A Drosophila KCNQ Channel Essential for Early Embryonic Development

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The mammalian voltage-dependent KCNQ channels are responsible for distinct types of native potassium currents and are associated with several human diseases. We cloned a novel Drosophila KCNQ channel (dKCNQ) based on its sequence homology to the mammalian KCNQ genes. When expressed in Chinese hamster ovary cells, dKCNQ gives rise to a slowly activating and slowly deactivating current that activates in the subthreshold voltage range. Like the M-current produced by mammalian KCNQ channels, dKCNQ current is sensitive to the KCNQ-specific blocker linopirdine and is suppressed by activation of a muscarinic receptor. dKCNQ is also similar to the mammalian channels in that it binds calmodulin (CaM), and CaM binding is necessary to produce functional currents. In situ hybridization analysis demonstrates that dKCNQ mRNA is present in brain cortical neurons, the cardia (proventriculus), and the nurse cells and oocytes of the ovary. We generated mutant flies with deletions in the genomic sequence of dKCNQ. Embryos produced by homozygous deletion females exhibit disorganized nuclei and fail to hatch, suggesting strongly that a maternal contribution of dKCNQ protein and/or mRNA is essential for early embryonic development.

Key words: KCNQ channels; M-current; Drosophila ion channels; embryonic development; fly mutants; maternal effect

Introduction

KCNQs are relatively new additions to the voltage-dependent potassium (Kv) channel superfamily. The functional significance of KCNQ channels is highlighted by their relevance to native currents and their association with human diseases. Five KCNQ genes have been cloned to date, all from mammals. The first member, KCNQ1, coassembles with a regulatory β-subunit, KCNE1, to produce the slow delayed rectifier current (I_Ks) in cardiomyocytes (Wang et al., 1996; Yang et al., 1997). Mutations in KCNQ1 cause prolongation of cardiac action potentials and are responsible for one form of long QT syndrome (Sanguinetti et al., 1996). KCNQ1/KCNE1 is also expressed in the cochlea, and mutations of either gene are associated with hearing loss (Neyroud et al., 1997; Schulze-Bahr et al., 1997). KCNQ2 and KCNQ3 were cloned by linkage to a form of human epilepsy (Biervert et al., 1997; Schulze-Bahr et al., 1997). KCNQ4 and KCNQ5 were identified by their sequence homology to other family members (Kubisch et al., 1999; Lerche et al., 2000; Schroeder et al., 2000a). KCNQ4 mutations are associated with a form of dominant deafness in humans (Kubisch et al., 1999). KCNQ5 is found in high levels in the cortex and hippocampus of the mouse, and its activity is inhibited by the M-current blockers linopirdine and XE-991, suggesting that it may contribute to the M-current in some neurons (Schroeder et al., 2000a; Jensen et al., 2005).

The structure and function of major classes of Kv channels are generally conserved between mammals and invertebrates. The genetically accessible fruit fly Drosophila has served as an excellent model system for Kv channel studies. For example, several Kv channels, including the prototypical Shaker, EAG, and Slo, were first cloned from flies based on their mutant behavioral phenotypes (Kamb et al., 1987; Papazian et al., 1987; Pongs et al., 1988; Atkinson et al., 1991; Warmke et al., 1991; Adelman et al., 1992). Lesions in Shaker (Wu et al., 1983), Slo (Elkins et al., 1986; Zhong and Wu, 1991), and Shab (Hegele et al., 1999) genes selectively affect distinct types of potassium currents in the larval body wall muscle of mutant flies.

Kv channels have been divided into seven families based on sequence distances (Hille, 2001), and six of them have been cloned in Drosophila. An analysis of the complete Drosophila genome has predicted a seventh Kv family, composed of a single KCNQ-related gene (Littleton and Ganetzky, 2000). We cloned the novel Drosophila KCNQ gene dKCNQ. When it is expressed in heterologous cells, dKCNQ forms a functional channel that resembles the mammalian KCNQ channels in several aspects. To study in vivo function, we generated several independent fly lines with deletions in the dKCNQ gene locus and analyzed mutant...
phenotypes. Surprisingly, we find that maternally contributed dKCNQ protein and/or mRNA is essential for early embryonic development.

Materials and Methods

Cloning of dKCNQ. Database searches were performed using the BLAST (basic local alignment search tool) server (National Center for Biotechnology Information). AE003830, a genomic scaffold clone from the right arm of chromosome 2, was sequenced by the Drosophila Genome Project (Adams et al., 2000). Genomic sequences that show significant homology with mouse KCNQ2 (mKCNQ2) on the protein level lie in the chromosomal region 46F3 (23506–31191 of AE003830. Drosophila genome annotation, release 3.0). Primers were designed according to the two candidate protein sequences in that region (Flybase accession number FBgn0033494, KCNQ-PA and KCNQ-PB; GenBank accession numbers NM_176120 and NM_176119) and used to amplify dKCNQ from an embryonic cDNA library (Drosophila Quick-clone cDNA; Clontech, Palo Alto, CA), Drosophila melanogaster RE26469 full insert cDNA [GenBank accession numbers BT001588, an embryonic cDNA expressed sequence tag (EST), kindly generated by Piero Carninci at RIKEN Genome Institute (University of California at Berkeley, Berkeley, CA)], and adult Canton S and y w fly cDNA. Adult fly cDNA was extracted by creating mRNA from 25 flies using Ultraspec RNA Isolation System (Biotex, Houston, TX) and was reverse transcribed using Superscript First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA) and was reverse transcribed using Supernatant First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA). The primer pair was as follows: coding primer, 5′-GTGTGGAATAGACGAAGATGAT-3′; and noncoding primer, 5′-CGCTAAATGTTAGTCTGCT-3′.

The PCR product was cloned into the pcDNA3.1-Topo-V5-His mammalian expression vector with the V5 tag at the C terminus (Invitrogen, San Diego, CA). The sequence of dKCNQ has been deposited into GenBank (accession numbers ATY23300 for Canton S and ATY23301 for y w). Although the PCR products were also cloned into pCMV-PA and pCMV-Myc (Clontech), we found that these N-terminal tags abolish the functional activity of the channel.

Electrophysiology constructs and reagents. dKCNQ cDNA was cloned into pIRE2-EGFP, a bicistronic vector that allows coexpression of the channel and the enhanced green fluorescent protein (EGFP) in the same channel and the enhanced green fluorescent protein (EGFP) in the same cell (Clontech). dKCNQ (R326E) in pIRE2-EGFP was generated by site-directed mutagenesis using Quik-Change system (Stratagene, La Jolla, CA) according to the instructions of the manufacturer, and the mutation was confirmed by sequencing through the entire coding region. CaM cDNA (the kind gift from Dr. John Lowenstein, Brandeis University, Waltham, MA) was subcloned into a modified version of the mammalian expression vector pcDNA3.1 to give it a HA-epitope tag at the N terminus. cDNA for mouse M3 receptor was kindly provided by Neil Nathanson (University of Washington, Seattle, WA) and was subcloned into the EcoRI and BamHI sites of pIRE2-EGFP.

Oxotremorine M (Oxo M) and linopirdine (DuP 996) were from Research Biochemicals (Natick, MA). All other chemicals were obtained from Sigma (St. Louis, MO).

Antibody preparation, coimmunoprecipitation, and Western blotting. Anti-dKCNQ rabbit antisem 1–67 was generated against amino acids 691–741 in the C terminus of dKCNQ, expressed as a glutathione-S transferase fusion protein. The antibody was then affinity purified against a maltose-binding protein fusion protein containing the same dKCNQ fragment.

Coimmunoprecipitation and Western blotting were done as described previously (Wang et al., 1999). In brief, ts2001 cells were maintained in MEM supplemented with 10% fetal bovine serum (FBS). Forty-eight hours after transfection with a calcium phosphate protocol, cells were lysed in a buffer containing 1% CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 120 mM NaCl, 50 mM KCl, 50 mM NaF, 2 mM DTT, and protease inhibitors (1 mM PMSF and 1 μg/ml each aprotinin, leupeptin, and pepstatin A). Cleared lysates were incubated overnight at 4°C with J-67, and the immunocomplexes were precipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins in the cell lysates or immunoprecipitates were separated on polyacrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and then incubated with anti-dKCNQ C-67 or anti-CalM (Upstate Biotechnology, Lake Placid, NY) in blocking buffer at 4°C overnight. After three washes with TBST, blots were incubated with HRP-conjugated anti-rabbit or anti-mouse IgG (Amersham Biosciences, Arlington Heights, IL) for 1 h at room temperature. Proteins were visualized with an enhanced chemiluminescence detection system (Amersham Biosciences).

Electrophysiology. Chinese hamster ovary (CHO) cells were maintained in Ham’s F-12 medium containing 10% FBS and 1% penicillin/streptomycin. They were transfected using Lipofectamine 2000 (Invitrogen, Gaithersburg, MD) according to the instructions of the manufacturer. Cells were either transfected with pIRE2-EGFP dKCNQ constructs or cotransfected with V5-tagged dKCNQ channel construct and EGFP. Currents produced by the C-terminal-tagged channel are indistinguishable from those produced by the untaged version.

Twenty-four to 48 h after transfection, dKCNQ currents were recorded in the whole-cell configuration using an Axo-patch 200A amplifier (Molecular Devices, Union City, CA). Pipette electrodes were pulled from borosilicate glass and had resistances of 2–3 MΩ. The bath solution consisted of the following (in mM): 145 NaCl, 5 KCl, 2 MgCl2, 2 CaCl2, and 10 HEPES, pH 7.2. The electrode solution consisted of the following (in mM): 140 KCl, 0.5 MgCl2, 2 CaCl2, 5 EGTA, and 10 HEPES, pH 7.2. This gives an internal solution containing 100 mM free Ca2+1, as calculated with Easy software (Biosoft, Cambridge, UK). Two millimolar ATP was included in the electrode solution for some experiments, with similar results. Data were acquired and analyzed with pClamp 8 software (Axon Instruments). All results are shown as mean ± SEM, and statistical significance was assessed by Student’s t-test (Origin software, Microcal Software, Northampton, MA).

Fly stocks. Flies were raised on standard malasses food at 25°C. EP(2)2074 insertion line (w; EP2074/Cyo +) was from the Berkeley Drosophila Genome Project collection of EP lines from the controlled misexpression project (Rorth, 1996; Rorth et al., 1998). Wild-type Canton S flies, the transposase line (w; wg/Cyo; Δ2–3Dr/TM6), and other fly lines were generously provided by Leslie Griffith (Brandeis University, Waltham, MA).

Generation and characterization of dKCNQ-deletion lines. The precise insertion site of the EP(2)2074 element within the dKCNQ gene was confirmed by inverse PCR and direct sequencing of genomic DNA flanking the insertion site. Precise and imprecise excision lines were generated by mobilizing the P-element using Δ2–3 as the source of transposase (Robertson et al., 1988). The Δ2–3 line has the Drop marker gene on the chromosome so that recombination between the transposase insertion site and the genome of the transposase can be followed easily. The jumps were generated as follows. First, w; EP(2)2074/Cyo + males were crossed with w; wg/Cyo; Δ2–3Dr/TM6 females. Second, F1 male progenies with genotype w; EP(2)2074/Cyo; Δ2–3 Dr/+ were selected and crossed with w; Xa/Cyo; Xa/+ females. EP(2)2074/Cyo balancer heterozygotes were chosen rather than EP/wg flies, because imprecise excision is favored by the absence of a homologous wild-type chromosome at the insertion site (O’Kane, 1998). Third, excision of the P-element from the genome (EP(2)2074) was judged by the loss of eye color carried by the w+ mC minigene on the P-element. Single white-eyed F2 males with genotype w; EP(2074/Cyo; Δ2–3 Dr/+ were selected and crossed with w; Xa/Cyo; Xa/+ females. EP(2)2074/Cyo balancer heterozygotes were chosen rather than EP/wg flies, because imprecise excision is favored by the absence of a homologous wild-type chromosome at the insertion site (O’Kane, 1998). Third, excision of the P-element from the genome (EP(2)2074) was judged by the loss of eye color carried by the w+ mC minigene on the P-element. Single white-eyed F2 males with genotype w; EP(2074/Cyo; Δ2–3 Dr/+ were selected and crossed with w; Xa/Cyo; Xa/+ females. EP(2)2074/Cyo balancer heterozygotes were chosen rather than EP/wg flies, because imprecise excision is favored by the absence of a homologous wild-type chromosome at the insertion site (O’Kane, 1998). Third, excision of the P-element from the genome (EP(2)2074) was judged by the loss of eye color carried by the w+ mC minigene on the P-element. Single white-eyed F2 males with genotype w; EP(2074/Cyo; Δ2–3 Dr/+ were selected and crossed with w; Xa/Cyo; Xa/+ females. EP(2)2074/Cyo balancer heterozygotes were chosen rather than EP/wg flies, because imprecise excision is favored by the absence of a homologous wild-type chromosome at the insertion site (O’Kane, 1998).
tion occurred in the region between the primers. Excision lines in which this primer pair did not produce a PCR product presumably contain larger deletions that remove at least one of the primer sites, so they were put to the next round of PCR testing, with another pair of primers farther away from the original insertion site. The process was repeated until a genomic fragment was PCR amplified from each deletion line. The PCR products were then sequenced to determine the deletion breakpoints on the dKCNQ genomic DNA. Primer pair L50 and R026 was used to sequence the deletion line dKCNQ169, L50 and R026 for line dKCNQ528, L3274 and R50 for line dKCNQ428, L50 and R80 for line dKCNQ538 and L3274 and R3847 for line dKCNQ460, respectively. The sequences of these primers are as follows: L50, 5′-GTGAGGTTGCTAAAAGATCGAGG-3′; L80, 5′-AGACGAGTATGAAACAGTGCCG-3′; L3274, 5′-AGAGCTCCTTGTAGACTTACCGTCTG-3′; L50, 5′-ATAGAAGCGCAGCCCAACAGT-3′; R50, 5′-ATCAAGGAGCAGGACCCGCTG-3′; R80, 5′-ATCAAGGAGACGGACCCGCTG-3′; R026, 5′-CTCCCCCTTCCACATAACTTACG-3′; and R3847, 5′-CGAATTGCTACTCTGCTTGAAGAACGGCCGTTGACTAC-3′.

In four of the five deletion lines we recovered, there are stretches of short sequences left between the deletion breakpoints. They are 1–3 bp in length and probably are derived from the original P-element insert. We also observed excisions that occurred internally within the deletion lines and probably are derived from the original P-element insert. Short sequences left between the deletion breakpoints. They are 1–33 bp in length and probably are derived from the original P-element insert.

Results

Cloning of a novel KCNQ gene from Drosophila

We used the protein sequence of mouse KCNQ2 to search the Drosophila genome and identified a genomic sequence in 46F3 on the right arm of chromosome 2 that shares significant homology with the mammalian channel on the protein level. We used PCR to amplify and clone cDNA from an embryonic cDNA library, an embryonic EST cDNA, and adult cDNA to test the existence of the two predicted Drosophila KCNQ transcript sequences. The difference between the two predicted transcripts, PA (long) and PB (short), is the presence or absence of two exons found in the C-terminal region past the last predicted transmembrane domain. In both embryo and adult, we were only able to find the PB, or shorter version of the transcript, which we now refer to as dKCNQ.

dKCNQ cDNA encodes a novel protein of 809 amino acids (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Alignment of dKCNQ cDNA and genomic sequences indicates that the dKCNQ coding region is composed of 17 exons. Similar to mammalian KCNQ channels, dKCNQ protein has six putative transmembrane domains, followed by a long cytoplasmic C-terminal domain. The Drosophila protein is 36% identical and 48% similar to the mKCNQ2 channel (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The S1 through S6 transmembrane domains are highly conserved, with 63% identical and 77% similar amino acid residues. Hence, dKCNQ has all the elements for a voltage-dependent potassium channel, including the string of positive charges in S4 that is believed to be the voltage sensor and the GYGD signature sequence in the pore region that constitutes the potassium selectivity filter when four subunits come together to form a functional channel. Conversely, the conservation in the C-terminal domain is low (24% identity and 33% similarity). Interestingly, there is far greater homology in two domains that correspond to the two CaM binding sites we identified in mouse KCNQ2 (supplemental Fig. 1, available at www.jneurosci.org as supplemental
dKCNQ forms a functional KCNQ-like channel in CHO cells

We tested the possible function of dKCNQ as a potassium channel by whole-cell recording from CHO cells transfected with dKCNQ cDNA. CHO cells have been shown to lack endogenous potassium current (Yu and Kerchner, 1998), and we did not observe any current from untransfected cells under our recording conditions. Outward currents were detected 1–2 d after transfection of dKCNQ (Fig. 1A). These currents show several features that are shared by both the native M-current and recombinant KCNQ2/3 channels recorded under the same conditions. First, dKCNQ currents are activated at subthreshold membrane potentials; the voltage activation curve (Fig. 1B) reaches half-maximum activation at approximately −20 mV. Compared with KCNQ2/3 channels, dKCNQ appears to open at more hyperpolarized membrane potentials, but its voltage dependence is weaker. The depolarization required for an e-fold increase in conductance is 21 mV for dKCNQ (Fig. 1B) compared with 12 mV for KCNQ2/3 (Selyanko et al., 2000; Wen and Levitan, 2002). Second, dKCNQ currents have slow activation and deactivation kinetics. The activation can be described by a sum of two exponentials, with time constants of 1748 ± 362 and 151 ± 16 ms (n = 5), at 0 mV. The deactivation can also be fitted to a second-order exponential function. When recorded at −120 mV after a depolarizing step to 0 mV for 1 s, the slow component of deactivation has a time constant of 137 ± 13 ms and the fast component of 105 ± 75 ms (n = 5).

dKCNQ is inhibited by a M-current-specific blocker

Linopirdine and its analog XE-991 have been shown to inhibit both native M-current and cloned mammalian KCNQ channels in the micromolar to submicromolar concentration range (Costa and Brown, 1997; Lamas et al., 1997; Wang et al., 1998; Kubisch et al., 1999; Lerche et al., 2000; Schroeder et al., 2000). Evidence supports the notion that linopirdine acts extracellularly, probably by binding directly to the channel (Costa and Brown, 1997). Considering the sequence similarity between the mammalian channels and the fly channel in the N-terminal region, including the putative transmembrane domains and extracellular loops, we tested whether dKCNQ current is also blocked by linopirdine. When 100 μM linopirdine is applied to the bath solution, 90% of the whole-cell dKCNQ current is blocked (Fig. 2A). The dose–response curve shows that linopirdine inhibits the fly KCNQ channel with an IC50 value of 10.8 μM (Fig. 2B), similar to that for the mammalian channels. The Hill slope of the curve is 1.1, indicating a one-to-one stoichiometry of the inhibitor with the channel.

dKCNQ is inhibited by muscarinic receptor activation

Activation of an array of G-protein-coupled receptors modulates the native M-current. M-current was given its name because it was first shown to be inhibited by agonists for the M1 muscarinic receptor (Brown and Adams, 1980). Muscarinic suppression of the cloned KCNQ1–KCNQ5 channels has been reconstituted in heterologous cells coexpressing the receptor and appears to use a similar intracellular signaling mechanism as that for the native current (Schroeder et al., 2000b; Selyanko et al., 2000; Shapiro et al., 2000). Recent studies support the hypothesis that depletion of the essential membrane lipid phosphatidylinositol-4,5-bisphosphate during receptor activation contributes to the suppression of channel activity (Suh and Hille, 2002; Zhang et al., 2003).

We coexpressed the dKCNQ channel and an M1 receptor in CHO cells. Whole-cell currents were measured before and 1–2 min after stimulation of the receptor with 10 mM Oxo M (Fig. 2C). In cells expressing dKCNQ alone, 100 ± 5.2% (n = 11) of the current remains 1–2 min after Oxo M application, whereas currents are reduced to 28 ± 5% (n = 11) of the starting level by Oxo M in cells coexpressing the M1 receptor (p < 0.05; Student’s t test). Thus, dKCNQ shares this defining feature of M-current, modulation by muscarinic receptor activation.

Calmodulin binding is necessary for dKCNQ activity

We and others have shown using biochemical assays that the highly conserved Ca2+-binding protein calmodulin binds to mammalian KCNQ channels constitutively (Wen and Levitan, 2002; Yus-Najera et al., 2002; Gamper and Shapiro, 2003). Our mutagenesis and competition experiments provided evidence that CaM binding is essential for the functional expression of M-like current (Wen and Levitan, 2002). We tested whether this also applies to the Drosophila channel.

The two CaM–binding motifs in the proximal portion of the C-terminal domain of the mammalian channels are also conserved in the fly channel (supplemental Fig. 1, site 1 and site 2, available at www.jneurosci.org as supplemental material). A dKCNQ fragment containing the two CaM–binding sites and the connecting sequences between them (amino acids 300–463) interacts with CaM when tested by the yeast two-hybrid interaction assay (data not shown). In addition, both endogenous (Fig. 3A, lane 3) and overexpressed (Fig. 3A, lane 2) CaM coimmunoprecipitate with the full-length dKCNQ channel in heterologous cells. We have shown previously that changing a conserved arginine residue in CaM-binding site 1 of KCNQ2 to a glutamate abolishes CaM binding. This mutant KCNQ2 channel does not produce measurable current when coexpressed with KCNQ3, although it is targeted to the cell surface and heteromerizes with
When a similar arginine residue is mutated in the dKCNQ channel (R326E), CaM no longer coimmunoprecipitates with the channel protein (Fig. 3A, lane 4). This mutant dKCNQ (R326E) channel produces small currents in some transfected CHO cells (Fig. 3B). The average current density in cells expressing dKCNQ (R326E) is 16.4 ± 6.2 pA/pF (n = 13), which is only 7% of that in cells expressing wild-type dKCNQ (234.8 ± 43.5 pA/pF; n = 9; p < 0.05, Student’s t test). The small remaining current could be attributable to some residual binding of CaM to the mutant channel, because we observed a weak signal above background for CaM in the mutant channel immunoprecipitates after long exposure of the blots (20 min to 1 h; data not shown).

Together, our data show that dKCNQ is a functional KCNQ channel. Although the fly equivalent of the mammalian neuronal M-current has not been described, dKCNQ could be a component of a similar current in vivo. We next studied the physiolog-
critical role of dKCNQ in vivo by examining animals with deletions in dKCNQ genomic DNA.

**Generation of dKCNQ deletion lines**

We identified a P-element line, EP(2)2074, in the BDGP database, in which a P-element is inserted 191 bp downstream of the seventh exon of dKCNQ (Fig. 4). To generate deletions in the dKCNQ gene, we used a strategy that takes advantage of the high frequency of imprecise excisions during mobilization of P-elements. EP(2)2074 was remobilized with a stable source of transposase, Δ2–3, and 103 independent excision lines (referred to as EP<sub>lines</sub>) were selected. Using a PCR screening method, we recovered five homozygous viable lines that contain deletions of various lengths in the first half of the dKCNQ gene (referred to as dKCNQ deletion lines). Sequencing across the breakpoints of the deletions showed that these lines can be divided into two groups based on the location of the lesions in the transcripts. The first group, including dKCNQ<sub>18B</sub>, dKCNQ<sub>349</sub>, dKCNQ<sub>53B</sub>, and dKCNQ<sub>24B</sub>, lacks the sequence coding for part or all of the S1–S6 transmembrane domains and the conserved CaM-binding site 1 right after S6 (Table 1, Fig. 4). Because they lack the membrane-spanning domains that are essential for potassium channel function, these four lines are functional null alleles for the channel activity of dKCNQ. The second group consists of one line, dKCNQ<sub>42B</sub>, in which the deletion removes part of exon 8 (Table 1, Fig. 4). RT-PCR experiments revealed three dKCNQ transcripts in these flies arising from alternative splicing (data not shown). Because all three transcripts retain part of the intronic sequences between exon 7 and exon 8, premature stop codons are introduced several residues downstream of S353 (4 and 22 residues, respectively). Hence, dKCNQ<sub>24B</sub> is an allele expressing a mutant dKCNQ, with a C-terminal tail truncated after the conserved CaM-binding site 1. It remains to be tested whether this shortened protein lacking CaM-binding site 2 still has channel activity. We favor the idea that dKCNQ<sub>24B</sub> is also a functional null allele, because our previous study of CaM interaction with the mammalian channels suggests that both CaM-binding sites are necessary for CaM binding and that CaM binding is essential for channel function (Wen and Levitan, 2002).

We also recovered several lines with precise excision of EP(2)2074. DNA sequencing across the original P-element insertion site in one of them, dKCNQ<sub>42B</sub>, confirmed that the dKCNQ locus in this fly is identical to that of wild-type flies. We then used dKCNQ<sub>24B</sub> as the wild-type control for all the experiments described below.

**dKCNQ deletion flies have a maternal effect phenotype**

Homozygous dKCNQ deletion flies develop to adulthood, with a viability close to those of dKCNQ<sub>42B</sub> and EP(2)2074. Homozygous dKCNQ deletion adults exhibit an ~50:50 male to female ratio and have no gross anatomical abnormalities; they also exhibit no obvious defects in such basic motor skills as walking and flying. Mutations in other potassium channels have been shown to cause several characteristic behavioral phenotypes, including ether-induced leg shaking (Kaplan and Trout, 1969; Elkins et al., 1986) and temperature-sensitive paralysis (Elkins et al., 1986; Hegde et al., 1999). In contrast to Shaker, Slo, Shab, and EAG mutants, homozygous dKCNQ deletion flies are indistinguishable from the wild type in these behavioral tests (data not shown), suggesting that dKCNQ serves a different set of physiological functions than these other potassium channels. Interestingly, the deletion flies do appear to have a “restless” phenotype, in that they appear more active than wild-type or dKCNQ<sub>24B</sub> controls. We are currently investigating whether there may be a sleep phenotype associated with the dKCNQ gene, and our preliminary studies have shown that deletion flies do appear to exhibit a sleep defect when compared with controls.

In our attempt to establish a homozygous dKCNQ deletion stock by crossing homozygous males and females, we observed that the eggs laid by homozygous mothers failed to hatch into larvae (Table 2, Crosses 1 and 2). We found that this embryonic lethality was associated only with homozygous dKCNQ deletion mothers. The homozygous dKCNQ deletion males have normal fertility, because they can produce adult progeny when mated to wild-type females (Table 2, Cross 3). Therefore, the lethality phenotype suggests a maternal effect of dKCNQ products in embryonic development: dKCNQ is essential for early development, and dKCNQ mRNA or protein is provided maternally to the embryo. The seemingly normal viability of homozygous dKCNQ deletion adult flies, conversely, indicates that zygotic transcription of dKCNQ is not required for development of the animal. This explains why homozygous dKCNQ deletion embryos produced by heterozygous mothers (which can still make a maternal contribution of dKCNQ) can develop to adulthood. Heterozygous embryos from homozygous dKCNQ deletion females and wild-type males still fail to hatch (Table 2, Cross 2), even with a

**Table 1. dKCNQ deletion fly lines**

<table>
<thead>
<tr>
<th>Fly line</th>
<th>Deletion in genomic DNA</th>
<th>Deletion in cDNA</th>
<th>Predicted deletion in protein</th>
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<tbody>
<tr>
<td>24B</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>18B</td>
<td>+110 to +2053</td>
<td>Exon 2–7</td>
<td>S1–S6 + site 1</td>
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<tr>
<td>35B</td>
<td>−553 to −3368</td>
<td>Exon 1–7</td>
<td>S1–S6 + site 1</td>
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<tr>
<td>42B</td>
<td>+2062 to +3840</td>
<td>Part of exon 8</td>
<td>C-terminal sequences after site 1</td>
</tr>
<tr>
<td>53B</td>
<td>+1071 to +3303</td>
<td>Exon 5–7</td>
<td>55–56 + site 1</td>
</tr>
<tr>
<td>64B</td>
<td>+453 to +3493</td>
<td>Exon 3–7</td>
<td>Part of 51, 52–56, + site 1</td>
</tr>
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</table>

The first nucleotide of the ATG start codon for dKCNQ protein is numbered as +1. Compared with precise excision line 24B, some deletion lines have short P-element sequences left between the breakpoints of excision. In line 35B, there is 1 bp (A); in 42B, there are 33 bp (CGTCTTACTTATTTACTTACTTATTTCATCATG); in 53B, there are 22 bp (CATGATGAATACAGAATAAC); and in 64B, there are 4 bp (GTTA).

**Figure 4.** dKCNQ deletion mutants. The dKCNQ gene contains 17 coding exons, Exons of dKCNQ (gray boxes) and its neighboring gene CG12214 (large gray box) are shown. “ATG” and “Stop” indicate the translation initiation and termination sites, respectively. Arrows indicate the direction of transcription. The EP(2)2074 P-element (triangle) is 119 bp downstream of exon 7. Deletions in the dKCNQ genomic sequence for five independent lines generated by precise excision of the P-element are shown below. Line 24B resulted from a precise excision.
Adult progeny were produced from the cross; the embryos in Sp background. dKCNQ24B rescue indicates that the time window when copy of the wild-type gene from the male. The lack of paternal embryo (right) has a disorganized pattern of nuclei on the surface. Embryo staining with DAPI.

Table 2. Maternal effect of dKCNQ

<table>
<thead>
<tr>
<th>Fly line</th>
<th>Cross 1</th>
<th>Cross 2</th>
<th>Cross 3</th>
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<tr>
<td></td>
<td>Female</td>
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<td>Female</td>
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<td></td>
<td>Δ/Δ</td>
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<td>Δ/Δ</td>
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<tr>
<td>24B</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>18B</td>
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The dKCNQ locus on the second chromosome is represented as Δ. The precise excision line 24B was used as control. Sp/CyO was used as the wild-type background. Sp (Sternopleural) is a bristle marker gene located on the second chromosome, and CyO (Curly of Oster) is a second chromosome balancer. +, Adult progeny were produced from the cross; −, embryos failed to hatch. All deletion lines gave the same results.

Figure 5. Embryo staining with DAPI. A, Nuclear distribution in mid-cleavage stage dKCNQ53B precise excision (left) and dKCNQ24B deletion (right) embryos. B, Nuclear staining of the embryos in A, under higher magnification. In contrast to the wild type (left), the dKCNQ53B embryo (right) has a disorganized pattern of nuclei on the surface.

copy of the wild-type gene from the male. The lack of paternal rescue indicates that the time window when dKCNQ is critical lies before the zygotic expression of the gene.

We examined embryos produced by homozygous deletion females to further characterize the embryonic lethality phenotype. Using the DNA binding dye DAPI, we looked at the distribution of nuclei in these embryos 1–2 h after egg laying. The mutant embryos show clear nuclear staining (Fig. 5, right panels), suggesting that they have undergone multiple cycles of nuclear division and at least some of the nuclei have migrated to the surface of the embryo. However, the nuclei of the mutant embryos are not organized as in the wild-type embryos (Fig. 5, left panels). In contrast to the orderly packed nuclei seen in the wild type, in the mutants, some nuclei appear to have broken down into smaller fragments whereas others have clustered abnormally, indicating that development of the mutant embryo is arrested before the cellularization stage.

We ascribe this maternal effect phenotype solely to deletions in the dKCNQ gene, because the genomic sequences of genes upstream and downstream of dKCNQ were not affected in the deletion lines (Fig. 4). Because all five independently generated deletion lines display this phenotype although the precise jump-out does not, it is highly unlikely that the phenotype is attributable to some second-site mutation.

dKCNQ mRNA distribution

Because of the intriguing developmental phenotype seen in the offspring of homozygous deletion females, we asked whether dKCNQ mRNA is found in embryos. We collected embryos within 1 h of laying to ensure that zygotic transcription had not yet begun. RT-PCR reveals dKCNQ mRNA in early embryos from dKCNQ24B, Canton S, and yellow white females (data not shown) but not in the embryos from dKCNQ53B deletion females crossed with dKCNQ53B males (Fig. 6) or dKCNQ24B males (data not shown). To determine whether dKCNQ mRNA is also found in the ovary, we performed whole adult fly and whole ovary in situ hybridization. dKCNQ mRNA is present in brain cortical neurons (Fig. 7A, long arrows), optic lobe lamina (short arrows) and photoreceptors (arrowheads), and the cardia (proventriculus) of the thorax (data not shown). In addition, the nurse cells of the ovaries (Fig. 7C,D, arrow), as well as the maturing oocytes (Fig. 7C, arrowhead), contain high levels of dKCNQ mRNA. No message was noted in male reproductive tracts (data not shown) nor in brain or ovary of dKCNQ53B deletion lines (Fig. 7B,E). These data, combined with the embryo RT-PCR results and the fact that the offspring of dKCNQ deletion females do not develop into adults, support the idea that dKCNQ is a maternally contributed gene and is essential for early embryonic development.

Discussion

Cloning and electrophysiological analysis of dKCNQ

We cloned from Drosophila a single KCNQ-related gene, dKCNQ, based on its sequence homology to mammalian KCNQ channels. The cloned channel produces a KCNQ-like current in CHO cells with subthreshold voltage activation and slow kinetics. This current is sensitive to the M-current selective blocker linopirdine and is modulated by activation of a muscarinic receptor. Like KCNQ2, the fly channel binds CaM and requires CaM binding for its functional expression. Therefore, dKCNQ exhibits many characteristics of the mammalian channels. Especially interesting is the modulation of dKCNQ by activation of a muscarinic receptor, the defining feature of the M-current. It is important to point out that, although cDNA for a mammalian M1 receptor was used in this study, activation of the Drosophila muscarinic receptor leads to apparently similar downstream signaling events, including stimulation of phosphatidylinositol metabolism (Shapiro et al., 1989; Chyb et al., 1999). Therefore, muscarinic modulation of the dKCNQ channel could occur in the fly, but its physiological significance is yet to be identified. It also remains to be determined whether other modulators of the mammalian M-current can modulate dKCNQ.
Indeed, most patients with mutated forms of KCNQ2 or KCNQ3 have seizures only during the first few weeks after birth and then become apparently normal, except for a slightly higher susceptibility to seizures later in life (Ronen et al., 1993; Psenka and Holden, 1996). Because dKCNQ is the only KCNQ-like gene in Drosophila, a dKCNQ mutant can provide fundamental insight into the requirement of KCNQ-like channels in processes as diverse as differentiation, development, synaptic plasticity, and regulation of neuronal excitability.

Indeed, we observed an unexpected embryonic lethal phenotype in dKCNQ deletion flies. Our results show that embryos produced by homozygous deletion females fail to survive, strongly suggesting that dKCNQ protein or mRNA is required in early developmental events and is provided maternally to the embryo. The fact that dKCNQ mRNA is found in the ovary and in the early embryo (before zygotic transcription) further supports this hypothesis. Even more intriguingly, homozygous dKCNQ deletion embryos produced by heterozygous females can develop into healthy adults. Thus, beyond the lifetime of the maternally contributed products, dKCNQ can be completely absent without destructive consequences on the development of the animal. This observation supports the idea that maternally deposited dKCNQ is sufficient to sustain the development of the embryo, and zygotic expression of the dKCNQ gene is dispensable. This could be attributable to compensation of dKCNQ function by other Kv channels in later developmental stages.

As illustrated by the nuclear staining, the orderly nuclear distribution characteristic of a mid- to late-cleavage stage wild-type embryo is disrupted in embryos without dKCNQ products, suggesting that these mutant embryos die before cell formation (cell formation in the fly embryo starts $\sim 2.5$ h after egg laying). It is not clear whether Drosophila expresses potassium currents at such an early stage of development. The first currents that can be

**Figure 6.** RT-PCR of dKCNQ24B and dKCNQ53B early embryos. mRNA for dKCNQ is seen in embryos from dKCNQ24B females crossed with dKCNQ24B males (left lane, arrow). In dKCNQ53B females crossed with dKCNQ53B males, no message is found (right lane). Predicted band size, 571 bp. L, Ladder.

$dKCNQ$ is the only gene in the Drosophila genome that has significant homology with mammalian KCNQ channels. The five KCNQ genes that have been identified in mammals have overlapping expression patterns in the nervous system and other tissues. They contribute to several distinct native potassium currents of great physiological importance. It will be interesting to see whether a single Drosophila gene can fulfill the functions of these multiple mammalian genes. Mammalian KCNQs can associate with other $\alpha$-subunits within the family, or $\beta$-subunits, to form heteromeric channels that have distinct functional properties. For instance, two $\beta$-subunits with a single membrane-spanning domain, KCNE1 and KCNE3, have profound effects on KCNQ1 channel properties (Sanguinetti et al., 1996; Schroeder et al., 2000b). Although homology search reveals no KCNE-related $\beta$-subunit in the Drosophila genome (Littleton and Ganetzky, 2000), dKCNQ could use some as yet unidentified protein as its auxiliary subunit. We detected at least three alternative-spliced transcripts in RT-PCR products from wild-type flies (data not shown). It hence seems likely that isoforms of dKCNQ channels exist in vivo, and functional diversity from a single $dKCNQ$ gene is plausible.

**A developmental role for dKCNQ**

Because multiple KCNQ subunits exist in mammals, and they express in overlapping areas and often interact with each other to form heteromeric channels, mutations in one subunit may not produce easily detectable phenotypes attributable to functional compensation by other subunits. Indeed, most patients with mutated forms
recorded from Drosophila embryonic central neurons include a delayed potassium current that appears during mid-embryogenesis (13–14 h after egg laying) (Baines and Bate, 1998). Does the essential role of dKCNQ for early embryonic development require its channel activity? Ionic flow and resting membrane potential have been implicated in the regulation of embryonic morphogenesis, best studied in chick and Xenopus (Rutenberg et al., 2002). Consistent with this idea, several ion channel and ion pump mRNAs are present in specific and dynamic expression patterns in early embryos in both species, well before neuralization. These include maternal KCNQ1 mRNA, which is present in unfertilized embryos and in cells at early-cleavage stages in chick (Rutenberg et al., 2002). It is conceivable that ionic currents and membrane potential could also play important roles in early embryonic development in the fly. Sustained potassium current at subthreshold membrane potentials produced by mammalian KCNQ channels has been shown to be a key regulator of resting potential (Brown and Yu, 2000; Oliver et al., 2003). Alternatively, dKCNQ could serve some critical function other than as an ion channel protein. For example, dKCNQ may be a component of a signaling complex that transduces external cues into cellular responses in the developing embryo. As a membrane protein with a long cytoplasmic tail containing signaling protein binding sites, dKCNQ is well suited for such a role.

References


