Expression of a poriferan potassium channel: insights into the evolution of ion channels in metazoans

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SUMMARY

Ion channels establish and regulate membrane potentials in excitable and non-excitable cells. How functional diversification of ion channels contributed to the evolution of nervous systems may be understood by studying organisms at key positions in the evolution of animal multicellularity. We have carried out the first analysis of ion channels cloned from a marine sponge, *Amphimedon queenslandica*. Phylogenetic comparison of sequences encoding for poriferan inward-rectifier K^+ (Kir) channels suggests that Kir channels from sponges, cnidarians and triploblastic metazoans each arose from a single channel and that duplications arose independently in the different groups. In *Xenopus* oocytes, AmqKirA and AmqKirB produced K^+ currents with strong inward rectification, as seen in the mammalian Kir2 channels, which are found in excitable cells. The pore properties of AmqKir channels demonstrated strong K^+ selectivity and block by Cs^+ and Ba^{2+}. We present an original analysis of sponge ion channel physiology and an examination of the phylogenetic relationships of this channel with other cloned Kir channels.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/212/6/761/DC1

INTRODUCTION

Potassium (K^+) channels are found in cell membranes throughout all domains of life. They are important for essential cellular functions including the regulation of cell volume, growth, membrane excitability and K^+ secretion and recycling. Among the functionally diverse K^+ channels, inwardly rectifying (Kir) channels are unusual in that they preferentially conduct K^+ from the extracellular compartment into the cell. Channel subunits are comprised of two transmembrane domains (M1 and M2) flanking a central pore domain (P), which is highly conserved among the great variety of K^+ channels. Their presence in prokaryotes and a variety of eukaroytic cell types emphasizes their importance to cells. The mechanisms of permeation, selectivity and rectification are known from the structural analysis of K^+ channels in prokaryotes (Doyle et al., 1998; Jiang et al., 2002; Kuo et al., 2003; Nishida et al., 2007) and in mice (Nishida and MacKinnon, 2002). Knowledge of how membrane conduction properties changed during the evolution of multicellularity would be aided by the comparative analysis of K^+ channels from phyla occupying basal positions.

Relationships among the earliest diverging animal groups are still not clearly understood, with recent molecular evidence supporting one of placozoans, ctenophores or sponges as the sister group to all other metazoans (Dellaporta et al., 2006; Dunn et al., 2008; Srivastava et al., 2008). Most phylogenies, however, place sponges (suspension-feeding animals lacking nerves and muscle) as the most ancient multicellular metazoans. Analyses based on 18S rDNA suggest that sponges arose first but are paraphyletic. This suggests that a sponge-like animal gave rise to other metazoans (Borchiellini

et al., 2001; Collins, 1998; Sperling et al., 2007), and that critical metazoan innovations might, therefore, be reflected in modern sponges. Evidence for elements of the postsynaptic scaffold in sponge larvae suggests this might be true (Sakarya et al., 2007). Although sponges lack true nerves, they show many types of non-nervous behavior. Aneural coordination in sponges involves regulation of their feeding current by immediate flagella arrest (Hexactinellida) (Leys and Mackie, 1997) or contraction of canals (Demospongiae, Calcarea) (Elliott and Leys, 2007; Nickel, 2004). In the former, the mechanism is a calcium action potential (Leys et al., 1999; Leys and Eerkes-Medrano, 2006); in the latter it is thought to be by calcium waves triggered by local release of signaling molecules (Ellwanger and Nickel, 2006).

Intracellular recordings have not yet been obtained from any sponge due to the difficulty of penetrating the complex glycocalyx surface coat and the thin nature of cells and tissues lining the surface (Litchfield and Morales, 1976; Muller et al., 1998). Patch-clamp recordings of cation channels have been made from the demosponge *Axinella polypoides* (Carpaneto et al., 2003; Zocchi et al., 2001) but trivalent ions were required to obtain a seal and no electrical conduction was found. Similar difficulty has been encountered in patching glass sponge tissue and, given the extreme difficulty of collecting and maintaining these deep-water animals, an *ex situ* approach is considered to offer a better insight into ion channel function in sponges and the evolution of electrical signaling in this phylum.

In this study, we report the cloning and functional characterization of the first ion channels isolated from a sponge (*Amphimedon*

queenslandica, Demospongiae, Porifera). The newly available genome project of the tropical demosponge *A. queenslandica* (formerly *Reniera* sp.) (http://www.jgi.doe.gov/sequencing/why/3161.html) has facilitated progress in understanding the collection of metazoan homologues present in the earliest animals. We have prioritized the study of inward-rectifier potassium (Kir) channels for their basic structure and their important role both in stabilizing the resting potential and regulating cellular excitability. We present functional data on two clones, AmqKirA and AmqKirB, whose properties were studied in the *Xenopus* oocyte expression system. Sponge Kir channels share the phenotype and critical residues that regulate strong inward rectification with vertebrate Kir2 channels, which are typically expressed in excitable cells including neurons and skeletal and cardiac muscle cells (Doupnik et al., 1995). Phylogenetic comparison with other metazoan Kir channels suggests that Kir channels in sponges, cnidaria and triploblastic metazoans each arose from a single channel and that duplications arose independently in the different groups. Diversification into the channel subfamilies from Kir1 to Kir7 occurred independently in the chordates.

MATERIALS AND METHODS
Isolation of cDNAs and DNA sequencing

Amphimedon queenslandica larvae were procured from Heron Island Reef, Queensland, Australia and preserved in RNA later (Qiagen, Valencia, CA, USA). Total RNA was extracted with TRIZOL® according to Invitrogen specifications. Full-length cDNA was prepared in two ways: with the GeneRacer kit (Invitrogen, Carlsbad, CA, USA) and by rolling circle amplification (RCA) (Polidoros et al., 2006). For RCA, cDNA was synthesized from 5 μg total RNA using Superscript III Reverse Transcriptase Module (Invitrogen) with a 5′-phosphorylated OligodT primer (Ellwanger and Nickel, 2006). Following purification with the QIAquick PCR purification kit (Qiagen), full-length transcripts circularized with 150 units CircLigase enzyme (Epicentre, Madison, WI, USA) in 1× Circligase Buffer with 50 μmol l⁻¹ ATP. The reaction was incubated for 1 h at 60°C, inactivated at 80°C for 10 min and purified as above. Circularized templates were amplified using 10 units of phi29 DNA polymerase in 1× phi29 DNA Polymerase Reaction Buffer, supplemented with 200 μg ml⁻¹ bovine serum albumin, 200 μmol l⁻¹ dNTPs and 10 μmol l⁻¹ random heptamers, which were modified by 3′-phosphothiote linkages to make the primers resistant to phi29 exonuclease activity. Amplification proceeded for 20 h at 30°C and was inactivated by incubation at 70°C for 10 min.

Putative ion channel sequences were assembled from unannotated trace files of the *A. queenslandica* (formerly *Reniera* sp.) genome as previously described (Sakarya et al., 2007). Initial fragments were amplified using specific primers designed against these sequences. The complete cDNA sequences were determined using a combination of inverse PCR on RCA cDNA template (Polidoros et al., 2006) and RACE-PCR on GeneRACE-ready cDNA (Invitrogen GeneRacer kit). Products were cloned into the PCR-4-TOPO (Invitrogen) vector and sequenced. Open reading frames were amplified using primers modified with restriction sites to allow ligation into pXT7 expression plasmid (Dominguez et al., 1995). The full sequence was confirmed from uncloned PCR products from at least two independent PCR reactions, and clones free of PCR errors were selected for expression.

Amino acid sequence and phylogenetic analysis

Sponge inward-rectifier sequences were aligned and compared with amino acid sequences of both invertebrate and vertebrate inward-rectifier sequences compiled from NCBI, Joint Genome Institute

(http://www.jgi.doe.gov/sequencing/why/3161.html) and Ensembl databases. To determine pairwise identity values, AmqKirA and AmqKirB amino acid sequences were each in turn aligned using LALIGN online software (http://www.ch.embnet.org/software/LALIGN_form.html) and default parameters for global alignments. Multiple alignments were generated using MUSCLE (Edgar, 2004). Alignments used for phylogenetic analysis were manually adjusted in MacClade (Maddison and Maddison, 2003) and a PERL script was used to remove characters with more than 2% gaps to produce a trimmed alignment of 122 sequences and 280 positions. All data sets are available on request. The optimal tree topology and Bayesian posterior values were obtained using MrBayes v.3.1.2 (Ronquist and Huelsenbeck, 2003) with the default parameters for amino acid sequences. The same data set was also analyzed with RaxML (Stamatakis, 2006), as implemented on the CIPRES server (http://www.phylo.org/) to obtain maximum likelihood bootstrap values.

Functional expression in oocytes

RNA preparation, oocyte isolation and injection were done using standard methods (Boland et al., 2003). In brief, plasmids containing sponge channel cDNAs were linearized and capped RNAs synthesized *in vitro* using Ambion (Austin, TX, USA) mMessage Machine RNA polymerase kits. RNA was purified using the RNAid kit (Bio 101, Vista, CA, USA) and concentrations were determined by spectrophotometry. Oocytes were surgically harvested from female *Xenopus laevis* (Xenopus I, Dexter, MI, USA) frogs after anesthesia by immersion in buffered 0.1% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St Louis, MO, USA). Oocytes were released and defolliculated by gentle agitation for 1 h or less in 0.5 mg ml⁻¹ collagenase A (Sigma Chemical Co.) dissolved in a Ca²⁺-free solution containing (in mmol l⁻¹): 96 NaCl, 2 KCl, 1 MgCl₂, 5 Hepes, pH 7.4 with NaOH. Oocytes were then washed and stage V/VI oocytes were injected with 50 nl of cRNA dissolved in DEPC-treated water (5–45 ng cRNA/oocyte). Oocytes were maintained at 19°C in a frog Ringer solution of (in mmol l⁻¹): 96 NaCl, 1 KCl, 1 CaCl₂, 2 MgCl₂, 10 Hepes, 5 sucrose and 2 Na pyruvate, pH 7.4 with NaOH with 50 μml⁻¹ penicillin G and 50 μg ml⁻¹ streptomycin. Electrophysiological recordings were done 1–6 days post-injection.

Electrophysiology

K⁺ currents were recorded from oocytes using standard methods of two-electrode voltage clamp (Boland et al., 2003). We used a Geneclamp 500B amplifier (Axon Instruments, Foster City, CA, USA) and an OC-725C amplifier (Warner Instruments, Hamden, CT, USA). Voltage-measuring and current-passing electrodes were backfilled with 3 mol l⁻¹ KCl and had resistances between 0.3–1.0 MΩ. Currents were sampled at 5–10 kHz and filtered at 1–2 kHz. Recordings were done at room temperature (about 23°C) and oocytes were perfused continuously during recordings. Solution composition varied by experiment. The standard, low-chloride, external solution contained (in mmol l⁻¹): 5 K⁺ methanesulfonate (MES), 95 NMDG–MES, 2 CaCl₂, 2 MgCl₂, 10 Hepes, pH 7.3 with methanesulfonic acid. K⁺ concentrations were adjusted by changing the ratios of K⁺ and NMDG-containing solutions using either the MES or chloride salt of K⁺. Other solutions are noted in figure legends. Data were recorded on Pentium computers equipped with Digidata 1320A (Axon Instruments) A/D hardware. Axon's Clampex aquisition and Clampfit analysis software (v. 9) were used. Data were also transferred to Microsoft Excel and Microcal Origin (Northampton, MA, USA) for additional analysis, curve-fitting and

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AmqKirA    MEPAHKNVNYELVSNSNENIHR---DDVP--ITFRGTREARRR-ERLVKRSNGRSLVHHH    54
AmqKirB    MSVQFSN-SYEKISETEGTVREEATDAAPTIVSFQPTVPTRLRPERLVKRSTARSILHHK    59
consensus  M    N YE S        D P    F T   R R ERLVKRS  RS HH

                                         M1
AmqKirA    NIPPLSLLGYISDGFTTVLNARWIVIILLFAAMYVLSWLLFGFIWWGIDSAYVAVTNSSC   114
AmqKirB    NIPPLSWLAYVSDGFTTLINAEWYIIIGLFSAVYLSSWLLFTFMWWSFDAAYVSVTNNSC   119
consensus  NIPPLS L Y SDGFTT  NA W  II LF A Y  SWLLF F WW  D AYV VTN SC

              P                                    M2
AmqKirA    VSNIDGFSASFLFSIETQVTIGYGYRFVADDCSFGILILVIQCLVGLVIDSFLLGLIFAK   174
AmqKirB    IENVGGFSSSFLFSLETQVTIGYGHRYIQSTCHFGIFLLVVQSLIGLFIDSFLLGLIFAK   179
consensus    N  GFS SFLFSL TQVTIGYG R    C FGI  LV Q L GL IDSFLLGLIFAK

AmqKirA    ITRPRNRRKTILFSDTACINVNNKGEKRLQFRIGDVRSRSSLVEAHVRVQLYWNRKDGET   234
AmqKirB    ISRPRNRRKTILFSDIACINVNAKGERCLQFRVADVRKNSSLVEAHVRVQLYWHKKDGAT   239
consensus  I RPRNRRKTILFSD ACINVN KGE  LQFR  DVR  SSLVEAHVRVQLYW  KDG T

AmqKirA    DEYRLEQNDLEVGYDSGTDRIILLTPVVITHIIKETSPLYTVTNDSILNEDIEIVIILEA   294
AmqKirB    DEYRLEQNDLEVGYDSGTDRIILLTPVVITHIIKETSPLYAVTNDSILNEDIEIVIILEA   299
consensus  DEYRLEQNDLEVGYDSGTDRI ILLTPVVITHIIKETSPLY VTNDSILNEDIEIVIILEA

AmqKirA    IVESTGLTAQALWSYTEREILFNYKFKPMIYRQSDAKGTWEVDFNRLSSI---EPCSC   349
AmqKirB    IVESTGLTAQALWSYTEREILFGRKFIPMTNREKTAKGTWEVDFKKLSDVVTSEGTNM   357
consensus  IVESTGLTAQALWSYTEREILF KFIPM R  AKGTWEVDF LS     E
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Fig. 1. LALIGN global alignment of predicted amino acid sequences of two sponge inward-rectifier potassium (Kir) channels, AmqKirA and AmqKirB, cloned from *Amphimedon queenslandica.* Membrane-spanning regions deduced from hydropathy analysis and comparison with vertebrate Kir channels are noted by bars (M1, M2). Putative protein kinase A (PKA) (●) and protein kinase C (PKC) (▲) phosphorylation sites were predicted by two out of three prediction programs used (KinasePhos, PredPhospho and Group-based Phosphorylation Scoring).

the production of figures. Results were calculated from cells with negligible background currents and only for stable cells with membrane potentials not more depolarized than –50 mV in 5 mmol l^{-1} external K$^+$.

RESULTS AND DISCUSSION
Phylogenetic analysis and primary structure

Two inward-rectifier K$^+$ channels, AmqKirA and AmqKirB, were cloned from the demosponge *A. queenslandica.* Analysis of the genome assembly indicated that these are single exon, tandemly duplicated genes. The cDNAs had open reading frames of 1047 and 1071 bp, respectively, corresponding to predicted amino acid sequences of 349 and 357 residues with 68% shared identity (Fig. 1). As with other Kir channels, AmqKirA and AmqKirB each contain two transmembrane helices M1 and M2 flanking a conserved pore structure containing the TXGYG signature sequence for K$^+$ channels. We identified five and four consensus protein kinase A (PKA) phosphorylation sites in the cytoplasmic domains of AmqKirA and AmqKirB, respectively (Fig. 1). Additionally, in AmqKirB, there are two consensus protein kinase C (PKC) phosphorylation sites. Sequence identity for the full-length clones was 29–35% with *Nematostella vectensis* (Cnidaria) and 23–30% with mammalian Kir channels; well below the identity levels found across known Kir subfamilies (50–60% with the exception of Kir7, which shares only 38% identity with other mammalian subfamilies) (Doupnik et al., 1995; Krapivinsky et al., 1998). The channel core (M1, P and M2) had higher amino acid identity with mammalian (38–51%) and cnidarian (48–54%) inward-rectifiers (Table 1; supplementary material Fig. S1).

The phylogenetic relationships between sponge and other metazoan inward-rectifiers are shown in Fig. 2. The overall tree topology shows that Kir diversification occurred independently in sponges, cnidarians, invertebrates and chordates and indicates that a single ancestral gene gave rise to inward-rectifiers in the metazoa.

We rooted the tree between poriferan and cnidarian sequences based on current knowledge of relationships among metazoan phyla (Borchiellini et al., 2001; Collins, 1998; Sperling et al., 2007). Computer-predicted inward-rectifier sequences from the choanoflagellate *Monosiga brevicolis* (gi167515450 and gi167522960) were highly divergent and their inclusion in the analysis destabilized all branches, suggesting that protozoan and metazoan Kir sequences are distinct. Lack of available sequences for ctenophores prevented a test of the recent hypothesis that this group is the sister to other metazoans (Dunn et al., 2008).

The order of branching of vertebrate Kir subfamilies is consistent with the analysis by Tanaka-Kunishima and colleagues (Tanaka-Kunishima et al., 2007). Branching of vertebrate Kir6 after the triploblastic invertebrates (Fig. 2) was also not supported in their analysis. As shown previously for other ion channels (Okamura et al., 2005), we find that diversification of Kir channel subfamilies occurred in vertebrates and that urochortate Kirs lie at the base of this diversification. (A compressed archive of files used to analyze the phylogeny is available from the authors.)

Electrophysiological properties

To study the functional characteristics of AmqKirA and AmqKirB channels, we cloned full-length cDNAs into the pXT7 expression

Table 1. Percentage amino acid identity of AmqKirA and AmqKirB with *Nematostella vectensis* (Nv) and vertebrate Kir channels calculated from global alignments with LALIGN online software (http://www.ch.embnet.org/software/LALIGN_form.html) for the overall channel sequence and the channel core (transmembrane regions M1 and M2 and pore region)

Sequence	% Identity with AmqKirA		% Identity with AmqKirB	
	Overall	Core	Overall	Core
AmqKirA	–	–	67.8	69.2
AmqKirB	67.8	69.2	–	–
Nv gi156375411	35.4	46.8	34.6	46.8
Nv gi156372465	29.2	54.4	29.6	50.6
Nv gi156395183	32.7	50.6	33.5	50.6
Nv gi156387460	32.7	51.9	31.7	48.1
rKir1.1	28.4	44.3	28.7	48.1
mKir2.1	29.5	46.8	28.2	38.0
hKir2.2	30.1	51.3	29.5	46.2
hKir2.3	27.9	46.2	28.1	44.9
hKir2.4	26.9	44.9	25.7	38.5
hKir3.1	23.3	39.7	24.5	42.3
hKir3.2	30.2	47.4	28.9	43.6
hKir3.3	29.1	47.4	27.8	41.0
hKir3.4	29.3	44.9	28.6	44.9
hKir4.1	25.9	38.0	27.1	41.8
hKir4.2	25.3	42.3	26.6	42.3
hKir5.1	24.8	41.0	24.3	37.2
hKir6.1	25.1	43.0	26.6	44.3
hKir6.2	28.7	49.4	28.6	43.0
hKir7.1	25.0	43.6	23.2	39.7

Vertebrate Kir sequences are given for human (hKirx.x), rat (rKir1.1) and mouse (mKir2.1) channels, which were used as controls in our electrophysiology experiments.
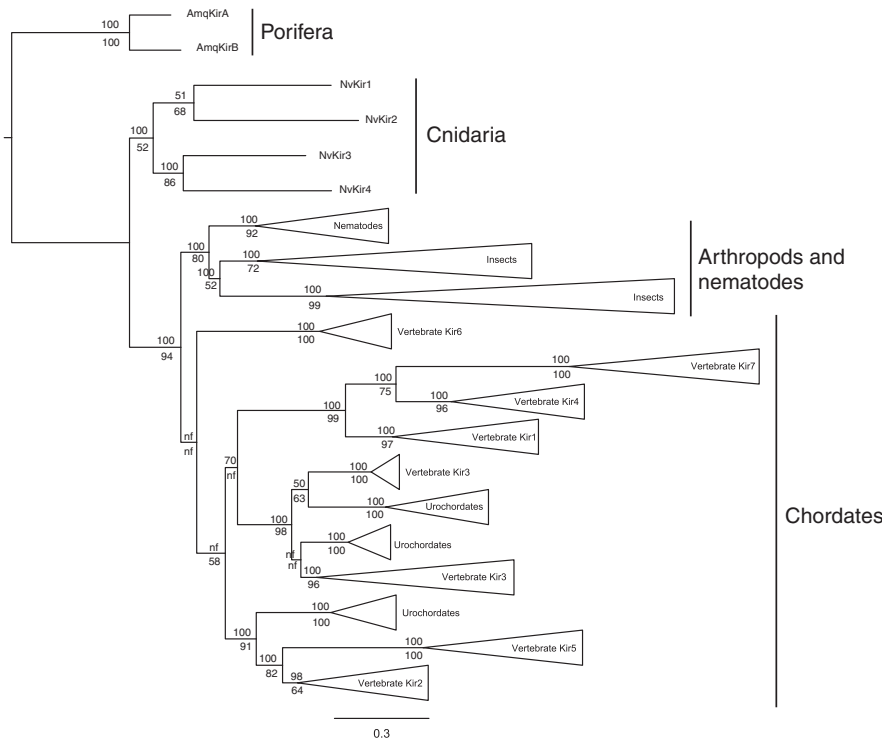
Fig. 2. Phylogenetic relationships between AmqKir channels and other metazoan inward-rectifier potassium (Kir) channels. The phylogenetic relationship was derived as described in Materials and methods. The best Maximum Likelihood tree is shown with Bayesian support values above and RaxML maximum likelihood bootstrap support values below each node. Relationships not recovered in the RaxML analysis are indicated by nf. The tree was simplified by collapsing vertebrate Kir subfamilies from one through to seven and urochordate, arthropod and nematode clades of inward-rectifiers (represented by open triangles). Complete datasets used in analysis are available upon request.
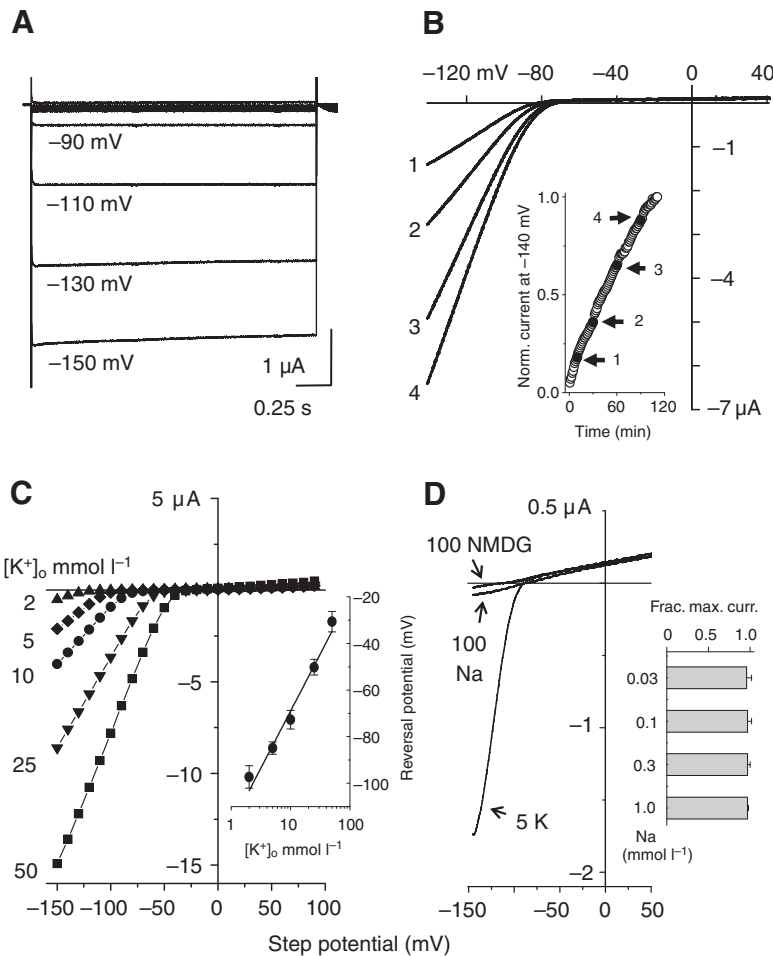
Fig. 3. Heterologous expression of sponge inward-rectifier potassium (Kir) channels. Strongly rectifying currents in $5\,mmol\,l^{-1}$ external K^+ (KMES) recorded from (A) AmqKirA using 2 s voltage steps from −150 to +100 mV in +20 mV increments from a holding potential −50 mV and (B) AmqKirB using a voltage ramp from a holding potential of −50 mV. Current–voltage plots for AmqKirB taken at 10 (#1), 30 (#2), 60 (#3) and 90 (#4) mins. The inset shows, in the same cell, the time-dependent change in the normalized current at −140 mV (until the recording ended). (C) Effect of increasing external K^+ on AmqKirA currents. Solutions of 2, 5, 10, 25 and $50\,mmol\,l^{-1}$ KMES were used. The inset is a semi-log plot of the reversal potential *versus* external K^+ concentration for AmqKirA (N=6–28 per concentration, means ± s.e.m.). The fitted line has a slope of 49 mV. (D) Representative AmqKirA current–voltage relationships recorded in $5\,mmol\,l^{-1}$ K^+, $100\,mmol\,l^{-1}$ NMDG, and $100\,mmol\,l^{-1}$ Na (all used as MES salts) showing no Na or NMDG permeability. The inset shows results of the test of Na (0.03–$1\,mmol\,l^{-1}$ NaCl) block of $5\,mmol\,l^{-1}$ KMES currents (N=5, means ± s.e.m.).

plasmid, prepared cRNAs by *in vitro* transcription and injected purified cRNAs into frog oocytes. From a holding potential of –50 or 0 mV, voltage steps or a ramp protocol were used to change the membrane potential. Oocytes injected with either AmqKirA or AmqKirB cRNAs demonstrated prominent inwardly rectifying currents, which did not inactivate during 2 s voltage pulses. Because we injected oocytes with only a single cRNA species, it is clear that AmqKir channels can exist as homo-multimers. Whether they naturally co-assemble with other Kir subunits is not yet known.

The current–voltage relationships for AmqKirA and AmqKirB are strongly rectifying with large inward currents at membrane potentials more hyperpolarized to the equilibrium potential for K^+ (E_K) and very small currents positive to E_K (Fig. 3A,B). In mammalian cells, this phenotype is determined by pore-lining residues, which facilitate voltage-dependent block by internal Mg^{2+} and polyamines. Mutagenesis of the strongly rectifying mammalian Kir2.1 channels has identified residues that control block by Mg^{2+} and polyamines, including S165 and D172 in the M2 region and E224, D255, D259 and E299 in the C-terminus (for a review, see Lu, 2004). These are largely conserved in AmqKirA and AmqKirB (supplementary material Fig. S1).

Sponge channels were also constitutively active without the addition of activators such as G-proteins or ATP, although we cannot rule out a requirement for an activator that is endogenous to the oocytes. A suggestion for an as yet unknown modulatory mechanism was found for AmqKirB currents, which demonstrated an unusual phenomenon of run-up of current amplitude during recordings. The example (Fig. 3B) shows 5-fold growth of the inward current without a significant change in the reversal potential and without an increase in the outward current over nearly 2 h. Similar results were obtained from all cells injected with AmqKirB although the rate of increase of inward current varied from cell-to-cell. Run-up has been reported for vertebrate Kir2.1 and Kir2.2 currents and may be explained by PKA activity whereas these currents were downregulated by activation of PKC (Fakler et al., 1994; Scherer et al., 2007; Zitron et al., 2004).
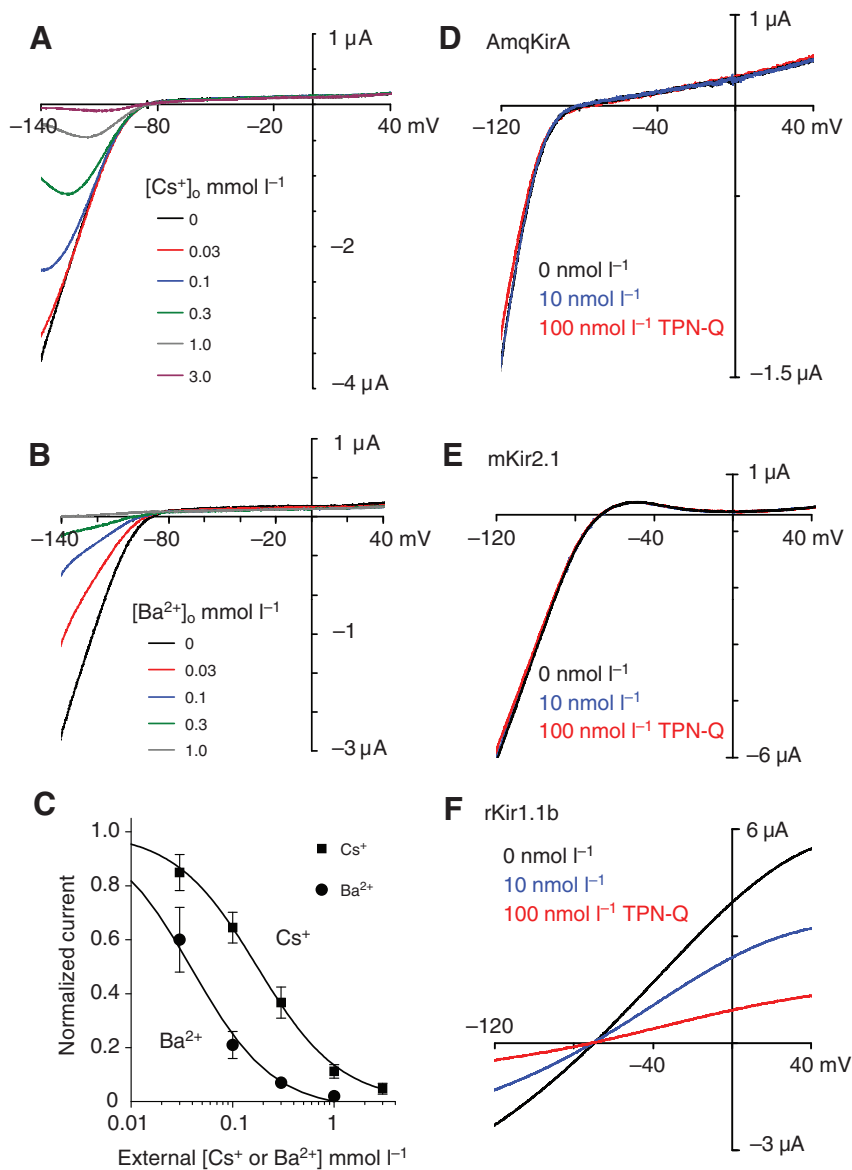

Fig. 4. Test of channel block of AmqKirA. Representative current–voltage relationships in 5 mmol l^{-1} KMES with increasing concentrations of (A) CsCl or (B) BaCl$_2$. (C) The normalized Kir current at –140 mV is plotted *versus* blocking ion concentration. The data were fit by a logistic equation with an IC$_{50}$ of 37 μmol l^{-1} for Ba^{2+} (*N*=5–8) and 173 μmol l^{-1} for Cs$^+$ (*N*=8, means ± s.e.m.). Test for tertiapin-Q (TPN-Q) toxin block of (D) AmqKirA, (E) mKir2.1 (mouse Kir) and (F) rKir1.1b (rat Kir) in the absence (0 nmol l^{-1}) or presence of 10 and 100 nmol l^{-1} TPN-Q. Recordings were done in 5 mmol l^{-1} KMES, pH 7.6. Currents were measured using 500 ms voltage ramps from a holding potential of –50 mV. The block of rKir1.1b was reversible.

Selectivity for K+

Increasing external K^+ concentration, $[K^+]_o$, caused the reversal potentials for AmqKir currents to shift in accordance with the E_K. The current amplitude also increased in a concentration-dependent manner (Fig. 3C). The relationship between reversal potential and $[K^+]_o$ was linear; the fitted slope was 49 mV, close to the theoretical value of 58 for a perfectly K^+ selective channel (Fig. 3C, inset). Selectivity was further tested by perfusing with $5\,mmol\,l^{-1}$ K^+ or solutions containing $100\,mmol\,l^{-1}$ Na^+ or $100\,mmol\,l^{-1}$ NMDG in the absence of K^+ (Fig. 3D). Currents were negligible in the Na^+ or NMDG-containing solutions. This high K^+ selectivity is typical of most K^+ channels (Hille, 2001). In addition, Na^+ $(0.03–1\,mmol\,l^{-1})$ was ineffective as a blocker of K^+ $(5\,mmol\,l^{-1})$ currents (Fig. 3D, inset).

Block by external Cs+ and Ba2+

To further characterize the electrophysiology of AmqKir channels, we tested the effect of Cs^+ and Ba^{2+} (monovalent and divalent cations that block K^+ current in other inward-rectifiers). Both AmqKirA and AmqKirB currents were blocked by Cs^+ and Ba^{2+} (Fig. 4) but we analyzed data for AmqKirA only because continual current run-up (Fig. 3B) precluded accurate measurement of the percentage block in AmqKirB. Oocytes expressing AmqKirA were perfused with solution containing $0.03–3\,mmol\,l^{-1}$ Cs^+ (Fig. 4A) and $0.01–1\,mmol\,l^{-1}$ Ba^{2+} (Fig. 4B), and current inhibition was assessed using voltage step and ramp protocols. Block by Cs^+ showed prominent voltage-dependence in that hyperpolarization intensified the block and depolarization relieved it. Block of AmqKirA currents in $5\,mmol\,l^{-1}$ K^+ was concentration-dependent with an IC_{50} of $37\,\mu mol\,l^{-1}$ for Ba^{2+} and $173\,\mu mol\,l^{-1}$ for Cs^+ when measured at $–140\,mV$ (Fig. 4C).

Test of block by tertiapin-Q

Tertiapin-Q (TPN-Q), a non-oxidizable form of honeybee toxin, has been shown to selectively inhibit a subset of Kir channels and is one of only a few blockers available to discriminate between the contributions of different Kir channels *in situ* (Ramu et al., 2004). We tested the ability of TPN-Q to block AmqKirA, relative to the toxin-sensitive rat Kir1.1b and toxin-insensitive mouse Kir2.1. Because the block of Kir1.1b by TPN-Q is pH-sensitive (Ramu et al., 2004), we performed these experiments at an external pH of 7.6. In our experiments, AmqKirA was insensitive to $100\,nmol\,l^{-1}$ TPN-Q (Fig. 4D) (N=3), similar to mouse Kir2.1 (Fig. 4E) (N=1). Rat Kir1.1b currents, however, were blocked in a concentration-dependent manner, with 80% block at $100\,nmol\,l^{-1}$ (Fig. 4F) (N=2). The structural basis of TPN-Q selectivity is a variable region between M1 and M2. It has been suggested that the variable region in Kir1.1 (11 residues) and Kir3.4 (10 residues) forms an alpha helix that interacts with the helical portion of TPN-Q. Insensitivity of Kir2.1 channels may be explained by a shorter variable region (eight residues) as a complete helical turn may not form (Jin and Lu, 1999; Ramu et al., 2004). The corresponding region in AmqKir channels has only four residues, suggesting that is too short to confer sensitivity.

Concluding remarks

The electrophysiological properties of sponge Kir channels including the absence of substantial outward current at membrane potentials positive to E_K, the high selectivity for K^+ and the voltage-dependent block by Ba^{2+} and Cs^+ indicate that these key features of strong inwardly rectifying K^+ channels are conserved throughout evolution. In mammals, strongly rectifying K^+ channels such as Kir2 channels are typically found in skeletal muscle, cardiac muscle and neurons (Kubo et al., 2005; Oliver et al., 2000). Kir2 channels function in setting the excitation threshold by permitting membrane

depolarization without much K^+ efflux. Cells expressing channels such as the AmqKir channels should be able to maintain a stable resting potential and be able to sustain prolonged depolarizations without massive loss of internal K^+. The strong rectification of AmqKir channels is consistent with the idea that some sponge cell membranes may be specialized for active signaling.

The essential structural regions that control K^+ selectivity have been conserved throughout the metazoa for more than 600 million years of evolution. Differences in the structures of the AmqKir channel and other metazoan Kir channels may impart functional differences not yet identified but clearly do not impact the fundamental properties of ion selectivity, block and rectification. Our results provide insight into the functional properties of the first ion channels cloned from the oldest lineage of metazoans and they shed light on properties that must have been present in the early evolution of multicellular organisms.

LIST OF ABBREVIATIONS

AmqKirA	*Amphimedon queenslandica* inward-rectifier K^+ channel A
AmqKirB	*Amphimedon queenslandica* inward-rectifier K^+ channel B
E_K	equilibrium potential for K^+
Kir	inward-rectifier K^+
$[K^+]_o$	external K^+ concentration
M1	transmembrane domain 1
M2	transmembrane domain 2
P	pore domain
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
TPN-Q	tertiapin-Q

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