CHAPTER

24

Calcium

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OUTLINE

THE CALCIUM SIGNAL IN CONTEXT

Calcium is ubiquitous in living systems, and diverse in its roles as an intracellular signal. The heterogeneity of calcium function can be appreciated in the elaborate neurons and glia of the vertebrate nervous system, where many intracellular signaling cascades are regionally distinct and can be studied in isolation. As one of the alkaline earth metals, calcium has a high dehydration rate, is extremely reactive, and possesses an irregular coordination sphere that forms numerous metal complexes with other neutral or negatively charged molecules (proteins, sugars, etc.). In eukaryotic signaling, perhaps the most familiar of these calcium coordination compounds are the members of more than 120 families of EF-hand proteins that contain a helix-loop-helix structure of 12 or 14 amino acids to bind calcium (Ca^{2+}) ions (for review see Permyakov & Kretsinger, 2011), the most familiar of which is calmodulin. After binding calcium, the conformation and function of EF-hand proteins such as calmodulin (CaM) are changed, and thus a calcium-triggered signal is born.

Given the number and diversity of potential partners, calcium within cells is rarely found in isolation. Though the total intracellular calcium may be a few millimolar (mM), in most cells the free cytoplasmic calcium, $[Ca^{2+}]_c$, is only tens of nanomolar (nM), which is four orders of magnitude less than the 2–2.5 mM extracellular calcium, $[Ca^{2+}]_o$, found in blood or CSF. This extraordinary difference between total and free $[Ca^{2+}]$ reflects in part the rapid sequestration of Ca^{2+} into

intracellular compartments, efflux through the plasma membrane via transporters or pumps, and interactions with the aforementioned binding proteins as well as other cytoskeletal and diffusible buffers. As a consequence of this highly reactive environment, the free path length for Ca^{2+} is often less than 75 nm. Its ability to serve as a signaling molecule in a cell that may be tens of microns in extent implies that there must be an array of cellular elements that allow transient local changes of $[Ca^{2+}]_c$ via routes other than its influx and efflux through the plasma membrane. In this chapter we will come to appreciate that at least two intracellular organelles have been implicated as local and highly dynamic reservoirs from which Ca^{2+} is released by signaling molecules that diffuse through the cytoplasm. Though far from being the sole intracellular second messenger, it is now clear that over the course of eukaryote evolution Ca^{2+} has been selected as an ideal, rapid and localized signaling component of many cellular processes.

CALCIUM MEASUREMENT

Much of our understanding of the essential role of Ca^{2+} in cellular physiology has been indirect

During extra-vital investigations of the perfused frog heart by Sydney Ringer in the 1880s, the accidental use of London tap water to make his experimental salt solutions led to an extraordinary discovery. The abnormal function of the heart observed in isotonic NaCl could be corrected and dramatically extended by inclusion of calcium in the bathing solution at a concentration similar to that found in blood (Ringer, 1883). The major technical challenge in the subsequent century was to develop methods by which $[Ca^{2+}]_c$ could be rapidly, reliably and non-destructively measured during physiological activity. Early attempts to measure calcium flux or concentration relied on monitoring the movement of radioactive $[^{45}Ca^{2+}]$, and the miniaturization of ion-sensitive electrodes whose sub-micron tips could impale cells and be used to estimate the average $[Ca^{2+}]_c$ (Marban et al., 1980). Higher spatial resolution was achieved by electron microscopy using energy-dispersive X-ray microanalysis of neuronal compartments, but this provided only static information. To examine $[Ca^{2+}]$ dynamics, tissue was prepared by rapid freezing and fixation at precise moments following physiological stimulation. When combined with fluorescent imaging, the results from these heroic experiments supported the notion that the neuronal endoplasmic reticulum (ER) acts as a rapid source and sink of cytoplasmic Ca^{2+} following activation of voltage- or ligand-operated Ca^{2+}entry at synapses (Pozzo-Miller et al., 1999).

Current optical methods to measure calcium use chemical or protein-based fluorescent indicators

Most recent efforts to demonstrate variations in $[Ca^{2+}]_c$ are inspired by early imaging experiments on muscle and nerve using aequorin, a calcium-sensitive protein from a luminous jellyfish that emitted light in response to Ca^{2+} binding (Ridgway & Ashley, 1967). Based on the power of this approach, the revolution over the last 30 years in characterizing calcium signaling at the subcellular level originated with the pioneering work of Nobel laureate Roger Tsien and colleagues (Miyawaki et al., 1997; Tsien, 1980). By clever chemical and molecular tricks they created chemical or protein-based compounds that permit optical detection of $[Ca^{2+}]$ even within cellular micro-domains.

The optical monitoring of $[Ca^{2+}]$ relies on indicators whose fluorescence changes upon binding to calcium

Many of the chemical probes used to measure $[Ca^{2+}]$ are tetracarboxylate dyes, derivatives of the well-known calcium chelator EGTA [ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] that have been hybridized with

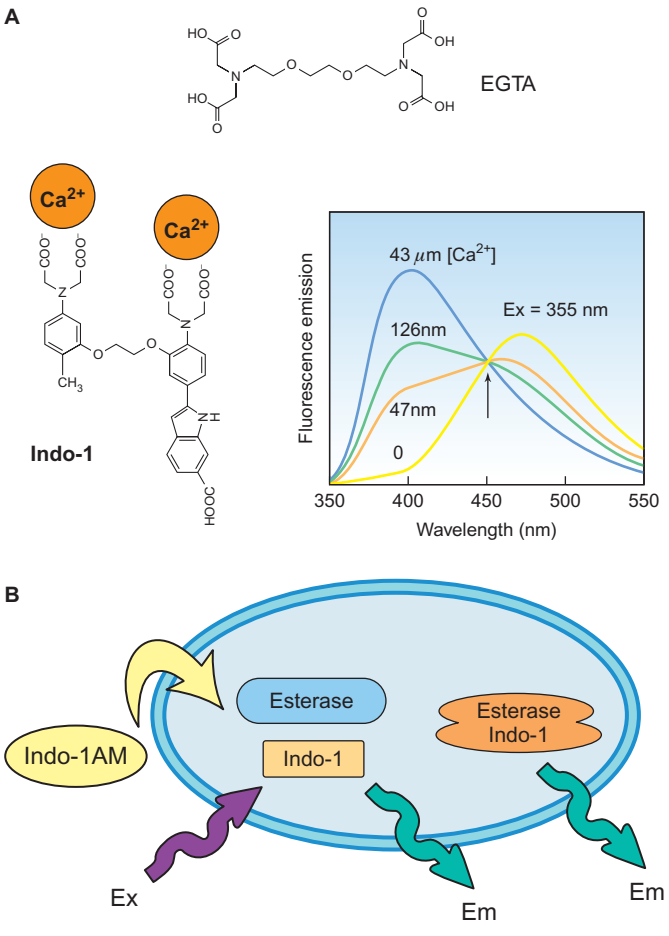

FIGURE 24-1 A. Many chemical indicators of $[Ca^{2+}]$ are based on EGTA. A. One example, Indo-1, has two Ca-binding sites, and when excited with 355 nm light the resulting fluorescence spectra is the sum of the curves for Ca-Indo-1 (peak, 398 nm) and apo-Indo-1 (peak, 482 nm). The ratio of the fluorescence at these wavelengths is used to estimate free Ca^{2+}. All spectra regardless of $[Ca^{2+}]$ cross at 447 nm, the isobestic point (arrow). **B.** Membrane-permeant Indo-1 AM is free to diffuse across membranes and is trapped inside cells and their organelles by endogenous esterases. The fluorescence, shown emanating from the cytoplasm and the endoplasmic reticulum, can be used to estimate the $[Ca^{2+}]$ in each region. AM, acetoxymethyl; Ex, excitation; Em, emission.

aromatic rings to impart fluorescence (Figure 1). The enormous utility and popularity of these dyes is due largely to the fact that they can be made membrane permeable by esterification of the calcium-binding sites, and loaded by diffusion into cells of any size. Once inside, the dyes are trapped by endogenous esterases that remove the masking acetoxymethyl groups and render them functional indicators again. Substitution of different functional groups on the aromatic rings creates indicators with a wide range of spectral properties and disassociation constants, k_d, for calcium binding that range from 100 nM to 400 µM. A rule of thumb is that indicators can be used to quantify $[Ca^{2+}]$ over a range from one-tenth to 10 times their k_d. So in principle it should be possible to measure the full range of intracellular $[Ca^{2+}]$ by using multiple dyes of overlapping affinities. Some care needs to be taken in following rapid changes in Ca^{2+} in neurons and glia, however, since the observed changes in fluorescence will be slower with higher-affinity dyes. Variations of this technique use the salts or dextran-bound versions of the dyes introduced into individual cells via patch pipette. This can be used to enhance the detectability of the cellular fluorescence against a dark field, and to avoid the uptake of dye into organelles. Further, the use of patch pipettes to load the dye makes it possible to simultaneously measure $[Ca^{2+}]$ while recording ionic currents through voltage- or ligand-operated channels.

Increased resolution can be accomplished optically or by targeting indicator proteins

In conventional fluorescence microscopy, the spatial resolution is limited to an oblate spheroid roughly 200 nm in diameter in the plane of view, and extending 600 nm along the optical axis. These dimensions are much larger than the granularity required to measure Ca^{2+} dynamics within synaptic boutons, dendritic spines, astrocytic processes, or even the domain immediately subjacent to the plasma membrane. In special cases when cells and their regions of interest are adherent to a flat optical surface, total internal reflectance fluorescence (TIRF) microscopy can be used to image restricted regions that extend less than 100 nm into the sample. As useful as this is, it fails to solve the problem of tracking calcium dynamics in even smaller volumes within a particular organelle or region, particularly those that are not adherent to the surface.

This problem is currently addressed by using one of the genetically encoded Ca^{2+} indicators (GECIs) (Mank & Griesbeck, 2008). These indicators can be selectively targeted to a precise locale (plasma membrane, nucleus, ER, mitochondria, Golgi, secretory vesicles or gap junctions) by inclusion of an appropriate localization signal sequence as part of the indicator. Alternatively, GECIs can be fused to a protein of interest and report the $[Ca^{2+}]$ in neighboring nanodomains. These indicators are generally of three types. Some are derivatives of aequorin, and generate their bioluminescence via a chemical reaction upon binding to calcium. Others, like camgaroos and pericams, use Ca^{2+}-response elements such as calmodulin linked to circularly permuted green fluorescent protein (GFP); Ca^{2+} binding changes the protonation and spectral properties of the indicator. In the final category of GECIs a calcium response element is inserted between two different fluorescent proteins chosen so that the emission spectra of one overlap the excitation spectra of the other. Upon Ca^{2+} binding the distance between the two proteins changes, and the indicator fluorescence is altered by changes in the efficiency of Förster resonance energy transfer (FRET) between two chromophores; for example, cyan and yellow fluorescent proteins (Demaurex & Frieden, 2003). In principle these gene-product based systems make it possible to detect $[Ca^{2+}]$ in any subcellular domain. Ultimately, the experimental challenge in the analysis of smaller and smaller volumes will reduce to whether it is possible to target and express sufficient dye to provide a detectable signal at the targeted location (Shigetomi et al., 2010).

CALCIUM HOMEOSTASIS AT THE PLASMA MEMBRANE

The balance between calcium efflux and influx at the plasma membrane determines $[Ca^{2+}]$

The plasma membrane separates the cell interior from the external environment, and free intracellular calcium is an

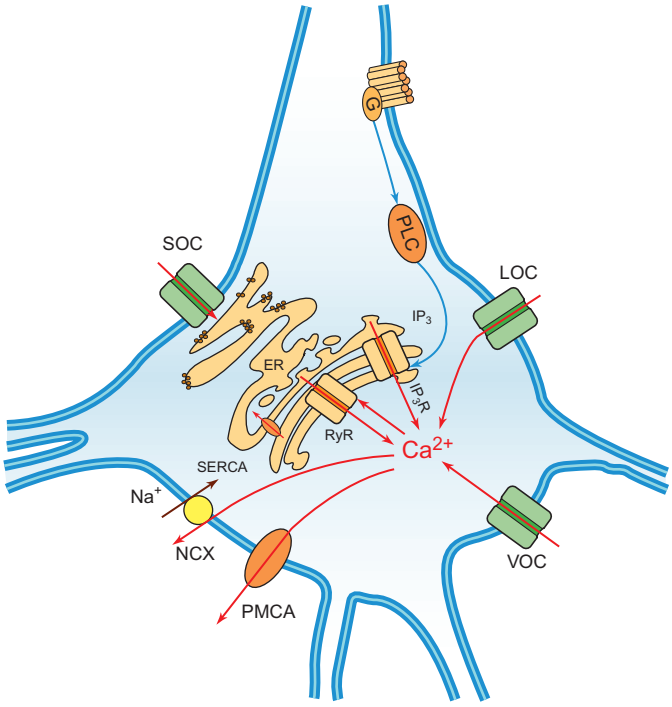

FIGURE 24-2 **Ca^{2+} can enter a cell through store-operated channels (SOC), ligand-operated channels (LOC) and voltage-operated channels (VOC).** Ca^{2+} is transported out of the cytoplasm into the endoplasmic reticulum (ER) by the sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase (SERCA), or to the extracellular space by the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+–Ca^{2+} exchanger (NCX). Ca^{2+} is released from the ER via either ryanodine receptor (RyR) activation by Ca^{2+}, or inositol-trisphosphate (IP_3) activation of the inositol-trisphosphate receptor (IP_3R). The IP_3 is generated by phospholipase C (PLC) upon its activation by G-protein–coupled receptors in the plasma membrane.

equilibrium established by the balance of efflux and influx pathways. The pumps and transporters in the plasma membrane export the calcium to the external environment, and a variety of channels allow Ca^{2+} to flow back into the cell. Similarly, as far as the cytoplasm is concerned the interior of an organelle is topologically equivalent to the extracellular space. Like the plasma membrane, organelles have pumps that transport Ca^{2+} into them, and channels that allow Ca^{2+} to flow back into the cytoplasm. But there the parallel ends, since nothing done transiently within the cell, such as the uptake or release of Ca^{2+} from these organelles, changes the equilibrium concentration of free Ca^{2+} established by proteins in the plasma membrane. Similarly the addition of mM concentrations of buffers and dyes to track $[Ca^{2+}]_c$ as outlined above does nothing to change the final Ca^{2+} equilibrium, though their presence may have a dramatic effect on the speed of recorded signals.

Efflux pathways — pumps and transporters

The two major Ca^{2+} efflux pathways in the nervous system are pumps and transporters. The former are found in all cells and depend on the hydrolysis of ATP to pump Ca^{2+} from the cell. The latter are usually restricted to excitable cells, and Ca^{2+} exchange is an electrogenic process driven by ion gradients (see also in Ch. 3).

The plasma membrane calcium ATPases (PMCAs) are P-type ion transporters that form phosphorylated intermediates during the pump cycle (Ch. 3). In mammals there are four major isoforms (PMCA 1, 2, 3, 4) encoded on separate genes. There are more than 30 PMCA splice variants, but all have intracellular N- and C- termini separated by 10 membrane-spanning segments. The tails are the least conserved regions, and are the loci of many protein–protein interactions that modulate pump function. The most prominent of these associations is the binding of CaM to the C-terminus. In different splice variants the auto-inhibition of PMCA in the resting state is removed following binding of Ca^{2+}–CaM. The subsequent conformational change in the C-terminus then exposes the catalytic domain of the pump. Differences in binding kinetics and CaM affinity in the different variants result in functional diversity. The differential distribution of these transporters presumably reflects local needs in Ca^{2+} signal processing (Strehler et al., 2007).

In addition to the PMCAs, multiple isoforms of the electrogenic Na^+/Ca^{2+} (NCX) exchanger are used to regulate $[Ca^{2+}]$. Under resting conditions the NCXs export a single Ca^{2+} and import three Na^+ into the cell during each transport cycle. In the face of the large electrochemical gradient it is not obvious whether the NCXs play a major role in determining the equilibrium $[Ca^{2+}]_c$. The NCXs are voltage-sensitive and bidirectional: when the cell is depolarized during an action potential, the transport is reversed, and Na^{2+} is extruded while Ca^{2+} is imported into the cell. The impact of the NCX family on calcium homeostasis is therefore complex. Their presence is extremely important for Ca^{2+} clearance should cytoplasmic ATP levels drop, but they become a liability if a cell undergoes prolonged depolarization such as during ischemia. Under that condition the NCX may be a major contributor to a Ca^{2+} influx that ultimately leads to cell death (see in Chs. 35, 37).

Influx pathways — Ca enters the cell through four major routes

The large difference between intracellular and extracellular $[Ca^{2+}]$ creates a large electrochemical gradient that serves as the driving force for ions through the channels in the plasma membrane. The most significant route is through the Ca^{2+}-selective voltage-activated ion channels (Figure 24-2) (see Ch. 4). They serve as the primary route for rapid Ca^{2+} influx in excitable cells, and are responsible for rapid, sustained membrane depolarization as well as synaptic vesicle fusion and the triggering of muscle contraction. A second route includes the vast array of ligand-operated ionotropic channels (dealt with in other chapters) many of which are relatively non-selective to cations, and have significant Ca^{2+} permeability. As examples, the glutamate (NMDA, AMPA and kainate: see Ch. 17), as well as the ACh (α7, α9: see Ch. 13) and ATP (P2X: see Ch. 19) receptors fall into this category (Rogers & Dani, 1995). Ca^{2+} influx through these channels can produce effects as diverse as activation of the ryanodine receptor (RyR) on the endoplasmic reticulum (ER) to release Ca^{2+}, or the gating of adjacent Ca^{2+}-activated K^+ channels in the plasma membrane. The third route of Ca^{2+} entry is through store-operated channels (SOC) activated upon emptying of the ER Ca^{2+} store (see below). The fourth route of entry is through the transient receptor potential (TRP) channel family members, most of which are also nonspecific cation channels. They can be activated by intracellular ligands, and respond to hormones and transmitters through phospholipase C (PLC) isoform PLCβ activation and second messengers like arachidonic acid (AA), diacylglycerol (DAG), sphingosine and others (see Chs 5 and 23).

CELLULAR ORGANELLES AND CALCIUM POOLS

Membrane cisternae, tubular networks and vesicular pools are found throughout the perinuclear region, dendritic arbors and synaptic endings in the nervous system (see Chs. 1 and 7). In some sensory cells, such as the receptor cells of the inner ear, the total membrane area of the organelles may be up to 70-fold the area of the plasma membrane (see Ch. 53). Most organelles have elevated $[Ca^{2+}]$ compared to the cytoplasm. Yet in this vast array of organelles, only the ER and mitochondria are known to have significant impact on Ca^{2+} dynamics. The role of the ER is relatively clear, and in the brain it is a dynamic Ca^{2+} store much like the sarcoplasmic reticulum in muscle. Our understanding of the role of mitochondria as an effective Ca^{2+} store is less secure. Recent experiments suggest that mitochondria may have significant impact in particular nanodomains of the cell (Filippin et al., 2005), but much of the Ca^{2+} signaling detected in mitochondria is likely associated with either the need for ATP generation, or the initiation of the cellular apoptotic pathway (see Ch. 37).

The endoplasmic reticulum is the primary intracellular calcium store

The ER is structurally complex. It can be divided into three morphologically distinct regions consisting of the smooth ER, the rough ER and the nuclear envelope. Calcium can be stored and released throughout the network since the pumps and Ca^{2+} release channels are found in all three regions, and Ca^{2+}-binding proteins are distributed throughout the lumen (for review see Rizzuto & Pozzan, 2006). As a result of these cisternae, Ca^{2+} signaling over long distances within cells is possible. Privileged Ca^{2+} domains are established, for example, in pancreatic acinar cells by using the ER to transport Ca^{2+} from one end of the cell to the other without elevating $[Ca^{2+}]_c$. The Ca^{2+} effectively tunnels through the cell, and the apical ER is reloaded by elevating the $[Ca^{2+}]_{ER}$ at the basal end of the cell (Mogami et al., 1997). The notion that the lumen of the ER is a single compartment is based largely on *in vitro* experiments on solitary HeLa or COS cells that had dye loaded into the lumen. Dye freely diffused within ER, and prolonged illumination in a small region resulted in complete bleaching of the dye throughout the network (Lippincott-Schwartz & Patterson, 2003; Montero et al., 1997). These experiments have yet to be extended to the nervous system *in vivo*, but the situation is more complex in neurons and glia. Electron micrographic reconstruction of different cisternae and regions of the ER suggest that the extensive tubular network neurons and glia may be more than a single entity with a continuous lumen. Further support for this notion is provided by experiments in which physiological agonists activate distinct regions of ER in cultured astrocytes and myocytes. This suggests that individual regions of ER can generate separate Ca^{2+}signals to control specific calcium-dependent processes (Golovina & Blaustein, 1997).

The ER has pumps, storage buffers and Ca^{2+} release channels

The Ca^{2+} within the ER is very high, with estimates of $[Ca^{2+}]_{ER}$ ranging from 100 to 800μM (Solovyova & Verkhratsky, 2002). Given that the ER tubular network extends through vast regions of a cell, it is unlikely that any subcellular domain of cytoplasm is far from a potent source of Ca^{2+} either from the plasma membrane or ER (Figure 24-3). The high $[Ca^{2+}]_{ER}$ is achieved using the sarco- and endoplasmic reticulum Ca^{2+} ATPase (SERCA). The SERCA family has three gene products. SERCA1 and SERCA2 are found in muscle and the six SERCA3 isoforms are found in non-muscle cells (Bobe et al., 2004). In each pump cycle two Ca^{2+} are sequestered and a single molecule of ATP is hydrolyzed. What is perhaps most remarkable is that in each case, at the ER and the plasma membrane, the pumping is accomplished against an enormous nanomolar-to-millimolar Ca^{2+} concentration gradient (see also in Ch. 3).

Within the ER, Ca^{2+} is buffered by multiple binding proteins. In striated muscle calsequestrin (CS) is the primary buffer in the sarcoplasmic reticulum, but calreticulin (CR) predominates in the ER of non-muscle cells. Both proteins have acidic C-terminal domains, with up to 50 low-affinity (k_d~1 mM) binding sites. Other luminal Ca^{2+}-binding proteins in the ER, such as reticulocalbin and calstorin, may have higher affinity binding as suggested by their typical EF hand domains. This cornucopia of buffers has a varied distribution, and in avian cerebellar Purkinje cells, CS is heterogeneously distributed (Takei et al., 1992), while CR and other binding proteins (BiP, endoplasmin, Erp72, PDI) are found homogenously and diffusely distributed throughout the ER (Villa et al., 1991). As a result we would expect the $[Ca^{2+}]_{ER}$ to show variations between different regions of the organelle, and perhaps different flux through the ER release channels.

Ca^{2+} release from the ER is regulated primarily by Ca^{2+}-dependent and inositol (1,4,5)-trisphosphate-dependent signaling cascades (Ch. 23). Other messengers, such as cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) can modulate or stimulate receptors, but their contributions are just beginning to be understood, and will not be considered further. The ryanodine (RyR) receptor and inositol 1,4,5-trisphosphate (IP_3R) receptor are very large, with molecular weights of about 2.3 MD and 1.3 MD respectively. They both form release channels from tetramers of identical subunits, and their long N-termini have numerous sites for binding ATP, Ca^{2+}, and proteins such as CaM. In addition they can be phosphorylated by PKA, PKC, PKG, CaM kinase II, and protein tyrosine kinases, to name but a few (see Chs. 25 and 26). Possible changes at these sites means that release of Ca^{2+} from the ER can be modulated by other signaling cascades operating in parallel with the Ca^{2+} and IP_3 signals. The differential distribution of the RyR and IP_3R release channels in the ER is also important since their distribution determines the particulars of regional variations in calcium signaling through the creation of ligand-dependent Ca^{2+} microdomains.

Activation of different ER signaling pathways elicit different responses

There is ample diversity in the expression of both receptors. For the RyR, three major isoforms (RyR1-3) are expressed in brain (for review see Bouchard et al., 2003), and for IP_3R, three gene products, IP_3R1-3 are expressed as well. Additional diversity for the IP_3Rs is imparted by alternative splicing of the IP_3R-1 and IP_3R-2 genes. Since the isoforms have different kinetics, we await mapping of their subcellular distribution to understand the functional significance of their diversity.

Activation of these receptors impacts neuronal physiology in a variety of ways. For the RyR, Ca^{2+} induced Ca^{2+} release (CICR) has been implicated in synaptic plasticity. It has been argued that plasticity is associated with a large rise in Ca^{2+} in the postsynaptic microdomain. In the hippocampus there is evidence that Ca^{2+} entry through NMDA receptors on dendritic spines initiates the process, and the high levels of $[Ca^{2+}]$ necessary to promote plasticity result from CICR from RyR-sensitive stores (Emptage et al., 1999). Activation of IP_3 as a second messenger shows diversity in responses as well depending on the receptor activated. In the cerebellum, glutamate activation of the metabotropic glutamate receptor type I (mGluR1) at the synapse between parallel fibers and Purkinje cells generates IP_3 by PLCβ4. In the hippocampus glutamate activates the mGluR5 receptor and IP_3 is produced by PLCβ1. These two

different pathways produce radically different results in cell expression systems. Glutamate binding to the mGluR1 results in a single Ca^{2+} spike, whereas the mGluR5 activation can produce Ca^{2+} oscillations that can be modulated by phosphorylation of the mGluR5 by PKC (Kawabata et al., 1996).

Store-operated Ca^{2+} entry: The ER signals when empty to open channels in the plasma membrane

At rest SERCA activity is sufficient to maintain high $[Ca^{2+}]_{ER}$, but after elevated Ca^{2+}signaling and release of Ca^{2+} into the cytoplasm, the ER store may become depleted. Over time the ER is reloaded to normal levels. The fundamental questions are how is the emptying sensed, and how does it signal and facilitate the reloading of the ER? RNA_i knockdown experiments have identified stromal interaction molecule 1 (STIM1) as the sensor of ER depletion (Liou et al., 2005), and Orai1 (Feske et al., 2006) and TRP canonical 1 (TRPC1) (Huang et al., 2006) as two of the plasma membrane integral proteins implicated in store-operated Ca^{2+} (SOCE) entry.

STIM1 is an ER membrane protein with a luminal EF-hand domain that senses $[Ca^{2+}]_{ER}$. Depletion releases Ca^{2+} from the low-affinity binding domain, and STIM1 is then able to migrate to puncta in the ER membrane that are within 10–20 nm of the plasma membrane. There, in the case of Orai1, the pore of the calcium-release activated current (CRAC) store operated channel (SOC), it associates with the N and C termini and gates open the channel, allowing Ca^{2+} influx. Nevertheless, complete understanding of STIM1 function is likely to be even more complex since it has been shown to not only activate Orai1, but to simultaneously inhibit neighboring voltage-activated $Ca_V1.2$ channels (Wang et al., 2010).

Mitochondria have a complex impact on Ca^{2+} dynamics

Mitochondria are implicated to varying degrees in energy production, cell signaling and calcium homeostasis (Figure 24-3). It is incontrovertible that mitochondria can transiently

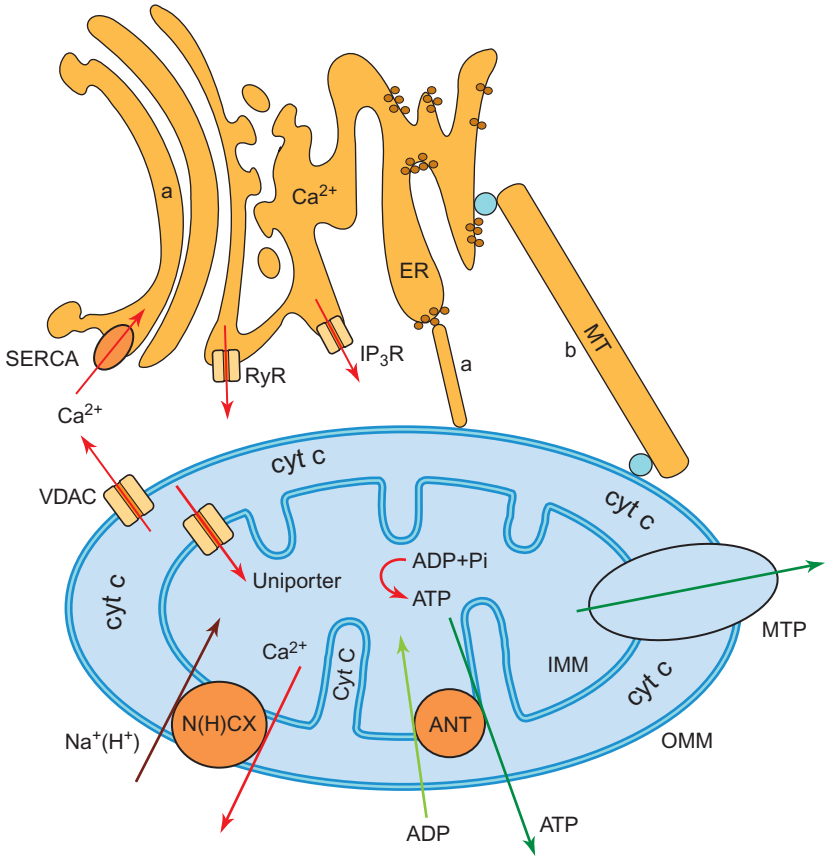

FIGURE 24-3 **Mitochondria are often positioned in the cytoplasm at sites of high transient $[Ca^{2+}]$ near the plasma membrane or ER, and elevated $[Ca^{2+}]$ serves as a signal for energy production**. In the case of the ER the close association with the mitochondria is maintained by tethering proteins (a), or by binding both the ER and the mitochondria to cytoskeletal elements such as microtubules (MT) (b). Ca^{2+} released from the ER via activation of the RyR or IP_3R in close proximity to the mitochondria create local domains of elevated Ca^{2+}. The voltage-dependent anion-selective channels (VDAC) in the outer mitochondrial membrane (OMM) supply Ca^{2+} to the electrogenic uniporter in the inner mitochondrial membrane (IMM) that accumulates Ca^{2+} into the mitochondria. The elevated mitochondrial $[Ca^{2+}]_m$ activates the rate limiting steps in the TCA cycle, elevates oxidative phosphorylation and promotes ATP synthesis. ATP is ultimately exported from the mitochondria through the adenine nucleotide translocase (ANT) in exchange for ADP. Ca^{2+} exits the mitochondria by Na^+–Ca^{2+} or H^+–Ca^{2+} exchangers (N(H)CX), and can be sequestered in the ER by the SERCA. Necrosis occurs when high $[Ca^{2+}]_m$ is combined with oxidative or nitrosative stress that promotes the opening of the mitochondrial permeability transition pore M(PTP) with a subsequent loss of ATP.

accumulate massive amounts of calcium, but it would be a mistake to view them as simple stores an alogous to the ER, designed to take up and release Ca^{2+} on demand. In fact, excessive Ca^{2+} uptake into mitochondria leads to an overload that triggers overproduction of reactive oxygen species (ROS), mitochondrial depolarization, and the inhibition of ATP production.

Normal mitochondrial functions are subtle, and to a first approximation they are ideally suited to link cell activity to energy production. They are known to migrate towards microdomains of high Ca^{2+} flux near the plasma membrane and ER. As might be expected, they congregate at presynaptic endings where the metabolic requirements of vesicle recycling and Ca^{2+} extrusion through the PMCA are most severe. What may be unclear is how at low $[Ca^{2+}]_c$ of less that 100 nM, the intramitochondrial Ca^{2+} could become elevated sufficiently to activate the rate-limiting enzymes of the TCA cycle, increasing oxidative phosphorylation and thus ATP production, given that the mitochondrial Ca^{2+} uniporter is known to have low affinity (K_d~20–50 µM).

The answer may lie in measurements of $[Ca^{2+}]$ in the relevant microdomains. Measurements of the matrix Ca^{2+} concentration at rest reveal that $[Ca^{2+}]_m$ is indistinguishable from the resting cytoplasmic $[Ca^{2+}]_c$. Upon stimulation, however, the $[Ca^{2+}]_m$ may be 10- to 100-fold higher than $[Ca^{2+}]_c$ (Rizzuto et al., 2004). Experiments using GFP-based indicators localized on the cytosolic surface of the outer mitochondrial membrane (OMM) have demonstrated that upon Ca^{2+} mobilization the $[Ca^{2+}]_{om}$ may be 5 to 10 times higher than the bulk $[Ca^{2+}]_c$, lending support to the notion that mitochondria that have migrated near release sites have privileged access to microdomains of elevated Ca^{2+} (Giacomello et al., 2010).

This also suggests a mechanism by which mitochondria may be intimately involved in the fine-tuning of propagating Ca^{2+} signals. Positioning of mitochondria near points of influx from the ER would allow transient buffering of Ca^{2+} near the pore of the RyRs and IP_3Rs and prevent Ca^{2+}-mediated inactivation of these channels, thereby prolonging the response. Similarly, positioning near the Ca_v channels in the plasma membrane could change the $[Ca^{2+}]_c$ equilibrium by preventing the equivalent Ca^{2+}-inactivation of the voltage-sensitive channels. From these examples, it should be apparent that the subtleties of Ca^{2+} buffering in microdomains of elevated Ca^{2+} distinguishes mitochondrial buffering from the roles played by the ER.

CA^{2+} SIGNALING BEGINS IN MICRODOMAINS

The wide array of potential partners for Ca^{2+} raises the question of how the different signaling paths are separated. An elevation of intracellular Ca^{2+} throughout the cytoplasm would trigger numerous signaling cascades, and such crosstalk would compromise cell function. Further, studies that use genetically targeted indicators to specific subcellular locations suggest that the bulk cytoplasmic signal may be irrelevant, and very different from the signal at an organelle or near a source in the ER or plasma membrane. These theoretical and experimental concerns gave rise to the notion of Ca^{2+} microdomains within cells. The logical corollary was the associated idea that the signaling cascades requiring highest $[Ca^{2+}]_c$ would be arrayed nearest to local sources (Berridge et al., 2003). Microdomains are thought to be roughly hemispheric gradients of calcium created near the mouth of RyR and IP_3R channels in the ER or the voltage-operated, ligand-operated, SOC and TRP channels in the plasma membrane. Recall that the electrochemical gradient between cytoplasm and the ER lumen or the extracellular space is the driving force for Ca^{2+} flux into the cytoplasm. The particular shape and size of the elevated Ca^{2+} domain is determined by the magnitude of the Ca^{2+} influx, and the concentrations of fixed and diffusible buffers in the cytoplasm. The RyR and the IP_3R channels are large, with conductances of 100–200 pS, while those at the plasma membrane may be relatively small, with the SOC estimated to be five orders of magnitude smaller, with a chord conductance of only 9 fS (Zweifach & Lewis, 1993). The $[Ca^{2+}]_c$ at the cytoplasmic face of a pore depends on channel opening, but for the plasma membrane channels it has been estimated as being between 10 and 300 µM (Art et al., 1995; Llinas et al., 1992).

This dramatic increase from basal $[Ca^{2+}]_c$ at a channel pore is the common beginning of most intracellular signaling cascades, but there is wide variation in the length of the subsequent path. The shortest is perhaps the Ca^{2+}-mediated inactivation of the Ca^{2+} channel itself. Elevation of $[Ca^{2+}]_c$ is known to inhibit the channels and thus serve as a regulatory mechanism that limits total Ca^{2+} influx. In other cascades the Ca^{2+} plume may extend further outward from the channel pore to the cytoplasmic domains of neighboring integral membrane proteins. The large (BK) and small (SK) Ca^{2+}-activated K^+ channels in vertebrate hair cells are found in close approximation to either L-type Ca^{2+} channels or ACh channels with high Ca^{2+} permeability (Art et al., 1995), (Yuhas & Fuchs, 1999). Strong Ca^{2+} buffering with rapid chelators such as BAPTA uncouples the Ca^{2+} activation of the associated K^+ channels, whereas more sluggish buffers such as EGTA do not. These results suggest that the source of Ca^{2+} for gating is physically close to the K^+ channels. For other functions the Ca^{2+} signal initiating a cellular response is not from a single source, but may result from the coordinated action of multiple channels. The N-type Ca_V channels are often found concentrated in the presynaptic terminal, and the total Ca^{2+} that evokes the response is the sum of multiple microdomains providing Ca^{2+} to bind to synaptotagmin and promote vesicle fusion to the plasma membrane. Similarly, Ca^{2+} signals are implicated in changes in cell morphology in the nervous system, and Ca^{2+} influx through TRPC channels in growth cones participates in neurite development and guidance (Greka et al., 2003). Focal release of Ca^{2+} in the growth cone results in axonal growth towards the side with elevated $[Ca^{2+}]_c$ (Zheng, 2000).

LOCAL AND GLOBAL CA^{2+} SIGNALING: INTEGRATIVE ROLES FOR ASTROCYTES?

Electrically silent astrocytes use Ca^{2+} as a signaling molecule

With the advent of membrane-permeable calcium indicators, a window opened into one of the most durable and problematic

issues in neuroscience. Glia constitute the majority of cells in the brain, but since they are nonexcitable, techniques developed to study the electrical properties of neurons and muscle yielded little insight into their physiology or pathology. The watershed was a paper by Cornell-Bell et al. (1990) demonstrating that glutamate induces calcium waves in cultured astrocytes. Propagation of the wave through neighboring cells of the coupled network suggested that in principle the brain might possess a long-range Ca^{2+}signaling system that was electrically mute and therefore enigmatic. Truly, prior to the advent of chemical indicators and natural gene products that could reveal and follow the Ca^{2+} dynamics within the glial syncytium, it was difficult to imagine the extent of neuronal and glial interaction at each synapse. Of course as the initial observations were extended to *ex vivo* and *in vivo* experiments, the picture became more complex. In the intact brain, 2-photon imaging of the Ca^{2+} signal suggested that the extent of the calcium wave was more restricted than had been imagined from the initial results of the *in vitro* experiments on cultured cells (Fiacco & McCarthy, 2006).

The notion that the Ca^{2+} signal in astrocytes is functionally significant and correlated with neuronal activity is supported by imaging experiments that record $[Ca^{2+}]_c$ elevation during natural stimuli. An elevation in $[Ca^{2+}]_c$ has been observed in restricted groups of astrocytes in the mouse barrel cortex in response to stimulation of individual whiskers (Wang et al., 2006). Similarly, visual stimuli whose orientation and spatial frequency drive neurons in the visual cortex elevate the $[Ca^{2+}]_c$ in neighboring astrocytes (Schummers et al., 2008). Finally, in response to odorants a calcium signal is seen correlated with the vascular response in the ferret olfactory bulb (Petzold et al., 2008).

Intriguing as these results are, how and why would Ca^{2+} signals in astrocytes be related to brain function? Astrocytes are the predominant macroglia in the brain, and they serve at least two functions based on their morphology and their relation to neurons and the vasculature. First, each astrocyte with its fine filopodial processes may envelop more than 100,000 synaptic neighbors. Each astrocytic process serves as an intimate chaperone for a synapse, and could potentially monitor neuronal activity and modulate synaptic function (for review see Agulhon et al., 2008). Second, astrocytes are known to release cyclooxygenase products from their perivascular endfeet that dilate or constrict the adjacent vasculature and modulate cerebral blood flow, allowing them to have a profound influence on cerebral metabolism (Gordon et al., 2009). Given this, it is plausible that Ca^{2+} signaling in astrocytes coordinates and not only integrates the activity at individual synapses, but may also drive changes in metabolism in response to regional demand.

It is clear from the array of integral membrane proteins expressed in their plasma membrane that astrocytes are far from passive bystanders. They express receptors for a variety of neurotransmitters and neuromodulators. In cortex, hippocampus, cerebellum and retina there is strong evidence that Ca^{2+} signals have been initiated or modulated not only by glutamate, but ACh, GABA, dopamine, norepinephrine, ATP, adenosine and histamine, to name a few (for review see Fiacco & McCarthy, 2006). In some astrocytes there is direct Ca^{2+} entry from the extracellular space through the TRPC1 channel. There is also indirect calcium elevation from the internal stores since many of the transmitters act via G protein–coupled receptors (GPCRs). On the whole, current evidence suggests that the neuron–astrocyte conversation is often initiated by the release of neuronal transmitters that bind to astrocytic receptors resulting in Gq stimulation of phospholipase C and the formation of IP_3 and diacylglycerol. The release of Ca^{2+} from the ER follows, but it is not yet obvious which patterns of neuronal activity will elevate Ca^{2+} only at a single astrocytic process, and which patterns result in a Ca^{2+} wave that propagates to the cell body, to the vascular end-feet, or to other astrocytes in the syncytium.

The tripartite synapse: gliotransmitters and modulation of transmission at the synapse

A presynaptic bouton and paired postsynaptic dendritic spine are often associated with an astrocytic process (FIgure 24-4). This anatomical arrangement has focused research on the possibility that the interaction between neurons and glia is a true conversation. Neuronal transmitter release onto astrocytes elevates their $[Ca^{2+}]_c$, and that in turn releases gliotransmitters from the astrocyte back onto the enclosed neurons. It is known that astrocytes in culture will release ATP, D-serine and glutamate upon Ca^{2+} elevation. The degree to which they do so in the intact brain is an outstanding question of vital importance, since gliotransmission would be another means of dynamically altering neuronal transmission or generating plasticity at the synapse (for review see Halassa & Haydon, 2010).

To address one of these questions, hippocampal slices from transgenic mice were used to demonstrate dynamic control of synaptic transmission at the Schaffer collateral-CA1 synapse by adenosine, a metabolite of the gliotransmitter ATP (produced by ectonucleotidases expressed on astrocytes). Using conditional expression of the cytoplasmic tail of synaptobrevin II researchers were able to create a dominant negative inhibitor of exocytosis (dnSNARE) in astrocytes (Pascual et al., 2005). In wild type mice, high-frequency stimulation of a small band of Schaffer collaterals will elicit potentiation at innervated synapses and an adenosine-mediated depression of neighboring, non-innervated ones. It was known that adenosine of unknown origin was acting on the presynaptic neuronal A1 receptor. In the transgenic mice, the astrocyte-specific dnSNARE blocked exocytosis of ATP from astrocytic lysosomes, and stimulation of the Schaffer collaterals failed to cause the adenosine-mediated depression in the neighboring synapses observed in wild-type mice. These results strongly suggest that astrocytes can actively participate in the dynamic control of synaptic transmission.

The question of the role played by astrocytes in plasticity was examined for the long-term potentiation (LTP) and long-term depression (LTD) in the supraoptic nucleus (SON) of the hypothalamus (Panatier et al., 2006). LTP and LTD are two opposing forms of plasticity, and they rely on differential activation of the NMDA receptor. As highlighted in Chapter 17, the NMDA receptor function is complex. In this context we are particularly interested in the regulation of the receptor at the glycine-binding site. D-serine, released

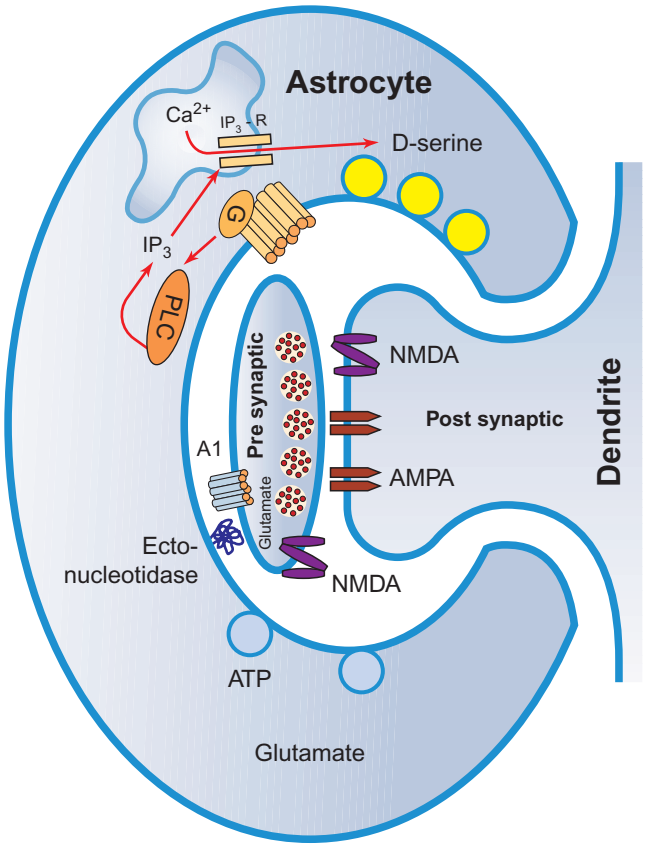

FIGURE 24-4 **Astrocytic filopodia envelop neuronal pre- and postsynaptic processes to form a tripartite synapse**. The release of glutamate from the presynaptic neuron activates AMPA and NMDA receptors in the postsynaptic neuron, and diffuses to the G-coupled receptors in the astrocyte leading to an activation of phospholipase C and the creation of IP_3. Binding of IP_3 to its receptor in the ER leads to Ca^{2+} elevation in the filopodia and the release of D-serine into the synaptic space where it binds to NMDA receptors. Current evidence suggests that Ca^{2+} elevation in the astrocyte leads to glutamate release and activation of extrasynaptic NMDA receptors, as well as the release of ATP that is converted by ectonucleotidases to adenosine to gate presynaptic A1 receptors.

by Ca^{2+}-dependent exocytosis from astroglia, is the naturally occurring ligand for this site. In the SON the synaptic pairs in virgin females have substantial astrocyte coverage, and exhibit LTP, but in lactating rats the synaptic endings have reduced astrocyte coverage and exhibit LTD. This would be consistent with the notion that when synapses are substantially covered by astrocytes, there is a relatively high concentration of D-serine available to bind to the NMDA receptor, and the synapses exhibit LTP. Conversely, when the synapses are relatively bare, the D-serine concentration is low and NMDA receptor activation exhibits LTD. These results are intriguing since they may reveal the true role of D-serine as a gliotransmitter released from neighboring astrocytes. They also suggest that plasticity at the receptor might result from a highly dynamic process that changes the anatomical relationship between neurons and glia, and thereby impacts synaptic plasticity.

Astrocyte control of cerebral vasculature

An important role for the Ca^{2+} signal in astrocytes is as a means of integrating the metabolic demand of neurons with changes in regional blood flow (Figure 24-5). Astrocytes have the necessary signaling cascades to produce either dilation or constriction of vessels, and these effects can be elicited by natural stimuli, synaptic inputs onto the astrocytes, or photolytic release of caged Ca^{2+}in the end-feet (Gordon et al., 2009). There are several routes by which a Ca^{2+} signal could effect changes in the vascular tone. Elevated Ca^{2+} in the astrocyte endfeet may directly activate ion channels such as the large Ca^{2+}-activated K^+ (BK) channel. It has been proposed that the K^+ efflux through these channels increases $[K^+]_o$ near the juxtaposed smooth muscle cells (SMC). This would generate an influx of K^+ through the SMC K_{IR} channels and hyperpolarize the cell, decreasing Ca^{2+} influx. As a result the SMC would relax and the vessel dilate.

An alternative and more complex Ca^{2+}-initiated cascade begins with activation of the Ca^{2+}-sensitive phospholipase A_2 and the generation of arachidonic acid (AA). The vasoactive compounds then released from astrocytes onto the

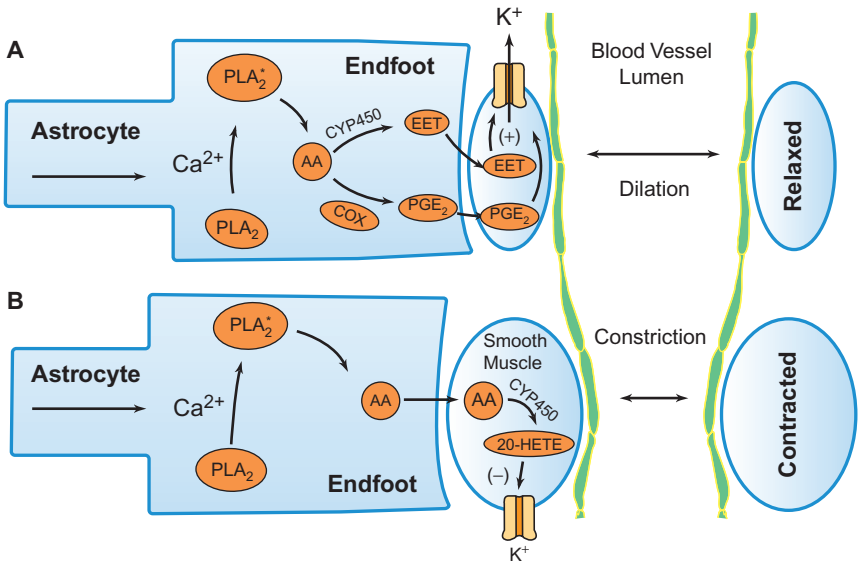

FIGURE 24-5 **Elevating Ca^{2+} in astrocyte end-feet leads to either arteriole dilation or constriction by way of Ca^{2+}-sensitive phospholipase A_2 (PLA2) production of arachidonic acid (AA)**. **A**. Under conditions of reduced nitric oxide (NO), AA production leads to dilation of the vessel following production of epoxyeicosatrienoic acid (EET) or prostaglandin E_2 (PGE_2), both of which increase the K^+ channel open probability which hyperpolarizes and relaxes the vascular smooth muscle. **B**. In elevated NO the production of EET is inhibited in the astrocyte relative to the generation of 20-hydroxyeicosatetraenoic acid (20-HETE) in the smooth muscle, and on balance the K^+ channel open probability decreases leading to depolarization and contraction of the smooth muscle.

SMC depend on the activated enzymatic cascade. Vessel dilation follows from release of either prostaglandin E_2 (PGE_2), produced by the cyclooxygenase (COX_1) pathway, or one of several epoxyeicosatrienoic acids (EETs) generated by the activity of particular cytochrome P450s. Vessel constriction is observed following release of PGF_2 and even AA itself, which can be converted by another variant of the cytochrome P450 in the SMC into 20-hydroxyeicosatetraenoic acid (20-HETE). The conditions under which either pathway is chosen remain unclear. Current speculation is that the conversion of AA to EET or 20-HETE is dependent on the level of nitric oxide (NO) present. Enzymes in the EET cascade may be inhibited by an elevation in NO, and on balance, increasing NO will result in 20-HETE–induced vessel constriction. Similarly, depleting NO might result in an increased production of EET, resulting in vessel dilation. Present controversy aside, it is clear that astrocytes have the signaling cascades necessary for both vessel dilation and constriction, and it remains an active question as to the conditions under which an elevation in Ca^{2+} signals either.

CONCLUSIONS

The advent of fluorescent Ca^{2+} probes made possible the direct exploration of a wide range of intracellular Ca^{2+}

ISCHEMIA AND TOXICITY, WHEN CA^{2+} SIGNALING IS TOO MUCH OF A GOOD THING

Jonathan Art

With myriad pathways involving Ca^{2+}, it is not surprising that a loss of Ca^{2+} homeostasis is implicated in cell death in response to trauma or chronic brain disease. For trauma, the therapeutic question is one of how to develop appropriate neuroprotective pharmacological cocktails to block necrosis and apoptosis. Experimental results of the last three decades suggest that it will be necessary to reexamine the simple "calcium overload hypothesis" and determine the degree to which Ca^{2+} elevation by different routes is involved in cell death, and how associated damage from each can be minimized.

Ischemic stroke interrupts the blood supply and results in oxygen and glucose deprivation to both neurons and glia (see Ch. 35). At the core of the insult is necrosis of all cell types, and extending outward is a variable region of compromised cells. Since much of brain metabolism has been taken over by astrocytes, the glia are more likely to survive even in regions where neurons are lost. The glia are far from benign, and there is evidence in model systems that they contribute to neuronal death in the region surrounding the initial infarct. Elevated Ca^{2+} waves propagate through the astrocyte syncytium either by the serial release of ATP on neighboring cells, and activation of the P2X receptors, or by Ca^{2+} carried through gap junctions between cells. As a result glutamate is released from the astrocytes and gates the neuronal NMDARs. The metabolically compromised neurons are depolarized, the Mg^{2+} block of the channel is largely absent, and the result is massive Ca^{2+} influx. The neuronal Ca^{2+} elevation by this pathway is the best studied, but numerous clinical approaches targeting these receptors have largely failed to be neuroprotective (Kalia et al., 2008). These results force us to reexamine alternative therapeutic strategies.

Since the goal is to avoid cell death, a logical place to start is the mitochondria. It is known that neuronal Ca^{2+} overload leads to mitochondrial Ca^{2+} accumulation, which decreases the electrochemical gradient and the production of ATP at precisely the moment when PMCA and SERCA are most needed to effectively export or sequester Ca^{2+}. In addition prolonged mitochondrial Ca^{2+} elevation leads to generation of reactive oxygen species and evokes opening of the mitochondrial permeability transition pore, with a subsequent release of cytochrome c and activation of caspases resulting in apoptosis (Murphy et al., 1999) (see Ch. 37). Thus neuroprotection may ultimately rely on preventing mitochondrial damage by Ca^{2+} to block the release of mediators of necrosis and apoptosis. In addition, the neuronal Ca^{2+} elevation will activate calpains, the Ca^{2+}-dependent proteases that cleave the NCX, SERCA, IP_3R, RyR, etc., all proteins involved in regulating Ca^{2+} homeostasis. This effectively locks the neurons into a state of elevated Ca^{2+}. Since influx and efflux pathways are both disabled by the calpains, one can imagine that blocking proteolysis of the efflux pathways might be neuroprotective. Finally, as noted above, many therapies target voltage- and ligand-operated influx, but efflux via transporters and pumps should be examined as well. During prolonged depolarization the neuronal NCX would be lethal, since it loads the cell with Ca^{2+} as it extrudes Na^+. Upon repolarization, under hypoxic conditions and low ATP, the NCX is neuroprotective since it is still able to extrude Ca^{2+} when activity of the PMCA pump has been compromised. This suggests transporter therapies would be complex, but a voltage-dependent block might be in order, so at depolarized potentials NCX would be blocked to prevent damage, but would be unblocked and active to export Ca^{2+} when the cell repolarizes.

As suggested by this brief list, the targets for potential neuroprotective therapies are vast, and the ultimate cocktail will likely address multiple Ca^{2+} cascades. But in closing it bears emphasizing that the most obvious targets for potential therapy may not be neurons, but instead the glia, the metabolic support for the brain. Pharmacologically limiting the propagating Ca^{2+} waves may in the end be the best means of protecting their excitable neuronal neighbors and restricting the long-term damage to the circumscribed region of the initial insult.

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signaling. Indirect evidence that fueled past speculation has been replaced by quantitative measurements to test competing hypotheses. In the subsequent period, the questions asked about the signaling cascades have also grown more sophisticated. From humble beginnings, when it was interesting to know simply whether Ca^{2+} was elevated or depressed within the cytoplasm upon application of an agonist, we have entered a period of far greater specificity. It is now realized that for a given biological question the global cytoplasmic signal may not be the one of interest, and a region of physiological import may be restricted to a few nm in extent. The creation of additional probes targeted to specific organelles and splice variants of channels, pumps and transporters holds the potential to systematically reveal the underlying logic of different cascades and regional variations within a cell. Moreover, the introduction of this technology has given voice to activity of the majority of cells in the nervous system that are electrically mute, and it has given us tools to explore the ongoing conversations between neurons, glia and the vasculature.

> "Molecules respond to [calcium] as do people to music. There are some who are unaffected and absorb nothing, there are some who react by the degraded vibration of foot or finger, but some there are who rise and dance and change partners."
>
> —W. A. H. RUSHTON, 1962.

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