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# The Interaction between PSD-95 and $Ca^{2+}$/Calmodulin Is Enhanced by PDZ-Binding Proteins

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**In this study, we evaluate the interaction between the postsynaptic scaffolding protein, PSD-95, and calmodulin. Surface plasmon resonance spectroscopy was used to characterize the binding of PSD-95 to calmodulin that had been immobilized on a sensor chip. Additionally, soluble calmodulin was found to inhibit the binding of PSD-95 to immobilized calmodulin. The HOOK region of PSD-95, which is located between the *src* homology 3 domain and the guanylate kinase–like domain, was determined to be involved in the binding of PSD-95 to calmodulin. We also found that C-terminal peptides from proteins such as CRIPT and the *N*-methyl-D-aspartate receptor NR2B subunit, which associate with the PDZ domain of PSD-95, enhanced the affinity of PSD-95 for calmodulin. The binding of ligands to the PDZ domain may change the conformation of PSD-95 and affect the interaction between PSD-95 and calmodulin.**

**Key words: calmodulin, MAGUK, PDZ domain, PSD-95, surface plasmon resonance spectroscopy.**

Abbreviations: APC, adenomatous polyposis coli; Kv1.4, Shaker voltage-gated $K^+$ channel; GK, guanylate kinase; MAGUK, membrane-associated guanylate kinase–like domain; MAP1A, microtubule-associated protein 1A; NMDA, *N*-methyl-D-aspartate; SH3, *src* homology 3; SPR, surface plasmon resonance.

PSD-95 is a member of the membrane-associated guanylate kinase (MAGUK) family, the members of which consist of multiple protein–protein interaction motifs, including one or more copies of a PDZ domain, an *src* homology 3 (SH3) domain, a bridging region known as a HOOK region, and a guanylate kinase (GK)–like domain (*1–4*). PSD-95 associates with *N*-methyl-D-aspartate (NMDA) receptors and downstream signaling molecules at the postsynaptic density (*5–8*), suggesting that PSD-95 contributes to specific signaling pathways coupled to NMDA receptors. $Ca^{2+}$ influx through NMDA receptors induces various intracellular phenomena, including calmodulin activation. $Ca^{2+}$/calmodulin activates several intracellular molecules, such as $Ca^{2+}$/calmodulin-dependent protein kinase II and neuronal nitric oxide synthase (*9*). Several recent reports have described how certain MAGUK proteins associate with calmodulin in the presence of $Ca^{2+}$ (*10*, *11*). SAP102 binds calmodulin, and this interaction is involved in the formation of the SAP102/calmodulin/PSD-95 complex (*10*). Calmodulin also binds SAP97 and regulates the intramolecular interactions between the SH3, HOOK, and GK domains of SAP97 (*11*). On the other hand, no interaction between PSD-95 and calmodulin has been identified (*3*, *4*). Tavares *et al.* reported that calmodulin fails to bind the SH3-HOOK-GK fragment of PSD-95 (*3*). An alignment of the putative calmodulin-binding regions of PSD-95, SAP97, and SAP102 reveals some similarities; however, not all the putative recognition residues of PSD-95 share homology with SAP97 and SAP102 (*10*, *11*), indicating that PSD-95 may bind calmodulin with low affinity. As a result, traditional techniques may not be sufficient to detect these interactions. We therefore used surface plasmon resonance (SPR) spectroscopy to evaluate the characteristics of the PSD-95/calmodulin interaction. SPR can detect interactions with rapid kinetics that can be difficult to detect by traditional techniques. We also examined the possibility that the binding partners for the PDZ domain of PSD-95 promote its interaction with calmodulin.

## EXPERIMENTAL PROCEDURES

*Protein Purification*—Recombinant human calmodulin expressed in *Escherichia coli* was purified to homogeneity using phenyl Sepharose chromatographic columns, as described previously (*12*).

A full-length murine PSD-95 clone was a generous gift from Prof. Y. Fujiyoshi (Kyoto University). PSD-95 fragments were subcloned upstream of the His-tag of the pET-22b(+) expression plasmid (Novagen). This PSD-95 fusion protein was expressed in *Escherichia coli*, purified on a ProBond column (Invitrogen), and eluted in buffer containing imidazole. The obtained PSD-95 protein was then dialyzed against the purification buffer before it was loaded onto a gel-filtration column (HiLoad 26/60 Superdex 200 pg; Amersham Biosciences).

*BIAcore Analysis*—Using a BIAcore 3000 SPR system, the binding of an analyte to the immobilized ligand was measured in arbitrary units (resonance unit; RU). Purified proteins were immobilized on CM5 sensor chips to final resonance values of ~1,000 RU for calmodulin. Various concentrations of analytes in HBS-P (10 mM HEPES, 150 mM NaCl, 0.005% surfactant P20, pH 7.4) containing 5 mM $CaCl_2$ or 5 mM EGTA were injected for

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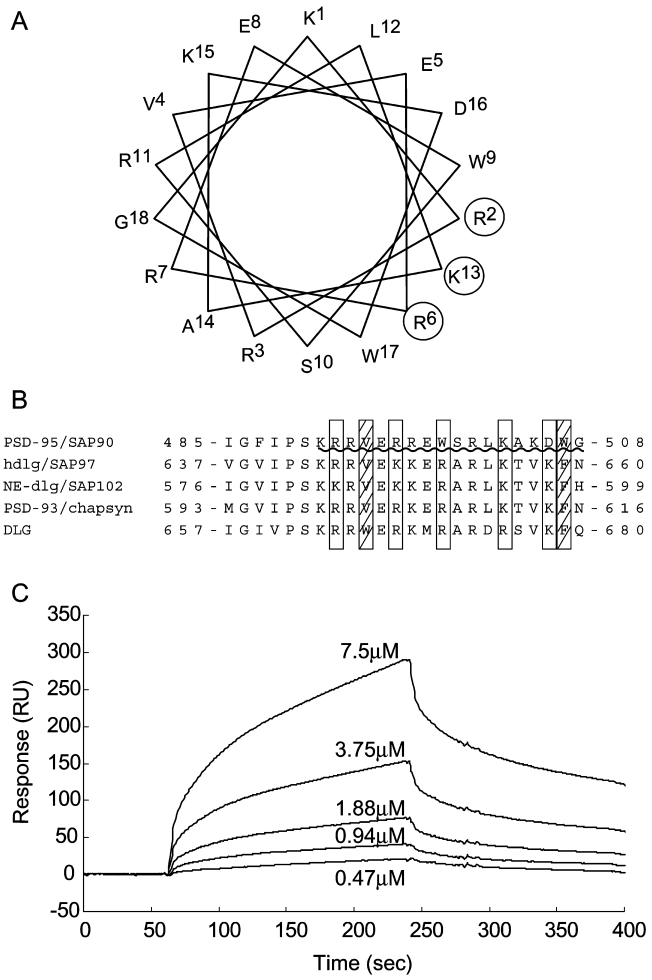


Fig. 1. **Analysis of the putative calmodulin-binding region of MAGUK family proteins.** (A) A helical wheel representation of the putative calmodulin-binding region of PSD-95. The basic residues that cluster on one side of the helical wheel are circled. (B) An amino-acid sequence alignment of the putative calmodulin-binding domains of MAGUK family proteins. The basic residues of the MAGUK family proteins that are predicted to cluster at one side of the α-helix are boxed. The hydrophobic amino acids of the 1–14 motif for calmodulin-binding are boxed in dark gray. The wavy line underscores the amino-acid sequence of the synthetic HOOK peptide. (C) Calmodulin was immobilized on a CM5 sensor chip to investigate the interaction of calmodulin with the HOOK peptide. The HOOK peptide was injected at 0.47, 0.94, 1.88, 3.75 and 7.5 μM for 3 min, at a flow rate of 15 μl/min.

2 or 3 min (20 or 15 μl/min, respectively). 2-Mercaptoethanol was added to the running buffer at 0.1% when PSD-95 was an analyte. Each series of experiments was performed in duplicate. The sensor chips were regenerated by a 10-s injection of 50 mM NaOH. The sensorgrams were analyzed using BIAevaluation software (version 3.1; BIAcore). All of the binding curves were collected by subtraction of reference flow cells. Large spikes at the beginning and the end of an injection are due to a difference in the refractive indices of the buffer alone and the buffer containing an analyte protein. The model used was the 1:1 Langmuir binding interaction model. The association rate constant, ka, and the dissociation rate constant, $k_d$, were determined by global fitting of the curves. The dissociation constant was determined by $K_D = k_d/k_a$. The mean values of the rate constants and the dissociation constants were obtained from duplicate experiments for each concentration.

Table 1. **Rate constants and dissociation constants of PSD-95 for calmodulin in the presence and absence of C-terminal peptides.**

| C-terminal peptides | $k_a$ ($M^{-1}$ $s^{-1}$) | $k_d$ ($s^{-1}$) | $K_D$ (M) |
|---|---|---|---|
| none | $7.10 \times 10^2$ | $3.94 \times 10^{-3}$ | $5.55 \times 10^{-6}$ |
| CRIPT (400 μM) | $1.38 \times 10^3$ | $3.93 \times 10^{-3}$ | $2.85 \times 10^{-6}$ |
| NR2B (800 μM) | $1.62 \times 10^3$ | $3.95 \times 10^{-3}$ | $2.43 \times 10^{-6}$ |

## RESULTS AND DISCUSSION

*Putative Calmodulin-Binding Region of PSD-95*—The HOOK region between the SH3 and GK domains of MAGUK proteins is reportedly composed of a basic amphiphilic α-helix. Charged and hydrophobic residues reside on opposite sides of the helix (*10*, *11*), creating an ideal calmodulin-binding domain. A helical wheel representation of the HOOK region of PSD-95 showed a cluster of basic amino acids on one side of this helix (Fig. 1A). The number of basic residues that form the cluster in PSD-95, however, was smaller than those in SAP97 and SAP102. In addition, hydrophobic residues were not clustered on the helical wheel. Based on the conserved positions of the hydrophobic residues, PSD-95 has a 1-14 motif, which is one of the characteristic recognition motifs for calmodulin (Fig. 1B). To evaluate the interaction between PSD-95 and calmodulin, we synthesized a peptide corresponding to amino-acid residues 491–508 of PSD-95 (HOOK peptide), which form the α-helix (*3*, *4*). To monitor the binding of this peptide to calmodulin, we performed SPR with calmodulin that had been immobilized on the surface of a CM5 sensor chip. In the SPR analyses, the HOOK peptide bound to the immobilized calmodulin in a dose-dependent manner (Fig. 1C). The $K_D$ value between the HOOK peptide and calmodulin was calculated to be 17.4 μM ($k_a = 7.15 \times 10^2$ $M^{-1}$ $s^{-1}$, $k_d = 1.24 \times 10^{-2}$ $s^{-1}$). This relatively low affinity might be explained by the smaller cluster of basic amino acids in PSD-95 than in other MAGUK proteins, or by the scattered hydrophobic residues in this protein (Fig. 1B).

*Interaction between PSD-95 and Calmodulin*—We analyzed the binding of full-length PSD-95 to calmodulin that had been immobilized on the surface of a CM5 sensor chip. PSD-95 bound to the immobilized calmodulin in a dose-dependent manner (Fig. 2A). The calculated $K_D$ value for full-length PSD-95 and calmodulin was 5.55 μM (Table 1). The affinity of PSD-95 for calmodulin is lower than that of other MAGUK proteins, such as SAP97 and SAP102 (*10*, *11*). A key question is whether this interaction occurs *in vivo*. The concentration of calmodulin in the soluble fraction of neural tissues is about 20 μM (*13*). The $K_D$ value determined here is therefore not sufficient to produce a physiological interaction between PSD-95 and calmodulin. The local concentration of endogenous calmodulin near L-type calcium channel $\alpha_{1C}$ subunits in HEK293 cells, however, was estimated to be about 2.5 mM (*14*), despite the low concentration of free $Ca^{2+}$/calmodulin in these cells (45 nM) (*15*). Several lines of

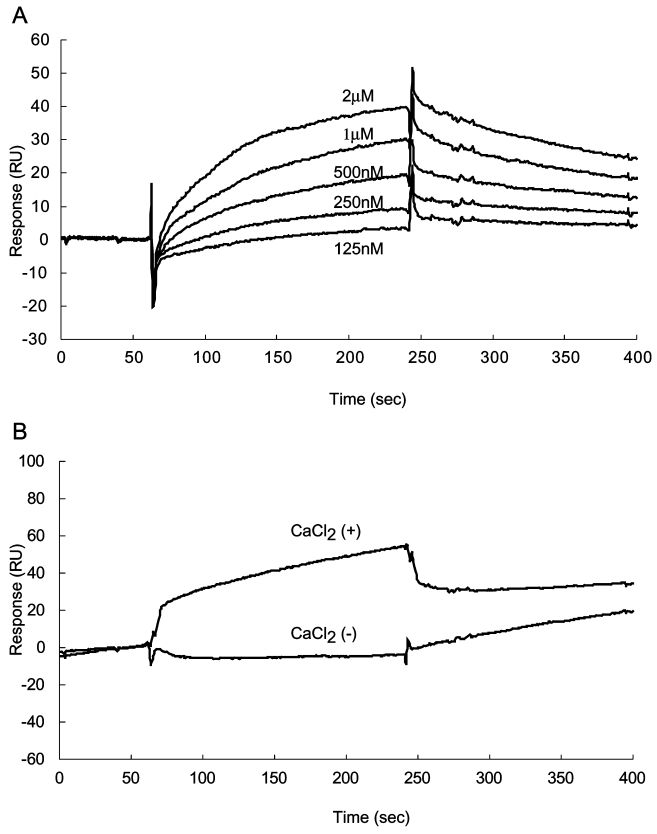


Fig. 2. **Interaction between full-length PSD-95 and calmodulin.** (A) PSD-95 (125 nM, 250 nM, 500 nM, 1 μM and 2 μM) was injected over calmodulin that had been immobilized on a sensor chip for 3 min at a flow rate of 15 μl/min. (B) The calcium dependence of the interaction between PSD-95 and calmodulin was determined. PSD-95 was injected over immobilized calmodulin. To remove $Ca^{2+}$, 5 mM EGTA replaced the 5 mM $CaCl_2$ in the running buffer.

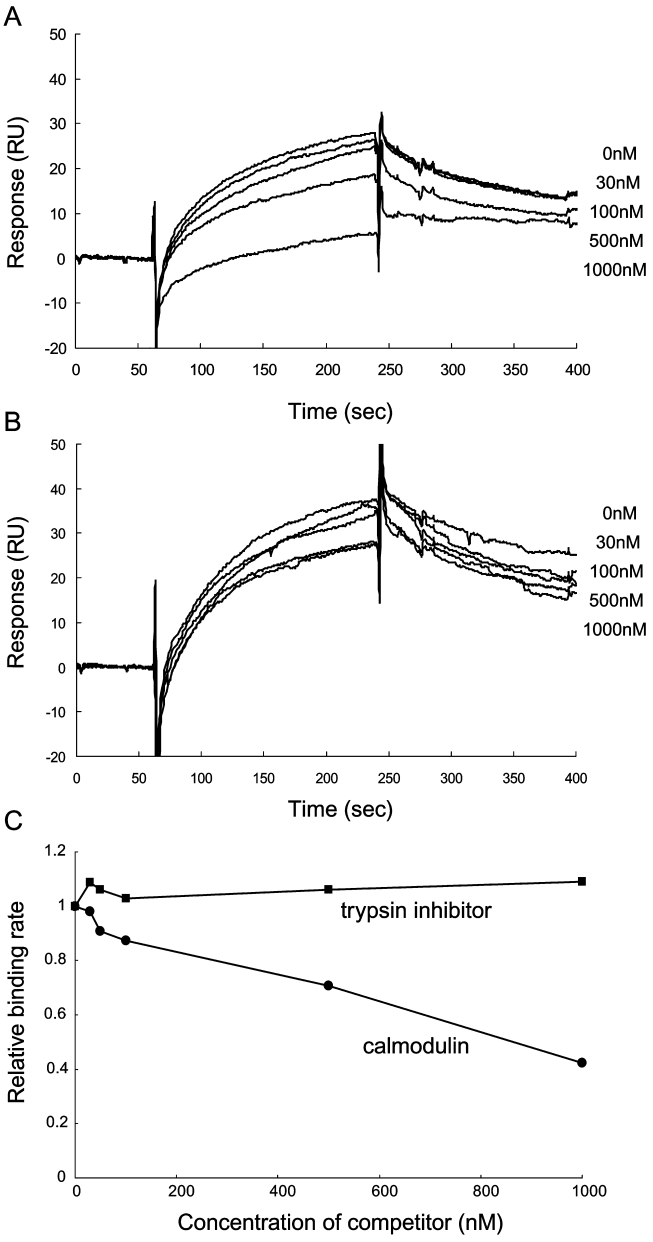


Fig. 3. **Specific binding of PSD-95 to calmodulin.** (A) PSD-95 was coinjected with soluble calmodulin (0 to 1 μM) over calmodulin that had been immobilized on a CM5 sensor chip for 3 min at a flow rate of 15 μl/min. (B) PSD-95 was coinjected with soluble soybean trypsin inhibitor (0 to 1 μM) over calmodulin that had been immobilized on a CM5 sensor chip for 3 min at a flow rate of 15 μl/min. (C) The binding rates were determined between 72 and 105 s of the sensorgrams in (A) and (B). All binding rates were normalized to the binding rate observed in the absence of competitors (relative binding rate).

evidence suggest that the concentration of calmodulin near PSD-95 may be greater than that of cytoplasmic calmodulin. First, immunohistochemistry has shown that a subset of $\alpha_{1C}$ subunit clusters colocalize with PSD-95 (*16*). Second, L-type calcium channel $\alpha_{1D}$ subunits bind Shank (*17*), which interacts with PSD-95 *via* GKAP (*18*). Third, a calcium microdomain was suggested to be present near NMDA receptors, which are known to bind PSD-95 (*19*). Consequently, the concentration of calmodulin near PSD-95 may be higher than in the rest of the cytoplasm. It seems likely that a high local calmodulin concentration enables a physiological interaction between PSD-95 and calmodulin, even though the affinity of PSD-95 for calmodulin measured *in vitro* is low. The affinity of full-length PSD-95 for calmodulin was higher than that of the HOOK peptide, indicating that the structure around the HOOK region might be essential for calmodulin recognition. Another possibility is the existence of additional calmodulin-binding sites outside of the HOOK region of PSD-95. A competition assay would allow us to examine this possibility. However, because the HOOK peptide bound to both PSD-95 (data not shown) and calmodulin, it was impossible to perform a competition assay using the SPR technique. The interaction between these proteins was observed in buffer containing 5 mM $CaCl_2$. This binding was abolished when $Ca^{2+}$ was removed with 5 mM EGTA (Fig. 2B), indicating that this interaction is dependent on $Ca^{2+}$. Further studies are needed to determine whether an increase in intracellular $Ca^{2+}$ during neuronal stimulation results in calmodulin binding to PSD-95.

To demonstrate the specific binding of PSD-95 to calmodulin, a competition assay was employed (Fig. 3). Soluble calmodulin at concentrations of 500–1,000 nM

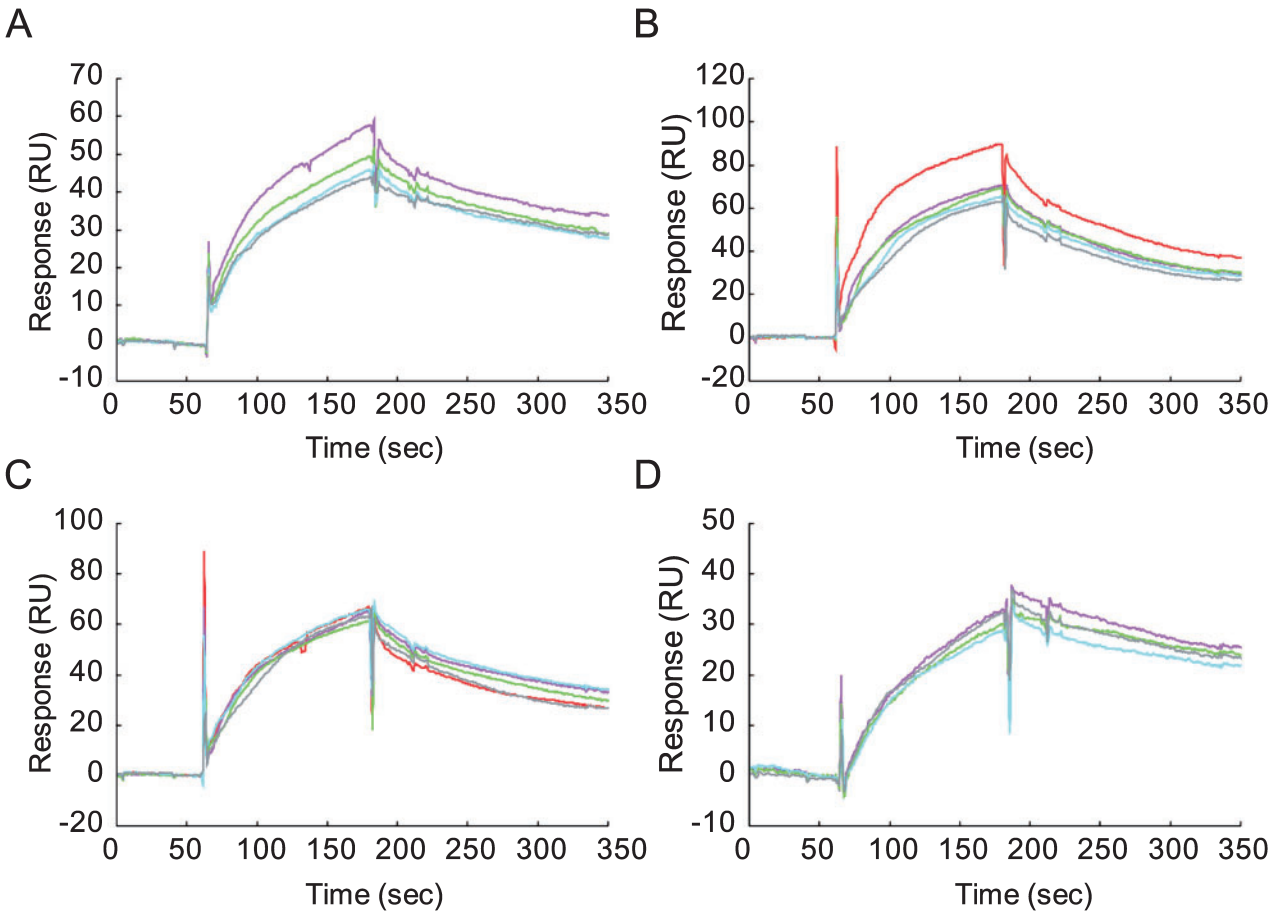


Fig. 4. **The effects of C-terminal peptides containing (S/T)X(V/I) motifs on the binding of calmodulin to PSD-95.** PSD-95 alone (gray line) or in combination with various concentrations of C-terminal peptides that contained a (S/T)X(V/I) motif (blue line, 100 μM; green line, 200 μM; purple line, 400 μM; red line, 800 μM ) was injected over calmodulin that had been immobilized on a CM5 sensor chip for 2 min at a flow rate of 20 μl/min. (A) CRIPT peptide (0, 100, 200 and 400 μM); (B) NR2B peptide (0, 100, 200, 400 and 800 μM); (C) Kv1.4 peptide (0, 100, 200, 400 and 800 μM); (D) APC peptide(0, 100, 200 and 400 μM).

competed with calmodulin that had been immobilized on a CM5 sensor chip for the interaction with PSD-95 (Fig. 3A). The apparent $EC_{50}$ value was approximately 800 nM (Fig. 3C). On the other hand, 30–1,000 nM soybean trypsin inhibitor, whose molecular weight and acidity are similar to those of calmodulin, did not affect the interaction between PSD-95 and calmodulin (Fig. 3, B and C). These results indicate that PSD-95 specifically associates with calmodulin.

Because the affinity of full-length PSD-95 for calmodulin was low, we were not able to detect this interaction with an immunoprecipitation procedure (data not shown). Our data are consistent with the observation reported by Paarmann *et al.* (*11*), which suggested that the affinity of calmodulin for PSD-95 is lower than its affinity for SAP97.

*The (S/T)X(V/I) Motif Modulates the PSD-95/Calmodulin Interaction*—At excitatory synapses, glutamate receptors, associated signaling proteins, and cytoskeletal proteins accumulate in the postsynaptic density and form a network of protein-protein interactions. Reportedly, calmodulin does not modify the interaction between SAP102 and the C terminus of the NMDA receptor NR2B subunit (*10*). In this study, we tried to identify proteins in the postsynaptic density that affected the interaction between PSD-95 and calmodulin. MAP1A has been reported to bind isolated GK domains of MAGUK proteins, but not full-length MAGUKs (*20*). The concomitant binding of PDZ-binding partners to MAGUK proteins enhanced the association of MAP1A and full-length MAGUK proteins (*20*). Therefore, we predicted that PDZ-binding partners would facilitate the binding of MAGUK proteins to SH3-GK domain binding proteins.

The C-terminal regions of several proteins that contain a (S/T)X(V/I) motif, including adenomatous polyposis coli (APC), CRIPT, Shaker voltage-gated $K^+$ channel (Kv1.4), and NR2B subunit, have been shown to associate with the PDZ domain of PSD-95 (*21–24*). We determined whether or not the interactions between peptides representing the C termini of these proteins and the PDZ domain of PSD-95 affected the binding of calmodulin to the HOOK region between the SH3 and GK domains of PSD-95. Peptides corresponding to the tails of APC, CRIPT, Kv1.4 and NR2B were combined with PSD-95 and applied to CM5 sensor chips on which calmodulin had been immobilized. Because a sample containing 800 μM of the CRIPT peptide produced a direct interaction with calmodulin (data not shown), the effect of 800 μM of

the CRIPT peptide was not determined. Coinjection of the C-terminal peptides of CRIPT or NR2B with PSD-95 resulted in a dose-dependent shift from the control sensorgram (no peptide) (Fig. 4, A and B). Kinetic analyses revealed that 400 μM CRIPT peptide and 800 μM NR2B peptide enhanced the interaction between PSD-95 and calmodulin (Table 1). The association rate constant, but not the dissociation rate constant, approximately doubled in the presence of the peptides (Table 1), indicating that the C-terminal peptides of CRIPT and NR2B facilitate the association of PSD-95 with calmodulin, but do not inhibit their dissociation. The C-terminal peptides of Kv1.4 and APC did not cause a dose-dependent shift of the sensorgram for the interaction between PSD-95 and calmodulin (Fig. 4, C and D), while the interaction between PSD-95 and MAP1A is enhanced by not only the C-terminal peptides of CRIPT and NR2B but also the C-terminal peptides of Kv1.4 and APC (*20*). MAP1A binds to the GK domain (*20*), whereas calmodulin binds to the HOOK region between the SH3 and GK domains of PSD-95. These different binding sites might account for the differences in PDZ-binding ligands to enhance their interactions.

Our results suggest that due to the intramolecular interactions in full-length PSD-95, some SH3-GK–binding ligands may not be able to approach these binding sites, and that PDZ-binding may disrupt the intramolecular interactions, thereby exposing the SH3-GK binding region. There are a number of ligands in the postsynaptic density for the PDZ domains of MAGUK proteins. Therefore, MAGUK proteins that are localized within the postsynaptic density would associate with these PDZ-binding proteins, thereby allowing calmodulin or MAP1A to approach their binding sites. Because calmodulin is reportedly involved in the heteromeric assembly of SAP102 and PSD-95 (*10*), the association of calmodulin with PSD-95 within neurons may play a role in switching the MAGUK proteins from intramolecular interactions to intermolecular interactions, which could induce a reorganization of postsynaptic density proteins. PSD-95 has lower affinity for calmodulin than other MAGUK proteins, indicating that higher concentration of $Ca^{2+}$/calmodulin is needed for binding between PSD-95 and calmodulin. The biological significance of this weak but novel interaction still remains to be determined, because no MAGUK protein which binds to calmodulin with low affinity, like PSD-95, has been reported yet. However, our results show that both low and high affinities for calmodulin are mixed together in the postsynaptic density, and this may be reflected in a variety of synaptic responses. Members of the MAGUK proteins, which bind calmodulin, may change in proportion to the concentration of $Ca^{2+}$/calmodulin; moderate and higher concentration of $Ca^{2+}$/calmodulin may promote homodimerization of SAP102 and heteromultimerization of PSD-95 and SAP102, respectively. Although the amino acid sequence is conserved between PSD-95 and SAP102, with 65% homology, several lines of evidence suggest MAGUK proteins have some different binding partners (*6*, *25–28*). These findings suggest that MAGUK proteins have individual roles in synaptic functions, including postsynaptic localization of membrane proteins and forming a functional protein complex. Further studies are needed to determine the specific role of the interaction between each MAGUK protein and calmodulin in postsynaptic multi-function.

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