Monomeric 14-3-3 Protein Is Sufficient to Modulate the Activity of the *Drosophila* Slowpoke Calcium-dependent Potassium Channel*

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*Drosophila* 14-3-3*ζ* (D14-3-3*ζ*) modulates the activity of the Slowpoke calcium-dependent potassium channel (dSlo) by interacting with the dSlo binding protein, Slob. We show here that D14-3-3*ζ* forms dimers in *vitro*. Site-directed mutations in its putative dimerization interface result in a dimerization-deficient form of D14-3-3*ζ*. Both the wild-type and dimerization-deficient forms of D14-3-3*ζ* bind to Slob with similar affinity and form complexes with dSlo. When dSlo and Slob are expressed in mammalian cells, the dSlo channel activity is similarly modulated by co-expression of either the wild-type or the dimerization-deficient form of D14-3-3*ζ*. In addition, dSlo is still modulated by wild-type D14-3-3*ζ* in the presence of a 14-3-3 mutant, which does not itself bind to Slob but forms heterodimers with the wild-type 14-3-3. These data, taken together, suggest that monomeric D14-3-3*ζ* is capable of modulating dSlo channel activity in this regulatory complex.

14-3-3 proteins comprise a ubiquitous family of highly conserved proteins. They bind specifically to a variety of target proteins containing phosphoserine motifs and function as intracellular regulator or adaptor proteins in diverse cellular functions (1, 2). The crystal structures of human 14-3-3 and 14-3-3*δ* reveal that 14-3-3 proteins share a similar dimeric structure, with each monomer consisting of nine α-helices organized in an antiparallel array (3, 4). Co-crystallization of several ligand-bound 14-3-3 complexes further demonstrates that the 14-3-3 dimer is arranged in such a way that the ligand binding groove runs in opposite directions in each monomer, allowing simultaneous binding of two ligands to one 14-3-3 dimer (5). The ability of 14-3-3 proteins to form homodimers or heterodimers in cells has also been demonstrated using biochemical approaches (6, 7). However, the significance of 14-3-3 dimerization for target protein regulation has only been shown in a few instances. For example, although Raf binds equally well to both the dimeric and monomeric forms of 14-3-3, only the dimeric form of 14-3-3 is able to maintain Raf in an inactive state in the absence of GTP-bound Ras and to stabilize an active conformation of Raf in *vitro* (8). Other evidence supporting the importance of 14-3-3 dimerization comes from studies with a mutant form of 14-3-3 that sequesters endogenous 14-3-3 into heterodimers, in which only the wild-type subunit is capable of binding to target proteins (9, 10).

There are only two isoforms of 14-3-3 in *Drosophila*, D14-3-3*ζ* and ε. D14-3-3*ζ* is highly enriched in brain and presynaptic termini of motor axons and plays important roles in regulating olfactory learning and synaptic functions (11, 12). In previous studies, we have shown that D14-3-3*ζ* forms a regulatory protein complex with the *Drosophila* calcium-dependent potassium channel (dSlo), mediated by its binding to a novel channel binding protein, Slob (13, 14). The interaction between D14-3-3*ζ* and Slob is dependent upon the phosphorylations of two serine residues located at the N-terminal domain of Slob. Co-expression of dSlo with D14-3-3*ζ* and Slob in transfected cells results in a dramatic decrease in dSlo channel activity (14). These results provide evidence that D14-3-3*ζ* may participate in the regulation of ion channel activity and synaptic transmission in the nervous system.

To investigate the molecular mechanism of this regulatory protein complex, we explored the role of 14-3-3 dimerization in regulating dSlo channel function. We report here that D14-3-3*ζ* forms dimers when expressed in cell lines. Similar to mammalian 14-3-3, substitution of key amino acids in the N-terminal domain of D14-3-3*ζ* abolishes its dimerization and produces a monomeric form of D14-3-3*ζ*. We found that the D14-3-3*ζ* monomer is capable of forming a complex with dSlo, via binding to Slob. Moreover, the dSlo channel activity, measured using the whole-cell voltage clamp technique, is similarly modulated when dSlo is co-expressed with Slob, together with either the wild-type or the dimerization-deficient mutant form of D14-3-3*ζ*. We also show that modulation of dSlo channel activity by wild-type D14-3-3*ζ* is not affected by overexpression of another 14-3-3*ζ* mutant, which can heterodimerize with wild-type 14-3-3 but does not itself bind to Slob. Taken together, these results suggest that monomeric D14-3-3*ζ* is capable of modulating dSlo channel activity in this regulatory complex.

**EXPERIMENTAL PROCEDURES**

*Antibodies—* Rabbit polyclonal anti-dSlo, anti-Slob, and anti-14-3-3 antibodies were generated and purified as described previously (13, 14). Monoclonal anti-FLAG antibody (M2) was purchased from Sigma. Anti-D14 monoclonal antibody was kindly provided by Dr. D. Oprian (Brandeis University).

*cdNA Constructs and Mutagenesis—* The 14-3-3 dimerization-deficient mutants were created using site-directed mutagenesis of the following D14-3-3*ζ* amino acids: L15AE to Q15QR and R88VE to N88VQ. A PCR strategy with appropriate primers was used to introduce the pertinent mutations and to generate 1D4 or FLAG epitope tags on the C terminus of D14-3-3*ζ* cDNA. The PCR products were then cloned into the mammalian expression vector, pC7DNA3. Wild-type and dimerization-deficient mutant 14-3-3*ζ*-EBFP cDNAs were constructed by cloning...

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1 The abbreviations used are: dSlo, *Drosophila* calcium-dependent potassium channel; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DSS, diisocinimidyloxy; BFP, blue fluorescent protein; EBFP, enhanced BFP; GFP, green fluorescent protein; EGFP, enhanced GFP; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid.

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the respective D14-3-3ζ cDNA into the vector pEBFP-N1 (Clontech). QuikChange strategy (Stratagene) was used to generate mutations in D14-3-3ζ at amino acid residues Arg-59 and Arg-63 (R59A/R63A). Construction of dSlo-pcDNA3, dSlo-EGFP, and Slob-pcDNA3 was as described previously (13, 14).

**Transfection and Immunoprecipitation—**tsA201 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. A calcium phosphate transfection protocol was used to introduce cDNAs into the cells. Forty-eight hours after transfection, cells were lysed in lysis buffer containing 1% CHAPS, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 2 mM diethioctet, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A (Sigma)). After a centrifugation to remove insoluble debris from the lysate, the supernatant was precleared with 50 μl protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA). 14-3-3 proteins were immunoprecipitated by incubation with anti-ID4 or anti-FLAG antibodies (4 μg/ml) for 2 h at 4°C, followed by overnight incubation at 4°C with 50 μl protein A/G plus-agarose. The immunoprecipitates were then washed with lysis buffer five times.

**Chemical Cross-linking—**Transfected cells were lysed in a HEPES-based lysis buffer containing 1% Triton, 50 mM HEPES (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM NaN3, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Disuccinimidyl suberate (DSS) was added to the lysates to a final concentration of 0.5 mM, and they were incubated at 4°C with slow rotation for 1 h. The reaction was quenched with 1× SDS gel-loading buffer. Western Blot—Proteins in the cell lysates or immunoprecipitates were separated on polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with 5% nonfat milk in TBST (0.1% Tween 20 in TBS), the blots were probed with appropriate primary antibodies in blocking buffer at 4°C overnight. The membranes were then washed with TBST and incubated with a 1:3000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse IgG (Amer- sham Biosciences, Arlington Heights, IL) for 1 h at room temperature. After three washes of the membrane with TBST and one wash with TBS, protein complexes were visualized using the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

**Whole-cell Recording—**dSlo channel activity was recorded in the whole-cell configuration from tsA201 cells expressing dSlo-EGFP and Slob, together with pEBFP vector, or wild-type or dimerization-deficient mutant D14-3-3ζ-EBFP. For experiments with R59A/R63A 14-3-3ζ, cells were transfected with dSlo-EGFP, Slob and WT D14-3-3ζ-EBFP together with pcDNA3 vector, or R59A/R63A 14-3-3ζ-ID4, in a 1:1:1:1 molar ratio. The FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) was used to transfect cells with these cDNAs. Recordings were done 1–2 days after transfection on a Zeiss Axiovert 25 inverted fluorescence microscope (Zeiss). Transfected cells bearing both GFP and BFP fluorescence were identified with the fluorescein isothiocyanate and BFP filter sets. Patch electrodes with resistances 1–3 MΩ were pulled from borosilicate glass and fire-polished.

The bath solution contained (in millimolar): 30 KCl, 120 NaCl, 2 MgCl2, 1 EGTA, and 10 HEPES (pH 7.2). The pipette solution contained (in millimolar): 150 KCl, 2 MgCl2, 0.5 BAPTA, and 10 HEPES (pH 7.2). Free calcium concentrations in the pipette solution were determined as described previously (15). Whole-cell currents were filtered at 1 kHz and digitized at 20 kHz with an Axopatch 200A amplifier. Data acquisition and analysis were performed with pCLAMP8 software. All results are shown as mean ± S.E. Statistical significance was assessed by one-way analysis of variance.

**RESULTS**

**Dimerization of D14-3-3ζ—**The crystal structure of human 14-3-3ζ shows that the dimerization interface is formed by interaction of helix A with helices C and D (3, 4). Substitutions of several key amino acids in both helices A and D result in a dimer-deficient form of human 14-3-3ζ (8). Because D14-3-3ζ is highly homologous to its mammalian counterparts, with an 81.2% amino acid sequence identity between human 14-3-3ζ and D14-3-3ζ (Fig. 1A), we used site-directed mutagenesis to assess the role of these key amino acids in dimer formation of D14-3-3ζ.

Mutations of D14-3-3ζ were made in helix A (L15AE to Q15QR, MW-14-3-3ζ), or helix D (R88VE to N88VQ, WM-14-3-3ζ), or both (MM-14-3-3ζ) (Fig. 1, A and B). For biochemical studies, cDNA constructs for both the wild-type and mutant forms of D14-3-3ζ were tagged with either a 1D4 or FLAG epitope (Fig. 1B).

The ability of D14-3-3ζ to dimerize was first tested using a co-immunoprecipitation approach. Wild-type D14-3-3ζ with the 1D4 and FLAG epitope tags were co-expressed in tsA201 cells. D14-3-3ζ-FLAG was immunoprecipitated from cell lysates with anti-FLAG antibody, and the immunoprecipitate was probed with anti-1D4 antibody on a Western blot. As shown in Fig. 2A, lane 1, D14-3-3ζ-1D4 co-immunoprecipitates with D14-3-3ζ-FLAG. In addition, we used a chemical cross-linking method to verify the dimerization of D14-3-3ζ. When cell lysates from wild-type D14-3-3ζ-transfected cells were incubated with DSS, two bands corresponding to the molecular weights of the monomeric and dimeric D14-3-3ζ were detected on the Western blot (Fig. 2B, lane 1). Taken together, these results show that wild-type D14-3-3ζ is capable of forming dimers in vitro.

All three D14-3-3ζ mutants are readily expressed in tsA201 cells. On a Western blot, they migrate at positions similar to that of wild-type D14-3-3ζ (Fig. 2A), suggesting that they are metabolically stable. We then examined the effect of these mutations on D14-3-3ζ’s ability to dimerize. As shown in Fig. 2 (A and B), although substitutions of the two amino acids in helix D (WM-14-3-3ζ) have little effect on the dimerization of

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**FIG. 1. Site-directed mutagenesis for dimerization of D14-3-3ζ.** A, amino acid alignment for human and Drosophila 14-3-3ζ isoforms. The conserved amino acids are shown as capital letters. The regions corresponding to helicesaa—of human (H) 14-3-3ζ are indicated with shaded bars. The amino acids mutated for dimerization are surrounded with solid boxes for D14-3-3ζ or dotted boxes for H14-3-3ζ. B, schematic representation of the wild-type (WT) and mutant forms of D14-3-3ζ.

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**FIG. 2.** A, Western blot analysis for D14-3-3ζ and 1D4 co-immunoprecipitation in tsA201 cells. Lane 1, D14-3-3ζ-1D4 co-immunoprecipitates with D14-3-3ζ-FLAG. Lane 2, wild-type D14-3-3ζ-1D4 is not co-immunoprecipitated with D14-3-3ζ-FLAG.
FIG. 2. Dimerization of D14-3-3ζ. A, tsA201 cells were co-transfected with WT-FLAG and WT-1D4 (lane 1), WM-FLAG and MW-1D4 (lane 2), MM-FLAG and MM-1D4 (lane 3), and WT-FLAG and WT-1D4 (lane 4) forms of D14-3-3ζ. Cell lysates were probed for the expression of wild-type and mutant forms of D14-3-3ζ with either the anti-1D4 (middle panel) or anti-FLAG (bottom panel) antibodies. FLAG immunoprecipitates were probed with anti-1D4 antibody to detect the presence of 1D4-tagged D14-3-3ζ. A band corresponding to the molecular weight of D14-3-3ζ is detected in immunoprecipitates of WT- and WM-D14-3-3ζ but not MW- and MM-D14-3-3ζ transfected cells (top panel). B, cell lysates from tsA201 cells co-transfected with WT-FLAG and WT-1D4 (lane 1), WM-FLAG and WM-1D4 (lane 2), MM-FLAG and MM-1D4 (lane 3), and WT-FLAG and vector (lane 5) were chemically cross-linked with DSS, and probed on Western blot with a polyclonal antibody that recognizes D14-3-3ζ. Bands corresponding to the dimer form of D14-3-3ζ are present in WT and WM (lanes 1 and 2) but not MW and MM (lanes 3 and 4). D14-3-3ζ-transfected cell lysates. The dimer band of D14-3-3ζ is also seen in lysates from WT-FLAG transfected cells (lane 5).

FIG. 3. Dimerization of D14-3-3ζ is not required for Slob binding. tsA201 cells were transfected with Slob together with one of the FLAG-tagged D14-3-3ζ constructs: WT (lane 1), WM (lane 2), MW (lane 3), MM (lane 4), or a control vector (lane 5). D14-3-3ζ was immunoprecipitated with anti-FLAG antibody and probed on Western blot with anti-Slob antibody (top panel). Slob is present in immunoprecipitates from the WT (lane 1) and all three variants of mutant D14-3-3ζ (lanes 2–4) but not in that from vector-transfected cells (lane 5). Expression of both Slob (bottom panel) and all forms of D14-3-3ζ (middle panel) is similar under all transfection conditions.
**Fig. 4.** The dimerization-deficient mutant D14-3-3ζ forms a complex with dSlo channels. tsA201 cells were transfected with dSlo and Slob together with FLAG-tagged WT (lane 1), MM-D14-3-3ζ (lane 2), or a control vector (lane 3). D14-3-3ζ was immunoprecipitated with anti-FLAG antibody and probed on Western blot with anti-dSlo antibody (top panel). dSlo is present in immunoprecipitates from both WT (lane 1) and the MM-D14-3-3ζ (lane 2) but not in that from vector-transfected cells (lane 3). Expression of dSlo (middle panel) and the WT and MM-D14-3-3ζ (bottom panel) is similar under all transfection conditions.

**Fig. 5.** dSlo channel activity is modulated by both dimeric and monomeric D14-3-3ζ. dSlo currents were evoked by 350-ms depolarizing voltage steps from −30 mV in 10-mV increments, in the whole-cell voltage clamp configuration. Representative current traces recorded from tsA201 cells transfected with dSlo and Slob together with a control vector (A), the WT-D14-3-3ζ (B), or the MM-D14-3-3ζ (C). D, the activation time constant (τ) (mean ± S.E.), from single-exponential fits of dSlo currents evoked at +150 mV, is significantly increased with co-transfection of either the WT or MM-D14-3-3ζ (p < 0.05). E, the peak dSlo current density at +150 mV (mean ± S.E.), normalized to membrane capacitance, is significantly lower in cells co-transfected with either the WT or MM D14-3-3ζ (p < 0.05).
D14-3-3ζ (lane 2), mutations of the three amino acids in helix A (MW-D14-3-3ζ) completely abolish the formation of a D14-3-3ζ dimer in vitro, as determined by both co-immunoprecipitation and cross-linking assays (lane 3). In addition, substitution of all five amino acids in both helices A and D (MM-D14-3-3ζ) also results in a total loss of D14-3-3ζ dimerization (Fig. 2, A and B, lane 4). These data indicate that the key amino acids identified in human 14-3-3ζ are conserved and critical for dimerization of D14-3-3ζ as well (8).

Dimerization-deficient Mutants of D14-3-3ζ Bind Slob and Form Complexes with dSlo—To determine if the dimerization-deficient mutants of D14-3-3ζ are capable of binding Slob, we again utilized the co-immunoprecipitation strategy. Wild-type or dimerization-deficient mutants of D14-3-3ζ were co-expressed with Slob in tsA201 cells, and lysates were immuno-precipitated with antibodies specific for 14-3-3. The immunoprecipitates were probed with anti-Slob antibody for 14-3-3-bound Slob. As shown in Fig. 3, all three mutants of D14-3-3ζ bind Slob as well as does wild-type D14-3-3ζ, suggesting that these mutations in D14-3-3ζ do not affect its ability to interact with Slob. These results suggest that the D14-3-3ζ monomer is sufficient to bind Slob.

Wild-type D14-3-3ζ interacts with the dSlo channel, via its binding to Slob (14). We asked whether the dSlo/Slob-D14-3-3ζ protein complex persists with monomeric D14-3-3ζ. Either the wild-type or dimerization-deficient mutant D14-3-3ζ (MM-D14-3-3ζ) was co-expressed with both Slob and dSlo in tsA201 cells, and the 14-3-3 immunoprecipitate was probed on a Western blot with anti-dSlo antibody for dSlo protein present in the complex. As shown in Fig. 4, dSlo was co-immunoprecipitated equally well with both the wild-type (lane 1) and dimerization-deficient mutant MM-D14-3-3ζ (lane 2). These results show that, in the presence of Slob, the monomeric D14-3-3ζ is able to enter into a protein complex with the dSlo channel.

Monomeric Form of D14-3-3ζ Modulates dSlo Channel Activity—We have shown previously that co-transfection of D14-3-3ζ, together with Slob, dramatically decreases the activity of dSlo channels (14). To investigate whether dimerization of D14-3-3ζ is necessary for modulation of the dSlo channel by D14-3-3ζ, we measured dSlo currents using the whole-cell voltage-clamp configuration. dSlo-EGFP and D14-3-3ζ-EBFP were used in these experiments to identify co-transfected cells by fluorescence. In tsA201 cells transfected with cDNAs for both dSlo and Slob, whole-cell K+ currents were elicited by depolarizing voltage steps from a holding potential of −80 mV, with 30 μM intracellular free Ca2+ (Fig. 5A). When wild-type D14-3-3ζ was co-expressed with Slob and dSlo, dSlo channel activity was decreased dramatically (Fig. 5B), as evidenced by a slowdown of activation kinetics (Fig. 5D) and a reduction of peak current amplitude (Fig. 5E) evoked by the same depolarizations. This is consistent with our previous study in detached membrane patches (14).

To determine if the D14-3-3ζ dimer is required to modulate the dSlo channel activity in this protein complex, we fused EBFP to one of the dimerization-deficient mutants of D14-3-3ζ, MM-D14-3-3ζ, which does not dimerize but interacts with dSlo via Slob (Figs. 2–4). Fusion of EBFP to wild-type and mutant D14-3-3ζ does not interfere with their binding to Slob (data not shown). In addition, we used chemical cross-linking to confirm that EBFP-tagged wild-type I4-3-3 proteins still form dimers, but MM-D14-3-3ζ-EBFP does not dimerize (data not shown). As shown in Fig. 5C, co-transfection of MM-D14-3-3ζ, together with Slob and dSlo, also decreases the whole-cell current of dSlo channels. The extent of reduction of current amplitude and the slowdown of activation by co-transfection of D14-3-3ζ is not significantly different between wild-type and dimeriza-

dition-deficient forms of D14-3-3ζ (Fig. 5, D and E). At +150 mV, the time constant for activation (τ) of dSlo current is 7.8 ± 4.3 ms in the absence of D14-3-3ζ and 120.9 ± 43.2 ms or 134.9 ± 71.5 ms in the presence of wild-type or mutant D14-3-3ζ, respectively (Fig. 5D). The dSlo current density at +150 mV is 805 ± 397 pA/pF in control and 353 ± 80 pA/pF in WT co-transfected and 334 ± 126 pA/pF in MM-D14-3-3ζ co-transfected cells (Fig. 5E). Both parameters are significantly different between control and either the wild-type or mutant D14-3-3ζ co-transfected cells but not between wild-type and mutant D14-3-3ζ groups. This suggests that the monomeric D14-3-3ζ is sufficient to modulate dSlo channel activity.
Modulation of dSlo Channel Activity by D14-3-3ζ Is Not Affected by Overexpression of a D14-3-3ζ Mutant That Does Not Bind Slob—To explore further the role of dimerization of D14-3-3ζ in the modulation of dSlo channel activity, we constructed another mutant, R59A/R63A D14-3-3ζ, which no longer binds Slob (Fig 6A) but still forms a dimer with wild-type D14-3-3ζ (Fig. 6B, lane 1). It has been reported that similar mutants of mammalian 14-3-3 isoforms can form a heterodimer, in which only the wild-type subunit is able to bind the target protein. This mutant thus can function as a dominant negative when dimer binding is required for the targeting of 14-3-3 proteins (9, 16).

We first asked whether R59A/R63A D14-3-3ζ can influence the binding of WT-D14-3-3ζ homodimers to Slob, via forming heterodimers with WT-D14-3-3ζ. To demonstrate that Slob is able to bind 14-3-3 homodimers, we used a different approach to detect the 14-3-3 proteins in this protein complex. Slob was co-expressed with either wild-type or the dimerization-deficient mutant MM-D14-3-3ζ, and lysates were immunoprecipitated with antibody specific for Slob. The immunoprecipitates were then probed with anti-14-3-3 antibody for Slob-bound 14-3-3. As shown in Fig. 7, there is more 14-3-3 protein in Slob immunoprecipitates from cells co-transfected with WT-D14-3-3ζ than with MM-D14-3-3ζ (compare lanes 2 and 4 in the top panel). Because both WT- and MM-D14-3-3ζ have similar affinity for Slob (Fig. 3), this result suggests that Slob can bind to either 14-3-3 homodimers or monomers.

Moreover, when an excess of R59A/R63A D14-3-3ζ is co-expressed with either wild-type or the dimerization-deficient mutant D14-3-3ζ (Fig. 7), the amount of D14-3-3ζ detected in Slob immunoprecipitates is reduced only in WT (compare lanes 1 and 2) but not in MM-D14-3-3ζ (compare lanes 3 and 4) co-transfected cells. Because R59A/R63A D14-3-3ζ does not directly bind to Slob and only dimerizes with WT-D14-3-3ζ but not with MM-D14-3-3ζ (Fig. 6), the reduction of WT-D14-3-3ζ in the Slob-D14-3-3ζ complex suggests that R59A/R63A D14-3-3ζ heterodimers with WT 14-3-3ζ compete with WT 14-3-3 homodimers for binding to Slob.

We next investigated whether R59A/R63A D14-3-3ζ has an effect on the modulation of dSlo channel activity by D14-3-3ζ. Whole-cell dSlo currents were recorded from cells transfected with dSlo, Slob, and WT D14-3-3ζ in the absence or presence of R59A/R63A D14-3-3ζ. We found no significant difference in either current amplitude or activation kinetics between cells with (Fig. 8B) or without (Fig. 8A) co-transfection of R59A/R63A D14-3-3ζ. At +150 mV, the time constant for activation of dSlo current is 122.3 ± 104.2 ms in the absence and 111.2 ± 17.9 ms in the presence of R59A/R63A D14-3-3ζ (Fig. 8C), and the dSlo current density is 392 ± 201 pA/pF in control and 343 ± 118 pA/pF in R59A/R63A D14-3-3ζ co-transfected cells (Fig. 8D). These data show that, although R59A/R63A D14-3-3ζ can heterodimerize with wild-type D14-3-3ζ, it does not interfere with the ability of D14-3-3ζ to modulate dSlo channel activity via Slob. That is, R59A/R63A D14-3-3ζ does not act as a dominant negative for this response. Taken together with the results in Fig. 6, these data provide further evidence that dimer binding of D14-3-3ζ to Slob is not required for the modulation.

DISCUSSION

Since their rediscovery in the late 1980s, 14-3-3 proteins have been recognized as a family of multifunctional proteins that bind to and regulate the function of a variety of cellular proteins (1, 7, 17). While efforts continue to identify novel 14-3-3 binding partners, recent attention has focused on investigating the molecular mechanism of 14-3-3 function, especially the role of 14-3-3 dimerization (2, 18). Two modes of action have been attributed to the dimeric nature of 14-3-3 proteins. First, because one 14-3-3 dimer allows simultaneous binding of two ligands, 14-3-3 proteins can serve as a scaffold to bridge the interaction between two different proteins. Such 14-3-3-mediated association has been reported for several proteins (19–21). On the other hand, 14-3-3 proteins can cause conformational changes in their binding partners via interaction of the 14-3-3 dimers with multiple sites in the target protein (8, 22).

We demonstrated previously that D14-3-3ζ modulates the activity of the dSlo channel through its interaction with the dSlo-binding protein, Slob (13, 14). The interaction between
D14-3-3 and Slob requires phosphorylation of Slob by calcium/calmodulin-dependent kinase II and is abolished by mutations in two phosphoserine motifs in Slob (14). Based on these observations, we asked whether modulation of dSlo channel activity requires dimerization of D14-3-3. This may involve one of the following mechanisms: 1) 14-3-3 dimers may bring other proteins into this complex, thereby allowing these proteins to modulate dSlo or 2) 14-3-3 may change the conformation of Slob upon simultaneous binding of the 14-3-3 dimer to the two phosphoserine motifs in Slob.

Using a combination of molecular biological and biochemical methods, we demonstrate here that D14-3-3 proteins form dimers in a heterologous expression system, and the amino acid residues crucial for dimerization are conserved between Drosophila and human 14-3-3 proteins, indicating that they have similar dimerization interfaces (4). The dimerization-deficient mutants of D14-3-3 can be stably expressed in transfected cells. They not only bind well to Slob but also form complexes with dSlo. This is similar to reports from other groups showing that other proteins bind monomeric and dimeric forms of 14-3-3 with similar efficiency (23, 24). We show further that co-expression of either the dimeric or the dimerization-deficient form of D14-3-3 with Slob, decreases the activity of dSlo channels in transfected cells. No significant difference was observed between wild-type and the dimerization-deficient mutant of D14-3-3 in modulating dSlo channel activity, as measured by changes in both the activation kinetics and the peak current density of whole-cell dSlo currents. In addition, we found that modulation of dSlo channel activity by wild-type D14-3-3 is not affected by overexpression of a mutant, R59A/R63A D14-3-3, which interferes with the binding of 14-3-3 homodimers to Slob. These results, taken together, indicate that the modulation of dSlo channel activity does not require the formation of 14-3-3 dimers.

It is not uncommon to find examples in which dimerization of 14-3-3 is not essential for its function (25, 26). Our studies provide evidence that binding of monomeric D14-3-3 is sufficient to change dSlo channel properties. It rules out the possibility that the modulation observed in this overexpression system is dependent upon other regulatory proteins that might have been bridged by a 14-3-3 dimer. However, the precise molecular mechanism underlying the profound modulation of dSlo channel activity by Slob and D14-3-3 remains to be determined.

It is well-established that ion channels are subject to modulation by intimately associated proteins, including auxiliary channel subunits, protein kinases, phosphatases, and other regulatory proteins. Although 14-3-3 proteins are abundant in brain, exploration of the function of 14-3-3 proteins in the nervous system has just begun. Several groups have reported

**Fig. 8.** Modulation of dSlo by D14-3-3 is not affected by overexpression of R59A/R63A D14-3-3. Whole-cell dSlo currents were recorded from tsA201 cells using recording conditions identical to those described for Fig. 5. Representative current traces recorded from tsA201 cells transfected with dSlo, Slob, and WT-D14-3-3 together with a control vector (A) or with R59A/R63A D14-3-3 (B) in a 1:1:1:2 molar ratio. Co-transfection of R59A/R63A D14-3-3 does not significantly change either the activation time constant (mean ± S.E.) (C) (p > 0.5) or the peak dSlo current density (mean ± S.E.) (D) (p > 0.5), at +150 mV.
that 14-3-3 proteins interact with various types of ion channels and membrane receptors, including Ca\(^{2+}\)-activated Cl\(^{-}\) channels, growth factor receptors, and γ-aminobutyric acid, type B and α2-adrenergic receptors (27–30). Moreover, a very recent report shows that 14-3-3 proteins associate with the human ether-a-go-go-related channel and that this protein-protein interaction amplifies and prolongs adrenergic stimulation of human ether-a-go-go-related K\(^{+}\) channel activity (16). Taken together with our finding that 14-3-3 modulates dSlo channel activity in *Drosophila*, these data strongly suggest that the 14-3-3 protein family plays important roles in regulating diverse neuronal functions.

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