Drosophila Ortholog of Succinyl-CoA Synthetase β Subunit: A Novel Modulator of Drosophila KCNQ Channels

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Gao L, Fei H, Connors NC, Zhang J, Levitan IB. Drosophila ortholog of succinyl-CoA synthetase β subunit: a novel modulator of Drosophila KCNQ channels. J Neurophysiol 99: 2736–2740, 2008. First published April 2, 2008; doi:10.1152/jn.01314.2007. Voltage-gated KCNQ potassium channels are responsible for slowly activating potassium currents in heart, brain, and other tissues. Functional defects of KCNQ channels are linked with many diseases, including epilepsy and cardiac arrhythmias. Therefore KCNQ potassium channels have been widely studied, especially in the CNS. We have identified Drosophila CG11963, which encodes a protein orthologous to the β subunit of mammalian succinyl-CoA synthetase (SCS, also known as succinate thiokinase), as a novel modulator of Drosophila KCNQ channels. Direct interaction of CG11963 and dKCNQ was demonstrated by yeast two-hybrid screen and communoprecipitation. Cell surface biotinylation experiments further confirmed that CG11963 resides on the plasma membrane of tsA-201 cells. Coexpression of CG11963 with dKCNQ shifts the conductance–voltage (G–V) relationship of dKCNQ channels to more positive membrane potentials in Chinese hamster ovary (CHO) cells. Moreover, directly dialyzing glutathione S-transferase fusion CG11963 protein into CHO cells also shifts the dKCNQ G–V curve rightward. The effect of CG11963 persists in the presence of 1 mM adenosine triphosphate (ATP), a substrate of SCS. Taken together, our data define CG11963 as a new dKCNQ-binding protein capable of modulating the properties of the channel. Our evidence suggests that this modulation is mediated by direct interaction of CG11963 with the channel and is not dependent on ATP.

INTRODUCTION

KCNQ channels are among the most extensively studied voltage-gated potassium channels and play a critical role in cellular excitability (Delmas and Brown 2005). Mammalian KCNQ channel genes encode five known isoforms (KCNQ1–KCNQ5), mutations in which underlie human cardiac and neurological diseases (Biervert et al. 1998; Chouabe et al. 2000; Neyroud et al. 1997; Saplowski et al. 1997; Wang et al. 1996). Mammalian “M-current,” which is a slow, nonactivating potassium conductance first identified by Brown and Adams (1980) in superior cervical ganglion (SCG) neurons, is mediated by KCNQ2/3 channels (Wang et al. 1998). M-current is found in many neuronal cell types and dramatically regulates the firing rate of neurons. A KCNQ-like channel from Drosophila, termed dKCNQ, was recently cloned and characterized in our lab (Wen et al. 2005). There is only one KCNQ-like gene in the fly. Many aspects of the functional properties of dKCNQ have striking parallels with their mammalian orthologs, such as slow activation and deactivation kinetics, and no discernable inactivation. Calmodulin can form a stable interaction with dKCNQ through two binding sites on the C-terminus of the channel and is likely required for channel activity (Wen et al. 2005).

To search for other binding partners and modulators of KCNQ channels, we performed a yeast two-hybrid screen using the C-terminus of dKCNQ as bait against a Drosophila melanogaster adult Matchmaker cDNA library. We recovered a number of interacting proteins and focus here on one of them, a protein named CG11963, which is orthologous to the beta subunit of mammalian succinyl-CoA synthetase (SCS). Our data identify CG11963 as a novel modulator of the Drosophila KCNQ channel.

METHODS

Yeast two-hybrid screen

The yeast two-hybrid screen was performed using the Matchmaker Two-Hybrid System 3 (Clontech), using the entire 543 amino acid C-terminal tail of the Drosophila dKCNQ channel as bait against a Drosophila adult Matchmaker cDNA library. Positive clones were selected on plates lacking adenine, histidine, leucine, and tryptophan and were assayed for α-galactosidase activity. The plasmids of positive clones were isolated from yeast cells and the sequences of the cDNAs were determined.

Cloning of Drosophila CG11963

CG11963 cDNA was cloned and amplified by polymerase chain reaction (PCR) analysis from a Drosophila whole body cDNA library. Primers were designed according to the sequence in GenBank that corresponds to Drosophila CG11963. The forward primer was 5’-CCC CCC GAA TTC ACC ATG GCT TCA TTC TTG GCA CGA ACT GGC-3’ and the reverse primer was 5’-TCA GCC GAT CTT GGG AGC GCT GTG-3’. PCR products were sequenced from both directions and cloned into the mammalian expression vectors pcDNA3.1/Myc-his (Invitrogen) for biochemical assays and the pIRE2-EGFP vector (Clontech) for electrophysiological studies. cDNAs encoding dKCNQ were in the pcDNA3.1(+) (Invitrogen) expression vector for electrophysiology and in the pcDNA3.1(+) vector with a FLAG tag at the C-terminus for biochemistry.

Construction and purification of glutathione S-transferase–CG11963 proteins

cDNAs encoding full-length CG11963 were fused to glutathione S-transferase (GST) in the pGEX-4T-1 vector and expressed in Escherichia coli BL21(DE3). Fusion protein was purified using glutathione-

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Biochemistry

tsA-201 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (Invitrogen) plus penicillin and streptomycin (Invitrogen) in 100-cm² culture dishes. DNA encoding FLAG-tagged dKCNQ in pcDNA3.1(+) and/or Myc-tagged CG11963 in pcDNA3.1/Myc-his were transfected using Lipofectamine 2000 reagent, according to the manufacturer’s protocol (Invitrogen). For all constructs, the appropriate empty vector was used as a negative control.

Transfected cells were lysed and proteins were immunoprecipitated from lysates, separated by gel electrophoresis, and transferred to nitrocellulose membranes for Western blotting as described previously (Zeng et al. 2006). Equal amounts of protein were loaded in each well. Antibodies used for Western blotting and coimmunoprecipitation were purchased from Sigma (anti-Myc M5546 and anti-FLAG F3165).

For biotinylation of cell surface protein, transfected tsA-201 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with gentle agitation for 30 min at 4°C with 1.0 mg/ml EZ-linkTM sulfo-N-hydroxysulfosuccinimide (NHS)-S-S-biotin (Pierce Biotechnology) in cold PBS as described previously (Wen and Levitan 2002). Biotinylated proteins (equal amounts loaded in each well) were separated by SDS-PAGE and immunoblotted as above.

Electrophysiology

Chinese hamster ovary (CHO) cells were maintained in F-12K nutrient mixture (Invitrogen) with 10% fetal bovine serum plus penicillin and streptomycin. They were seeded onto glass coverslips in a 35-mm cell-culture dish and transiently transfected using Lipofectamine 2000. Transfections consisted of combinations of dKCNQ in pcDNA3.1(+) and CG11963 in pIRE2-eGFP or pIRE2-eGFP vector alone.

Standard whole cell patch recordings were performed with CHO cells exhibiting green fluorescence 1 to 2 days after transfection, with an Axopatch 200A amplifier (Axon) and an Axiovert 25 inverted fluorescence microscope (Carl Zeiss MicroImaging). Standard extracellular saline contained (in mM): 140 NaCl, 2.8 KCl, 2 CaCl₂, 2 MgCl₂, 10 D-glucose, and 10 HEPES (pH 7.2). Standard intracellular saline contained (in mM): 140 KCl, 10 BAPTA, and 10 HEPES (pH 7.2).

Results

Our laboratory recently cloned the only known Drosophila ortholog of KCNQ channels, dKCNQ, and identified CaM as one of its binding partners (Wen et al. 2005). To search for other proteins that interact with dKCNQ, we performed a yeast two-hybrid screen using the intracellular C-terminal tail (543 amino acids) of dKCNQ as bait against a Drosophila adult cDNA library.

The screen of 3,500,000 mating yeast colonies revealed that the C-terminus of dKCNQ interacts with a number of unexpected proteins in the fly proteome. Among them, one was identified as CG11963, the gene for which can be found on Drosophila Chromosome 3R at location 4,764,405–4,768,750. CG11963 encodes a protein with a calculated molecular mass of 54,808 Da that exhibits 59% amino acid identity with the human SCS β subunit. The role of Drosophila CG11963 protein has not been characterized. Moreover, there is no information to suggest that this protein is involved in ion channel modulation, so its binding to dKCNQ is unexpected.
and of great interest. We cloned CG11963 and tested its ability to modulate dKCNQ.

To confirm and characterize the interaction between CG11963 and full-length dKCNQ channels, we cotransfected FLAG-tagged dKCNQ channels with Myc-tagged CG11963 in tsA-201 cells and performed coimmunoprecipitation experiments. The results of such experiments demonstrate that dKCNQ does interact with CG11963 in transfected tsA-201 cells (Fig. 1A). Considering that CG11963 is orthologous to a mitochondrial matrix enzyme subunit, these results are striking and novel.

To determine whether CG11963 associates with channels residing in the plasma membrane, a surface biotinylation assay was used. We found that Myc-tagged CG11963 and FLAG-tagged dKCNQ proteins both are present in the pool of biotinylated membrane proteins (Fig. 1B). Surprisingly, our data also show that CG11963 resides in the plasma membrane even if dKCNQ is not present (Fig. 1B). These findings are consistent with the idea that CG11963 not only interacts with dKCNQ channels, but also itself is an integral plasma membrane protein.

We next investigated the functional consequences of the interaction between dKCNQ and CG11963. We coexpressed CG11963 with dKCNQ channels in CHO cells and recorded dKCNQ whole cell currents. We found that, in the presence of CG11963, the dKCNQ conductance–voltage (G–V) curve is right-shifted by about 10 mV compared with control cells in which only dKCNQ is expressed [from −24.5 mV (n = 18) to −14.6 mV (n = 13) P < 0.05] (Fig. 2, A–D). This change is specific to dKCNQ because CG11963 does not shift the G–V relationship of Drosophila Slowpoke BK (dSlo) (Fig. 2E) or human EAG1 (hEAG) (Fig. 2F) potassium channels. We also used a standard protocol to measure the deactivation of the dKCNQ channel. Channel-expressing cells were initially held under voltage clamp at a relatively depolarized membrane potential (+30 mV in this experiment) and then hyperpolarized to −40 and −60 mV, respectively (Brown and Adams 1980). Under these conditions, dKCNQ currents deactivate slowly, showing a slow inward current relaxation. We analyzed the closing kinetics of dKCNQ by fitting the deactivating current with two exponential time constants at each voltage. We found no difference between the controls and the cells that were cotransfected with CG11963 (Table 1).

To determine whether CG11963 acts intracellularly to modulate dKCNQ we constructed a GST fusion CG11963 (GST–CG11963) and diluted it into the recording pipette solution at a final concentration of 400 nM. As shown in Fig. 3A, both GST and GST–CG11963 migrate on SDS-PAGE gels according to their predicted molecular weights (28 and 83 kDa, respectively). We compared initial currents, which were recorded immediately after patch break-in, with those recorded 5 min later after the fusion protein had diffused into the cell. Cells dialyzed with GST–CG11963 (Fig. 3B) display a small but significant rightward shift of the G–V curve from −22.3 mV (n = 11) to −17.5 mV (n = 10) (P < 0.05); the controls, which were dialyzed with GST protein, do not show such a shift. This implies that direct interaction between dKCNQ and CG11963, the dKCNQ conductance–voltage (G–V) curve is

FIG. 2. CC11963 shifts the conductance–voltage relationship (G–V) of Drosophila KCNQ potassium channels to the right. Representative whole cell currents of dKCNQ channels in Chinese hamster ovary (CHO) cells transfected with dKCNQ channels alone (A) or dKCNQ channels together with CG11963 (B) were recorded under standard whole cell voltage clamp conditions (10 mV steps; 500 ms). C: current–voltage (I–V) relationship of dKCNQ channels. Coexpression of CG11963 slightly inhibits the peak current of dKCNQ. D: G–V relationship for dKCNQ with or without CG11963. Data were fitted to a single Boltzmann function with the follow parameters: dKCNQ alone, V <sub>50</sub> = −24.5 mV, slope = 15.7 (n = 18); dKCNQ + CG11963, V <sub>50</sub> = −14.6 mV, slope = 16.0 (n = 13). E: G–V curves derived from standard whole cell currents recorded from CHO cells expressing Drosophila Slowpoke BK (dSlo) potassium channels with or without CG11963. There is no significant difference between the control currents (dSlo alone), and dSlo currents in the presence of CG11963. F: coexpressing CG11963 with human EAG1 (hEAG) potassium channels does not affect the G–V relationship of hEAG.
physical binding of CG11963 to the intracellular portion of dKCNQ is responsible for modulating the channel's voltage dependence.

Adenosine triphosphate (ATP) is a substrate of SCS and there is an ATP-grasp domain within the CG11963 sequence, which raised the possibility that ATP might be involved in its modulation of dKCNQ. To test this possibility, we used 1 mM ATP in the internal solution, and still observed CG11963 modulation of dKCNQ (Fig. 3D). These results suggest that this effect of CG11963 on dKCNQ channels is independent of ATP.

### TABLE 1. Deactivation of dKCNQ is not changed by CG11963

<table>
<thead>
<tr>
<th>Time Constant</th>
<th>dKCNQ Alone</th>
<th>dKCNQ + CG11963</th>
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<tr>
<td>(\tau_{\text{slow}}) at -40 mV, ms</td>
<td>3,104 ± 191</td>
<td>3,269 ± 429</td>
</tr>
<tr>
<td>(\tau_{\text{fast}}) at -40 mV, ms</td>
<td>466 ± 22</td>
<td>499 ± 27</td>
</tr>
<tr>
<td>(\tau_{\text{slow}}) at -60 mV, ms</td>
<td>2,719 ± 240</td>
<td>2,308 ± 311</td>
</tr>
<tr>
<td>(\tau_{\text{fast}}) at -60 mV, ms</td>
<td>475 ± 23</td>
<td>427 ± 34</td>
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Deactivation of the dKCNQ channel was measured as follows: Slowly deactivating whole cell dKCNQ currents were recorded during hyperpolarizing steps from a holding potential of +30 mV to potentials of -40 and -60 mV, respectively. The deactivation was then fitted with two exponential time constants (\(\tau\)), fast and slow, at each voltage.

### DISCUSSION

This study describes a novel binding partner and modulator of Drosophila KCNQ channels. Our data show that CG11963, a fly ortholog of the mammalian SCS \(\beta\) subunit, interacts with Drosophila KCNQ channels and modulates the \(G-V\) relationship of these channels. The specificity of this modulation is demonstrated by the inability of CG11963 to modulate the activities of dSlo and hEAG1 potassium channels. Direct application of GST-fusion CG11963 protein in cells shifts the \(G-V\) curve in the same direction as does coexpression of CG11963 with the channel. Moreover, 1 mM ATP does not influence this effect of CG11963. Given the fact that only the intracellular C-terminal region of dKCNQ was used as bait in the yeast two-hybrid screen, and that intracellular application of GST–CG11963 protein causes a similar modulatory effect, it seems possible that CG11963 protein binds to a site or sites within the C-terminal region of dKCNQ to change the function of the channel.

CG11963 encodes a protein orthologous to the \(\beta\) subunit of mammalian SCS, a mitochondrial matrix enzyme that participates in the citric acid cycle. SCS catalyzes the reversible synthesis of succinyl-CoA from succinate and CoA (Nishimura 1986) and its activity is important for metabolism and mito-
chondrial function. A homozygous disruption in the SUCLA2 (human SCS β subunit) gene was identified in patients with encephalomyopathy and mitochondrial DNA depletion syndrome (Elpeleg et al. 2005). Surprisingly, surface biotinylation experiments demonstrate that CG11963 protein is located in the plasma membrane even without coexpression of dKCNQ channels. This finding implies a role for CG11963 as a membrane protein. Consistent with our result, analysis of the primary amino acid sequence of CG11963 predicts two potential transmembrane segments [amino acids 294–317 and 348–369; predicted by TMPred—Prediction of Transmembrane Regions and Protein Orientation (EMBnet-CH); Fig. 1C]. Interestingly, CG11963 has an extended C-terminus compared with its mammalian orthologs and there are 23 lysines within this 54 amino acid region (Fig. 1C). This extended C-terminal domain might also contribute to the plasma membrane targeting of CG11963 or its interaction with dKCNQ channels. The β subunit of human SCS does not contain such a lysine-rich C-terminus and we find that it does not modulate human KCNQ2 channels (data not shown).

The function of Drosophila CG11963 has not previously been determined, but our evidence suggests that CG11963 might be a membrane protein capable of modulating the activity of dKCNQ potassium channels. It is interesting that the modulation does not require coexpression of the SCS α subunit, suggesting that SCS enzymatic activity is not involved. This is not the first example of a new function being assigned to an old protein. For example, calreticulin was initially identified as a calcium-binding protein regulating Ca²⁺ homeostasis in the ER lumen, where the majority of cellular calreticulin is located, but growing evidence indicates that calreticulin is also involved in many other critical functions such as mediating mRNA destabilization in cytoplasm, exerting antithrombotic effects at the cell surface, and modulating cell adhesion (Johnson et al. 2001; Opas et al. 1996; Totary-Jain et al. 2005; Yokoyama and Hirata 2005).

In summary, we demonstrate here the presence of CG11963 in the plasma membrane and its modulation of dKCNQ channels. Although it is still not known whether CG11963 modulates native dKCNQ currents in neurons, further studies of how the modulation of dKCNQ by CG11963 affects neuronal physiology will lead to better understanding of how neuronal excitability is regulated. Since KCNQ channels are so intimately involved in cardiac and neuronal function, our identification of a completely novel KCNQ modulatory protein extends our understanding of the regulation of membrane excitability and may ultimately have clinical implications.

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References


