Purification of an EH Domain-binding Protein from Rat Brain That Modulates the Gating of the Rat ether-à-go-go Channel*

Elemor T. Pirost, Lijian Shen, and Xin-Yun Huang§

From the Department of Physiology, Cornell University Medical College, New York, New York 10021

Mutations in the gene encoding ether-à-go-go (EAG) potassium channel impair the function of several classes of potassium currents, synaptic transmission, and learning in Drosophila. Absence of EAG abolishes the modulation of a broad group of potassium currents. EAG has been proposed to be a regulatory subunit of different potassium channels. To further explore this regulatory role we searched for signaling molecules that associate with EAG protein. We have purified a ~95-kDa protein from rat brain membranes that binds to EAG. When co-expressed in mammalian cells this protein coimmunoprecipitates with EAG and alters the gating of EAG channels. Expression of this protein is regulated during neuronal differentiation. The protein is identical to the recently reported rat protein epsin, which is an EH domain-binding protein similar to the Xenopus mitotic phosphoprotein MP90. These results show that proteins of the epsin family are modulators of channel activity that may link signaling pathways, or the cell cycle, to EAG and thus to various potassium channel functions.

Potassium channels play essential roles in neuronal functions and contribute to the signaling capacity of excitable cells (1). Modification of potassium channel activity has been implicated in cellular mechanisms of learning and memory. Depending on their specific properties, potassium channels could maintain resting membrane potentials, determine the repolarization phase of action potentials, modulate neurotransmitter/hormone release, modulate firing frequency, and participate in posttranslational integration (1, 2). The ether-à-go-go (EAG) potassium channel is unique because mutations in the Drosophila EAG gene have profound effects on more than one type of potassium current (3–5).

EAG was first identified by a neurological mutant in Drosophila that causes a leg-shaking phenotype (6). Electrophysiological studies revealed that EAG mutations cause spontaneous repetitive firing in motor axons and elevated transmitter release at the larval neuromuscular junction (3). EAG mutant flies have defects in learning (7) and in responses to certain odors (8). All identified K⁺ currents in larval muscle were affected by EAG mutations including the fast (I_{CF}) and delayed non-inactivating (I_{N}) voltage-gated K⁺ currents, the fast (I_{CF}) and slow (I_{CS}) Ca²⁺-activated K⁺ currents. Furthermore, mutations in the EAG gene block the modulation of different K⁺ currents by several signaling pathways (9). The mechanism how EAG mutations affect different K⁺ currents has not been defined, however. Interactions between EAG and shaker potassium channels has been tested in Xenopus oocytes with conflicting results as to whether these proteins coassemble into functional heteromultimeric channels (10, 11). Based on genetic evidence, the EAG protein was postulated to be a regulatory subunit of or to modulate the assembly of a broad group of potassium channels (5). It also has been suggested that EAG may act downstream of calcium/calmodulin-dependent protein kinase II in modulating neuronal plasticity (7).

To explore the regulatory role of EAG, we decided to identify signaling molecules that might associate with EAG protein to receive cellular signals to modulate the function of EAG and possibly other K⁺ currents. We report here the purification of a protein from rat brain membranes that interacts with rat EAG. We used overlay assays to monitor the purification of EAG-binding proteins and characterized one of several EAG-binding proteins, with an apparent molecular mass of 95 kDa, in further biochemical and electrophysiological experiments. This protein is identical to the recently identified rat epsin (12), a protein that is similar to the Xenopus mitotic phosphoprotein MP90 (13). When coexpressed in HEK 293 cells, epsin coimmunoprecipitates with EAG and EAG interacts with epsin through its carboxyl-terminal domain. Epsin decelerates both activation and deactivation processes of EAG channels in HEK 293 cells as well as in neuronal NG108-15 cells. Given the multifunctionality of the epsin family of proteins, such as binding to EH (for Eps15 homology; a ~70 amino acid long protein module (14)) domain containing proteins, involved in endocytosis and cell cycle regulation, these proteins could be a new class of modulators of channel activity that link various signaling pathways, and may be the cell cycle, to EAG and thus to other potassium channel functions.

EXPERIMENTAL PROCEDURES

Subcloning—The full-length cDNA encoding rat EAG (from Dr. Olaf Pongs) was subcloned into the KpnI and NotI restriction sites of pcDNA3 (Invitrogen). NH₂- and COOH-terminal fragments (624 and 1443 base pairs, respectively) of EAG were generated using polymerase chain reaction. These fragments were subcloned into pcDNA3 for use in in vitro translation. Both NH₂- and COOH-terminal constructs were confirmed by DNA sequencing. Rat epsin cDNA in pcDNA3.1/His B (Invitrogen) was obtained from Hong Chen and Pietro De Camilli (Yale University) and used in our transfection studies.

Overlay Assay and Western Blot—Overlay assays were performed according to the procedures of Guichet and co-workers (15) with some modifications. Briefly, samples were separated on 8% SDS-PAGE and transferred to nitrocellulose membrane filters. Blots were incubated for 60 min (4 °C) with a buffer (AC) containing 10% (v/v) glycerol, 100 mM NaCl, 1 mM EDTA, 0.1% (v/v) Tween 20, 2% (w/v) milk powder, and 20 mM Tris-HCl (pH 7.6). Following quick rinses with AC buffer (without

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‡ Norman and Rosita Winston Foundation Biomedical Fellow.

§ To whom correspondence should be addressed. Tel.: 212-746-6362; Fax: 212-746-8690; E-mail: xyhuang@mail.med.cornell.edu.

1 The abbreviations used are: EAG, ether-à-go-go; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; AS, ammonium sulfate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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milk), filters were probed with [35S]methionine-labeled EAG for 2 h (4 °C). Probes were generated with the TNT T7 in vitro transcription/translation kit following the manufacturer's instructions (Promega). Filters were washed extensively with AC buffer, dried, and exposed to PhosphorImager plates (4–12 h).

In some experiments, nitrocellulose filters were probed with a rabbit anti-epsin polyclonal antibody (raised against the DFW and NPF domains of epsin), 1:1000 dilution, from Hong Chen and Pietro De Camilli (Yale University), followed by incubation with a horseradish peroxidase-linked secondary goat anti-rabbit antibody (1:4000 dilution) and enhanced chemiluminescence detection (NEN Life Science Products Inc.). An affinity purified rabbit anti-EAG antibody (Genemed Synthesis), raised against an EAG peptide (NGSGSKGWGPPKSNK), was used to immunoprecipitate EAG from transfected 293 cell lysates. Cell lysates were prepared, and proteins were immunoprecipitated as described previously (16–19). Binding assays were performed with GST fusion protein fragments of EAG and rat brain homogenates as described (20).

**Protein Purification**—Ten frozen adult rat brains (Pol Freeze Biologics) were thawed on ice and homogenized in 150 ml of homogenization buffer containing (in mM) HEPES (20), NaCl (20), KCl (100), EDTA (1), EGTA (1) (pH 7.4), and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 16 µg/ml benzamidine, 200 µg/ml phenanthroline, 0.7 µg/ml pepstatin A, and 2 µg/ml aprotonin). After an initial homogenization (850 × g, 10 min), cell membranes were sedimented (100,000 × g, 60 min). Membrane proteins were extracted by 15 ml of homogenization buffer containing 2.5% CHAPS (w/v), followed by centrifugation (100,000 × g, 60 min). Proteins (160 mg) from the resulting supernatant were sequentially precipitated with 10 and 25% (w/v) ammonium sulfate (AS). Precipitated proteins were resuspended in 20 ml of buffer (RB) containing 20 mM HEPES, 20 mM NaCl (pH 7.4), 1 µg/ml leupeptin, 16 µg/ml benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.25% (w/v) CHAPS. Following overlay analysis, 25% AS-precipitated samples were centrifuged at a 10-kDa nominal molecular mass limit filter (Millipore) and applied (0.3 mg of proteins) to a Mono-Q anion exchange column (Amersham Pharmacia Biotech). Proteins were eluted with a linear NaCl gradient (100–750 mM), collected in 0.5 ml/min fractions and frozen in liquid nitrogen (storage at −80 °C). Samples of these fractions were analyzed by overlay assay, silver, and Coomassie Blue staining. All assays were performed at least three times, and representative data are shown in the figures.

**Peptide Sequencing**—Internal sequence analysis was performed on proteins excised from an SDS-PAGE gel, based on the procedures of Fernandez et al. (21). Briefly, proteins were digested with trypsin and separated by microbore high performance liquid chromatography purification. Following mass spectrometric analysis, one peptide fragment was subjected to sequencing by Edman degradation.

**Cell Transfection**—Both NG108-15 and HEK 293 cells were grown as described previously (16). For transient expression of epsin or EAG, cells grown in 10-cm (for biochemistry) or 55-mm dishes (for electrophysiology) were transfected, using the calcium phosphate precipitation method, with 10 µg and 2 µg of cDNA, respectively. For electrophysiological experiments, 0.2 µg of green fluorescent protein encoding cDNA was co-transfected with epsin and EAG. Coexpression of green fluorescent protein did not alter EAG channel activity and kinetics. Stable epsin and EAG expressing HEK 293 cell lines were also established by selection with G418 (0.5 mg/ml).

**Electrophysiology**—Potassium currents from single voltage-clamped cells were recorded (at room temperature) in the whole cell configuration of the patch clamp technique using an Axopatch 200A amplifier (Axon Instruments Inc.) (22). The recording pipette solution contained (in mM): potassium aspartate (105), KCl (20), MgCl2 (1.5), CaCl2 (0.1), HEPES (10), EGTA (11), Na-ATP (3) (pH 7.2) with KOH. The bath solution consisted of (in mM): NaCl (118), KCl (6), CaCl2 (2.5), glucose (10), HEPES (20), MgCl2 (1.5) (pH 7.4) with NaOH. Micro-Hematocrit capillary tubes (VWR) were used as recording electrodes (resistance: 1–4 MΩ). The current signal was filtered with a low pass Bessel filter (1–2 KHz), digitally acquired at 5–10 KHz and analyzed using pCLAMP 6.0 (Axon Instruments Inc.). When necessary, series resistance errors were compensated by 70–80%. While recording current-voltage relations employing step depolarizing pulses from a holding potential of −80 mV leakage and capacitative currents were subtracted by a P/4 routine. In experiments where hyperpolarizing pulses (from a 0 mV holding potential) were used to assess the deactivation, and subsequent reactivation properties of EAG currents, leak subtraction was performed off-line (by scaling leak currents obtained with a step to −80 mV from −70 mV). Due to technical limitations, we were unable to construct the relative G(V) curves by measuring tail currents with higher concentrations of extracellular potassium.

Steady-state conductance was calculated using the following equation,

\[ G = I(V - E_k) \]  

where \( I \) is the current measured at the end of the depolarizing voltage pulse, \( V \) is the membrane potential, and \( E_k \) was −78.9 mV in our experiments.

The voltage dependence of EAG channel activation was determined by measuring currents elicited by voltage steps to 0 mV, from various (−110 to −10 mV) prepsules (23). The resulting currents were best described by the sum of two exponentials,

\[ I = I_d [1 - \exp(-t/\tau_d)] + I_s [1 - \exp(-t/\tau_s)] \]

where \( I_d \) and \( I_s \) is the amplitude of the fast and slow current components, respectively. The fast component could be best described with first-order exponentials. The slow component behaved similarly (\( m = 1 \), except in the −80 to −110 mV prepulse potential range, where it could be fitted only by a second-order exponential. In all cases, the exponential functions were extrapolated back to the time point when the voltage change occurred.

The normalized conductance-voltage relationship (Fig. 3, B and C) and the relationship describing the proportion of the current activated in the slow mode versus voltage (Figs. 4E and 6B) were described with Boltzmann functions,

\[ Y = 1/\left(1 + \exp\left(V_{mid} - V/k\right)\right) \]

where \( Y \) denotes either the normalized conductance (\( G/G_{max} \)), or the proportion of the current in the slowly activating mode (\( I/I_d + I_s \)), \( V_{mid} \) is the midpoint potential (in mV), and \( k \) is the slope factor (in mV).

Data values were compared using Student's \( t \) test and considered significantly different if \( p < 0.05 \).

**RESULTS**

**Purification of EAG-binding Proteins from Rat Brain**—To identify proteins interacting with EAG protein, we used an overlay assay to monitor the purification of potential EAG-binding proteins. Rat brain membranes were chosen as starting material because brain expresses EAG most abundantly (23). In *vitro* translated [35S]methionine-labeled EAG polypeptide was used as probe to identify proteins that bind to EAG. In *vitro* translated rat EAG polypeptide has an apparent molecular mass of −108 kDa when assessed by SDS-PAGE and autoradiography (Fig. 1A) or Western blot with an anti-EAG antibody (results not shown). Several proteins from rat brain membranes interacting with the EAG probe (Fig. 1C). We chose the −95-kDa protein, which had the highest intensity in overlay autoradiography, for large-scale purification. Brain membrane proteins were first fractionated by ammonium sulfate precipitation. Putative EAG-binding protein(s) were precipitated by 25% ammonium sulfate (25% AS; Fig. 1C, right), these proteins were further purified with ion-exchange chromatography. Two fractions (numbers 22 and 23) contained the −95-kDa EAG-binding protein (Fig. 1B, left). After SDS-PAGE separation, the band containing the −95-kDa protein was excised and subjected to mass spectrometry and amino acid sequencing analyses (see “Experimental Procedures”).

**Identification of an EAG-binding Protein**—The following peptide sequence, determined by microsequencing, was searched against the protein database: NIVHNYSEAEIK. A recently identified Eps15-interacting 94-kDa protein, epsin, contained this peptide sequence, determined by microsequencing, was searched against the protein data base: NIVHNYSEAEIK. A overlapping region of the DPW and NPF domains of epsin, we analyzed all fractions at all stages of purification (Fig. 2A). After SDS-PAGE and autoradiography, for large-scale purification. Brain membrane proteins were first fractionated by ammonium sulfate precipitation. Putative EAG-binding protein(s) were precipitated by 25% ammonium sulfate (25% AS; Fig. 1C, right), these proteins were further purified with ion-exchange chromatography. Two fractions (numbers 22 and 23) contained the −95-kDa EAG-binding protein (Fig. 1B, left). After SDS-PAGE separation, the band containing the −95-kDa protein was excised and subjected to mass spectrometry and amino acid sequencing analyses (see “Experimental Procedures”).

**Identification of an EAG-binding Protein**—The following peptide sequence, determined by microsequencing, was searched against the protein database: NIVHNYSEAEIK. A recently identified Eps15-interacting 94-kDa protein, epsin, contained this peptide sequence at its NH2 terminus (amino acid residues 12–23) (22). The molecular structure of epsin is depicted in Fig. 2A. To determine that this EAG-binding protein is epsin, we analyzed all fractions at all stages of purification with an anti-epsin antibody (Fig. 2B), which was raised against an overlapping region of the DPW and NPF domains of epsin (Ref. 12; Fig. 2A). Beforehand, the antibody was tested in HEK 293 cells, which were transfected with His-tagged rat epsin
pcDNA3, was transcribed and translated in vitro. Intensity (arrow) proteins purified by AS (position of a band (electrophoresed on an 8% SDS-PAGE gel. Subsequent autoradiography revealed a 95-kDa band (95 kDa) in fraction 22 and 23, which was identified by EAG.

FIG. 1. Purification of EAG-binding proteins from the rat brain. A, the rat EAG expressed in vitro. EAG encoding cDNA, subcloned into pcDNA3, was transcribed and translated in vitro in the presence of [35S]methionine. The probe was then purified on a G-25 microspin column and electrophoresed on an 8% SDS-PAGE gel. Subsequent autoradiography revealed a ~108-kDa translation product. Numbers on the left indicate molecular mass standards (in kDa). B, purification scheme of EAG-binding proteins. C, overlay analyses of rat brain membrane proteins (left) and proteins purified by AS (right) precipitation. Several proteins were detected by the in vitro expressed EAG probe in the unpurified membrane fraction (brain) and the fraction precipitated by 25% ammonium sulfate (25% AS). The arrow indicates the position of a band with the highest intensity (~95 kDa). Overlay assays were performed, as described under “Experimental Procedures,” on 400 μg of total brain proteins, 6 μg of 10 and 25% AS-precipitated proteins, and on 200 μg of proteins remaining in the supernatant following 25% AS precipitation (25% Sup). D, overlay (left) and silver staining (right) assays, following ion-exchange chromatography. Proteins (300 μg) precipitated by 25% AS were further purified using a Mono-Q anion-exchange column. Prior to loading, the column was equilibrated with a buffer containing 20 mM HEPES, 0.25% CHAPS (w/v), and 100 mM NaCl (pH 7.4). Samples were applied to the column in the same buffer and were eluted (100–750 mM NaCl gradient) into 50 × 0.5-ml fractions (flow rate: 0.5 ml/min). Two aliquots (10 μl each) of each fraction was separated on 8% SDS-PAGE gels, and either silver stained (right) or transferred to nitrocellulose filters and overlaid with the EAG probe (left). Results for lanes 20–27 are shown here. Arrow indicates the position of a band (~95 kDa) in fraction 22 and 23, which was identified by EAG.

cDNA. This antibody recognized a distinct ~105-kDa band in epsin-transfected cells (Fig. 2B, left, single arrow), and a band (~95 kDa) in brain membranes (Fig. 2B, left, double arrow). Furthermore, the 95-kDa band was detected by this antibody in the 25% AS fraction and in fraction 23 (IEX number 23) of the ion-exchange column (Fig. 2B, right), while it was absent in untransfected HEK 293 cells and in the supernatant (25% Sup) of the protein fraction precipitated by 25% AS (Fig. 2B). An additional band (~83 kDa) was present in all fractions tested, except in IEX number 23, which is probably due to antibody cross-reaction with another protein (Fig. 2B). Therefore, the ~95-kDa EAG-binding protein purified from rat brain membrane is epsin.

Interaction of EAG and Epsin—To determine if there is an in vivo association between epsin and EAG, we performed co-immunoprecipitation studies. In epsin and EAG co-transfected HEK 293 cells, we found that an anti-EAG antibody was able to immunoprecipitate epsin from transfected cell lysates (Fig. 2C, left). As a control, no epsin was detected in immunoprecipitates with preimmune serum (Fig. 2C, right). In cell lysates from epsin or EAG singly transfected cells, no epsin was detected in the immunoprecipitates with an anti-EAG antibody (Fig. 2C, left, and data not shown). Furthermore, epsin from rat brain extracts was co-immunoprecipitated with anti-EAG antibody (Fig. 2C, left). Therefore, together with the above results, EAG associates with epsin in vivo, as well as in vitro. To further confirm the interaction and to determine whether the amino- or carboxyl-terminal domains of EAG protein are involved in this interaction, we tested the NH2- and COOH-terminal domains of EAG for their interaction with epsin. The GST fusion protein of the COOH-terminal region (residues from 482 to 962) of EAG specifically pulled down epsin from rat brain homogenates, as evidenced by Western blot (Fig. 2C, right). As control, GST alone or GST fusion protein of the NH2-terminal domain of EAG did not pull down epsin from rat brain extracts (Fig. 2C, right). In addition, both NH2- and COOH-terminal domains of EAG were translated in vitro and used as probes in overlay assays on brain proteins. The same ~95-kDa protein band, previously identified by the full-length EAG probe, was detected with the COOH-terminal fragment (Fig. 2D, arrow), but not with the NH2-terminal fragment of EAG (Fig. 2D). Thus, the carboxyl-terminal region of EAG mediates the interaction with epsin.

Functional Interaction between EAG and Epsin—To investigate possible physiological effects of epsin on EAG channels, whole cell voltage-clamp technique was used to compare potassium currents through EAG channels in the absence or presence of epsin. As reported before, EAG can function as a structural subunit, when expressed heterologously in cell lines. Transfection of HEK 293 cells with EAG plasmid DNA yielded robust channel expression. Cells transfected with epsin alone did not generate any currents (results not shown). Currents were evoked by depolarizing to various potentials (from −70 to +60 mV) from a −80 mV holding potential. At +40 mV the outward, non-inactivating currents had similar steady-state amplitudes of 3637.2 ± 940.4 pA (n = 8) and 2600.7 ± 692.8 pA (n = 5) in cells transfected with EAG and EAG + epsin, respectively. The midpoint of activation (V1/2) was shifted to the right (p < 0.05) in EAG + epsin-transfected cells, as compared with cells expressing EAG alone (Fig. 3B; V1/2 = 19.6 ± 3.7 mV, n = 5, for EAG + epsin transfected cells, and V1/2 = −0.7 ± 3.5 mV, n = 8, for EAG-transfected cells). The slope
Epsin Decreases the Rate of Deactivation of EAG Channels—

The rate of closure (deactivation) of the EAG channel was also modified by epsin. EAG channels deactivate very rapidly, which makes it difficult to evaluate the time course of deactivation (Fig. 5A, top; also see Ref. 24). Nevertheless, we find that epsin slowed down the rate of deactivation of EAG channels (Fig. 5A). The time to reach 80% of the steady-state current value (during deactivation) increased significantly (from 10–15 to 20–25 ms) in cells coexpressing epsin and EAG, relative to cells transfected with EAG alone (Fig. 5B).

Epsin Modulation of EAG Channel Activity in NG108-15 Cells—To confirm the results in HEK 293 cells, we evaluated the effect of epsin on EAG channels in NG108-15 cells. In undifferentiated NG108-15 cells, only low levels of endogenous epsin are expressed (Fig. 6D). When co-transfected into undifferentiated NG108-15 cells, epsin modulates both the activation and deactivation of EAG channels (Fig. 6). EAG channel activation was significantly delayed by epsin (in the −40 to −80 mV prepulse potential range) in co-transfected NG108-15 cells (Fig. 6A). This was accompanied by a significant shift in the midpoint voltage for the slow current component of activating EAG channel ($V_{\text{mid}} = -47.1 \pm 2.9$ mV, $n = 4$ and $V_{\text{mid}} = -37.4 \pm 2.1$ mV, $n = 10$ for EAG and EAG + epsin-transfected cells, respectively; Fig. 6B). This shift was smaller than the one observed in HEK 293 cells (−20 mV). Low levels of endogenous epsin in undifferentiated NG108-15 cells may be responsible for these differences. In addition, epsin caused a significant delay in the deactivation of EAG channels in NG108-15 cells (Fig. 6C).
Experimental Procedures). Each point represents mean values (6±S.E.).

Epsin does not alter the activation curve of Kv1.2 channels. Normalized conductance-voltage curves were obtained for HEK 293 cells expressing Kv1.2 with or without epsin. Representative leak-subtracted potassium currents are shown for EAG (top) and EAG + epsin-transfected (bottom) HEK 293 cells. Currents were evoked by depolarizing pulses to 0 mV, from various (−110 to −10 mV; 1-s duration) prepulses in 10 mV increments. B, the time necessary to reach 80% of maximum current (I80) values (in experiments depicted in A) are plotted as a function of prepulse (EAG: filled circles; EAG + epsin: open circles). Values at each voltage were compared using unpaired Student’s t test (n ≥ 6), and deemed significantly different if p < 0.05 (indicated by asterisks). C and D, current traces of activating K+ channels in HEK 293 cells transfected with EAG (C) or EAG + epsin (D), in response to a voltage step from −60 to 0 mV. The traces were fitted with two exponentials (the continuous line depicts the slow component). From such fits, the amplitude of the fast (Ips) and slow (Is) current components were calculated. E, the fraction of the slow current component (Is/Ips + Is) is altered in EAG + epsin (open circles) versus EAG expressing HEK 293 cells (filled circles). The relative contribution of the slow component was calculated from the biexponential fits (see C and D) of the currents evoked from various prepulse voltages (V) to 0 mV. Results are from the same cells as in B. Mean values (n ≥ 6) were approximated with Boltzmann functions. Vertical bars, when bigger than symbols, represent S.E. F, slow (squares) and fast (circles) time constants of K+ channel activation are not significantly different in EAG (filled symbols) and in EAG + epsin-transfected (open symbols) HEK 293 cells (n ≥ 6; p < 0.05). Values for time constants were obtained from exponential fits to data described in E. Fast activation was only observed in the −10 to −60 mV prepulse voltage range. Data points (mean ± S.E.) were connected with straight lines.

FIG. 3. Epsin modulates the activation of EAG channels. A, current transients in HEK 293 cells transfected with EAG alone (left) or together with epsin (right). These currents transients were elicited by depolarizing pulses to between −70 and +60 mV (10 mV increments) from a −80 mV holding potential. B, epsin shifts the conductance-voltage relationship of EAG channels. Currents were measured at the end of 1.5-s depolarizing pulses, and these values were divided by the driving force (V − Ek) to obtain the steady-state conductance. The conductance values were normalized to the conductance measured at +60 mV (G/Gm) and plotted as a function of the test potential. Data for conductances from EAG- (filled circles) and EAG + epsin- (open circles) transfected cells (n ≥ 5) were fitted by Boltzmann functions (see “Experimental Procedures”). Each point represents mean values (± S.E.). C, epsin does not alter the activation curve of Kv1.2 channels. Normalized conductance-voltage curves were obtained for HEK 293 cells expressing either Kv1.2 (filled circles, n = 5) or coexpressing Kv1.2 and epsin (open circles, n = 5), using the same recording protocols and analysis as in B (mean ± S.E.).

DISCUSSION

We have identified a protein, epsin, that binds directly to EAG potassium channel proteins. A functional consequence of the epsin-EAG interaction is that both activation and deactivation of EAG channels are altered. Coexpression of epsin resulted in a shift in the midpoint of activation of EAG channels.

EAG channels are activated in a voltage-dependent manner (23, 25). Currents activate slower with hyperpolarizing prepulses and conversely, faster with depolarizing prepulse voltages (Fig. 4). During activation, EAG channels pass through several closed states prior to opening. Depolarizing prepulses “advance” an increasing number of channels to the “final” closed state, just preceding opening. Therefore, under these conditions, opening is a relatively rapid process, compared with a state (with hyperpolarizing prepulses), where a large number of channels have to pass through multiple closed states. The process described by fast time constants dominates the activation close to the threshold of opening, while the slow opening mode is prevalent for very negative holding potentials. Between the extreme voltages, some channels are found at remote closed states, while others are just “ready” to be opened. Hence, the activation process can be described by a mixture of fast and slow time constants. While observing the opening of EAG channels using prepulses of various strengths, we noticed that epsin significantly decelerated the activation process in the −30 to −70 mV prepulse range (Fig. 4B). As shown in Fig. 4E, this is the approximate voltage range, where some EAG channels are in the slow, and some are in the fast opening mode. Deceleration of the activation process was actually due to the presence
of a higher proportion of slowly activating current in epsin + EAG-transfected HEK 293 cells (Fig. 4E). The time constants for the fast and slow activating processes remained unchanged by epsin. Overall, epsin retards the transition of EAG channels toward opening, which leads to a decreased probability of opening at a certain voltage (Fig. 3B).

Recently two other proteins were reported to bind to EAG channel proteins: Slob (26) and KCR1 (27). Slob was identified as binding to Drosophila Slowpoke calcium-dependent potassium channels though a yeast two-hybrid screen. Furthermore, Slob could increase the activity of Slowpoke channels. While Slob also communoprecipitates with the Drosophila EAG (26), it is not known, however, whether it has any effect on EAG channel activity. Another EAG-binding protein is KCR1 (27). KCR1 cDNA was isolated from rat cerebellum through a suppression cloning strategy (inhibition of the expression of a low-threshold non-inactivating outward potassium current generated from the rat cerebellar poly(A)+ RNA). KCR1 has 12 proposed transmembrane domains and binds to the COOH-terminal tail of the rat EAG. When coexpressed with rat EAG, KCR1 causes faster EAG channel activation. Interestingly, KCR1 shifts the midpoint potential (of the relative amplitude of the slow current component) 10 mV in the hyperpolarizing direction (27).

Proteins of the epsin family are multifunctional. Epsin is an EH domain-binding protein. Chen et al. (12) identified epsin based on its binding to the EH domain of Eps15. Epsin was suggested to play a role in endocytosis, by virtue of binding to both Eps15 and to the α-adaptin subunit of AP-2 (12). Clathrin assembly is facilitated by AP-1 (in the trans-Golgi network) and AP-2 (at the plasma membrane) (reviewed in Ref. 28). Interestingly, AP-2 and AP-3 (the heart-specific AP complex) form potassium channels in planar lipid bilayers (29, 30). Epsin uses different binding sites for AP-2 and the EH domain (12). Visual examination of the rat EAG sequence did not reveal an obvious EH domain. Similarly to its binding to AP-2, epsin likely uses a different domain for binding to the rat EAG. It is possible that epsin regulates the endocytosis of channel proteins, an unexplored topic, that deserves further investigation.

Epsin is enriched in presynaptic nerve terminals and may be involved in synaptic vesicle endocytosis. Modulation of potassium channel activity and regulation of transmitter release at nerve terminals have emerged as essential sites of regulation in synaptic plasticity (1, 31, 32). Thus, epsin might connect EAG to the control of neurotransmitter release, which underlies the defect of EAG mutants in Drosophila.

Epsin is homologous to the Xenopus mitotic protein MP90 (13). This raises the possibility that epsin could couple cell cycle regulation to ion channel modulation. Indeed, in Xenopus oocytes expressing rat EAG, mitosis promoting factors induce a reduction of EAG current amplitude, and it has been suggested that EAG channels may play a role in the cell cycle process (33). These possibilities warrant further investigation.

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