Intracellular Ca\(^{2+}\) regulation is an intensely studied field in physiology due to the ubiquitous nature of Ca\(^{2+}\) and its prominence as a signaling molecule. Because changes, small or large, in the cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) can alter a tremendous number of cellular processes, a complete understanding of the regulatory subtleties is essential. A component of this regulation is the capacitative Ca\(^{2+}\) entry pathway that provides for a Ca\(^{2+}\) influx into the cytoplasm in response to a loss of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) storage compartments (for review, see Refs. 1, 12).

Capacitative Ca\(^{2+}\) entry

Many cells rely on intracellular stores of Ca\(^{2+}\), typically a specialized subcompartment of the endoplasmic reticulum, to promote specific signaling pathways. Release of Ca\(^{2+}\) into the cytoplasm from an intracellular storage site provides a means to elevate the [Ca\(^{2+}\)]\(_i\), at discrete subcellular locations or in a spatially uniform pattern throughout the interior of even very large cells. In contrast, Ca\(^{2+}\) entry from the extracellular environment would yield pronounced gradients, with the concentration being highest near the plasma membrane. Both routes, release and entry, can be utilized to dictate Ca\(^{2+}\) levels within specific domains of the cell. Most nonexcitable cells are dependent on inositol 1,4,5-trisphosphate (IP\(_3\))-sensitive intracellular stores to increase the [Ca\(^{2+}\)]\(_i\), in response to physiological stimuli. As an example, a typical ATP-induced [Ca\(^{2+}\)]\(_i\), transient in a single, cultured vascular endothelial cell is shown in Fig. 1A. In the presence of extracellular Ca\(^{2+}\), the transient is characterized by a rapid upstroke, followed by a gradual decline to a plateau level of elevated Ca\(^{2+}\) (Fig. 1A). However, after removal of extracellular Ca\(^{2+}\), the plateau phase of elevated Ca\(^{2+}\) is abolished (Fig. 1B). Figure 1 demonstrates the combined effects of agonist stimulation triggering Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores (the rapid upstroke of [Ca\(^{2+}\)]\(_i\)) and extracellular Ca\(^{2+}\) entry to maintain the elevated plateau phase of Ca\(^{2+}\). The experiment performed in Ca\(^{2+}\)-free conditions shows that the increase in the intracellular Ca\(^{2+}\) signal can be reasonably short lived (Fig. 1B). To achieve this return to baseline on a fast time scale, cells use an endoplasmic reticulum Ca\(^{2+}\)-adenosine 5\(^{-}\)triphosphatase (ATPase) to recycle Ca\(^{2+}\) back into the stores, making it available for subsequent release. In addition, cells also utilize the plasma membrane to transport Ca\(^{2+}\) out of the cell and decrease [Ca\(^{2+}\)]\(_i\) (via Na\(^+\)/Ca\(^{2+}\) exchange and plasma membrane Ca\(^{2+}\)-ATPase). This makes the reliance on an intracellular store to generate transient increases in [Ca\(^{2+}\)] problematic because the amount of Ca\(^{2+}\) within the stores is finite compared with the extracellular environment. Indeed, for every [Ca\(^{2+}\)]\(_i\), transient generated by release from intracellular stores, a fraction of that load is lost to the extracellular environment.

In the absence of compensatory mechanisms, it then seems inevitable that repetitive stimulation of cells in vivo would result in the depletion of the finite reserves in the intracellular stores. Clearly, the cell’s physiological dependence on these stores makes it necessary for it to also have a mechanism to quickly replenish them. Because the extracellular environment serves as the ultimate Ca\(^{2+}\) reservoir, refilling of these stores must employ a plasma membrane influx pathway to deliver Ca\(^{2+}\) from the extracellular environment to the cytoplasm and subsequently to the internal...
compartments. This ability to generate a unique plasma membrane Ca\(^{2+}\) influx as a means to replenish depleted intracellular storage compartments is a recognized role of the capacitative Ca\(^{2+}\) entry pathway. Beyond store refilling, capacitative Ca\(^{2+}\) entry may serve to prolong the initial agonist-induced Ca\(^{2+}\) signal (Fig. 1A) and to sustain the propagation of Ca\(^{2+}\) waves and oscillations (for review, see Refs. 1 and 3). Recent findings have also provided increasing evidence that the Ca\(^{2+}\) influx through this pathway may serve to regulate cellular functions such as adenylate cyclase activity, phototransduction in Drosophila photoreceptors, and mitogenesis during conditions of low or depleted Ca\(^{2+}\) stores (for review, see Ref. 1).

Capacitative Ca\(^{2+}\) entry, first proposed by Putney (11) and recently reviewed in great and thorough detail (12), is a term based on its analogy to a capacitor in an electrical circuit. The pathway is characterized by the fact that charged or full intracellular stores prevent Ca\(^{2+}\) current flow through this pathway, whereas discharged or empty stores promote Ca\(^{2+}\) flux into the cytoplasm. Evidence is accumulating that these capacitative transport pathways may be a family of channels, on the basis of the various kinetics reported for Ca\(^{2+}\) influx (for review, see Ref. 3). These entry channels do not conform to the typical characteristics of other well-recognized Ca\(^{2+}\) influx pathways, such as voltage-operated channels, second mes-

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**FIGURE 1.** Capacitative Ca\(^{2+}\) entry signals. Prolonged stimulation of a single vascular endothelial cell (CPAE cell; cell line derived from calf pulmonary artery endothelium) with ATP in the presence (A) or absence (B) of 2 mM extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{o}\)]). Cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{i}\)] \(_{i}\)) was measured with the Ca\(^{2+}\)-sensitive dye indo 1. Sustained Ca\(^{2+}\) influx that occurred in the presence of extracellular Ca\(^{2+}\) (A) is thought to be due to activation of capacitative Ca\(^{2+}\) entry. C: standard protocol to activate capacitative Ca\(^{2+}\) entry consisted of Ca\(^{2+}\) store depletion with thapsigargin in the absence of extracellular Ca\(^{2+}\). Ca\(^{2+}\) was returned to the surrounding bath solution, and subsequent rise in [Ca\(^{2+}\)\(_{i}\)] (overshoot) was due to capacitative Ca\(^{2+}\) entry. D: subcellular [Ca\(^{2+}\)\(_{i}\)] gradients during capacitative Ca\(^{2+}\) entry were measured using confocal laser scanning microscopy in a fluo 3-loaded, thapsigargin-treated cell. At left, a–c mark locations in cell.
senger-operated channels, or receptor-operated channels (for review, see Ref. 4). This unique capacitative Ca\textsuperscript{2+} entry family of influx pathways has been demonstrated in numerous cell types, including endothelial cells, exocrine acinar cells, lymphocytes, oocytes, and hepatocytes to name a few. In general, excitable cells do not appear to generate a “traditional” capacitative Ca\textsuperscript{2+} entry. This may be because these cells generate significant voltage-dependent Ca\textsuperscript{2+} influx across the plasma membrane during excitation, which may make the sustaining action of the capacitative Ca\textsuperscript{2+} entry redundant. In addition, most excitable cells are much less dependent on IP\textsubscript{3}-sensitive stores to increase the [Ca\textsuperscript{2+}]i. That being said, some smooth muscle cells, chromaffin cells, and adrenal glomerulosa cells have been shown to generate capacitative Ca\textsuperscript{2+} entry, and this may be attributed to their partial reliance on IP\textsubscript{3}-sensitive intracellular stores.

Evidence for store dependence

Although many aspects of capacitative Ca\textsuperscript{2+} entry remain unknown or controversial, the store-dependent generation of the plasma membrane Ca\textsuperscript{2+} influx appears to be certain and well documented. The first direct experimental proof of the capacitative Ca\textsuperscript{2+} entry model was demonstrated by Hallam et al. (5). They determined that depletion of the intracellular stores activated Ca\textsuperscript{2+} entry by a mechanism independent of receptor occupation or inositol phosphates. Depletion of the intracellular stores by repetitive agonist stimulation in a Ca\textsuperscript{2+}-free environment triggered a large influx of Ca\textsuperscript{2+} when the ion was returned to the extracellular environment. This large Ca\textsuperscript{2+} influx or “overshoot” is now the characteristic trademark of capacitative Ca\textsuperscript{2+} entry. This influx pathway can also be activated by inhibition of the sarcoplasmic-endoplasmic reticulum Ca\textsuperscript{2+}-ATPase pump using thapsigargin, cyclopiazonic acid, or 2,5-di-tert-butyl-1,4-benzohydroquinone. These inhibitors block the sequestration of Ca\textsuperscript{2+}, thereby promoting the depletion of the stores and triggering capacitative Ca\textsuperscript{2+} entry. Figure 1C illustrates a typical protocol for depleting the intracellular stores of Ca\textsuperscript{2+} with thapsigargin in a Ca\textsuperscript{2+}-free extracellular solution. The subsequent readdition of Ca\textsuperscript{2+} to the surrounding bath induces a large increase in [Ca\textsuperscript{2+}]i, (overshoot), indicative of capacitative Ca\textsuperscript{2+} entry. Prolonged incubation or perfusion of cells in Ca\textsuperscript{2+}-free solution alone will also trigger capacitative Ca\textsuperscript{2+} entry, following the return of extracellular Ca\textsuperscript{2+}. This demonstrates that a rise in intracellular Ca\textsuperscript{2+} is not a prerequisite to the activation of this pathway in cultured vascular endothelial cells.

Regardless of the mechanism used to generate the capacitative Ca\textsuperscript{2+} influx in these cells, spatially resolved measurements show distinct cellular gradients (Fig. 1D). Activation of the capacitative pathway causes the [Ca\textsuperscript{2+}], to rise faster and to higher levels in the cell periphery compared with the perinuclear region or the nucleus (Fig. 1D). The most likely explanation for these differences in the subcellular capacitative Ca\textsuperscript{2+} entry transients is regional differences in the membrane surface area-to-volume ratio. The thickness of the cultured vascular endothelial cell dramatically decreases from the nuclear region toward the cell’s periphery. Thus the surface-to-volume ratio is highest in the periphery and decreases toward the nucleus. As a result, during Ca\textsuperscript{2+} entry across the plasma membrane, [Ca\textsuperscript{2+}], rose faster and to higher levels in the periphery (Fig. 1Da). In contrast, the Ca\textsuperscript{2+} signal was slower and the initial overshoot smaller close to or in the cell nucleus (Fig. 1D, b and c, respectively). These marked [Ca\textsuperscript{2+}], gradients during capacitative entry slowly disappeared as [Ca\textsuperscript{2+}], relaxed to a sustained plateau level in the continuous presence of extracellular Ca\textsuperscript{2+}. Together, this influx pathway can be activated in the absence of agonist, IP\textsubscript{3}, or elevation of [Ca\textsuperscript{2+}],. Therefore, depletion of the intracellular stores appears to be the sole requirement for stimulation of the mechanism inducing capacitative Ca\textsuperscript{2+} entry.

How is the intracellular store status detected and transmitted to the plasma membrane influx channel?

In contrast to the relative certainty associated with the store-dependent control of the capacitative Ca\textsuperscript{2+} entry channel, the means by which the level of Ca\textsuperscript{2+} in the stores is monitored and communicated to the plasma membrane is very unclear. Any model of capacitative Ca\textsuperscript{2+} entry requires that the intracellular compartment communicates the status of its filling to the plasma membrane influx channels. This particular aspect of the process has received considerable attention. To date, no clear mechanism has been established and very little precedence exists for the biological monitoring of an intracellular storage compartment. However, there are a number of noteworthy theories about capacitative Ca\textsuperscript{2+} entry that are in various stages of development. One of the original suggestions held that depleted storage compartments release a low-molecular-weight factor or diffusible signal, the so-called “Ca\textsuperscript{2+} influx factor,” into the cytoplasm (Fig. 2A). This factor was described as being a phosphorylated compound with a molecular mass of <500 Da (13). Evidence exists to suggest
that this factor is responsible for activating the Ca\(^{2+}\) influx at the plasma membrane. However, other diffusible signals have received attention, including inositol 1,3,4,5-tetrakisphosphate (IP\(_4\)), guanosine \(3'\)'\(,5'\)'-cyclic monophosphate, and metabolites of the cytochrome P-450 system. The activation of a tyrosine kinase is also suggested to occur in a parallel fashion with store Ca\(^{2+}\) depletion. Indeed, it has been demonstrated that depletion-induced Ca\(^{2+}\) influx is associated with tyrosine phosphorylation in endothelial cells. On the basis of the known influence of tyrosine phosphorylation on many different Ca\(^{2+}\)-transport pathways, this is an attractive hypothesis. However, a consensus on the factors or molecules involved in capacitative Ca\(^{2+}\) entry has not been reached (for further references to this topic, see Ref. 12).

Another model to explain detection and communication of capacitative Ca\(^{2+}\) entry is conformational coupling. This model suggests that there is a direct (protein-protein) interaction between the intracellular Ca\(^{2+}\) storage compartment and the capacitative Ca\(^{2+}\) entry channel in the plasma membrane (Fig. 2B). This direct interaction is an analogy taken from skeletal muscle. The plasma membrane Ca\(^{2+}\) channels in the transverse tubules of skeletal muscle are closely apposed to the Ca\(^{2+}\) release channels of the sarcoplasmic reticulum membrane, allowing for functional coupling between the two structures. The conformational coupling or protein-protein interaction hypothesis attempts to delineate a capacitative Ca\(^{2+}\) entry mechanism based on a similar interaction between intracellular Ca\(^{2+}\) stores and the plasma membrane (for review, see Ref. 12). The coupling model holds that the large cytoplasmic head of the IP\(_3\) receptor on the endoplasmic reticulum, located and held in close proximity to the plasma membrane, conformationally responds to the store Ca\(^{2+}\) filling status and transmits this information directly to the plasma membrane Ca\(^{2+}\) entry channel (8).

Of course, this model assumes that the required capacitative Ca\(^{2+}\) entry channels tonically reside at the plasma membrane. However, Fasolo et al. (4) and Somasundaram et al. (15) have proposed that the channels reside in intracellular vesicles and are inserted exocytotically in response to the appropriate signal (Fig. 2C). Downregulation of capacitative Ca\(^{2+}\) entry could occur by endocytotic recycling of the capacitative Ca\(^{2+}\) entry pathways from the plasma membrane. This channel insertion process is similar to other well-characterized channel insertion mechanisms, including the insulin-responsive glucose transporter, the antidiuretic hormone-responsive water channel, the urinary bladder H\(^+\)-ATPase, and the cystic fibrosis transmembrane conductance regulator Cl\(^{-}\) channel. A significant body of literature lends conditional support for and against all the various capacitative Ca\(^{2+}\) entry theories listed above; however, to date, clear and decisive evidence remains elusive.

**What is the influx pathway?**

The specific channel or transporter that allows for capacitative Ca\(^{2+}\) entry remains a mystery. Capacitative currents, however, have been measured electrophysiologically, with Hoth and Penner (7) being the first to demonstrate a Ca\(^{2+}\) current, in mast cells, associated with store depletion. They termed this current a Ca\(^{2+}\) release-activated Ca\(^{2+}\) current (I\(_{\text{CRAC}}\)), to distinguish it from other plasma membrane Ca\(^{2+}\) influx pathways. The CRAC channel is likely to be a subset of the capacitative Ca\(^{2+}\) entry channels. I\(_{\text{CRAC}}\) is characterized by inward rectification, high Ca\(^{2+}\) selectivity, inhibition by intracellular Ca\(^{2+}\), and a very low unitary conductance (~0.02 pS). Other studies have suggested that IP\(_3\), IP\(_4\), Ca\(^{2+}\), or ATP may directly activate capacitative Ca\(^{2+}\) entry channels in different cell types. The conductance of these channels, however.
ever, is much larger than the original $I_{\text{CRAC}}$ (ranging from 2 to 20 pS; for review, see Ref. 3) and may represent other members belonging to the family of capacitative Ca$^{2+}$ channels. $I_{\text{CRAC}}$ is frequently used as a synonym for capacitative Ca$^{2+}$ entry, even in studies in which the nature of the influx pathway is not unequivocally established. Because of its well-defined electrophysiological characteristics, however, the terminology $I_{\text{CRAC}}$ should be restricted to store-operated Ca$^{2+}$ currents with the aforementioned properties.

Although the actual channel responsible for these currents has not been identified, there is considerable interest in the Drosophila TRP and TRPL proteins and their mammalian homologues. These proteins are involved in Drosophila phototransduction and display characteristics similar to capacitative Ca$^{2+}$ entry. Another channel protein with regard to the study of capacitative Ca$^{2+}$ entry that is receiving attention is the IP$_3$ receptor. This protein is expressed as three different isoforms (types 1, 2, and 3) and has been shown in some cell types to be localized to the plasma membrane. It has been suggested that an isoform of the IP$_3$ receptor could be the capacitative Ca$^{2+}$ entry channel, based on receptor expression studies in Xenopus oocytes (for review, see Ref. 12). Putney (12) has speculated that the type 3 IP$_3$ receptor may be the capacitative Ca$^{2+}$ entry channel on the basis of its electrophysiological characteristics. However, cultured vascular endothelial cells (e.g., CPAE cells, cell line derived from calf pulmonary artery endothelium) generate a capacitative Ca$^{2+}$ entry transient but fail to express the type 3 IP$_3$ receptor, based on reverse transcriptase-polymerase chain reaction experiments in our laboratory. Through the combined use of molecular biology and electrophysiological techniques, the identification and characterization of the channel should be imminent. This information will be necessary to efficiently determine the precise signaling mechanisms involved in the activation and regulation of this unique Ca$^{2+}$ influx pathway.

Interaction of capacitative Ca$^{2+}$ entry with cytoskeletal microfilaments

Our laboratory has focused on the dynamic nature of intracellular Ca$^{2+}$ signaling, including the refilling of intracellular Ca$^{2+}$ stores (2), and the activation/regulation of capacitative Ca$^{2+}$ entry (6). We have specifically investigated the cellular microfilament network and whether or not it is a requirement for capacitative Ca$^{2+}$ entry in endothelial cells. This approach is based on our increasing understanding of the roles of various cytoskeletal elements in intracellular communication as well as the possibility that the microfilaments may serve as a structural or communication link between the intracellular stores and the plasma membrane influx pathway (for review, see Ref. 1).

Because the microfilament network (Fig. 3A) is a dynamic structure, inhibition of new microfilament formation with cytochalasin D leads to the complete degradation of this network (Fig. 3B) and significant alteration of the gross morphology of the cell. After cytochalasin D treatment, the endothelial cell was able to generate a typical ATP-induced [Ca$^{2+}$]i transient (Fig. 3C), demonstrating that IP$_3$-dependent release of Ca$^{2+}$ remained intact. However, after thapsigargin application to deplete the intracellular stores, the capacitative Ca$^{2+}$ entry transient was almost completely abolished (Fig. 3C) compared with control (Fig. 3D). Because IP$_3$ activation of the IP$_3$ receptor was functional following microfilament disruption, we speculate that a conformational coupling, rather than a diffusible factor, is the more likely model to describe activation of capacitative Ca$^{2+}$ entry in this cell type. However, the cytoskeleton is known to guide exocytic/endocytic vesicle trafficking, thereby lending conditional support for the channel insertion hypothesis as well.

In contrast, Ribeiro et al. (14) recently reported the opposite effect of cytochalasin D disruption of the microfilament network. In their studies on NIH/3T3 cells, ATP- and platelet-derived growth factor-induced [Ca$^{2+}$]i transients were abolished, but capacitative Ca$^{2+}$ entry remained operative. They assumed the diffusible factor theory, since uncoupling the endoplasmic reticulum from the plasma membrane with cytochalasin D did not inhibit the capacitative Ca$^{2+}$ entry in their cell system. These conflicting results present a conundrum regarding the mechanism of capacitative Ca$^{2+}$ entry. It is likely that some aspects of the diffusible factor, conformational coupling, and channel insertion theories all might have validity in the final description of the true mechanism. Therefore, it is probably appropriate not to view these as mutually exclusive models.

Despite some controversy on the interpretation of results with cytochalasin D, these studies demonstrated unequivocally that spatial organization is essential for Ca$^{2+}$ signaling in general and, in particular, capacitative Ca$^{2+}$ entry. This is consistent with recent evidence (10) suggesting that store depletion activates Ca$^{2+}$ entry in a spatially restricted fashion, i.e., Ca$^{2+}$ influx is restricted to membrane regions colocalized with Ca$^{2+}$ stores that have been selectively depleted. From these experiments, it is apparent that the ultrastructural arrangement of the two membranes involved in this signaling pathway is of crucial importance. An even higher level of complexity, in regard to the
spatial organization of capacitative Ca^{2+} entry, can be encountered in polarized cell types. In some epithelial cells, capacitative Ca^{2+} entry appears to be restricted to the basolateral membrane. Mogami et al. (9), for example, recently demonstrated that in pancreatic acinar cells Ca^{2+} entry through a basolateral membrane patch was able to recharge Ca^{2+} stores located close to the apical membrane. The authors could not detect any discernible changes in [Ca^{2+}]_{i} during the refilling period. They suggested that Ca^{2+} was rapidly sequestered by Ca^{2+} stores at the site of entry and was subsequently transported through the endoplasmic reticulum network to release sites located in the cell's apical pole. Thus activation of the capacitative Ca^{2+} entry pathway, Ca^{2+} sequestration into the intracellular stores, and the possible subsequent redistribution within the endoplasmic reticulum network are dependent on the subcellular microarchitecture of the membrane, the cytoskeleton, and Ca^{2+} transport proteins involved in this process.

**Outlook for the future**

Recent findings have provided increasing evidence that the Ca^{2+} influx due to activation of capacitative Ca^{2+} entry may be important in many cell signaling functions while promoting the refilling of the intracellular Ca^{2+} stores. Nonetheless, this entry pathway remains enigmatic in many aspects of its regulation and function, despite enormous research progress that has uncovered many tantalizing facets of this Ca^{2+} signaling pathway. Crucial issues that remain to be resolved relate to its molecular identity, its biophysical properties, and the identification of the store depletion signal. Research on capacita-
tive Ca\textsuperscript{2+} entry has progressed rapidly. We therefore have great optimism that the near future will shed light on some of these unsolved mysteries.

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References

8. Irvine, R. F. “Quantal” Ca\textsuperscript{2+} release and the control of Ca\textsuperscript{2+} entry by inositol phosphates—a possible mechanism. FEBS Lett. 263: 5–9, 1990.