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ONE-TRIAL *IN VITRO* CONDITIONING REGULATES AN ASSOCIATION BETWEEN THE β-THYMOSIN REPEAT PROTEIN Csp24 AND ACTIN

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Abstract—One-trial conditioning in *Hermissenda* results in enhanced intrinsic cellular excitability of sensory neurons in the conditioned stimulus pathway, and the phosphorylation of several proteins. Previous results demonstrated that the development of enhanced intrinsic excitability was dependent on the expression of conditioned stimulus pathway phosphoprotein-24 (Csp24), an intracellular protein containing four repeated β-thymosin homology domains. Consistent with this, antisense oligonucleotide-mediated inhibition of Csp24 expression prevents the reduction in amplitude of the A-type transient K^+ current (I_A) and the depolarized shift in the steady-state activation curve normally produced by one-trial *in vitro* conditioning of isolated photoreceptors. One-trial conditioning also regulates Csp24 phosphorylation. We now show that purified recombinant Csp24 sequesters G-actin *in vitro* with an approximate K_d value of 2.8 μM. We also observed a significant increase in the coprecipitation of actin with Csp24 after one-trial *in vitro* conditioning using antibodies directed toward either Csp24 or phospho-Csp24. Preincubation with protein kinase C (PKC) selective inhibitors attenuated the increase in Csp24 phosphorylation and coprecipitated actin observed after one-trial conditioning. Our findings indicate that the PKC signaling pathway contributes to the phosphorylation of Csp24 after one-trial conditioning, and that PKC activity modulates an association between Csp24 and actin. These data suggest Csp24 may influence intrinsic excitability by regulating cytoskeletal dynamics.

Key words: actin-binding, excitability, protein kinase C, phosphorylation, beta-thymosin repeat protein.

One-trial conditioning of *Hermissenda* results in long-term increased intrinsic cellular excitability of type B photoreceptor neurons in the conditioned stimulus (CS) pathway (Crow and Forrester, 1991; Crow and Siddiqi, 1997). The induction and maintenance of this increased excitability is dependent on the regulated expression and phosphorylation of 24 kDa conditioned stimulus pathway phosphoprotein (Csp24) (Crow and Xue-Bian, 2002; Crow et al., 2003). Csp24 exhibits significant amino acid sequence homology to members of the actin-binding β-thymosin protein family, and colocalizes with actin *in vivo* (Crow and Xue-Bian, 2000, 2002; Crow et al., 2003). β-Thymosins are the major G-actin sequestering proteins in vertebrate cells, and homologues have been identified in many vertebrate and invertebrate species. Although the best known role of the β-thymosins is sequestering G-actin, there is evidence indicating that they may also function in more diverse cellular pathways than only sequestering monomeric actin (Sanger et al., 1995; Carlier et al., 1996; Sun et al., 1996). Other functions that have recently been attributed to the β-thymosins include roles in cancer, apoptosis, inflammation, wound healing and stem cell proliferation, although the mechanisms by which the β-thymosins affect these processes have yet to be fully elucidated (reviewed in Huff et al., 2001).

The actin cytoskeleton is a critical component for establishing cellular polarity and morphology, as well as mediating motility, protein transport and localization. The actin cytoskeleton is actively remodeled according to the ongoing demands of the cell, and remodeling of its underlying structure depends on the dynamic interplay between a wide variety of actin-binding proteins. These actin-binding proteins are critical components of the cytoskeleton, serving a wide range of functions such as mediating signal transduction to the actin cytoskeleton, regulating actin filament polymerization/depolymerization, attaching actin filaments to the cell membrane, organizing the three-dimensional actin scaffold, and linking associated proteins to the actin network (Revenu et al., 2004; Stossel et al., 2001; Meyer and Feldman, 2002; Paavilainen et al., 2004; Sun et al., 1995, 1996).

Multiple lines of evidence highlight the importance of the cytoskeleton and actin-binding proteins for precise ion channel localization and regulation. For instance filamin, which crosslinks actin and induces the formation of orthogonal F-actin structures, is also directly involved in the binding and localization of the potassium channels Kv4.2, Kir2.1, and the cationic pacemaker channel HCN1 (hyperpolarization-activated, cyclic nucleotide-gated potassium channel 1), to specific regions of the cell membrane (Gravante et al., 2004; Petrecca et al., 2000; Sampson et al., 2003). The actin network has also been implicated in maintaining Kv2.1 clustering (O'Connell et al., 2006), which

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Abbreviations: ASW, artificial sea water; CS, conditioned stimulus; Csp24, 24 kDa conditioned stimulus pathway phosphoprotein (accession number AAN08024); CV, column volume; GF109203X, bisindolylmaleimide I; I_A, A-type transient K^+ current; IMAC, immobilized metal affinity chromatography; PB, phosphate buffer; PKC, protein kinase C; RIPA, radioimmunoprecipitation; βTH, beta-thymosin homology domain; 2-D, two-dimensional.

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may be an important factor in determining the intrinsic excitability of hippocampal pyramidal neurons (Misonou et al., 2004).

In addition to protein localization, actin-binding proteins can also influence channel activity. Tyrosine phosphorylation regulates the interaction of cortactin with Kv1.2, and channel activity is regulated by this interaction (Hattan et al., 2002). Similarly, the activities of Kv1.5, cystic fibrosis transmembrane conductance regulator, and polycystin-2 are influenced by the actin cytoskeleton (Maruoka et al., 2000; Cantiello, 1996; Montalbetti et al., 2005). In *Hermissenda*, we have shown that one-trial *in vitro* conditioning of isolated type B photoreceptors results in a reduction in the A-type K^+ current (I_A) amplitude, as well as a reduction in the depolarized shift in the steady-state activation curve of I_A. These changes in I_A are dependent on the expression of Csp24, as evidenced by their blockade after *Csp24* antisense oligonucleotide treatment (Yamoah et al., 2005). In addition to Csp24, protein kinase C (PKC) activation is also known to contribute to enhanced excitability underlying memory in *Hermissenda* (Farley and Auerbach, 1986; Matzel et al., 1990; Crow, 2004).

Here, we report that recombinant Csp24 sequesters G-actin with an approximate K_d value of 2.8 μM. In addition, one-trial *in vitro* conditioning of isolated circumesophageal nervous systems significantly increases the coprecipitation of actin with anti-Csp24 antibodies. Increased Csp24 phosphorylation, as well as actin coprecipitation, is significantly reduced by PKC-selective inhibitors. These data suggest that Csp24 may affect cytoskeletal dynamics through a PKC-dependent pathway, providing a potential mechanism for influencing intrinsic excitability in *Hermissenda*.

EXPERIMENTAL PROCEDURES

Expression and purification of Csp24

A bacterial expression plasmid containing full-length *Csp24* (Crow et al., 2003) fused at the carboxy terminal to a thrombin cleavage site (MYPRGNG) and a His_6 purification tag was generated by standard PCR methods using the oligonucleotides 5′-TCTGCCGCACCCCAAGGACTCAT-3′, and 5′-TTATTAATGATGATGATGATGATGACCATTCCCACGTGGATACATGGATCCGGAGCTGGCCTTCTCTGC-3′. The PCR product was cloned into pIVEX2.3 restricted at *Nco*I and *Bam*HI to yield the bacterial expression plasmid pIVEX2.3 CSP24-TH_6. JM109(*DE3*) *E. coli* were transformed with pIVEX2.3 CSP24-TH_6, grown in Luria broth supplemented with 55 mM glucose and 25 mM Tris pH 8.0 to OD_{600} ~0.4, and induced with 0.5 mM IPTG for 2–4 h to stimulate CSP24-TH_6 expression. Pelleted bacteria were weighed and resuspended at 10 ml/g in lysis buffer [50 mM phosphate buffer (PB) pH 7.0, 300 mM NaCl, 10 mM imidazole, 100 μl/g immobilized metal affinity chromatography (IMAC) protease inhibitor cocktail (Sigma, St. Louis, MO, USA), 1 mM DFP, 250 μg/ml lysozyme, 20 U/ml DNase I, 1× Bugbuster (EMD Biosciences, San Diego, CA, USA)], and incubated for 30 min at room temperature. The lysate was centrifuged 25,000×*g* for 30 min. at 4 °C, diluted 1:1 with 50 mM PB pH 7.0, 300 mM NaCl, 10 mM imidazole, 1 mM DFP, 1 mM benzamidine and mixed with 1–2 ml TALON IMAC resin (Clonetech, Mountain View, CA, USA) for 1 h at 4 °C to bind the recombinant protein. Columns were packed by gravity flow, rinsed with 10 column volumes (CV) 50 mM PB pH 7.0, 300 mM NaCl, 10 mM imidazole, washed with 10 CV 50 mM PB pH 7.0, 750 mM NaCl, 10 mM imidazole, and eluted with 50 mM PB pH 7.0, 300 mM NaCl, 200 mM imidazole. Fractions containing eluted CSP24-TH_6 were pooled and the buffer exchanged with 20 mM Tris pH 8.4, 150 mM NaCl using PD-10 columns (GE Healthcare, Piscataway, NJ, USA). To cleave the C-terminal His_6 purification tag, 2.5 mM $CaCl_2$ and 1.7 U/ml biotinylated thrombin were added and the solution digested overnight at 30 °C. Biotinylated thrombin was removed using excess streptavidin–agarose, and undigested protein and cleaved His_6 were removed by IMAC as described above. The purified Csp24 (flow-through) was exchanged into 10 mM Tris pH 7.8, 0.2 mM DTT using PD-10 columns, and further purified by size exclusion chromatography to remove any remaining truncated products. The purity and quantity of each preparation were assessed by SDS-PAGE and SYPRO Ruby fluorescent stain versus a known BSA standard. After purification, no visible protein bands other than full-length Csp24 were detected.

One-trial *in vitro* conditioning

Adult *Hermissenda crassicornis* were obtained from Sea Life Supply (Sand City, CA, USA) and maintained in artificial seawater (ASW) aquaria at 14±1 °C on a 12-h light/dark schedule. The one-trial *in vitro* conditioning procedure using isolated circumesophageal nervous systems has been described in detail previously (Crow and Forrester, 1991; Crow et al., 1996; Crow and Siddiqi, 1997; Crow and Xue-Bian, 2002). Briefly, the conditioning trial consisted of a 5 min presentation of 10^{-4} W/cm^2 light (conditioned stimulus, or CS) paired with the application of 5-HT to the isolated nervous system. The final concentration of 5-HT in the ASW was 10^{-4} M. Unpaired control groups received the CS and 5-HT separated by 5 min. For the unpaired control groups, the 5-HT was applied in red light and washed out after the 5 min exposure. For conditioned groups the 5-HT was washed out after the conditioning trial.

For experiments examining PKC, isolated circumesophageal nervous systems were incubated in ASW containing 5 μM of the PKC inhibitor bisindolylmaleimide I (GF109203X, final DMSO in ASW was 0.01%) or 2 μM of chelerythrine (final DMSO in ASW was 0.01%) 30 min before one-trial *in vitro* conditioning. Following *in vitro* conditioning the nervous systems were washed in fresh ASW containing the PKC inhibitor. Fifteen minutes after the conditioning trial, the nervous systems were prepared for $^{32}PO_4$-labeling and two-dimensional (2-D) gel separation of phosphoproteins as described previously (Crow et al., 1996, 1999; Crow and Xue-Bian, 2000). To minimize potential animal-to-animal variability, circumesophageal nervous systems from eight animals were pooled and used for each experimental treatment and control procedure in each experimental replication involving 2-D gel analysis. Immunoprecipitation experiments with conditioned and unpaired controls used three pooled nervous system preparations for each replication.

Protein phosphorylation, 2-D gel electrophoresis and immunoprecipitation

Protein phosphorylation after exposure to GF109203X or chelerythrine was examined in isolated circumesophageal nervous systems of conditioned preparations. Samples were analyzed by 2-D gel electrophoresis using a first dimension isoelectric focusing (IEF) gel with an immobilized pH gradient (pH 4–7) and a precast 8–18% linear gradient SDS–polyacrylamide gel for the second dimension as described in (Crow and Xue-Bian, 2000). For quantitative analysis of $^{32}PO_4$-labeled proteins, gels were exposed to phosphorimaging screens for 24 h, scanned, and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Densitometric analysis of Csp24 from SYPRO Ruby–stained gels was used to assess protein loading for the different