cAMP, NO, etc.) effects will depend on its on and off rates of binding and if in the right time frame will accumulate to encode frequency, as shown for NFAT translocation (Dolmetsch et al., 1997). The large number of Ca^{2+} -binding proteins with unique on and off rates of Ca^{2+} binding and associated conformational protein changes dictates that regenerative Ca^{2+} changes will have widespread effects in cells. Just as for action potentials, Ca^{2+} waves are signals whose mechanisms and actions must be defined for each cell and tissue type.

Transcription Extends the Temporal Reach of Ca^{2+}

Gene transcription extends the impact of Ca^{2+} signaling into long-term changes in the life of the cell. Most Ca^{2+} -regulated transcription factors are in coincidence switching networks that lead to unique endpoints. Both short- and long-term changes are evoked by experience-driven synaptic activity and localized Ca^{2+} changes that alter synaptic connectivity within a neural circuit (Flavell and Greenberg, 2007). In lymphocytes, a repetitive or prolonged increase in $[\text{Ca}^{2+}]$, in combination with other signals, is needed for calcineurin-mediated progressive dephosphorylation of NFAT serines to expose a buried nuclear localization sequence. Importin then brings NFAT into the nucleus, where it can activate hundreds of immunity-related genes. Kinases rapidly phosphorylate NFAT, and it is returned to the cytoplasm via Crm1 exportin. During myogenesis, Ca^{2+} -dependent CaMK prevents formation of complexes of the transcription factor MEF2 and histone deacetylases (HDACs) but also phosphorylates HDAC4 and HDAC5 to induce nuclear

export of these transcriptional repressors (McKinsey et al., 2000). TORC2, a CREB coactivator that regulates the viability of pancreatic islets, is restricted to the cytoplasm by a phosphorylation-dependent interaction with 14-3-3 protein. Increasing $[\text{Ca}^{2+}]$ activates calcineurin to dephosphorylate TORC2, while increasing cAMP inhibits SIK2 (SNF1-like kinase 2). These coincident signals enable dephosphorylated TORC2 to enter the nucleus (Screaton et al., 2004). Mitogens activate $\text{PLC}\gamma$ to release Ca^{2+} and DAG that in turn coactivate PKC. Ca^{2+} also binds RasGRF, activating RasGRP; PKC and RasGRP then coactivate Ras/Raf1 to initiate the MAPK cascade. These parallel switching networks are inputs to other coincidence counters, eventually activating genes that initiate proliferation or differentiation. In adult cardiomyocytes, proliferation and differentiation are not usually options; most of these cells undergo hypertrophy or die. Hypertrophy as a recognizable endpoint has led to the identification of many muscle proteins that regulate chromatin accessibility, gene transcription, and even microRNAs (Olson, 2006; van Rooij and Olson, 2007).

As with all intracellular compartments, accurate measurements of nuclear $[\text{Ca}^{2+}]$ are lacking. Ca^{2+} and other molecules smaller than ~ 50 kDa can diffuse directly into the nucleus from the cytoplasm through the ~ 50 nm (~ 30 Ca^{2+} diameters) nuclear pore, where enzymes such as CaMKIV can directly regulate transcription factors and coregulators. Larger molecules are actively transported by the GTP-hydrolyzing Ran cycle. Ca^{2+} block of small-molecule diffusion has been observed in isolated nuclei, but there is little evidence to support physiological gating of the pore to small molecules. Ca^{2+} gradients may exist in folds of the nuclear membrane or specialized Ca^{2+} release sites on the inner nuclear membrane. Unique intranuclear buffers, nuclear geometry, and the lack of organelles dictates that intranuclear Ca^{2+} sequestration will be distinct from that found in the cytoplasm, but whether this has any biological consequence is an outstanding question.

Conclusion

Evolution has adopted positively charged Ca^{2+} and negatively charged phosphate ions as the two primary signaling elements of cells. Ca^{2+} signaling affects every aspect of a cell's life and death. The most tightly regulated ion within all membrane-bound organisms, Ca^{2+} binds to thousands of proteins to effect changes in localization, association, and function. Questions of current interest are the identification of new Ca^{2+} compartments and their Ca^{2+} release receptors, gating of TRP and SOC/Orai channels, regulatory networks surrounding IP_3 and RyR channels, the regulation of Golgi, endosomal, and vesicular fusion events, and increased understanding of Ca^{2+} regulation of mitochondrial shuttling, fission, fusion, energy production, and death. Longer-term questions that require development of new technologies surround Ca^{2+} changes within nuclei and organelles, vesicles, highly localized gradients, and their consequences.

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