

The neuronal connexin36 interacts with and is phosphorylated by CaMKII in a way similar to CaMKII interaction with glutamate receptors

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Electrical synapses can undergo activity-dependent plasticity. The calcium/calmodulin-dependent kinase II (CaMKII) appears to play a critical role in this phenomenon, but the underlying mechanisms of how CaMKII affects the neuronal gap junction protein connexin36 (Cx36) are unknown. Here we demonstrate effective binding of ³⁵S-labeled CaMKII to 2 juxtamembrane cytoplasmic domains of Cx36 and in vitro phosphorylation of this protein by the kinase. Both domains reveal striking similarities with segments of the regulatory subunit of CaMKII, which include the pseudosubstrate and pseudotarget sites of the kinase. Similar to the NR2B subunit of the NMDA receptor both Cx36 binding sites exhibit phosphorylation-dependent interaction and autonomous activation of CaMKII. CaMKII and Cx36 were shown to be significantly colocalized in the inferior olive, a brainstem nucleus highly enriched in electrical synapses, indicating physical proximity of these proteins. In analogy to the current notion of NR2B interaction with CaMKII, we propose a model that provides a mechanistic framework for CaMKII and Cx36 interaction at electrical synapses.

brain | electrical synapse | gap junction | protein-protein interaction | synaptic plasticity

The glutamatergic synapse exhibits well-known long-term plasticity, where activity-dependent modulation has been attributed to the calcium/calmodulin (CaM)-dependent protein kinase CaMKII (in the following CaMKII refers to the chemical α -isoform) (for review see ref. 1). Electrical coupling has been reported to exhibit activity-dependent plasticity as well, in particular at the mixed-electrical synapse of the VIII nerve-Mauthner cell synapse of goldfish, which exhibits long-term potentiation of both electrical and chemical components after tetanic stimulation (2–4) and in response to activation of the cannabinoid type 1 receptor (5). Sustained changes of electrical activity have also been documented at electrical synapses in the reticular nucleus of the thalamus, where interneuronal gap junctions are frequent (6).

The gap junction protein involved in the electrical synapse of the fish Mauthner cell is connexin35 (Cx35) (7); the mammalian ortholog is Cx36 (8, 9), which is the major gap junctional component of electrical and mixed synapses in the CNS. Activation of the NMDA receptor and subsequent increase in intracellular Ca^{2+} were reported to be essential for the induction of changes in gap junction conductance at the mixed Mauthner cell synapse involving modifications of gap junction channels, and CaMKII was considered to play a key role in this electrical synaptic plasticity (4, 10). However, mechanistic aspects of this putative interaction and whether it exists in mammalian systems are unknown. We therefore have examined whether Cx36 may provide a functional target for changes occurring during activation of CaMKII.

CaMKII is a multimeric holoenzyme having 8–12 subunits. Each subunit has kinase activity. Central to the ability of CaMKII to generate sustained changes in postsynaptic efficacy after stimulation is interaction of the subunits with the calmodulin/ Ca^{2+} complex ($\text{Ca}^{2+}/\text{CaM}$) (see ref. 1 for a recent review). In the “off state,” the enzyme is inactive because an autoinhibitory regulatory domain of the enzyme binds to its catalytic region preventing autoactivation and substrate binding. Binding of $\text{Ca}^{2+}/\text{CaM}$ is the primary event for release from autoinhibition by exposing a target site (T-site) and a substrate binding site (S-site) of the enzyme (1, 11). This opening of the CaMKII generates an autonomous kinase by an intraholoenzyme, intersubunit phosphorylation of a threonine residue at position T(286) (1). Once phosphorylated at T(286) (see Fig. 5 for additional information) the autoinhibitory domain with its pseudotarget and pseudosubstrate sites can no longer interact with the corresponding domains of the catalytic site, allowing the catalytic domain to access and phosphorylate substrates (12, 13). Remarkably, sustained autonomous activity is independent of $\text{CaM}/\text{Ca}^{2+}$ and keeps the kinase active after the initial Ca^{2+} stimulus has subsided (see ref. 1 for review). Interaction of CaMKII with 1 of its most prominent target proteins at postsynaptic sites, the NR2B subunit of the NMDA receptor, has been scrutinized within recent years (12, 14), identifying several amino acid residues in the autoinhibitory domain of CaMKII that are critical for the interaction with this target protein (14, 15).

To elucidate mechanistic aspects of the interaction of the neuronal gap junction protein Cx36 with CaMKII, we first used in silico screens to identify putative binding and phosphorylation sites. These sites were further verified by pull-down experiments and in vitro phosphorylation assays. Two cytoplasmic CaMKII binding sequences of Cx36 were discovered. One site resides at the cytoplasmic loop of Cx36 (CLB), and another resides nearer to the carboxyl-terminal region of the protein (CTB). Surprisingly, both putative binding sites revealed sequences that are similar to those within the autoinhibitory domain of CaMKII. The CLB site contains core residues similar to a sequence

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surrounding the autoinhibitory domain around T(286) (pseudo-target site), and the CTB site reveals a conserved motif, which is central to the pseudosubstrate site of the kinase. In vitro binding and phosphorylation studies of Cx36-GST fusion proteins indicated striking similarities with those of carboxyl-terminal domains of the NR2B subunit of the NMDA receptor in terms of their dependence on autophosphorylation of CaMKII. In addition, fragments of the Cx36 cytoplasmic binding sites generate autonomous activation akin to residues of the carboxyl terminus of the NR2B subunit. In situ immunocytochemistry of the inferior olive, a brainstem nucleus highly enriched with Cx36 at sites of electrical synapses, indicated physical proximity of the 2 proteins. In accordance with these findings, we suggest that Cx36, the most common connexin at electrical synapses in mammals, interacts with and is phosphorylated by CaMKII in a way similar to CaMKII phosphorylation of glutamate receptors.

Results

In Vitro Analysis Indicates Binding of CaMKII to Cytoplasmic Domains of Cx36. In silico analysis [[supporting information \(SI\) Text](#)] yielded 2 potential consensus motifs for CaM/CaMKII binding within the cytoplasmic domains of Cx36. The first site resides at a juxtamembrane position of the cytoplasmic loop (CLB, amino acids 175–195) between the second and third transmembrane domains of Cx36, and the second domain corresponds to a juxtamembrane position of the carboxyl terminus (CTB, amino acids 272–292) (Fig. 1A and [SI Text](#)). In addition to the predicted binding sites, 4 putative CaMKII phosphorylation motifs ([SI Text](#) and [Table S1](#)) were identified in 2 regions in the cytoplasmic loop.

To validate the direct binding of CaMKII to Cx36, we used pull-down strategies with Cx36 fusion proteins as baits: For this purpose we incubated a GST fusion protein consisting of the cytoplasmic loop site (GST-Cx36CL, amino acids 100–201) or the carboxyl terminus (GST-Cx36CT, amino acids 272–321) with in vitro translated ³⁵S-labeled CaMKII under conditions where Ca²⁺ and CaM were either present or absent. As shown in Fig. 1B and C, Ca²⁺/CaM is sufficient to promote binding of CaMKII to GST-Cx36CL and GST-Cx36CT. Note that the addition of Ca²⁺ alone is effective presumably because of the presence of CaM in the erythrocyte lysate from which CaMKII was translated in vitro (as assessed by Western blotting; data not shown). Because autophosphorylation of CaMKII is crucial for its interaction with target receptor proteins (16), we also evaluated binding efficiency of both fusion proteins to autophosphorylated CaMKII. Autophosphorylation of [³⁵S] CaMKII was achieved by incubation with ATP in the presence of Ca²⁺/CaM at 30 °C for 15 min (in the control sample ATP was added after incubation at 30 °C). Autophosphorylation increased affinity of CaMKII to GST-Cx36 fusion proteins of both cytoplasmic fragments with a more pronounced effect on GST-Cx36CT (Fig. 1B and C, last 2 lanes each for original autoradiograms). Deletion mutants of the GST fusion proteins lacking the presumptive binding sites [GST-Cx36CL-Del (Δ 175–195) and GST-Cx36CT-Del (Δ 272–292)] showed significantly weaker binding to CaMKII (Δ 175–195 by \approx 40% and Δ 272–292 by 25%) than did the full-length GST proteins (Fig. 1D), confirming the interaction of CaMKII with both binding domains.

Additionally, affinities of Cx36 domains to CaMKII were quantified by using surface plasmon resonance spectroscopy and peptides (20–22mers) containing the binding sites (see above) and the phosphorylation domains (amino acids 99–119 for the cytoplasmic loop site and amino acids 300–321 for the carboxyl-terminal domain). The two peptides containing the CaMKII binding sites showed a stronger affinity to CaMKII than those corresponding to the phosphorylation regions. Application of 25 μ M peptides corresponding to the putative Cx36 binding and

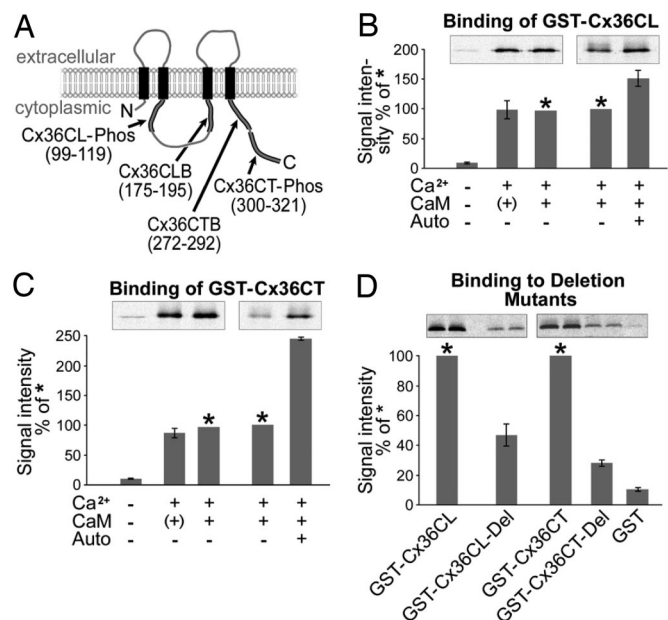


Fig. 1. CaMKII binds effectively to cytoplasmic loop (GST-Cx36CL) and carboxyl-terminal (GST-Cx36CT) fusion proteins immobilized on GST agarose beads. (A) Schematic representation of the putative CaMKII binding (CB) and phosphorylation domains (C-Phos) of Cx36 as indicated by in silico screens. (B) Representative autoradiographs and quantitative densitometry of binding of in vitro translated [³⁵S]-CaMKII to the GST-Cx36CL fusion protein. Binding is effective with translated CaMKII protein plus Ca²⁺ (second lane with CaM in brackets) because in vitro translated CaMKII contains sufficient amounts of calmodulin from the erythrocyte lysate as assessed by Western blotting (data not shown). Addition of Ca²⁺/CaMKII does not increase binding significantly. (See the third lane in B labeled with an asterisk. This band was used for quantification of signal intensity set to 100%; see also in C.) Autophosphorylation was achieved by incubation of CaMKII with Ca²⁺/CaM at 30 °C in the presence of ATP. In the control, ATP was added after incubation. Autophosphorylated CaMKII binds more effectively than the unphosphorylated CaMKII form. (C) Binding of [³⁵S]-CaMKII to the carboxyl-terminal Cx36 fusion protein (GST-Cx36CT). Autophosphorylation leads to a 2.5-fold increase of CaMKII binding. (D) Deletion of the putative CaMKII binding domains (Del) of Cx36 leads to a clearly reduced affinity of CaMKII. Quantification of signal intensity was in relation to the respective wild-type constructs (100%, indicated with *). Incubation with GST alone served for control. Note that the autoradiograph shows duplicate lanes from the same experiment.

phosphorylation domains to immobilized CaMKII resulted in the following sequence of affinities: CTB > CLB > CLP > CTP (Fig. S1). Concentration/response curves for CTB and CLB peptides indicated EC₅₀ values of \approx 8 μ M for CTB and >30 μ M for CLB, respectively (data not shown).

Cx36 Is Phosphorylated by CaMKII. We and others have recently shown that Cx36 and the fish ortholog Cx35 are targets for multiple kinases (17, 18). Because our in silico screen indicated 4 putative cytoplasmic phosphorylation motifs, we investigated whether the consensus sequences are actually targets for phosphorylation. To quantitatively assess the autoradiography, we immobilized GST-Cx36CL and GST-Cx36CT fusion proteins on a solid-phase medium coated with glutathione (glutathione-agarose beads). After clearing the solid phase from unbound fusion proteins, beads were incubated with either nonphosphorylated or autophosphorylated CaMKII in the presence or absence of Ca²⁺/CaM. Whereas without Ca²⁺/CaM induction of phosphorylation of either fusion protein did not achieve levels above background, Ca²⁺/CaM had a major effect on GST-Cx36CL (Fig. 2A) and a small effect on GST-Cx36CT (Fig. 2B). Autophosphorylation of CaMKII strongly increased the phos-

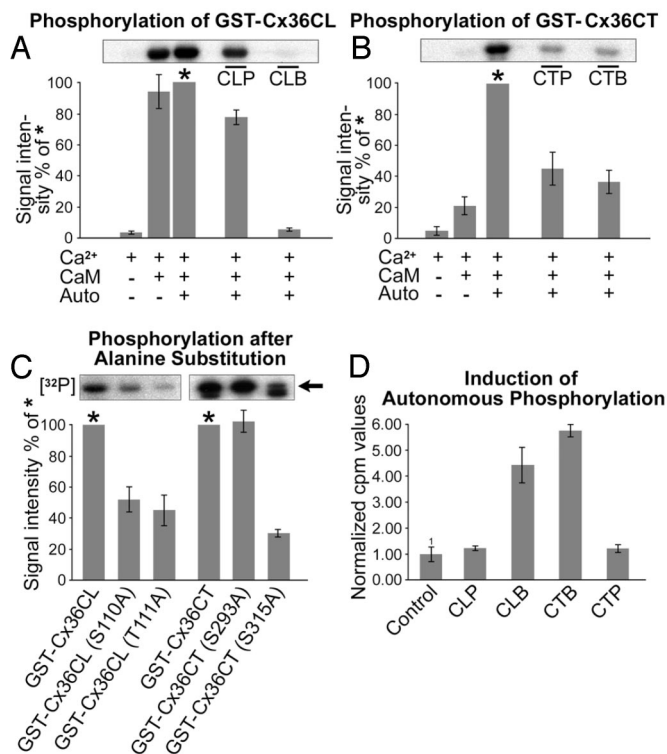


Fig. 2. Activated CaMKII phosphorylates Cx36 and exhibits autonomous kinase activity by interacting with binding fragments of Cx36. (A) GST fusion protein of the cytoplasmic loop site (GST-Cx36CL) was in vitro phosphorylated by CaMKII in the presence of Ca²⁺/CaM. Ca²⁺/CaM is sufficient to phosphorylate the cytoplasmic loop fusion protein. Autophosphorylation of CaMKII reveals no significant effect (set to 100% for quantification and labeled with an asterisk). Peptide inhibition using a sequence corresponding to the Cx36 binding site (CLB) inhibits phosphorylation almost completely, whereas peptide CLP corresponding to the phosphorylation domain of Cx36 shows a lower effect. (B) Autophosphorylation of CaMKII is essential for phosphorylation of the Cx36CT fusion protein (Cx36CT-GST). Phosphorylation is reduced by competition with both cognate peptides (CTP and CTB). (C) Point mutations, including (S110A) and (T111A) for the cytoplasmic loop and (S293A) and (S315A) of the carboxyl-terminal fusion proteins, were studied. Densitometric quantification revealed a significant reduction in phosphorylation at the cytoplasmic loop residues after alanine substitution. Note that the carboxyl-terminal GST-Cx36CT revealed a lower band in the autoradiogram, which is considered to represent a degradation product. The specific band is indicated by an arrow. In the carboxyl terminus, mutation of S293 had no effect on the phosphorylation efficacy. S315A mutation severely reduced phosphorylation. (D) Immobilized biotinylated peptides of cytoplasmic binding sites (CLB and CTB) and phosphorylation sites (CLP and CTP) were incubated with CaMKII without Ca²⁺ and calmodulin. Trapped CaMKII was then tested for activity to phosphorylate the CaMKII substrate autocomamide in the presence of [³²P]ATP. Both peptides derived from the Cx36 binding sites exhibited a profound effect on autonomous activity of CaMKII, whereas the peptides covering the phosphorylation sites did not.

phosphorylation of the GSTC₃₆CT, indicating that phosphorylation is significantly more effective at the carboxyl-terminal region when CaMKII is autophosphorylated (Fig. 2*B*). To test whether phosphorylation requires both binding and phosphorylation sites, we performed competition experiments with peptides corresponding to the binding and phosphorylation sites of Cx36. GST-Cx36CL phosphorylation by CaMKII was almost completely inhibited by competing with the mimetic peptide corresponding to the cytoplasmic loop binding site. The effect was less pronounced when GST-Cx36CL was exposed to the peptide corresponding to the cytoplasmic loop phosphorylation site (Fig. 2*A*, last 2 lanes). In contrast, GST-Cx36CT phosphorylation

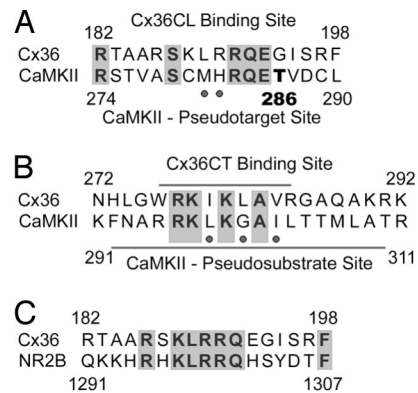


Fig. 3. Sequence similarities between the Cx36 binding domains and the autoinhibitory region of CaMKII. (A) The cytoplasmic loop (CL) binding site of Cx36 reveals similarities to core residues of the pseudotarget site of CaMKII surrounding T(286). (B) The carboxyl-terminal binding site of Cx36 reveals a conserved motif, which is similar to the pseudosubstrate binding site of CaMKII. Notably, both the CT binding site of Cx36 and the pseudosubstrate binding site of CaMKII contain consensus motifs for calmodulin binding, indicated with underlying bars. (C) The CL binding site of Cx36 is also highly similar to the CaMKII binding sequence of the NR2B subunit of the NMDA receptor. Neutral amino acid exchanges are indicated by dots.

could be substantially reduced by both cognate peptides of the binding and the phosphorylation domains of the carboxyl terminus of Cx36 (Fig. 2*B*, last 2 lanes). The minor effect of the CLP peptide to block phosphorylation of the GST-CL protein may be explained by steric hindrance within the cytoplasmic loop fragment, which does not allow access of the peptide to its target sequence. Alternatively, the CLP peptide may become phosphorylated and no longer prevent phosphorylation of the corresponding Cx36 site.

Additionally, mass spectroscopy was applied on digested GST-Cx36CL and GST-Cx36CT proteins to determine the phosphorylated amino acid residues within the cytoplasmic loop and carboxyl-terminal phosphorylation motifs. Tryptic digests of the carboxyl terminus were found to be phosphorylated in vitro, at residues serine S293 and S315. Digests of the cytoplasmic loop fusion protein indicated phosphorylation of residues serine S110 and threonine T111 (*SI Text* and Fig. S2). To confirm the usage of the individual phosphorylation residues, GST fusion proteins carrying a point mutation of serine to alanine at the sites of interest were devised by using overlap extension PCR. Mutations of phosphorylation consensus motifs for GST-Cx36CL included (S110A) and (T111A), and those for GST-Cx36CT included (S293A) and (S315A). Mutations in the cytoplasmic loop, S110A and T111A, each led to $\approx 50\%$ reduction in phosphorylation of GST-Cx36CL (Fig. 2*C*). In the carboxyl-terminal region, mutation of S293 had no effect on the phosphorylation efficacy, whereas it was strongly reduced after mutation at S315.

CaMKII Reveals Autonomous Activation by Cx36 Binding Sites. Sequence analyses of the CaMKII binding sites of Cx36CL and Cx36CT revealed striking similarities with the pseudotarget and pseudosubstrate binding sites of the kinase. When CLB was compared with the autoinhibitory region surrounding T(286) of CaMKII, a central core consisting of the R(283)QE285 motif, which has been shown to be necessary for target binding by CaMKII (14, 15), became apparent (Fig. 3*A*). Notably, in the carboxyl-terminal binding domain of Cx36 core residues R(277)KIKLAV(283) of the pseudosubstrate binding site of CaMKII are also similar (Fig. 3*B*). Even more remarkably, comparison of the Cx36CLB sequence to that of the CaMKII binding domain of the NMDA receptor subunit NR2B (Fig. 3*C*)

Whereas the central core of the CLB domain reveals amino acid residues resembling the pseudotarget binding site of CaMKII, the CTB site shows core residues similar to the pseudosubstrate binding region. We assume these similarities as a result of convergence because the intron/exon boundaries of CaMKII and Cx36 are different indicating that the domains may not have developed from a single ancestral gene. Apparently, the Cx36 cytoplasmic domain is endowed with 2 peptide sequences, which have been suggested to be crucial for the regulation of CaMKII activity (14, 15). According to similarities of interaction of the Cx36 binding sites with properties of the NR2B (13) subunit and the striking sequence similarity of both target binding sites we suggest a model where Cx36 may function in a bipartite fashion similar to the interaction of the NR2B subunit with CaMKII (1, 11). This model (Fig. 5) entails the autoinhibitory “gate” hypothesis of the regulatory and catalytic domains of CaMKII as described above. With respect to interactions with Cx36 the sequences of events may follow the following pathway: binding of Ca^{2+} /CaM to the autoinhibitory domains displaces the gate and allows the CLB domain with its very similar pseudosubstrate binding site to interact with the target site of the kinase. Autophosphorylation of CaMKII is not required for this initial step, as indicated by the properties of the CLB fragment. Subsequent autophosphorylation at T(286), which enables the kinase to interact with further targets and/or substrate proteins (14, 15, 23), may then facilitate the binding of CTB to its corresponding substrate recognition motif at the catalytic segment of the kinase. Of note, the carboxyl-terminal binding site of Cx36 overlaps entirely with a sequence identified as a CaM binding site (24). This fact offers a further argument for a subsequential processing of both Cx36 segments during interaction with the kinase. Because CaM bound to the carboxyl terminus of Cx36 would inhibit the interaction with the substrate binding site of the kinase, the increasing affinity of the enzyme to Ca^{2+} /CaM, which was found to increase 1,000 times (25) after autophosphorylation of T286 (so-called Ca^{2+} /CaM trapping), could compete for CaM with Cx36. When CaM is displaced from the CTB site, it is released from inhibition and able to bind to the kinase.

It is interesting that both binding sites reveal autonomous activation of CaMKII, a feature that has been described for a peptide fragment of the NR2B subunit homologous to the autoinhibitory domain of the kinase (13). Autonomous activation of CaMKII is thought to convey a further activity-dependent mechanism that works synergistically with binding and autophosphorylation of the kinase and seems to be an essential feature responsible for sustained synaptic strength (for a review see refs. 1 and 12). In this context, Cx36 binding sites seem to share mechanisms with the NR2B subunit that have been proven to be essential for enhancing synaptic efficacy. Interspecies comparison shows that the CLB and CTB motifs of Cx36 are evolutionarily highly conserved among the Cx36 orthologs (data not shown), including the fish Cx35, for which most of the data on functional interaction of CaMKII with this neuronal gap junction protein have been obtained (26, 27). Protein–protein interaction, in the described way, requires physical association of the interacting partners. CaMKII is known to display translocation from the cytosol to postsynaptic sites in response to Ca^{2+} increase through activation of NMDA receptors (28). Subcellular association of Cx35 and CaMKII has recently been demonstrated in the mixed synapses of the Mauthner neuron of goldfish, implying a physical interaction of both proteins (29). Our finding of a colocalization of CaMKII and Cx36 in the inferior olive is a first hint that the electrical synapse constitutes a subcellular complex where dynamically regulated interaction with CaMKII could play a role in tuning electrical synapse activity. A feasible functional effect could be an increase in synaptic efficacy as has previously described by us (30) in the

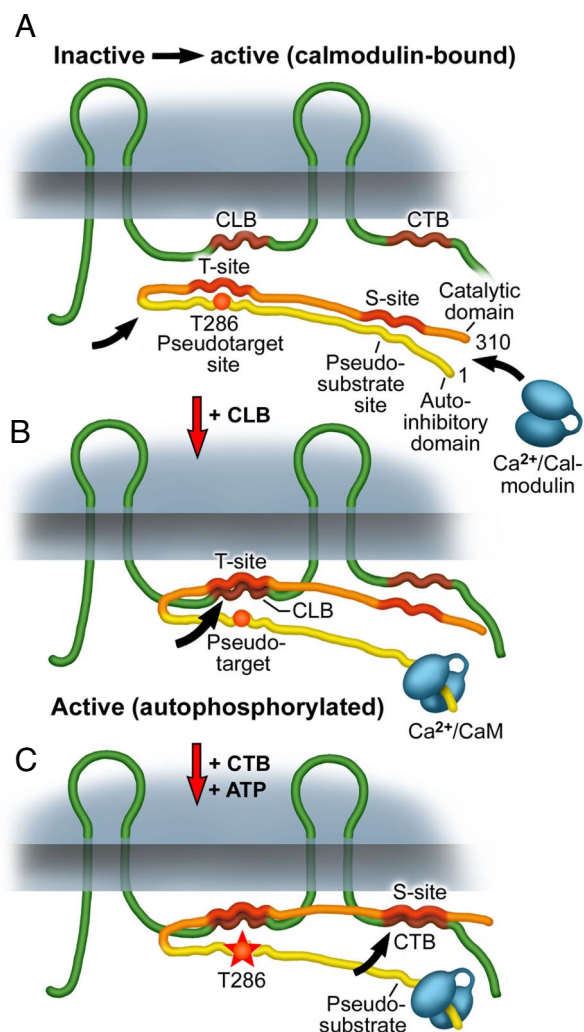


Fig. 5. Model for the interaction of CaMKII with Cx36. The model follows the “gate” concept developed for interaction of CaMKII with the NMDA receptor (see also text). (A) Upon local Ca^{2+} elevation Ca^{2+} /calmodulin, either from cytosolic sources or Cx36 bound, binds to the calmodulin binding site of CaMKII that overlaps with its pseudosubstrate site. (B) Binding of Ca^{2+} /CaM to CaMKII opens the gate and exposes the target site (T-site) and the substrate site (S-site) of the kinase. Exposure of the T-site allows binding of the cytoplasmic loop binding site (CLB) of Cx36, a process that does not require autophosphorylation of the kinase. (C) Autophosphorylation of CaMKII at T(286) leads to enhanced binding of the carboxyl-terminal binding site (CTB) of Cx36 to the S-site of the activated kinase. Increasing autophosphorylation of CaMKII may also facilitate shifting of Ca/CaM from a Cx36-bound position to kinase binding because of an increase in CaM affinity (trapping phenomenon) of CaMKII.

form of “run-up” of junctional conductance in N2A cells expressing Cx36.

In conclusion, the data described in this paper provide clues regarding mechanistic aspects underlying molecular interaction of CaMKII and the electrical synaptic protein Cx36. We uncovered similarities between this mechanism and regulation at chemical synapses, which have been described for the CaMKII/NR2B interaction (1, 11, 13). We thus suggest that both modes of interneuronal transmission may share common molecular features in modulating activity-dependent functional efficacy.

Materials and Methods

Binding of in Vitro Translated CaMKII to Fusion Proteins and Peptides. CaMKII was in vitro translated in the presence of [^{35}S]methionine (Amersham) by

using pCMV-SPORT6-CaMKII (RZPD) and TNT T7 quick coupled transcription–translation kit (Promega). Labeled [³⁵S] CaMKII was used directly in CaMK buffer without Ca²⁺/CaM, with Ca²⁺ and Ca²⁺/CaM (New England Biolabs), or after 15 min of incubation at 30 °C with calmodulin and Ca²⁺-containing CaMK buffer resulting in autophosphorylation of CaMKII. Fifty micrograms of GST Cx36 fusion proteins [GST-Cx36CL (aa100–201) and GST-Cx36CT (aa272–321)] were immobilized on 30 μL of GST-agarose beads (Pierce). Beads were washed 3 times with PBS containing Ca²⁺/Mg²⁺ and once in PBS without Ca²⁺/Mg²⁺. Both washing solutions contained 1% BSA and 1% fish gelatin (Sigma). Immobilized GST fusion proteins were incubated for 2 h with CaMKII at 4 °C. After 30 min, BSA and fish gelatin were added to a final concentration of 1%. Subsequently, the beads were washed 4 times with the CaMK buffers as described for autophosphorylation (see below). Beads were incubated with SDS sample buffer for 5 min at 65 °C, and supernatants were separated via SDS/PAGE. Binding and phosphorylation experiments (see below) were all done in triplicate. Data are presented as means ± SD.

In Vitro Phosphorylation of Fusion Proteins by CaMKII. Fifty micrograms of fusion protein (GST-Cx36CL, GST-Cx36CT) was immobilized on 30 μL of GST-agarose beads (Pierce) and washed 4 times with PBS. Two-hundred-fifty units of CaMKII were incubated in CaMKII buffer containing Ca²⁺ or Ca²⁺/CaM (New England Biolabs). To activate the CaMKII via autophosphorylation, the enzyme was incubated at 30 °C in the presence of 150 μM ATP. To the nonautophosphorylated probes, ATP was added after incubation at 30 °C. The immobilized fusion proteins were incubated with either nonphosphorylated or autophosphorylated CaMKII in the presence of [³²P]ATP for 30 min at 30 °C. One hundred μg of peptides (see *SI Text*) containing the predicted phosphorylation sites and binding regions of Cx36 were added to autophosphorylated probes to test competition for binding and phosphorylation of

CaMKII. Beads were washed 5 times with PBS and incubated with SDS sample buffer for 5 min at 65 °C. The supernatants were separated via SDS/PAGE. [³²P]-Phosphorylated fusion proteins were visualized via autoradiography.

Induction of CaMKII Autonomous Activity by Cx36 Binding Peptides. Twenty micrograms of peptides (CLB, CTB, CLP, and CTP) were biotinylated as described previously (31) and bound to 15 μL of neutravidin beads (Pierce) according to the instructions of the manufacturer. A control sample without peptide was also prepared. The beads were washed 3 times with CaMKII reaction buffer (New England Biolabs) and incubated with 250 units of CaMKII (New England Biolabs). After 30 min at 4 °C, 300 μM autocalmitide 2 (New England Biolabs), 100 μM [³²P]ATP (Amersham Biosciences), and 0.5 mM EGTA were added and incubated for 8 min at 30 °C for phosphorylation. Thereafter, 2× SDS sample buffer was added and the beads were heated (65 °C) for 5 min to stop the reaction. The supernatants were spotted on phosphocellulose squares (Upstate), and the washed squares were measured in a Beckman scintillation counter. Values were normalized with respect to the control sample.

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