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Calcium Signaling

Review

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Ionized calcium (Ca^{2+}) is the most common signal transduction element in cells ranging from bacteria to specialized neurons. Unlike many other second-messenger molecules, Ca^{2+} is required for life, yet prolonged high intracellular Ca^{2+} levels lead to cell death. Ca^{2+} cannot be metabolized like other second-messenger molecules, so cells tightly regulate intracellular levels through numerous binding and specialized extrusion proteins. Normal intracellular Ca^{2+} levels at ~100 nM are 20,000-fold lower than the 2 mM concentration found extracellularly. Scores of cellular proteins have been adapted to bind Ca^{2+} tightly, in some cases simply to buffer or lower free Ca^{2+} levels, and in others to trigger second-messenger pathways. The well-known basic elements in Ca^{2+} signal transduction have been extensively reviewed (Berridge, 1993; Carafoli, 1992; Clapham and Neer, 1993; Pozzan et al., 1994; Rhee, 1991; Tsien and Tsien, 1990). The aim of this review is to highlight recent findings in Ca^{2+} signaling and to discuss their implications.

Ca^{2+} Binding

Why is Ca^{2+} so important? Seawater magnesium (Mg^{2+}) is higher in concentration than Ca^{2+}, but unlike Ca^{2+} is not excluded from the cytosol. One likely argument for the uniqueness of Ca^{2+} is that it must be maintained at low levels, since it precipitates phosphate, the established energy currency of cells. Ca^{2+} has a lower affinity for water than Mg^{2+}. Cells evolved strategies for binding Ca^{2+}, perhaps at first simply to reduce its cytosolic levels, but later for signal transduction.

Ca^{2+} ions are able to accomodate 4–12 oxygen atoms in their primary coordination sphere, but coordination numbers of 6–8 are most common (McPhalen et al., 1991). The artificial chelator EDTA binds divalents through its high local concentration of combining groups, two nitrogen groups and four oxygen groups, which form an approximate octahedral arrangement around Ca^{2+} (Pauling, 1947). Proteins often bind Ca^{2+} through ~6 oxygen atoms, which are provided by glutamate and aspartate residues that are charged at most biologically relevant pH (Fasman, 1989). Many Ca^{2+}-binding sites contain an inner shell of oxygen atoms clustered 2.1–2.7 Å from the Ca^{2+} ion, a second shell populated largely by carbon atoms to support the coordinating oxygen atoms of the inner shell, and a third shell 4–5 Å away from the Ca^{2+} ion that contains nitrogen atoms (Nayal and Di Cera, 1994). With our current understanding of protein structures, we cannot always predict Ca^{2+}-binding sites on the basis of amino acid sequence. Nevertheless, an important motif is the EF hand, named arbitrarily after the E and F regions of parvalbumin. This helix-loop-helix Ca^{2+}-binding motif is characterized by two α helices separated by a Ca^{2+}-binding loop. The Ca^{2+}-binding loop is composed of residues containing side chain oxygen groups, aspartate and glutamate, and a glycine required for the loop structure (Kretsinger, 1980). Cooperative binding of multiple Ca^{2+} ions is not unusual, and more than one Ca^{2+}-binding motif can be found within the same protein.

It is useful to classify binding proteins as trigger or buffer proteins (Table 1; Baimbridge et al., 1992; Heizmann and Hunziker, 1991). Trigger proteins (e.g., calmodulin) change their conformation upon binding Ca^{2+} and modulate effector molecules such as enzymes and ion channels. Buffering Ca^{2+}-binding proteins, such as calsequestrin, may simply bind Ca^{2+} as its concentration increases within a cell or organelle. However, putative buffer proteins may possess as yet undiscovered trigger functions.

Ca^{2+} Action Is Local

As would be expected from the number, affinity, and specificity of Ca^{2+}-binding proteins, Ca^{2+} can be an extremely localized second messenger. Ca^{2+} diffuses much more slowly than predicted simply from its ionic (~1 Å, diffusion coefficient [D] = 1000 $\mu m^2/s$) or even hydrated (D = 800 $\mu m^2/s$) radius. It is estimated that a Ca^{2+} atom migrates no further than 0.1–0.5 μm, lasting only ~50 μS before encountering a binding protein (assuming 10^8 M-1s-1 k_{on} and 300 μM binding protein concentration [Allbritton et al., 1992]). Ca^{2+} diffusion also depends on the degree of saturation of existing Ca^{2+}-buffering proteins and varies, for example, between 15 and 65 $\mu m^2/s$ (Allbritton et al., 1992). However, the image of a cell as a uniform volume for diffusion is a gross simplification, since cellular buffers are undoubtedly distributed in a functional and nonuniform manner. Furthermore, Ca^{2+} buffers may be either mobile or immobile. Around the mouth of Ca^{2+} permeant channels, Ca^{2+} ions exit the 5 Å pore at rates exceeding 10^6 ions per second and rapidly reach high concentrations in the immediate surrounding volume.

Sequestration of Ca^{2+} in the Endoplasmic Reticulum

Since mammalian cells are several micrometers thick and usually >10 μm in length, other Ca^{2+}-sequestering mechanisms are needed. The endoplasmic reticulum (ER) spreads like a vast three-dimensional spider web within cells, acting as a framework for Ca^{2+}-binding proteins and actively sequestering Ca^{2+} into its intraorganellar space. Ca^{2+} pumps in the ER membrane (SERCA pumps) use ATP to pump Ca^{2+} ions into the ER, where they are sequestered by high concentrations of specialized buffer molecules, such as calsequestrin, that have no known trigger function but act as dynamic storage molecules for low affinity, high capacity Ca^{2+} uptake. One important question facing cell biologists is whether the high ER [Ca^{2+}] has a function beyond warehousing Ca^{2+}. Is protein sorting conducted in this strange environment, or are there separate and dynamically rearranged ER pools? Do high free and buf-

Table 1. Examples of Mammalian Proteins Triggered by Ca^{2+}

Protein	Ca^{2+}-Binding Site	Protein Function
Troponin C	EF hand	Modulator of muscle contraction
Calmodulin	EF hand	Ubiquitous modulator of protein kinases and other enzymes (MLCK, CaM kinase II, adenylyl cyclase I)
Calretinin, retinin, visinin	EF hand	Activator of guanylyl cyclase
Calcineurin B	EF hand	Phosphatase
Calpain	EF hand	Protease
Inositol phospholipid-specific PLC	EF hand	Generator of $InsP_3$ and diacylglycerol
α-Actinin	EF hand	Actin-bundling protein
Annexin		Implicated in endo- and exocytosis, inhibition of PLA_2; ion channel?
Phospholipase A2		Producer of arachidonic acid
Protein kinase C		Ubiquitous protein kinase
Gelsolin		Actin-severing protein
Ca^{2+}-activated K^+ channel		Effector of membrane hyperpolarization
$InsP_3$ Receptor		Effector of intracellular Ca^{2+} release
Ryanodine receptor		Effector of intracellular Ca^{2+} release
Na^+/Ca^{2+} exchanger		Effector of the exchange of Ca^{2+} for Na^{2+} across the plasma membrane
Ca^{2+} ATPase		Pump of Ca^{2+} across membranes
Ca^{2+} antiporters		Exchanger of Ca^{2+} for monovalent ions
BoPCAR		G protein–linked Ca^{2+}-sensing receptor
Caldesmon		Regulator of muscle contraction
Villin		Actin organizer
Arrestin		Terminator of photoreceptor response
S100β		Unknown
Calreticulin		Ca^{2+} buffer/modulator of nuclear hormone receptor
Parvalbumin	EF hand	Ca^{2+} buffer
Calbindin	EF hand	Ca^{2+} buffer
Calsequestrin		Ca^{2+} buffer

fered concentrations of Ca^{2+} in the ER and Golgi network contribute to condensation of proteins observed in protein sorting, and if so, what happens to protein processing when stores are emptied?

Increases in Cytosolic Ca^{2+} for Signal Transduction

There are several mechanisms to introduce small bursts of Ca^{2+} into the cytosol for signal transduction. Ca^{2+} ions from the two largest Ca^{2+} sinks, the extracellular space and the ER, are injected into the cytosol either across the plasma membrane or from the ER through ion channels. There are two common motifs for Ca^{2+} signaling, illustrated in Figure 1.

Nonexcitable Cells: Overview

In nonexcitable cells such as blood cells, hepatocytes, and endothelia, the slow inositol (1,4,5)-trisphosphate ($InsP_3$)-mediated pathway predominates. Two receptor classes, the G protein–coupled receptor class of seven transmembrane–spanning receptors (GCRs) and the receptor tyrosine kinases (RTKs), release $InsP_3$ via the pathways shown in Figure 1a. GCRs activate phospholipase Cβ (PLCβ), while RTKs stimulate phospholipase Cγ (PLCγ) to convert phosphatidylinositol (4,5)-bisphosphate ($PtdInsP_2$) into $InsP_3$ and diacylglycerol (Berridge and Irvine, 1989). $InsP_3$ acts as an intracellular second messenger by binding to the specialized tetrameric $InsP_3$ receptor that spans the endoplasmic reticular membrane and triggering release of Ca^{2+} from the ER. Table 2 lists common G protein–linked and tyrosine kinase–linked receptors that stimulate intracellular increases in Ca^{2+} levels. Either of these $InsP_3$-mediated signal transduction pathways can increase intracellular $[Ca^{2+}]$ from ~100 nM to ~1 μM.

Ca^{2+} can also enter nonexcitable cells by crossing the plasma membrane. Nonexcitable cells enhance Ca^{2+} entry by hyperpolarization. Open potassium (K^+) channels force the membrane potential to more negative potentials, drawing Ca^{2+} more rapidly across the plasma membrane. Ca^{2+} enters through specialized voltage-independent Ca^{2+}-selective channels triggered by second-messenger molecules. Ca^{2+} selectivity is ensured by the structure of the channel pores, which strain out all other ions.

Excitable Cells: Overview

In addition to the system described for nonexcitable cells, excitable cells contain voltage-dependent Ca^{2+} channels that enable these cells to increase cytosolic Ca^{2+} levels dramatically. Specialized Ca^{2+} trigger proteins near the plasma membrane inner surface initiate functions as diverse as exocytosis in neurons and contraction in muscle. In excitable cells, depolarization from the resting membrane potential (~−70 mV) initiates conformational changes in Ca^{2+}-selective ion channels (voltage-dependent Ca^{2+} channels) via special voltage-sensing regions (S4) of these molecules, catalyzing the flood of Ca^{2+} across the membrane. Since the forces between chemical and electrical balance for Ca^{2+} are equal at ~+150 mV, Ca^{2+} flows into the cell at all physiological membrane potentials (−90 to +60 mV). Voltage-dependent Ca^{2+} channel activity is self-limiting—the Ca^{2+} channel itself closes in a time-dependent fashion, while further depolarization only decreases the electrochemical driving force for Ca^{2+} entry.

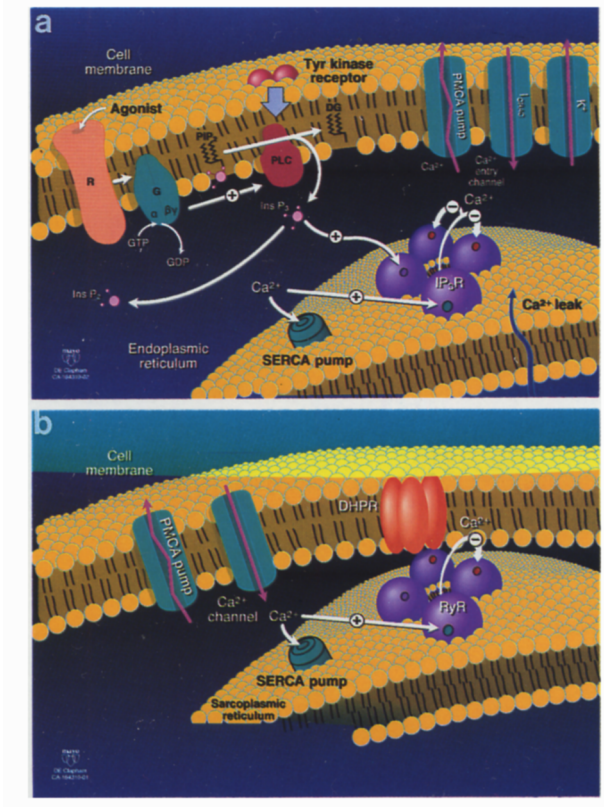

Figure 1. Ca^{2+} Signaling in Nonexcitable and Excitable Cells

(A) Nonexcitable cells. Abbreviations are as follows: R, G protein–linked receptor; G, G protein; PLC, phospholipase C or $PtdInsP_2$ phosphodiesterase; $InsP_3$, inositol (1,4,5)trisphosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; DG, diacylglycerol; PMCA, plasma membrane Ca^{2+} ATPase pump; ICRAC, Ca^{2+} release–activated current; K^+, potassium-selective channel; $InsP_3R$, $InsP_3$ receptor; SERCA, smooth endoplasmic reticulum Ca^{2+} ATPase pump. Plus indicates stimulatory regulation; minus indicates inhibition.

(B) Excitable cells. The system described in (A) coexists with the present system in most excitable cells. Ca^{2+} channel indicates a voltage-dependent Ca^{2+} channel. RyR, ryanodine (Ca^{2+}-sensitive) receptor; DHPR, dihydropyridine receptor. PMCA and SERCA as in (A).

In excitable cells (e.g., neurons), Ca^{2+} entering through voltage-dependent Ca^{2+} channels may directly activate ryanodine receptors (RyR), the excitable cell counterparts to the $InsP_3$ receptor, to release Ca^{2+} from intracellular stores (Figure 1b). Skeletal muscle is a specialized case of this theme, in which dihydropyridine receptors on the surface of the plasma membrane and in T tubules abut the ER tetrameric RyR. Conformational changes induced by voltage in the dihydropyridine receptor result in Ca^{2+} influx and perhaps directly modulate the RyR to release Ca^{2+} from intracellular stores (McPherson and Campbell, 1993).

G Proteins

At least 30 seven transmembrane–spanning receptors initiate Ca^{2+} release through the activation of PLCβ (Table 2). Experiments using pertussis toxin (which disrupts coupling between receptors and $G_{t\alpha}$, $G_{i\alpha}$, and $G_{o\alpha}$) demonstrate that both pertussis toxin–sensitive and –insensitive G proteins transduce signals between receptors and PLCβ (Sternweis and Smrcka, 1992). The most well-established path for activation of PLCβ is through the pertussis toxin–insensitive $G_{q\alpha}$ (and presumably others of the family, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 16}$; Lee et al., 1992; Smrcka et al., 1991; Taylor and Marshall, 1992). $G_{q\alpha}$-linked receptors, such as the muscarinic type 3 and serotonin 5HT1C, rapidly increase intracellular Ca^{2+}. The pertussis toxin–sensitive G proteins that mediate Ca^{2+} release are less well established, but there is evidence that both $G_{o\alpha}$ and $G_{i\alpha}$ subunits are involved (Moriarty et al., 1990).

It is now firmly established that $G_{\beta\gamma}$ subunits also activate PLCβ (Clapham and Neer, 1993; Neer, 1995 [this issue of *Cell*]), giving rise to speculation that pertussis toxin–sensitive PLCβ activation may result from $G_{\beta\gamma}$ rather than $G_{o\alpha}$ and $G_{i\alpha}$. G_α and $G_{\beta\gamma}$ subunits appear to interact with separate domains of the PLCβ molecule, implicating independent regulation by both effector arms of the heterotrimeric G protein. As yet, very little is known about the specificity of $G_{\beta\gamma}$ subunits in activating PLCβ subtypes, except

Table 2. Plasma Membrane Receptors Increasing Intracellular Ca^{2+}

Via PLCβ	Via PLCγ	Directly
α1-Adrenergic		
Muscarinic m1, m3, m5	Epidermal growth factor receptor	Nicotinic ACh channels
Purinergic P2y P2u, P2t	Platelet-derived growth factor receptor	Glutamate receptor family of ion channels
Serotonin 5HT1C	Fibroblast growth factor receptor	
H1	ErbB2	
GnRH	T cell receptor	
TRH		
Glucagon		
Cholecystokinin		
Vasopressin V-1a, V-1b		
Oxytocin		
Angiotensin II		
Thrombin		
Bombesin		
Vasoactive intestinal peptide		
Bradykinin		
Tachykinin		
Thromboxanes		
Platelet-activating factor		
F-Met-Leu-Phe		
Endothelin opiate		
BoPCAR		

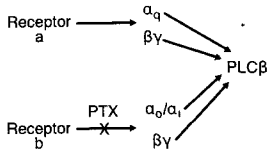

Figure 2. Potential G Protein Subunit Signaling Pathways Stimulating PLCβ

Abbreviation: PTX, pertussis toxin.

that transducin $G_{\beta\gamma}$ ($G_{\beta1\gamma1}$) is less effective than other dimer combinations.

The fact that both G_α and $G_{\beta\gamma}$ can modulate PLCβ greatly increases the potential complexity of signal transduction (Figure 2). Ca^{2+} is an important modulator of PLCβ, adenylyl cyclase, nitric oxide synthase, phospholipases, and calmodulin (CaM) kinases. The multiple convergent and divergent pathways make defininings the steps in signal transduction difficult. When distal and complex endpoints in cell function, such as cell division, are assayed in complex intact systems, such as with overexpression of single proteins, one can predict a dizzying number of conclusions (and papers).

Receptor Tyrosine Kinases

Tyrosine kinase receptors activate PLCγ. In brief, single transmembrane–spanning receptor molecules, such as the platelet-derived growth factor receptor, dimerize and autophosphorylate on tyrosines upon ligand binding. The phosphorylated tyrosines form docking sites for the PLCγ SH2 domains, bringing PLCγ into proximity with $PtdInsP_2$. Again, $InsP_3$ and diacylglycerol are formed. In general, tyrosine kinase–activated PLCγs increase Ca^{2+} more slowly and for longer durations than do G-mediated PLCβs.

PtdIns

PLC hydrolyzes phosphatidylinositol (PtdIns) phosphatidylinositol 4-phosphate (PtdInsP), and $PtdInsP_2$. Once PLC splits $PtdInsP_2$ into $InsP_3$ and diacylglycerol, a complex set of enzymes mediates the generation of multiple inositol phosphates (Berridge and Irvine, 1989). It is clear that $InsP_3$ is the dominant second-messenger molecule for release of intracellular Ca^{2+} (Berridge, 1993). However, inositol (1,3,4,5)tetrakisphosphate ($Ins(1,3,4,5)P_4$) enhances Ca^{2+}-induced Ca^{2+} influx (Lückhoff and Clapham, 1992), inhibits Ca^{2+} ATPase pumps (Yoo, 1991), and may bind specific intracellular receptors (Theibert et al., 1991). Inositol (3,4,5,6)tetrakisphosphate ($Ins(3,4,5,6)P_4$) apparently uncouples muscarinic stimulation of [Ca^{2+}] from chloride secretion (Vajanaphanich et al., 1994).

$InsP_3$ and Ryanodine Receptors

The $InsP_3$ receptor is a homotetramer of ~310 kDa subunits surrounding a relatively nonselective cationic pore (Mikoshiba, 1993). $InsP_3$ receptor expression varies from millions per cell in cerebellar Purkinje neurons to a few hundred in other cells and is ubiquitous. Each subunit binds one $InsP_3$ molecule in a positively charged, Arg/Lys-rich N-terminal region. $InsP_3$ binding is blocked by heparin, but there are to date no other known effective antagonists. Multiple isoforms are encoded by at least four genes, all of which share significant similarity to each other, partial homology with the RyR, and no significant homology with voltage-dependent Ca^{2+} channels. The cytoplasmic N-terminal region also has two ATP-binding sites and at least one Ca^{2+}-binding site (Mignery and Sudhof, 1990). Regulation of the $InsP_3$ receptor is complex in that it binds multiple $InsP_3$ molecules, is desensitized by $InsP_3$ itself (Hajnóczky and Thomas, 1994), is phosphorylated by protein kinase A, and has a biphasic sensitivity to cytoplasmic Ca^{2+} levels (Bezprozvanny et al., 1991; Finch and Goldin, 1994; Iino, 1990; Parys et al., 1992). At low and high Ca^{2+} levels, the $InsP_3$ receptor is relatively insensitive to $InsP_3$. The sensitivity of the receptor to $InsP_3$, which is also biphasic, is greatest in the physiological range between 0.5 μM and 1.0 μM $InsP_3$. Properties of the $InsP_3$ receptor in vivo have been confirmed by direct patch–clamp recordings of the outer nuclear membrane (Stehno-Bittel et al., 1995).

The phenomenon of quantal relase (Bootman, 1994), in which repetitive applications of low concentrations of intracellular $InsP_3$ release quanta, or fractions of the Ca^{2+} pool, will continue to puzzle Ca^{2+} researchers until basic regulatory properties of the channel are understood. Such properties as cytoplasmic [Ca^{2+}] or $InsP_3$-induced desensitization, differing receptor isoform affinity for $InsP_3$, $InsP_3$ receptor distribution, and intralumenal Ca^{2+} regulation of the $InsP_3$ receptor need further quantitation. There is evidence both for and against significant intralumenal control of $InsP_3$ receptor gating (Missiaen et al., 1991; Shuttleworth, 1992). Another area of controversy has been the significance of $InsP_3$ receptors found in the plasma membrane. Although the ER contains by far the highest number of $InsP_3$ receptors, $InsP_3$ receptors have also been identified in the plasma membrane (Khan et al., 1992).

The RyR (the excitable cell counterpart of the $InsP_3$ receptor) is composed of a tetramer of four ~560 kDa subunits and is gated either by electromechanical coupling to the plasma membrane dihydropyridine receptor in skeletal muscle (see Figure 1b), by Ca^{2+}, or by cADP-ribose in some cell types (Coronado et al., 1994; Ehrlich et al., 1994). Like the $InsP_3$ receptor, it is modulated by Mg^{2+}, ATP, and Ca^{2+}, although Ca^{2+} and Mg^{2+} inhibition occurs in the mM range. Similarly, the RyR is relatively nonselective for cations, although it excludes all anions. Three separate genes (*ryr-1*, *ryr-2*, and *ryr-3*) encode RyRs expressed predominantly in skeletal muscle, cardiac muscle, and brain or smooth muscle, respectively. FKBP12, a *cis-trans* peptidylprolylisomerase that binds the immunosuppressants FK506 and rapamycin, copurifies with RyR and modulates RyR channel opening in lipid bilayers (Brillantes et al., 1994). A newly discovered second messenger, cADP-ribose, releases Ca^{2+} in sea urchin eggs and may be a physiologically relevant RyR agonist in cardiac and pancreatic cells (*ryr2*, *ryr3*; Mészáros et al., 1993; Thorn et al., 1994). cADP-ribose is synthesized from nicotinamide adenine dinucleotide (NAD^+) by ADP-ribosyl cyclase, an enzyme found in both invertebrate and mammalian cells. Interestingly, the second-messenger cyclic GMP regulates cADP-ribose levels in some cells (Galione, 1993). One mystery of neuronal Ca^{2+} signaling is the func-

tion of the large numbers of low affinity $InsP_3$ receptors in Purkinje cells of the cerebellum. The receptors seem to be too dense and too ineffective at Ca^{2+} release to play the usual role of receptor-mediated intracellular Ca^{2+} release. $InsP_3$ receptors are dense but RyRs sparse in cerebellar Purkinje and hippocampal CA1 pyramidal cells. In contrast, RyRs are dense and $InsP_3$ receptors sparse in the dentate gyrus and CA3/4 areas of the hippocampus (Sharp et al., 1993). A second area ripe for exploration is the physiological role of unique distributions of RyRs and $InsP_3$ receptors in axons, dendritic spines and shafts, and cell bodies. Electron microscopy of hippocampus showed RyR in axons, dendritic spines, and dendritic shafts near the spines, while the $InsP_3$ receptor was more prominent in dendritic shafts and cell bodies (Sharp et al., 1993).

Ca^{2+} Pumps

Although buffers help maintain low cytosolic Ca^{2+} ions, they alone cannot combat the infinite source of high Ca^{2+} surrounding the cell. For this purpose, Ca^{2+} pumps transport Ca^{2+} ions into the ER or extracellular space at the cost of 1 to 2 ATPs per Ca^{2+} ion removed. Both smooth ER (SERCA) and plasma membrane (PMCA) Ca^{2+} pumps are P type ATPases (Pederson and Carafoli, 1987), defined by an obligatory phosphorylated intermediate in the pump cycle. Although both have ten putative transmembrane-spanning domains, homology between the two classes is surprisingly low. The sarcoplasmic reticulum Ca^{2+} ATPase pumps are the products of three different genes, known as SERCA1, SERCA2, and SERCA3. SERCA1 pumps are exclusively expressed in fast-twitch skeletal muscle, while SERCA2 pumps are expressed in cardiac and slow-twitch skeletal muscle, and SERCA3 pumps are expressed in nonmuscle tissues (Pozzan et al., 1994). Although pharmacological tools for studying Ca^{2+} pumps are generally lacking, thapsigargin, a tumor-promoting sesquiterpene lactone, irreversibly inhibits the SERCA pump in a highly specific manner by trapping it in its Ca^{2+}-free state. Thapsigargin has been used extensively to deplete Ca^{2+} stores and raise cytoplasmic $[Ca^{2+}]$. Extensive site-directed mutagenesis studies and high resolution electron microscopy of sarcoplasmic reticulum Ca^{2+} ATPase suggests a large cytoplasmic ATPase head gating a cylindrical Ca^{2+} translocation or channel domain (MacLennan et al., 1985, 1992; Toyoshima et al., 1993). PMCA pumps are expressed by four genes in a tissue-specific manner, with multiple alternatively spliced versions. Na^+/Ca^{2+} exchange pumps also regulate intracellular Ca^{2+} levels but are not reviewed here (see Strehler, 1995).

Mitochondria

Mitochondria accumulate Ca^{2+} at up to 0.5 mM levels in the mitochondrial matrix owing to a large electrochemical gradient created by mitochondrial hydrogen exchange. Mitochondrial Ca^{2+} uniporters have lower affinities for Ca^{2+} than SERCA pumps and probably are only significant when cytosolic Ca^{2+} rises above ~ 0.5 μM (Pozzan et al., 1994). Under pathological conditions, mitochondria are capable of absorbing large amounts of Ca^{2+}. Clever experiments using mitochondrial-targeted aequorin (a Ca^{2+}-sensing photoprotein [Rizzuto et al., 1993]) suggest that mitochondrial Ca^{2+} transients can be evoked under physiological conditions, but until the properties of aequorin in its local environment are known, the results must be interpreted with caution.

Capacitative Entry

One of the most exciting areas in Ca^{2+} signal transduction in recent years has been the discovery of gating of Ca^{2+} entry across the plasma membrane by depletion of intracellular stores (Putney, 1990). Stimulation of receptors such as muscarinic type 3 result in relatively rapid rises in cytoplasmic Ca^{2+} levels (Lechleiter et al., 1991a), effectively depleting endoplasmic reticular stores and somehow activating Ca^{2+} entry across the plasma membrane. Thus, although the PtdIns-linked receptor releases Ca^{2+} only transiently, cytoplasmic Ca^{2+} increases are prolonged substantially by capacitative entry mechanisms. The membrane potential regulates the magnitude of Ca^{2+} entry simply by controlling the driving force for Ca^{2+}; hyperpolarization increases cytoplasmic Ca^{2+} levels. Besides simply repleting Ca^{2+} stores, capacitative entry undoubtedly plays a physiological role. Ca^{2+}-dependent processes, such as Ca^{2+} waves (see below), are dramatically accelerated through the capacitative entry mechanism (Girard and Clapham, 1993).

Work in this area has proceeded along two paths. First, voltage-independent Ca^{2+} entry mechanisms have been intensively studied, and there are several Ca^{2+}-permeant channels that may serve this function (Table 3). None of these putative ion channels have been purified or cloned. The most well-established pathway in this regard is $I_{CRAC/DAC}$ (Ca^{2+} release–activated or depletion-activated current; for review, see Fasolato et al., 1994). I_{CRAC} has an extremely low conductance (~ 20 fS), approximately 1,000-fold lower than the conductance of most ion channels. The net current passing through the Ca^{2+} entry pathways in an entire cell is ~ 5 pA; in comparison, the net current activated in neurons by voltage-dependent Ca^{2+} channels is hundreds of pA. I_{CRAC} is activated by several experimental procedures that result in depletion of stores, probably the most reliable of which is thapsigargin, the inhibitor of the SERCA pump. I_{CRAC} is highly Ca^{2+}-selective and, like many other Ca^{2+} trigger proteins, is inactivated by high intracellular Ca^{2+} levels.

Several other Ca^{2+} entry pathways have been proposed but are probably of even lower density and more localized in function (Table 3). These include a Ca^{2+}-activated entry pathway modulated by $Ins(1,3,4,5)P_4$ (Lückhoff and Clapham, 1992), $InsP_3$-modulated Ca^{2+} entry pathways (Kuno and Gardner, 1987; Vaca and Kunze, 1994), and a higher conductance depletion-activated entry channel found in epithelial cells (Lückhoff and Clapham, 1994). Candidate genes for the Ca^{2+} entry pathways include the Drosophila gene products trp and trpl (Hardie and Minke, 1993). In Drosophila, light-induced PtdIns hydrolysis by PLC activates a Ca^{2+}-selective channel absent in the *trp* mutant. However, it is far from established that *trp* or *trpl* produce the protein responsible for I_{CRAC}, and no mammalian homo-

Table 3. Calcium Entry Currents

Current	Conductance at 22°C	Open Time (ms)	Selectivity	Mechanism	Reference
I_{CRAC}, I_{DAC}	0.02 pS (110 Ca^{2+})	Unknown	$Ca^{2+} > Ba^{2+} > Mn^{2+}$	Ionomycin, $InsP_3$/BAPTA/0 Ca^{2+} thapsigargin	Hoth and Penner, 1992 Zweifach and Lewis, 1993
I_{DC}	2 pS (160 Ca^{2+})	8	$Ba^{2+} >> Ca^{2+} \approx Mn^{2+}$	BAPTA/0 Ca^{2+}/thapsigargin	Lückhoff and Clapham, 1994
	20 pS (160 Ba^{2+})	16			
$I_{Ca.InsP_3}$	7 pS (100 Ba^{2+})	2	$Ba^{2+} = Ca^{2+}$	$[InsP_3]_i$	Kuno and Gardner, 1987
	8 pS (110 Ca^{2+})	20,200	$Ca^{2+} > Ba^{2+} > Na^{+}$	$[InsP_3]_i$	Vaca and Kunze, 1994
$I_{Ca.InsP_4}$	2 pS (100 Mn^{2+})	200	$Ca^{2+} = Ba^{2+} = Mn^{2+}$	$Ca^{2+}{}_i$ required, $InsP_4$ potentiates	Lückhoff and Clapham, 1992
$I_{Ca.ATP}$	5 pS (130 Ca^{2+})		$Ca^{2+} = Ba^{2+} > Na^{+}$	ATP receptor	Benham and Tsien, 1987
$I_{Ca.Ca}$	5 pS (90 Ca^{2+})		$Ca^{2+} = Na^{+} = K^{+}$	$Ca^{2+}{}_i$	Tscharner et al., 1986
	20 pS (90 Ca^{2+})				

logs of *trp* or *trpl* have yet been reported. A brief report linked recombinant *trpl* expression in sf9 insect cells with a Ca^{2+}-permeant but nonselective cation conductance (Hu et al., 1994).

The second major question in the field of capacitative entry is the mechanism by which depleted stores signal the Ca^{2+} entry channel. Numerous second messengers have been proposed to initiate I_{CRAC}, including small G proteins, pertussis toxin–sensitive heterotrimeric G proteins, cGMP, a product of cytochrome P450 activity, various lipids, tyrosine phosphorylation, and $InsP_3$, but to date none has been demonstrated to do so convincingly. The most interesting and controversial candidate second messenger is Ca^{2+} influx factor (CIF). CIF was initially isolated from Jurkat T cells stimulated to deplete their Ca^{2+} stores by phytohemagglutinin treatment (Randriamampita and Tsien, 1993). It has been partially characterized as a <500 Da phosphorylated pH-stable anion that induces Ca^{2+} influx when applied externally to macrophage, astrocytoma, and fibroblast cell lines. Presumably CIF is released or generated from the ER or adjacent regions after $InsP_3$ induces Ca^{2+} release from stores (Clapham, 1994). Support for a diffusable phosphorylated intermediate initiating Ca^{2+} entry came from experiments in Xenopus oocytes (Parekh et al., 1993), but not all properties of the oocyte current matched those of I_{CRAC}. Key issues to be settled in this field are whether there is more than one Ca^{2+} entry pathway mediated by store depletion and concrete identification of either a second messenger or direct signaling (analagous to the dihydropyridine–RyR) that mediates the store depletion signal. These efforts would be aided considerably by cloning and characterization of the protein responsible for I_{CRAC} and identification of CIF.

Ca^{2+} Waves

Ca^{2+} waves and oscillations are commonly observed in cells. The time and spatial variance of Ca^{2+} waves potentially contain much more information than simple static levels of intracellular Ca^{2+}. The large number of Ca^{2+}-binding proteins with unique Ca^{2+} binding rates and affinities dictates that waves and oscillations will have widespread effects in cells (Clapham and Sneyd, 1995). Like early recordings of action potentials, these waves are signals whose mechanisms and purposes must be defined for each tissue type; there is no one unique set of steps underlying all regenerative Ca^{2+} phenomena. In each cell type, the contribution and kinetics of the elements must be measured and incorporated into a mathematically defined model, ideally a set of partial differential equations that reproduce the observations (Atri et al., 1993).

Two Ca^{2+} wave models will be briefly discussed, representing regenerative Ca^{2+} release in some nonexcitable cells (e.g., Xenopus oocytes) and some excitable cells (e.g., sympathetic neurons). In immature Xenopus oocytes, $InsP_3$ receptors, but not RyRs, gate Ca^{2+} release from the ER. In this system (Atri et al., 1993; Lechleiter and Clapham, 1992), $InsP_3$ is generated by G protein–linked receptor stimulation of PLCβ. $InsP_3$ diffuses rapidly at speeds of ~230 $\mu m^2/s$ throughout the cell and occupies receptors for minutes (Allbritton and Meyer, 1993) before being degraded. $InsP_3$ receptors release Ca^{2+} in so-called hotspots (Lechleiter et al., 1991b). These hotspots may be due to high local concentrations of Ca^{2+}, $InsP_3$ receptors, or $InsP_3$. In this model, Ca^{2+} released from the ER diffuses to adjacent sites, where it increases the sensitivity of the $InsP_3$ receptor, inducing further Ca^{2+} release (the Ca^{2+} wavefront). Local Ca^{2+} release generates high $[Ca^{2+}]$ at the mouth of the $InsP_3$ receptor channel and directly inhibits the channel. Ca^{2+} ATPase pumps then remove Ca^{2+} from the cytoplasm. Overexpression of the SERCA1 pump increases the frequency of Ca^{2+} waves in Xenopus oocytes (Camacho and Lechleiter, 1993). When diffusion in two dimensions is incorporated, this model (Atri et al., 1993) reproduces complex patterns seen in large cells such as oocytes (Figure 3A).

In sympathetic neurons, Ca^{2+} entering via voltage-dependent Ca^{2+} channels triggers RyRs to release ER Ca^{2+}. The Ca^{2+} wavefront is maintained by spreading Ca^{2+}-induced Ca^{2+} release at RyRs. Ca^{2+} declines behind the wavefront as the ER Ca^{2+} is depleted and as pumps remove free Ca^{2+} (Friel, 1995). In contrast with nonexcitable cells, inhibition of channel activity at high $[Ca^{2+}]$ does not appear to be important for sympathetic neuronal Ca^{2+} oscillations. This difference may result in different types of Ca^{2+} wavefronts, i.e., more sharply defined wavefronts in nonexcitable cells. cADP-ribose increases the Ca^{2+} sensitivity of the nonskeletal RyR in sympathetic neurons, perhaps mediating the physiological counterpart of observed caffeine-induced Ca^{2+} oscillations in neurons (Hua et al., 1994).

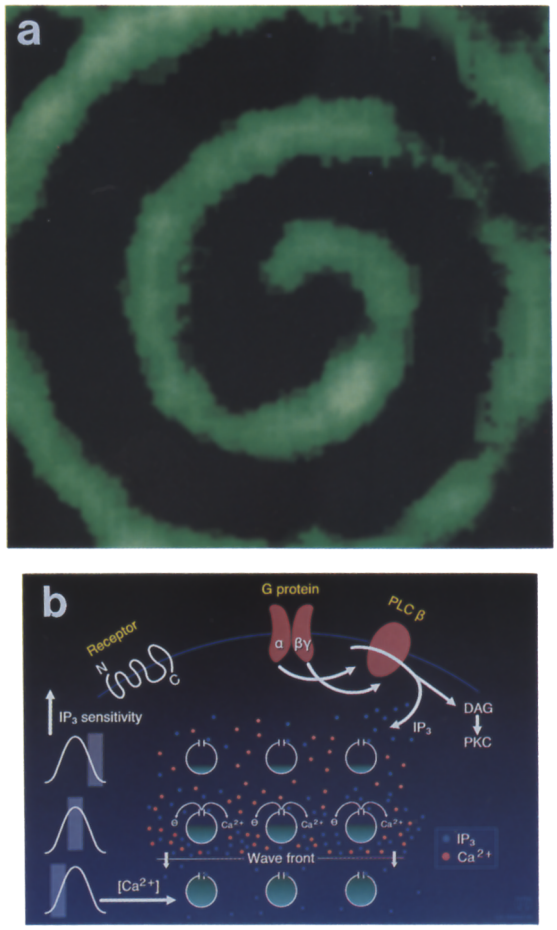

Figure 3. Ca^{2+} Waves

(A) Ca^{2+} wave observed in an intact Xenopus oocyte. Green indicates high $[Ca^{2+}]$ (peak level, approximately 0.5 μM). An oocyte was loaded with $InsP_3$ (10 μM) to evoke Ca^{2+} release and with the Ca^{2+}-sensitive fluorescent dye Fluo3 (15 μM) prior to confocal imaging. The distance between expanding wavefronts, or wavelength, is 250 μm, and waves travel 15–30 μm/s. Many other complex patterns, as well as simple planar waves, can be observed within the same oocyte.

(B) How Ca^{2+} waves are generated. The bell-shaped dependence of the $InsP_3$ receptor on $[Ca^{2+}]$, shown at the left side of the figure, is the key to understanding the model. The activated receptor initiates a signal transduction cascade to produce $InsP_3$, which diffuses rapidly throughout the cell. Where $[InsP_3]$ is very high, ER Ca^{2+} stores release Ca^{2+}. High $[Ca^{2+}]$ increases the sensitivity of $InsP_3$ receptor to $InsP_3$, rapidly releasing Ca^{2+} from stores and forming the wavefront (middle row). The very high $[Ca^{2+}]$ generated by Ca^{2+} pouring out of the $InsP_3$ channel decreases the sensitivity of the channel to $InsP_3$ and terminates Ca^{2+} release (top row, behind the Ca^{2+} wavefront). Full stores ahead of the diffusing Ca^{2+} wave (bottom row) have not yet released and are still relatively insensitive to $InsP_3$. Ca^{2+} pumps replete stores and lower cytoplasmic Ca^{2+} levels. The model in sympathetic neurons is similar, except that RyRs replace the $InsP_3$ receptors, and only the rising phase of the bell-shaped dependence on Ca^{2+} has significance. Pump activity and store depletion account for return of Ca^{2+} levels to normal in the neuronal model.

Ca^{2+} gradients within cells have been proposed to initiate cell migration, exocytosis, lymphocyte killer cell activity, acid secretion, transcellular ion transport, neurotransmitter release, gap junction regulation, and numerous other functions (Tsien and Tsien, 1990). The most complex wave patterns, exhibiting hotspots and spherical, spiral, and planar waves, were demonstrated in Xenopus oocytes (Lechleiter et al., 1991b). There appears to be insufficient space within a single 10–20 μm mammalian cell for such complex patterns, but similar patterns have been observed in larger cardiac cells and in networks of astrocytes and glia. Ca^{2+} signals between cells have also been identified in brain and epithelial function. Our understanding of the brain may be radically changed by observations of Ca^{2+} waves spreading across astrocytes and exciting overlying neuronal cells, either through gap junctions (Nedergaard, 1994) or via glutamate neurotransmisson between astrocytes and neurons (Parpura et al., 1994).

Life, Death, and Ca^{2+}

Ca^{2+} is essential for cell growth and survival, although its effects are so widespread that it has been difficult to pin down specific mechanisms. Ca^{2+} affects the cell cycle in more than one way: depletion of the $InsP_3$ receptor–gated Ca^{2+} pool results in cell cycle arrest at G0/G1 and S phases, and Ca^{2+} is necessary and sufficient for resumption of meiosis in marine eggs; a spike of Ca^{2+} triggers completion of meiosis and initiation of mitosis (Means, 1994). However, our understanding of the specific mechanisms for these effects is still at an early stage. In Xenopus oocytes, resumption of meiosis is mediated by Ca^{2+}/calmodulin stimulation of CaM kinase IIα to phosphorylate as yet unidentified targets (Lorca et al., 1993). G0 to G1 transitions in yeast and mammalian cells appear to require calcineurin, the Ca^{2+}-dependent Ser/Thr phosphatase 2B (Means, 1994).

A more defined endpoint than cell division is activation of a specific transcription factor. For example, Ca^{2+} stimulates CaM kinase–dependent phosphorylation of the cAMP response element–binding protein (CREB) (Sheng et al., 1991). The local nature of Ca^{2+} action is again demonstrated by the finding that gene transcription depends on how Ca^{2+} enters the cell. Ca^{2+} entry through voltage-dependent L type Ca^{2+} channels and N-methyl-D-aspartic acid (NMDA) receptors initiates gene transcription through distinct DNA-regulatory elements (Bading et al., 1993). Not only does the route of Ca^{2+} entry affect which genes are transcribed, but cellular $[Ca^{2+}]$ levels quantitatively correlate with transcription factor expression in single cells (Negulescu et al., 1994). More surprises on the far-reaching effects of Ca^{2+} on gene transcription are undoubtedly in store. For example, calreticulin, a molecule previously thought to act only as a Ca^{2+} buffer, appears to regulate the glucocorticoid nuclear hormone receptor (Burns et al., 1994).

Intranuclear Ca^{2+} increases initiate gene expression and cell cycle progression, but also can activate degradative processes in programmed cell death, or apoptosis. Prolonged high $[Ca^{2+}]$ activates nucleases that cleave DNA and degrade cell chromatin. Ca^{2+} promotes DNA digestion by direct stimulation of endonucleases, or indirectly by its activation of Ca^{2+}-dependent proteases, phosphatases, and phospholipases, resulting in a loss of chromatin structural integrity (Nicotera et al., 1994).

Although many loose correlations can be drawn be-

tween Ca^{2+}, transcriptional control, and cell division, the intervening details beg further study. In particular, a quantitative correlation between Ca^{2+} wave oscillation frequency and amplitude and transcription of a particular element would be an exciting development. Such a correlation would imply that receptors encode specific information in the frequency and amplitude of induced Ca^{2+} oscillations.

Conclusion

Evolution of molecular strategies to buffer cytosolic Ca^{2+} levels resulted in specialized Ca^{2+}-binding regions in proteins. These Ca^{2+}-binding motifs have been incorporated in many proteins, enabling Ca^{2+} to act as a triggering second-messenger element that induces conformational changes in effector molecules. Scores of receptors and ion channels use the Ca^{2+} signal to initiate events as basic as cell motility, contraction, secretion, and division. Ca^{2+} as a signal transduction element in excitable cells is controlled by depolarization of membrane potential, inducing conformational changes in Ca^{2+}-selective voltage-dependent channels. Ion channels raise cytosolic Ca^{2+} levels just under the plasma membrane within milliseconds, rapidly initiating events such as neurotransmission and muscle contraction. In nonexcitable cells, G protein–linked receptors and receptor tyrosine kinases initiate slower Ca^{2+} release at the endoplasmic reticulum/cytosolic interface. In nonexcitable cells, these events are potentiated by hyperpolarization rather than depolarization. Complex mechanisms for Ca^{2+} release from the ER and for Ca^{2+} entry across the plasma membrane, and for sequestering released Ca^{2+}, lead to an inherently oscillatory system. Whether these oscillations specify receptor or cell-specific information is one of the important questions yet to be answered in biology.

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