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POSTSYNAPTIC CALCIUM SIGNALING MICRODOMAINS IN NEURONS

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1. ABSTRACT

Calcium ions are crucial messengers in the regulation of synaptic efficacy. In the postsynaptic neuron, this is exemplified by the tight temporal and spatial co-segregation of calcium ions with calcium-dependent signal transduction protein complexes in dendritic spines. Over the last several years optical imaging, physiological, structural, and biological studies have clarified the molecular mechanisms underlying differential calcium signaling within the spine. In this review, we discuss how calcium signaling "microdomains" are organized and regulated. We emphasize the structural and functional features of precisely regulated supramolecular complexes incorporating proteins involved in calcium influx, calcium efflux, and signal transduction. These complexes act in concert to orchestrate the sophisticated postsynaptic calcium signaling that underlies synaptic plasticity.

2. INTRODUCTION

Neurons are the most polarized cells in the body, with structural and functional domains comprising the cell body, dendrites, and axons as well as specializations within these compartments. This unique arrangement has evolved to allow neurons to form complex signaling networks that underlie central nervous system function. The somatodendritic compartment of neurons, which receives inputs from many presynaptic axon terminals, can be further divided into multiple subdomains specialized to transduce and integrate signals from these various inputs (1). Individual synapses exemplify such subdomains; though numerous and close together, they retain the ability to function both independently and in concert with one another.

Synapses harness sophisticated, precisely localized calcium-dependent signaling mechanisms to regulate diverse processes, ranging from neurotransmitter release to synapse-specific changes such as long-term potentiation (LTP) and long-term depression (LTD) (2, 3, 4). At rest, cells typically have a cytosolic calcium concentration of about 100 nM, but upon stimulation this can rise to the micromolar range, regulating the functions of a number of different calcium-dependent cellular proteins. Why calcium? There are three main reasons. First, calcium flux is ultra-rapid, permitting signaling that is not only amplitude-dependent but also frequency-dependent. Second, neurons have multiple means of exquisitely controlling cytoplasmic calcium levels in a highly localized fashion. Finally, calcium is a universal and versatile messenger, regulating the functions of a

number of structural, modulatory, and signaling proteins (5).

In neurons, the segregation of calcium ions in the cytoplasm, both temporally and spatially, is accomplished in large part through the dynamic regulation of different modes of influx and efflux. Calcium can enter the cytoplasm through a number of pathways. Excitatory stimuli trigger calcium influx through voltage-dependent calcium channels (VDCCs), and synaptic currents are carried partly by calcium entering the neuron through glutamate-gated ion channels. Calcium is also released from intracellular stores, where concentrations can be in the 100 micromolar range, through ryanodine and inositol-1,4,5-trisphosphate (IP_3) receptor channels (2, 5, 6). Cytoplasmic calcium concentrations are reduced by regulated extrusion, as occurs through Ca^{2+}-ATPases situated within both the smooth endoplasmic reticulum (SER) and plasma membrane (7, 8). Though less well understood, calcium-buffering proteins such as parvalbumin and calbindin may be important in shaping the duration and amplitude of calcium signals, as well as limiting calcium diffusion. In turn, calcium-dependent processes are modulated via structural and functional specializations within neurons on both sides of the synapse. For instance, calcium entry through VDCCs in the presynaptic terminal is closely coupled to vesicle exocytotic machinery, allowing for rapid, calcium-dependent neurotransmitter release. Several reviews discuss these presynaptic processes in detail (2, 9), and they will not be discussed in this review. Here we will focus on postsynaptic calcium signaling microdomains, and how such complexes are assembled, localized, and regulated in neurons.

3. MORPHOLOGICAL BASIS FOR POSTSYNAPTIC CALCIUM MICRODOMAINS: DENDRITIC SPINES

Major dendritic specializations crucial in the organization of postsynaptic microdomains are the spines, small protrusions from dendritic shafts that represent the primary postsynaptic targets of excitatory synapses in the CNS (10). Spines represent basic units of neuronal integration and constitute individual calcium compartments, with different pathways of calcium influx and efflux predominating among different spines (11, 12, 13). They are typically found at a density of about 4-5 spines per micron of dendritic shaft *in vivo* (14). Morphologically, spines are quite variable -- with stubby, thin, mushroom or cup shapes. They range in volume from less than 0.01 $micron^3$ for small, thin spines to 0.8 $micron^3$ for large, mushroom-shaped spines (10); these small volumes permit large changes in calcium concentration in response to only small amounts of calcium flux. Spines are also highly dynamic, changing in shape and number during development and in response to changes in synaptic activity (10, 14-19).

Despite their variability in size and shape, dendritic spines have several signature features. Directly apposed to the presynaptic terminal active zone, and occupying about 10% of the spine surface, is the postsynaptic density (PSD). Biochemically isolated PSDs resemble semicircular discs, measuring about 40-50 nm thick and 400-500 nm in diameter. Their dense, static appearance in electron micrographs belies a dynamic structure (20), and in fact within this specialization is a web-like matrix of interacting neurotransmitter receptor, signaling, and structural proteins in constant flux (21-24). Spines contain a variety of organelles, including an assortment of smooth or coated vesicles, multivesicular bodies, polyribosomes, and SER that in some cases is specialized to form a distinct 'spine apparatus.' The SER snakes up into the spines from the dendritic shafts, abutting signaling proteins at the plasma membrane. SER is found in about 50% of dendritic spines, and its presence correlates with spine size; though about 80% of large mushroom spines have SER, it is present in only 20% of small thin spines (10, 14, 25). As the SER is a major source of intracellular calcium stores (8), this points to the importance of spine size in calcium signaling.

4. ORGANIZATION OF CALCIUM SIGNALING COMPLEXES IN DENDRITIC SPINES

The spine architecture described above is supported by a highly-regulated lattice of interacting proteins which can accommodate the precise coordination of calcium influx and efflux with the regulation of calcium-dependent, synaptic signaling proteins, many residing within or at the edge of the PSD. Many of these signaling pathways can regulate spine morphology as well. Much is known about the structure and regulation of calcium influx proteins, and these will be discussed first. Later we will discuss mechanisms of calcium efflux.

4.1. Mechanisms of calcium influx in spines

Calcium enters the spine cytoplasm primarily though three main processes: neurotransmitter-gated cation channels, VDCCs, and from internal stores though IP_3 receptor channels. The neurotransmitter- and voltage-gated ion channels are embedded within the plasma membrane, and IP_3 receptors reside in the SER (figure 1). All are members of protein complexes that are optimized to bring signaling proteins into close proximity to the regions of calcium flux, and often in proximity to one another, thus further increasing the range and complexity of calcium signaling.

4.1.1. Neurotransmitter-gated ion channels: NMDA receptors

The predominant excitatory neurotransmitter that mediates synaptic transmission to dendritic spines is glutamate, which binds to two major types of receptors: ionotropic and metabotropic. Ionotropic receptors are multimeric glutamate-gated cation channels, and are divided into AMPA, kainate, and NMDA-preferring receptors based on differences in pharmacology, subunit composition, and channel properties (26). Although some AMPA receptors form Ca^{2+}-permeable channels, the preponderance of glutamate-gated calcium influx into spines flows through NMDA receptors, which are major constituents of the PSD. The NMDA receptor channels are most likely tetramers of NR1 and various NR2A-D

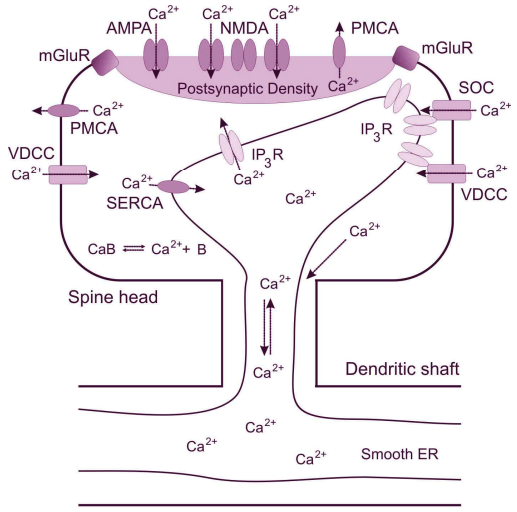

Figure 1. Major determinants of calcium distribution within the dendritic spine. This schematic diagram shows key proteins involved in calcium influx and efflux. Abbreviations: B, endogenous calcium buffer; IP_3R, IP_3 receptor; mGluR, metabotropic glutamate receptor, type 1; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SOC, store-operated channel; VDCC, voltage-dependent calcium channel.

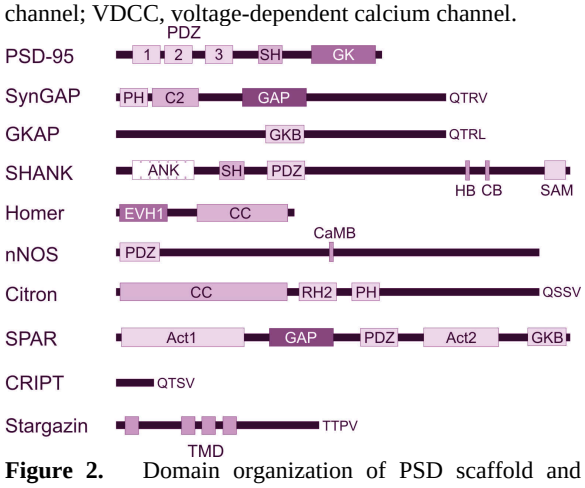

Figure 2. Domain organization of PSD scaffold and calcium signaling proteins. The modular structures of many postsynaptic proteins involved in the formation of targeting or signaling protein complexes within the dendritic spines are depicted schematically. For GKAP, the $GKAP_L$ variant is depicted. The immediate early gene form of Homer (Homer 1a) lacks the CC domain. C-terminal motifs involved in binding to PDZ-containing proteins are indicated using the one-letter code for amino acid residues. Abbreviations: 1-3, PDZ1-3, respectively; Act, F-actin reorganizing domain; ANK, ankyrin repeats; CaMB, calcium-calmodulin binding domain; CB, cortactin-binding domain; CC, coiled-coil region; C2, phospholipid-dependent calcium binding domain; EVH1, enabled/VASP homology domain; GAP, GTPase-activating protein domain; GK, guanylate kinase-like domain; GKB, GK-binding domain; HB, Homer-binding domain; nNOS, neuronal nitric oxide synthase; PH, pleckstrin homology domain; RH2, ring-H2 finger domain; SAM, sterile alpha motif; SH, SH3 domain; TMD, transmembrane domain.

subunits. Additional diversity is generated through alternative RNA splicing of several subunit genes. Each subunit shares a similar membrane topology, with three transmembrane domains, a large extracellular N-terminal domain, and an intracellular C-terminal domain. NR2A-D subunits have particularly large intracellular cytoplasmic tails, with C-termini ending in the conserved sequences -E-S-D-V or -E-S-E-V. This short peptide motif directs binding to a PSD protein called PSD-95/SAP90 through interaction with a ~90 amino acid residue interaction domain in PSD-95 termed a PDZ domain (27-29).

The PSD-95 protein is a member of the *m*embrane-*a*ssociated *gu*anylate *k*inase (MAGUK) family, forming a subfamily with other synaptic proteins in mammals such as PSD-93/chapsyn-110, SAP97/hDlg, and SAP102. All except SAP97 appear to be components of the PSD and associated with NMDA receptors. The PSD-95 family is characterized by a modular organization, with 3 PDZ domains, a Src-homology 3 (SH3) domain, and a catalytically-inactive guanylate kinase (GK) domain (figure 2). The PDZ, GK and SH3 domains are demonstrated sites for protein-protein interactions. Several PSD-95 family members multimerize in a head-to-head fashion, enhancing their ability to act as organizers of the NMDA signaling complex (30, 31).

Scaffolding functions of PSD-95 in NMDA receptor signaling have been confirmed by identification of several interacting proteins (figure 3). One of these is the enzyme neuronal nitric oxide synthase (nNOS), which catalyzes the formation of nitric oxide from L-arginine. Neuronal NOS binds to PSD-95 via a PDZ-β-finger interaction (32, 33), bringing NMDA receptors, which allow calcium influx, into close proximity of nNOS, which is regulated by calcium-calmodulin (32). In fact, suppressing the expression of PSD-95 in cultured neurons blocks calcium-activated nitric oxide production by NMDA receptors selectively, without affecting NMDA receptor or nNOS expression or function (34). Furthermore, disrupting PSD-95 PDZ interactions in cerebellar granule cells prevents NMDA-receptor mediated stimulation of nNOS, but does not affect calcium influx through NMDA receptors (35). Thus, PSD-95 appears to mediate protein-protein interactions that couple a calcium channel (NMDA receptor) to a calcium-regulated enzyme (nNOS) to form a signaling complex. Further regulation may be achieved through alternative splicing; a splice variant of nNOS lacking the PDZ domain no longer associates with PSD-95 (32), and thus may not be regulated by NMDA receptors. Finally, the cytoplasmic protein CAPON competes with PSD-95 for interaction with nNOS and may regulate nNOS activity by preventing binding of PSD-95 to nNOS (figure 4) (36).

In addition to nNOS, PSD-95 has been found to interact with other calcium-dependent signaling proteins, such as regulators or effectors of small GTPases (figures 2 and 3). SynGAP, a synaptic GTPase-activating protein (GAP) for Ras, interacts through its C-terminus (ending in –Q-T-R-V) with each of the 3 PDZ domains of PSD-95 (37, 38). Its interaction with PSD-95 positions it to

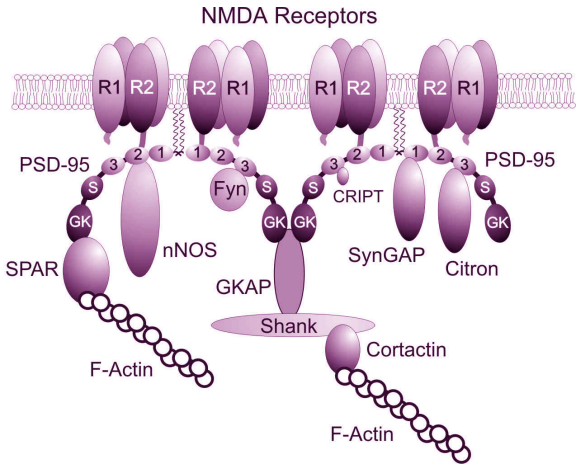

Figure 3. NMDA receptor/PSD-95 supramolecular protein complex. C-termini of NR2 NMDA receptor subunits bind to PDZ1 and PDZ2 of PSD-95. PSD-95 is linked to the plasma membrane through palmitoylation at the N-terminus (wavy lines). PSD-95 also forms multimers, as indicated, with itself and other MAGUKs. Other components of the PSD-95 complex and their sites of interaction are indicated. Abbreviations: 1-3, PDZ1-3; S, SH3 domain; GK, guanylate kinase-like domain.

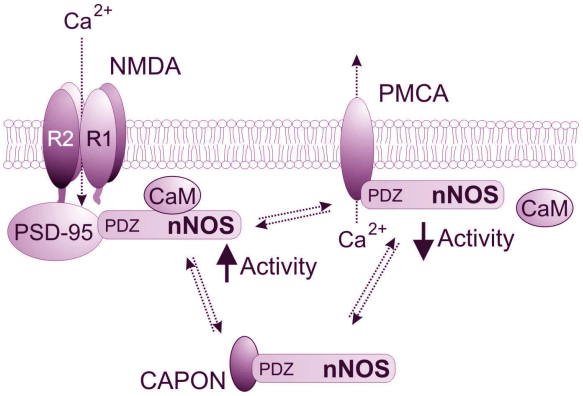

Figure 4. Multimodal regulation of nNOS. PSD-95 brings nNOS into the vicinity of calcium influx through NMDA receptors, increasing its activity. CAPON competes with PSD-95 for binding to the nNOS PDZ domain, and can prevent the calcium-dependent activation of nNOS. The plasma membrane calcium ATPase PMCA 4b also can bind to the PDZ domain of nNOS, and the resultant extrusion of calcium by PMCA 4b decreases nNOS activity. Arrows indicate an expected increase or decrease in nNOS activity in these situations. Abbreviations: CaM, Ca^{2+}-calmodulin; nNOS, neuronal nitric oxide synthase; PMCA, plasma membrane Ca^{2+}-ATPase.

inactivate Ras, which is activated in response to NMDA receptor stimulation (39). In fact, there is strong evidence that calcium activates Ras; though the links in neurons remain unclear, nNOS, protein kinase C, pyk2, and Ras-GRF (guanine nucleotide exchange factor) have been suggested as 'calcium sensors' for this activation (39, 40). The SynGAP protein harbors both a pleckstrin homology domain as well as a C2 domain that binds calcium in a phospholipid-dependent manner (figure 2), suggesting that it may be responsive to both phospholipid and calcium signaling. Furthermore, its activity is inhibited by calcium-calmodulin-dependent protein kinase II (CaMKII) phosphorylation (37). Thus, SynGAP may play a key role in NMDA receptor-dependent calcium signaling through Ras. The Rho/Rac and Rap GTPases may be involved in NMDA receptor signaling as well. The Rho effector citron binds preferentially to the third PDZ domain of PSD-95 (41, 42). Citron is a brain-specific variant (lacking the protein kinase domain) of citron kinase, a Rho effector expressed outside the central nervous system. Also, a synaptic GTPase-activating protein for the small GTPase Rap, termed Spar, has been identified which interacts with the GK domain of PSD-95 (43).

NMDA receptors themselves are modulated by protein tyrosine kinases (44, 45). Non-receptor tyrosine kinases of the Src family –which includes Fyn, Lyk, Lyn, and Yes -- may be associated with NMDA receptors through interactions with PSD-95 (46). NMDA receptors are stimulated by Src or Fyn (47, 48), and induction of hippocampal LTP is associated with Src activation and NR2B tyrosine phosphorylation (49-51). Mutant mice lacking Fyn, though not those lacking Src or Yes, display deficits in LTP and spatial memory (52). Interestingly, Fyn binds to PDZ3 of PSD-95 through its SH2 domain (figure 2), which may be particularly relevant as Fyn mutant mice display markedly decreased levels of NR2A phosphorylation (53). Since PSD-95 increases Fyn phosphorylation of NR2A in heterologous cells, PSD-95 may bring Fyn into close proximity of NMDA receptors. Other Src family tyrosine kinases can be co-immunoprecipitated with NMDA receptors, and may also be interacting with PSD-95 (53, 54). Regulation of NMDA receptors by tyrosine kinases may thus be facilitated by the formation of an NMDA receptor/tyrosine kinase complex, likely with PSD-95 as an intermediary.

The calcium-binding protein calmodulin is highly enriched in the PSD, and it has been shown to bind directly and regulate NMDA receptors. Calmodulin binds in a calcium-dependent manner to two distinct sites in the C-terminal region of the NR1 subunit (figure 5). One site is contained within the alternatively spliced C1 exon cassette, while the other, termed C0, is common to all known NR1 variants. Calcium-calmodulin binding reduces both the probability of channel opening and the mean open time of NMDA receptor channels (55, 56), providing a possible mechanism for activity-dependent feedback inhibition and calcium-dependent inactivation of NMDA receptors. The calcium-dependent inactivation of NMDA receptors, however, likely also depends on factors such as subunit composition. For example, in transfected cells the calcium-dependent inactivation of NR1/NR2 receptors requires NR2A or NR2D; inactivation is not observed in NR2B- and NR2C-containing receptors (57). NMDA receptors are also regulated by the Ca^{2+}-calmodulin dependent protein kinase CaMKII, which is itself a very abundant PSD protein activated by NMDA receptor stimulation. Activation of CaMKII by NMDA receptor stimulation increases the association of CaMKII with NR2B, possibly through the translocation of CaMKII from an F-actin to PSD-bound

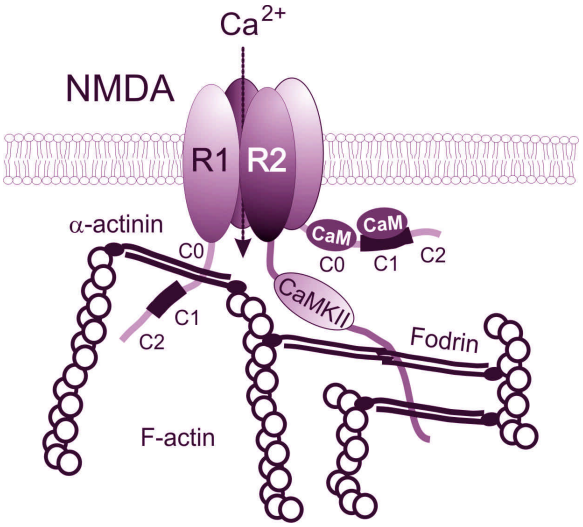

Figure 5. NMDA receptor interactions with the actin cytoskeleton. The indicated cytoskeletal interactions of the NMDA receptor subunits NR1 and NR2 are independent of PSD-95. Such interactions can modify the structure of NMDA receptor-containing macromolecular signaling complexes as well as spine structure. C0 is common to all NR1 subunits; C1 and C2 are alternatively spliced cassettes of the NR1 subunit. Abbreviations: CaM, Ca^{2+}-calmodulin; CaMKII; Ca^{2+}-calmodulin-dependent protein kinase type II.

state (58). In fact, the cytoplasmic tails of NMDA receptor subunits interact directly with CaMKII, and these interactions can lock CaMKII in an active state (59). Since CaMKII has many actions, including regulation of glutamate receptors, inhibition of the synaptic RasGAP SynGAP, and disruption of the interactions between microtubule-associated protein (MAP)-2 and actin, it clearly plays a critical role in calcium-dependent synaptic signaling.

Several major themes emerge from these studies of NMDA receptor interactions with signaling pathways involving nNOS, GAPs, non-receptor tyrosine kinases, calcium-calmodulin, and CaMKII. First, PSD-95 plays a key role in the organization of these diverse signaling pathways, linking them to NMDA receptors. Next, some of these interactions provide for regulation of the NMDA receptors themselves, and thus of calcium influx through these channels. Finally, many proteins in these signaling pathways are calcium-regulated. Thus by binding to PSD-95, or directly to NMDA receptors themselves, calcium-responsive proteins from a number of different signaling pathways can be positioned close to the mouth of the NMDA receptor channel, thereby specifying the nature of the responses.

4.1.2. Voltage-dependent calcium channel complexes

In addition to calcium influx through neurotransmitter-gated channels, the voltage-dependent control of intracellular calcium levels is crucial for many neuronal processes. Multiple types of plasma membrane VDCCs that differ in their pharmacological and physiological properties are expressed in neurons (60, 61), including the L-type, N-type, P/Q-type, R-type, and T-types. These channels are multisubunit complexes composed of pore-forming $alpha_1$ and accessory beta, $alpha_2$delta, and gamma subunit proteins. The $alpha_1$ subunit spans the membrane six times within each of four domains, and can sometimes form functional channels when expressed alone. Pharmacological diversity is generated through multiple different $alpha_1$ subtypes, termed $alpha_{1A-E}$. Additional fine-tuning is achieved through the various modulatory subunits. The number and types of VDCCs in spines has recently been evaluated using optical fluctuation analysis, and was estimated at 1-20 VDCCs per spine, primarily of the R-type, in hippocampal CA1 neurons (62). However, it has been suggested that N/P/Q types predominate in neocortical spines. Clearly then, with such small numbers of channels and variations in VDCC subtypes, the function and specificity of VDCC-dependent responses may differ among spines (62).

VDCC function is under tight regulation, comprising both channel inactivation and facilitation linked to the entry of calcium. Recently, it has been shown that calmodulin is a critical calcium sensor for both calcium-dependent inactivation and facilitation of the L-type channel, likely via calcium-dependent interaction of calcium/calmodulin with a calmodulin-binding isoleucine-glutamine (IQ)-like domain on the carboxy-terminal tail of the $alpha_1$ subunit (63, 64). This calcium-sensing is most likely explained by large, rapid local increases in calcium. Furthermore, it appears that calmodulin has a tethering site, which anchors it constitutively to the $alpha_1$ subunit within the calcium 'hotspot'; upon channel activation and calcium influx, the now calcium-associated calmodulin then binds to the effector site, modulating channel function. Similar mechanisms may be found for other subtypes which also have the IQ domain, and other sites on VDCCs may be involved in inactivation/facilitation for those subtypes that lack an IQ domain (65). In support of this notion, calcium hotspots have been noted at the bases of proximal dendrites by optical imaging (66), where a selective clustering of L-type channels has been demonstrated by immunocytochemistry (67). Another study reported the clustering of L-type calcium channels in growth cones, resulting in local increases in calcium concentration near the location of morphological changes. These microdomains were about 7 microns in diameter; at the 'hotspot' a single action potential raised the intracellular calcium concentration by about 90 nM, compared with an average cytosolic increase of around 10 nM (68). Together, these studies support the existence of functional calcium microdomains due to clustering of VDCCs.

VDCCs are also regulated through interaction with the aforementioned accessory proteins. For instance, the beta subunits are cytoplasmic proteins that stimulate activity of the channel-forming $alpha_1$ subunit through direct interactions. More recently, a particularly interesting regulatory mechanism was described involving the stargazin protein, which has structural similarity to the

skeletal muscle gamma subunit, with an additional C-terminal extension (69). This protein is mutated in the *stargazer* mouse, which has distinctive head movements (presumably due to defective vestibular function), ataxia and seizures. The ataxia is persistent but the seizures are episodic, disparate features foreshadowing possible dual functions of the stargazin protein (70). Akin to the effects of the skeletal muscle gamma subunit on L-type VDCC function, stargazin increases the steady-state inactivation of neuronal P/Q-type ($alpha_{1A}$) channels in heterologous cells (69). Interestingly, the C-terminal extension of stargazin contains a PDZ binding motif through which it interacts with PSD-95 family members (71). In this regard, stargazin has been shown to have two additional functions; it regulates both the delivery of AMPA receptors to the membrane surface and their synaptic targeting, the latter dependent on the C-terminus of stargazin (71). Remarkably, then, stargazin has the potential of regulating calcium influx in multiple ways by virtue of its interactions with both calcium channels and AMPA-preferring glutamate receptors.

4.1.3. IP_3 receptor complexes

In contrast to NMDA receptors and VDCCs, IP_3 and ryanodine receptor channels are located in the SER, although IP_3 receptors predominate in spines. Both IP_3 and ryanodine receptors are tetramers of large, membrane-spanning subunits (about 300-500 kDa each) that form a central calcium-conducting pore in the SER. One difference is that while both are activated by calcium, the IP_3 receptor channel is gated primarily by IP_3, a messenger generated from phospholipids through the action of phospholipase C. In turn, phospholipase C is activated by certain G-protein coupled neurotransmitter receptors. In spines, the most characterized receptors to date are the metabotropic glutamate receptors (mGluRs).

The mGluRs are seven transmembrane-domain proteins linked through G proteins to second messenger systems such as the phosphoinositide (PI) and adenylyl cyclase cascades. Eight subtypes (mGluR1-8) have been identified by molecular cloning, with different pharmacological and signaling properties (72). They can be divided into three groups based on G protein coupling, pharmacology, and sequence similarity. Group 1 (mGluR1 and mGluR5) receptors are postsynaptic and activate phospholipase C (PLC) whereas group 2 (mGluR2 and 3) and group 3 (mGluR4, 6, 7, and 8) receptors can function at pre-or postsynaptic sites and negatively couple to adenylyl cyclase. Of these, the mGluR1alpha (a splice variant of mGluR1) and mGluR5 receptors are unique in that they have long intracellular C-terminal tails and are G-protein-coupled to phospholipase C, with generation of IP_3 and subsequent IP_3 receptor-mediated release of calcium from intracellular stores. Unlike NMDA receptors, which are located centrally in the PSD, these postsynaptic mGluRs are situated at the periphery of the PSD.

Recent studies have provided insights into the interactions of the group 1 mGluRs, mGluR1alpha and mGluR5. Each contains a -P-P-X-X-F- motif, which has been shown to interact with an enabled/VASP homology (EVH1) domain in a 28-kDa protein, Homer, which was originally identified as an immediate early gene responsive to synaptic activity (figure 2) (73). Subsequently, a family of Homer proteins has been identified, all of which bind to the group 1 mGluRs (74, 75). Interestingly, unlike the original immediate early gene form of Homer (termed Homer 1a), the other members contain a C-terminal coiled-coil domain that mediates self-multimerization, rendering Homer multivalent and thus able to link mGluRs to other Homer-binding proteins. Since Homer 1a is unable to multimerize, it can competitively interfere with the multimerization of coiled-coil Homer, thereby acting as a natural, inducible dominant-negative (76).

The generation of IP_3 near plasma membrane mGluRs and the need to activate IP_3 receptors in the SER begs the question of whether mGluR and IP_3 receptors share an IP_3 microdomain. In fact, this has been suggested by physiological studies (77). The arrangement of SER in the spine provides structural support for this concept; SER can be found within spines, often abutting the lateral margins of the PSD, where mGluRs are preferentially located (figure 1) (2, 25, 75). Thus, a close association of mGluRs with IP_3 receptors would facilitate tight spatial and temporal calcium regulation. Indeed, Tu et al. (76) found that the Homer protein forms a physical tether linking the group 1 mGluRs to IP_3 receptors through -P-P-X-X-F- motifs, which are present in both the metabotropic glutamate and IP_3 receptors. Expression of the immediate early gene form of Homer (Homer 1a), which lacks the ability to self-multimerize, inhibits glutamate-induced intracellular calcium mobilization by de-coupling mGluRs and IP_3 receptors (figure 6) (76). This provides a mechanism for activity-dependent regulation of intracellular calcium release, as synaptic activity increases production of Homer 1a. Interestingly, the Homer-binding motif (-P-P-X-X-F-) has also been found in a number of other proteins including the ryanodine receptor, dynamin III, and several VDCC $alpha_1$ subunits (3, 76), extending the potential functions of Homer as a synaptic organizer.

IP_3 receptors are modulated through interactions with other proteins as well; IP_3 receptors bind calmodulin and the immunophilin FKBP12, and possibly calcineurin (78). Interactions of the IP_3 receptor have also been described with store-operated calcium channels (SOC). These channels are opened upon unloading of intracellular calcium stores in the SER through the activation of IP_3 receptors. This calcium-release activated calcium current appears to be involved in refilling intracellular stores through a process called capacitative calcium entry. There is a tight functional association with IP_3 receptors and SOCs (79), an association dependent on the interaction of the N-terminus of the IP_3 receptor with the SOC (80). In this case, gating of the SOCs by IP_3 receptors is expected to refill the depleted intracellular stores (8).

4.2. Mechanisms of calcium removal from spines

Influx is just one half of the calcium equation in spines; the other is efflux. As for calcium entering the spine, there are several ways for calcium to leave the spine. First, we will introduce the ATP-dependent calcium pumps,

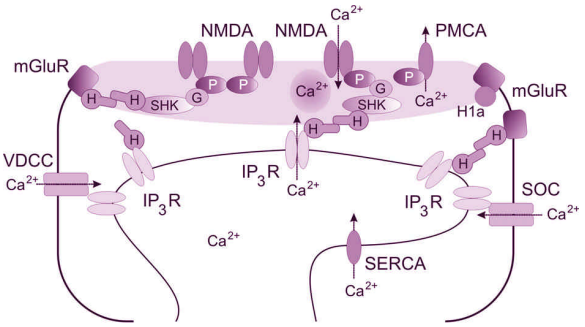

Figure 6. Overview of functional interactions among different modes of calcium influx and efflux. A dendritic spine is schematically depicted, with the PSD shaded. NMDA receptors are centrally located within the PSD, and mGluRs at the PSD periphery. VDCCs, PMCAs, and SERCAs are present in spines as well. SER harbors extensive intracellular calcium stores, which are released through IP_3 receptor channels. Functional interactions among the VDCCs, IP_3 receptors, and mGluRs are depicted. Homer 1a lacks the coiled-coil multimerization domain, and can uncouple mGluRs from IP_3 receptors. Full descriptions and details of these interactions are found in the text. Abbreviations: G, GKAP; H, Homer; IP_3R, IP_3 receptor; mGluR, metabotropic glutamate receptor, group 1; P, PSD-95; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SHK, SHANK; SOC, store-operated calcium channel; VDCC, voltage-dependent calcium channel.

which sequester calcium from the cytoplasm to internal stores such as SER and extrude calcium from the spine to the extracellular milieu. Next, we will discuss changes in spine morphology that can alter calcium dynamics within the spine, including the flow of calcium through the spine neck to the dendritic cytoplasm. Finally, we will explore the potential roles of calcium-buffering proteins in shaping spine calcium responses.

4.2.1. Calcium-ATPases

Ca^{2+}-ATPases are found in both the plasma membrane and SER. The Ca^{2+}-ATPases in the plasma membrane (PMCA1-4) are proteins of about 125-140 kDa, with 10 transmembrane domains and both N- and C-termini situated intracellularly. However, there are differences among the various forms with regard to regulation, including that by calmodulin. PMCA 2b and 4b interact with PSD-95 family members (81), which may bring them into complexes with multiple calcium influx proteins, such as NMDA receptors. Direct interactions of PMCA with calcium-dependent signaling proteins have recently been described through the C-termini of PMCA 4b and the PDZ domain of nNOS. PMCA 4b co-precipitates with nNOS, and nNOS activity is reduced by calcium extrusion via PMCA (82).

Another means of lowering cytoplasmic calcium concentrations is through sarco-endoplasmic reticulum Ca^{2+}-ATPase pumps (SERCA). Several isoforms of these membrane-spanning proteins, which drive calcium into the SER from the cytoplasm, have been identified in neurons (8). Importantly, these proteins are likely the primary means of refilling depleted SER calcium stores. At the same time, they are able to lower local cytoplasmic calcium concentrations; pharmacological blockade of SERCA alters the calcium decay kinetics in spines (83). As the presence of SER in spines correlates with spine size, SERCA pumps may be particularly important in regulating calcium dynamics in larger spines.

4.2.2. Spine neck calcium diffusion

In addition to the regulation of these calcium pumps, morphological changes in the spine may be important in changing calcium dynamics. In this regard, Majewska et al. (83) found that the spine calcium decay after action potentials depends on spine neck length. Also, long-necked spines have slower kinetics of refilling their internal stores from dendritic calcium, again suggesting that spine necks can function as bottlenecks in the diffusion of calcium between dendrite and spine head (13). This reinforces the importance of spine morphology, which changes during development and in response to synaptic activity, in the regulation of spine calcium dynamics (10, 14-19).

Not surprisingly, the cytoskeleton is a crucial structural element in regulating changes in spine morphology. Elements of the actin-based cytoskeleton predominate within spines, and although tubulin is present in PSD fractions and MAP proteins have been localized at synapses, microtubules appear absent from spines (84). Thus, it was unexpected when PSD-95 was found to bind to a small polypeptide called CRIPT through its third PDZ domain. CRIPT binds microtubules, and its overexpression in heterologous cells causes a redistribution of PSD-95 to microtubules. Lastly, MAP-1A binds to PSD-95 through the GK domain, an interaction stimulated by occupancy of neighboring PDZ domains. These interactions may permit the linkage of NMDA receptors to the tubulin-based cytoskeleton through PSD-95 (3), or alternatively may be involved in microtubule-dependent transport of PSD-95 complexes.

Extensive protein links from glutamate receptors in the PSD to the actin cytoskeleton have been described, both PSD-95-dependent and -independent. The actin-binding protein alpha-actinin binds to the cytoplasmic tails of both NR1 and NR2B; this interaction is regulated by calcium-calmodulin, which competes with alpha-actinin for binding to NR1 (figure 5) (85). Brain spectrin, or fodrin, binds to several NMDA subunits *in vitro*. NR1 binding to spectrin is inhibited by calcium-calmodulin, and the interaction between spectrin and NR2B is sensitive to both calcium-calmodulin and Fyn-mediated tyrosine phosphorylation (86). Also, fodrin undergoes proteolysis by calpain following increases in intracellular calcium concentration produced by activation of glutamate receptors (84). PSD-95-linked actin cytoskeleton interactions are also known. PSD-95 interacts with GKAP through its GK domain (87). In turn, the C-terminus of GKAP binds to a protein dubbed Shank (for *SH*3 domain, *ank*yrin repeats) (88, 89), that has multiple domains for

interaction, including the SH3, ankyrin repeats, PDZ domain, proline-rich Homer-binding motif, and a SAM domain; in addition, it interacts with the actin cross-linking protein cortactin. Finally, the RapGAP Spar also interacts with the GK domain of PSD-95, and Spar has two domains, Act1 and Act2, that induce remodeling of the actin cytoskeleton (figures 2 and 3) (43). Thus, there are extensive interactions of PSD proteins with the actin cytoskeleton, the regulation of which could have roles in the remodeling of spines.

So how are these proteins involved in regulating spine shape? Several recent studies have addressed the regulation of spine shape by proteins within the spine, such as those of the PSD-95 supramolecular complex. Proteins including Shank, Spar, drebrin, and the Rac1 GEF kalirin-7 alter spine size and/or shape when overexpressed in neurons (18, 43, 90, 91). When overexpressed in neurons, Shank is able to increase spine head size in a manner that depends on Homer (90). Spar also causes enlargement of spine heads; dominant-interference of its interactions causes spines to become more narrow and elongated. These effects depend on both the actin-binding and RapGAP domains, implicating Rap signaling in the regulation of postsynaptic structure (43). Other small GTPases have been implicated in the regulation of spine morphology; it has been suggested that Rac promotes spine formation while Rho prevents it (92). Changes in spine shape appear to depend in part on calcium release from SER. In neurons treated with caffeine, which triggers calcium release from intracellular stores, most of the dendritic spines elongated over the next several hours (14, 93). Thus, both calcium release as well as calcium-dependent enzymes appear involved in modifying spine size and shape. The effects of VDCCs on spine morphology are less clear, though clustering of VDCCs and the resultant calcium 'hotspots' promote morphological changes in neuronal growth cones (68).

4.2.3. Cytoplasmic calcium-buffering proteins

In addition to their roles in enzymatic and signaling mechanisms, calcium-binding proteins also serve as buffers. Such buffering proteins include calbindin, parvalbumin, and calretinin, among others (5, 13). These can reduce the amplitude of calcium signals, alter decay kinetics, and limit diffusion. Though calcium-buffering capacity has been evaluated in a variety of cell types, including neurons, spine calcium buffering proteins and their buffering capacities are not well understood. Still, it seems likely that spine calcium buffering proteins play key roles in shaping calcium responses, for instance by limiting the spatial spreading of calcium to local signaling microdomains (5).

5. SUPRAMOLECULAR PROTEIN COMPLEXES AND SYNAPTIC REGULATION

5.1. Multimodal proteins organizing supramolecular complexes

We have emphasized the structural and functional features of protein complexes involving VDCCs, mGluRs, and NMDA receptors that impart a high degree of signaling selectivity through the formation of signaling microdomains. However, opportunities exist for the synergistic interaction of signaling among these diverse pathways (94), increasing the specificity of calcium signaling within the spine. Three proteins appear particularly important in such multimodal organization: the 'master scaffolds' PSD-95, Homer, and Shank. These proteins possess several features that enhance their regulatory power. First, they are all members of larger families of similar proteins, providing for a diversity of responses. Second, they each have interactions with multiple signaling and structural proteins, including one another. Along these same lines, they are each able to self-multimerize, extending their potential reach and opportunities for cross-talk. Third, they have features suggesting they are dynamically regulated. Finally, their relative localizations within the PSDs of spines are suggestive of distinct but interrelated functions.

Multiple protein family members and alternative splicing expand the potential for differential regulation. The Shanks are large proteins, over 200 kDa in some cases. There are three forms -- Shank 1, 2 and 3 --plus multiple splice variants. Though the functional significance of these variants is unclear, they have distinct distribution patterns (89). Homer also has multiple family members, Homer 1-3, all of which except for Homer-1a (and the Homer 1 variant Ania-3) have the coiled-coil domain (75). As mentioned previously, PSD-95 is a member of the MAGUK family, which includes the postsynaptic proteins PSD-93/chapsyn-110, SAP97/hDlg, and SAP102. Though in most cases any distinct roles for members of these families remain unknown, they clearly expand the potential diversity of responses.

Interactions of PSD-95- and Homer-based complexes have been discussed extensively due to their key roles in the organization of NMDA and mGluR signaling complexes, respectively. Importantly, Shank has the potential to link such complexes to one another; Shank binds Homer directly and is linked to PDS-95-based complexes through GKAP (88, 95). Such links expand the calcium signaling repertoire, and present the possibility for linking IP_3 receptor channel complexes in the SER to NMDA receptor channels in the plasma membrane (figure 6). An additional interaction each of the master scaffold proteins possesses is the ability to oligomerize. The SAM domain of Shank is involved in tail-tail multimerization of Shank, PSD-95 multimerizes in a head-to-head fashion, and Homer proteins interact with one another through the coiled-coil domains (31, 75, 89).

The dynamic regulation of these proteins likely plays a key role in specifying the nature of calcium responses in spines. Time-lapse studies of tagged PSD-95 show an activity-dependent turnover of over 20% of PSD-95 clusters over 24 hours (96); Homer 1c (PSD-Zip45) shows even higher steady-state turnover rates (97), and Homer 1a was initially identified by its expression in response to sustained seizure activity (73). Shank has several features suggesting it may also be dynamically regulated, and in fact its mRNA is found in dendrites,

suggesting translation may be under local control in response to synaptic activity (98).

Lastly, the relative localizations of these proteins are suggestive of specialized functions. PSD-95 may organize elements within the PSD close to the membrane, whereas Homer and Shank lie more at the cytoplasmic face of the PSD, and may mediate interactions with cytoskeletal and cytosolic proteins (99). The Shanks may be particularly versatile organizers of the PSD, linking PSD-95 and Homer-based complexes and integrating signals from mGluRs and NMDA receptors by coordinating the influx of calcium through NMDA receptor channels and the mGluR-dependent release of calcium from intracellular stores. Shank may also provide a means for NMDA receptors to couple to intracellular calcium stores independent of mGluRs by linking NMDA receptor/PSD-95 complexes to Homer/IP_3 receptor complexes (figure 6).

Taken together, studies suggest that these 'master scaffolding' proteins -- Shank, Homer and PSD-95 -- confer on the postsynaptic neuron the potential for organizing and regulating sophisticated calcium-dependent processes within spines. Furthermore, the ability to regulate interactions among these proteins in an activity-dependent fashion makes these supramolecular complexes well-suited for key roles in mechanisms of synaptic plasticity.

5.2. Calcium microdomains and synaptic regulation

Given the tight spatial and temporal organization of calcium microdomains in the vicinity of calcium-dependent signaling proteins, what are the roles of such complexes in mechanisms of synaptic plasticity? Genetic approaches have begun to be reported for several of the main players. “Knock out” mice lacking PSD-95 had an enhancement of LTP at the expense of LTD, and impaired spatial learning (100). Targeted removal of the NR2 NMDA receptor subunits C-terminal tail (which contain the PDZ-binding motif) causes impaired receptor signaling without affecting NMDA channel function. Mice lacking the C-terminus of NR2A had impaired synaptic plasticity and contextual memory, while mice lacking the C-terminus of NR2C had deficits in motor coordination (101). These findings suggest that protein locations or interactions are important, not just the function of the channels. Though genetic studies evaluating the function of key proteins such as Homer and SHANK are not yet available, a number of cellular studies emphasize the likelihood that these processes are regulated and involved in mechanism of plasticity, in particular demonstrating the functional importance of these microdomains.

We have highlighted the precise co-localization of signaling proteins in the spine with distinct calcium sources. Importantly, evidence exists that not only the elevation of intracellular calcium but also the mode of calcium entry is critical for the specificity of calcium responses in neurons. For example, nNOS is selectively stimulated by calcium influx through NMDA receptors, while other modes of entry are not so effective (35). In hippocampal neurons the stimulation of various K^+ channels by calcium depends upon the subtype of calcium channel involved: influx through L-type calcium channels activates SK calcium-activated K^+ channels, influx through N-type calcium channels activates BK channels, and influx through P/Q-type calcium channels activates neither (102). Also, increases in intracellular calcium through VDCCs induce Homer 1c clustering, while calcium influx through NMDA receptors results in the disassembly of Homer 1c complexes (97). Finally, even at distant sites such specificity can be preserved. In hippocampal neurons calcium entry through NMDA-preferring glutamate receptors and L-type VDCCs is equally effective in activating serum response element-mediated transcription, but only calcium flux through L-type calcium channels stimulates the cAMP response element (103). Furthermore, a calcium microdomain near NMDA receptors is the ‘on switch’ for extracellular signal-regulated kinase (ERK)-mediated synapse to nucleus signaling (104), a signal which propagates to the nucleus independent of global increase in calcium concentration. On the other hand, Dolmetsch et al. (105) found that the CaM-binding IQ domain of L-type VDCCs was necessary for the RAS/mitogen-activated protein kinase pathway, which conveys signals from the base of the L-type channel to the nucleus. These studies point to the importance of local calcium microdomains not only in local calcium-dependent signaling, but also, and perhaps unexpectedly, in long-range signaling to the nucleus.

Another feature we have stressed is the web-like interaction of multimodal structural and signaling proteins with one another, increasing the power and complexity of calcium signaling. For instance, stimulation of a few parallel fibers, which emanate from cerebellar granule cells, results in a biphasic pattern of calcium accumulation in the dendritic tree that is highly localized to the region of transmitter release (77, 106). The fast component is reminiscent of depolarization-induced calcium influx through voltage-gated calcium channels; the slow component is mediated through mGluR stimulation, and is required for LTD (107). This latter mechanism requires repetitive stimulation, most likely since the IP_3 concentration must reach 10 micromolar for appreciable calcium release (6). Thus, the tight physical coupling between mGluRs and IP_3 receptors may be a reflection of the need to achieve a high concentration of IP_3 within a microdomain, sufficient to generate the local release of calcium from intracellular stores. It is likely that such a signaling microdomain is based on a protein complex involving mGluRs, Homer, and IP_3 receptors in the dendritic spine. Also, short forms of Homer that do not multimerize enhance coupling of mGluRs to VDCCs while at the same time disrupting coupling to the IP_3-sensitive SER pools (108). Thus, different Homer isoforms appear capable of specifying the calcium signaling output of the mGluRs.

Intriguing, though less direct, was the finding by Emptage et al. (109) of NMDA receptor-dependent calcium release from intracellular stores. Fast synaptically-evoked calcium transients are localized to individual spines and blocked by not only the AMPA receptor antagonist CNQX, but also the NMDA receptor antagonist APV, suggesting

that NMDA receptors are required. However, antagonists of calcium-induced calcium release abolish these synaptically-evoked calcium transients. As only small amounts of calcium are carried through NMDA receptors, but the calcium concentration required for ryanodine receptor activation is large, the two proteins must be very close to one another. As outlined previously, this could be explained by the ability of SHANK to bridge Homer to the NMDA receptor/PSD-95 complex through GKAP (figure 6). In such a scenario, Homer could then bind to a Homer binding-motif on the ryanodine receptor, bringing NMDA and ryanodine receptors into close proximity. Akin to the work of Emptage, Nishiyama et al. (110) recently showed that postsynaptic calcium, derived from influx through NMDA receptors and differential release from intracellular stores through ryanodine and IP_3 receptors, regulates both the polarity and input specificity of activity-dependent synaptic modification. In the CA1 hippocampus, partial blockade of NMDA receptors results in the conversion of LTP to LTD, while the induction of LTD at hetero- and homo-synaptic sites requires functional IP_3 and ryanodine receptor channels, respectively (110). This study underscores the importance of the spatiotemporal patterns of differential calcium release from internal stores for mechanisms of synaptic plasticity.

6. CONCLUSIONS AND PERSPECTIVE

Clearly, then, there is cross-talk among postsynaptic calcium signaling complexes that is functionally important. Specificity of responses is increased through the modular organization within and among the discrete calcium signaling complexes. We have discussed the major features of calcium signaling microdomains, emphasizing the specializations that optimize the temporal and spatial segregation of calcium ions in dendritic spines. The spine in fact seems uniquely endowed to orchestrate the fine-tuning of calcium responses. However, exploiting the versatility and universality of calcium-dependent processes is not without risk. As other reviews in this series will address, calcium is also involved in multiple mechanisms of cell injury and death. In fact, it is tempting to speculate that spines may have evolved for just this reason. At the same time the spine neck compartmentalizes calcium signaling, it may also have the ability to act as a firewall by isolating local disruptions in calcium regulation, protecting the integrity of the neuron. Future studies on calcium dynamics of spines will likely stress both their involvement in higher order neuronal functions as well as their possible roles in disease.

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