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RESEARCH ARTICLE

A unique alkaline pH-regulated and fatty acid-activated tandem pore domain potassium channel (K_{2P}) from a marine sponge

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SUMMARY

A cDNA encoding a potassium channel of the two-pore domain family (K_{2P}, KCNK) of leak channels was cloned from the marine sponge *Amphimedon queenslandica*. Phylogenetic analysis indicated that AquK_{2P} cannot be placed into any of the established functional groups of mammalian K_{2P} channels. We used the *Xenopus* oocyte expression system, a two-electrode voltage clamp and inside-out patch clamp electrophysiology to determine the physiological properties of AquK_{2P}. In whole cells, non-inactivating, voltage-independent, outwardly rectifying K^+ currents were generated by external application of micromolar concentrations of arachidonic acid (AA; EC_{50} ~30 μmol l^{-1}), when applied in an alkaline solution (≥pH 8.0). Prior activation of channels facilitated the pH-regulated, AA-dependent activation of AquK_{2P} but external pH changes alone did not activate the channels. Unlike certain mammalian fatty-acid-activated K_{2P} channels, the sponge K_{2P} channel was not activated by temperature and was insensitive to osmotically induced membrane distortion. In inside-out patch recordings, alkalinization of the internal pH (pK_a 8.18) activated the AquK_{2P} channels independently of AA and also facilitated activation by internally applied AA. The gating of the sponge K_{2P} channel suggests that voltage-independent outward rectification and sensitivity to pH and AA are ancient and fundamental properties of animal K_{2P} channels. In addition, the membrane potential of some poriferan cells may be dynamically regulated by pH and AA.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/14/2435/DC1

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INTRODUCTION

Tandem pore domain K^+ channels (K_{2P}; KCNK) are a family of ion channels involved in the control of background or leak membrane conductances (Bayliss and Barrett, 2008; Enyedi and Czirják, 2010). Background K^+ conductances are a major determinant of resting membrane potential and input resistance, two key components of electrical signaling in animal cells. In mammals, there are 15 K_{2P} genes that encode for six subfamilies that group together functionally, based on the mechanisms of channel activation and inhibition (Enyedi and Czirják, 2010). Members of the K_{2P} family of channels are regulated in variable ways by diverse chemical, physical and pharmacologic agents, including arachidonic acid (AA), internal and/or external protons, membrane stretch, temperature and anesthetic agents (Bayliss and Barrett, 2008; Dedman et al., 2009). A structural understanding for the distinct mechanisms of activation is still incomplete, although the highly variable C-terminal region is implicated in the regulation of some K_{2P} channels by AA, internal pH and mechanical stimuli (Kim et al., 2001b) and specific residues near the channel pore regulate external or internal proton sensitivity in some channels (Rajan et al., 2000; Kim et al., 2001a; Morton et al., 2003; Niemeyer et al., 2007; Sandoz et al., 2009; Niemeyer et al., 2010). A better understanding of the molecular determinants of K_{2P} channel activation might be achieved by the isolation of more diverse members of this important family of ion channels.

We report the first identification of a novel K_{2P} channel cloned from the marine sponge *Amphimedon queenslandica* (Hooper and Van Soest, 2006) a demosponge from the Great Barrier Reef. Sponges are sessile, aquatic, multicellular animals that lack a nervous system, but display contractions to expel water (Nickel, 2004; Elliott and Leys, 2007). There is limited information available on membrane conductances in sponges (Zocchi et al., 2001; Tompkins-MacDonald et al., 2009), but hexactinellid (glass) sponges are known to coordinate flagellar movement and demonstrate calcium-dependent action potentials (Leys et al., 1999). Studying ion channels in native sponges using fine electrodes is impeded by the complex glycocalyx and high membrane fluidity (Carpaneto et al., 2003) and thus requires an alternative approach such as the cloning and functional expression of ion channels. Because the phylum Porifera may share a common ancestor with all other metazoans (Philippe et al., 2009), insight into the structure and function of ion channels from this unusual animal (*A. queenslandica*) may be a key to understanding the genetic repertoire present at the origin of metazoan evolution. Moreover, an understanding of the activation mechanisms for channels cloned from diverse species can provide comparative data that will be useful in determining structure–function relationships of ion channel proteins and understanding their physiological roles in different organisms.

As shown in this study, a K^+ channel subunit cloned from *A. queenslandica* (AquK_{2P}) possesses two pore-forming domains and

four transmembrane segments, features common to all K_{2P} channels. The deduced amino acid sequence for $AquK_{2P}$ fails to classify the channel into a known subfamily of K_{2P} channels. We find that the sponge channel is activated by AA, a polyunsaturated fatty acid, in an external pH-dependent fashion. The sponge K_{2P} channel is also activated by internal alkalinization with or without AA but its gating is insensitive to temperature changes as well as membrane stretch. Overall, the conservation in sponge and some mammalian K_{2P} channels of voltage-independent outward rectification and sensitivity to pH and AA suggest that these are ancient elements of animal K_{2P} channel gating.

MATERIALS AND METHODS

Isolation of cDNAs and DNA sequencing

Amphimedon queenslandica larvae were procured from Heron Island Reef, Queensland, Australia, and preserved in RNAlater stabilization solution (Qiagen, Valencia, CA, USA). RNA extraction and cDNA preparation and cloning methods have been described previously (Tompkins-MacDonald et al., 2009). The primer pair used for amplification of a predicted full-length K_{2P} subunit was based on sequence data in the *A. queenslandica* genome (Srivastava et al., 2010) and was composed of 5′-GGCTCGAGCCACCATGGAGAAA-GAGGTCGAG-3′ (forward) and 5′-GGACAGTTCACTCCT-TCTCTACTTCAG-3′ (reverse). After cloning into the PCR-4-TOPO vector (Invitrogen, Carlsbad, CA, USA), the $AquK_{2P}$ cDNA was ligated into a pXT7 expression plasmid. To ensure the integrity of the sequence data and that the cDNA was complete, we subjected two clones to complete sequencing of both DNA stands in both directions and obtained identical results. We compared our sequence data with the genomic database for *A. queenslandica* and detected only a single K_{2P} channel sequence at contig 13452:297098..298220; the open reading frame was interrupted by a single intron and the coding region of 1059 nucleotides predicted a 353 amino acid peptide. The exon sequences in the genomic database and two cDNA clones were identical except for the codon predicting residue 350. At this position, our results predicted a valine whereas the genomic database predicts a glycine.

Phylogenetic analysis and alignments

The translated sponge K_{2P} sequence was aligned and compared with amino acid sequences of K_{2P} (KCNK) sequences obtained from NCBI. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Alignments were performed in MAFFT (http://mafft.cbrc.jp/alignment/server/) using default parameters and viewed in BioEdit version 7.0.5.3 (Ibis Biosciences, Carlsbad, CA, USA). Membrane topology was predicted by the ExPASy TMHMM server v2.0 (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and adjusted by alignment with channels with published topology profiles. Sequence data for $AquK_{2P}$ have been deposited in GenBank (accession number JN165777).

RNA and oocyte preparation and injection

RNA preparation, *Xenopus laevis* oocyte isolation and oocyte injection were carried out using methods previously described in detail (Boland et al., 2009; Tompkins-MacDonald et al., 2009). Animal protocols were approved by the University of Richmond and the Virginia Commonwealth University Institutional Animal Care and Use Committees. Individual oocytes (stages V and VI) were injected with 40–60 nl RNA (0.25–1.0 ng nl^{-1}) in RNAse-free water. Oocytes were maintained at 17–19°C for 1–5 days in an ND-96 solution containing (in mmol l^{-1}): 96 NaCl, 1 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, 5 sucrose and 2 Na pyruvate, with 50 U ml^{-1} penicillin G, 50 μg ml^{-1} streptomycin and, in most cases, 50 μg ml^{-1} tetracycline. Solution pH was adjusted with NaOH to 7.4 using an Orion 310 pH meter (Thermo Fisher Scientific, Pittsburgh, PA, USA).

Electrophysiology

Two-electrode voltage clamp (TEVC) methods were used to record whole oocyte potassium currents using standard methods (Boland et al., 2009) with a Geneclamp 500B (Molecular Devices, Sunnyvale, CA, USA) or an OC-725C amplifier (Warner Instruments, Hamden, CT, USA). Currents were sampled at 5–10 kHz and filtered at 1–2 kHz. Experiments were performed at room temperature (21–23°C), and a small volume bath chamber was perfused continuously during recordings. Bath temperature was changed by cooling or heating the solution from the reservoir to the recording chamber and was measured with a calibrated digital thermometer containing a small probe that was placed next to the oocyte in the recording chamber. The standard TEVC external recording solution contained (in mmol l^{-1}): 2 KCl, 98 NaCl, 2 $MgCl_2$, 0.3 $CaCl_2$ and 5 Hepes with the pH adjusted with NaOH to 6.5–9.0. Solution osmolality was adjusted by adding sucrose or eliminating some of the NaCl and was measured using a Fiske 210 osmometer (Advanced Instruments, Norwood, MA, USA).

For inside-out patch recordings, we used a Dagan 3900A amplifier (Dagan Corporation, Minneapolis, MN, USA). Patch recording pipettes had resistances of approximately 0.5–1.5 Mohm after fire-polishing and were backfilled with a standard extracellular solution that was composed of (in mmol l^{-1}): 96 Na methanesulfonate (MES), 2 KMES, 1 $MgCl_2$, 1 $CaCl_2$ and 5 Hepes with the pH adjusted to 7.4 with NaOH. The bath solution (facing the internal surface of the membrane) was composed of (in mmol l^{-1}): 96 KMES, 10 Hepes, 5 EGTA and 5 EDTA. The pH of the bath solution for patches was modified with the addition of KOH, which summed to 3 mmol l^{-1} K^+ at the highest pH values tested. Inside-out macropatch recordings were obtained at room temperature (20–23°C) with continual perfusion of the internal surface of the membrane patch.

Electrophysiological data were recorded on Pentium computers equipped with Digidata A/D hardware (Molecular Devices). Molecular Device's Clampex and Clampfit analysis software were used. Data were also transferred to Microsoft Excel (Microsoft, Redmond, WA, USA) or Microcal Origin (OriginLab, Northampton, MA, USA) for additional analysis and the production of figures. Results are expressed as means ± s.e.m.; N is the number of cells or patches tested. Statistical significance was evaluated by paired or unpaired Student's two-tailed t-tests with $P<0.05$ considered to be significant.

Reagents

Reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) or Nu-Chek Prep Lipids (Elysian, MN, USA). AA was dissolved in ethanol or DMSO at 1000–3000×, stored at –20°C and diluted into recording solution immediately before use. Vehicle controls were determined to be without effect at the highest concentrations of diluents. Fatty-acid-free bovine serum albumin (BSA) was dissolved into recording solutions at 0.5 mg ml^{-1}.

RESULTS

$AquK_{2P}$ amino acid sequence and homology

$AquK_{2P}$ is the first K_{2P} (KCNK) channel to be identified from poriferans and the subunit shares the common features of this family of channels: two pore-forming loops (P1 and P2) and four

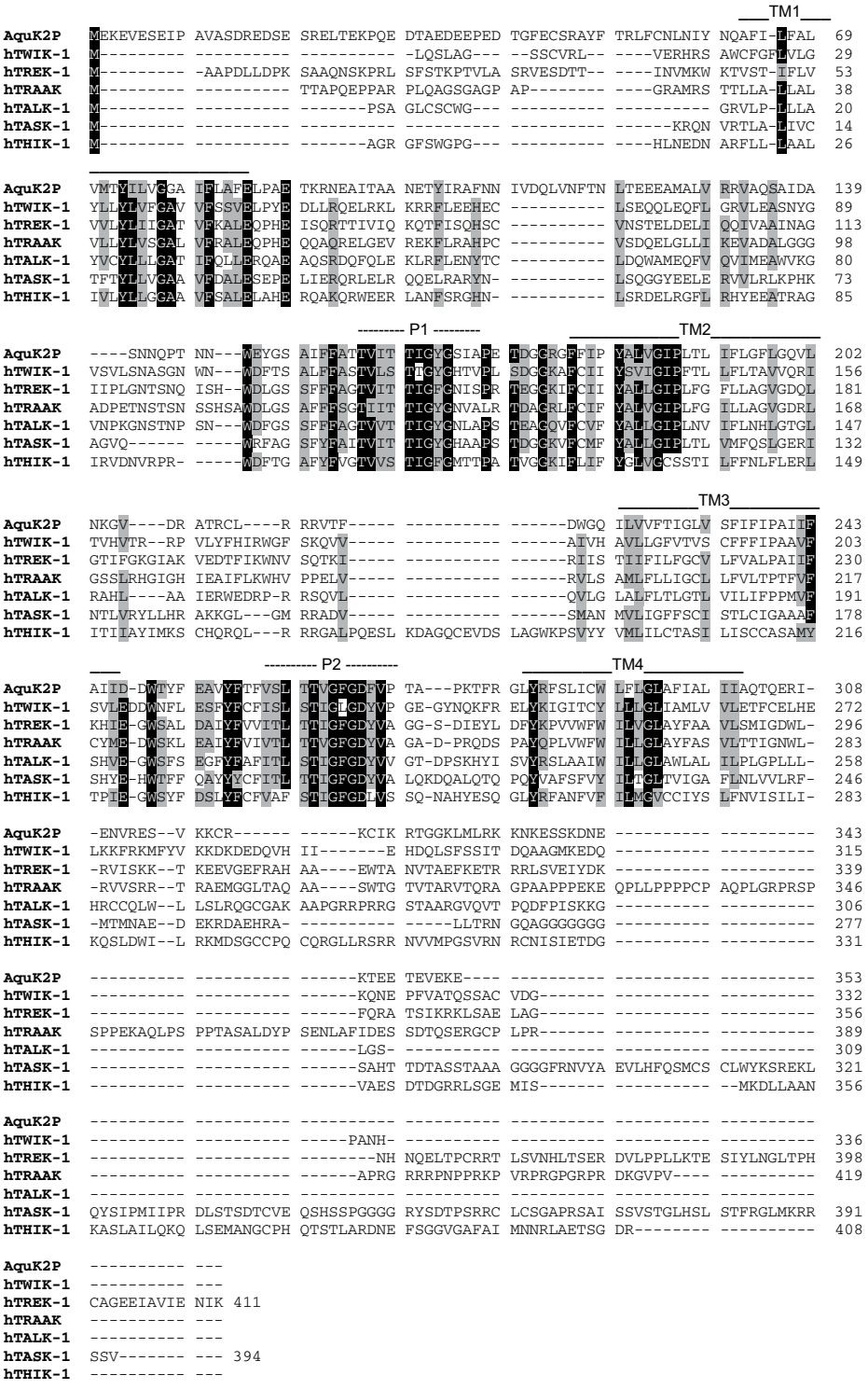

Fig. 1. Amino acid sequence comparison of $AquK_{2P}$ and mammalian K_{2P} channels. The deduced amino acid sequence of the *Amphimedon queenslandica* $AquK_{2P}$ subunit was aligned with a representative from each of the major classes of human (h) K_{2P} channels. Dashes indicate gaps inserted for a better alignment. Identical and conserved residues are boxed in black and gray, respectively. The locations of the pore-forming regions (P1, P2) and the transmembrane alpha helices (TM1–TM4) are shown as dashed and solid lines, respectively. Numbers refer to the last residue in each line.

transmembrane domains (TM1–TM4; Fig. 1). The pore-forming loops contain a K^+ selectivity filter with the conserved residues GYG in the P1 region and GFG in the P2 region. Overall, $AquK_{2P}$ shows low (18–25%) amino acid identity to human K_{2P} channels but even the mammalian K_{2P} channels have only 25–38% overall amino acid identity between subfamilies. Residues that have remained conserved over evolutionary time are likely to be crucial to channel function (black and gray boxes, Fig. 1). For example, the predicted pore regions are 53% (P1) and 82% (P2) conserved among $AquK_{2P}$ and TREK-1 (Fig. 1).

The six subfamilies of K_{2P} channels – TREK/TRAAK, TALK/TASK-2, TWIK, THIK, TASK-1,-3,-5 and TRESK – can be discriminated based on functional differences (Bayliss and Barrett, 2008; Enyedi and Czirják, 2010; Mathie et al., 2010). However, phylogenetic comparison of $AquK_{2P}$ with known human K_{2P} channels shows that the molecular composition of the sponge variant is sufficiently different that it does not group into these known functional classifications. An unrooted phylogenetic tree shows a separate branch-point for $AquK_{2P}$ whereas six major branches cluster the human K_{2P} subfamilies both structurally and functionally (Fig. 2).

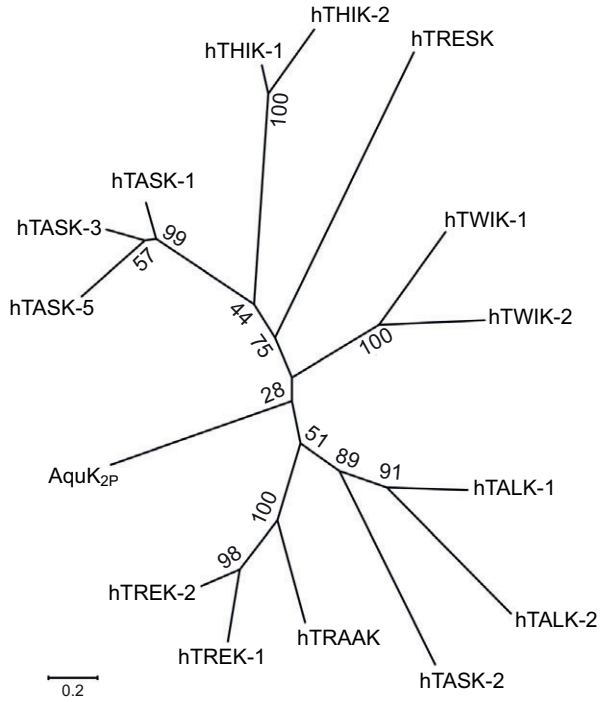

Fig. 2. Phylogenetic analysis of $AquK_{2P}$. An unrooted tree comparing $AquK_{2P}$ to human (h) K_{2P} channels. The evolutionary history was inferred using the maximum likelihood method (Jones et al., 1992). The tree with the highest log likelihood (–5892.4037) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Scale bar indicates the number of amino acid substitutions per site. Branch numbers represent bootstrap values of 100 replicates. The analysis involved 15 amino acid sequences and a total of 213 positions in the final data set.

External mechanisms of $AquK_{2P}$ channel activation

Non-inactivating K^+ channel currents were generated by external application of the polyunsaturated fatty acid AA (5,8,11,14-eicosatetraenoic acid) to oocytes injected with RNA prepared from the $AquK_{2P}$ cDNA (Fig. 3). We discovered that the activation by AA depended on an external alkaline pH (pH_o; see Fig. 4); therefore, we studied the AA-dependent current activation and assessed K^+ selectivity using pH_o 8.5 or 9.0. $AquK_{2P}$ currents are largely time independent, but at strong depolarizations they were not instantaneous (Fig. 3A, see top three traces at +20, +40 and +60 mV and 3C, inset). Resolution of the time dependence of activation was better achieved in inside-out patch recordings (supplementary material Fig. S1A). The rapid current activation was best fit by a two-exponential function, but the fitted time constants (τ_1~0.8 ms and τ_2~9 ms) were not voltage or pH dependent (supplementary material Fig. S1B). There was also no saturation of the outward current at strong depolarizations (Fig. 2A, supplementary material Fig. S1A,C).

In TEVC recordings, there was almost no inward current in an external solution containing 2 mmol l^{-1} K^+ and 98 mmol l^{-1} Na^+ (Fig. 3A,B and 3D, inset) and, even in elevated external K^+, the currents did not show inward rectification (Fig. 3D, inset). Macropatch recordings in symmetrical K^+ also failed to show significant inward current at pH 6.0 to 8.0; however, at hyperpolarized membrane potentials of –50 to –180 mV and pH 8.5, a small inward current developed (supplementary material Fig. S1B) but the current–voltage curve was still outwardly rectifying. Our data support the conclusion that $AquK_{2P}$ is an outwardly rectifying channel with no apparent voltage dependence to the activation mechanism. Mechanisms of outward rectification have been revealed in some channel types, including the external Mg^{2+} block of NMDA receptor channels (Nowak et al., 1984), the differential influx or efflux of cations in TRPM7 channels (Penner and Fleig, 2007) and a mechanism intrinsic to the channel protein in TWIK-1 channels (Lesage et al., 1996).

To study the concentration–response relationship for $AquK_{2P}$ current activation by AA, we applied increasingly higher concentrations of AA without recovery in between the concentrations; the dose–response relationship has a fitted EC_{50} value of 30.6 μmol l^{-1} with a Hill coefficient of 4.0 (Fig. 3C). Because there is a sensitization to prior AA applications (see Fig. 4), the steep slope could be explained by increasing levels of sensitization or perhaps a cooperative effect of AA on channel opening. The channels are mainly permeable to K^+ because a plot of the current reversal potential *versus* the log of the K^+ concentration yielded a linear relationship with a slope of 46 mV $decade^{-1}$, which is close to the theoretical value of 58 mV $decade^{-1}$ for a K^+ selective channel (Fig. 3D). Even in elevated external K^+, the currents did not show inward rectification (Fig. 3D, inset). The dose-dependent activation of $AquK_{2P}$ by AA (Fig. 3C) did not change the current reversal potential (Fig. 3B).

As noted, the activation of $AquK_{2P}$ by AA was regulated by external alkalinization (Fig. 4). $AquK_{2P}$ currents were always activated by 10–100 μmol l^{-1} AA when applied in an external recording solution of pH 8.5 or 9.0 and sometimes even at pH 8.0, if the AA was applied for a long duration (>5 min) or if this followed a prior activating event at alkaline pH values. However, only rarely (<5% of cells) were currents detected when up to 200 μmol l^{-1} AA was applied at an external pH of 7.4. Independent of pH_o, $AquK_{2P}$ current activation was reversed completely by external application of bovine serum albumin (BSA), a fatty acid binding protein, confirming that AA was required for activation (Fig. 4A,B).

A sensitization effect was apparent in all cells tested with external AA and alkaline pH. For example, the representative time course in Fig. 4A shows that AA-dependent current activation at pH_o 9.0 was enhanced following prior activation at pH_o 8.5 or 9.0 when compared with the effect of the first application of pH_o 9.0 to the cell. These changes occurred with complete washout of the pH effects and in the constant presence of AA. We also documented this sensitization using three successive applications of AA at pH_o 9.0, with interruptions by non-activating applications of AA at pH_o 7.4. The time course of the response to AA (Fig. 4B) shows that the K^+ current is activated more quickly and the steady-state current amplitude is significantly increased by each successive application of alkaline pH (Fig. 4C), even with complete washout of the effect in between the applications of alkaline pH. The time course and reversibility of these effects argue against a perfusion artifact as an explanation for the sensitization. However, changes in external pH alone (in the range of 6.5 to 9.0) failed to activate any current in $AquK_{2P}$-expressing oocytes (Fig. 4D, N=5). None of the pH_o-regulated, AA-dependent $AquK_{2P}$ currents showed differences in the current reversal potential or rectification in 2 mmol l^{-1} external K^+, even when the currents were sensitized by prior activation, as for the example shown in Fig. 4E. The most important conclusion is that $AquK_{2P}$ subunits assemble to make functional K^+ channels whose AA-dependent activation is regulated by external alkaline pH, but $AquK_{2P}$ is not activated by external alkalinization alone.

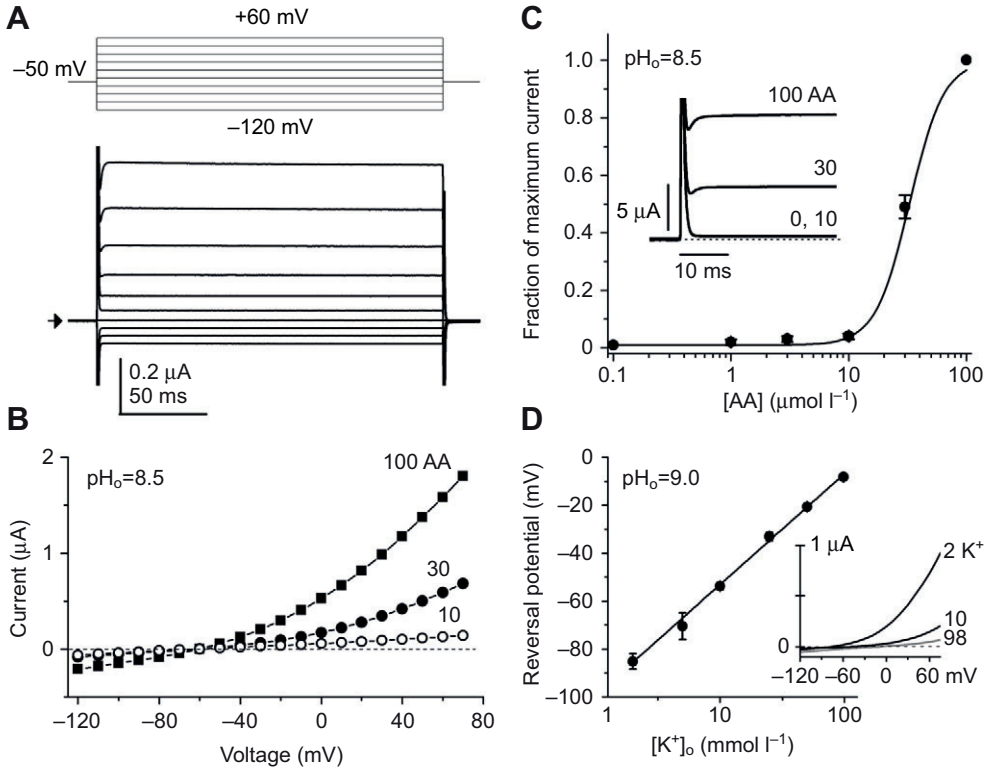

Fig. 3. Biophysical properties of $AquK_{2P}$ channels. (A) Two-electrode voltage clamp (TEVC) $AquK_{2P}$ currents elicited by external application of 30 µmol l^{-1} arachidonic acid (AA) with the indicated voltage protocol; for clarity, only 20 mV increments are shown although the voltage family in the experiment used 10 mV increments (as plotted in B). The whole oocyte capacitative transients at the onset and offset of the voltage step are truncated in this image. The arrow indicates the zero current level. (B) Current–voltage plots for the $AquK_{2P}$ currents elicited by 10, 30 or 100 µmol l^{-1} AA in the same recording as shown in A. Currents were measured at steady-state, at the end of each voltage step. The dashed line indicates the zero current level. (C) Concentration–response data for AA activation of $AquK_{2P}$. Steady-state current amplitudes were measured at +60 mV for a given concentration and were normalized to the current activated by 100 µmol l^{-1} AA in the same cell (means ± s.e.m., N=10). The inset shows the first 30 ms of current from a representative cell; the dashed line indicates zero current level and whole oocyte capacitative transients are truncated. Doses were applied in order of increasing concentration for 5 min each. The solid curve is fitted to the normalized data in Origin v.8 using a logistic equation: $y=[(A_1-A_2)/\{1+([AA]/EC_{50})^b\}]+A_2$, where A_1 and A_2 represent the maximum and minimum responses, respectively, and b is the slope. The fitted EC_{50} was 30.6 µmol l^{-1} with a fitted slope of 4.0. The external recording solution for A–C contained (in mmol l^{-1}): 2 KCl with 98 NaCl and the pH was adjusted to 8.5 with NaOH. (D) A semi-logarithmic plot of the measured reversal potential (zero current level) of $AquK_{2P}$ currents as a function of external K^+ concentration. Cells were tested at pH_o 9.0 with 10 µmol l^{-1} AA and the pH was adjusted with NaOH to avoid changing the K^+ concentration. Data are means ± s.e.m. (N=3–9 cells). The solid line is fitted by linear regression to y=46.4x–99.5. The inset shows current–voltage plots from a representative cell in 2, 10 and 98 mmol l^{-1} K^+; the dashed line indicates the zero current level.

Internal mechanisms of $AquK_{2P}$ channel activation

Using inside-out macropatches, we tested the internal pH (pH_i) sensitivity of $AquK_{2P}$ in the absence of AA. Fig. 5 shows that the channel is regulated by pH_i alone. Activity was very low at pH_i values that are expected in a resting oocyte (~7.4) and was enhanced at alkaline pH_i values. An internal pH of 6.0 largely blocked the current. Although the alkaline-activated currents showed sensitization (see Fig. 6B), the current amplitude eventually reached a plateau level for each pH_i value (Fig. 5A). The alkaline pH_i-activated currents were outwardly rectifying in 2 mmol l^{-1} external K^+ and there was no change in the reversal potential upon activation by pH_i (Fig. 5B). A concentration–response curve for the pH_i sensitivity, determined after alkaline pH_i-activated currents had stabilized and sensitization was no longer apparent, had a pK_a of 8.18±0.05 (Fig. 5C).

In inside-out patches, $AquK_{2P}$ channels were also activated by AA applied directly to the internal surface of the membrane (Fig. 6). The patches were first tested at pH 7.4 to mimic the expected internal pH of the oocyte in the TEVC recordings. The time course of current activation in membrane patches showed a delay upon first application of AA with a much shorter delay upon reapplication of AA (Fig. 6A). The second application of AA followed a reversal of the AA effect by BSA and then washout of BSA from the chamber.

The onset of activation of $AquK_{2P}$ by AA was faster in the membrane patch (Fig. 6A) when compared with the whole cell (Fig. 4A), but the sensitization to a second application of AA was observed both in whole-oocyte recordings with AA applied externally and in excised inside-out patches with AA applied internally. We also tested for sensitization following internal pH-dependent gating in the absence of AA. As shown in Fig. 6B, $AquK_{2P}$ channels became activated in a solution of pH_i 8.5 (time point 1 in Fig. 6B) and were then de-activated by the return to pH_i 6.0. The second and third application of pH_i 8.5 (time points 3 and 4 in Fig. 6B) yielded a faster onset of current and an increase in current amplitude with each application when compared with the first exposure to the alkaline internal solution. The sensitization was not accompanied by any changes in rectification or reversal potential (Fig. 6C). Most cells eventually reached a stable level of activation by alkaline pH_i and, under these conditions, could then be used for the dose–response relationship already presented in Fig. 5C.

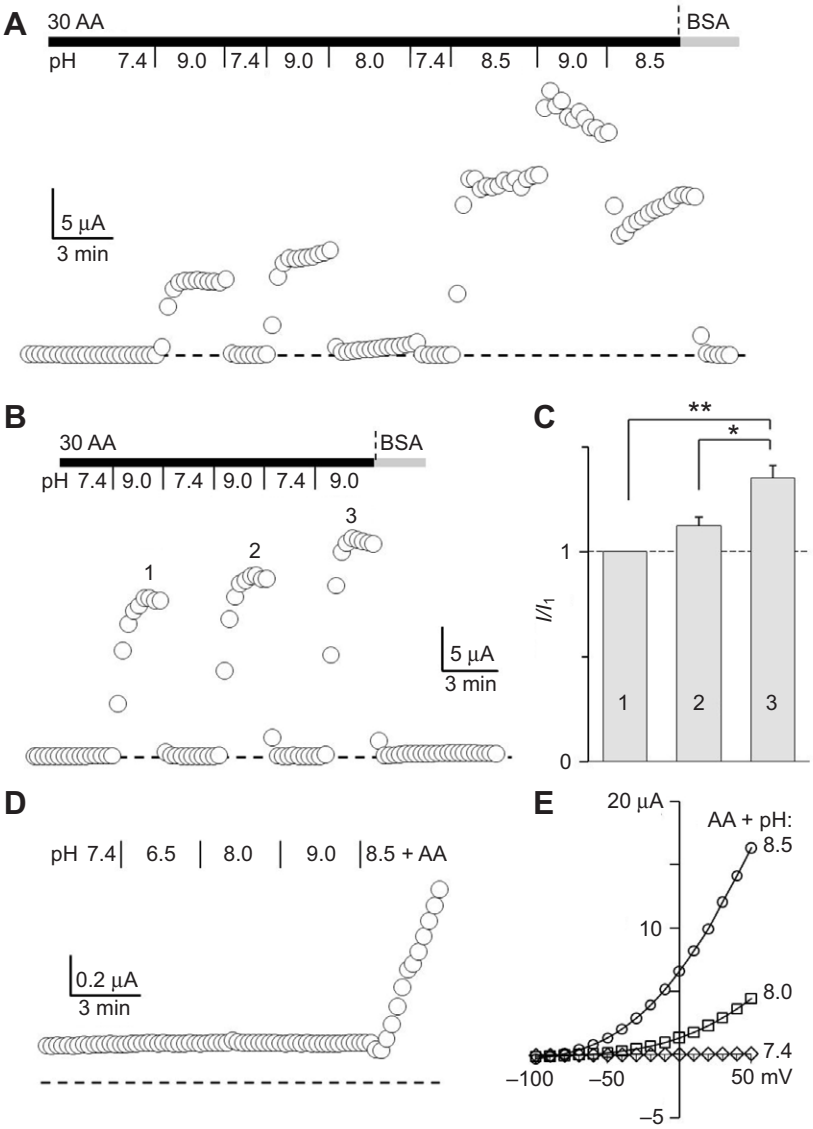

Fig. 4. $AquK_{2P}$ channels are activated by externally applied AA. (A,B,D) Representative time courses from TEVC recordings of $AquK_{2P}$-expressing oocytes. Current amplitudes were measured at a step potential of +60 mV from a holding potential of –50 mV; dashed lines indicate the zero current level. (A) External AA (30 µmol l^{-1}) was applied in recording solutions of the indicated external pH (pH_o) values. BSA (0.5 mg ml^{-1}) at pH_o 8.5 was used to reverse the AA effect. Note that the recording began in the absence of AA at pH_o 7.4. (B) Demonstration of a sensitization of the activation by repeated applications of AA in alkaline pH_o. (C) Composite data showing the current magnitude activated by three successive applications of AA at pH_o 9.0 (means ± s.e.m., N=4). The currents for the last two measurements (2,3) were normalized (I/I_1) to the steady-state level reached with the first (1) application of AA in each cell. Brackets with asterisks indicate values that are significantly different: *P<0.05; **P<0.01. (D) A representative time course to test the effect of external pH alone on current activation. Note the confirmation of channel expression at the end by application of 30 µmol l^{-1} AA in a pH_o 8.5 recording solution. (E) Representative current–voltage plots for 30 µmol l^{-1} AA-activated currents at three different pH_o values; this is a different cell than that shown in A–D. Steady-state currents were measured from voltage steps elicited from a holding potential of –50 mV; all recording solutions contained 2 mmol l^{-1} external K^+. In this recording, the order of application was pH_o 7.4, 8.5 and then 8.0; thus the current elicited at pH_o 8.0 + AA may represent a sensitized response.

Effects of osmotic and thermal stimuli on $AquK_{2P}$

Compared with AA-dependent activation, $AquK_{2P}$ channels were relatively insensitive to mechanical forces within the membrane that were induced by osmotic shrinking or swelling of the oocytes (Fig. 7A,B), which we confirmed with a dissecting microscope. The small changes in $AquK_{2P}$ current coincident with cell swelling (142 mOsm kg^{-1}) or shrinking (400 mOsm kg^{-1}) are not significantly different from each other and are only a small fraction of the current amplitude measured after 5 min of AA (Fig. 7A), a time point we selected for quantitation of the results. The same results were obtained in nine of nine cells (Fig. 7B) in which we always confirmed the ability to activate the sponge K^+ channels by application of external AA (pH_o 9.0, 215 mOsm kg^{-1}) at the end of the experiment, as shown in Fig. 7A. In four additional cells (data not shown), we first reversibly activated $AquK_{2P}$ currents with external AA (pH_o 9.0, 215 mOsm kg^{-1}) and then applied the same osmotic challenges at pH_o 7.4 and 9.0. Prior activation of $AquK_{2P}$ current by AA did not reveal a mechanosensitivity of the sponge K_{2P} channels. To confirm that the brief osmotic challenges were sufficient to modify the activation of channels that are known to be mechanosensitive, we also tested oocytes expressing TREK-1 or TRAAK (Patel et al., 1998; Maingret et al., 1999a; Maingret et al., 1999b; Bang et al., 2000; Lesage et al., 2000; Dedman et al., 2009) and observed activation of the mammalian K_{2P} channels in hypoosmotic solutions and deactivation in hyperosmotic solutions (data not shown). Based on these results, we conclude that $AquK_{2P}$ harbors little or no mechanosensitivity.

$AquK_{2P}$ channels were not sensitive to temperature in the range of 14–32°C at pH 8.5 or 9.0. An example of the failure to activate $AquK_{2P}$ currents with cool or warm temperatures is shown in Fig. 7C; the same observations were made in four of four cells in which we always confirmed expression of the K^+ channels by activation with external AA at alkaline pH_o. For comparison, elevating the temperature from 24°C to 32°C results in a fivefold increase in mammalian TREK-1, TREK-2 and TRAAK currents (Kang et al., 2005).

DISCUSSION

We completed a physiological study of a structurally distinct sponge K_{2P} channel to learn about its mechanisms of activation and to compare it with other members of the K_{2P} channel family. K_{2P} channels are present throughout the animal kingdom, although we

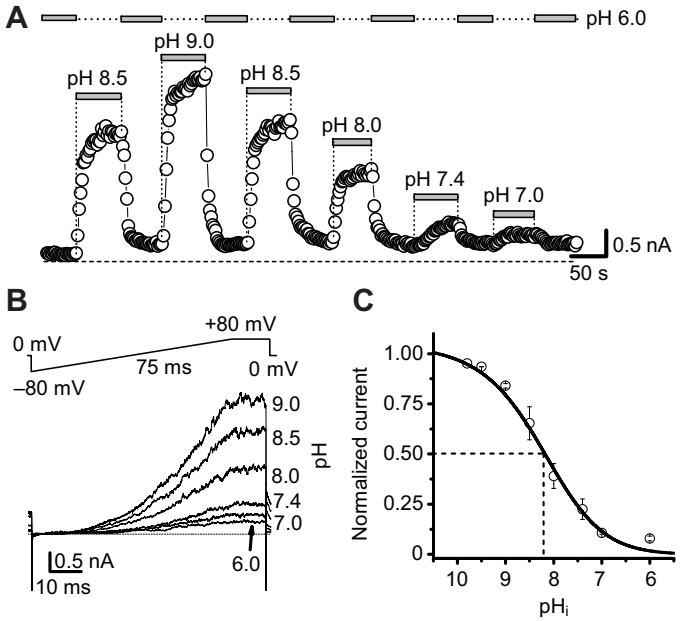

Fig. 5. $AquK_{2P}$ channels are activated by internal alkalinization. (A) Representative time course of inside-out macropatch recordings from oocytes injected with $AquK_{2P}$ RNA. Current amplitudes were measured at +80 mV using a 75 ms voltage ramp from –80 to +80 mV. The dashed line on the current time course indicates the zero current level. The horizontal bars at the top indicate the changes in internal pH (pH_i) values. (B) Representative $AquK_{2P}$ currents evoked by various pH_i values (6.0–9.0). Traces are averages from three runs. (C) A pH_i dose–response curve for normalized $AquK_{2P}$ currents (means ± s.e.m., N=4–6). Data were normalized to the maximum value obtained by fitting with the Hill equation for each patch. The solid line was fitted with the Hill equation $I=1/[1+(pK_a/pH_i)^h]$, where pK_a is the pH_i value at which the current was 50% inhibited and h is the Hill coefficient. The pK_a was determined to be 8.18±0.05 and h=0.75±0.06.

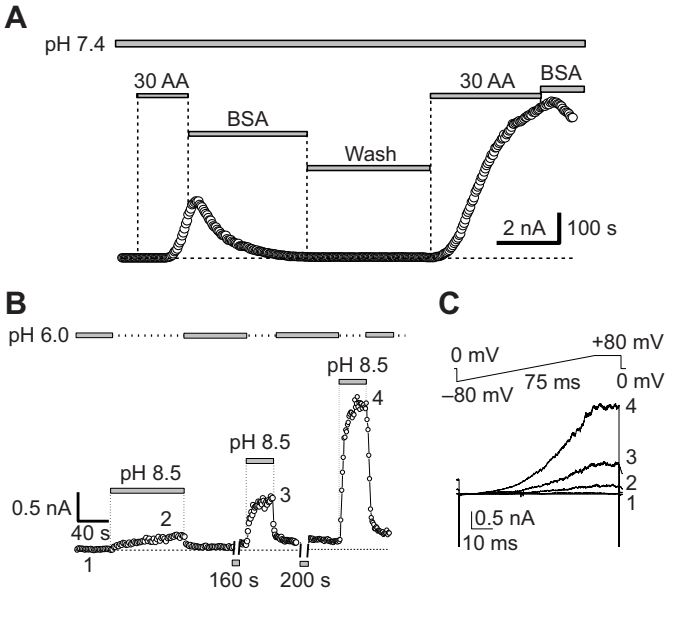

Fig. 6. Activation of $AquK_{2P}$ by internal AA and sensitization of current activation. Representative time courses of inside-out macropatch recordings from oocytes injected with $AquK_{2P}$. Current amplitudes were measured at +80 mV using a 75 ms voltage ramp from –80 to +80 mV. Dashed line indicates the zero current level. (A) Demonstration of the activation of $AquK_{2P}$ by two successive applications of AA (30 μmol l^{-1}) to the internal side of the membrane patch. BSA (0.5 mg ml^{-1}) reversed the AA effect and the wash solution removed the BSA prior to the second application of AA. Horizontal bars above the current data indicate the internal pH value and the time of application of different solutions. (B) Representative time course from inside-out macropatch recordings using repeated applications of pH_i 8.5, all in the absence of AA. (C) Representative $AquK_{2P}$ currents sampled at the time points noted in B (1–4) using the indicated ramp protocol with pH_i=6.0 (trace 1; baseline) and three consecutive applications of pH_i=8.5 (traces 2–4) to the same patch.

know very little about the functional properties of invertebrate and non-mammalian vertebrate channels. Outside the highly conserved TM1–TM4 domains and the two P regions (Fig. 1), $AquK_{2P}$ is considerably different from any known K_{2P} channels and appears to be a distinct class, based on phylogenetic analysis (Fig. 2). The presence of a K_{2P} subunit in choanoflagellates (http://genome.jgi-psf.org/Monbr1/Monbr1.home.html; data not shown) suggests that these channels were present before the advent of multicellularity. Given the important role of K_{2P} channels in epithelia (Davis and Cowley, 2006) and the proposal that epithelial cells were the first specialized cells in the evolution of multicellularity (Adams et al., 2010; Fahey and Degnan, 2010), it is not surprising to find K_{2P} subunits represented in all animal phyla. Presently, functional expression of K_{2P} channels from non-mammalian species is lacking but could be useful in providing greater insight into the evolutionary relationships of these important ion channels.

Like many of the mammalian K_{2P} channels (Bayliss and Barrett, 2008; Enyedi and Czirják, 2010), the sponge channel likely plays a major role in setting the resting membrane potential in some poriferan cells. We found that $AquK_{2P}$ channel activation has a requirement for internal alkaline pH or external alkaline pH plus AA, information that is biologically relevant because seawater has a pH of approximately 8.1 (Orr et al., 2005). Thus, the marine sponge's native environment provides an opportunity for basal activity of this potassium channel *in situ* and the coupling of changes in the cellular membrane potential to small changes in pH and the presence of polyunsaturated fatty acids. Furthermore, the outwardly rectifying nature of the $AquK_{2P}$ currents suggests a possible role in the repolarization of action potentials, which could provide a physiological link between pH, AA and the frequency of firing of calcium-dependent action potentials in sponges (Leys et al., 1999).

AA sensitivity of $AquK_{2P}$

The activation of $AquK_{2P}$ by externally or internally applied AA (Figs 3, 4, 6) shows that the poriferan channel shares a functional feature unique to the TREK/TRAAK subfamily of K_{2P} channels (Fink et al., 1998; Enyedi and Czirják, 2010). Furthermore, the activity of $AquK_{2P}$ does not require additional subunits, which differentiates it from K_{2P}5.1 (Duprat et al., 2007) and the electrically silent members K_{2P}7.1 (Salinas et al., 1999) and K_{2P}12.1 (Rajan et al., 2001). Our results are also consistent with prior work that attributes the structural basis for AA activation of mammalian TREK/TRAAK channels to an internally facing region of the channel (Patel et al., 1998; Kim et al., 2001a; Kim et al., 2001b) because the time course of $AquK_{2P}$ activation was faster when the lipid-soluble AA was applied directly to the internal face of the membrane (Fig. 6) than when it was applied outside the whole cell (Fig. 4). The concentration dependence of $AquK_{2P}$ channel activation by AA, determined with external application of the fatty acid, showed an EC_{50} of ~30 μmol l^{-1} (Fig. 3C). The presence and concentration of endogenous AA in sponges is unknown, but free (non-esterified) AA

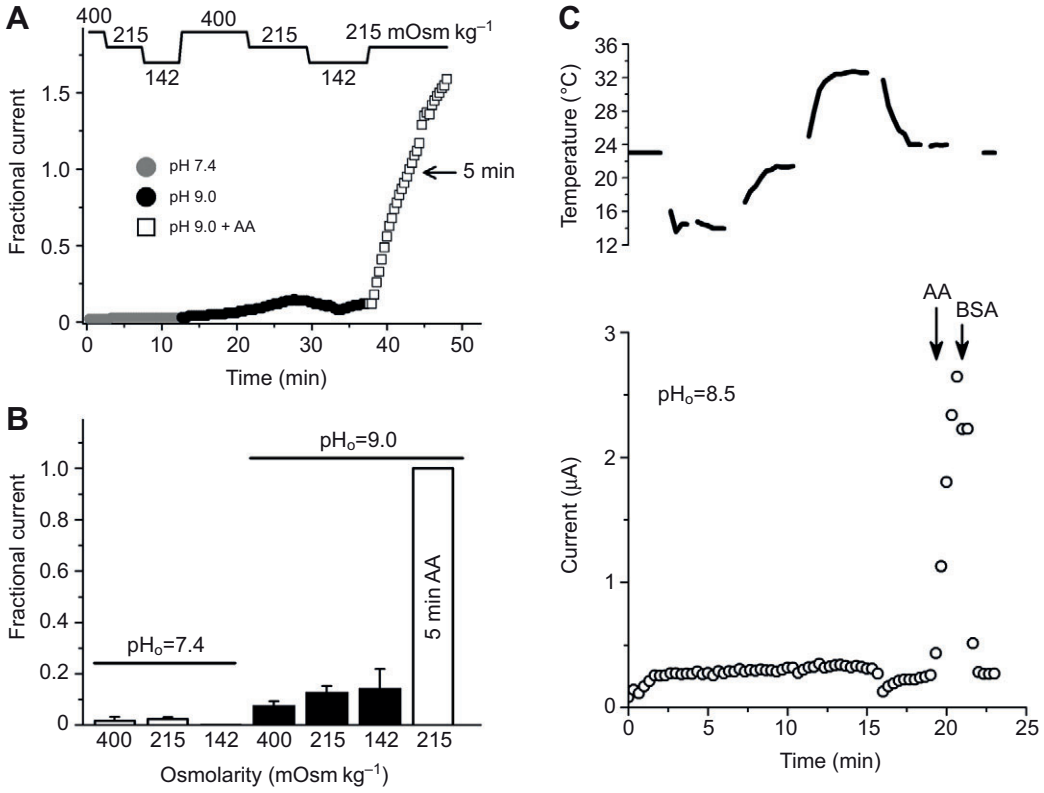

Fig. 7. $AquK_{2P}$ is insensitive to osmotic and thermal stimuli. (A) In a representative cell, the $AquK_{2P}$ outward current at +60 mV was measured and normalized to the current measured 5 min (arrow) after the application of 30 μmol l^{-1} AA at pH_o 9.0 at the end of the experiment. Applications of solutions at pH_o 7.4 or 9.0 and of different osmolarity (142, 215 and 400 mOsm kg^{-1}) are noted on the plot. (B) Bar graph showing composite results (means ± s.e.m., *N*=9) for the effects of osmotic stimuli at pH_o 7.4 or 9.0 in recordings made as in A. (C) Top: temperature changes in the oocyte recording chamber measured during application of a 2 mmol l^{-1} K^+, pH_o 8.5 external solution. Interruptions are times during which the temperature was not recorded. Bottom: the corresponding time dependence of the outward $AquK_{2P}$ K^+ current measured at +50 mV; bath temperature and current are plotted on the same time scale. The arrows denote the brief application of AA (30 μmol l^{-1}) or BSA to activate or reverse the activation of the $AquK_{2P}$ channels, respectively.

in human tissues ranges from 1 to 100 μmol l^{-1} as determined by mass spectrometry (Brash, 2001). Few examples of fitted EC_{50} values for AA activation of mammalian K_{2P} channels exist in the literature, but TREK-2 channels are reported to have an EC_{50} of 7 μmol l^{-1} AA (Bang et al., 2000) and AA facilitates the constitutive activation of THIK-1 channels with an EC_{50} of 1 μmol l^{-1} (Rajan et al., 2001). The extracellular AA concentrations used in the present study were necessary to activate the $AquK_{2P}$ channels and are not unreasonable given the concentrations utilized in other cellular physiology experiments and that be available in biological tissues.

Activating stimuli are more limited for $AquK_{2P}$ than related K_{2P} channels

Under the conditions tested, the AA-dependent activation of $AquK_{2P}$ occurs in the absence of constitutive activation and without significant mechanosensitivity (Fig. 7A,B). We also found no substantial effects of temperature changes on $AquK_{2P}$ current activation (Fig. 7C). Thus, compared with certain polymodal mammalian K_{2P} channels, particularly those stimulated by AA (Patel et al., 1998; Duprat et al., 2007; Lotshaw, 2007; Mathie et al., 2010), the mechanism of activation of $AquK_{2P}$ is more restricted. The internally facing C termini of TREK/TRAAK channels are thought to provide both AA sensitivity and mechanosensitivity (Patel et al., 1998; Kim et al., 2001a; Kim et al., 2001b). Notably, the C-terminal region is structurally divergent among K_{2P} channels and $AquK_{2P}$ has one of the shortest C termini (Fig. 1, from Q303 to E353). The identification in this paper of a K_{2P} channel that shows AA sensitivity but little or no mechanosensitivity could provide useful comparisons for future studies on the unique structural determinants for these two types of stimuli.

Extracellular alkalinization regulates AA-dependent activation of $AquK_{2P}$

Extracellular pH is a common regulator of mammalian K_{2P} channels (Bayliss and Barrett, 2008; Enyedi and Czirják, 2010). For example, extracellular acidification inhibits TREK-1 with a pK_a of 7.35, inhibits TASK-3 with a pK_a of 5.96 (Rajan et al., 2000), and activates TREK-2 with a pK_a of 7.3 (Sandoz et al., 2009). Despite the differences in the pK_a values for proton inhibition or activation, each of these extracellular pH-regulated K_{2P} channel displays a resting K^+ current at standard recording conditions of pH_o 7.4. Because we never observed constitutive activity of $AquK_{2P}$ at pH_o 7.4 and the sponge channel current was only rarely activated by AA at pH_o 7.4, the effect of pH_o on $AquK_{2P}$ can be described as alkaline regulation of channel activation by AA. Although TASK-2 (Morton et al., 2003; Zúñiga et al., 2011) and TALK channels (Bayliss and Barrett, 2008) are also activated by external alkalinization, they do not require a polyunsaturated fatty acid, which differs from the mechanism of pH_o regulation of $AquK_{2P}$ (Fig. 3). The pK_a for external pH regulation of AA-dependent activation of $AquK_{2P}$ is undetermined because of the sensitization to repeated activation of the channels. However, our data suggest that the midpoint of the pH_o sensitivity of the lipid activation is likely to be greater than 8.0. For example, we always observed activation by

AA when pH_o was raised to 8.5, but AA-activated currents at pH_o 8.0 were very small unless tested after an initial sensitizing stimulus. The sponge K_{2P} channel would exist in a favorable environment of ~pH 8.1 in seawater (Orr et al., 2005), in which small changes in pH_o could dynamically regulate channel opening by AA.

Sensitization of the activation of $AquK_{2P}$ by AA in alkaline solutions was prominent in TEVC recordings (Fig. 4) and was present in excised patch recordings in which AA was applied rapidly and directly to the internal side of the membrane (Fig. 6A). Repeated applications of alkaline pH (Fig. 6B) to the inside surface of a membrane patch also induced a sensitized response, although current amplitudes in the macropatch recordings tended to reach a stable amplitude over time (often 5–30 min). Because sensitization eventually stabilized following inside-out patch excision, cytoplasmic agents or cytoskeletal elements that are lost upon excision may contribute to this phenomenon. However, the molecular mechanism for the sensitization is not yet defined.

Intracellular alkalinization and AA are activators of $AquK_{2P}$

$AquK_{2P}$ currents are activated by alkaline internal pH with a pK_a of 8.18 (Fig. 5). The sensitivity to internal pH was measured with an external pH of 7.4, which is non-activating for $AquK_{2P}$. Under these conditions, internal alkalinization is sufficient to open the channels and AA is not required. Mammalian TREK/TRAAK channels are also regulated by internal pH. Internal alkalinization stimulates TRAAK whereas acidification stimulates TREK-1 and TREK-2 (Maingret et al., 1999b; Kim et al., 2001a; Honoré et al., 2002). However, whereas TREK and TRAAK channels demonstrate constitutive activity in whole oocytes studied by TEVC at pH_o 7.4, $AquK_{2P}$ currents were negligible under the same conditions and less than 5% of the cells showed AA-dependent current activation at pH_o 7.4. The heterogeneity of cytosolic pH in intact oocytes and the internal pH sensitivity of $AquK_{2P}$ may explain these observations. Collagenase-treated *Xenopus* oocytes were shown to have a resting cytosolic pH range of 7.06–7.93, with an average pH of 7.43 (Cicirelli et al., 1983). At an average internal pH of 7.4, we would expect 25% of the maximum current activation based on our inside-out patch clamp recordings (Fig. 5C). Thus, we hypothesize that most of the intact oocytes tested in the TEVC recordings in this study had a resting cytosolic pH less than 7.4, which was insufficient to reveal constitutive channel activation. Further investigation will be needed to determine the mechanisms by which internal alkalinization and AA gate the $AquK_{2P}$ channel.

Conclusions

The marine sponge $AquK_{2P}$ channel is AA activated and pH sensitive, properties that are shared by certain mammalian K_{2P} channels and highlight the conservation of basic gating mechanisms for animal K_{2P} channels. A requirement for $AquK_{2P}$ activation by internal alkalinization or the modulatory effects of external alkalinization is also biologically relevant because seawater has a pH of ~8.1. Thus, the native environment of the marine sponge may provide an opportunity for basal activity of this potassium channel *in situ* and the coupling of small changes in pH and the presence of polyunsaturated fatty acids to regulation of the cellular membrane potential and action potential repolarization. Finally, AA-dependent channel activation without osmotically induced activation makes $AquK_{2P}$ unique among K_{2P} channels and provides an opportunity for the future determination of the structural regions of the channel that are necessary for AA sensitivity in the absence of mechanosensitivity.

LIST OF ABBREVIATIONS

$AquK_{2P}$	*Amphimedon queenslandica* K_{2P} channel
AA	arachidonic acid
BSA	bovine serum albumin
K_{2P}	tandem pore domain potassium channel
MES	methansulfonate
pH_i	internal/intracellular pH
pH_o	external/extracellular pH
TEVC	two-electrode voltage clamp

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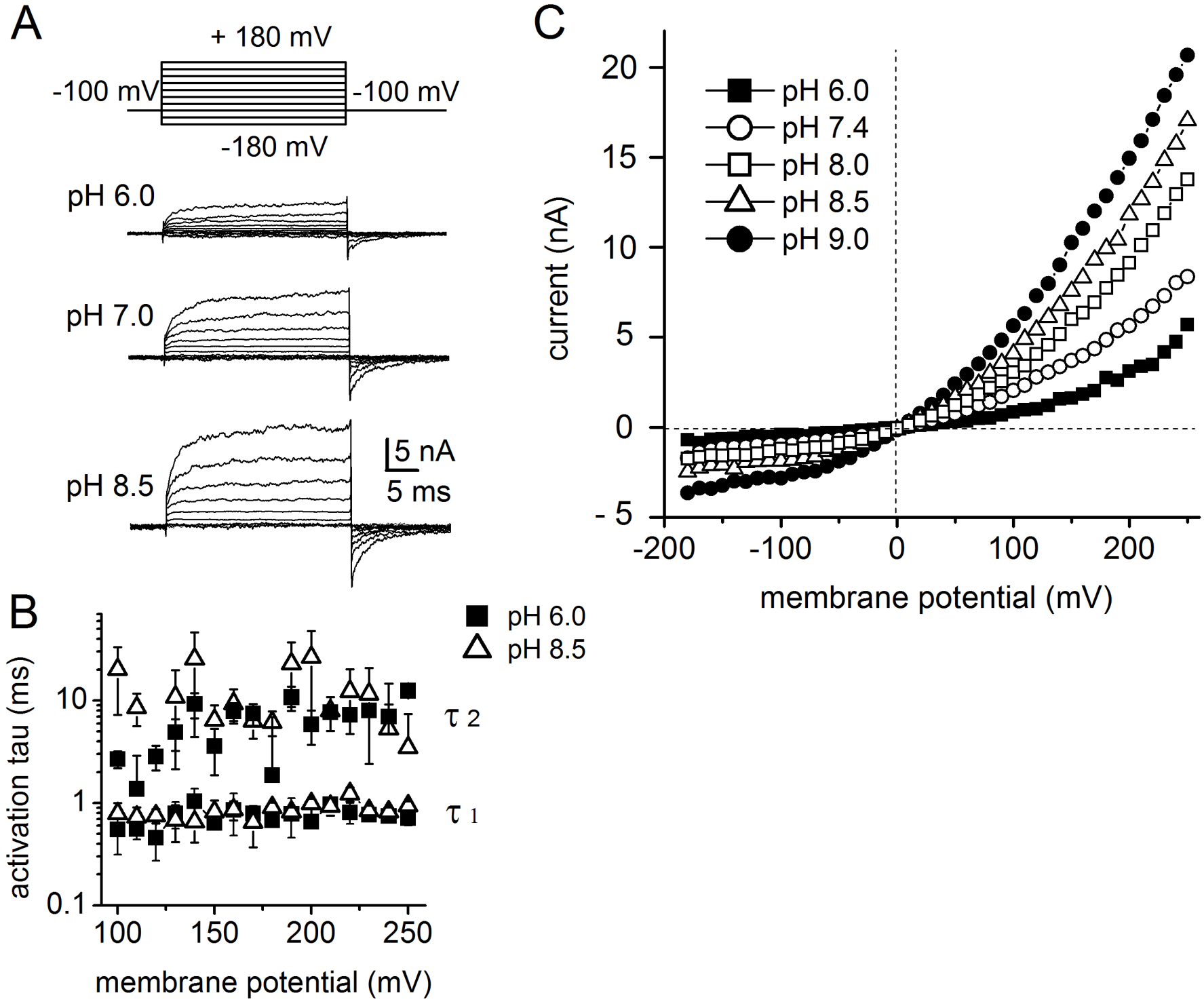
A
+ 180 mV
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-100 mV
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pH 6.0
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pH 8.5
5 nA
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activation tau (ms)
10
1
0.1
100
150
200
250
membrane potential (mV)
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current (nA)
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- 5
-200
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200
membrane potential (mV)
pH 6.0
pH 7.4
pH 8.0
pH 8.5
pH 9.0