Pickpocket1 is an ionotropic molecular sensory transducer

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Running title: Ppk1 functions as a molecular signal transducer

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Keywords: ENaC, ASIC, degenerin, mechanosensory transduction, sensory neuron

BACKGROUND: Ion channels are candidate molecules for transforming external stimuli into neural activity during sensory perception.

RESULTS: Pickpocket1 encodes an acid sensing ion channel (ASIC) that is sufficient to drive neural activity in sensory neurons.

CONCLUSION: The perception of external acid by Pickpocket1 channels is sufficient to produce phasic sensory neuron activity.

SIGNIFICANCE: ASIC channels can function as molecular sensory transducers in sensory neurons.

SUMMARY
The molecular transformation of an external stimulus into changes in sensory neuron activity is incompletely described. Although a number of molecules have been identified that can respond to stimuli, demonstration that these molecules can transduce stimulation into useful neural activity is lacking. Here we demonstrate that pickpocket1 (ppk1), a Drosophila homologue of mammalian Degenerin/ENaC channels, encodes an acid-sensing sodium channel that conducts a transient depolarizing current in multidendritic (md) sensory neurons of Drosophila melanogaster. Stimulation of Ppk1 is sufficient to bring these sensory neurons to threshold eliciting a burst of action potentials. The transient nature of the neural activity produced by Ppk1 activation is the result of Ppk1 channel gating properties. This model is supported by the observation of enhanced bursting activity in neurons expressing a gain of function ppk1 mutant harboring the degenerin mutation. These findings demonstrate that Ppk1 can function as an ionotropic molecular sensory transducer capable of transforming the perception of a stimulus into phasic neuronal activity in sensory neurons.

Periphera sensory neurons are capable of sensing and responding to a
broad range of stimuli including mechanical, chemical and thermal cues. These neurons are thought to express molecular sensory transducers that transform stimuli into changes in neuronal activity. For a protein to function as a molecular sensory transducer, it must not only be able to sense and respond to a stimulus, but also be able to transform the sensing of the stimulus into an electrical signal capable of evoking the firing of action potentials in the sensory neuron.

Ion channel proteins are particularly strong candidates to serve as molecular sensory transducers because their ion channel function enables them to transform the sensing of a stimulus, upon activation, into an electrical current that can alter neuron activity. While there are many examples of ion channel receptors that can respond to sensory stimuli (1-8), there are few examples where activation of these channels can drive neuronal activity.

The sub-group of mammalian Deg/ENaC channels activated by extracellular acidic pH represent attractive candidates for functioning in mammalian sensory neurons as molecular sensory transducers. These acid-sensing ion channels (ASICs) are expressed in sensory neurons, including DRG and have been implicated in mechanosensation and proprioception (9-15). The Drosophila Deg/ENaC homolog, ppk1, is expressed in a restrictive manner in class IV multidentritic (md) sensory neurons (14;16-18). These polymodal sensory neurons form extensive dendritic networks that ramify beneath the epidermis and are required for normal proprioception and nociception (16;19;20). In consideration of such findings, we hypothesized that md neurons in Drosophila, in which Ppk1 is restrictively expressed, may harbor acid-sensitive channels that can function as ionotropic molecular sensory transducers.

In the current study, we demonstrate that ppk1 encodes an acid-sensing ion channel in Drosophila class IV md sensory neurons. Activation of the transient depolarizing sodium current conducted by Ppk1 is sufficient to drive md neurons to threshold, eliciting a burst of stimulus-dependent action potentials. Furthermore, this burst of action potentials is sensitive to changes in gating as stimulation of a Ppk1 protein harboring the gain-of-function degenerin mutation results in sustained bursting and loss of normal sensory neuron function. Together, these findings demonstrate that Ppk1 can function as a physiologically relevant ionotropic molecular sensory transducer capable of transforming stimuli into changes in neuronal activity.

**Experimental Procedures**

*Fly stocks and husbandry*

All fly stocks were maintained on standard food at 25 °C and a 12 hour light cycle. Stocks harboring the f07052 and d02171 P-elements were obtained from the Bloomington Stock Center (University of Indiana). The ppk1-Gal4,UAS-mCD8-GFP fly line was a gift from Darren Williams (King’s College London, UK).

*Generation of ppk1 deletion and transgenic flies*

FRT/flippase recombinase targeted recombination was used to generate flies harboring the ppk1 gene deletion (ppk1ESB (21;22)). Specifically, the ppk1 gene was deleted using Flippase...
mediated recombination between the PBac insertion lines f07052 and d02171 (www.drosdel.org.uk). The elB gene was also deleted in the recombination event but ruled out due to functional rescue by the ppk1 encoding transgene (Figure 1a). The ppk1 transgene used in rescue experiments was generated by cloning the coding sequence of ppk1 from the cDNA RE19290 (DGRC) into the pUAST vector in front of a C-terminal flag-epitope. The degenerin gain-of-function mutant, ppk1deg, was engineered by incorporating the S551F substitution into this transgene. UAS-ppk1-FLAG and UAS-ppk1deg-FLAG flies were generated through P-element-mediated transformation (Rainbow Transgenic). Rescue flies of the genotype ppk1ESB;ppk1-Gal4,UAS-CD8-GFP/UAS-ppk1-FLAG were generated by crossing homozygotic ppk1ESB;ppk1-Gal4,UAS-CD8-GFP females to homozygotic ppk1ESB;UAS-ppk1-FLAG males. All genotypes were verified using PCR.

Genotyping
Single fly genomic DNA preparations for the polymerase chain reaction were performed as described previously (23). The ppk1 gene, including the first two introns, and the UAS-ppk1-FLAG (Ppk1-transgene), which contains no introns, were identified with a standard PCR using the forward 5’-GGGAGGATGAGGAGAAGG-3’ and reverse 5’-ACTCCATTGCTATCGCAGCT-3’ primers at an annealing temperature of 68 ºC with 3 mM Mg2+.

Larvae crawling assay
Early third-instar larvae in the foraging stage were used in all crawling assays (16). Larvae were derived from 10 hour-old embryo collections and aged for 80 hours at 25°C and 60% humidity. Prior to the assay, larvae were washed three times with distilled water to remove food particles. Larvae then were placed on 1% agarose plates and allowed to acclimate to testing conditions for 10 minutes. Larval crawling was assayed at 25 ºC. For video capture of movement during the assay, three individual larvae were placed on the center of a fresh 150 mm agarose plate and recorded for 1.5 minutes or until they moved out of range. Video recordings of larvae crawling were captured at 30 frames per second with a Sony HDV1080i digital high definition video camera and analyzed with DIAS 3.4.2 software (Soll Technologies; Iowa City, IA; (24).

Noceception assay
Noceptive responses to noxious mechanical stimiuli were assayed using a standard protocol (2;18). In brief, third-instar larvae in the wandering stage were exposed to a transient, noxious mechanical stimulus applied to the 3rd, 4th or 5th segment of the abdominal region with a 46 mN Von Frey filament. Positive responses were scored only when larvae performed at least one 360º rotation around the anterior-posterior axis. Each larva was stimulated only once.

Primary neuronal culture
Neuronal cultures from midgastrula stage embryos were prepared as described previously (25). Briefly, 3-4 hour-old embryos were collected and dechorionated with 50% bleach. The content of three to four embryos was removed and dispersed onto a glass coverslip in a drop of culture medium. Neurons were grown in culture in a
defined bicarbonate based medium in 5% CO₂ at 23 °C for 2-3 days. In these midgastrula stage embryo cultures, neurons arise from neuroblast precursors.

**Electrophysiology**

Class IV md sensory neurons were identified in primary midgastrula neuronal cultures with epi-fluorescence for GFP expression, as driven by ppk1-Gal4,UAS-mCD8-GFP. Acid-sensing macroscopic currents from these neurons were recorded in voltage-clamp experiments using the whole-cell patch clamp configuration. Neurons were clamped to -60 mV (unless noted otherwise) and currents were activated by rapid solution exchange from control (7.4) to acidic pH. For most experiments, the extracellular bath solution contained (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 10 D-glucose, 10 HEPES and 10 MES (pH was adjusted with HCl before each experiment). The pipette solution contained (in mM) 120 Cs-glucuronate, 20 NaCl, 0.1 CaCl₂, 1 EGTA, and 10 HEPES (pH was adjusted to 7.2 with CsOH). In paired patch-clamp experiments examining P<sub>K</sub>/P<sub>Na</sub> selectivity, the extracellular bath solution initially presented to the neuron contained 110 mM NaCl plus 40 mM TEA-Cl. The NaCl was subsequently replaced with 110 mM KCl. Under both conditions, the pipette contained 140 mM Cs-glucuronate with current evoked with pH 4.5.

Currents were filtered at 1 kHz and acquired at 2 kHz and analyzed with an Axopatch 200B interfaced via a Digidata 1440A to a PC running the pClamp 10.2 software suite (Molecular Devices). Recording pipettes had resistances of 10–13 MΩ. Acidic pH was applied using a computer controlled fast perfusion system (AutoMate Scientific). All electrophysiological experiments were performed at room temperature.

Action potentials (AP) from class IV md neurons were recorded using the whole-cell current-clamp configuration. Action potentials were evoked with 600 ms intracellular injections of suprathreshold depolarizing current pulses or by relief from amiloride blockade subsequent to an acid challenge. For these experiments, the extracellular bath solution was (in mM) 140 NaCl, 5 KCl, 1 CaCl₂, 10 D-glucose, and 10 HEPES (pH was adjusted to 7.4 with NaOH). The pipette solution was (in mM) 120 K-glucuronate, 20 NaCl, 0.1 CaCl₂, 1 EGTA, and 10 HEPES (pH was adjusted to 7.2 with KOH). The first action potentials evoked with minimal current excitation were used to determine AP threshold, amplitude, half-width and maximal rates of depolarization and repolarization. Firing frequencies were compared at 2 pA/pF current injection. Cell capacitance was determined by integrating the area of capacitive transients from the average of 10 ramps from -60 to -100 mV.

**Data Analysis and Statistics**

For the analytical description of block by amiloride, the dose-response curve was fitted with the Hill equation:

\[
\delta = \frac{1}{1 + \left( \frac{IC_{50}}{[X]} \right)^n},
\]

where \( \delta \) is the blocked fraction of the current in the presence of amiloride at the concentration \([X]\), \( IC_{50} \) is the half maximal inhibitory concentration of amiloride, and \( n \) is the Hill coefficient. The pH-response curve for the sustained acid-sensing current was fitted with the alternative formulation of the Hill
equation: \[ \theta = \frac{1}{1 + 10^{\text{pH} - \text{pK}_d}} \], where \( \theta \) is the fraction of current activated at pH, and \( n \) is the Hill coefficient.

All summarized data reported as mean ± SEM. Summarized data compared with a two-tailed Student’s t-test. Proportions compared with a z-test. \( P \leq 0.05 \) considered significant.

RESULTS

Class IV md sensory neurons contain multiple acid-sensing currents

The mammalian homologs of \( \text{ppk1} \) that are expressed in sensory receptors of the peripheral nervous system, the ASICs, are activated by acidic pH (9-15). Thus, we voltage-clamped \( \text{Ppk1} \)-expressing md sensory neurons and probed the effects of an acid-stimulus on macroscopic current. For these studies, primary neuronal cultures were generated from embryos expressing GFP in Class IV md neurons (Figure 1A). As shown in Figure 1, decreasing extracellular pH rapidly evoked a sustained inward Na\(^+\) current \( (I_s) \) in md sensory neurons. A distinct transient inward Na\(^+\) current \( (I_t) \) was also observed in these neurons. This transient current, though, was only observed following relief from amiloride-blockade subsequent to an activating acid-challenge with application of amiloride alone in the absence of an acid stimulus not provoking \( I_t \). These data demonstrate for the first time the existence of acid-sensitive currents in \textit{Drosophila} sensory neurons.

Further analysis of the sustained current \( (I_s) \) in voltage-clamped class IV md sensory neurons determined, as shown in figure 2, that the half-activation pH (pH\(_{50}\)) for \( I_s \) is 4.1 ± 0.08. The majority of this sustained, acid-sensing current, moreover, is sensitive to the Deg/ENaC channel inhibitor amiloride, IC\(_{50}\) = 230 ± 20 \( \mu \)M, with ~20% residual current remaining in the presence of a saturating dose of amiloride. In contrast, \( I_s \) is refractory to the broad-spectrum TRP channel inhibitor ruthenium red. \( I_s \) is also equally selective for Na\(^+\) and K\(^+\). This pharmacological and biophysical fingerprint is consistent with a Deg/ENaC ion channel mediating the bulk of this current. Similar to \( I_s \), the transient acid-sensing current \( I_t \) identified in class IV md neurons, as shown in Figure 3 is sensitive to amiloride, refractory to ruthenium red and carried by inward Na\(^+\) flux. Thus, \( I_s \) and \( I_t \) are acid-activated depolarizing currents, likely conducted by Deg/ENaC but not TRP channels.

The \textit{Drosophila} Deg/ENaC protein, \( \text{Ppk1} \), contributes to an acid-sensing ion channel that conducts a transient depolarizing current in class IV md sensory neurons

The simplified kinetic scheme shown in Figure 3a can be used to rationalize the transient nature of \( I_t \) and the appearance of this current only after relief from blockade following an activating acid-challenge, with the condition that the open channel blocker, amiloride, is able to protect the activated channel from inactivation. While open channel blockers do not always function in this manner, this is the most reasonable explanation of these results and is consistent with additional experiments designed to test this possibility. For example this model predicts that the magnitude of \( I_s \) should depend on the concentration of blocker as it controls the capture of channels in the activated-but-blocked state. (Conversely, \( I_s \) decreases with increasing [amiloride] as
described in Figure 2c). Moreover, the magnitude of $I_t$ is expected to decline as a function of the latency period separating the removal of blocker from the activating acid pulse. As shown in Figures 3d-f, both expectations are met by $I_t$. Because $I_t$ increases as a function of the [amiloride] used to capture the channel in the activated-but-blocked state and is evoked by relief from inhibition, this current must be amiloride-sensitive.

A $ppk1$ null mutant ($ppk1^{ESB}$) and rescue flies were used to determine the relation between the gene-product of $ppk1$ and the acid-sensing currents identified above in class IV md neurons. To verify our $ppk1$ genetic approaches, we first confirmed the effects of our $ppk1$ loss-of-function ($ppk1^{ESB}$) mutants on previously published behavioral phenotypes (Ainsley et al., 2003; Tracey et al., 2003). As shown in Figure 4, $ppk1^{ESB}$ null mutant larvae have a defect in larval locomotion and a reduction in a characteristic motor response to mechanical nociception similar to what has been previously published for $ppk1$ mutants. Both defects are rescued by the expression of wild type Ppk1 in md neurons of $ppk1^{ESB}$ mutants consistent with Ppk1 function being disrupted in the $ppk1^{ESB}$ mutant md neuron.

As shown by the representative current traces from voltage-clamped class IV md neurons and the corresponding summary graphs in Figure 5, deletion of $ppk1$ abolishes $I_t$ but is without effect on $I_s$. Moreover, targeted expression of wild type Ppk1 in class IV md neurons of $ppk1^{ESB}$ mutant flies rescues $I_t$, having no effect on $I_s$. These results demonstrate that $ppk1$ encodes a non-redundant component of an acid-sensing Na$^+$ channel in md neurons responsible for $I_t$ but not $I_s$.

Importantly, it also identifies a paradigm by which relief from blockade following activation by an acid stimulus can be used as a means for the targeted manipulation of the transient current conducted by Ppk1 channels in md neurons. Such targeted activation is critical for testing whether a certain channel is capable of driving stimulus-dependent action potential firing: a requirement for establishing the channel as an ionotropic molecular sensory transducer. Use of relief from inhibition following an acid-stimulus to specifically activate Ppk1 versus applying a mechanical stimulus, which is known to activate many mechanosensitive ion channels in md and other sensory neurons allowed us to focus specifically on the cellular function of Ppk1. The relationship of this heterotypic stimulation to the endogenous stimuli remains to be determined, nonetheless this approach is valid for determining Ppk1 channel properties.

Additional support for the conclusion that Ppk1 is a channel protein responsible for $I_t$ comes from study of md neurons expressing the gain-of-function mutant $ppk1^{deg}$. The gain-of-function $degenerin$ mutation increases the activity of Deg/ENaC channels (26-29). As shown in the representative current trace and summary graphs in Figure 6 from voltage-clamped class IV md neurons, expression of the $ppk1^{deg}$ mutant significantly increases the magnitude of the amiloride-sensitive (depolarizing) leak current, and $I_t$, as well as slows inactivation of $I_t$. These results are consistent with the $ppk1^{deg}$ mutant acting as a gain-of-function $ppk1$ mutation.

The mechanosensory behavior of larvae harboring the $ppk1^{deg}$ mutation is similarly compromised to what was
observed in loss-of-function ppk1ESB mutants (Figure 4). Our behavioral results support that manipulation of Ppk1 activity in md neurons is sufficient to alter normal sensory function.

**Ppk1 does not influence the inherent excitability of class IV md sensory neurons**

Electrical and cellular ablation studies demonstrate that complete loss of neuronal activity results in similar effects on behavior as the ppk1ESB mutation (16;19;30). Therefore it is possible that the intrinsic activity of class IV md neurons could be dramatically altered in ppk1ESB null mutants. This was tested in current-clamp experiments on class IV md neurons as shown in Figure 7. Surprisingly, the intrinsic characteristics and firing frequency of action potentials, evoked by supra-threshold depolarizing currents, in current-clamped ppk1-expressing class IV md sensory neurons is similar in wild type and ppk1ESB flies. As expected, the voltage-gated Na+ channel inhibitor, tetrodotoxin, abolished all action potential firing. These observations are consistent with Ppk1 not contributing to the shape of the action potential or being required for the inherent excitability of md neuron, but rather being involved in the sensing and/or transformation of a stimulus into changes in neuronal activity.

**Ppk1 containing channels can function as an ionotropic molecular sensory transducer**

To determine if Ppk1 functions as an ionotropic molecular sensory transducer in md neurons we tested the ability of Ii to depolarize md neurons, resulting in the generation of action potentials. This was accomplished by selectively activating Ii using the relief from blockade following an acid-challenge paradigm. As shown in Figure 8, targeted activation of Ii drove current-clamped md neurons to threshold, resulting in a burst of action potentials. This movement to threshold and the dependent train of action potentials is absent in md neurons from ppk1ESB flies, but is rescued to wild type by the targeted expression of the wild type ppk1 transgene in these neurons of ppk1ESB flies. These results are consistent with activation of Ppk1 in md neurons being sufficient to transiently depolarize membrane potential to threshold, evoking a burst of action potentials. That the move to threshold and associated firing of action potentials is transient reflects the transient nature of Ii. Moreover, these results are consistent with Ppk1 being capable of transforming external stimuli into neuronal activity.

Normally, Ppk1 contributes little to the resting membrane potential (Figure 8c) of md neurons allowing this channel to function as an incidence sensor. This is not the case in md neurons expressing ppk1deg where the constitutively-active Ppk1 makes a notable contribution to leak current at rest (Figure 6b). As shown in Figure 8, this robust depolarizing leak current in md neurons containing the ppk1deg mutant results in marked depolarization of the resting membrane potential, causing these neurons to sit near threshold (which is -35 mV; see fig. 7c). This change in the resting membrane potential combined with a larger and more prolonged Ii (see fig. 6) results in md neurons expressing ppk1deg having abnormal electrical activity and responses to relief from amiloride blockade following an acid stimulus. As shown in the example current trace from...
a representative md neuron expressing \textit{ppk1}^{deg} in Figure 8a (bottom), membrane potential is depolarized near threshold at rest. Addition of amiloride drives membrane potential below threshold nearer the normal resting membrane potential for (wild type) class IV md neurons by blocking the robust depolarizing leak current arising from the constitutively-active \textit{Ppk1}^{deg} channel. This enables the firing of action potentials upon relief from amiloride-blockade subsequent to an acid challenge. Activation of the robust and prolonged \textit{I_\text{L}} following wash rapidly drives membrane potential back towards \textit{E}_{\text{Na}^+} evoking a sustained (instead of a transient) train of action potentials in md neurons from \textit{ppk1}^{deg} flies: there is no adaptation but rather action potentials continue firing until membrane potential reaches \textit{E}_{\text{Na}^+}.

**DISCUSSION**

The current results demonstrate that the \textit{Drosophila} Deg/ENaC protein, Ppk1, is a key component of an acid-sensing ion channel expressed in class IV md sensory neurons. Activated Ppk1 channels conduct a transient depolarizing sodium current in md neurons. Ppk1 is not active at rest, though, and consequently contributes little to the resting membrane potential, shape of the action potential and inherent excitability of md neurons. Rather stimulus-dependent activation of Ppk1 channels drives md neurons to threshold evoking a phasic burst of action potentials. In this regard, Ppk1 channels serve as ionotropic molecular sensory transducers functioning as incidence detectors capable of transforming an external stimulus into an electrical signal able to evoke action potential firing in class IV md neurons.

This function of Ppk1 (in md neurons) is critical for normal sensory perception.

\textit{Ppk1 is a critical component of an acid-sensing Deg/ENaC channel}

Results from the current electrophysiology studies provide compelling evidence that Ppk1 is a key component of an ion channel in md neurons: normal \textit{ppk1} expression and function are required for a transient, acid-sensing, amiloride-sensitive depolarizing sodium current in these sensory neurons. In this regard, this \textit{Drosophila} homolog mirrors the ion channel function of other members of the Deg/ENaC channel family, in particular, the mammalian ASIC and \textit{C. elegans degenerin} channel proteins, also expressed in sensory neurons (9;11;31). Sequence homology and structural similarity with ASIC proteins, which contribute to a conductive pore and for which the structure has been resolved (32;33), are consistent with Ppk1 being a pore-forming subunit. This is supported by our findings that a gain-of-function mutation in Ppk1 changes the biophysical properties of the transient current associated with the expression and function of this channel protein.

Importantly, the alteration in gating by the gain-of-function mutation was sufficient to alter sensory responses, consistent with Ppk1 functioning during signal transduction in sensory neurons. Moreover, the observation that the behavioral phenotype of larvae harboring a gain-of-function \textit{ppk1} mutation in their md neurons phenocopies larvae harboring loss-of-function \textit{ppk1} mutations is similar to observations made for loss- and gain-of-function mutations in Deg/ENaC channels expressed in \textit{C. elegans} touch
receptors (26-29). These results support that any change in normal Ppk1 activity, be it an increase or a decrease, disrupts the ability of md neurons to transform sensory information appropriately, compromising normal behavioral responses. This emphasizes the importance of the appropriate activation of Deg/ENaC channels to the process of changing a stimulus into a properly graded electrical signal during sensory transduction.

Deg/ENaC channels function as molecular signal transducers

The current studies demonstrate that decreases in pH activate and then quickly inactivate Ppk1 channels. This is similar to the effects of decreases in pH on mammalian ASIC channels (9;10;15) with the exception that the inactivation of Ppk1 is particularly rapid. Importantly, the ability to relieve activated-Ppk1 from blockade following stimulation by an acid challenge allowed us to test if the activation of Deg/ENaC proteins is sufficient to drive sensory neurons to threshold; and thus, function as ionotropic molecular sensory transducers. These results help define the role that Deg/ENaC channels play in the mechanistic and ionic basis of sensory transduction. They serve two discrete functions: 1) during the initial phase of sensory transduction they act as protein sensors involved in the direct perception of the stimulus; and 2) through their ability to conduct a depolarizing current upon stimulus-dependent activation capable of bringing the membrane potential to threshold, transform the sensing of a stimulus into an electrical signal that results in changes in neuronal activity. Interestingly, the neuronal activity resulting from the activation of Ppk1 is shaped by the gating properties of the channel suggesting that these channels can also play an important role in the encoding of the stimuli. This idea is supported by the observation of the deleterious effects of the degenerin mutation on mechanosensory behavior.

The current results provide support for the idea that Deg/ENaC channels function as ionotropic molecular sensory transducers important for sensory transduction in peripheral sensory neurons. This solidly places Deg/ENaC channels, along with TRP and Piezo channels (1-3;7;8;34;35), into a small group of ion channels capable of sensing and transforming stimuli into electrical signals. The current results extend understanding significantly by identifying Deg/ENaC channel proteins as essential elements in sensory transduction, being important in both the early formative events encoding sensory stimuli and the transformative events resulting in changes in sensory neuron activity upon channel activation. The mechanistic paradigm emerging from the current studies, in consideration of earlier findings regarding the function of Deg/ENaC channels, has stimulus dependent activation of Deg/ENaC channels in sensory dendrites resulting in a depolarization of the plasma membrane via a transient inward sodium current. The resulting transient depolarization to threshold evokes a burst of action potentials, defined by channel gating properties, resulting in the transformation of the stimulus into an electrical signal in sensory neurons that carries instructive information into the nervous system allowing for the appropriate behavioral response.
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Acknowledgements

This work was supported by an American Heart Association Established Investigator Award (0640054N) to JDS and an NIH RO1 (NS062811) to BAE.
FIGURE LEGENDS

Figure 1. Acid-sensing currents in class IV md neurons. Inverted fluorescent image (a) of dissected ppk1-Gal4, UAS-mCD*GFP larvae stained for GFP expression. The ventral nerve cord (vnc) and a GFP-expressing class IV md neuron (ddaC) are indicated (circle). (b) The ddaC neuron indicated in a is shown in high magnification stained for GFP (arrow in i) and HRP (ii). The identity of the dorsal da neurons shown in i and ii are indicated (see asterisks in i and iii). (c) Fluorescent image of ddaC neuron from a. (d) Fluorescent image of a cultured embryonic neuron derived from ppk1-Gal4, UAS-mCD8-GFP embryos. Scale bar in c and d = 30 um. (e) Representative sustained (I_s) and transient (I_t) acid-sensing macroscopic Na⁺ currents in a typical voltage-clamped class IV md neuron. Inward Na⁺ current is downwards. Cells were clamped to -60 mV with current evoked by rapidly lowering bath pH from 7.4 to 4.5 (noted with gray bar); and for I_t subsequently washing 1 mM amiloride (noted with black bar). (f) Summary graph of the mean magnitude of I_s (evoked by pH 4.5; n = 20) and I_t (evoked by washing 1 mM amiloride following an acid challenge provided in the presence of the inhibitor; n = 20) in md neurons at -60 mV.

Figure 2. Characterization of I_s. (a) Representative macroscopic current trace of I_s repetitively evoked with pH 4.5 in a voltage-clamped class IV md neuron at different test potentials (as indicated above) and the corresponding I-V relation (shown in the inset below and to the left; n ≥ 3). Bath and pipette solutions contained 140 mM NaCl, and 20 mM NaCl plus 120 mM Cs-gluconate, respectively. Also shown in the inset below and to the right is the relative magnitude of I_s (at -60 mV) upon replacing bath [Na⁺] with [K⁺], n = 4. (b) The dose-response curve (n ≥ 6) for pH activation of I_s as fitted with a standard Hill equation: pH₅₀ = 4.1 ± 0.08. (c) The dose-response curve (n ≥ 3) for percent amiloride inhibition of I_s: IC₅₀ = 230 ± 20 µM. The corresponding I/I_max values are shown in the inset. (d) Typical acid-evoked I_s in a voltage-clamped class IV md neuron in the absence and presence of 20 µM ruthenium red. A summary graph of relative current in the presence of ruthenium red (RR) for I_s is shown to the right (n = 4).

Figure 3. Characterization of I_t. (a) This simple kinetic scheme rationalizes activation of I_t by relief from blockade following removal of blocker subsequent to activation by an acid-challenge, and explains why I_t is transient. C indicates closed channels; A indicates activated channels; A_B indicates activated-but-blocked channels; and I indicates inactivated channels. Transitions from C to A and A to I are pH-dependent and that from A to A_B is [amiloride]-dependent where activated-but-blocked channels can be captured in this state as a function of the [amiloride] provided during an activating acidic pH pulse. (b) Macroscopic I-V relation for I_t as determined from voltage-clamp experiments, n ≥ 3. Bath and pipette solutions contained 140 mM NaCl, and 140 mM Cs-gluconate, respectively. (c) Representative sustained (I_s) and transient (I_t) acid-sensing macroscopic Na⁺ currents in a typical voltage-clamped class IV md neuron in the
presence of 20 μM ruthenium red (n = 7; all other conditions the same as 3a). Representative macroscopic currents from a typical voltage-clamped md neuron (d) and corresponding summary graph (e; n = 8) demonstrating that the magnitude of I_b is dependent on relief from blockade, increasing as a function of the [amiloride] used to captured the channel in the A_B state during the activating pH pulse. *Significantly greater compared to 100 μM amiloride. (f) A representative current trace of I_b sequentially evoked with different latency periods prior to washing amiloride and the corresponding summary graph (inset; n ≥ 3) showing that I_b decreases in magnitude as a function of the latency period between the cessation of the activating acid pulse and washing of the blocker. The hash marks in this continuous current trace represent regions removed to generate the figure. In each of these deleted regions an activating acid pre-pulse of consistent time was provided in the presence of amiloride.

Figure 4. Normal ppk1 function in class IV md neurons is necessary for typical larval locomotion and nociceptive responses to stimuli. (a) The centroid paths of three representative wild type (black; left), ppk1^{ESB} null mutant (blue; middle left), ppk1^{ESB} rescued with expression of the wt transgene in md neurons (red, middle right), and gain-of-function ppk1^{deg} mutant (green, right) larvae. The open dots indicate starting points. The degree of direction change per 1 sec is shown below (b) for the corresponding larvae. (c) Summary graph of mean direction change of larval movement for wild type, ppk1^{ESB} (null), rescued ppk1^{ESB} and ppk1^{deg} larvae. Data collected from experiments identical to those in 1a with n ≥ 49 larvae for each condition. (d) Summary graph of the percentage of responding larvae to harsh touch (46 mN) for wild type, ppk1^{ESB}, rescued ppk1^{ESB} and ppk1^{deg} larvae (n ≥ 168). *Significant decrease compared to wt; ** significant increase compared to ppk1^{ESB}.

Figure 5. Ppk1 conducts I_b but not I_s. (a) Typical acid-evoked macroscopic Na^+ currents in voltage-clamped (holding potential -60 mV) class IV md neurons from wild type (left; n = 4), ppk1^{ESB} mutant (middle; n = 3) and ppk1^{ESB} mutant rescued with expression of the wt ppk1 transgene in class IV md neurons (right, n = 4) flies. In these experiments, neurons were treated with 1 mM amiloride prior to providing the activating acid pulse with blocker and low pH subsequently washed simultaneously to evoke I_b. (b) Summary graph of the probability of observing I_b in wild type, ppk1^{ESB} (null) and rescued ppk1^{ESB} voltage-clamped md neurons. (c) Summary graph of the magnitude of I_s in voltage-clamped class IV md neurons from wild-type (n=22), ppk1^{ESB} (n=13) and rescue ppk1^{ESB} (n=12) flies. Data presented in b and c from experiments identical to that in a.

Figure 6. The gain-of-function ppk1^{deg} mutation increases the amiloride-sensitive leak current in md neurons at rest and I_b. (a) Representative acid-evoked macroscopic Na^+ currents in voltage-clamped (holding potential -60 mV) class IV md neurons from wild type (left; n = 4), ppk1^{ESB} (middle; n = 3) and ppk1^{deg} (right; n = 4) flies. In these experiments, neurons were treated with 1 mM amiloride prior to providing the activating acid pulse with blocker washed in the
middle of the activating acid pulse. (b) Summary graph of the amiloride-sensitive (depolarizing) leak current in voltage-clamped (-60 mV) md neurons from wild type (n = 15) and \(ppk1^{\text{deg}}\) (n = 4) flies. (c) Summary graph of the magnitude of \(I_t\) evoked by relief from amiloride-inhibition following an acid stimulus in voltage-clamped md neurons from wt (n = 22) and \(ppk1^{\text{deg}}\) (n = 4) flies. Data from experiments are similar to those in 7a. (d) The time constants of inactivation \(\tau_{\text{inac}}\) at pH 4.5) for \(I_t\) in md neurons from wt and \(ppk1^{\text{deg}}\) flies. Time constants calculated by fitting \(I_t\) from experiments identical to that in 7a. *Significantly greater/slower compared to wt.

**Figure 7.** \(ppk1\) does not influence the inherent excitability or the intrinsic characteristics of action potentials in class IV md neurons. Representative action potentials are shown from from wild type (a) and \(ppk1^{\text{ESB}}\) (b) current-clamped class IV md neurons. Action potentials were evoked with suprathreshold depolarizing currents for 600 ms. The effects of 50 nM TTX on action potentials in wild type md neurons is shown in the inset at a condensed scale. Summary graphs of the threshold (c), amplitude (d) and half-width (e) of the first action potential (evoked with a suprathreshold current as in a and b), and firing frequency (with injection of 2 pA/pF; f) from wild type (n = 12) and \(ppk1^{\text{ESB}}\) (n = 16) class IV md neurons. No values significantly different.

**Figure 8.** Ppk1 is an ionotropic molecular sensory transducer capable of bringing sensory neurons to threshold. (a) Representative action potentials evoked by relief from amiloride inhibition following an acid stimulus from current-clamped class IV md neurons from wild type (top), \(ppk1^{\text{ESB}}\) (upper middle), rescued \(ppk1^{\text{ESB}}\) (lower middle) and \(ppk1^{\text{deg}}\) (bottom) flies. The trains of stimulus-dependent action potentials are shown at an expanded time scale to the right (noted with dashed line) for each group. The typical action potential threshold (see fig. 2c) and resting membrane potential \(V_{\text{rest}}\); from 8c) of md neurons from wild type flies is also indicated in the bottom trace for \(ppk1^{\text{deg}}\) neurons. (b) Summary graph of the probability of action potential firing in response to relief from amiloride inhibition following an acid stimulus in class IV md neurons from wild type (n = 10), \(ppk1^{\text{ESB}}\) (n = 7), rescued \(ppk1^{\text{ESB}}\) (n = 7) and \(ppk1^{\text{deg}}\) (n = 9) flies. Data from experiments are similar to those in Fig. 8a. *Significant decrease compared to wt; ** significant increase compared to the null \(ppk1^{\text{ESB}}\). (c) Summary graph of the resting membrane potential \(V_{\text{rest}}\) in voltage-clamped md neurons from wild type (n = 12), \(ppk1^{\text{ESB}}\) (n = 8) and \(ppk1^{\text{deg}}\) (n = 19) flies. *Significantly greater than wt and the null \(ppk1^{\text{ESB}}\).
Figure 4:

(a) Schematic representation of wild type, ppk1^{ESB}, rescue, and ppk1^{deg} larvae, showing the directional changes over time.

(b) Graphs depicting the direction change in degrees over time for each genotype:
- Wild type
- ppk1^{ESB}
- Rescue
- ppk1^{deg}

(c) Bar charts showing the direction change in degrees for each genotype:
- Wild type
- Null
- Rescue
- ppk1^{deg}

(d) Bar charts showing the percentage of responding larvae:
- Wild type
- Null
- Rescue
- ppk1^{deg}

Note: The graphs and charts illustrate the behavioral responses of the larvae under different conditions, with ppk1^{ESB} and ppk1^{deg} showing altered responses compared to wild type and rescue.
a  wild-type  

\[ \text{pH 4.5} \]

amiloride wash

\[ I_t \]

\[ 100 \text{ pA} \]

10 sec

\[ I_t \]

\[ \text{ppk1}^{ESB} \]

amiloride wash

\[ I_t \]

\[ \text{rescue} \]

amiloride wash

\[ I_t \]

b

\begin{align*}
\text{wt} & \quad \text{ppk1}^{ESB} & \quad \text{rescue} \\
\text{Probability of } I_t & \quad 0.8 \quad \ast & \quad 0.4 \quad \text{bar} \\
\end{align*}

\[ I_s \text{, pA} \]

\begin{align*}
\text{wt} & \quad \text{ppk1}^{ESB} & \quad \text{rescue} \\
\text{I_s, pA} & \quad 200 \quad \text{bar} & \quad 200 \quad \text{bar} \\
\end{align*}

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**Figure 6**

(a) Graphical representation of wild-type and mutant strains under different conditions.

- **Wild-type**
  - pH 4.5
  - Amiloride wash
  - Current trace labeled $I_t$
  - 100 pA, 20 sec

- **ppk1^{ESB}**
  - pH 4.5
  - Amiloride wash
  - Current trace labeled $I_t$

- **ppk1^{deg}**
  - pH 4.5
  - Amiloride wash
  - Current trace labeled $I_t$
  - 200 pA, 20 sec

(b) Bar graph showing amiloride-sensitive $I_{leak}$ for wild-type (wt) and ppk1^{deg}.

(c) Bar graph showing $I_t$ for wild-type (wt) and ppk1^{deg}.

(d) Bar graph showing $\tau_{inac}$ for wild-type (wt) and ppk1^{deg}.

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a

wild-type

pH6.0

+ amiloride

wash

ppk1ESB

pH6.0

+ amiloride

wash

rescue

pH6.0

+ amiloride

wash

ppk1deg

pH6.0

+ amiloride

wash

[caption]

[figure]

b

Probability of firing

wt

null

rescue

deg

[chart]

C

V_{rest} (mV)

wt

null

deg

[chart]
Pickpocket1 is an ionotropic molecular sensory transducer
Nina Boiko, Volodymyr Kucher, James D. Stockand and Benjamin A. Eaton

*J. Biol. Chem.* published online October 1, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.411736

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