Structure and Regulation of Calcium/Calmodulin-Dependent Protein Kinases

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I. Introduction

The concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) is highly regulated because Ca^{2+} is an important cellular signaling molecule.[1] Basal levels of intracellular Ca^{2+} in most mammalian cells is in the 10−50 nM range, and stimulated concentrations are generally 500−1000 nM. Since concentrations of Ca^{2+} in the extracellular space or intracellular organelles (endoplasmic and sarcoplasmic reticulum and mitochondria) are millimolar, the cell has numerous mechanisms for modulating $[Ca^{2+}]_i$ both temporally and spatially.[2] Such mechanisms include ligand- (IP_3, cADP-ribose, cGMP, glutamate, etc.) and voltage- (cell depolarization and hyperpolarization) gated ion channels, Ca^{2+} pumps, and exchangers in both the plasma membrane and intracellular Ca^{2+} storage-organelles. These ion channels, pumps, and exchangers are subject to complex regulation by cellular signaling pathways. For example, the L-type Ca^{2+} channel which is primarily activated by membrane depolarization (i.e., voltage-gated) is also subject to additional modulation through covalent phosphorylation by protein kinases, which are stimulated by cAMP or calcium.[3,4]

Temporal and spatial changes in $[Ca^{2+}]_i$ mediate responses of many agonists such as hormones, growth factors, and neurotransmitters. Mammalian cells contain a large number of proteins that bind $[Ca^{2+}]_i$ with varying specificity and affinity. Some of these proteins have low affinity and act primarily as "buffers" that limit Ca^{2+} diffusion. Other proteins bind Ca^{2+} with high affinity and specificity and are responsible for producing the multiple biochemical changes and physiological responses characteristic of elevated $[Ca^{2+}]_i$. Some of these proteins, such as protein kinase C, bind Ca^{2+} directly, whereas others are regulated indirectly through a "transducer" such as calmodulin (CaM). CaM is a ubiquitous 16−17 kD protein containing four helix−loop−helix motifs that bind Ca^{2+} with high specificity and affinity (0.5−5 μM K_d values).[5] CaM has two globular domains, each of which contains two Ca^{2+} binding sites; these domains are connected by a flexible α-helix. Binding of Ca^{2+} to CaM induces a conformational change exposing hydrophobic residues that promote interaction of the Ca^{2+}/CaM complex with a large number of proteins including a family of Ser/Thr protein kinases. This family of Ca^{2+}/CaM-dependent protein kinases (CaM-Ks) is the subject of this review.

A. CaM-Ks: General Characteristics

CaM-Ks can be divided into two general categories: those with very restricted substrate specificity (restricted) and those with broad substrate specificity (multifunctional). Table 1 lists members of these two groupings with some of their general characteristics. Note that they all have similar domain organizations (Figure 1) with the exception of CaM-KIII (eEF-2K), which belongs to a unique family of Ser/Thr protein kinases (see below). As discussed for each kinase, they are autoinhibited in the absence of Ca^{2+}/CaM. In addition to activation by Ca^{2+}/CaM, all of these CaM-Ks are further regulated by phosphorylation—either autophosphorylation or phosphorylation by some other kinase. In some cases this phosphorylation activates the CaM-K, whereas in other cases it is inhibitory.

II. Multifunctional CaM-Kinases

A. The CaM-Kinase Cascade

Many protein kinases form cascades where one protein kinase activates a downstream protein kinase.[6] Some of these cascades, like the MAP kinases, have multiple members, whereas others like cAMP-dependent protein kinase/phosphorylase kinase are more limited. Enzyme cascades, including protein

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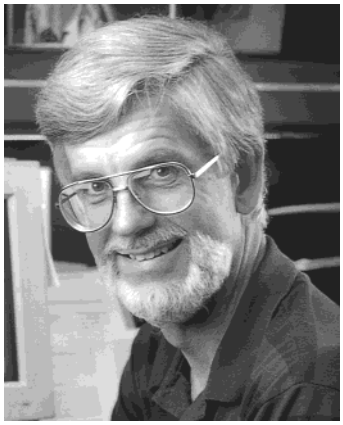
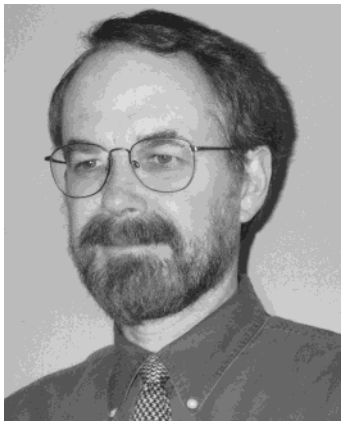


Thomas R. Soderling, a native of Northern Idaho, received his B.S. degree in Chemistry from the University of Idaho in 1966. His PhD. degree work in Biochemistry at the Unversity of Washington under Professor Edwin Krebs was on regulation of glycogen synthase by cAMP-dependent protein kinase. Following a postdoctoral stay at Vanderbilt University, he remained for 17 years as a faculty member in the Molecular Physiology Department and an investigator of the Howard Hughes Medical Institute. In 1991 he moved to the Oregon Health Sciences University, where he is Associate Director of the Vollum Institute and Professor of Biochemistry and Molecular Biology. His work for the past 20 years has focused on structure/function and neuronal roles of protein kinases regulated by calcium/calmodulin.



James T. Stull studied pharmacology at Emory University and the University of California, San Diego. After receiving his Ph.D. degree under Professor Steven Mayer in 1971, he spent 3 years as a postdoctoral fellow with Professor Edwin Krebs in Davis, CA. He was then appointed Assistant Professor at the University of Calirornia, San Diego, and subsequently moved to the University of Texas Southwestern Medical Center at Dallas, where he is the Bashour Distinguished Professor, Chair of the Physiology Department, and Chair of the interdepartmental integrative Biology Graduate Program. He is also an Associate Editor of the *Jouranl of Biological Chemistry*. His research interests include mechanisms of regulation of smooth and nonmuscle contractility by calmodulin-dependent phosphorylation mechanisms with an emphasis on integrating molecular, biochemical, and physiological experimental approaches.

kinases, exhibit several unique regulatory aspects: (1) amplification of the input to output signal, (2) opportunities for independent regulation of each member of the cascade, and (3) potential for cross-talk with other signaling pathways.

Over the past six years a CaM-K cascade has been described that contains three members: CaM-KK, CaM-KI, and CaM-KIV.[7,8] CaM-KK is the upstream member of the cascade which activates CaM-KI and CaM-KIV through phosphorylation of their "activation loops". At the base of their catalytic cleft, many protein kinases, including CaM-KI and CaM-KIV, have an "activation loop" containing a Ser/Thr in which phosphorylation strongly augments kinase activity. A somewhat unusual feature of the CaM-K cascade is that binding of Ca^{2+}/CaM to both CaM-KK and its substrates, CaM-KI and CaM-KIV, is required for phosphorylation of the activation loop sites.[9,10] It is not readily apparent if there is some unique advantage of binding of the same allosteric activator to adjacent members of a cascade, but other examples do exist (e.g., the AMP-kinase cascade). Each member of this CaM-K cascade will be described.

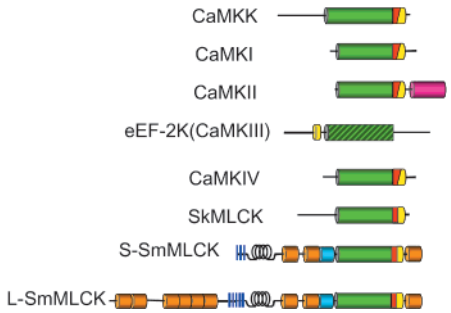


Figure 1. Domain organization for members of the Ca^{2+}/calmodulin (CaM)-dependent protein kinases. The domains are shown as as follows: catalytic domains, dark green; autoinhibitory domains, red; Ca^{2+}/CaM-binding domains, yellow; association domain of CaM-KII, magenta; Ig modules, gold; fibronectin modules, blue; actin binding motifs, blue bars; PEVK repeats, black springs; and nonconserved regions without identified structural or functional domains, black lines. Isoform variations for each type of kinase are not shown. The catalytic core of eEF-2K belongs to a protein kinase family significantly different from the other Ca^{2+}/CaM-dependent protein kinases. See text for references.

B. CaM-Kinase Kinase

Initial studies on CaM-KI and CaM-KIV indicated the existence of an "activating factor" in brain extracts that, upon incubation with Ca^{2+}/CaM plus Mg^{2+}/ATP, could dramatically increase their activities.[11] An α CaM kinase kinase (CaM-KK), which can activate both CaM-KI and CaM-KIV, was cloned[12] as

Table 1. Summary of Properties of Major Mammalian Ca^{2+}/CaM-Dependent Protein Kinases. Details and References Are Given in the Sections Describing Each Kinase

CaM-kinase	subunit Mr	holoenzyme	substrates	regulation by phosphorylation
CaM-KI	42 kD	monomer	unknown	CaM-KK: increased total activity
CaM-KII	50−60 kD	dodecamer	multiple	autophosphorylation: constitutive activity : enhanced binding of Ca^{2+}/CaM
CaM-KIII (eEF-2K)	100 kD	monomer	eEF-2	autophosphorylation: constitutive activity PKA: constitutive activity
CaM-KIV	65−67 kD	monomer	multiple	CaM-KK: increased total and constitutive activity autophosphorylation: activation
CaM-KK	55−65 kD	monomer	CaM-KI, CaM-KIV PKB	PKA: inhibitory
SkMLCK	67−150 kD	monomer	RLC	
SmMLCK	130−210	monomer	RLC	PKA, PKC, CaM-KII, PAK: increases K_{CaM} or V_{max}

well as more recently a β CaM-KK isoform.[13,14] These monomeric CaM-KKs have general domain organizations common to other CaM-Ks (Figure 1). Many protein kinases have a primary amino acid sequence (AID) that interacts with and inhibits the catalytic domain.[15] In many cases AIDs are "pseudosubstrates" which contain substrate recognition determinants except they lack a phosphorylatable Ser/Thr. The Ca^{2+}/CaM complex binds to a sequence (CBD) which partially overlaps the AID, presumably causing a comformational change and thereby disrupting interaction of the AID with the catalytic domain and inducing kinase activity. CaM-KKs also have an AID and CBD,[16] but their catalytic domains have two unique properties. The first is the absence of the conserved acidic residues in catalytic subdomains V and VI that, in many other kinases, recognize basic residues in substrates; the second is an unusual Arg-Pro-rich insert (RP-insert). The absence of acidic residues suggests that CaM-KK does not recognize basic residues in the primary sequence around the phosphorylation sites in the activation loops in CaM-KI (Thr177)[9] and CaM-KIV (Thr196).[17] For many substrates of protein kinases, the primary sequence around the phosphorylation site is important for substrate recognition. However, studies on the MAP kinases suggest that the primary sequence in the activation loop may not dictate the specificity of the activating kinase.[18] A recent study has shown that the RP-insert in the catalytic domain of CaM-KK is important in recognition of CaM-KI and CaM-KIV as substrates. Deletion of the RP-insert in CaM-KK abolishes its activity toward CaM-KI and CaM-KIV.[19] Interestingly, the RP deletion mutation has no effect on the ability of CaM-KK to activate protein kinase B (PKB). Activation of PKB through phosphorylation of its activation loop by CaM-KK was shown to occur both in vitro with recombinant enzymes as well as in NG108 cells upon elevation of intracellular Ca^{2+}.[20] Compared to CaM-KI and CaM-KIV, PKB is a relatively poor substrate for CaM-KK,[21] suggesting that different substrate recognition determinants may be involved. This could explain why activation of PKB is not altered by deletion of the RP insert in CaM-KK where the same mutation obviates activity toward CaM-KI and CaM-KIV. Activation of PKB by CaM-KK may contribute to protection against cellular apoptosis.[20] In certain cells, such as cerebellar granule cells, small elevations of intracellular Ca^{2+} prevents apoptosis.[22]

Cross-talk between different signaling cascades or pathways is rather common, and this is also true for the CaM-K cascade. For example, cAMP-dependent protein kinase (PKA) can phosphorylate two sites in CaM-KK and inhibit its activity.[23,24] One of these phosphorylation sites is in the CBD of CaM-KK. This inhibition depends on the temporal sequence of the cellular stimulation. For example, treatment of cultured cells with forskolin, which elevates cAMP levels and stimulates PKA, results in strong inhibition of CaM-KK activation upon subsequent Ca^{2+} mobilization as well as suppression of activation of its downstream target CaM-KIV.[23] However, if Ca^{2+} mobilization occurs prior to elevation of cAMP levels, inhibition of CaM-KK by PKA is reduced because Ca^{2+}-CaM blocks the access of PKA to phosphorylate the CBD.[16] Cross-talk with the cAMP system can also occur at the level of type I adenylate cyclase, which itself can be stimulated by the binding of Ca^{2+}/CaM. CaM-KIV appears to phosphorylate the CaM-binding domain and inhibit activation of the type I adenylate cyclase by Ca^{2+}/CaM.[25]

C. CaM-Kinase I

CaM-KI is a monomeric 42 kD enzyme that is widely distributed in most mammalian cell types.[26] There are three genes encoding α, β, and γ isoforms with alternative splicing to form β1 and β2.[27,28] Although physiological substrates and therefore functions of this kinase are not well characterized, it is the only CaM-K for which a crystal structure is known.[29] Therefore, the main focus in this section will be CaM-KI structure and the role of its AID in inhibiting the kinase.

CaM-KI, like other protein kinases (see review by Taylor and co-workers in this issue), has a bilobal catalytic domain with the upper lobe primarily binding ATP and the lower lobe binding protein substrates and performing catalysis. In the basal inhibited state, multiple residues in the AID of CaM-KI interact with both surfaces of the upper and lower lobes of the catalytic domain.[29] The AID contains two α-helices, αR1 and αR2, which interact with the lower and upper lobes, respectively. For example, residues 305−316 in αR2 interact with the outer surface of the upper catalytic lobe, resulting in distortion of the ATP-binding pocket. Multiple residues in αR1 of the AID form hydrophobic and ionic interactions with the lower catalytic lobe that binds protein/peptide substrates. The best peptide substrates of CaM-KI have a basic residue at the P-3 position (i.e., three residues N-terminal of the phosphorylated Ser/Thr) and a hydrophobic residue at P-5.[30] Glu102, which is predicted to interact with the P-3 basic residue in substrates of CaM-KI, interacts with Lys300 in αR1. Likewise, Phe298 in αR1 may mimic the P-5 residue in substrates, and it is buried in a hydrophobic pocket formed by residues Phe104, Ile210, and Pro216 of the lower catalytic lobe. Thus, due to interactions of residues in the AID with key residues in both lobes of the catalytic domain, basal CaM-KI cannot effectively bind ATP or protein substrates and is inactive.[29] Initial binding of Ca^{2+}/CaM to the CBD in αR2 presumably disrupts its interactions with the upper catalytic lobe and allows the ATP-binding domain to adopt its proper conformation.[31,32] A resultant conformational change in αR1 or perhaps subsequent interaction of Ca^{2+}/CaM with αR1 displaces its "pseudosubstrate" inhibitory interactions with the lower lobe. The activated kinase can then bind both substrates and catalyze phosphorylation.

This mechanism of autoinhibition of CaM-KI is quite different from an analogous kinase, twitchin kinase, which is activated by binding of Ca^{2+}/S100.[33] S100, like CaM, is an "EF hand" Ca^{2+} binding protein. In twitchin kinase, the AID physically occupies the catalytic cleft rather than interacting with

the outer surfaces of the two catalytic lobes.[34] Residue Glu6023 in the catalytic domain of twitchin kinase would normally interact with a basic residue in substrates, but it binds to Arg 6237 in the AID. Residues 6236−6240 in the AID form a short helix which occupies the ATP-binding pocket. These comparisons of CaM-KI and twitchin kinase indicate that although the overall structures of their bilobal catalytic domains are highly conserved, the mechanisms by which their AIDs inhibit kinase activities differ markedly. One caveat of these results is that the crystal structures of both CaM-KI and twitchin kinase were obtained with truncated enzymes. Although it seems unlikely that truncations would alter the mode of interaction of the AID with the catalytic domain, it remains a possibility. It will be important to obtain crystal structures for other CaM-Ks to determine their mechanisms of basal inhibition.

In addition to activation by binding of Ca^{2+}/CaM, CaM-KI is further activated through a phosphorylation mechanism. Like many protein kinases, CaM-KI has an "activation loop" containing a phosphorylation site, Thr177.[9] Phosphorylation of Thr177 in CaM-KI is catalyzed by CaM-KK, resulting in a 10- to 20-fold increase in CaM-KI activity toward most of its substrates. As stated above, activation of CaM-KI by CaM-KK only occurs when Ca^{2+}/CaM is bound to both kinases.[35] Presumable binding of Ca^{2+}/CaM to CaM-KI is required to present a proper conformation of the activation loop to CaM-KK. Interestingly, the activation loop in CaM-KI in the absence of bound Ca^{2+}/CaM is not highly structured and cannot be visualized in the crystal structure.[29] CaM-KI activated by CaM-KK still has an absolute requirement for Ca^{2+}/CaM for activity. Phosphorylation of Thr177 in CaM-KI results in a 40-fold decrease in its K_m value for phosphorylation of site 1 in synapsin.[30] However, it has recently been reported that certain synthetic peptides that contain basic residues in the P-6 and/or P-7 positions are phosphorylated equally well by CaM-KI whether it was activated by CaM-KK or not.[30] The physiological relevance of this observation is not known, but it could suggest the existence of CaM-KI substrates whose phosphorylations are not dependent on CaM-KK activation of CaM-KI.

Little is known about physiological functions of CaM-KI. In vitro CaM-KI can phosphorylate synapsin[27] and the cystic fibrosis transmembrane regulator,[36] but its physiological substrates are not established. It is expressed in most mammalian cells,[37] predominantly in the cytoplasm although nuclear isoforms may exist.[38] When truncated CaM-KI is overexpressed in a mammalian cell, it can phosphorylate nuclear transcription factors[39] but the physiological relevance is not clear. However, CaM-KI in *C. elegans* does have a nuclear localization signal and may participate in regulation of gene transcription through phosphorylation of transcription factors.[40]

D. CaM-Kinase IV

Expression of monomeric mammalian αCaM-KIV is largely limited to neural tissues, T cells, and testis.[7,8] A β splice variant, containing an additional 28 N-terminal residues, is differentially expressed during development and is strongly expressed in cerebellar granule cells of mature rats.[41] Unlike CaM-KI and CaM-KK, CaM-KIV has not been identified in *Drosophila* or *C. elegans*.

General domain organization of CaM-KIV is similar to CaM-KI, but it has extended N- and C-termini. Like CaM-KI, CaM-KIV is activated by binding of Ca^{2+}/CaM and by phosphorylation of its activation loop (Thr196) by CaM-KK. However, CaM-KIV also appears to be regulated through autophosphorylation after its activation by CaM-KK. Autophosphorylation of the CaM-KIV Ser/Thr-rich N-terminus is required for full activation.[42,43] For example, the Ser12Ala plus Ser13Ala mutations prevent activation of CaM-KIV by CaM-KK, but a mutant that lacks the N-terminal 20 residues is activated normally by CaM-KK.[43] This suggests that the N-terminus has an AID that can be neutralized by autophosphorylation or by truncation. One consequence of activation of CaM-KIV, but not of CaM-KI, by CaM-KK is the generation of considerable Ca^{2+}-independent activity. Since mutation of Thr196 to Ala obviates both the increase in total as well as Ca^{2+}-independent activity,[17] the Ca^{2+}-independent activity could be a direct consequence of Thr196 phosphorylation or, more likely, it could be due to subsequent autophosphorylation. This issue has not been resolved. Generation of Ca^{2+}-independent activity might be an important regulatory mechanism; it would allow a transient elevation in intracellular Ca^{2+} levels to produce a prolonged CaM-KIV activation to regulate gene transcription through phosphorylation of transcription factors. Such a mechanism would be analogous to CaM-KII where autophosphorylation of Thr286 generates prolonged Ca^{2+}-independent activity, which is essential for long-term potentiation in hippocampal region CA1.[44] Once CaM-KIV has been activated by CaM-KK, Ser332 in the CBD is autophosphorylated in a Ca^{2+}-independent reaction[45] and prevents subsequent binding and activation by Ca^{2+}/CaM.

CaM-KIV shows very strong nuclear localization,[46] and good evidence supports the idea that it is responsible for Ca^{2+}-dependent gene transcription through phosphorylation of several transcription factors: CREB, SRF, and MEF2 phosphorylation.[47−49] CaM-KIV phosphorylates Ser133 in CREB, the site phosphorylated by PKA. Transfected CaM-KIV alone is a relatively poor stimulator of transcriptional activation by CREB, perhaps because its V_{max} value for CREB phosphorylation is low.[47] Upon activation by CaM-KK, however, CaM-KIV has V_{max} and K_m values equivalent to those of PKA[50] and cotransfection of CaM-KK with CaM-KIV gives a 14-fold enhancement of transcription.[12] Studies in cultured hippocampal neurons indicate that CaM-KIV regulates CREB-dependent gene transcription in response to electrical stimulation or KCl depolarization.[51] This role of CaM-KIV in CREB-mediated transcription has been confirmed in CaM-KIV knockout mice. In one study, CREB phosphorylation in the knockout mice was strongly suppressed in several brain regions by paradigms which elicited CREB phosphorylation in wild-type mice.[52] In another study, the knockout mice

showed impaired cerebellar Purkinje neuron development and reduced CREB phosphorylation in cerebellum extracts.[53] Recent studies suggest that in addition to phosphorylation of CREB, CaM-KIV exerts a further regulatory role through phosphorylation of CBP (CREB binding protein).[54,55] Given that CBP is a transcriptional co-activator for many transcription factors, including CREB, its phosphorylation by CaM-KIV presents a mechanism for regulating the expression of numerous genes by Ca^{2+}.

CaM-KIV is present in the cytoplasm as well as the nucleus; therefore, it probably has physiological functions in addition to phosphorylation of transcription factors. One probable substrate is oncoprotein 18 which negatively regulates microtubule assembly. Oncoprotein 18 can be phosphorylated on multiple sites by several protein kinases; CaM-KIV phosphorylates Ser16 of oncoprotein 18.[56] This phosphorylation, which inhibits the interaction between oncoprotein 18 and tubulin, is observed in Jurkat cells upon stimulation of the CD3 receptor,[57] which activates CaM-KIV.[58]

E. CaM-KII

The most intensely investigated member of the multifunctional CaM-Ks is CaM-KII. The CaM-KII family (reviewed in ref 59) is encoded by four genes (α, β, γ, and δ) which also exhibit alternative splicing. The γ and δ isoforms are expressed in most tissues, whereas the α and β isoforms are most prominent in neural tissues and comprise up to 2% of total protein in hippocampus. The overall subunit domain organization is similar to the other CaM-Ks except for a C-terminal extension that is involved in subunit association. The holoenzyme is an oligomeric protein comprised of 12 50−60 kD subunits arranged as two stacked hexameric rings.[60,61] The C-terminal association domains form the central core of each ring with the N-terminal catalytic domains projecting outward. In the absence of bound Ca^{2+}/CaM, the CaM-KII is maintained in an inactive conformation due to an interaction of an AID with the catalytic domain of its own subunit. Interestingly, the sensitivity of CaM-KII to activation by Ca^{2+}/CaM is dictated by the subunit composition of the holoenzyme.[62]

Unlike many other protein kinases, CaM-KII does not contain a phosphorylatable residue analogous to the activation domains of CaM-KI and CaM-KIV. Thus, CaM-KII is not phosphorylated or activated by CaM-KK.[12] However, the unique holoenzyme structure of CaM-KII endows it with unusual regulatory properties required for sensing and transducing various types of intracellular Ca^{2+} signals. Upon activation by Ca^{2+}/CaM binding, the kinase undergoes an immediate autophosphorylation on Thr286 (numbering based on the α isoform).[59] This autophosphorylation occurs within the oligomeric complex (i.e., intramolecular) but between adjacent subunits (intersubunit) that have bound Ca^{2+}/CaM.[63,64] This rapid autophosphorylation on Thr286 has two important regulatory consequences: (1) the subsequent dissociation rate for Ca^{2+}/CaM upon removal of Ca^{2+} is decreased by several orders of magnitude,[65] and (2) even after full dissociation of Ca^{2+}/CaM, the kinase retains partial activity (i.e., Ca^{2+}/CaM-independent or constitutive activity). Presumably the stacked hexameric ring structure of the CaM-KII holoenzyme restricts intersubunit autophosphorylation to within each ring.

Thus, transient elevation of intracellular Ca^{2+} can give a prolonged response through the constitutive activity of autophosphorylated CaM-KII, and this property appears to be critical for certain physiological functions of CaM-KII. This is especially pertinent in certain regions of the brain where the frequency of afferent pathway stimulation can alter the efficiency of transmission at glutamatergic synapses. This process, known as synaptic plasticity, is triggered by elevated postsynaptic $[Ca^{2+}]_i$. CaM-KII has been hypothesized as a decoding mechanism for synaptic frequencies[66] because of its unique activation properties as discussed above and its localization in dendritic spines in an organelle called the postsynaptic density (PSD).[67] The magnitude of constitutive CaM-KII activity, due to autophosphorylation of Thr286 on adjacent subunits in the oligomeric holoenzyme, should depend on the duration, amplitude, and frequency of elevated Ca^{2+} entering dendritic spines due to afferent stimulation. A recent in vitro study[68] shows that CaM-KII activation is regulated by these parameters. The abilities of CaM-KII to decode the frequency of synaptic stimulation and to give a prolonged readout beyond the initial stimulus are two characteristics required for a molecule involved in generation of synaptic plasticity.[69]

In many cells CaM-KII is largely soluble and widely distributed throughout the cell, but discrete subcellular pools of CaM-KII have recently been identified. Alternative splice varients of α, δ, and γ isoforms contain a nuclear localization signal (NLS),[70,71] and nuclear CaM-KII is likely to play a role in Ca^{2+} mediated gene transcription through phosphorylation of transcription factors such as CCAAT/enhancer-binding protein (C/EBP).[72,73] It is intriguing that CaM-kinases I and IV can phosphorylate a Ser adjacent to the NLS in CaM-KII and prevent its nuclear localization, but whether this occurs physiologically is uncertain.[74] Recent studies indicate that muscle CaM-KII may also be localized in muscle to the sarcoplasmic reticulum through a unique protein, αKAP. αKAP contains a hydrophobic N-terminus fused to the C-terminal association domain of CaM-KII.[75] The C-terminal association domain of αKAP can form heteromers with full-length CaM-KII subunit, and the hydrophobic N-terminus of αKAP directs the resulting kinase complex to the sarcoplasmic reticulum membrane.[76] Likely substrates for CaM-KII in the sarcoplasmic reticulum include the ryanodine receptor,[77] phospholamban,[78] and the Ca^{2+} ATPase pump.[79]

Brain is particularly abundant in CaM-KII, where it appears to be associated with specialized structures. For example, there is evidence for co-localization of the CaM-KII β isoform with the cytoskeleton. Upon stimulation of the Ca^{2+}-permeable NMDA-R ion channel in hippocampal neurons, CaM-KII dissociates from F-actin and undergoes translocation to membraneous fractions including the PSD.[80] The

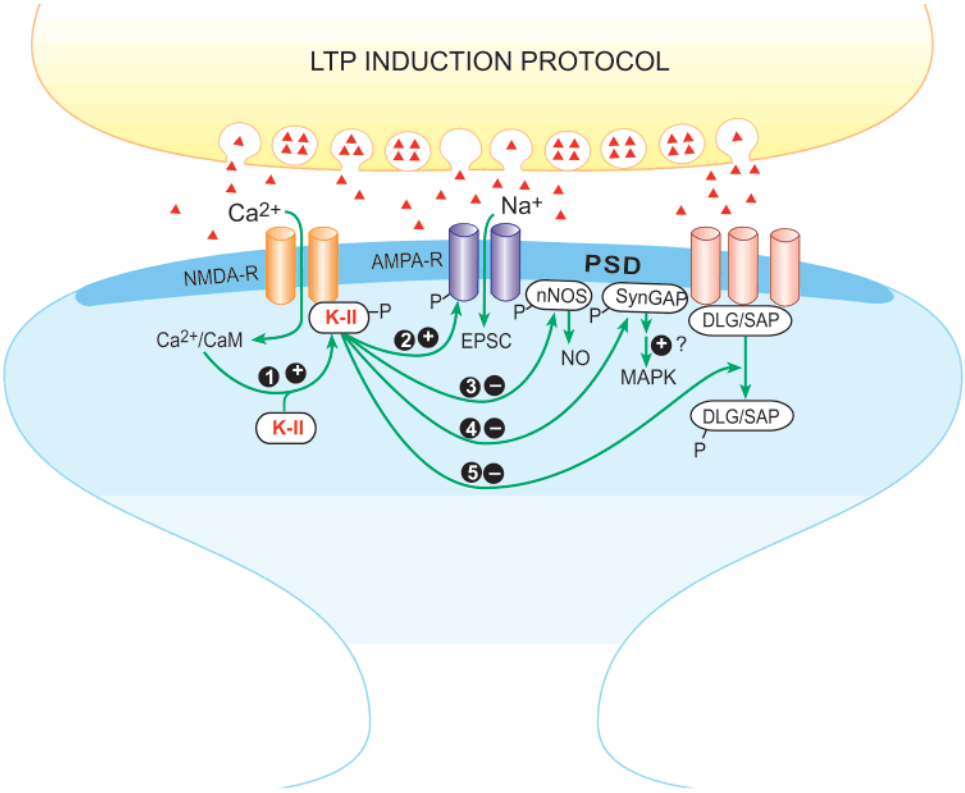


Figure 2. Neuronal synaptic substrates of CaM-KII. Activation of CaM-KII during LTP, due to Ca^{2+} influx through the NMDA-R, leads to autophosphorylation (reaction 1) and generation of constitutive activity. Constitutive CaM-KII can phosphorylate and potentiate AMPA-Rs (reaction 2) to strengthen the excitatory postsynaptic current (EPSP). Activated CaM-KII can also phosphorylate and inhibit nNOS (reaction 3) and SynGAP (reaction 4), resulting in possible activation of MAPK, and phosphorylate the scaffold protein DLG/SAP (reaction 5), causing disruption or remodeling of synaptic structures. See text for references. (Reprinted with permission from ref 86. Copyright 2000 Elsevier Science.)

PSD, a complex of postsynaptic membrane proteins involved in mediating and modulating synaptic transmission, is associated with the cytoskeleton through anchoring proteins.[67,81] CaM-KII is a major constituent of the PSD, where it is anchored in part through the protein densin-180.[82] This interaction of CaM-KII with densin-180 does not appear to depend on the activation state (i.e., Thr286 autophosphorylation) of the kinase. In contrast, additional CaM-KII can associate with the PSD through interaction with the NMDA-type glutamate-gated ion channel, but this translocation appears to require activation of the kinase and perhaps its autophosphorylation on Thr286.[83,84] Translocation to the PSD occurs in hippocampal slices upon treatments which activate CaM-KII, and it promotes the phosphorylation of CaM-KII substrates in the PSD such as the AMPA-R.[83,85] Translocation would localize CaM-KII at a critical site of Ca^{2+} influx into the dendritic spine since the NMDA-R ion channel has considerable Ca^{2+} permeability, and its activation is required for several types of synaptic plasticity. In addition to localizing CaM-KII to a site of Ca^{2+} influx, this translocation also situates the activated kinase in close proximity to several very important neuronal substrates.[86] It is intriguing that the stacked hexameric ring structure of CaM-KII may have implications for this subcellular targeting. For example, one of the hexameric rings may have a localization function through multisite interactions with PSD proteins, such as densin-180 and/or the NMDA-R, whereas the other hexameric ring may catalyze phosphorylation of neighboring substrates.

CaM-KII can phosphorylate a large number of proteins in vitro,[59] and recently several substrates that may be involved in synaptic plasticity have been identified (Figure 2).[86] One of the most intensely studied forms of synaptic plasticity is long-term potentiation (LTP) in region CA1 of hippocampus.[87,88] Brief high-frequency stimulation of the afferent pathway causes a long-lasting increase in the efficiency of transmission at these excitatory synapses that use glutamate as the neurotransmitter. The glutamate released by the afferent neuron into the synaptic cleft can bind to several specific transmembrane receptor proteins in the postsynaptic neuron. One of these receptors, the AMPA-type glutamate receptor (AMPA-R), is an ion channel that upon activation by binding of glutamate allows Na^+ influx to depolarize the postsynaptic neuron. LTP at these synapses is detected electrophysiologically as a postsynaptic depolarization due to increased Na^+ current through predominantly AMPA-Rs. AMPA-Rs are thought to be tetramers of various combinations of subunits GluR1-GluR4.

Numerous studies have documented that activated CaM-KII can phosphorylate the GluR1 subunit of the AMPA-R and enhance its current.[69] The AMPA-R is phosphorylated by CaM-KII during LTP in region CA1 of hippocampus.[89,90] Phosphorylation of Ser831 in GluR1 by CaM-KII potentiates AMPA-R current by increasing single-channel conductance.[91] Indeed,

about 60% of CA1 neurons that exhibit potentiation during LTP show an increase in unitary conductance.[92] These biochemical and electrophysiological results are supported by studies in transgenic mice. For example, adult mice lacking GluR1, the AMPA-R subunit phosphorylated by CaM-KII, show a specific deficit in LTP.[93] Likewise, a single-site mutation in αCaM-KII (Thr286Ala) results in a mouse which is deficient in CA1 LTP.[44] This subtle mutation does not effect activation of CaM-KII through binding of Ca^{2+}/CaM, but it precludes generation of constitutive activity by autophosphorylation. Thus, CaM-KII phosphorylation of AMPA-Rs with resultant potentiation of current is thought to contribute prominently to LTP at the CA1 synapse of hippocampus.[69,94]

Several other PSD proteins can also be phosphorylated by CaM-KII (Figure 2). Indeed, the NMDA-R which acts as an anchor for activated CaM-KII can be phosphorylated by CaM-KII.[95] Although phosphorylation of Ser1303 in the NR2B subunit has not been reported to directly regulate channel properties, it appears to decrease its binding affinity for CaM-KII.[96] Another substrate is a novel Ras-GTPase activating protein (SynGAP) localized at the PSD of hippocampal neurons through its interaction with the scaffold proteins PSD-95 and SAP102.[97,98] SynGAP is phosphorylated and potently inhibited by CaM-KII. This suggests that activation of the NMDA-R, which is also part of the PSD-95 complex, may result in activation of CaM-KII, which in turn phosphorylates and inhibits SynGAP, thereby potentiating activation of the MAP kinase pathway that appears to be important in some forms of synaptic plasticity. Regulation of MAP kinase through CaM-KII-mediated phosphorylation of SynGAP needs to be verified in intact neurons. Another CaM-KII substrate anchored to PSD-95 is neuronal nitric oxide synthase (nNOS). Phosphorylation of nNOS at Ser847 results in partial inhibition of its activity.[99] Nitric oxide, the product of NOS, appears to be an important signaling molecule in brain.[100] Thus, it is clear that numerous targets of CaM-KII are important in cellular regulation, and the recent discovery of a specific inhibitor protein for CaM-KII [101,102] should facilitate identification of additional substrates. This inhibitor protein, CaM-KIIN, acts noncompetitively with respect to substrates and bears no sequence similarity to the AID of CaM-KII. Peptides corresponding to the AID of CaM-KII can inhibit CaM-KII, but they act competitively with substrate and are not specific in that they also inhibit several other protein kinases.[103]

III. Restricted CaM-Kinases

A. CaM-Kinase III or Elongation Factor-2 Kinase

CaM-KIII was originally identified as a Ca^{2+}/CaM-dependent kinase that phosphorylated an unidentified protein of 100 kD.[104,105] This substrate was subsequently identified as elongation factor-2,[106] and this kinase is now referred to as elongation factor-2 kinase (eEF-2K) as eEF-2 is the only known substrate. eEF-2K is a monomeric 100 kD enzyme present in most mammalian tissues as well as in many invertebrates. It has been cloned from several mammalian species, and it shows very little homology to the highly conserved catalytic 12 subdomains of other mammalian Ser/Thr protein kinases.[107,108] However, eEF-2K does show higher homology to several *Dictyostelium* protein kinases, and it has been proposed that these novel Ser/Thr protein kinases represent a new family that recognizes α-helical structure in its substrates.[109] A recent mutagenesis analysis of functional domains in eEF-2K indicates an N-terminal catalytic core (residues 75−335) and a C-terminal domain that binds eEF-2 with a central linker region. The CaM-binding domain is between residues 51−96, probably comprised of a putative amphipathic α-helix of residues 81−94.[110]

eEF-2K can be regulated by both intramolecular autophosphorylation as well as phosphorylation by PKA.[111,112] Both of these phosphorylations generate Ca^{2+}-independent activity and therefore have the potential for prolonging a transient Ca^{2+} signal. Regulation by PKA appears to be physiologically relevant since treatments of various cell types with agonists which elevate cAMP produce Ca^{2+}-independent eEF-2K activity and subsequent phosphorylation of it substrate eEF-2.[113,114] There also appear to be cellular mechanisms for regulating the protein levels of eEF-2K. Treatment of PC12 cells with NGF results in a slow (max. effect at 12 h) loss of eEF-2K protein. Phosphorylation by eEF-2K of Thr56 and Thr58 in eEF-2 inhibits the function of eEF-2. eEF-2 mediates the translocation step in peptide chain elongation and is therefore intimately involved in protein synthesis. Thus, eEF-2K is thought to be an important negative regulatory of protein synthesis by agonists that elevate Ca^{2+} and/or cAMP.[115]

B. Myosin Light Chain Kinases

Myosin light chain kinase (MLCK) is a Ca^{2+}/CaM-dependent protein kinase that phosphorylates a specific serine at the N-terminus of the regulatory light chain (RLC) of myosin II.[116,117] This is the only identified physiological substrate, so it is a dedicated protein kinase in contrast to the other multifunctional CaM-dependent protein kinases. RLC phosphorylation initiates contractions in smooth muscle due to its stimulatory activity on the actin-activated myosin MgATPase activity.[118,119] In contrast, RLC phosphorylation potentiates contraction in striated muscles by increasing the formation of force-bearing cross-bridges.[120] Although MLCKs are dedicated kinases, they participate in many cellular activities due to the diverse roles of its substrate, myosin II.[117]

There are two genes for MLCK in vertebrates.[121] The skeletal muscle MLCK gene encodes a kinase catalytic core and regulatory segment containing autoinhibitory and CaM-binding domains (AID and CBD) that are distinct from the smooth muscle MLCK (Figure 1). However, the smooth muscle kinase gene expresses a short and long MLCK in a cell-specific manner due to alternate promoters.[122,123] The short MLCK is expressed in differentiated smooth muscle tissues and some nonmuscle cells, whereas long MLCK is found in some nonmuscle cells and injured vascular smooth muscle cells.[117] Both forms of the enzyme have additional structural motifs

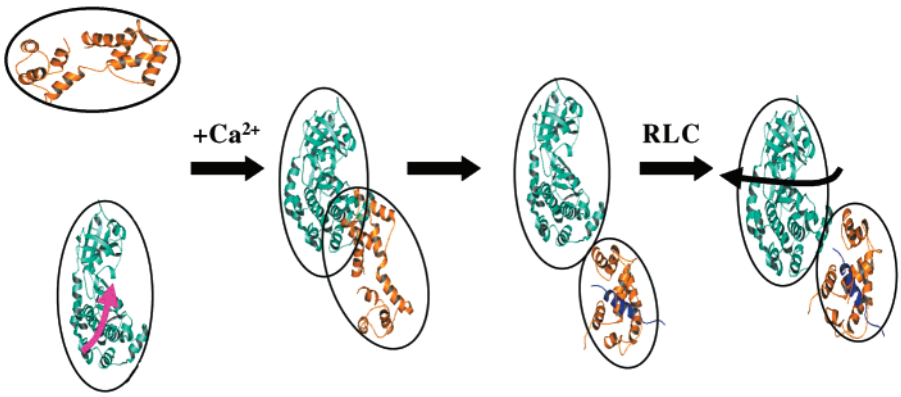


Figure 3. Model for CaM activation of MLCK. CaM and the catalytic core of MLCK are ellipsoid molecules with similar maximal dimensions. The AID of the kinase (arrow) converges to the catalytic cleft to inhibit RLC, but not ATP, binding. When Ca^{2+} binds to CaM, the protein initially binds with its C-domain to the CBD of MLCK. Calmodulin then collapses and translocates the regulatory segment, exposing the catalytic cleft. Binding of substrate (arrow) results in closure of the cleft and reorientation of calmodulin relative to the catalytic core. Calmodulin structures are shown in gold, modeled structures of kinase core are in green, and the MLCK calmodulin-binding peptide is in blue. The ellipsoids represent the structures of MLCK and calmodulin obtained from X-ray and neutron scattering experiments. See text for references.

not found in skeletal muscle MLCK (Figure 1). These include Ig and Fn modules as well as a PEVK repeat-rich region; the functional roles of these structural modules in MLCK is not clear. Both smooth muscle MLCKs contain an actin-binding sequence consisting of repeat motifs (DFRXXL), each of which may bind a single actin monomer in an actin filament.[124,125] The N-terminal extension of long MLCK may be responsible for an increased affinity for actin-containing filaments[126] and localization to the cleavage furrow of dividing cells.[127] Additionally, the N-terminus of long MLCK contains a SH2-binding domain and a consensus tyrosine phosphorylation site for Src which may also provide dynamic localization signals in cells.[128,129]

An AID folds back on the surface of the large domain of MLCK catalytic core similar to other CaM-dependent protein kinases and prevents RLC but not ATP binding in the catalytic cleft (Figure 3). Protein fragmentation complementation analyses show that the primary autoinhibitory motif is contained within the sequence between the catalytic core and the CaM-binding sequence,[130] consistent with previous results obtained with truncation mutants.[131] In smooth muscle MLCK residues C-terminal to the CaM-binding sequence, including the Ig module, do not appear to be functional components of the regulatory segment.[130]

When Ca^{2+}/CaM binds to a synthetic peptide based on the CBD of MLCK, it undergoes a conformational collapse with its two domains encompassing the peptide by bending of a flexible interconnecting helix.[132−134] This complex results in the peptide forming a single α-helix in an antiparallel orientation, i.e., the C-terminal domain of CaM binds to the N-terminus of the peptide and vice versa. Two hydrophobic residues in the peptide separated by 12 intervening residues bind to two hydrophobic clefts in the respective CaM domains. One side of the formed tunnel contains a hydrophobic surface that interacts with the hydrophobic surface of the α-helical peptide.

The two domains of CaM each are effectively independent structures and capable of activating target enzymes, including MLCK.[135] However, when both domains are present, there is a greater extent of activation than either domain alone, indicating an ideal spatial arrangement of the two CaM domains for activation. MLCK activation involves an ordered sequence of binding by Ca^{2+}/CaM (Figure 3) with an average K_{CaM} value of 1 nM.[136−139] The C-terminal domain of CaM binds to the N-terminus of the CBD with the subsequent binding of the N-terminal domain of CaM. CaM thus initially binds near the catalytic cleft of MLCK in an elongated form.[140] The extended CaM (68 Å maximum linear dimension) then collapses to 49 Å with a size and shape on MLCK similar to that reported for Ca^{2+}/CaM in complex with peptides containing the CBD of MLCKs.[141] Ca^{2+}/CaM binds at a position distant from the equatorial plane that is perpendicular to the long axis of MLCK, which has a maximal linear dimension of 78 Å for its catalytic domain (Figure 3). Thus, Ca^{2+}/CaM bound to the kinase appears to result in a substantial movement of the regulatory segment away from the catalytic cleft.

In addition to binding to the CBD in the regulatory segment, ensuing CaM interactions with the catalytic core per se may be necessary for activation.[142−144] Although CaM binding and activation processes are basically similar for both smooth and skeletal muscle MLCKs, selective mutated CaMs have abilities to activate one or the other kinase, indicating significant distinct differences in target-specific interactions.[5,144] Subsequent binding of myosin RLC induces additional conformational changes resulting in closure of the cleft and reorientation of CaM with transfer of phosphate from ATP to RLC (Figure 3).[145,146]

The smaller N-terminal domain of the catalytic core of MLCK binds MgATP under a glycine-rich flap in the presence or absence of Ca^{2+}/CaM, indicating that the ATP-binding pocket is exposed and competent during autoinhibition;[147] however, the protein substrate, RLC, does not bind.[148] The orientation of the N-terminus of RLC in the cleft may be similar to the orientation of the substrate recognition fragment of the inhibitor peptide of cAMP-dependent protein kinase.[148] Similar to the catalytic subunit of the

cAMP-dependent protein kinase, there are two acidic residues in the catalytic cleft of MLCK that appear to bind basic residues of the AID in the regulatory segment.[121] When the AID is displaced by CaM binding, arginine at the P-3 position (three residues N-terminal of the phosphorylatable serine 19) binds to the two glutamate residues in the cleft which are close to the catalytic aspartate. Additionally, hydrophobic residues immediately C-terminal of serine 19 (p+1, p+2, and p+3) contribute to substrate recognition, but additional residues in the N-terminal half of RLC are also important for substrate recognition.[149]

The activity of smooth muscle MLCK can be modulated by phosphorylation at specific sites that lead to increased K_{CaM} or V_{max} values; however, both nonphosphorylated and phosphorylated MLCKs are tightly regulated by Ca^{2+}/CaM binding, and no evidence exists for physiologically relevant Ca^{2+}/CaM-independent activity. The most well-documented effect of MLCK phosphorylation is a 10-fold increase in K_{CaM} that occurs upon phosphorylation of one of two serine residues in the C-terminus of the CaM-binding sequence (Figure 3). Protein kinases that phosphorylate this site in vitro include PKA,[150] PKC,[151] CaM-KII,[152] and PAK.[153] Phosphorylation of MLCK at this site by CaM-KII in smooth muscle tissues and cells in culture is initiated in a Ca^{2+}-dependent manner during smooth muscle contractions, resulting in a decrease in the sensitivity of RLC phosphorylation to $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ thus may act in two ways to regulate Ca^{2+}-dependent RLC phosphorylation. As smooth muscle contracts, it acts positively to increase RLC phosphorylation by activating MLCK via Ca^{2+}/CaM binding. At greater concentrations, $[Ca^{2+}]_i$ acts negatively on RLC phosphorylation by stimulating CaM KII phosphorylation of MLCK. Interestingly, cAMP-dependent protein kinase does not appear to significantly phosphorylate this site in smooth muscle tissues,[154] although it may in nonmuscle cells.[155] Although PKC phosphorylates a serine residue in the C-terminus of the CBD in vitro, stimulation of PKC activity with phorbol esters in smooth muscle tissues in vivo does not result in its phosphorylation.[154] MLCK phosphorylation by PAK is associated with decreased MLCK activity, inhibition of RLC phosphorylation, and inhibition of cell spreading or contraction. Results from studies on phosphorylation of short smooth muscle MLCK would also be applicable to the long MLCK, although direct studies are yet to be performed. The long MLCK has a Src tyrosine phosphorylation site in the extended N-terminus that may also be important in regulation of the kinase.

MLCK also contains several phosphorylation consensus sites for proline-directed protein kinases. Phosphorylation of two sites external to the catalytic core and regulatory segment in vitro by members of the MAPK family increase V_{max} with no change in the K_{CaM} value.[156,157] Treatment of MCF-7 cells with uPA, an activator of the MAPK pathway, stimulates cell migration, phosphorylation of MLCK, and myosin RLC, all of which were inhibited by a MEK inhibitor.[158] These results are consistent with a MAPK-stimulation of MLCK activity by phosphorylation with resultant increases in RLC phosphorylation and cell migration. The potential importance of many of these phosphorylation sites on short and long smooth muscle MLCK and regulation of diverse cellular functions has yet to be fully explored.

IV. Summary

The CaM-K family represents a diverse group of protein kinases that regulate a variety of cellular functions in response to elevated intracellular Ca^{2+}. These cellular functions include muscle contraction, ion channel permeability, gene transcription, and protein synthesis. Although these CaM-Ks have conserved overall domain organizations (Figure 1) and are activated by binding of Ca^{2+}/CaM, they each exhibit novel structural and regulatory properties essential for their unique physiological functions. A crystal structure for a portion of CaM-KI is known, and it will be interesting to see whether key regulatory interactions are conserved in the other CaM-Ks or whether there is divergence. In addition to being regulated by intracellular Ca^{2+}, many of the CaM-Ks are also activated or inhibited through phosphorylation by other protein kinases. This cross-talk with other signaling pathways allows integration of Ca^{2+} signals with other signaling pathways to modulate the physiological output of the cell.

V. Abbreviations

AID	autoinhibitory domain
αKAP	αCaM-KII-associated protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
$[Ca^{2+}]_i$	intracellular free calcium concentration
CaM	calmodulin
CaM-K	calmodulin-dependent protein kinase
CBD	calmodulin-binding domain
CREB	cAMP-responsive element binding protein
eEF-2K	eukaryotic elongation factor-2 kinase
GluR1	subunit of AMPA receptor
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MLCK	myosin light chain kinase
nNOS	neuronal nitric oxide synthase
NMDA	*N*-methyl-D-aspartate
PDZ	PSD-95/DLG/ZO-1
PKA	cAMP-dependent protein kinase
PKB	protein kinase B
PSD	postsynaptic density
RLC	regulatory light chain
synGAP	synaptic Ras-GTPase-activating protein

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