Shaker and Ether-à-Go-Go K^+ Channel Subunits Fail to Coassemble in *Xenopus* Oocytes

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ABSTRACT Members of different voltage-gated K^+ channel subfamilies usually do not form heteromultimers. However, coassembly between *Shaker* and ether-à-go-go (eag) subunits, members of two distinct K^+ channel subfamilies, was suggested by genetic and functional studies (Zhong and Wu. 1991. *Science*. 252:1562–1564; Chen, M.-L., T. Hoshi, and C.-F. Wu. 1996. *Neuron*. 17:535–542). We investigated whether *Shaker* and eag form heteromultimers in *Xenopus laevis* oocytes using electrophysiological and biochemical approaches. Coexpression of *Shaker* and eag subunits produced K^+ currents that were virtually identical to the sum of separate *Shaker* and eag currents, with no change in the kinetics of *Shaker* inactivation. According to the results of dominant negative and reciprocal coimmunoprecipitation experiments, the *Shaker* and eag proteins do not interact. We conclude that *Shaker* and eag do not coassemble to form heteromultimers in *Xenopus* oocytes.

INTRODUCTION

Neurons are capable of firing action potentials in diverse patterns largely due to the complement of K^+ channels they contain (Hille, 1992). One major group of K^+ channels comprises those that are gated by changes in the membrane potential. Voltage-dependent K^+ channels include four membrane-associated α subunits that contain the voltage sensor and form the pore (MacKinnon, 1991; Hartmann et al., 1991; Liman et al., 1992; Li et al., 1994; Schulteis et al., 1996; Seoh et al., 1996). In neurons, these α subunits may be identical or may be different members of a subfamily of closely related proteins (Sheng et al., 1993; Wang et al., 1993). Because channels containing mixtures of α subunits often have functional properties distinct from channels composed of identical subunits, differences in subunit composition contribute to K^+ channel diversity (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). As a result, the regulation of subunit composition has important functional consequences for neurons.

K^+ channel α subunits have been divided into subfamilies on the basis of sequence analysis (Warmke and Ganetzky, 1994; Chandy and Gutman, 1995; Hugnot et al., 1996; Wei et al., 1996; Jan and Jan, 1997). To determine whether members of different subfamilies can coassemble to form functional channels, electrophysiological and biochemical methods have been applied (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; McCormack et al., 1990; Covarrubias et al., 1991; Li et al., 1992; Sheng et al., 1993; Wang et al., 1993; Deal et al., 1994). For instance, coexpression of two different α subunits from the Kv1 subfamily, Kv1.1 and Kv1.4, generates a current with novel inactivation kinetics, single channel conductance, and pharmacology, suggesting that the Kv1.1 and Kv1.4 proteins assemble into heteromultimeric K^+ channels (Ruppersberg et al., 1990). Heteromultimers form between members of the same K^+ channel subfamily but, in general, members of different subfamilies do not coassemble (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; McCormack et al., 1990; Covarrubias et al., 1991; Li et al., 1992; Sheng et al., 1993; Wang et al., 1993; Deal et al., 1994). Recently, some exceptions to this rule have been reported (Hugnot et al., 1996; Post et al., 1996). For example, Kv6.1, which does not form functional channels when expressed alone, associates with Kv2.1 to generate a novel current (Post et al., 1996).

The *Drosophila Shaker* and ether-à-go-go (eag) K^+ channel subunits are members of two distinct subfamilies (Guy et al., 1991; Chandy and Gutman, 1995; Wei et al., 1996). Whereas the activity of *Shaker* channels is controlled primarily by voltage, the activity of the voltage-dependent eag channel is modulated by cyclic nucleotides (Brüggemann et al., 1993). A possible association between *Shaker* and eag subunits has been suggested on the basis of genetic and functional experiments (Zhong and Wu, 1991, 1993). Voltage clamp studies in *Drosophila* larval muscle fibers indicate that mutations at the *eag* locus affect all identified K^+ currents, including those specifically eliminated by mutations in the *Shaker* and *slowpoke* genes (Zhong and Wu, 1991). This observation led to the proposal that eag subunits coassemble with a wide variety of K^+ channel subunits, thereby contributing to the diversity of K^+ channels in vivo (Zhong and Wu, 1993). Recently, the same group reported that upon coexpression of *Shaker* and eag subunits in *Xenopus laevis* oocytes, the time course of inactivation becomes faster (Chen et al., 1996), raising the possibility that *Shaker* and eag coassemble to form functional channels.

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We have reexamined this possibility by using both electrophysiological and biochemical approaches. We report that coexpression of *Shaker* and eag subunits results in a K^+ current virtually identical to a summation of *Shaker* and eag current traces, with no change in inactivation kinetics. In addition, we find no evidence for interaction between the *Shaker* and eag proteins in dominant negative and reciprocal coimmunoprecipitation experiments. Therefore, we conclude that *Shaker* and eag subunits do not coassemble in *Xenopus* oocytes.

MATERIALS AND METHODS

Molecular biology

The *Shaker* B cDNA (Schwarz et al., 1988) was subcloned into the Bluescript II KS(+) vector (Stratagene, La Jolla, CA) and linearized with *Eco*RI. The Kv2.1 cDNA (Frech et al., 1989) was subcloned into the Bluescript II SK(−) vector (Stratagene, La Jolla, CA) and linearized with *Not*I. The eag cDNA (Warmke et al., 1991) was subcloned into the pGEMHE vector (Liman et al., 1992) and linearized with *Not*I. RNA was transcribed using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). To construct an epitope-tagged eag (eag-AU5), the six amino acid (TDFYLK) AU5 sequence was inserted immediately after the initiation methionine using a four-primer PCR strategy on the eag cDNA template (Horton et al., 1989; Lim et al., 1990). To generate a truncated, amino-terminal fragment of the *Shaker* protein (Sh1–246), the *Shaker* cDNA was digested with *Xba*I and *Spe*I, and the compatible ends were religated. This produced a large deletion and a frame shift in the sequence, resulting in a protein that consists of amino acids 1 to 246 of *Shaker*, plus eight additional amino acids before termination by a stop codon.

Electrophysiology

Oocytes were obtained from *Xenopus* frogs as previously described (Papazian et al., 1991). The total amount of *Shaker* cRNA injected was 0.1–0.5 ng per cell, which resulted in current amplitudes ranging from 0.5 to 50 μA at +80 mV. Only experiments with peak current amplitudes of 15 μA or less were used for analysis. *Shaker*, eag, eag-AU5, or Kv2.1 cRNAs were injected separately or in combination in the indicated molar ratio. Ionic currents were recorded 24–48 h after injection using a two-electrode voltage clamp (Warner Electronics, Hamden, CT). The bath solution was modified Barth's saline containing 1 mM KCl and 88 mM NaCl (Timpe et al., 1988). Linear leak and capacitive currents were subtracted using the P/-4 protocol (Bezanilla and Armstrong, 1977). Data were sampled at 30 μs per point and subjected to low-pass filtering at 1 kHz. All recordings were made at room temperature (20–22°C). The time course of inactivation was fitted with one exponential function using CLAMPFIT software (Axon Instrument, Foster City, CA). For dominant-negative experiments, Sh-IR, which contains a deletion of amino acids 6–46 to remove N-type inactivation, was used instead of wild-type *Shaker* (Hoshi et al., 1990). Sh1–246 cRNA was coinjected with Sh-IR, eag, or Kv2.1 cRNAs in the indicated molar ratios.

Biochemistry

For metabolic labeling of proteins, oocytes were coinjected with in vitro translation grade [^{35}S]-methionine and cRNA as previously described (Santacruz-Toloza et al., 1994b). *Shaker* (75 ng per cell), eag-AU5, or an equimolar mixture of *Shaker* and eag-AU5 cRNAs was injected into oocytes, keeping the total molar amount of cRNA constant. After 48 h, oocytes were disrupted in the presence of protease inhibitors either by brief sonication in 10% sucrose solution as previously described (Santacruz-Toloza et al., 1994b), or by brief homogenization in buffer H (100 mM NaCl, 20 mM Tris-HCl, 1% Triton X-100, pH 7.4) (Hollmann et al., 1994). Membrane proteins were solubilized in buffer H and subjected to centrifugation at 100,000 × *g* for 30 min at 4°C to remove insoluble material. Immunoprecipitations were performed by using antisera against a *Shaker*-β-galactosidase fusion protein (kind gift of Dr. Lily Jan), or AU5-specific monoclonal antibodies (Berkeley Antibody Company, Richmond, CA). For sucrose density gradient sedimentation, eag-AU5 or *Shaker* protein was separately expressed and labeled, solubilized in 1% Triton or 1% Zwittergent 3–12, and loaded on a 5–20% sucrose gradient (11 ml) containing either 1% Triton or Zwittergent (Nagaya and Papazian, 1997). Gradients were centrifuged at 36,000 rpm in a SW41 rotor for 20 h at 20°C. Fractions were collected from the bottom of each gradient and subjected to immunoprecipitation (Santacruz-Toloza et al., 1994b). Proteins were subjected to electrophoresis on 7.5% denaturing polyacrylamide gels followed by fluorography. Fluorographs were scanned and analyzed using a Model GS-700 scanning densitometer and Molecular Analyst Software version 1.5 (Bio-Rad, Hercules, CA).

Alternatively, proteins were expressed in oocytes without metabolic labeling. After immunoprecipitation and electrophoresis, proteins were transferred to nitrocellulose and the resulting immunoblots were probed with *Shaker* antibodies (1:250 dilution), followed by goat anti-rabbit IgG coupled to horseradish peroxidase (1:5000 dilution). Labeling was detected by enhanced chemiluminescence according to the manufacturer's protocol (Amersham Life Science, Buckinghamshire, UK).

RESULTS

Coexpression of *Shaker* and eag subunits does not alter inactivation kinetics

Shaker and eag cRNAs were injected into *Xenopus* oocytes separately and in mixtures containing different molar ratios of *Shaker* to eag cRNA (1:1, 1:2, and 1:3). K^+ currents were recorded using a two-electrode voltage clamp (Fig. 1 *A*). As expected, *Shaker* currents were characterized by rapid activation and nearly complete inactivation, whereas eag currents activated more slowly and did not inactivate significantly (Brüggemann et al., 1993; Robertson et al., 1996; Tang and Papazian, 1997). Currents recorded after coexpression of *Shaker* and eag subunits contained a fast, inactivating component, followed by a prominent sustained component. In oocytes expressing an excess of eag subunits (cRNA ratios 1:2 and 1:3), the slow activation kinetics of the sustained component were apparent. Coinjection with eag did not significantly change the amplitude of the peak *Shaker* current (data not shown).

If the current resulting from coexpression represents the activity of separate populations of *Shaker* and eag channels, then the shape of the current should correspond to a sum of *Shaker* and eag currents. Separate *Shaker* and eag currents were added and compared to scaled current traces obtained after coexpression (Fig. 1 *B*). The shapes of the summed currents were virtually identical to those obtained from coexpression of *Shaker* and eag at each injection ratio.

To compare the time course of inactivation, the inactivating component at +60 mV was fitted with a single exponential function (Fig. 2 *A*). We found no statistically significant difference between the inactivation time constant for *Shaker* expressed alone or in the presence of eag at three different molar ratios (Fig. 2 *B*). In each case, the time constant was between 2 and 4 ms, with a mean value of ~3

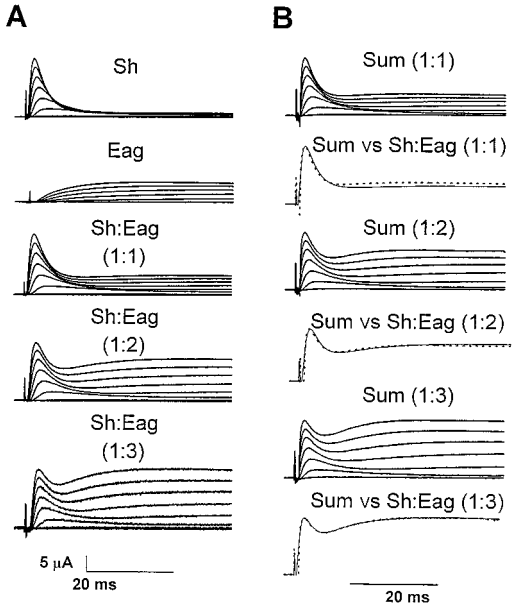

FIGURE 1 Coexpression of *Shaker* and eag subunits in *Xenopus* oocytes. (*A*) *Shaker* and eag cRNAs were injected separately or in the indicated molar ratios, keeping the amount of *Shaker* cRNA constant. Current traces were recorded using a two-electrode voltage clamp. From a holding potential of −80 mV, 48 ms test pulses were applied from −60 to +80 mV in 20 mV increments. (*B*) Separate *Shaker* and eag currents at similar expression levels were summed (*sum*) and compared to currents obtained from coexpression of *Shaker* and eag at ratios of 1:1, 1:2, or 1:3, as indicated. After scaling the summed traces, the summed (*dashed lines*) and coexpressed (*solid lines*) currents at +80 mV were superimposed.

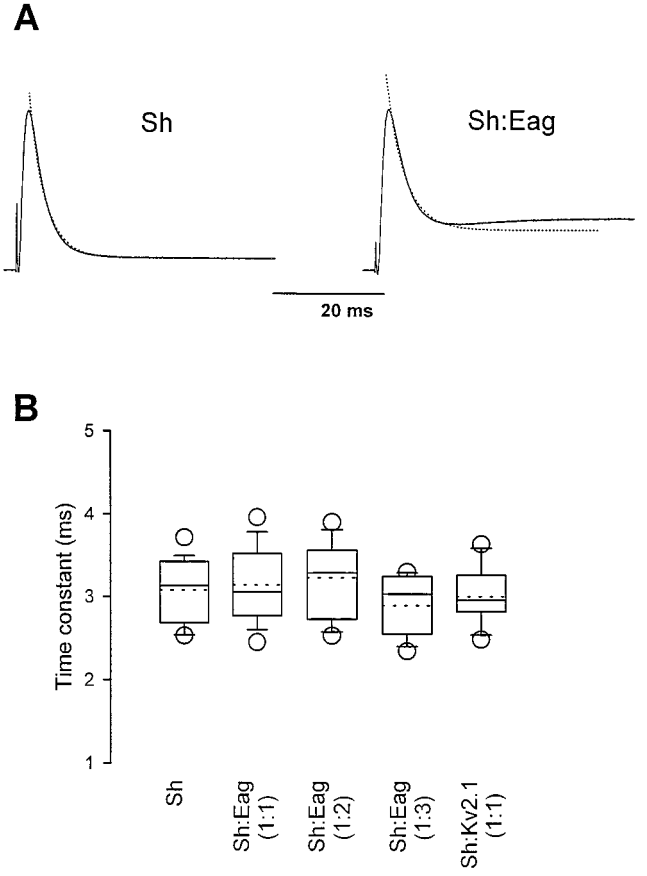

FIGURE 2 The kinetics of *Shaker* inactivation are unchanged upon coexpression with eag. (*A*) The kinetics of inactivation at +60 mV were fitted with a single exponential function (*dashed line*) for *Shaker* expressed alone (*left*) or with eag (1:1 ratio) (*right*). Representative fits are shown. The current traces have been scaled for comparison. (*B*) Box plots of the inactivation time constant at +60 mV for *Shaker* expressed alone or in combination with eag or Kv2.1 at the indicated molar ratios. The fitted time constant for *Shaker* was 3.1 ± 0.4 ms, $n = 19$. Using the two-sample Student's *t*-test, the time constant derived from each coexpression condition was found not to differ significantly from that of *Shaker*: *Shaker*/eag (1:1), $p = 0.66$, $n = 18$; *Shaker*/eag (1:2), $p = 0.37$, $n = 10$; *Shaker*/eag (1:3), $p = 0.23$, $n = 11$; *Shaker*/Kv2.1 (1:1), $p = 0.59$, $n = 14$. The box plot depicts the statistical distribution of the data: open circles represent the 95th (*top*) and 5th (*bottom*) percentile points; error bars indicate the 90th (*top*) and 10th (*bottom*) percentiles; the upper and lower margins of the box correspond to the 75th and 25th percentiles, respectively; the horizontal lines within the box mark the median (*solid line*) and mean (*dashed line*) values.

ms. Therefore, inactivation of homotetrameric *Shaker* channels can account for the kinetics of inactivation seen upon coexpression of *Shaker* and eag subunits.

For comparison, cRNA for Kv2.1, which forms a noninactivating channel, was coinjected with *Shaker* cRNA at a 1:1 molar ratio. Previous functional and biochemical experiments have demonstrated that Kv2.1 subunits do not coassemble with members of the Kv1 subfamily, which includes *Shaker* (Li et al., 1992). Upon coexpression, *Shaker* and Kv2.1 subunits generated currents that were virtually identical to the sum of *Shaker* and Kv2.1 currents expressed separately (data not shown). As was observed with eag, the time course of *Shaker* inactivation was unaffected by coexpression with Kv2.1 (Fig. 2 *B*).

Shaker assembly domain does not exert a dominant negative effect on eag expression

Subfamily-specific assembly of *Shaker* with other Kv1 subunits is mediated by a domain in the amino terminus of the protein (Li et al., 1992; Shen et al., 1993; Shen and Pfaffinger, 1995; Xu et al., 1995). A fragment containing amino acids 1 through 246 of *Shaker*, Sh1–246, which includes the assembly domain, has a strong dominant negative effect on the expression of *Shaker* channels (Fig. 3) (Li et al., 1992; Babila et al., 1994). This is because the amino-terminal fragment associates with the full-length *Shaker* protein, preventing its incorporation into active, cell surface channels. The assembly domain is required for the formation of *Shaker* tetramers (Li et al., 1992; Shen et al., 1993; C. T. Schulteis, N. Nagaya, and D. M. Papazian, submitted for publication), and is involved in the coassembly of *Shaker* and non-*Shaker* subunits (Yu et al., 1996; Sewing et al., 1996). Therefore, we investigated whether the *Shaker* assembly domain interacts with the eag subunit. Upon coexpression of the *Shaker* amino-terminal fragment Sh1–246 with full-length eag subunits over a wide range of molar ratios, no dominant negative effect on eag expression was observed (Fig. 3). Similarly, the fragment had no dominant

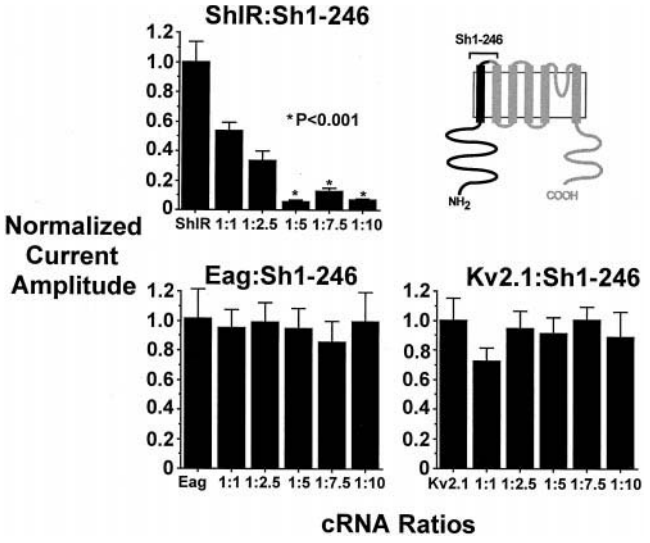

FIGURE 3 Sh1–246 does not exert a dominant negative effect on eag expression. The location of the Sh1–246 fragment is indicated in bold on the topology cartoon, *top right*. A fixed amount of Sh-IR, eag, or Kv2.1 cRNA was injected alone or with an increasing amount of Sh1–246 cRNA to achieve the indicated molar ratios. After 48 h, ionic currents were recorded with a two-electrode voltage clamp by pulsing for 94 ms from a holding potential of −80 mV to +40 (Kv2.1/Sh1–246 and Sh-IR/Sh1–246) or +60 mV (eag/Sh1–246). The steady-state current amplitude, measured starting at 75 ms, was averaged over an interval of 12.5 ms and normalized with respect to the control amplitude obtained in the absence of the Sh1–246 fragment. Histogram bars show the mean ± SE, $n = 5$ to 20 per coinjection ratio. The Sh-IR control bars show a normalized SEM as an indication of the variability in control measurements. Statistical significance was determined by a nonparametric analysis of variance (Kruskal-Wallis), followed by Dunn's multiple comparisons where appropriate. Each experiment shown was obtained using a single batch of oocytes, and is representative of 2 or 3 experiments performed with different batches.

negative effect on the expression of Kv2.1 channels, as expected, because *Shaker* and Kv2.1 subunits fail to coassemble (Fig. 3) (Li et al., 1992).

Shaker and eag proteins do not coassemble in *Xenopus* oocytes

To determine directly whether the *Shaker* and eag proteins coassemble, reciprocal coimmunoprecipitation experiments were performed. Antibodies directed against the *Shaker* protein (kind gift of Dr. L. Jan) have been described previously (Schwarz et al., 1990). To immunoprecipitate the eag protein, the amino-terminus was tagged with an AU5 epitope (Fig. 4 *A*) (Lim et al., 1990). The eag-AU5 construct produced functional channels with currents similar to that of wild-type eag, although activation was slightly slower (Fig. 4 *B*). As with wild-type eag, coexpression of *Shaker* and eag-AU5 did not alter the kinetics of *Shaker* inactivation (Fig. 4 *B*). A protein with an apparent molecular weight of ~150,000, close to that expected for eag (~130,000) (Warmke et al., 1991), was immunoprecipitated with a monoclonal antibody directed against the AU5 epitope (Fig. 4 *C*). This protein was present in oocytes injected with eag-AU5 cRNA, but not in H_2O-injected oocytes, identifying it as eag-AU5. N-linked glycosylation of the protein contributed to its broad appearance on SDS gels (data not shown).

Shaker, eag-AU5, or an equimolar mixture of *Shaker* and eag-AU5 cRNAs was injected into oocytes, keeping the total molar amount of cRNA constant. In vitro translation grade [^{35}S]-methionine was injected at the same time to label newly synthesized proteins. After 48 h, membrane proteins were solubilized in 1% Triton X-100 under conditions that maintain subunit associations (see Fig. 6) and subjected to immunoprecipitation with *Shaker*- or AU5-specific antibodies (Fig. 5 *A*). The mature *Shaker* protein, which migrates as a broad band of ~115 kDa (Santacruz-Toloza et al., 1994b), was immunoprecipitated by *Shaker* antibodies after expression alone or with eag-AU5. Some immature *Shaker* protein (~83 kDa) was also detected. Significantly, the *Shaker* protein was not detected after immunoprecipitation with AU5 antibodies. Similarly, the eag-AU5 protein was immunoprecipitated by AU5, but not *Shaker* antibodies. In the experiment shown, the AU5 antibody brought down several bands in addition to full-length eag-AU5. However, they were present when eag-AU5 was expressed alone and are likely to represent aggregated or degraded forms of eag (Fig. 5 *A*). Such bands were not present in all experiments (see Figs. 4 *C* and 5 *B*).

Alternatively, *Shaker*, eag-AU5, or an equimolar mixture of *Shaker* and eag-AU5 cRNAs was injected into oocytes in the absence of radioactive methionine. After immunoprecipitation with *Shaker* or AU5 antibodies, proteins were separated by electrophoresis, blotted to nitrocellulose, and probed with *Shaker* antibodies (Fig. 5 *B*). *Shaker* protein was readily detected after precipitation by *Shaker* antibodies, but not after precipitation with AU5 antibodies. That eag-AU5 was precipitated in this experiment was shown in a parallel immunoprecipitation of metabolically labeled eag-AU5 protein. Thus, no interaction between the eag-AU5 and *Shaker* proteins was detected in our reciprocal coimmunoprecipitation experiments.

Attempts to detect the eag-AU5 protein on immunoblots using the AU5 antibody were unsuccessful. The eag protein was also tagged at the carboxyl terminus with myc and his_6 epitopes, but antibodies directed against these tags were also unable to detect eag protein on immunoblots (data not shown).

As shown in Fig. 5 *C*, a reciprocal coimmunoprecipitation experiment was performed after coexpressing *Shaker* and wild-type eag. After immunoprecipitation with *Shaker* antibodies, the *Shaker* protein was apparent, but no protein corresponding to eag was detected. For comparison, *Shaker* was coexpressed with Kv2.1 (Fig. 5 *C*). Again, the *Shaker* protein was apparent, but no protein corresponding to Kv2.1 (expected molecular mass ~95 kDa) was detected. Coexpression with eag, Kv2.1, or eag-AU5 did, however, reduce the amount of *Shaker* protein precipitated compared to expression of *Shaker* alone (Fig. 5). Because the amount of

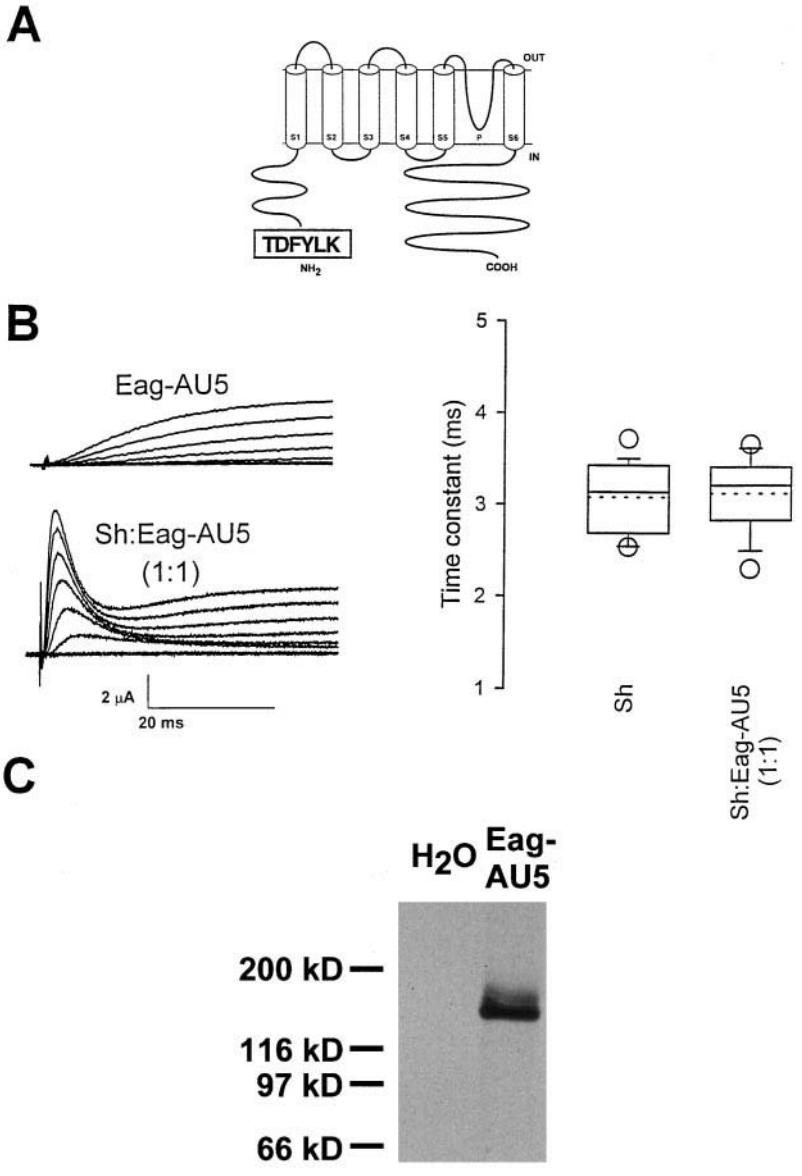

FIGURE 4 Functional and biochemical properties of an epitope-tagged eag, eag-AU5. (*A*) A model for the topology of the eag subunit indicates the approximate location of the six amino acid AU5 epitope (*boxed*). (*B*) Currents were recorded from eag-AU5 alone or after coexpression with *Shaker* using a 1:1 molar ratio of cRNA. *Left:* From a holding potential of −80 mV, 48 ms test pulses were applied from −60 to +80 mV in 20 mV increments. *Right:* The time constant of inactivation at +60 mV was fitted and displayed as described in Fig. 2. No statistically significant difference was detected in the presence or absence of eag-AU5 (Student's *t*-test, $p = 0.78$). Sample sizes were 19 and 12 for *Shaker* and *Shaker*/eag-AU5 (1:1), respectively. (*C*) Metabolically labeled proteins from water- or eag-AU5-injected oocytes were subjected to immunoprecipitation with an AU5 monoclonal antibody (1:1000 dilution), electrophoresis, and fluorography.

RNA injected was kept constant, a 50% reduction was expected. In these biochemical experiments, the level of reduction was variable and occasionally larger than 50%. Significantly, at low levels of expression, such as those used in electrophysiological experiments, coexpression of *Shaker* and eag or *Shaker* and Kv2.1 subunits did not significantly affect the size of the current. Both the inactivating and sustained components of the current attained the expected amplitudes. However, to optimize detection of the metabolically labeled proteins, much higher levels of expression were used for immunoprecipitation experiments than for functional analysis. Therefore, it is likely that nonspecific competition for cellular factors affected protein production in the biochemical experiments.

To immunoprecipitate intact oligomeric membrane proteins, it is important to solubilize under conditions that maintain specific subunit associations. The state of assembly of eag and *Shaker* proteins in 1% Triton was assessed by sucrose density gradient centrifugation. The majority of *Shaker* protein solubilized in Triton sedimented to a dense region of the gradient, consistent with a multimeric state of assembly (Fig. 6). A similar pattern has been obtained after solubilization in Chaps, a detergent that maintains the tetrameric structure of *Shaker* channels (Santacruz-Toloza et al., 1994a; Nagaya and Papazian, 1997). In contrast, the *Shaker* protein sedimented to a lighter region of the gradient after solubilization in Zwittergent, consistent with dissociation of the subunits in this detergent (Fig. 6) (Nagaya and Papazian, 1997). A fraction of the *Shaker* protein solubilized in Triton was also found in this region. The results indicate that the majority of specific associations between *Shaker* subunits are maintained upon solubilization in Triton. Similarly, eag-AU5 protein solubilized in Triton sedimented to a dense region of the gradient, consistent with the preservation of specific eag-AU5 subunit interactions under the conditions of our immunoprecipitation experiments.

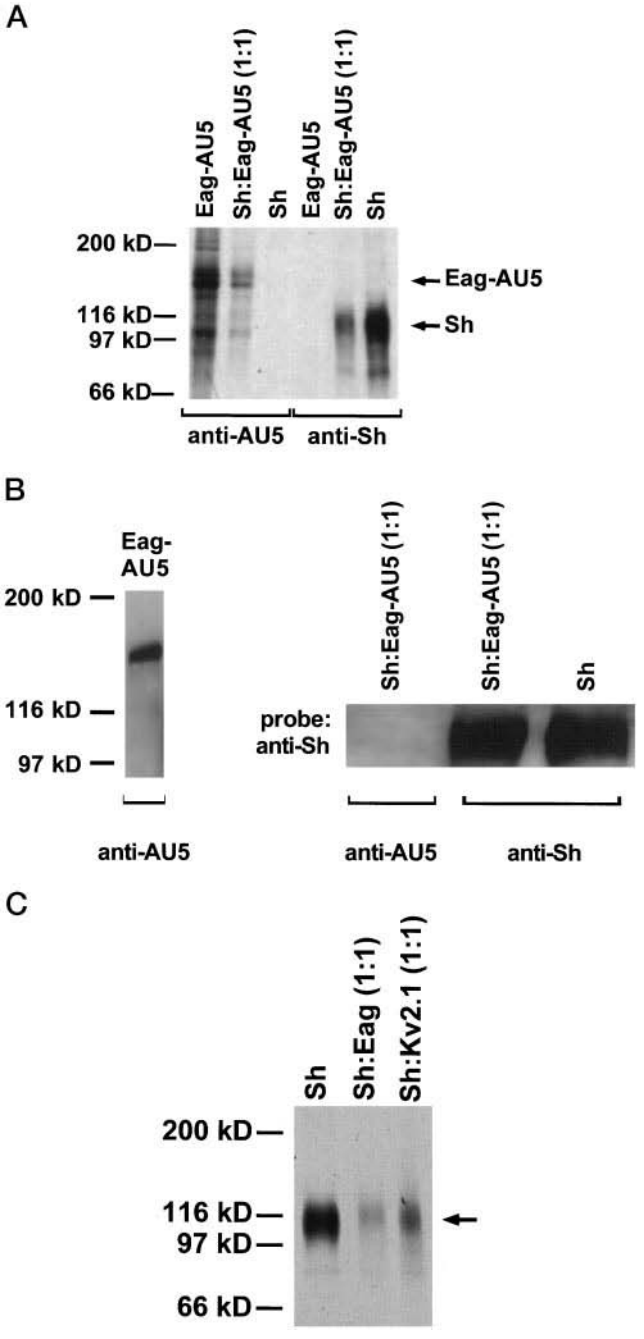

FIGURE 5 Lack of interaction between solubilized *Shaker* and eag subunits. (*A*) *Shaker* and eag-AU5 were expressed alone or together, as indicated, metabolically labeled, solubilized in Triton, and subjected to immunoprecipitation with anti-AU5 (1:200 dilution) or anti-*Shaker* (1:1000 dilution) antibodies, as noted at the bottom of the gel. Arrows at the right denote the eag-AU5 and mature *Shaker* (Sh) proteins. (*B*) *Right panel: Shaker* was expressed alone or in the presence of eag-AU5, solubilized, and immunoprecipitated with anti-AU5 or anti-*Shaker* antibodies as noted at the bottom of the gel. Proteins were separated by electrophoresis, blotted to nitrocellulose, and probed with anti-*Shaker* antibodies. *Left panel:* In parallel, eag-AU5 was metabolically labeled and immunoprecipitated with anti-AU5 antibodies, demonstrating that eag-AU5 protein was made and immunoprecipitated in this experiment. (*C*) *Shaker* was expressed alone or with wild-type eag or Kv2.1, as indicated, metabolically labeled, solubilized in Triton, and subjected to immunoprecipitation with anti-*Shaker* antibodies. The arrow denotes the mature *Shaker* protein.

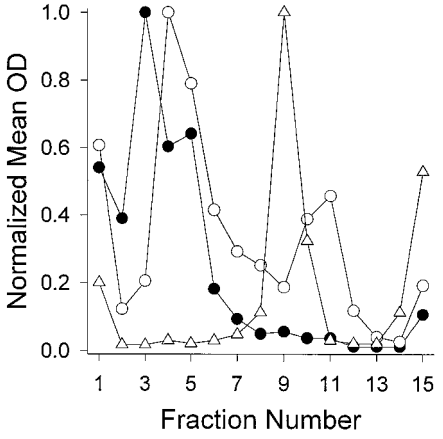

FIGURE 6 State of assembly of the *Shaker* and eag-AU5 proteins after solubilization. The eag (*filled circles*) or *Shaker* (*open circles*) proteins were solubilized in Triton, or *Shaker* protein (*open triangles*) was solubilized in Zwittergent, followed by sedimentation on linear 5–20% sucrose gradients. Fractions were collected and subjected to immunoprecipitation, electrophoresis, fluorography, and densitometric analysis. Mean optical density values (OD) for each fraction were normalized to the maximum value for the gradient. Lower fraction numbers correspond to denser gradient fractions.

DISCUSSION

We have presented three lines of evidence that *Shaker* and eag subunits do not coassemble in *Xenopus* oocytes. First, currents obtained upon coexpression of *Shaker* and eag subunits were virtually identical to the sum of separate *Shaker* and eag currents. Second, the domain that mediates incorporation of *Shaker* subunits into channels did not associate with eag subunits. Third, after solubilization under conditions that maintain subunit interactions, the *Shaker* and eag proteins could not be coimmunoprecipitated with either *Shaker*-specific or eag-specific antibodies.

Our conclusion differs from that of Chen et al. (1996) who reported that coexpression with an unspecified ratio of eag increased the rate of *Shaker* inactivation, leading to the suggestion that *Shaker* and eag subunits interact. In contrast to their results, however, channels with fewer than four *Shaker* inactivation particles are expected to inactivate more slowly than *Shaker* wild-type tetramers (MacKinnon et al., 1993). Whereas the eag channel lacks a prominent fast-inactivation mechanism (Fig. 1; see also Chen et al., 1996; Robertson et al., 1996; Tang and Papazian, 1997), the *Shaker* channel inactivates by a ball-and-chain mechanism, in which an amino-terminal ball inserts into the open mouth of the channel, preventing further conduction (Hoshi et al., 1990; Demo and Yellen, 1991). The rate of inactivation depends on the number of ball-containing subunits present in the tetrameric channel, and occurs more slowly as the number of balls is reduced (MacKinnon et al., 1993). We found no significant difference between the time constant of inactivation whether *Shaker* was expressed alone or in combination with eag at several molar ratios. Importantly, increasing the proportion of eag subunits did not reduce the

rate of inactivation, as would be expected if eag and *Shaker* formed heteromultimers.

By using a two-electrode voltage clamp, we obtained a mean value of 3 ms for the *Shaker* inactivation time constant at +60 mV in the presence and absence of eag. Only experiments in which the peak current amplitude at +80 mV was between 1 and 15 μA were analyzed. The time constant value that we obtained is in excellent agreement with two previous reports (MacKinnon et al., 1993; Shih and Goldin, 1997). In similar experiments, in contrast, Chen et al. (1996) obtained a larger inactivation time constant +50 mV for *Shaker* expressed alone. However, current amplitudes were as large as 50 μA, which might generate series resistance errors. Interestingly, the time constant value obtained by Chen et al. (1996) from macropatch experiments for *Shaker* plus eag (~3 ms) was quite similar to those obtained by us for *Shaker* plus or minus eag. Chen et al. (1996) reported a 1-ms increase in the time constant when *Shaker* subunits were expressed alone. However, current amplitudes were not provided for the macropatch experiments, leaving open the possibility that series resistance errors contributed to their results.

Although an exception to the subfamily specific assembly rule would be extremely significant, our evidence argues strongly that *Shaker* and eag subunits do not coassemble in *Xenopus* oocytes. Much remains to be learned about the assembly of subunits in the eag subfamily. A recent study suggests that an amino-terminal region may mediate subunit interactions in a human eag-related K^+ channel, h-erg (Li et al., 1997), whereas a carboxyl-terminal domain has been implicated in the assembly of the rat ether-à-go-go homolog, r-eag (Ludwig et al., 1997). Significantly, a fragment derived from the carboxyl terminus of r-eag exerts a dominant negative effect on r-eag expression, but not on the expression of the *Shaker* family member Kv1.5 (Ludwig et al., 1997). This result is consistent with our conclusion that *Shaker* and eag subunits do not form heteromultimers.

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