Calcium/Calmodulin-dependent Protein Kinase II Phosphorylates and Regulates the Drosophila Eag Potassium Channel*

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Modulation of neuronal excitability is believed to be an important mechanism of plasticity in the nervous system. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been postulated to regulate the ether-a-go-go (eag) potassium channel in Drosophila. Inhibition of CaMKII and mutation of the eag gene both cause hyperexcitability at the larval neuromuscular junction (NMJ) and memory formation defects in the adult. In this study, we identify a single site, threonine 787, as the major CaMKII phosphorylation site in Eag. This site can be phosphorylated by CaMKII both in a heterologous cell system and in vivo at the larval NMJ. Expression of Eag in Xenopus oocytes was used to assess the function of phosphorylation. Injection of either a specific CaMKII inhibitor peptide or lavendustin C, another CaMKII inhibitor, reduced Eag current amplitude acutely. Mutation of threonine 787 to alanine also reduced amplitude. Moreover, both CaMKII inhibition and the alanine mutation accelerated inactivation. The reduction in current amplitudes and the accelerated inactivation of dephosphorylated Eag channels would result in decreased outward potassium currents and hyperexcitability at presynaptic terminals and, thus, are consistent with the NMJ phenotype observed when CaMKII is inhibited. These results show that Eag is a substrate of CaMKII and suggest that direct modulation of potassium channels may be an important function of this kinase.

Numerous studies have suggested that ion channels are an important class of neuronal targets for protein kinases. Modulation of potassium channel activity by kinases has key importance in regulation of neuronal excitability and synaptic plasticity (1). The ether-a-go-go (eag) gene in Drosophila encodes a voltage-gated potassium channel that has a C-terminal cytoplasmic domain with homology to cyclic nucleotide-gated channels (2, 3). This channel is the founding member of a superfamily of vertebrate and invertebrate genes, which include eag, elk, and erg subfamilies (4). In flies, mutation of the eag gene causes shaking after exposure to ether (5) and hyperexcitability at the NMJ (6). These phenotypes have been demonstrated with both hypomorphic and null alleles of eag (see, for example, the NMJ phenotypes of eag<sup>2</sup>, a hypomorph, and eag<sup>e29</sup>, a null, in Ref. 7). In humans, mutation of the HERG gene is one cause of long QT syndrome (8).

Genetic interactions between Drosophila calcium/calmodulin-dependent protein kinase II (CaMKII) and Eag have suggested the hypothesis that Eag is a downstream target for CaMKII-mediated modulation of neuronal function (7). Inhibition of CaMKII by expression of a kinase-specific autoinhibitory domain peptide produced supernumerary excitatory junctional potentials at the third instar NMJ. This excitability defect was similar in many respects to that of eag mutants, and expression of the inhibitor on an eag background produced nonadditive effects on excitability. Both expression of inhibitory peptide and eag mutations blocked memory formation of courtship conditioning (7). These data suggested that CaMKII and Eag may function in a common pathway that regulates neuronal plasticity.

To investigate the direct interaction of Eag and CaMKII, we have identified a CaMKII phosphorylation site on Eag. We find that Eag is expressed in both the axon and the terminal boutons of motor neurons, where it is phosphorylated in vivo. We also present evidence that phosphorylation of this site can modulate channel function.

EXPERIMENTAL PROCEDURES

GST Fusion Protein Constructs—Fragments of the Eag cDNA were cloned into pGEX-2T (Amersham Biosciences) to make GST fusion proteins. The corresponding starting and ending amino acids of the fragments are as listed: pGEX-GST-Eag-N, amino acids 44–210; pGEX-GST-Eag-C1, amino acids 556–802; and pGEX-GST-Eag-C2, amino acids 556–1174.

Site-directed Mutagenesis—PCR was used to create Eag mutants. Oligonucleotide sequences of primers used for generating the mutants are as follows: S576A, 5′-GACATATGCTGTCATCTAAATGCAAAGTT-ATTGAGCAGCATCCGACATTTCGTCTGGCCGCGGATGGT-3′; TG55A, 5′-TCTGGCCTATTGTGATTTG-3′ and 5′-CACAAATGCCCCACG-AGCC-3′; TG787A, 5′-GGGAATTCTCGCGACTTGGCGGCGAGGAGT-GACAC-3′; TG11032 and TG11032-AGCAC-3′. Mutations are underlined. The double mutants S576A/T787A and S576A/T655A were made by subcloning fragments containing one mutation into pGEX-GST-Eag-C1 that contains the other mutation. Sequences were verified by DNA sequencing (Brandeis Biochemistry Core Facility).

Expression and Purification of Fusion Proteins—GST fusion proteins are expressed and purified by the protocol described in the manufacture instructions for the GST fusion protein purification kit (Amersham Biosciences). The expression level of fusion proteins was determined by SDS-PAGE and Coomassie staining.

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1 The abbreviations used are: NMJ, neuromuscular junction; CaMKII, calcium/calmodulin-dependent protein kinase II; WT, wild type; GFP, green fluorescent protein; GST, glutathione S-transferase; PMS, phosphate-buffered saline; PBT, phosphate-buffered saline, Triton X-100, and bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PIPES, 1,4-piperazineethanesulfonic acid.
culture was allowed to grow at 37 °C for another 2 h. Cells were harvested by centrifugation at 5,000 × g for 10 min.

The protocol for purification was modified from the manufacturer instructions from the glutathione-Sepharose-4B column (Amersham Biosciences). To purify the fusion proteins, cell pellets were resuspended in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM benzamidine, 1 μM microcystin-LR, and 1 μM dithiothreitol). 0.5 mg/ml lysozyme (Sigma) was added, and the mixture was incubated for 10 min. Triton X-100 was added to the reaction mixture and heated at 100 °C. The samples were added to the column and equilibrated with the lysis buffer to make a 50% suspension. After incubating with affinity-purified anti-Thr(P)-787 antibody (1:500) in PBS with 0.05% Tween 20 plus 10% normal goat serum for 60 min at 37 °C. After washing five times, the beads were incubated with 1:200 anti-rabbit rhodamine-conjugated secondary antibody (Jackson Immunolog Inc) for 30 min at room temperature. After five washes, the coverslips were mounted onto slides. The samples were examined using confocal microscopy (Bio-Rad 600 system).

Drosophila Stocks—Lines with mutations in the eag gene (eag1 and eag279) were a gift of Barry Ganetzky (University of Wisconsin, Madison, WI). The eag1 allele encodes a protein with a truncated C terminus2 and is a hypomorph, whereas the eag279 mutant is a null for the full-length mRNA (13) and there is no detectable full-length protein in head extracts (data not shown). The pan-neural GAL4 driver line C155 (14) was used to drive transgene expression in fly neurons. This line contains a GAL4 gene inserted into the elav locus and expresses in all neurons (15). UAS-CaMKII-T287D was made as described (16). This transgene encodes a constitutively active form of Drosophila CaMKII (17).

Preparation and Phosphorylation of Fly Head Extracts—Canton-S flies were collected and quickly frozen in liquid N2. Heads were sepa-
 rated from bodies with a sizing sieve. Approximately 250 heads were homogenized in the solubilization buffer and spun for 10 min at 4 °C. The supernatant was divided into four aliquots and mixed with CaMKII, phosphorylation assay reagents, and [γ-32P]ATP. Calcium-activated kinase was added to an assay mix containing 150 mM PIPES, pH 7.0, 15 mM MgCl2, 1 mM CaCl2, 10 μM calmodulin, and 50 μM [γ-32P]ATP. In control reactions, 0.5 mM EGTA was used and CaCl2 and CaM were omitted. CaMKII (R3 isofrom) was purified as described from transfected COS cells (18). The reactions were allowed to proceed at 30 °C for 10 min and terminated by adding 200 μl of solubilization buffer and 5 μl of the anti-Eag antibody. Immunoprecipitation was done by rotating tubes for 2 h at 4 °C. The Eag-IgG complex was pulled down with protein A beads. The beads were washed, and proteins were boiled off in SDS sample loading buffer and separated on SDS gels. Gels were dried and subjected to autoradiography.

Immunohistochemistry of Third Instar Larvae—Male third instar larvae were immobilized in a chamber cut out on a Sylgard-covered glass slide with insect pins (Fine Science Tool, Inc.) and cut open along their dorsal surface with Aesculap Micro Scissors. Gut and brain were removed. Larval body wall muscles were dissected in low calcium saline (5 mM HEPES, pH 7.2, 128 mM NaCl, 2 mM KCl, 35.5 mM sucrose, 4 mM MgCl2, 2 mM EGTA) and fixed in PBS with 4% paraformaldehyde for 30 min. Preparations were washed three times (15 min each) with PBT (PBS, 0.3% Triton X-100, 0.1% bovine serum albumin) and incubated in 1.50 affinity-purified anti-Thr(P)-787 antibody overnight at 4 °C, followed by incubation with a rhodamine-conjugated anti-rabbit IgG secondary antibody (Cappel, 1:200, 60 min at 37 °C). The samples were washed three times with PBT and mounted using Vectorshield (Vector Laboratories).

Electrophysiology—For expression in Xenopus oocytes, SpH1 sites flanking the Eag cDNA were used to subclone single-site mutations into the pGH19-eag construct (12). Plasmids were linearized using NotI and capped RNAs transcribed in vitro using T7 RNA polymerase according to manufacturer instructions (Message Machine, Ambion). RNA concentrations were quantified with spectrophotometric

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2 B. Ganetzky, personal communication.
readings. Stage V-VI oocytes were defolliculated by incubation in Ca\(^{2+}\)-
free OR2 solution (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl\(_2\), 5 mM 
Hepes, pH to 7.6 with NaOH) containing 2 mg/ml collagenase (Sigma, 
type 1A) for 1–3 h with gentle agitation. Oocytes were injected with 
0.1–0.2 ng of RNA/oocyte and were maintained in L-15 medium (con-
taining: 50% L-15 (Invitrogen), 15 mM Hepes, 50 mg/ml gentamycin, 
and 5 mg/ml bovine serum albumin, pH to 7.4 with NaOH) at 18 °C for 
2–5 days. Recordings were performed using a Turbo TEC-10C amplifier 
(NPI Electronics) and pCLAMP8 software (Axon Instruments). The 
extracellular recording solution typically contained 140 mM NaCl, 2 mM 
KCl, 1 mM MgCl\(_2\), 10 mM Hepes (pH to 7.1 with NaOH). An extracel-
lar solution high in K\(^{+}\) was used to enhance tail currents during 
measurements of the voltage dependence of activation; this solution 
contained 100 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5 mM Hepes (pH 7.4 
with KOH). Pipettes were filled with 2 M KCl and had resistances of 
0.3–0.6 megohms. Experiments were performed at room temperature 
(19–22 °C).

Unless otherwise noted, amplitude measurements refer to the peak 
currents observed during test pulses to the indicated voltages. Activat-
ation and inactivation time constants were determined by fitting traces 
(excluding capacitative transients) with two exponentials and a steady 
state. Conductance-voltage (G-V) relations were obtained from meas-
urements of instantaneous tails currents. Curves were normalized by 
the maximum conductance and fit with a Boltzmann distribution. 
Measurements were statistically compared using unpaired Student’s t 
tests with the Welch correction for unequal variances.

CaMKII autoinhibitory peptide (CaMKII inhibitor 271–301, Calbio-
chem) and lavendustin C (Calbiochem) were dissolved in water and 
Me\(_2\)SO, respectively. Equal volumes of the stock solutions or their 
corresponding buffers were injected using a Nanoject (Drummond) to 
obtain the final concentrations noted. Oocyte volume was estimated 
assuming a diameter of 1 mm.

RESULTS

Native Eag Is a Substrate for CaMKII—Previous work had 
shown that CaMKII could phosphorylate a bacterially pro-
duced fragment of Eag in vitro (7). To determine whether 
native Eag proteins in Drosophila were substrates for CaMKII, 
we prepared Canton-S fly head extracts. Radioactive ATP, pu-
 rified CaMKII, Ca\(^{2+}\)/CaM, and/or EGTA were added and the 
mixture incubated for 10 min. After the phosphorylation reac-
tion was complete, Eag was immunoprecipitated. Fig. 1 shows 
\(^{32}\)P incorporation into immunoprecipitated Eag protein visual-
ized by autoradiography. The presence of Ca\(^{2+}\)/CaM and 
exogenous pure CaMKII, Eag proteins from Canton-S fly heads 
were phosphorylated. The Eag band was not seen when either 
Ca\(^{2+}\)/CaM or CaMKII or both were absent in the reactions, 
suggesting that the phosphorylation was Ca\(^{2+}\)/CaM- 
and CaMKII-dependent.

Mapping of the CaMKII Phosphorylation Site on Eag in 
Vitro—Eag is a transmembrane protein with cytoplasmic 
N and C termini. To localize the site(s) of Eag phosphorylation, 
we made GST fusion proteins containing both cytoplasmic do-
mains. Fig. 2A shows a schematic diagram of Eag. GST-Eag-N 
is a 45-kDa fusion protein containing the N terminus of Eag 
from amino acid 44 to 210. This protein includes three CaMKII 
consensus sites (RXX[S/T]) at Thr-154, Thr-182, and Ser-188. 
GST-Eag-C1 is a 54-kDa fusion protein containing the C ter-
minus of Eag from amino acid 556–602. This fusion protein 
includes three CaMKII consensus sites at Ser-576, Thr-655, 
and Thr-787. Phosphorylation of fusion proteins. Purified GST fusion proteins or GST 
were phosphorylated in a reaction with [\(^{32}\)P]ATP and purified 
CaMKII in the presence (+) or absence (−) of calcium and calmodulin at 
30 °C for 2 min. Proteins were separated by SDS-PAGE. Phosphate 
incorporation was visualized by autoradiography. This experiment 
was performed four times with equivalent results.

CaMKII phosphorylates the C terminus of Eag. A, schematic 
diagram of Eag and the GST fusion proteins used in this study. B, 
phosphorylation of fusion proteins. Purified GST fusion proteins or GST 
were phosphorylated in a reaction with [\(^{32}\)P]ATP and purified 
CaMKII in the presence (+) or absence (−) of calcium and calmodulin at 
30 °C for 2 min. Proteins were separated by SDS-PAGE. Phosphate 
incorporation was visualized by autoradiography. This experiment 
was performed four times with equivalent results.
We conclude that Thr-787 of Eag is the only site that is phosphorylated by CaMKII. GST-Eag-C2 fusion proteins were phosphorylated in a reaction with [γ-32P]ATP and purified CaMKII in the presence of calcium and calmodulin, separated by SDS-PAGE, and subjected to autoradiography to visualize phosphate incorporation. This experiment was done four times with equivalent results. B, time course of GST-Eag-C2 phosphorylation. Purified GST-Eag-C2 and GST-Eag-C2-T787A fusion proteins were phosphorylated in a reaction with [γ-32P]ATP and purified CaMKII in the presence of calcium and calmodulin for the indicated times, separated by SDS-PAGE, and subjected to autoradiography to visualize phosphate incorporation. The arrowhead indicates the position of the full-length fusion protein. Lower molecular mass bands are degradation products. This experiment was performed four times with equivalent results.

Phosphorylation of Eag by CaMKII

Fig. 3. Threonine 787 is the major CaMKII site in Eag. A, phosphorylation of Eag wild type and mutant fusion proteins. Purified GST-Eag-C1 fusion proteins were phosphorylated in a reaction with [γ-32P]ATP and purified CaMKII in the presence of calcium and calmodulin, separated by SDS-PAGE, and subjected to autoradiography to visualize phosphate incorporation. The experiment was done four times with equivalent results. B, time course of GST-Eag-C2 phosphorylation. Purified GST-Eag-C2 and GST-Eag-C2-T787A fusion proteins were phosphorylated in a reaction with [γ-32P]ATP and purified CaMKII in the presence of calcium and calmodulin for the indicated times, separated by SDS-PAGE, and subjected to autoradiography to visualize phosphate incorporation. The arrowhead indicates the position of the full-length fusion protein. Lower molecular mass bands are degradation products. This experiment was performed four times with equivalent results.

Threonine 787 of Eag Is Phosphorylated in Transfected tsA201 Cells—To allow us to determine whether Thr-787 was a consensus phosphorylation site for PKA, the only such site in Eag, the mammalian tsA201 cell line has been used to express potassium channel proteins at relatively high levels (19). We used anti-Thr(P)-787 to probe the phosphorylation state of Eag in tsA201 cells, which had been transiently transfected with Myc-tagged Eag-WT or Eag-T787A cDNA. To test the ability of CaMKII to phosphorylate Eag, a cDNA encoding a constitutively active form of CaMKII (CaMKII-T287D) was cotransfected. Eag proteins in cell extracts were immunoprecipitated with the anti-Eag antibody and analyzed by immunoblotting (Fig. 4B). Equal amounts of Eag were precipitated from each cell extract, as shown by Western blots using an antibody that recognizes the Myc epitope tag present on the channel. When the same immunoprecipitations were probed with the anti-Thr(P)-787 phosphospecific antibody, there was a 13-fold increase in the immunoreactivity in cells cotransfected with Eag-WT and CaMKII-T287D, compared with those transfected with Eag-WT alone. The basal signal seen with the wild type alone was probably a result of its phosphorylation by endogenous kinase. We and others (20) have observed a low level of CaMKII activity in this cell line. There was no signal detected in cells transfected with the mutant Myc-Eag-T787A, even in the presence of the constitutively active CaMKII.

Thr-787 can also be shown to be an in vivo CaMKII site if the anti-Thr(P)-787 antibody is used to directly stain transfected tsA201 cells. For these experiments, cells were transfected with GFP-tagged Eag cDNAs, with or without the constitutively active form of CaMKII. After fixation, cells were stained with the anti-Thr(P)-787 antibody. Confocal images are shown in Fig. 5. Images on the left show GFP fluorescence, which represents Eag expression level and localization in these cells. Images of the same field on the right side of Fig. 5 show rhodamine fluorescence, which represents anti-Thr(P)-787 staining. Eag appears to be on plasma membranes as well as in internal membrane compartments, consistent with the fact that Eag is a membrane protein. The pattern of Eag localization does not
Phosphorylation of Eag by CaMKII

**Phosphorylation of Thr-787 Modulates Eag Activity**—To determine the functional consequences of Thr-787 phosphorylation, we expressed Eag in *Xenopus* oocytes. Because the level of activity of CaMKII endogenous to oocytes is unknown, we considered the possibility that endogenous CaMKII already might have phosphorylated the oocyte-expressed channels. If so, it should be possible to modulate Eag activity by inhibiting the endogenous CaMKII, providing there is an active phosphatase. Some of the most potent and specific inhibitors of CaMKII activity characterized to date are peptides with sequences corresponding to the autoinhibitory region of CaMKII (21). Fig. 7A (left) shows the current-voltage relations obtained for sham-versus autoinhibitory peptide-injected oocytes expressing wild type Eag channels. In the presence of the peptide, Eag peak current amplitudes, measured in response to test pulses to +40 mV (holding potential, −80 mV), decreased by nearly 50%, from 4.0 ± 0.1 μA to 2.1 ± 0.1 μA (n = 8, p < 0.001; actual current values for the normalized data shown in Fig. 7A, left). Comparison of the current-voltage relations shows that the percent decrease in amplitude appeared to be uniform for all voltages within the activation range of the channel (Fig. 7A, left). Peak amplitudes at +40 mV also were decreased in the presence of another inhibitor of CaMKII (22), lavendustin C (18 μM), from 4.1 ± 0.2 μA to 2.9 ± 0.1 μA (n = 9 for both conditions, p < 0.001; data not shown).

The inhibitory effects of autoinhibitory peptide and lavendustin C were dependent on Thr-787, the residue shown to be the above results suggest, the CaMKII endogenous to oo-

**Threonine 787 Is Phosphorylated in Vivo in Drosophila Neurons**—To determine whether Thr-787 was phosphorylated by CaMKII in vivo in flies, we employed the *Drosophila* GAL4-UAS system to drive overexpression of CaMKII in neurons and probed Eag phosphorylation with the phosphospecific anti-Thr(P)-787 antibody. C155, a GAL4 line that drives expression in the nervous system, was crossed to UAS-CaMKII-T287D transgene. There is almost no neuronal signal observed in *eag<sup>260</sup>* larvae stained with this antibody, which is not surprising, because this line does not make full-length *eag* mRNA. C155-GAL4 *eag<sup>+</sup>* larvae also show very little staining. These results suggest that anti-Thr(P)-787 antibody is specific for the phosphorylated Thr-787 in vivo in the nerve terminals. Staining in the nuclei of muscle cells is seen in both wild type and *eag<sup>260</sup>* mutant larvae. This may be nonspecific or it may be caused by the presence of a 50-kDa fragment of the C terminus that is still produced in *eag<sup>260</sup>* (data not shown). Overall, this result strongly suggests that Thr-787 of Eag is phosphorylated in vivo by CaMKII.

**Phosphorylation of Thr-787 Modulates Eag Activity**—To determine the functional consequences of Thr-787 phosphorylation, we expressed Eag in *Xenopus* oocytes. Because the level of activity of CaMKII endogenous to oocytes is unknown, we considered the possibility that endogenous CaMKII already might have phosphorylated the oocyte-expressed channels. If so, it should be possible to modulate Eag activity by inhibiting the endogenous CaMKII, providing there is an active phosphatase. Some of the most potent and specific inhibitors of CaMKII activity characterized to date are peptides with sequences corresponding to the autoinhibitory region of CaMKII (21). Fig. 7A (left) shows the current-voltage relations obtained for sham-versus autoinhibitory peptide-injected oocytes expressing wild type Eag channels. In the presence of the peptide, Eag peak current amplitudes, measured in response to test pulses to +40 mV (holding potential, −80 mV), decreased by nearly 50%, from 4.0 ± 0.1 μA to 2.1 ± 0.1 μA (n = 8, p < 0.001; actual current values for the normalized data shown in Fig. 7A, left). Comparison of the current-voltage relations shows that the percent decrease in amplitude appeared to be uniform for all voltages within the activation range of the channel (Fig. 7A, left). Peak amplitudes at +40 mV also were decreased in the presence of another inhibitor of CaMKII (22), lavendustin C (18 μM), from 4.1 ± 0.2 μA to 2.9 ± 0.1 μA (n = 9 for both conditions, p < 0.001; data not shown).

The inhibitory effects of autoinhibitory peptide and lavendustin C were dependent on Thr-787, the residue shown to be phosphorylated by CaMKII in our *in vitro* phosphorylation assays. Fig. 7A (right) shows the average current-voltage relations obtained for sham-versus inhibitory peptide-injected oocytes expressing Eag-T787A channels. To allow direct comparison with the effect of the peptide on wild type channels, the current-voltage relations were normalized to the maximum peak current observed in control oocytes in each case. Inhibitory peptide produced no change in the amplitudes of Eag-T787A currents at any voltage (Fig. 7A, right). Similarly, there was no change in the average current-voltage relations following treatment with lavendustin C (data not shown). Peak amplitudes of Eag-T787A currents elicited by test pulses to +40 mV were 1.1 ± 0.1 μA (n = 9) and 1.0 ± 0.1 μA (n = 7) for control and lavendustin C-treated oocytes, respectively.

Together, these results suggest that CaMKII endogenous to oocytes phosphorylates Eag channels and that phosphorylation at Thr-787 produces a substantial increase in Eag current. If, as the above results suggest, the CaMKII endogenous to oocytes is normally active in stage V and VI oocytes, one also would predict a difference in the current amplitudes of Eag-WT and Eag-T787A channels following injection of equal amounts of RNA. In agreement with this idea, Fig. 7B shows that the average peak current elicited by test pulses to +40 mV was 70% smaller for Eag-T787A than for the wild type channel (p < 0.001). Substitution of aspartate or glutamate residues, which adds a single negative charge at the phosphorylation sites, has been shown in several instances to totally or partially mimic the effects of phosphorylation (23, 24). As shown in Fig. 7B,
injection of an equal amount of Eag-T787D RNA produced current amplitudes that were larger than the Eag-T787A amplitudes by 1.5-fold (p < 0.001 for comparison to T787A) but not equivalent to wild type amplitudes. Given that phosphorylation results in the addition of more than one negative charge to the side chain, one interpretation of this result is that wild type channels expressed in oocytes are maximally phosphorylated. The addition of phosphate to the threonine residue may have a larger effect on amplitude than the substitution of aspartate because of the increased amount of negative charge. This type of quantitative difference between a phosphorylated residue and an acidic substitution has been seen in other mutants (24).

Finally, in addition to the above noted effects on amplitude, phosphorylation at Thr-787 also slows the inactivation of Eag channels. As shown in Fig. 7C and Table I, when compared with currents recorded from wild type channels, a larger proportion of the T787A current was inactivating and inactivation kinetics also were significantly faster. Similar trends also were observed in oocytes treated with inhibitory peptide and lavendustin C, although only the results for lavendustin C reached statistical significance (Table I). Alteration of Thr-787 did not appear to affect either the activation kinetics (Table I) or the voltage dependence of Eag channels (data not shown).

**DISCUSSION**

Previous studies from our laboratory (7) have shown that inhibition of CaMKII activity, by transgenic expression of a CaMKII autoinhibitory peptide (the ala transgene), results in electrophysiological and behavioral phenotypes that bear a striking similarity to those observed in Drosophila eag mutants. The simplest interpretation of these results is that CaMKII phosphorylation of Eag channels normally maintains or enhances Eag activity. In support of this hypothesis, the results of the present study demonstrate that CaMKII phosphorylates Eag at Thr-787 and that changes in phosphorylation at this site modulate current amplitude. Mutations of Eag at the CaMKII phosphorylation site that prevent phosphorylation (T787A) or mimic phosphorylation (T787D) result in significant changes in current amplitude. These changes must be the result of either alterations in channel properties or alterations in channel processing. The additional effect on channel inactivation, which also is consistent with the larval electrophysiological phenotype, suggests that effects on channel properties are the more likely interpretation.

This study also provides a likely explanation for one aspect of the eag phenotype at the NMJ. The eag phenotype observed in recordings at the larval NMJ is particularly distinctive, exhibiting several characteristics that are not mimicked by mutations of other potassium channel genes, such as Shaker (Sh) or slowpoke (6, 25). In particular, eag mutants have both spontaneous activity generated in the axon and an abnormal response to stimulation. Each stimulus is followed by multiple prolonged excitatory junctional potentials and an afterdischarge that continues for several minutes following the complete cessation of stimulation. These observations have suggested that one function of Eag channels is to limit excitability by maintaining the resting potential of presynaptic motor nerve terminals, although it has remained unclear whether the phenotype is a consequence of the loss of Eag channels directly from the terminal or whether, for example, the phenotype is a consequence of a more distant loss resulting in activity-dependent regulation of the expression of multiple other potassium channel types (26). Although not eliminating activity-dependent regulation as a possible contributor to the eag phenotype, our results provide the first demonstration of the localization of Eag directly at the presynaptic motor nerve terminals. In addition, we show that the level of Eag phosphorylation at this site is regulated by the activity of CaMKII. These data suggest that either the absence of Eag or the lack of CaMKII phosphorylation of an equal amount of Eag-T787D RNA produced current amplitudes that were larger than the Eag-T787A amplitudes by 1.5-fold (p < 0.001 for comparison to T787A) but not equivalent to wild type amplitudes. Given that phosphorylation results in the addition of more than one negative charge to the side chain, one interpretation of this result is that wild type channels expressed in oocytes are maximally phosphorylated. The addition of phosphate to the threonine residue may have a larger effect on amplitude than the substitution of aspartate because of the increased amount of negative charge. This type of quantitative difference between a phosphorylated residue and an acidic substitution has been seen in other mutants (24).

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Previous studies from our laboratory (7) have shown that inhibition of CaMKII activity, by transgenic expression of a CaMKII autoinhibitory peptide (the ala transgene), results in electrophysiological and behavioral phenotypes that bear a striking similarity to those observed in Drosophila eag mutants. The simplest interpretation of these results is that CaMKII phosphorylation of Eag channels normally maintains or enhances Eag activity. In support of this hypothesis, the results of the present study demonstrate that CaMKII phosphorylates Eag at Thr-787 and that changes in phosphorylation at this site modulate current amplitude. Mutations of Eag at the CaMKII phosphorylation site that prevent phosphorylation (T787A) or mimic phosphorylation (T787D) result in significant changes in current amplitude. These changes must be the result of either alterations in channel properties or alterations in channel processing. The additional effect on channel inactivation, which also is consistent with the larval electrophysiological phenotype, suggests that effects on channel properties are the more likely interpretation.

This study also provides a likely explanation for one aspect of the eag phenotype at the NMJ. The eag phenotype observed in recordings at the larval NMJ is particularly distinctive, exhibiting several characteristics that are not mimicked by mutations of other potassium channel genes, such as Shaker (Sh) or slowpoke (6, 25). In particular, eag mutants have both spontaneous activity generated in the axon and an abnormal response to stimulation. Each stimulus is followed by multiple prolonged excitatory junctional potentials and an afterdischarge that continues for several minutes following the complete cessation of stimulation. These observations have suggested that one function of Eag channels is to limit excitability by maintaining the resting potential of presynaptic motor nerve terminals, although it has remained unclear whether the phenotype is a consequence of the loss of Eag channels directly from the terminal or whether, for example, the phenotype is a consequence of a more distant loss resulting in activity-dependent regulation of the expression of multiple other potassium channel types (26). Although not eliminating activity-dependent regulation as a possible contributor to the eag phenotype, our results provide the first demonstration of the localization of Eag directly at the presynaptic motor nerve terminals. In addition, we show that the level of Eag phosphorylation at this site is regulated by the activity of CaMKII. These data suggest that either the absence of Eag or the lack of CaMKII phosphorylation...
RNA injection and were elicited by test pulses to superimposed traces obtained from Eag-WT and Eag-T787A expressing oocytes as indicated. In both no appreciable difference in capacity measurements (obtained using 20-mV pulses from frog on the same day following RNA injection. Oocytes were visually judged to be of the same stage and similar diameter. In addition, there was amounts of RNA (0.16 ng/oocyte) encoding either wild type, T787A, or T787D channels. Recordings were made from oocytes obtained from the same polarity preceding each test pulse (holding potential, 

This current also was induced in oocytes not expressing Eag. Leak subtraction was performed using a P/4 pulse protocol with pulses of opposite polarity preceding each test pulse (holding potential, −80 mV). In both B and C, currents were recorded on day 4 after RNA injection and were elicited by test pulses to +40 mV (holding potential, −80 mV).

**FIG. 7. CaMKII effects on Eag function.** A, comparison of current-voltage relations for sham- and inhibitory peptide-injected oocytes expressing Eag-WT (left) or Eag-T787A (right) channels. Oocytes were injected with 500 μM amounts of the inhibitory peptide 15−90 min prior to recording. Currents were elicited by a series of voltage steps from −100 to 80 mV (holding potential, −80 mV). Wild type and T787A amplitudes were normalized to the maximum peak current observed in control conditions in each case. Currents were leak subtracted to reduce contamination of measurements by a leak current that was often induced by treatment with either the inhibitory peptide or lavendustin C, but not buffer alone. This current also was induced in oocytes not expressing Eag. Leak subtraction was performed using a P/4 pulse protocol with pulses of opposite polarity preceding each test pulse (holding potential, −80 mV). B, average peak amplitudes observed in oocytes injected with equal amounts of RNA (0.16 ng/oocyte) encoding either wild type, T787A, or T787D channels. Recordings were made from oocytes obtained from the same frog on the same day following RNA injection. Oocytes were visually judged to be of the same stage and similar diameter. In addition, there was no appreciable difference in capacity measurements (obtained using 20-mV pulses from −80 mV) for the different conditions. C, scaled, superimposed traces obtained from Eag-WT and Eag-T787A expressing oocytes as indicated. In both B and C, currents were recorded on day 4 after RNA injection and were elicited by test pulses to +40 mV (holding potential, −80 mV).

**TABLE I**

**Eag kinetics**

Kinetic measurements were obtained from currents elicited by a test pulse to +40 mV (holding potential = −80 mV). The % inactivation was obtained using [(1 − Isteady state/Ifineak) × 100], where Isteady state was the mean current observed between 200 and 220 ms. This measurement could not be accurately determined (ND) for inhibitory peptide-injected oocytes due to contamination of the later portion of the traces by the appearance of a slow outward current. Measurements are the means ± S.E. with the number of observations given in parentheses. The p values refer to t test comparisons with the control (NS = not significant). Each grouping of data was obtained from recordings on the same day after RNA injection from oocytes of the same frog.

| Condition                      | Inactivation | p     | Activation tau | p     | Inactivation tau | p     |
|--------------------------------|--------------|-------|----------------|-------|-----------------|-------|
| WT                             | 13.1 ± 1.0 (9) | <0.01 | 4.3 ± 0.2 (9)  | NS    | 41.7 ± 2.3 (9)  | NS    |
| T787A                          | 17.7 ± 0.7 (9) |       | 4.9 ± 0.2 (9)  | NS    | 35.2 ± 1.0 (9)  | NS    |
| WT control                     | 10.8 ± 1.1 (5) | <0.01 | 5.5 ± 0.8 (5)  | NS    | 69.7 ± 10.4 (5) | NS    |
| WT plus inhibitory peptide     | ND           |       | 5.5 ± 0.5 (8)  | NS    | 60.2 ± 6.5 (8)  | NS    |
| WT control                     | 12.3 ± 0.9 (9) | <0.001| 5.0 ± 0.2 (9)  | NS    | 54.3 ± 5.7 (9)  | NS    |
| WT plus lavendustin C          | 17.3 ± 0.7 (9) | <0.001| 4.6 ± 0.1 (9)  | NS    | 37.8 ± 0.7 (9)  | <0.05 |

Eag. Expression of constitutively active CaMKII has effects on neuronal excitability (27). Some of these effects appear to be mediated by up-regulation of potassium conductances. Our data suggest that Eag is one of the channels that is modulated by CaMKII, but it may not be only one.

The direct interaction of Eag and CaMKII also supports the idea that the memory formation phenotype of *ala* transgensics may be a result of changes in excitability. Mutations in *Shaker*, *nap*, and *eag* have been shown to disrupt courtship conditioning (28). The biochemical connection between Eag and CaMKII demonstrated in this study suggests that at least part of the reason that *ala* transgensics fail in this behavior may be defective modulation of Eag. It is probable that there are other

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3 J. C. Choi, D. Park, and L. C. Griffith, unpublished results.
excitability-related targets of CaMKII in addition to Eag, but the identity of these targets and the nature of CaMKII’s interaction with them remain to be determined.

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