Title
CG16718, A Calcium-Activated Chloride Channel of the TMEM16 Family in Drosophila melanogaster

Author
Wong, Xiu Ming

Publication Date
2013

Peer reviewed|Thesis/dissertation

CG16718, A Calcium-Activated Chloride Channel of The TMEM16 Family in Drosophila melanogaster

by

Xiu Ming Wong

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Chemistry and Chemical Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

I would like to thank

Lily and Yuh Nung Jan

For your kind and patient mentorship,

Wei Zhou

For helping me start out,

Desiree Thayer and Hye Young Lee

My caring and considerate benchmates,

Jim Berg and Huanghe Yang

Who taught me electrophysiology,

Shi-Bing Yang

A brilliant mentor and a friend,

Jill Willdonger

For starting me out on flies,

Susan Younger

For generating the CG16718 knockout fly (and teaching me about flies and other things),

Christian Peters

For Figure 3D,

and all Jan lab members

For guidance and companionship.

Brian P. Lazzaro

For his gift of the Db11 strain,

Dominique Ferrandon

For invaluable and reassuring critique,

Robin and Bob Aglietti and Cheryl Tajon

For good food and friendship,

the twins Huibin and Weibin

The sunshine in my life,

and my mother

For letting me do whatever I want.

Abstract

TMEM16A and TMEM16B are calcium-activated chloride channels (CaCCs) with important functions in mammalian physiology. Whether distant relatives of the vertebrate TMEM16 families also form CaCCs is an intriguing open question. This thesis reports that a TMEM16 family member from *Drosophila melanogaster*, Subdued (CG16718), is a CaCC. Amino acid substitutions of Subdued alter the ion selectivity and kinetic properties of the CaCC channels heterologously expressed in HEK 293T cells. This *Drosophila* channel displays characteristics of classic CaCCs, thereby providing evidence for evolutionarily conserved biophysical properties in the TMEM16 family. Additionally, we show that knockout flies lacking *subdued* gene activity more readily succumb to death caused by ingesting the pathogenic bacteria *Serratia marcescens*, suggesting that *subdued* has novel functions in *Drosophila* host defense.

Contents

Introduction

TMEM16A [1, 2, 3] and a different family member, TMEM16B [4], encode the classic calcium-activated chloride channels (CaCCs) in various mammalian tissues. Previously observed in a variety of organisms from green algae [5, 6] to *Xenopus* [7, 8], these channels are activated by an increase in cytosolic calcium with outward rectification at low calcium levels, and they preferentially permeate larger anions [9, 10].

In mammals, TMEM16A regulates fluid secretion in submandibular glands [11, 3] as well as on the epithelia of airway surfaces [12]. This channel also modulates arterial [13, 14], tracheal [15] and gastrointestinal smooth muscle tone [16], and has been observed to play a role in noxious heat sensing in the peripheral nervous system [17]. TMEM16B is expressed in photoreceptor terminals [18] where CaCCs are hypothesized to stabilize presynaptic membrane potential [19]. This channel also gives rise to the majority of recorded CaCC current in hippocampal pyramidal neurons [20] and olfactory sensory neurons [21].

Besides TMEM16A and B, only one other mammalian family member, TMEM16F, has been biophysically characterized *in vitro* and *in vivo* [22], and functionally it plays a completely different role from its CaCC counterparts, playing a role in phospholipid scrambling and blood clotting [22]. TMEM16F is a small-conductance calcium-activated non-selective cation (SCAN) channel, which begs the question of whether or not the rest of the mammalian TMEM16 family encodes CaCCs (like TMEM16A and B) or SCAN channels like TMEM16F.

Outside of mammals, even less is known about the TMEM16 family. Ubiquitous in eukaryotes, TMEM16 family members regulate a bewildering variety of physiological functions. Ist2p, the single ortholog of the TMEM16 family in *Saccharomyces cerevisiae*, has been shown to function in endoplasmic reticulum-plasma membrane tethering [23]. A member of the TMEM16 family in *Drosophila*, Axs, is found on the meiotic spindle and regulates meiotic chromosomal segregation [24]. *Xenopus* TMEM16A functions to block polyspermy in fertilized oocytes and is to date the only non-mammalian TMEM16 member described as a CaCC [2]. We thus have a limited understanding of

both the biophysical and functional aspects of the TMEM16 family, and whether these properties are evolutionarily conserved.

In an attempt to uncover TMEM16 family members with CaCC or SCAN channel activity, we cloned and heterologously expressed TMEM16 members from various genetically tractable organisms for electrophysiological inspection. These included TMEM16 family members from *A. thaliana*, *C. elegans*, *S. cerevisiae*, *S. pombe* and *Drosophila melanogaster*. A major roadblock to this approach was the lack of plasma membrane localization, rendering electrophysiological study untenable. However a *Drosophila melanogaster* TMEM16 ortholog, CG16718, expressed on the plasma membrane, and was found to be a CaCC upon heterologous expression in HEK 293T cells. This channel shares various properties with its mammalian and *Xenopus* CaCC counterparts, as well as the SCAN channel TMEM16F. In addition, we observe that this channel plays a role in host defense in *Drosophila*, a function that has not been previously reported in the TMEM16 family. Given the physiological role of this newly identified CaCC, we chose to give CG16718 the name *subdued*.

Results

Subdued is an ortholog of mammalian TMEM16A and B

Multiple sequence alignment with the mammalian TMEM16 family shows that Subdued is most similar to TMEM16A and B (Figure 1A and B), sharing 32.8% and 31.7% identity with these channels respectively. Heterologous expression of Subdued would have been ideally done in the commonly used *Drosophila*-derived S2 cell line. However this cell line was reported to robustly express bestrophins which give rise to endogenous calcium-activated chloride currents [25], potentially confounding analysis. Alternatively, Subdued was expressed in human embryonic kidney (HEK) 293T cells, a common heterologous expression system for electrophysiological studies of CaCCs [1, 2, 3].

Subdued is a calcium and voltage dependent channel

Forty-eight hours post-transfection, HEK 293T cells expressing Subdued were recorded from using whole-cell patch clamp. Upon activation with 20-200 μM free intracellular calcium and symmetric NaCl solutions, the cells showed large time-dependent currents. In zero-calcium pipette solutions, no currents were observed in the transfected cells (Figure 2A). For this study all recordings were done with 200 μM calcium unless otherwise mentioned. Mock-transfected cells showed little to no CaCC (data not shown). Voltage ramps showed that Subdued was outwardly rectifying, giving rise to more current at depolarized potentials (Figure 2B). In these experiments with calcium infused from the whole-cell patch clamp pipette into the cytosol, rectification decreased over time as current amplitude increased. Run-up of current likely results from the process of calcium diffusing from the pipette solution into the cell, suggesting that rectification is also calcium-dependent, as has been shown for CaCCs [3].

Subdued primarily permeates chloride

Given that the mammalian TMEM16 family contains both anion and cation channels [22], we wanted

to determine the ionic selectivity of the channel. This was done by varying concentrations of NaCl externally while keeping the intracellular NaCl concentration constant at 140 mM. A series of positive reversal potentials (E_{rev}) was obtained upon decreasing the external NaCl concentration, indicating anionic selectivity (Figure 3A and B). P_{Na}/P_{Cl} was calculated to be 0.16, indicating an apparent permeability for cations that is smaller than the permeability for anions, as has been found for mammalian TMEM16A and B [4, 22]. Substitution of chloride with larger halide anions in the external solution revealed that Subdued preferentially permeates the larger anions SCN^-, I^- and Br^- relative to Cl^- (Figure 3C), another hallmark feature of classic CaCCs. However, classic CaCC blockers niflumic acid (NFA), flufenamic acid (FFA) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) as well as a more recently developed TMEM16A inhibitor T16Ainh-A01 [26] did not block the channel (data not shown). Benzbromarone, a TMEM16A blocker identified from a high throughput screen [15], blocked Subdued current significantly and reversibly (Figure 3D).

Subdued is a pore-forming subunit

To obtain evidence that Subdued is directly responsible for the observed currents, we introduced mutations onto the channel, and observed that the mutant channels produced currents that had different properties from the wildtype channel. A Q672K mutation produced currents that had significantly slower activation kinetics as compared to wildtype (Figure 4A and B). Interestingly, the corresponding mutation on mammalian TMEM16F produced a highly similar effect on channel kinetics [22]. A Y489H mutation decreased selectivity for chloride as evident from the decreased shift in E_{rev} upon introduction of a chloride gradient across the membrane (Figure 4C). The position of both mutations relative to the first 5 putative transmembrane domains can be seen on Figure 1B, where Y489 is marked in green and Q672 in red. Transmembrane domains are boxed and bolded, and were predicted using the TOPCONS program [27]).

Subdued plays a role in Drosophila host defense

FlyAtlas data shows that Subdued is expressed at moderate levels in a broad variety of tissues both in larvae and adults, making it difficult to predict a physiological function for this gene in *Drosophila* [28]. From FlyBase-curated data, we noticed that a genome-wide screen for genetic determinants of gut immunity in *Drosophila* reported subdued as a susceptibility hit [29]. In this study, ubiquitous RNAi of subdued caused increased lethality upon ingestion of Db11, a particular strain of *Serratia marcescens* isolated from moribund flies [30]. Serratia marcescens is a strain of Gram-negative

bacteria that is a common cause of nosocomial infections, and the Db11 strain has been shown to be virulent in flies. Importantly, although Db11 kills *Drosophila* in less than 24 hours when introduced via septic injury, its virulence is attenuated when introduced via ingestion [31]. Survival rates of different fly strains can thus be monitored over a span of a few days, allowing inspection of their relative host defense responses to Db11.

Subdued knockout flies are more susceptible to gut infection with *Serratia*

Wildtype flies and two knockout strains generated from independent crosses, KO2 and KO11, were fed Db11 mixed with sucrose solution, and their survival monitored for 8 days [29]. Confirming the susceptibility phenotype previously reported, the knockout strains had significantly higher lethality upon being fed Db11 (Figure 5A). Over the same timescale as the infection experiment, no significant lethality was observed for wildtype or knockout strains when the flies were only fed sucrose (data not shown).

We hypothesized that since the knockout flies did not display prominent structural abnormalities in the alimentary canal, it was possible that the susceptibility to Db11 infection arose from defects in host defense. At 48 hours post-infection, whole flies were homogenized and the homogenates were serially diluted and plated on LB agar plates with antibiotic selection. Colony forming units (CFU) were counted on each plate to estimate the number of Db11 bacteria present in the whole fly. Since only live flies were homogenized, this assay reports on the active host response the flies mount against Db11 infection. Significantly higher amounts of Db11 were isolated from knockout flies compared to wildtype (Figure 5B).

To control for the possibility that the higher titers of bacteria isolated from knockout flies could arise from increased feeding, we performed a feeding assay in which a food dye was introduced into the bacteria/sucrose solution [32]. After 72 hours of feeding, flies were dissected to isolate the intact guts and crop, which were homogenized and analyzed for food dye content as a read-out for food consumption. Knockout fly guts did not contain more food dye and surprisingly, slightly but significantly less food dye was recovered from the guts of knockout compared to the wildtype flies (Figure 5C). Thus we surmised that knockout flies are unlikely to consume more food than wildtype flies. Differences in whole animal bacterial titers are thus likely to result from disparities in host defense.

To see if bacteria also accumulated more in the guts as well as the whole animal, homogenates obtained from the feeding assay described above were serially diluted and plated on LB agar plates with antibiotic selection. Knockout fly guts had a significantly higher number of CFU of Db11 than

wildtype flies (Figure 5D), suggesting that *in vivo* proliferation of the bacteria in knockout flies is more pronounced in the gut as well as the whole animal.

Discussion

Here we report that Subdued (CG16718), an ortholog of the TMEM16 family in *Drosophila melanogaster*, is a calcium-activated chloride channel with biophysical properties resembling those of classic CaCCs. This channel is activated by internal calcium, with a lower bound of $[Ca^{2+}]_{in} = 20\ \mu M$ in whole cell patch clamp experiments in which current was observed. No significant currents were observed when an EGTA-buffered zero calcium solution was used as the internal solution. Relative to mammalian TMEM16A [3], TMEM16B [4] or *Xenopus* TMEM16A (H. Yang, personal communication, March 2013), Subdued is one to two orders of magnitude less calcium sensitive in whole cell patch clamp experiments. This could either reflect a true biophysical property of the channel, or could be an indication that the non-native HEK 293T expression system used in our experiments lacks auxiliary subunits required for higher calcium sensitivity. It would be interesting to test if directed mutagenesis of Subdued can tune its calcium sensitivity to within the realm of its mammalian and *Xenopus* counterparts.

Subdued rectifies outwardly, passing larger currents at more positive voltages. The channel permeates mainly chloride with a P_{Na}/P_{Cl} of 0.16, and preferentially permeates larger anions relative to smaller ones, giving the selectivity series $SCN^- > I^- > Br^- > Cl^-$. A Y489H mutation affected the ionic selectivity of the channel, making it more permeable to Na^+. This suggests that perhaps the Y489 residue is pore-lining, or has an allosteric effect on the structure of the pore. The Y489H mutant also gave rise to smaller currents compared to the wildtype channel, a reflection of either decreased unitary channel conductance or decreased membrane expression. Additionally, a Q672K mutation produced a dramatic slowing of activation kinetics, a phenomenon also observed when mutating the corresponding residue in mammalian TMEM16F. The observation that mutations to Subdued alter the properties of the currents strongly points to this protein as a pore-forming subunit of the recorded CaCCs.

Pharmacologically, Subdued is not blocked by the CaCC blockers NFA, FFA, NPPB or T16Ainh-A01. This might arise from structural differences in Subdued, perhaps in the pore, relative to

its mammalian and *Xenopus* counterparts. However benzbromarone blocks Subdued significantly, and could potentially be used to interrogate the location and properties of the channel pore. In conclusion, as a distantly related TMEM16 family member, Subdued will be useful as a tool in structure/function studies to parse out conserved or divergent biophysical properties such as calcium- and voltage-dependent gating, permeation and ion selectivity.

To study the function of the channel in *Drosophila*, we generated *subdued* knockout strains. Confirming results from a previous genome-wide RNAi study, the knockout was found to be more susceptible to gut infection by a strain of *Serratia marcescens*, Db11 [29]. An earlier study proposed that the cause of lethality is bacterial proliferation leading to invasion of gut tissue and subsequent gut distension and escape of bacteria into the hemolymph [31].

In the case of the *subdued* knockout, susceptibility arises, at least in part, by deficient host defense, since *in vivo* proliferation of Db11 was higher in knockout fly guts as well as in the whole animal as compared to wildtype flies. Additionally we observed that slightly but significantly less food dye was recovered from the guts of knockout flies. This might arise from lower food consumption by knockout flies, but could also be an indication of increased gut tissue damage due to greater numbers of Db11 in the gut, leading to leakage and diffusion of food dye into the hemolymph. It remains to be determined if higher Db11 titers in the whole animal also result from defective immune responses within the hemolymph. Tissue-specific RNAi of *subdued* using gut or hemocytes drivers did not recapitulate the whole animal RNAi phenotype [29]. Preliminary RNAi experiments performed in this study using tissue-specific drivers for the fat body, Malpighian tubules, gut and hemocytes failed to recapitulate the susceptibility phenotype (data not shown), suggesting that Subdued is likely to exert its protective function in a multitude of tissues.

One potential function for Subdued is in the regulation of the secretion of cationic antimicrobial peptides (AMPs), a process that occurs widely on epithelial surfaces and is known to play critical roles in host defense [33]. This hypothesis is consistent with the abundant mRNA expression of *subdued* in various epithelial tissues [28]. The susceptibility observed in *subdued* knockout flies could also be a consequence of a deficiency in dual oxidase (DUOX) mediated immunity. The DUOX system is reported to be critical in generating reactive oxygen species (ROS) in *Drosophila* gut epithelia [34]. This study reported that strong antimicrobial ROS species are generated by the peroxidase homology domain (PHD) of *Drosophila* DUOX in a chloride-dependent manner. These ROS species are likely to be the highly reactive hypohalites OCl or OSCN, the *in vivo* production of which requires trans-epithelial anion transport. A recent study identifying uracil as the bacterial effector of ROS production in *Drosophila* gut also reported that *Serratia marcescens*

secretes significant amounts of this compound [35]. Additionally, the *Drosophila* DUOX system has also been shown to mobilize downstream of the $G_{\alpha q}$-coupled signaling pathway [32], implicating other calcium-dependent responses in the *Drosophila* immune response. Following Db11 infection of subdued knockouts, it is possible that $G_{\alpha q}$ receptor stimulation fails to elicit sufficient amounts of halide transport onto gut epithelia due to a deficiency in CaCCs, reducing PHD-mediated generation of antimicrobial hypohalites and leading to increased bacterial proliferation and higher lethality.

There also remains the possibility that subtle structural deficits contribute to the susceptibility of Subdued knockouts to Db11 infection. Developmental defects in gut epithelial integrity [36] or the peritrophic matrix lining the gut [37] might result in the susceptibility phenotype. Subdued knockout flies did not have significant defects in gut epithelial polarity and integrity under basal conditions as observed by immunostaining for Armadillo and Discs Large (Dlg) to observe adherens and septate junction structure [38, 39] (data not shown). However, Subdued might function in gut epithelial or peritrophic matrix integrity only upon Db11 challenge to the gut, a possibility that will be explored in future study.

Given that Subdued is a CaCC, it remains to be shown if it has functions in *Drosophila* analogous to what is known about the functions of TMEM16A and B in mammals. If proven to be true, the function of CaCCs in ROS production would represent an evolutionarily conserved mechanism in epithelial immunity [40]. Additionally, FlyBase reports that mbn2 cells, an immortalized *Drosophila* hemocyte line, express abundant CG16718 transcript. Whether or not the channel regulates phospholipid scrambling in *Drosophila* hemocytes, like TMEM16F does in mammals, is an intriguing possibility. The mechanism of how TMEM16F regulates phospholipid scrambling remains elusive [22], and having shown that Subdued shares biophysical properties with TMEM16F, there remains the exciting possibility of evolutionary conservation of functional properties as well. At a biochemical level, this would help to determine the regions on these channels responsible for phospholipid scrambling.

In conclusion, the TMEM16 family is a diverse and exciting family of ion channels, and much remains to be learnt about the biology and biophysics of its numerous members.

Materials and Methods

Electrophysiology

CG16718 was subcloned from BDGP Drosophila Gene Collection cDNA clone LD10322, which yields the ORF of the RA splice variant of CG16718. Fresh HEK 293T cells were ordered from ATCC. Cells were transfected with CG16718-eGFPN1 (CG16718 tagged at the C-terminus with EGFP) for 24 hr (Fugene) and recovered for another 24 hr in fresh media. Patch pipets (World Precision Instruments) were pulled from a Sutter P-97 puller and fire-polished. Pipets had resistances of 3-5M Ω for whole cell patch clamp experiments. The bath was grounded via a 3M KCl agar bridge connected to a Ag-AgCl reference electrode. Data was acquired using a Multiclamp 700B amplifier controlled by Clampex 10.2 via Digidata 1440A (Axon Instruments). For NaCl gradient and anionic replacement experiments, the standard internal solution contained (in mM) 130 NaCl, 10 HEPES, 5.6 $CaCl_2$, 5 EGTA, 5 MgATP, 1 Na_2GTP, 10 phosphocreatine, pH 7.2. The free calcium concentration was calculated to be about 200 μM with WEBMAXC software (http://www.stanford.edu/cpatton/maxc.html) and was determined with a calcium electrode. External solutions contained various concentrations of NaCl or 140 NaX, 10 EGTA, 2 $MgCl_2$ and 10 HEPES, pH 7.2. Sucrose was added to balance osmolarity for the low NaCl solutions.

Drosophila genetics and fly stocks]

The CG16718 deficiency line was generated by following closely a heat-shock driven FLP recombinase protocol detailed in Parks et al. 2004. The fly strains used were PBac{RB}CG16718^{e02779} and P{XP}CG16718^{d03361} (Exelixis at Harvard Medical School).

Bacterial infection assays

Infection assays were performed as described in Cronin et al., with some modifications. Batches of 20 adult flies were used for each line assayed. Different genotypes were collected everyday from food bottles of identical age and kept in similarly time-matched food vials for 2 days before Db11 feeding

commenced. The food solution containing Db11 was prepared from liquid culture grown overnight at 37°C in LB (Luria Bertani) medium supplemented with 60 μg/ml ampicillin antibiotic . This starter culture was refreshed to log phase and diluted with a freshly prepared sterile-filtered 0.05 M sucrose solution to a final OD (600nm) = 0.1. Absorbent filters (37mm; Millipore) were thoroughly soaked with the bacteria/sucrose solution and one was placed into each vial. 20 flies were then put in each vial, which was then placed in a 29°C incubator. Lines were transferred to new vials with freshly prepared bacteria/sucrose solution every 4 days.

Colony-forming unit assay

Vials of 20 flies were fed with bacteria/sucrose solution as described above. After 48 hrs the flies were cold-anesthetized, transferred to eppendorf tubes and homogenized in 500 μL of LB media. Homogenates were serially diluted into LB and the serial dilutions plated onto LB-ampicillin plates (60 μg/mL). Plates were incubated at 37°C for 24 hrs and plates with a colony count between 100-500 were chosen for inspection. For gut dissections, the flies were fed 0.5% erioglaucine disodium salt (Sigma) together with the Db11/sucrose solution. 72 hours post-infection, four guts (including the crop) were homogenized in 50 μL PBS for each round of experiments and the homogenates were serially diluted and colony counts performed as described above. The remainder of the homogenates were spun down at 8000 g for 10 min and absorbance at 625 nm was taken as a readout for the amount of food consumed by the flies.

A

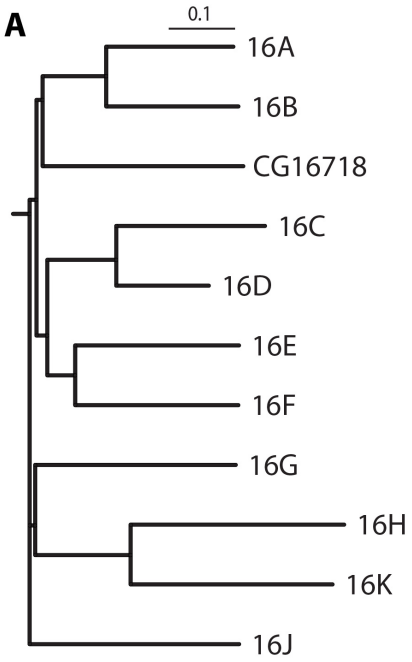

Figure 1. CG16718 (Subdued) aligns closely with mammalian CaCCs TMEM16A and B (*Mus musculus*)

(A) Multiple sequence alignment of protein sequences was done with ClustalW2 and phylogenetic tree construction was done in PHYLIP 3.67 (Drawgram).

(B) Putative transmembrane segments are highlighted with boxes and mutated residues (see Figure 4) featured in this report are marked in colour on the primary sequence alignment. Bracketed values at the end of the alignment indicate the number of residues in the whole channel.

B

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16A          VGFVKIHAPWHVLCREAEFLKLKMPTKKVYHISETRGLLKTINSVLQKITDPIQPKV--- 209
16B          SVFVRIHAPWQVLAREAEFLKIKVPTKKMYEIKAGGSIAKKFSAILQTLSSPLQPRV--- 240
CG16718      IWFVKIHAPLEVLRRYAEILKLRMPMKEIPGMSVVNRSTKSVFSSLKHVFQFFLRNIYVD 297

16A          AEHRPQTTKRLSYPFSREKQHLFDLTDRDSFFDSKTRSTIVYEILKR---TTCTKAKYSM 266
16B          PEHSNNRMKNLSYPFSREKMYLYNIQEKDTFFDNATRSRIVHEILKR---TACSRANNTM 297
CG16718      EEIFPKRAHRFTAIYSRDKEYLFDIR-QDCFFTTAVRSRIVEFILDRQRFPAKNQHDMAF 356

16A          GITSLLANGVYSAAYPLHDGDYEGDNVEFNDRKLLYEEWASYGVFYKYQPIDLVRKYFGE 326
16B          GINSLIANNIYEAAYPLHDGEYDSPGDDMNDRKLLYQEWARYGVFYKFQPIDLIRKYFGE 357
CG16718      GIERLIAEGVYSAAYPLHDGEITETG---TMRALLYKHWASVPKWYRYQPLDDIKEYFGV 413

16A          KVGLYFAWLGAYTQMLIPASIVGVIVFLYGCATVDENIPSMEMCDQRYN-ITMCPLCDKT 385
16B          KIGLYFAWLGLYTSFLIPSSVIGVIVFLYGCATIEEDIPSKEMCDHQNA-FTMCPLCDKS 416
CG16718      KIGLYFAWLGYYTYMLLLASIVGVICFLYSWFSLKNYVPVKDICQSGNTNITMCPLCD-W 472

16A          CSYWKMSSACATARASHLFDNPATVFFSVFMALWAATFMEHWKRKQMRLNYRWDLTGFEE 445
16B          CDYWNLSSACGTARASHLFDNPATVFFSIFMALWATMFLENWKRLQMRLGYFWDLTGIEE 476
CG16718      CNFWDLKETCNYAKVTYLIDNPSTVFFAVFMSFWATLFLELWKRYSAEITHRWDLTGFDV 532

16A          EED----HPRAEYEARVLEKSLRKESR------------NKETDKVKLTWRDRFPAYFTN 489
16B          EEERSQEHSRPEYETKVREKLLKESGKSAVQKLEANSPEDDEDDEDKLTWKDRFPGYLMN 536
CG16718      HEE----HPRPQYLARLEHIPPTRVDY-----------VTNIKEPTVHFWRMKLPATVFS 577

16A          LVSIIFMIAVTFAIVLGVIIYRISTAAALAMNSSPSVRSNIRVTVTATAVIINLVVIILL 549
16B          FASILFMIALTFSIVFGVIVYRITTAAALSLN--KATRSNVRVTVTATAVIINLVVILIL 594
CG16718      FSVVLLLIALAFVALLAVVVYRMSMLAALKVGASPMTTSSAIVLATASAAFVNLCLLYIL 637

16A          DEVYGCIARWLTKIEVPKTEKSFEERLTFKAFLLKFVNSYTPIFYVAFFKGRFVGRPGDY 609(956)
16B          DEIYGAVAKWLTKIEVPKTEQTFEERLLLKAFLLKFVNAYSPIFYVAFFKGRFVGRPGSY 654(1002)
CG16718      NYMYNHLAEYLTELEMWRTQTQFDDSLTLKIYLLQFVNYYASIFYIAFFKGKFVGHPGEY 697(1075)
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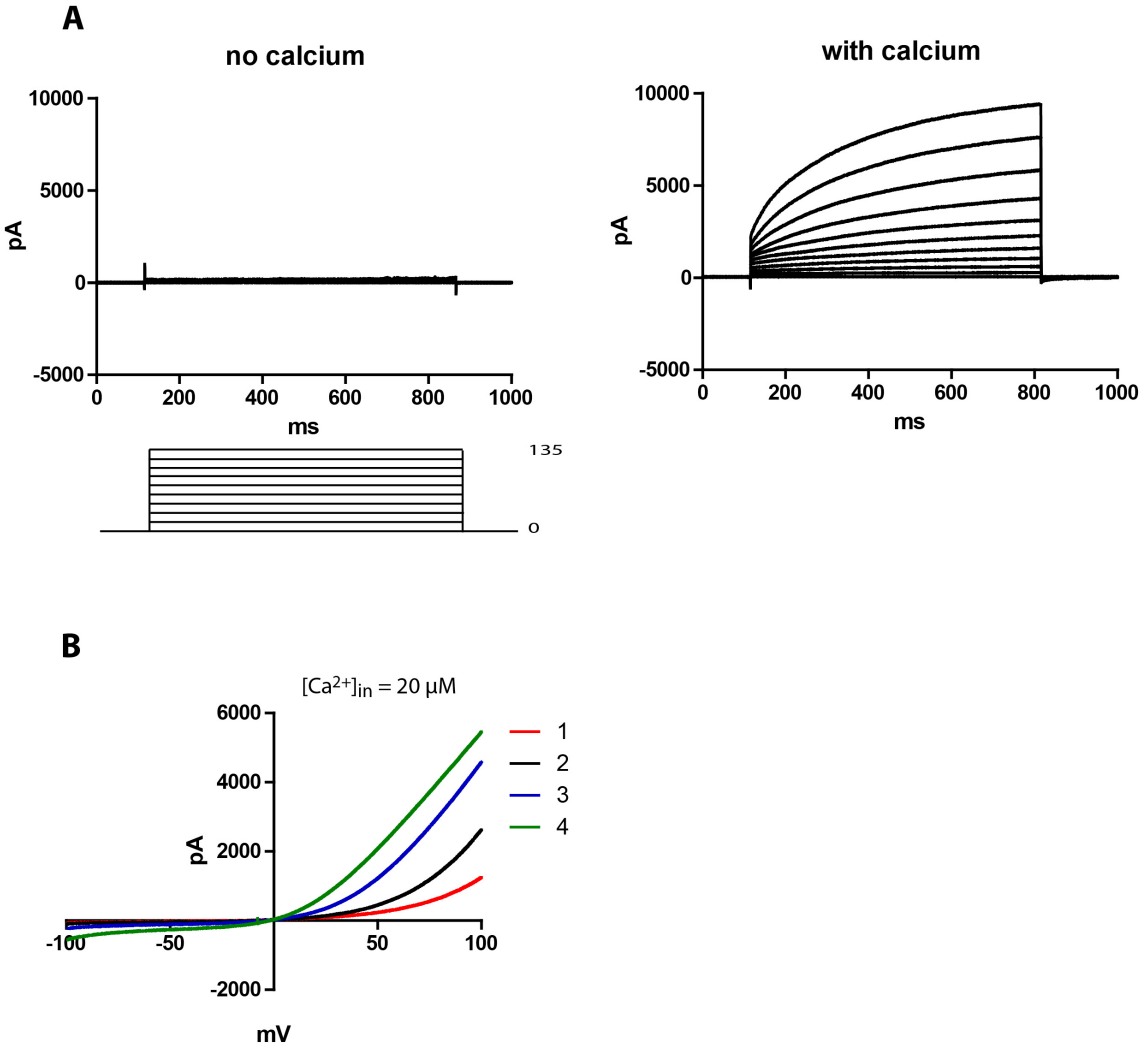
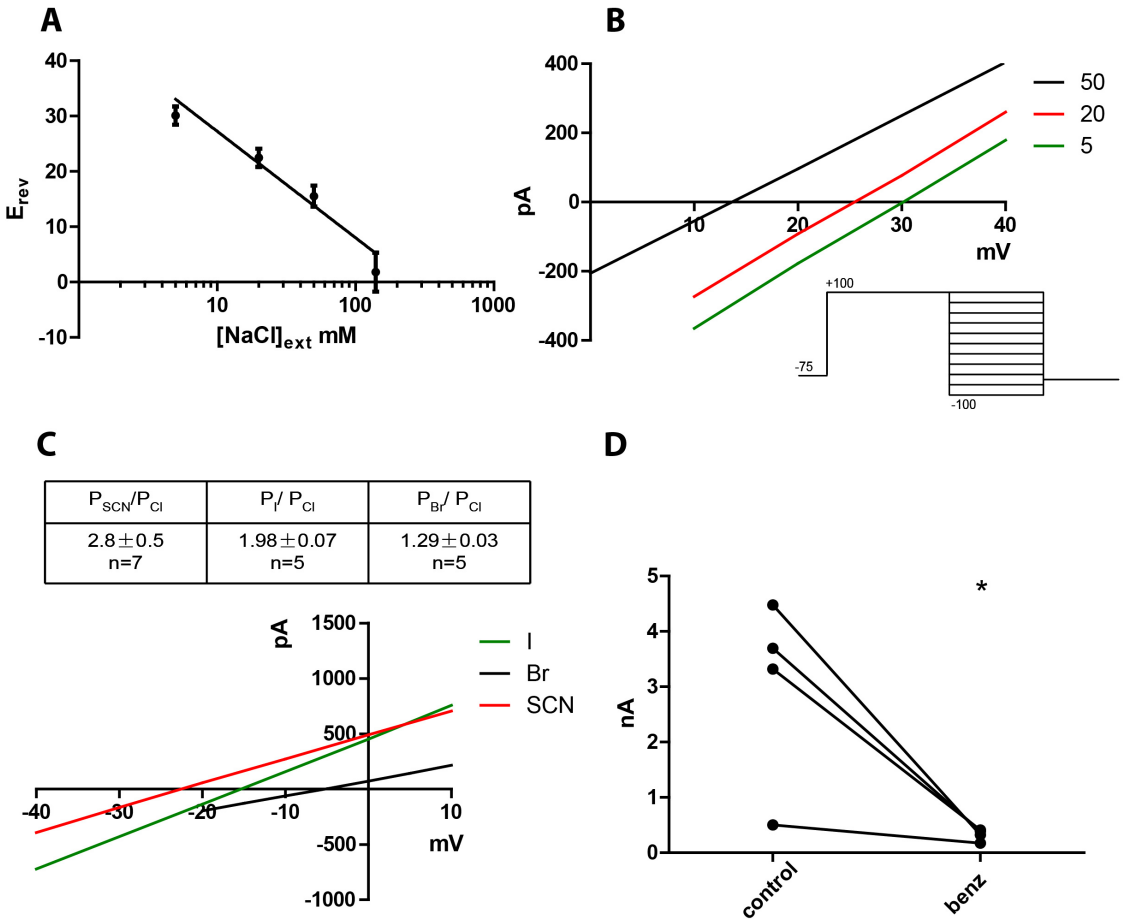


Figure 2. Subdued is a Calcium and Voltage Dependent Channel.

(A) Subdued-transfected HEK 293T cells were used for recording in whole cell patch clamp experiments. No current was observed without calcium in the pipette (left), but large time dependent currents were observed (right) when calcium was added to the pipette (200 µM free calcium). Unless otherwise mentioned, all recordings in this study were done with 200 µM free calcium in the pipette.

(B) Representative traces showing outward rectification of Subdued current in voltage ramps from -100 to +100 mV. Traces 1-4 were taken sequentially and show an increase in current and decrease in rectification over time (20 µM free calcium in pipette). The ramps were done at a rate (dV/dT) of 0.067 V/s.



P_{SCN}/P_{Cl}	P_I/P_{Cl}	P_{Br}/P_{Cl}
2.8±0.5 n=7	1.98±0.07 n=5	1.29±0.03 n=5

Figure 3. Subdued Displays Hallmark Ionic Selectivity of Classic CaCCs and is Blocked by a Known CaCC inhibitor.

(A) NaCl gradients were introduced across the membrane by varying NaCl concentrations of external solutions. The reversal potential (E_{rev}) at each concentration was obtained and fitted to the Goldman-Hodgkin-Katz (GHK) equation from which the P_{Na}/P_{Cl} was determined to be 0.16.

(B) Representative I/V plots obtained by varying external NaCl (in mM). A diagram of the voltage protocol used to measure E_{rev} is shown below. After a 750 ms activating pre-pulse to +100 mV, instantaneous tail currents were measured from test voltages -100 to +100 mV in 20 mV steps. After an initial estimate of E_{rev} using this protocol, test potentials and voltage increments were refined, while pre-pulse conditions and the length of voltage steps remained constant. For each NaCl concentration , n=4 to n=9.

(C) Subdued preferentially permeates larger anions with the selectivity sequence: SCN > I > Br > Cl. Bi-ionic conditions were introduced by varying the external solution. E_{rev} and permeability ratios were obtained by the same methodology as described for (B). Representative I/V plots obtained by varying the anion in external solutions using the same voltage protocols as described in (B) are shown.

(D) Subdued is significantly and reversibly blocked by 20 μM benzbromarone (n=4, $p < 0.05$, Student's t-test).

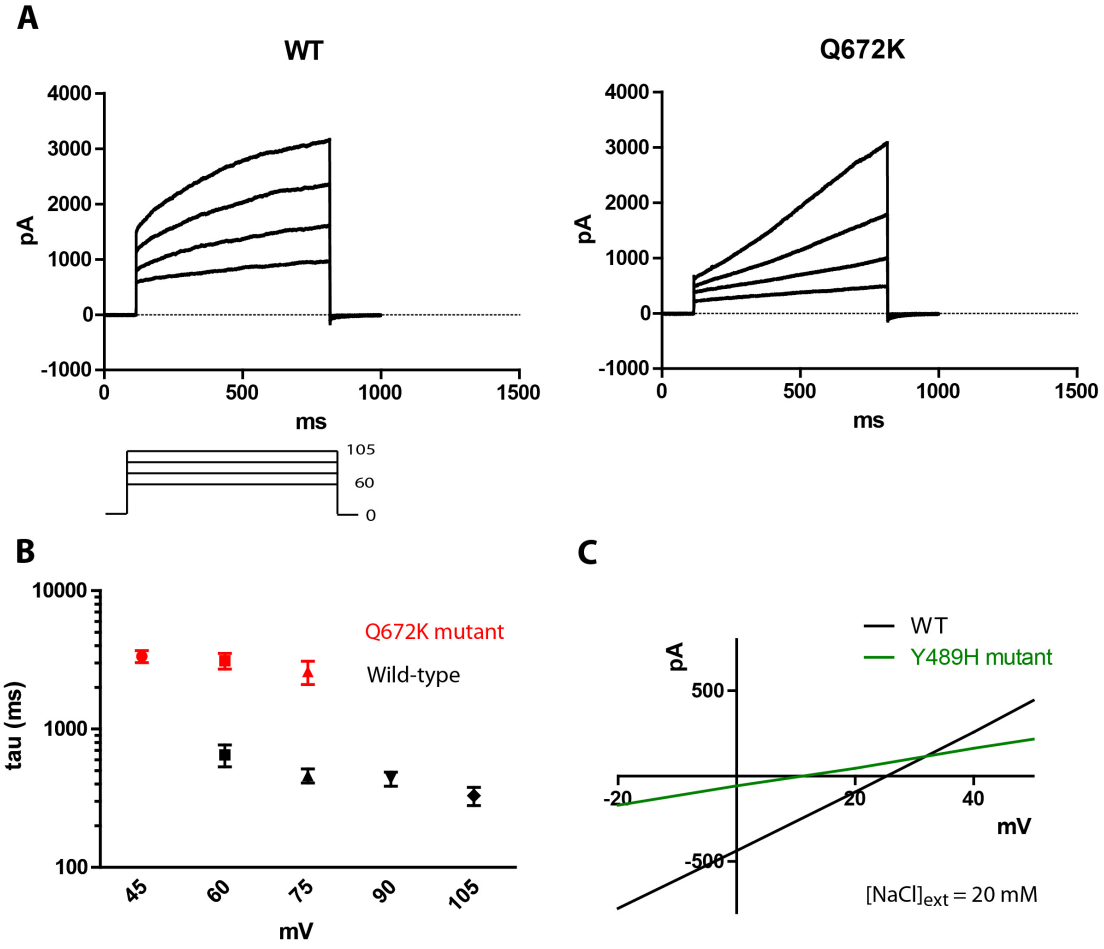


Figure 4. Mutations on Subdued Change Properties of Observed Currents.

(A) Different kinetic properties in the wildtype (WT) and Q672K mutant channel revealed by a voltage step protocol (750 ms in 15 mV increments).

(B) Semi-log plots of mean activation time constants (τ) as a function of voltage. τ was derived from the single exponential fitting of the current traces obtained from 750 ms (WT, n=6) and 5 s (Q672K, n=4) voltage steps. Time constants at 60 and 75 mV were significantly different for the two channels ($p < 0.001$, Student's t-test).

(C) A Y489H mutation decreases chloride selectivity compared to the WT channel. A representative I/V plot showing the shift in E_{rev} in a 20 mM external NaCl solution. WT E_{rev} was determined to be (25 ± 4) mV, n=5 ; the Y489H mutant E_{rev} was significantly different at (11 ± 2) mV, (n=4, $p < 0.05$, Student's t-test). Data was obtained using methodology described in Figure 2 (B).

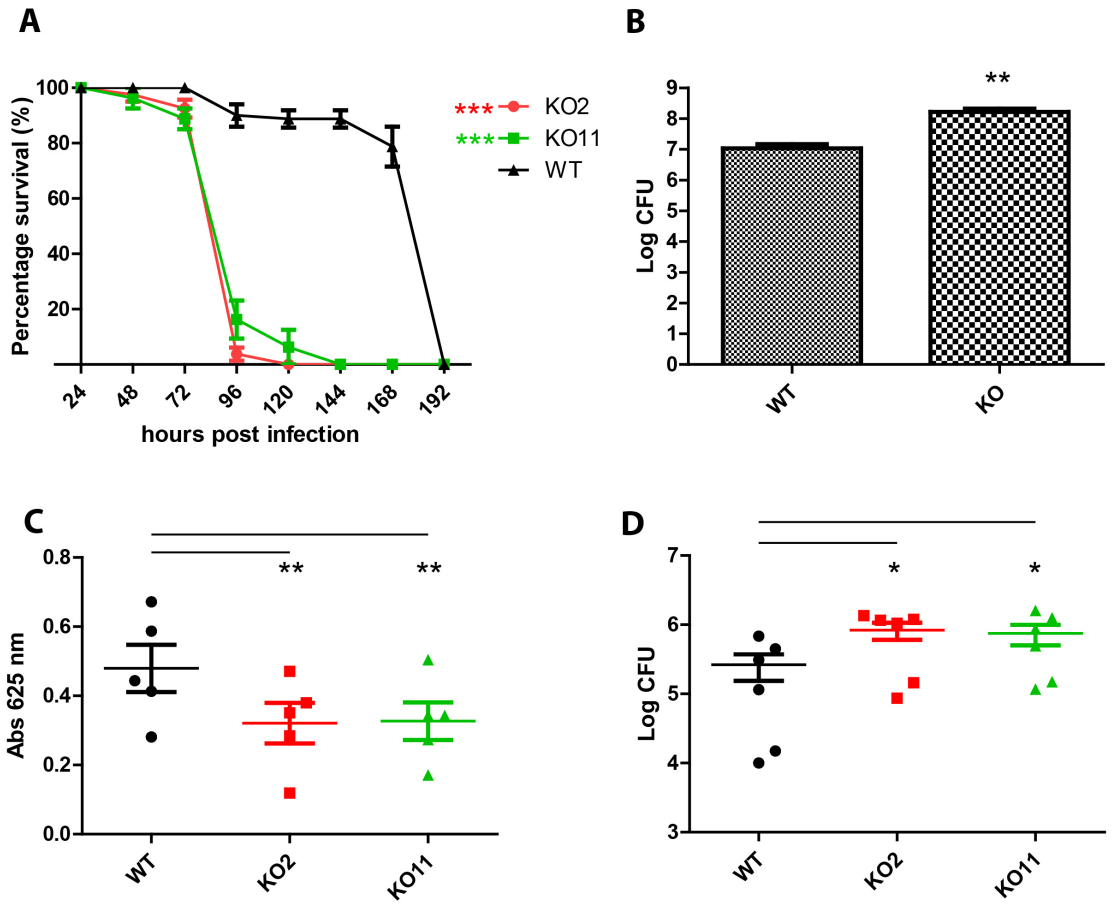


Figure 5. Subdued Plays a Role in Host Defense in Drosophila melanogaster.

(A) Subdued KO flies display susceptibility to Serratia infection. WT and KO flies were fed on a Db11/sucrose solution and their survival monitored for 192 hr post-infection. Two independently generated knockout strains, KO2 and KO11, were used. WT flies lived significantly longer compared to KO2 and KO11 (n=4, $p < 0.001$, two-way ANOVA).

(B) KO flies accumulate higher titers of bacteria in the whole animal. 20 whole flies were homogenized 48 hr post-infection. Serially diluted homogenates were plated on agar and inspected for Db11 colony forming units (CFU). Significantly more bacteria was recovered from the KO flies (n=7, Student's t-test, $p < 0.01$).

(C) KO flies do not consume more food than WT flies. Four fly guts were dissected and homogenized from vials of 20 flies fed with Db11/sucrose solution containing 0.5% w/v erioglaucine disodium salt (FDC Blue #1) 72 hours post-infection. The amount of food eaten by the flies was estimated by measuring absorbance of the dye. KO flies tended to consume significantly less food than WT flies (n=5, $p < 0.01$, repeated measures one-way ANOVA and Tukey's multiple comparison test). No significant difference was observed between the KO strains.

(D) Homogenate obtained from experiments described in (C) were inspected for Db11 colony forming units (CFU). Significantly higher amounts of bacteria were recovered from KO compared to WT fly guts (n=6, $p < 0.05$, repeated measures one-way ANOVA and Tukey's multiple comparison test). No significant difference was observed between KO strains.

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