Calcium Signaling

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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, 1983; Pozzan et al., 1994; Rheen, 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\(^{2+}\), Mg\(^{2+}\) 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 accommodate 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, 1969). 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 (Naylor 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 calsecquestrin, 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 μm²/s) or even hydrated (D = 800 μm²/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 10² M·s⁻¹ kᵩ and 300 μM binding protein concentration [Albritton 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 μM²/s (Albritton et al., 1992). However, the image of a cell as a uniform volume for diffusion is 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 10⁶ 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) stretches 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 calsecquestrin, that have unknown trigger functions 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 housing Ca\(^{2+}\). Is protein sorting conducted in this strange environment, or are there separate and dynamically rearranged ER pools? Do high free and buff-
Table 1. Examples of Mammalian Proteins Triggered by Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Ca(^{2+})-Binding Site</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin C</td>
<td>EF hand</td>
<td>Modulator of muscle contraction</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>EF hand</td>
<td>Ubiquitous modulator of protein kinases and other enzymes (MLCK, CAM kinase II, adenylyl cyclase I)</td>
</tr>
<tr>
<td>Calsemin, rilin, vishin</td>
<td>EF hand</td>
<td>Activator of guanylyl cyclase</td>
</tr>
<tr>
<td>CaClemine B</td>
<td>EF hand</td>
<td>Phosphatase</td>
</tr>
<tr>
<td>Calpain</td>
<td>EF hand</td>
<td>Protease</td>
</tr>
<tr>
<td>Inositol phospholipid-specific PLCα</td>
<td>EF hand</td>
<td>Generator of InsP(_2) and diacylglycerol</td>
</tr>
<tr>
<td>α-Adrenerg</td>
<td>EF hand</td>
<td>Actin-bundling protein</td>
</tr>
<tr>
<td>Annexin</td>
<td></td>
<td>Implicated in endo- and exocytosis, inhibition of PLA(_2) ion channel?</td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td></td>
<td>Producer of arachidonic acid</td>
</tr>
<tr>
<td>Protein kinase C</td>
<td></td>
<td>Ubiquitous protein kinase</td>
</tr>
<tr>
<td>Gelatin</td>
<td></td>
<td>Actin-severing protein</td>
</tr>
<tr>
<td>Ca(^{2+})-activated K(^+) channel</td>
<td></td>
<td>Effector of membrane hyperpolarization</td>
</tr>
<tr>
<td>InsP(_2) Receptor</td>
<td></td>
<td>Effector of intracellular Ca(^{2+}) release</td>
</tr>
<tr>
<td>Ryanodine receptor</td>
<td></td>
<td>Effector of intracellular Ca(^{2+}) release</td>
</tr>
<tr>
<td>Na(^+)/Ca(^{2+}) exchanger</td>
<td></td>
<td>Effector of the exchange of Ca(^{2+}) for Na(^+) across the plasma membrane</td>
</tr>
<tr>
<td>Ca(^{2+}) ATPase</td>
<td></td>
<td>Pump of Ca(^{2+}) across membranes</td>
</tr>
<tr>
<td>Ca(^{2+}) antiporator</td>
<td></td>
<td>Exchanger of Ca(^{2+}) for monovalent ions</td>
</tr>
<tr>
<td>BiP/Grp</td>
<td></td>
<td>G protein-linked Ca(^{2+})-sensing receptor</td>
</tr>
<tr>
<td>Caldesmon</td>
<td></td>
<td>Regulator of muscle contraction</td>
</tr>
<tr>
<td>Villin</td>
<td></td>
<td>Actin organizer</td>
</tr>
<tr>
<td>Anillin</td>
<td></td>
<td>Terminator of photoreceptor response</td>
</tr>
<tr>
<td>S100G</td>
<td></td>
<td>Unknown</td>
</tr>
<tr>
<td>Calpastatin</td>
<td></td>
<td>Ca(^{2+}) buffer/modulator of nuclear hormone receptor</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td>EF hand</td>
<td>Ca(^{2+}) buffer</td>
</tr>
<tr>
<td>Calbindin</td>
<td>EF hand</td>
<td>Ca(^{2+}) buffer</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>EF hand</td>
<td>Ca(^{2+}) buffer</td>
</tr>
</tbody>
</table>

Ferred 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 C\(_{β\text{1}}\) (PLC\(_{β\text{1}}\)), while RTKs stimulate phospholipase C\(_{β\text{2}}\) (PLC\(_{β\text{2}}\)) to convert phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P\(_2\)) into InsP\(_3\) and diacylglycerol (Berndige 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 channels near the plasma membrane inner surface initiate functions as diverse as excocytosis 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.
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 PLC\(_{β}\) (Table 2). Experiments using pertussis toxin (which disrupts coupling between receptors and G\(_{αi}\), G\(_{αo}\), and G\(_{αq}\)) demonstrate that both pertussis toxin–sensitive and –insensitive G proteins transduce signals between receptors and PLC\(_{β}\) (Sternweis and Smrcka, 1992). The most well-established path for activation of PLC\(_{β}\) is through the pertussis toxin–insensitive G\(_{αi}\) and presumptive others of the family, G\(_{αo}\), G\(_{αq}\), G\(_{αs}\), Lee et al., 1992; Smrcka et al., 1991; Taylor and Marshall, 1992). G\(_{αq}\)-linked receptors, such as the muscarinic type 3 and serotonin 5HT\(_{1C}\), 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\(_{αi}\) and G\(_{αo}\) subunits are involved (Moriarty et al., 1990).

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

| Table 2. Plasma Membrane Receptors Increasing Intracellular Ca\(^{2+}\) |
|--------------------------|--------------------------|--------------------------|
| Via PLC\(_{β}\) | Via PLC\(_{γ}\) | Directly |
| α1-Adrenergic | Epidermal growth factor receptor | Nicotinic ACh channels |
| Muscarinic m1, m3, m5 | Platelet-derived growth factor receptor | Glutamate receptor family of ion channels |
| Purinergic P2Y-P2U, P2T | Fibroblast growth factor receptor | |
| Serotonin 5HT\(_{1C}\) | Endothelin | |
| H1 | Beta3 | |
| GnRH | T cell receptor | |
| TRH | Glucagon | |
| Glucagon | Cholecystokinin | |
| Cholecystokinin | Vasoressin V-1a, V-1b | |
| Vasoressin V-1a, V-1b | Dihydonor | |
| Dihydonor | Angiotensin II | |
| Angiotensin II | Thrombin | |
| Thrombin | Bombesin | |
| Bombesin | Vasoactive intestinal peptide | |
| Vasoactive intestinal peptide | Bradykinin | |
| Bradykinin | Tachykinin | |
| Tachykinin | Thromboxanes | |
| Thromboxanes | Platelet-activating factor | |
| Platelet-activating factor | F-Met-Leu-Phe | |
| F-Met-Leu-Phe | Endothelin | |
| Endothelin | BoPCAR | |
that transducin Gβ (Gβγ) is less effective than other dimer combinations.

The fact that both Gα and Gβγ can modulate PLCγ greatly increases the potential complexity of signal transduction (Figure 2). Ca2+ is an important modulator of PLCβ, adenyl cyclase, nitric oxide synthase, phospholipases, and calmodulin (CaM) kinases. The multiple convergent and divergent pathways make defining 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 PtdIns(4,5)P2. Again, InsP3 and diacylglycerol are formed. In general, tyrosine kinase–activated PLCγ is the most stoichiometrically active and for longer durations than do G-mediated PLCγs.

PtdIns(4,5)P2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) into PtdInsP and PtdIns(4,5)P2. Once PLC splits PtdInsP into InsP3 and diacylglycerol, a complex set of enzymes mediates the generation of multiple inositol phosphates (Berridge and Irving, 1989). It is clear that InsP3 is the dominant second-messenger molecule for release of intracellular Ca2+ (Berridge, 1993). However, inositol (1,3,4,5)tetraakisphosphate (InsP1,3,4,5) enhances Ca2+-induced Ca2+ influx (Lückhoff and Clapham, 1992), inhibits Ca2+ ATPase pumps (Yoo, 1991), and may bind specific intracellular receptors (Theibert et al., 1991). Inositol (3,4,5,6)tetraakisphosphatidate (InsP3,4,5,6)P4 apparently uncouples muscarinic stimulation of Ca2+ release (Vagananichan et al., 1994).

InsP3 and Ryanodine Receptors

The InsP3 receptor is a homodimer of ~310 kDa subunits surrounding a relatively nonselective cationic pore (Mikoshiba, 1993). InsP3 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 InsP3 molecule in a positively charged, Arg/Lys-rich N-terminal region. InsP3 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 RyRs, and no significant homology with voltage-dependent Ca2+ channels. The cytoplasmic N-terminal region also has two ATP-binding sites and at least one Ca2+-binding site (Mignery and Sudhof, 1990). Regulation of the InsP3 receptor is complex in that it binds multiple InsP3 molecules, is desensitized by InsP3 itself (Hejnačzky and Thomas, 1994), is phosphorylated by protein kinase A, and has a biphasic sensitivity to cytoplasmic Ca2+ levels (Bezprozvanny et al., 1991; Finch and Goldin, 1994; Iino, 1990; Parys et al., 1992). At low and high Ca2+ levels, the InsP3 receptor is relatively insensitive to InsP3. The sensitivity of the receptor to InsP3, which is also biphasic, is greatest in the physiological range between 0.5 μM and 10 μM InsP3. Properties of the InsP3 receptor in vivo have been confirmed by direct patch-clamp recordings of the outer nuclear membrane (Siegho-Bittler et al., 1995).

The phenomenon of quantal release (Boothman, 1994), in which repetitive applications of low concentrations of intracellular InsP3 release quanta, or fractions of the Ca2+ pool, will continue to puzzle Ca2+ researchers until basic regulatory properties of the channel are understood. Such properties as cytoplasmic Ca2+ or InsP3-induced desensitization, differing receptor isoform affinity for InsP3, InsP3 receptor distribution, and intraluminal Ca2+ regulation of the InsP3 receptor need further quantitation. There is evidence both for and against significant intraluminal control of InsP3 receptor gating (Mossialos et al., 1991; Shuttleworth, 1992). Another area of controversy has been the significance of InsP3 receptors found in the plasma membrane. Although the ER contains by far the highest number of InsP3 receptors, InsP3 receptors have also been identified in the plasma membranes (Khan et al., 1992).

The RyR (the excitable cell counterpart of the InsP3 receptor) is composed of a tetramer of four ~580 kDa subunits and is gated either by electromechanical coupling to the plasma membrane dihydropyridine receptor in skeletal muscle (see Figure 1b), by Ca2+, or by cADPR-ribose in some cell types (Coronado et al., 1994; Ehrlich et al., 1994). Like the InsP3 receptor, it is modulated by Mg2+, ATP, and Ca2+, although Ca2+ and Mg2+ inhibition occurs in the mM range. Similarly, the RyR is relatively nonselective for cations, although it includes all anions. A separate gene (gp-1, gp-2, and gp-3) encodes RyRs expressed predominantly in skeletal muscle, cardiac muscle, and brain or smooth muscle, respectively. FKBP12, a cis-trans peptidylprolyl cis-trans isomerase 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, cADPR-ribose, releases Ca2+ in sea urchin eggs and may be a physiologically relevant RyR agonist in cardiac and pancreatic cells (Nyvold et al., 1993). Interestingly, the second-messenger cyclic GMP regulates cADPR-ribose levels in some cells (Galione, 1993). One mystery of neuronal Ca2+ 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 are 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$^{2+}$ concentrations.

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$^{2+}$ uniporers 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 receptor results in relatively rapid rises in cytoplasmic Ca$^{2+}$ levels (Lechleiter et al., 1991), 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 the NCX (Ca$^{2+}$ release-activated or depletion-activated current; for review, see Fasiolet al., 1994). NCX has an extremely low conductance (~200 Å), 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. NCX 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. NCX 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$_4$ (Luckhoff 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 (Luckhoff and Clapham, 1994). Candidate genes for the Ca$^{2+}$ entry pathways include the Drosophila gene products trp and trp (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 trp produce the protein responsible for NCX, and no mammalian homo-
Table 3. Calcium Entry Currents

<table>
<thead>
<tr>
<th>Current</th>
<th>Conductance at 22°C (pS)</th>
<th>Open Time (ms)</th>
<th>Selectivity</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>l_{Ca}</td>
<td>0.02 pS (110 Ca²⁺)</td>
<td>8</td>
<td>Ba²⁺ &gt; Na⁺ &gt; Mn²⁺</td>
<td>Ionomycin, InsP₃/BAPTA/Al Ca²⁺</td>
<td>Roth and Parmer, 1992</td>
</tr>
<tr>
<td>l_{Ca}</td>
<td>2 pS (160 Ca⁺⁺)</td>
<td>18</td>
<td>Ba²⁺ &gt; Ca²⁺ &gt; Mn²⁺</td>
<td>thapsigargin</td>
<td>Zwiller and Lewis, 1993</td>
</tr>
<tr>
<td>l_{KCa}</td>
<td>2 pS (100 Ba²⁺)</td>
<td>2</td>
<td>Ca²⁺ &gt; Ba²⁺ &gt; Na⁺</td>
<td>BAPTA/Al Ca²⁺ thapsigargin</td>
<td>Lückhoff and Clapham, 1994</td>
</tr>
<tr>
<td>l_{KCa}</td>
<td>6 pS (110 Ca²⁺)</td>
<td>20,200</td>
<td>Ca²⁺ &gt; Ba²⁺ &gt; Na⁺</td>
<td>[InsP₃]</td>
<td>Kuro and Gardiner, 1987</td>
</tr>
<tr>
<td>l_{KCa}</td>
<td>2 pS (100 Mn²⁺)</td>
<td>200</td>
<td>Ca²⁺ = Ba²⁺ &gt; Mn²⁺</td>
<td>[InsP₃]</td>
<td>Vaca and Kunze, 1994</td>
</tr>
<tr>
<td>l_{Ca}</td>
<td>5 pS (130 Ca⁺⁺)</td>
<td>200</td>
<td>Ca²⁺ = Ba²⁺ &gt; Na⁺</td>
<td>Ca²⁺, required, InsP₃, potentiates</td>
<td>Lückhoff and Clapham, 1992</td>
</tr>
<tr>
<td>l_{Ca}</td>
<td>8 pS (90 Ca⁺⁺)</td>
<td>200</td>
<td>Ca²⁺ = Na⁺ &gt; K⁺</td>
<td>ATP receptor</td>
<td>Benham and Tsien, 1987</td>
</tr>
<tr>
<td>l_{Ca}</td>
<td>20 pS (90 Ca⁺⁺)</td>
<td>200</td>
<td>Ca²⁺ = Na⁺ &gt; K⁺</td>
<td>Ca²⁺,</td>
<td>Tscharre et al., 1992</td>
</tr>
</tbody>
</table>

logks of trp or trp have yet been reported. A brief report linked recombinant trp expression in sf9 insect cells with a Ca²⁺-permeant but nonselective cation conductance (Hu et al., 1994).

The second major question in the field of capacitative entry is the mechanism by which depolarized stores signal the Ca²⁺ entry channel. Numerous second messenger signals have been proposed to initiate l_{Ca}, including small G proteins, pertussis toxin-sensitive heterotrimeric G proteins, cGMP, a product of cGMP-dependent protein kinase activity, various lipids, tyrosine phosphorylation, and InsP₃, but to date none has been demonstrated to do so convincingly. The most interesting and controversial candidate second messenger is Ca²⁺ influx factor (CIF). CIF was initially isolated from Jurkat T cells stimulated to deplete their Ca²⁺ stores by phorbol ester treatment (Randriamampita and Tsien, 1993). It has been partially characterized as a 50 Da phosphorylated acid-stable anion that induces Ca²⁺ influx when applied extracellularly to a high Na⁺ medium (Peter et al., 1993). However, not all properties of the current matched those of l_{Ca}. Key issues to be settled in this field are whether there is more than one Ca²⁺ entry pathway mediated by store depletion and concrete identification of either a second messenger or direct signaling (analogous 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 l_{Ca} and identification of CIF.

Ca²⁺ Waves

Ca²⁺ waves and oscillations are commonly observed in cells. The time and spatial variance of Ca²⁺ waves potentially contain much more information than simple static levels of intracellular Ca²⁺. The large number of Ca²⁺-binding proteins with unique Ca²⁺ 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 unique set of steps underlying all regenerative Ca²⁺ 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 Ca²⁺ wave models will be briefly discussed, representing regenerative Ca²⁺ release in some nonexcitable cells (e.g., Xenopus oocytes) and some excitable cells (e.g., sympathetic neurons). In immature Xenopus oocytes, InsP₃ receptors, but not RyRs, gate Ca²⁺ release from the ER. In this system (Atri et al., 1993; Lechleiter and Clapham, 1992), InsP₃ is generated by G protein-linked receptor stimulation of PLC. InsP₃ diffuses rapidly at speeds of ~200 μm/s throughout the cell and occupies receptors for minutes (Allbritton and Meyer, 1993) before being degraded. InsP₃ receptors release Ca²⁺ in so-called hotspots (Lechleiter et al., 1991b). These hotspots may be due to high local concentrations of Ca²⁺, InsP₃ receptors, or InsP₃ itself. In this model, Ca²⁺ released from the ER diffuses to adjacent sites, where it increases the sensitivity of the InsP₃ receptor, inducing further Ca²⁺ release (the Ca²⁺ wavefront). Local Ca²⁺ release generates high [Ca²⁺] at the mouth of the InsP₃ channel and directly inhibits the channel. Ca²⁺ ATPase pumps then remove Ca²⁺ from the cytoplasm. Overexpression of the SERCA1 pump increases the frequency of Ca²⁺ waves in Xenopus oocytes (Carmocho 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, Ca²⁺ entering via voltage-dependent Ca²⁺ channels triggers RyRs to release ER Ca²⁺. The Ca²⁺ wavefront is maintained by spreading Ca²⁺-induced Ca²⁺ release at RyRs. Ca²⁺ declines behind the wavefront as the ER Ca²⁺ is depleted and as pumps remove free Ca²⁺ (Friel, 1995). In contrast with nonexcitable cells, inhibition of channel activity at high [Ca²⁺] does not appear to be important for sympathetic neuronal Ca²⁺ oscillations. This difference may result in different types of Ca²⁺ waveforms, i.e., more sharply defined waveforms in nonexcitable cells. CAMP-elevates increases the Ca²⁺ sensitivity of the nonkeletal RyR in sympathetic neurons, perhaps mediating the physiological counterpart of observed caffeine-induced Ca²⁺ oscillations in neuronal cells (Hu et al., 1994).
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. Ca²⁺ signals between cells have also been identified in brain and epithelial function. Our understanding of the brain may be radically changed by observations of Ca²⁺ waves spreading across astrocytes and exciting overlying neuronal cells, either through gap junctions (Nedergaard, 1994) or via glutamate neurotransmission between astrocytes and neurons (Patapua et al., 1994).

Life, Death, and Ca²⁺
Ca²⁺ is essential for cell growth and survival, although its effects are so widespread that it has been difficult to pin down specific mechanisms. Ca²⁺ affects the cell cycle in more than one way: depletion of the InsP₃ receptor–gated Ca²⁺ pool results in cell cycle arrest at G0/G1 and S phases, and Ca²⁺ is necessary and sufficient for resumption of meiosis in marine eggs; a spike of Ca²⁺ 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 Ca²⁺/calmodulin stimulation of CAM kinase II to phosphorylate as yet unidentified targets (Lorca et al., 1993). G0 to G1 transitions in yeast and mammalian cells appear to require calcineurin, the Ca²⁺-dependent Ser/Thr phosphatase 2B (Means, 1994).

A more defined endpoint than cell division is activation of a specific transcription factor. For example, Ca²⁺ stimulates CAM kinase–dependent phosphorylation of the cAMP response element–binding protein (CREB) (Sheng et al., 1991). The local nature of Ca²⁺ action is again demonstrated by the finding that gene transcription depends on how Ca²⁺ enters the cell. Ca²⁺ entry through voltage-dependent L type Ca²⁺ channels and N-methyl-D-aspartate acid (NMDA) receptors initiates gene transcription through distinct DNA-regulatory elements (Bading et al., 1993). Not only does the route of Ca²⁺ entry affect which genes are transcribed, but cellular Ca²⁺ levels quantitatively correlate with transcription factor expression in single cells (Negulescu et al., 1994). More surprises on the far-reaching effects of Ca²⁺ on gene transcription are undoubtedly in store. For example, calcineurin, a molecule previously thought to act only as a Ca²⁺ buffer, appears to regulate the glucocorticoid nuclear hormone receptor (Burns et al., 1994).

Intracellular Ca²⁺ increases initiate gene expression and cell cycle progression, but also can activate degradative processes in programmed cell death, or apoptosis. Prolonged high Ca²⁺ activates nucleases that cleave DNA and degrade cell chromatin. Ca²⁺ promotes DNA digestion by direct stimulation of endonucleases, or indirectly by its activation of Ca²⁺–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-
between Ca²⁺, transcriptional control, and cell division, the 
intervening details beg further study. In particular, a quan-
titative correlation between Ca²⁺ 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 Ca²⁺ oscillations.

Conclusion
Evolution of molecular strategies to buffer cytosolic Ca²⁺ 
levels resulted in specialized Ca²⁺-binding regions in pro-
teins. These Ca²⁺-binding motifs have been incorporated in 
many proteins, enabling Ca²⁺ to act as a triggering 
second-messenger element that induces conformational 
changes in effector molecules. Scores of receptors and 
ion channels use the Ca²⁺ signal to initiate events as basic 
as cell motility, contraction, secretion, and division. Ca²⁺ 
as a signal transduction element in excitable cells is con-
trolled by depolarization of membrane potential, inducing 
conformational changes in Ca²⁺-selective voltage-depen-
dant channels. Ion channels raise cytosolic Ca²⁺ levels just 
under the plasma membrane within milliseconds, rapidly 
initiating events such as neurotransmission and muscle 
contraction. In nonexcitable cells, G protein–linked recep-
tors and receptor tyrosine kinases initiate slower Ca²⁺ re-
lease at the endoplasmic reticulum/cytosolic interface. In 
nonexcitable cells, these events are potentiated by hyper-
polarization rather than depolarization. Complex mechanis-
ms for Ca²⁺ release from the ER and for Ca²⁺ entry 
across the plasma membrane, and for sequestering re-
leased Ca²⁺, 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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