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Cysteine String Protein Regulates G Protein Modulation of N-Type Calcium Channels

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Summary

Cysteine string proteins (CSPs) are secretory vesicle proteins bearing a "J domain" and a palmitoylated cysteine-rich "string" region that are critical for neurotransmitter release. The precise role of CSP in neurotransmission is controversial. Here, we demonstrate a novel interaction between CSP, receptor-coupled trimeric GTP binding proteins (G proteins), and N-type Ca^{2+} channels. G_{α} subunits interact with the J domain of CSP in an ATP-dependent manner; in contrast, $G_{\beta\gamma}$ subunits interact with the C terminus of CSP in both the presence and absence of ATP. The interaction of CSP with both G proteins and N-type Ca^{2+} channels results in a tonic G protein inhibition of the channels. In view of the crucial importance of N-type Ca^{2+} channels in presynaptic vesicle release, our data attribute a key role to CSP in the fine tuning of neurotransmission.

Introduction

Cysteine string proteins (CSPs) are highly conserved secretory vesicle proteins. Deletion of CSP severely impairs presynaptic neuromuscular transmission in *Drosophila melanogaster*, indicating that CSP's function is critical to neurotransmission (Zinsmaier et al., 1994). The precise function of CSP in synaptic transmission is still enigmatic. CSP, so called because it contains a cysteine-rich domain that in rats consists of a string of nine cysteine residues flanked on the C-terminal side by three additional cysteines, is localized on synaptic vesicles (Mastrogiacomo et al., 1994), zymogen granules (Braun and Scheller, 1995), and chromaffin granules (Chamberlain et al., 1996). Another striking feature of CSP is that it contains a "J domain," which is a 70 amino acid region of homology shared by bacterial DnaJ and many otherwise unrelated eukaryotic proteins (Braun et al., 1996). Members of the DnaJ family are found in a wide variety of species from *E. coli* to man (reviewed in Silver and Way, 1993; Cyr et al., 1994). CSP interacts with and activates the ATPase activity of the heat shock cognate protein Hsc70 (Braun et al., 1996) as well as its closely related homolog Hsp70 (Chamberlain and Burgoyne, 1997) via its J domain (Braun et al., 1996). Although the domains essential for CSP/Hsc70 complex formation have been mapped (Braun et al., 1996; Stahl et al., 1999), the function of the CSP/Hsc70 cochaperone–chaperone complex is unknown.

There are conflicting reports supporting either a role for CSP in exocytosis or regulation of Ca^{2+} channel activity. The close proximity of fusion-competent synaptic vesicles to voltage-gated Ca^{2+} channels that trigger release at the active zone supports a possible CSP/Ca^{2+} channel interaction. Several lines of evidence suggest that CSP may indeed modulate Ca^{2+} channel activity. Injection of CSP antisense RNA into *Xenopus* oocytes inhibits the activity of omega-conotoxin-sensitive Ca^{2+} channels, implying that CSP modulates Ca^{2+} channels (Gundersen and Umbach, 1992). Studies in *Drosophila* CSP null mutants show normal induction of quantal release in response to α-latrotoxin and ionomycin, indicating that the exocytotic/endocytotic cycle of neurotransmitters is normal, consistent with the idea that CSP has a role in the upstream depolarization-dependent Ca^{2+} entry (Umbach and Gundersen, 1997; Ranjan et al., 1998). In addition, the idea that CSP may modulate Ca^{2+} channel activity is further strengthened by the report that CSP binds to α_{1A} subunits of P/Q channels (Leveque et al., 1998). Finally, it has recently been shown that, in *Drosophila*, overexpression of CSP suppresses the decrease of evoked release induced by the overexpression of syntaxin, suggesting that CSP could regulate a syntaxin-containing Ca^{2+} channel complex (Nie et al., 1999).

In contrast, other reports suggest a direct role for CSP in exocytosis independent of Ca^{2+} transmembrane fluxes. Overexpression of mammalian CSP in PC12 cells (Chamberlain and Burgoyne, 1998), insulin-secreting cells (Brown et al., 1998), and adrenal chromaffin cells (Graham and Burgoyne, 2000) reveals defects in stimulated exocytosis rather than defects in Ca^{2+} channel activity. Similarly, at peptidergic terminals of CSP null mutant *Drosophila*, Ca^{2+} currents are normal despite severely impaired neurotransmission (Morales et al., 1999).

It is possible that the role of CSP in Ca^{2+} channel function is due to an interaction with one of the channel's regulatory pathways. Presynaptic Ca^{2+} channels are subject to extensive modulation by protein kinase C (Swartz, 1993; Stea et al., 1995; Hamid et al., 1999), G protein $\beta\gamma$ subunits (Herlitze et al., 1996; Ikeda, 1996; Zamponi et al., 1997), and syntaxin (Bezprozvanny et al., 1995; Stanley and Mirotznik, 1997; Jarvis et al., 2000). To test the hypothesis that CSP influences the excitation–secretion coupling pathway by forming direct protein–protein interactions with receptor-coupled trimeric GTP binding proteins (G proteins) and thereby secondarily influencing Ca^{2+} channel activity (and possibly other G protein–mediated events), we examined the interaction of CSP with G proteins from several angles.

Results

Binding Properties of CSP, G Proteins, and N-Type Ca^{2+} Channels

In order to investigate the possibility of an in vitro association between G proteins and CSP, glutathione S-transferase (GST) fusion proteins consisting of full-length CSP or the amino terminus of CSP (amino acids 1–82,

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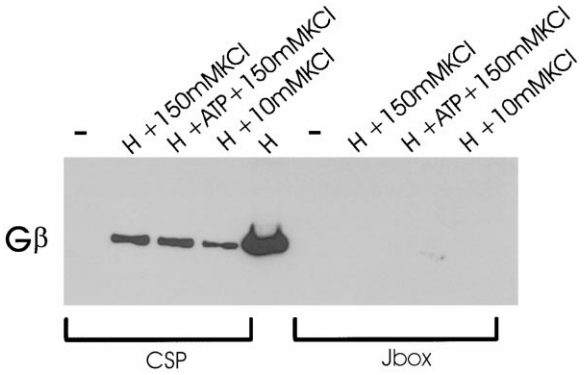

Figure 1. G Proteins Interact with CSP

Immunoblot analysis showing binding of G_β to CSP-GST fusion protein or J Box–GST fusion protein immobilized on agarose. Fusion proteins were incubated in the absence (−) or presence (H) of hippocampal homogenate (H). The effects of KCl concentration and ATP (2 mM) on the CSP/G_β interaction are shown. The lack of G_β association with the J Box indicates that G_β interacts with either the cysteine string domain or the C-terminal domain of the protein. The results shown are representative of eight independent experiments.

including the J domain) coupled to glutathione agarose beads were used in an in vitro binding assay. The beads were incubated with hippocampal homogenate, and washed, and bound proteins were eluted. The presence of $G_{\beta\gamma}$ was analyzed by Western blotting with anti-G_β monoclonal. $G_{\beta\gamma}$ is a highly stable complex such that immunodetection of G_β most likely reflects the presence of the intact $G_{\beta\gamma}$ complex (Clapham and Neer, 1997). Figure 1 shows that G_β associated with recombinant immobilized CSP in vitro. Conversely, no interaction of G_β was observed with the J domain, indicating that $G_{\beta\gamma}$ subunits specifically interact with the carboxyl terminus of CSP or that the complete structure of CSP is required for effective binding. The CSP/$G_{\beta\gamma}$ interaction was reduced in low (10 mM) KCl concentrations (Figure 1) and stable in high (300 mM) KCl concentrations (data not shown) and was not dependent on ATP. The maintenance of the CSP/$G_{\beta\gamma}$ interaction over the indicated salt concentrations reflects the stability of this protein–protein interaction.

Next, we investigated whether Hsc70 influenced the association of $G_{\beta\gamma}$ with CSP. Hsc70 is an abundant neural protein with coupled protein binding and ATPase activities. We have previously shown that CSP interacts with and activates the ATPase activity of Hsc70 (Braun et al., 1996). Although the function of the CSP/Hsc70 complex is unknown, regulation of the assembly/dissassembly of multimeric complexes such as presynaptic complexes is typical of this family of chaperone proteins. Figure 2 shows that Hsc70 as well as G_β associated with recombinant immobilized CSP in vitro. While the interaction of CSP with Hsc70 was increased in the presence of ATP, the interaction of CSP with G_β did not require ATP. Unlike G_β, which interacted with the carboxy-terminal domain of the protein, Hsc70 interacted with the amino-terminal J domain of CSP (Figures 2A and 2C). Greater association of Hsc70 with CSP was observed when purified Hsc70 was incubated with CSP-GST or J Box–GST beads compared to the solubilized hippocampal homogenate preparation (Figures 2A and 2C), perhaps due to the absence of detergent in the purified preparation. The minor amount of Hsc70 immunoreactivity in lanes 1 and 4 of Figure 2A likely reflects resence of small amounts of DnaK, a bacterial Hsc70 homolog that copurifies with recombinant proteins. The interaction of CSP with $G_{\beta\gamma}$ occurred both in the absence and presence of Hsc70, indicating that Hsc70 is neither required for nor does it interfere in the interaction between G_β and CSP. The G_β/CSP interaction was more stable than the Hsc70/CSP interaction (data not shown). We note that previous reports have indicated that CSP–protein interactions are typically transient and difficult to detect (Braun et al., 1996; Leveque et al., 1998; Nie et al., 1999). The relatively stable nature of the CSP/G_β complex is, therefore, unexpected, particularly in view of the notion that protein–protein interactions among molecular cochaperone–chaperone proteins like CSP/Hsc70 are typically of transient nature (Silver and Way, 1993; Cyr et al., 1994). No interaction between CSP and synaptophysin, Nsec-1, synaptotagmin, GAP43 (growth-

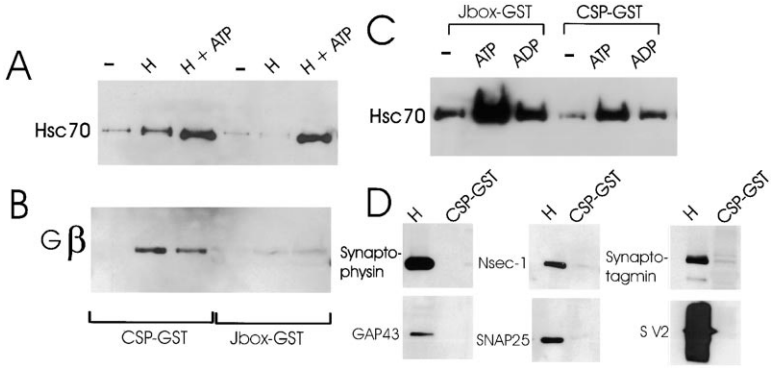

Figure 2. CSP Forms Direct Protein–Protein Interactions with G Proteins and Hsc70

Immunoblot analysis showing binding of (A and C) Hsc70 and (B) G_β to CSP-GST fusion protein or J Box–GST fusion protein immobilized on agarose. Fusion proteins were incubated in the absence (−) or presence (H) of hippocampal homogenate and 2 mM ATP at 37°C for 30 min. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with (A) anti-Hsc70-monoclonal (Sigma) or (B) anti-G_β monoclonal (Transduction Labs).

(C) Binding of purified bovine Hsc70 to an N-terminal construct of CSP (J Box) or CSP-GST fusion protein immobilized on agarose. Fusion proteins were incubated with purified bovine Hsc70 (2 μM) in the presence or absence of ATP (2 mM) or ADP (2 mM) for 1 hr at 22°C. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-Hsc70 monoclonal (Sigma). Hsc70 interaction with CSP is influenced by the presence of nucleotide.

(D) Immunoblot analysis of hippocampal homogenate (H) and CSP-GST fusion proteins incubated with hippocampal homogenate at 37°C for 30 min. The nitrocellulose was probed with anti-synaptophysin monoclonal, anti-Nsec-1 polyclonal, anti-synaptotagmin polyclonal, anti-GAP43 monoclonal, anti-SNAP25 monoclonal, and anti-SV2 monoclonal. The lack of binding of these synaptic proteins indicates the specificity of the CSP/$G_{\beta\gamma}$ interaction.

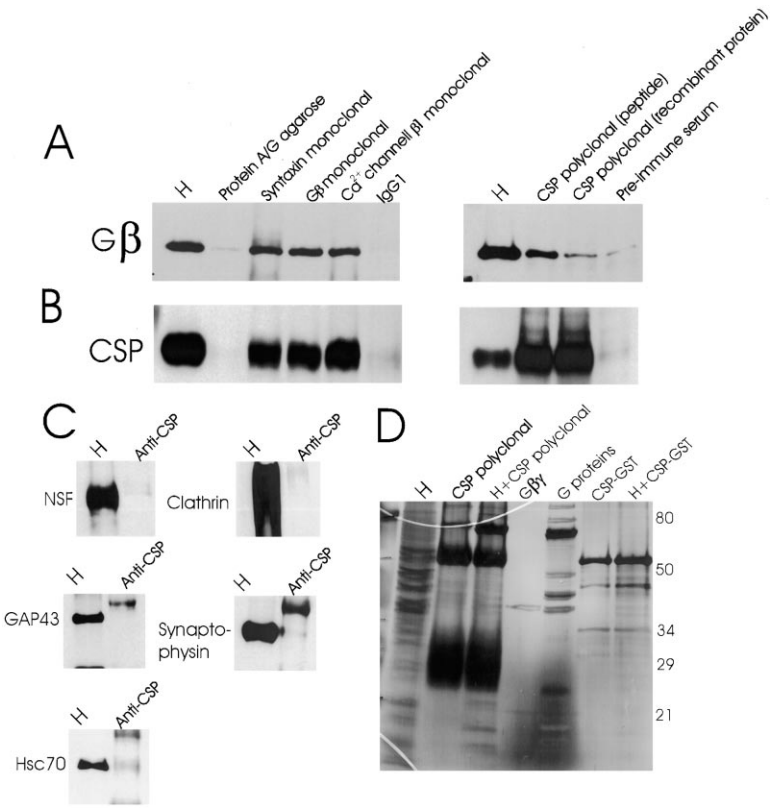

Figure 3. CSP and G_β Coimmunoprecipitate

Coimmunoprecipitation of CSP and G_β from rat hippocampal homogenate followed by immunoblot analysis (A) with anti-G_β monoclonal (Transduction Labs) and (B) anti-CSP polyclonal. Immunoprecipitation was achieved by incubating 190 μg of hippocampal homogenate with anti-syntaxin, anti-Ca^{2+} channel β1, anti-G_β, or anti-CSP (prepared either against a C-terminal peptide [amino acids 182–198] or the entire recombinant protein as indicated). The lanes are as indicated: (H) rat brain hippocampal homogenate (25 μg) loaded directly on the gel and incubation of hippocampal homogenate (190 μg) with protein A/G agarose, anti-syntaxin (Sigma) (syntaxin monoclonal), anti-G_β (Transduction Labs), anti-Ca^{2+} channel β1 (Transduction Labs), IgG1 immunoglobulin (Sigma), anti-CSP polyclonal (peptide), anti-CSP polyclonal (entire recombinant CSP), and nonimmune rabbit serum.

(C) Other synaptic proteins do not coimmunoprecipitate with CSP and are shown as controls. Immunoblot analysis of hippocampal homogenate and immunoprecipitation with anti-CSP polyclonal (peptide). The nitrocellulase was probed with anti-NSF polyclonal (StressGen), anti-clathrin monoclonal (Transduction Labs), anti-GAP43 monoclonal (Sigma), anti-synaptophysin monoclonal (Sigma), and anti-Hsc70 monoclonal (Sigma).

(D) Silver stain of proteins separated by SDS-PAGE. The lanes are as indicated: hippocampal homogenate (H), CSP polyclonal (peptide antibody), CSP polyclonal incubated in the presence of hippocampal homogenate, $G_{\beta\gamma}$ subunits (Calbiochem), G protein standards (Calbiochem), CSP-GST fusion protein, and CSP-GST fusion protein incubated in the presence of hippocampal homogenate. These results are representative of five independent experiments.

associated protein of 43 kDa), SNAP25 (synaptosomal-associated protein of 25 kDa), or SV2 was detected (Figure 2D), emphasizing the specificity of the CSP/$G_{\beta\gamma}$ protein interaction. Overall, these results suggest that the $G_{\beta\gamma}$/CSP dimer and/or a trimer of $G_{\beta\gamma}$/CSP/Hsc70 may, perhaps, contribute to the function of CSP in synaptic transmission.

To investigate which proteins CSP and $G_{\beta\gamma}$ associate with in brain tissue, we solubilized rat brain hippocampus in detergent and performed immunoprecipitations. The immunoprecipitations were resolved by polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed for the presence of CSP and G_β by Western blotting. Figure 3 demonstrates that G_β (Figure 3A) and CSP (Figure 3B) coprecipitated with anti-G_β monoclonal, anti-syntaxin monoclonal, and anti-Ca^{2+} channel β1 subunit but not anti-IgG control, nonimmune serum, or protein A/G agarose. The G_β subunit coimmunoprecipitated with anti-CSP generated against the a peptide corresponding to amino acids 182–198 but only slightly coprecipitates with a CSP antibody generated against the full-length recombinant CSP or preimmune serum. Both CSP polyclonal antibodies (i.e., peptide and full-length protein) immunoprecipitated CSP. The CSP antibody generated against the whole recombinant protein appeared to displace G_β from CSP, suggesting that this antibody may bind to the same region of CSP as $G_{\beta\gamma}$. In contrast, the anti-CSP (peptide) did not interfere with the CSP/$G_{\beta\gamma}$ interaction. Other synaptic proteins were not observed to precipitate with CSP, including NSF (soluble N-ethylmaleimide-sensitive factor), synaptophysin, clathrin, and GAP43 (Figure 3C), emphasizing the specificity of the CSP/Gβγ protein interaction. Only minor amounts of Hsc70 precipitated with CSP, suggesting that the CSP/Hsc70 complex is less stable than the CSP/$G_{\beta\gamma}$ complex. Nonspecific bands representing anti-CSP light and heavy chains are seen in the panels probed with synaptophysin, GAP43, and Hsc70. A silver stain of CSP polyclonal immunoprecipitations as well as CSP-GST fusion proteins demonstrates that G_β and, in the case of the immunoprecipitations, an ~75 kDa band that comigrates with G protein multimers represent the major CSP-associated proteins (Figure 3D). These interactions are specific in that they are enriched upon immunoprecipitation or protein binding assays. Taken together, these observations suggest that CSP and $G_{\beta\gamma}$ proteins form a physical complex.

To further evaluate the interaction between CSP, G proteins, and syntaxin, fusion proteins consisting of full-length CSP, the amino-terminal domain of CSP (J Box), the cytoplasmic domain of syntaxin, and the cytoplasmic II-III loop synprint motif of Ca^{2+} channels were immobilized on agarose beads (Sheng et al., 1996), incubated with hippocampal homogenate, and washed, and bound proteins were eluted. The presence of G_β, G_α, and Hsc70 was analyzed by Western blotting. Figure 4 demonstrates that G_β (Figure 4A) interacted with recombinant immobilized CSP, syntaxin, and the synprint motif of Ca^{2+} channels but not the amino-terminal domain of CSP (J Box). In contrast, G_α (Figure 4B) was observed

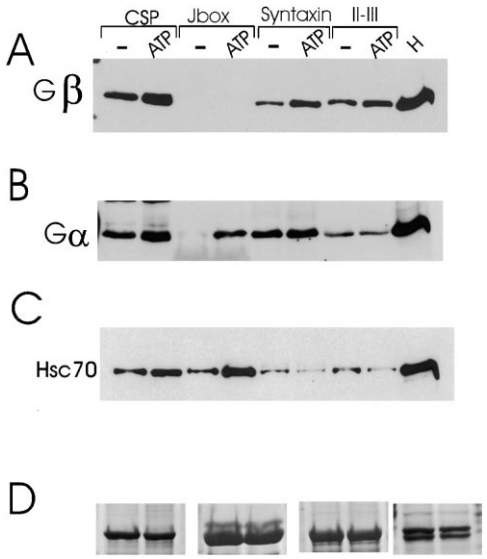

Figure 4. Immunoblot Analysis Showing Binding of Hsc70, G_{α}, and G_{β} to CSP-GST, J Box–GST, Syntaxin-GST, or His_6-α_{1B} Ca^{2+} Channel II-III Synprint Motif Fusion Proteins Immobilized on Agarose

Fusion proteins were incubated with hippocampal homogenate (190 μg) in the absence (−) or presence of ATP (2 mM) at 37°C for 30 min. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. Rat brain hippocampal homogenate (H) (25 μg) is shown in lane 9. The nitrocellulose membrane was probed with (A) anti-G_{β} monoclonal (Transduction Labs), (B) anti-G_{α} monoclonal (Calbiochem), and (C) anti-Hsc70 monoclonal (Sigma). Figure 4D shows the Coomassie staining profile of CSP-GST, J Box–GST, syntaxin-GST, and His_6 synprint motif. The results are representative of five experiments.

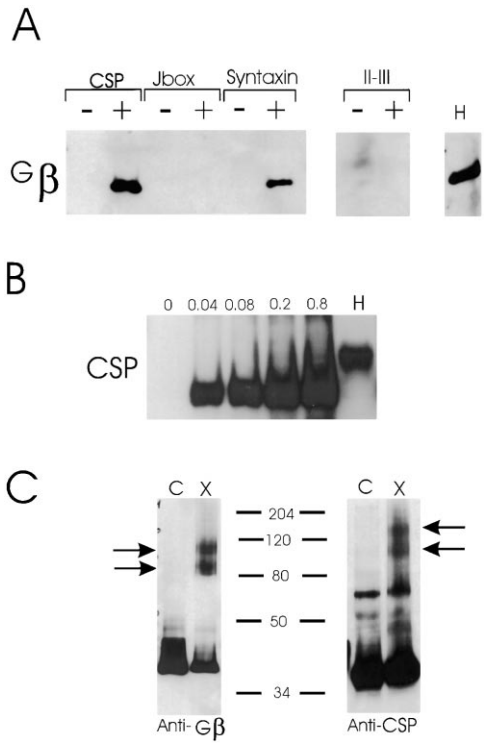

Figure 5. CSP Forms Direct Protein–Protein Interactions with $G_{\beta\gamma}$ and the Synprint Motif

(A) Immunoblot analysis showing binding of purified $G_{\beta\gamma}$ (Calbiochem) to recombinant CSP, syntaxin, and the synprint region immobilized on agarose. Fusion proteins were incubated in the presence (+) or absence (−) of 300 ng of purified $G_{\beta\gamma}$ (Calbiochem) at 37°C for 30 min. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. The nitrocellulose membrane was probed with anti-$G_{\beta\gamma}$ monoclonal (Transduction Labs). G_{β} binding was quantified (pixels) as CSP, 10,701; J Box, 0; syntaxin, 2804; and synprint motif, 0.

(B) Immunoblot analysis showing binding of purified CSP to α1B His_6 synprint motif fusion protein immobilized on agarose. The synprint fusion protein was incubated with increasing concentrations (0, 0.04, 0.08, 0.2, and 0.4 μM) of recombinant thrombin-cleaved CSP as indicated. The nitrocellulose membrane was probed with anti-CSP polyclonal. These results are representative of three independent experiments.

(C) CSP and G_{β} are rapidly cross-linked in intact hippocampal slices. Immunoblot analysis of intact hippocampal slices that were rapidly cross-linked and then detergent solubilized. Either 40 μl of control (C) or cross-linked (X) hippocampal homogenate was loaded on a 10% polyacrylamide gel and fractionated by PAGE. The membranes were probed with anti-CSP polyclonal and anti-G_{β} monoclonal (Transduction Labs). Prestained molecular mass markers were run simultaneously and are indicated. Cross-linked complexes containing G_{β} and CSP were observed to comigrate at ~110 kDa, suggesting that a single complex including both CSP and G_{β} exists in intact tissue. These results are representative of five independent experiments.

to interact with CSP and syntaxin and weakly with the synprint motif of the Ca^{2+} channel in an ATP-independent manner and with the amino-terminal J domain of CSP in an ATP-dependent manner. Hsc70 (Figure 4C) associated primarily with CSP and the amino-terminal J domain construct but also weakly with syntaxin and the cytoplasmic II-III loop synprint motif of Ca^{2+} channels. The observation that separate regions of CSP associate with G_{α} and G_{β} raises the possibility that CSP may chaperone $G_{\alpha}/G_{\beta\gamma}$ interactions in addition to $G_{\beta\gamma}/Ca^{2+}$ channel interactions.

While these data are consistent with a direct interaction between CSP and $G_{\beta\gamma}$, it does not permit us to rule out the possibility that $G_{\beta\gamma}$ and CSP interact indirectly. To investigate this possibility, we examined the ability of fusion proteins consisting of full-length CSP, the amino-terminal domain of CSP (J Box), the cytoplasmic domain of syntaxin, and the synprint motif of Ca^{2+} channels to interact with purified $G_{\beta\gamma}$. In each binding assay, an equal amount of fusion proteins was immobilized to agarose beads as confirmed by Ponceau S staining. The presence of G_{β} was analyzed by Western blotting with anti-G_{β} monoclonal. As shown in Figure 5A, the recombinant CSP/GST was able to bind purified $G_{\beta\gamma}$ subunits, indicating that the CSP/$G_{\beta\gamma}$ complex is indeed a direct physical interaction between $G_{\beta\gamma}$ and CSP. As we have shown previously, syntaxin was also capable of interacting with $G_{\beta\gamma}$ (Jarvis et al., 2000); however, this interaction appeared to be much weaker than that between CSP and $G_{\beta\gamma}$. Binding of G_{β} to CSP was four times greater than binding to syntaxin. In contrast, $G_{\beta\gamma}$ did not form a direct interaction with the synprint motif of the α_{1B} Ca^{2+} channel subunit or the amino-terminal domain of CSP (J Box). These results indicate that $G_{\beta\gamma}$ directly interacts with CSP and/or syntaxin and may be coupled to the synprint site of Ca^{2+} channels indirectly via CSP and/or syntaxin.

We also investigated the association in vitro of CSP and the synprint motif of the α_{1B} Ca^{2+} channel subunit. A fusion protein consisting of the synprint motif was attached to agarose beads and used in an in vitro binding assay. The beads were incubated with increasing concentrations of soluble recombinant CSP and washed; bound proteins were eluted and analyzed by Western blotting. In each binding assay, equal amounts of fusion proteins were immobilized to agarose beads as confirmed by Ponceau S staining. As seen in Figure 5B, CSP was observed to directly interact with the synprint motif of the Ca^{2+} channel. The binding was specific and saturable with a 50% effective concentration (EC_{50}) of about 350 nM CSP, based on quantitation of the binding by ECF (Amersham). As previously reported, bacterial expressed rat CSP is observed to migrate at a lower position upon SDS-PAGE than rat brain CSP, due to the posttranslational modification that is absent in the recombinant CSP (Braun and Scheller, 1995).

Overall, the data shown in Figures 1–5 strongly suggest that CSP is capable of binding to both the N-type Ca^{2+} channel and to $G_{\beta\gamma}$ in vitro and that a CSP/$G_{\beta\gamma}$ complex exists in brain.

CSP and G_β Are Rapidly Cross-Linked in Intact Hippocampal Slices

Rapid chemical cross-linking of intact hippocampal slices revealed two G_β-containing protein complexes (~90 kDa and ~110 kDa) and two CSP-containing protein complexes (~110 kDa and ~150 kDa) (Figure 5C). The protein complexes were resolved by SDS-PAGE and detected by immunoblot analysis. The 34 kDa CSP monomer as well as a 70 kDa CSP immunoreactive band were observed in un-cross-linked and cross-linked brain slice homogenates as previously reported (Braun and Scheller, 1995). No comparable protein complexes were observed in brain slices that were not exposed to cross-linking agents. Based on comigration, the 110 kDa complex likely contains both G_β (37 kDa) and CSP (34 kDa) in addition to other components. These results, together with our in vitro binding observations and coimmunoprecipitation experiments, suggest that the interaction of CSP and trimeric G proteins is indeed a physiological feature of neurons.

CSP Promotes G Protein Inhibition of N-Type Ca^{2+} Channels

To test whether the physical interaction between CSP and the N-type channel domain II-III linker region resulted in functional effects on the channel, we coexpressed CSP with N-type (α_{1B}, β_{1b}, and α_2-δ) Ca^{2+} channels in tsa-201 cells and assessed its effect on channel function via whole-cell patch clamp recordings. As shown in Figure 6A, upon coexpression with CSP, the channels exhibited a slowed current waveform compared to that typically observed with N-type Ca^{2+} channels (see Jarvis et al., 2000). Furthermore, current densities appeared reduced by ~2-fold in the presence of CSP (data not shown). Kinetic slowing observed in the presence of CSP is indicative of tonic inhibition of the channels by G protein $\beta\gamma$ subunits, and indeed, following application of a strong depolarizing prepulse (PP), peak current amplitude became dramatically increased, consistent with removal of a G protein–mediated inhibitory effect (i.e., Bean, 1989; Jarvis et al., 2000). To further examine this possibility, we cotransfected N-type channels together with CSP and the carboxy-terminal fragment of the β-adrenergic receptor kinase (βARK), an effective $G_{\beta\gamma}$ sponge (Koch et al., 1994). As seen in Figure 6A (see also Figure 6C), the degree of PP relief was greatly attenuated in the presence of the βARK fragment, further implicating the involvement of $G_{\beta\gamma}$ in this effect. In addition, the PP effect exhibited other hallmarks of G protein inhibition (Figure 6B). PP relief developed monoexponentially as a function of PP duration (top panel), reinhibition following the PP occurred rapidly (middle panel), and the degree of PP relief was

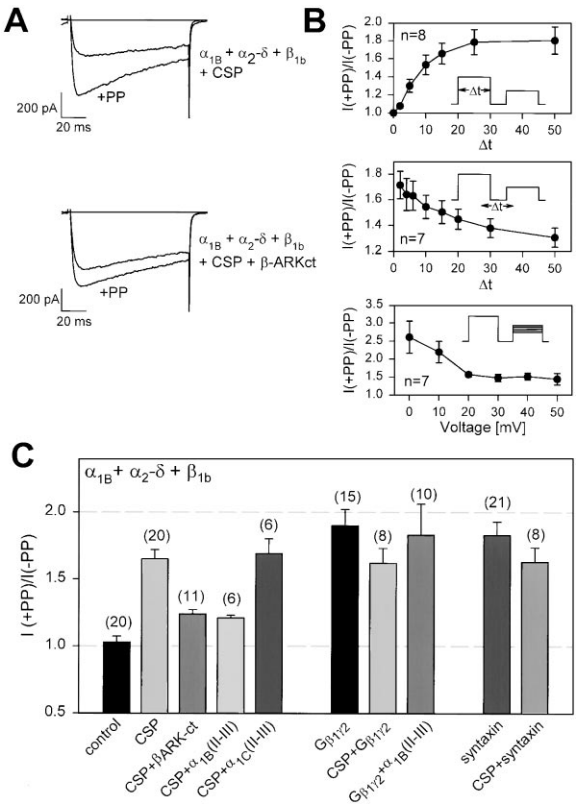

Figure 6. CSP-Mediated G Protein Inhibition of N-Type Calcium Channels

(A) Current records obtained from α_{1B}, β_{1b}, and α_2-δ N-type calcium channels transiently cotransfected with CSP into tsa-201 cells. (Top) Upon coexpression of CSP, N-type channels exhibit an atypically slowed current waveform, and current activity is increased following a 50 ms depolarizing prepulse (PP) to +150 mV. (Bottom) Additional coexpression of the carboxy-terminal fragment (residues 495–689) of the β-adrenergic receptor kinase (βARK) reduces the CSP-mediated current inhibition as evident from a reduced level of PP relief.

(B) Kinetic analysis of the CSP-mediated G protein inhibition. The error bars indicate standard errors. The insets illustrate the pulse paradigm used in each panel. (Top) Development of prepulse relief as a function of prepulse duration. (Middle) Reinhibition kinetics following a 50 ms PP to +150 mV. (Bottom) Voltage dependence of PP relief.

(C) Comparison of the magnitudes of PP relief assessed via application of a 100 ms PP to +150 mV, followed by a test depolarization to +20 mV 10 ms after the end of the PP. Note that coexpression of the synprint domain of the N-type channel (α_{1B} residues 718–963) but not the corresponding domain of the α_{1C} II-III linker region (residues 754–901) is able to reduce the CSP-induced G protein inhibition of the N-type channel. Numbers in parentheses indicate the numbers of experiments; error bars denote standard errors.

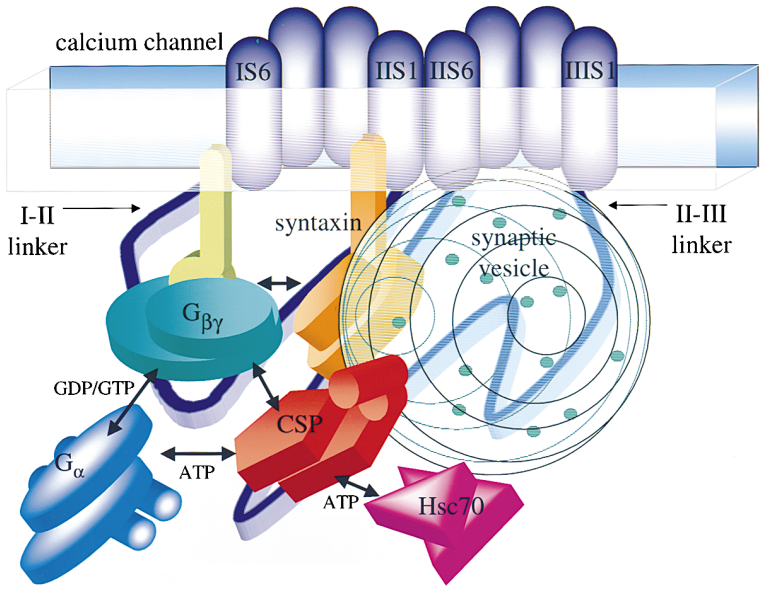

Figure 7. Model Depicting the Role of the CSP/Hsc70 Chaperone Machine in the Regulation of G Protein Modulation of Ca^{2+} Channel Activity

The synaptic vesicle protein CSP, by physically binding to the domain II-III linker region of the N-type Ca^{2+} channel subunit and to the $G_{\beta\gamma}$ protein dimer, may help target $G_{\beta\gamma}$ to its site of action on the N-type channel domain I-II linker. In addition, CSP may participate in the assembly/disassembly of the $G_{\alpha\beta\gamma}$ complex.

dependent on the test potential (bottom panel). Finally, as seen in Figure 6C, coexpression of CSP precluded an additional inhibitory effect of transiently overexpressed $G_{\beta\gamma}$ subunits as well as the tonic G protein inhibition of N-type calcium channels mediated by syntaxin 1A (Jarvis et al., 2000). The observed effects did not appear to depend on GDP/GTP exchange because inclusion of GDPβS in the patch pipette did not abolish the CSP-induced G protein inhibition (data not shown).

To assess whether the CSP-mediated G protein inhibition of N-type channel activity was dependent on an interaction between CSP and the channel rather than a general CSP-induced activation of G protein signaling in the tsa-201 cells, we coexpressed the complete synprint region of the α_{1B} II-III linker along with the channel and CSP to competitively inhibit CSP binding to the channel. If the G protein effect is indeed dependent on the physical interactions between CSP and the channel, then a reduction in CSP binding should be paralleled by a reduction in the G protein effect. As shown in Figure 6C, we observed a dramatic reduction (from 60% to 20%) in the degree of CSP-induced PP facilitation upon coexpression of the synprint domain. In contrast, modulation of channel activity by exogenously expressed $G_{\beta\gamma}$ was not affected by the presence of the synprint domain, indicating that the synprint domain did not generally interfere with G protein inhibition. A construct corresponding to the L-type channel II-III linker had no effect on the CSP-mediated G protein inhibition. However, given that different levels of $G_{\beta\gamma}$ may be expressed under different conditions, we cannot completely exclude the possibility that CSP might directly trigger the dissociation of the $G_{\alpha\beta\gamma}$ trimer, similar to what has been reported for certain types of RGS proteins (regulators of G protein signaling) (Bunemann and Hosey, 1998).

In summary, our experiments indicate that the interactions between CSP and the N-type calcium channels result in a robust tonic inhibition of channel activity by G protein $\beta\gamma$ subunits.

Discussion

Over the past 10 years, the molecular identification of a number of proteins essential for synaptic transmission has been realized. Many of the molecular determinants that govern the interaction between these proteins have been subject to intense study (reviewed in Scheller, 1995; Fernandez-Chacon and Sudhof, 1999). A major challenge is to define the biochemical pathway responsible for the exocytotic release of neurotransmitters, which constitutes the basis of most signaling between neurons of the central nervous system. Elucidation of this pathway must eventually explain the coupling of Ca^{2+} influx to synaptic vesicle fusion as well as the role of signal transduction pathways in the modulation of synaptic function. Although CSP is known to be essential for synaptic transmission, current genetic and biochemical analysis regarding the role of CSP in synaptic transmission has been controversial and has not permitted definitive functional conclusions. In this study, we provide evidence that CSP modulates G protein regulation of N-type Ca^{2+} channels and that CSP physiologically interacts with trimeric G proteins.

Our data suggest that CSP exists as a CSP/$G_{\beta\gamma}$ complex, a CSP/Ca^{2+} channel complex, a CSP/Hsc70 complex, and may well exist in larger multimers. G_{α} subunits interact with the J domain of CSP in an ATP-dependent manner; in contrast, $G_{\beta\gamma}$ subunits interact with the C terminus of CSP in both the presence and absence of ATP. The ATP-dependent interaction of G_{α} with the J domain of CSP does not involve $G_{\beta\gamma}$, and further investigation is required to establish the nature (i.e., direct or indirect) of this interaction. In addition, syntaxin also associates with these CSP-containing complexes through direct association with $G_{\beta\gamma}$ or the II-III linker synprint motif of the Ca^{2+} channel. Further investigation is required to establish the sequence of CSP–protein complex assembly/disassembly that takes place during synaptic transmission. Whereas the coprecipitation of CSP and Ca^{2+} channels is in accord with previous work (Leveque et al., 1998), reports concerning the coprecipitation of syntaxin and CSP are conflicting. For example, Leveque et al. (1998) did not observe the coimmunoprecipitation of CSP and syntaxin, while Nie et al. (1999) and Wu et al. (1999) found CSP and syntaxin to coimmunoprecipitate. In contrast, in *Drosophila*, a genetic interaction (Nie et al., 1999) and a biochemical interaction (Wu et al., 1999) between CSP and syntaxin has been re-

ported. Our observations indicate that a CSP/$G_{\beta\gamma}$ as well as a syntaxin/$G_{\beta\gamma}$ complex exists in brain tissue. The interaction of CSP with trimeric G proteins may explain the substantial diversity in reports concerning CSP function, since the experimental procedures utilized in these reports did not directly address the role of G proteins. Our results suggest that the effects of CSP on Ca^{2+} channel function may arise secondarily from the association with $G_{\beta\gamma}$ subunits. That current reports regarding the role of CSP in synaptic transmission are conflicting may be due to the fact that many of the previous experiments designed to address the role of CSP in synaptic transmission were not designed to evaluate the role of G proteins. In addition, conflicting reports in the literature may reflect different functional roles for G proteins and CSP in stimulated exocytosis.

Our observations indicate that CSP did indeed promote a tonic inhibition of N-type Ca^{2+} channel activity. In the presence of CSP, the channel became subject to substantial PP facilitation, one of the hallmarks of tonic $G_{\beta\gamma}$ modulation of voltage-dependent Ca^{2+} channels (i.e., Hille, 1994; Zamponi et al., 1997; Dolphin, 1998). In addition, the kinetic profile of this PP facilitation mirrored that typically observed with $G_{\beta\gamma}$ inhibition (i.e., Zhang et al., 1996; Zamponi and Snutch, 1998). Moreover, the degree of prepulse relief was greatly attenuated following coexpression of a known $G_{\beta\gamma}$ sponge, the carboxy-terminal fragment of the β-adrenergic receptor kinase (Koch et al., 1994). Finally, coexpression of CSP precluded the well-described inhibitory effects of exogenously expressed $G_{\beta\gamma}$ subunits on channel activity (see Herlitze et al., 1996; Ikeda, 1996; Zhang et al., 1996; Jarvis et al., 2000). Our in vitro binding observations, together with transient expression of key proteins in HEK cells and rapid chemical cross-linking intact hippocampal slices, strongly support a mechanism in which CSP promotes the inhibition of N-type Ca^{2+} channels by endogenously present G protein βγ subunits.

A current working model demonstrating the modulation of G protein inhibition of N-type Ca^{2+} channels by CSP is illustrated in Figure 7. In this model, CSP, when in close proximity to the active zone, associates with $G_{\beta\gamma}$ and Ca^{2+} channels, favoring a $G_{\beta\gamma}/Ca^{2+}$ channel complex and possibly preventing the formation of the $G_{\alpha}/G_{\beta\gamma}$ complex. The CSP-mediated promotion of $G_{\beta\gamma}$ binding to the N-type channel domain I-II linker region (De Waard et al., 1997; Herlitze et al., 1997; Zamponi et al., 1997) results in a tonic inhibition of presynaptic Ca^{2+} channels. In accordance with previous work (Rettig et al., 1996; Sheng et al., 1996), syntaxin is shown bound to the synprint motif of the Ca^{2+} channel. Since CSP also binds to the II-III cytoplasmic loop of presynaptic Ca^{2+} channels, we speculate that CSP may also chaperone interactions between Ca^{2+} channels and synprint-interacting proteins such as SNAP25 and syntaxin in addition to the $G_{\beta\gamma}$ association with the Ca^{2+} channel. The CSP-containing complexes shown are influenced by ATP and the localized cycling of synaptic vesicles (and therefore CSP) at the nerve terminal. Our model proposes that the synaptic vesicle protein CSP chaperones protein interactions with Ca^{2+} channels, thereby regulating Ca^{2+} entry through these channels. Given the current state of knowledge regarding the dynamic and cyclical nature of synaptic vesicles at the nerve terminals and the cyclical nature of receptor-coupled trimeric GTP binding proteins, we fully anticipate that CSP/G protein/Ca^{2+} channel interactions are dynamic. Further experimentation is required to reveal in "real time" the dynamic nature of these interactions. The regulation of Ca^{2+} channels by chaperones like CSP represents a novel concept with regard to the fine tuning of Ca^{2+} entry into presynaptic nerve terminals and a key factor in the control of neurotransmitter release and synaptic efficacy.

The interplay between SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) proteins, N-type Ca^{2+} channels, and cytoplasmic messenger molecules is becoming increasingly complex. For example, the binding of syntaxin to the N-type channel domain II-III linker appears to be antagonized by protein kinase C–dependent phosphorylation (Yokoyama et al., 1997). In addition, protein kinase C–dependent phosphorylation of the N-type channel domain I-II linker both upregulates channel activity (Hamid et al., 1999) and antagonizes G protein inhibition (Zamponi et al., 1997; Hamid et al., 1999). Both syntaxin (Stanley and Mirotznik, 1997; Jarvis et al., 2000) and CSP contribute to channel regulation by G protein βγ subunits. Recent evidence indicates that the extent of N-type channel modulation is dependent on the G_{β} subtype (Arnot et al., 2000; Ruiz-Velasco and Ikeda, 2000). Thus, nature has devised a multitude of avenues for the precise control of Ca^{2+} entry into presynaptic nerve terminals and, therefore, the fine tuning of neurotransmission (Wheeler et al., 1994). While CSP appears to contribute to this process, the tight interactions with heterotrimeric G proteins suggest a much broader role of this protein in cellular function.

Experimental Procedures

Preparation of Rat Hippocampal Homogenate

Rat hippocampi were hand homogenized with a teflon-coated homogenizer in 0.32 M sucrose, 10 mM HEPES KOH (pH 7.0), 1 mM EGTA, 0.1 mM EDTA, 0.5 mM PMSF, protease inhibitor cocktail (Bohringer Mannheim), 1 μM microcystin, 1 μM okadaic acid, and 1 mM sodium orthovanadate (2 ml/hippocampus). The homogenate was centrifuged for 10 min at 500 × g, and the supernatant was collected and subsequently centrifuged for 20 min at 20,000 × g (4°C). The pellet, containing the synaptic proteins, was resuspended in 1% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCl, 0.5 mM PMSF, protease inhibitor cocktail (Bohringer Mannheim), 1 μM microcystin, 1 μM okadaic acid, and 1 mM sodium orthovanadate and incubated for 30 min at 37°C. Following solubilization, large membrane fragments were removed by centrifugation at 1000 × g for 5 min. The resulting supernatant is a crude hippocampal homogenate that contains synaptic proteins. Protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin as the standard.

In Vitro Binding Assays

Glutathione S-transferase fusion proteins of CSP, J Box, and syntaxin and a His_6 α_{1B} Ca^{2+} channel II-III linker synprint motif fusion protein (amino acids 718–963) were prepared as described previously (Pevsner et al., 1994; Braun et al., 1996; Jarvis et al., 2000). Briefly, a GST fusion protein encoding a full-length CSP, the amino terminus of CSP (amino acids 1–82), or the cytoplasmic portion of syntaxin was constructed in the vector pGEX-KG (Guan and Dixon, 1991) and expressed in AB1899 strain of *E. coli*. DNA encoding residues 718–963 from the II-III linker of the α_{1B} Ca^{2+} channel subunit synprint region were amplified using PCR, subcloned into pTrcHisC (Invitrogen, CA), and expressed in *E. coli* TOP10. After induction of expression with 100 μM isopropyl-β-D-thiogalactopyranside for 5 hr, the bacteria were suspended in phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4), supplemented with 0.05% Tween 20, 2 mM EDTA, and 0.1% β-mercaptoethanol, and lysed by two passages through a French Press (Spectronic Instruments) or sonication (Branson Sonifier) in the case of the His_6 synprint protein. The fusion protein was recovered by binding of the GST domain to glutathione agarose beads

(Sigma) or Ni^{2+}-NTA agarose. The fusion protein beads were washed extensively and finally resuspended in 0.5% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCl, and 0.5 mM PMSF. Fusion proteins were incubated in the presence or absence of hippocampal homogenate, $G_{\beta\gamma}$ (Calbiochem), or recombinant CSP at 37°C for 30 min. The beads were washed, and bound proteins were eluted in sample buffer, fractionated by SDS-PAGE, and subjected to Western blot analysis. Recombinant CSP was purified from the GST fusion protein by cleavage with 0.2 μM thrombin in 0.5% Triton X-100, 20 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, and 150 mM KCl and followed by incubation in 0.3 mM PMSF. The protein concentration of recombinant proteins was estimated by Coomassie blue staining of protein bands after SDS-polyacrylamide gel electrophoresis using bovine serum albumin as a standard.

The EC_{50} was defined as the half-maximal binding of soluble CSP to the the immobilized synprint region, based on pixel intensity obtained by imaging (Amersham ECF; Storm Molecular Dynamics) and analyzed by ImageQuant software (Molecular Dynamics) (Pevsner et al., 1994). The wash procedure required ~4 min per sample, which may cause underestimates of both EC_{50} and the maximal level of binding. CSP was detected with anti-CSP polyclonal. The EC_{50} was estimated from plots of pixels versus concentration of protein added to beads.

Immunoprecipitation

CSP polyclonal antibodies raised against the peptide sequence CTQLTADSHPSYHTDGFN, corresponding to amino acids 182–198 of rat CSP, or full-length recombinant CSP were described previously (Braun and Scheller, 1995; Braun et al., 1996). Immunoprecipitation was achieved by incubating detergent-solubilized (1% Triton X-100) hippocampal homogenate with the indicated antibodies for 30 min at 37°C followed by protein A/G agarose (Santa Cruz Biotech) for 30 min, 22°C. Samples were washed, resuspended in 30 μl of sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies. Proteins were visualized using chemiluminescence (Amersham ECL) or fluorescence (Amersham ECF). In some cases, proteins were silver stained with Silver Stain Plus (Bio-Rad).

Cross-Linking of Intact Hippocampal Slices

Hippocampal slices were cross-linked with 4% paraformaldehyde as previously described (Braun and Madison, 2000). After cross-linking, hippocampal slices were hand homogenized (0.25 ml/slice) in 1% Triton X-100, 10 mM MOPS (pH 7.0), 4.5 mM $Mg(CH_3COO)_2$, 150 mM KCl, 10 mM glycine, and 0.5 mM PMSF (phenylmethylsulfonyl fluoride) and mixed end over end for 1 hr at 4°C. Sample buffer was added to the solubilized slice (final concentration 1% SDS, 42 mM Tris [pH 6.8], 7% glycerol, and 7% β- mercaptoethanol). Prior to SDS-PAGE, these samples were incubated at either 37°C, 80°C, or 100°C for 10 min. SDS-polyacrylamide electrophoresis was performed as described previously (Braun and Scheller, 1995).

Immunoblotting

Proteins were transferred electrophoretically at constant voltage from polyacrylamide gels to nitrocellulose (0.45 μm or 0.2 μm) in 20 mM Tris, 150 mM glycine, and 12% methanol. Transferred proteins were visualized by staining with Ponceau S. Nitrocellulose membranes were blocked for nonspecific binding using 5% milk, 0.1% Tween 20, and PBS solution (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 [pH 7.3]) and incubated overnight with primary antibody. The membranes were washed four times in the above milk/Tween/PBS solution and incubated for 30 min with goat anti-rabbit or goat anti-mouse IgG-coupled horseradish peroxidase. Antigen was detected using chemiluminescent horseradish peroxidase substrate (Amersham ECL). Immunoreactive bands were visualized following exposure of the membranes to Amersham Hyperfilm-MP.

Transient Transfection of HEK Cells

G protein subunits were expressed as described previously (Jarvis et al., 2000). cDNAs encoding the entire open reading frame of CSP, the carboxyl terminus of βARK (corresponding to residues 495–689), the α_{1B} II-III linker (corresponding to residues 718–963), and the entire α_{1C}II-III linker (corresponding to residues 754–901) were obtained by polymerase chain reaction from rat brain cDNA. Sequences were verified and subcloned into pMT2-SX for expression. PCR was used to form the full-length cDNAs encoding for the channels. The resultant DNA products were ligated into pGEM T-Easy vectors (Promega) and sequenced. G protein subunits and N-type protein Ca^{2+} channel subunits were prepared as described previously. Human embryonic kidney tsa-201 cells were grown in standard DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum and penicillin-streptomycin. The cells were grown to 85% confluency, split with trypsin-EDTA, and plated on glass coverslips at 10% confluency 12 hr before transfection. Immediately prior to transfection, the medium was exchanged, and a standard Ca^{2+} phosphate protocol was used to transfect the cells with cDNAs encoding for Ca^{2+} channel subunits (α_{1B}, α_2-δ, and β_{1b}), green fluorescent protein (EGFP-Clontech, CA), and as appropriate, cDNA constructs encoding CSP, J Box, βARKct, syntaxin, and/or Gβγ. After 12 hr, the cells were washed with fresh DMEM and allowed to recover for another 12 hr. The expression of transfected proteins was confirmed by Western blot analysis. Subsequently, the cells were incubated at 28°C in 5% CO_2 for 1–3 days prior to recording.

Patch Clamp Recordings

Immediately prior to recording, individual coverslips were transferred to a 3 cm culture dish containing recording solution comprised of 20 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM Tetraethylammonium chloride (TEA-Cl), 10 mM glucose, and 65 mM CsCl, (pH 7.2 with TEA-OH). Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) linked to a personal computer equipped with pCLAMP, version 6.0. Patch pipettes (Sutter borosilicate glass, BF150–86–15) were pulled using a Sutter P-87 microelectrode puller, fire polished using a Narashige microforge, and showed typical resistances of 3–4 MΩ. The internal pipette solution contained 108 mM CsMS, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES (pH 7.2). Data were filtered at 1 kHz and recorded directly onto the hard drive of the computer. Data were analyzed using Clampfit (Axon Instruments) and fitted using Sigmaplot 4.0 (Jandel Scientific). Unless stated otherwise, all error bars are standard errors, numbers in parentheses displayed in the figures reflect numbers of experiments, and p values given reflect Student's t tests.

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