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Identification of Putative Potassium Channel Homologues in Pathogenic Protozoa

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Abstract

K+ channels play a vital homeostatic role in cells and abnormal activity of these channels can dramatically alter cell function and survival, suggesting that they might be attractive drug targets in pathogenic organisms. Pathogenic protozoa lead to diseases such as malaria, leishmaniasis, trypanosomiasis and dysentery that are responsible for millions of deaths each year worldwide. The genomes of many protozoan parasites have recently been sequenced, allowing rational design of targeted therapies. We analyzed the genomes of pathogenic protozoa and show the existence within them of genes encoding putative homologues of K+ channels. These protozoan K+ channel homologues represent novel targets for anti-parasitic drugs. Differences in the sequences and diversity of human and parasite proteins may allow pathogen-specific targeting of these K+ channel homologues.

Introduction

Protozoan parasites are major contributors to worldwide disease [1]. They include apicomplexan parasites such as Plasmodium spp. (malaria), Toxoplasma gondii (toxoplasmosis), Cryptosporidium spp. (cryptosporidiosis, diarrhoea) and Babesia bovis (babesiosis), as well as the kinetoplastid parasites Trypanosoma spp. (sleeping sickness, Chagas’ disease) and Leishmania spp. (leishmaniasis). These parasites are together responsible for billions of infections and hundreds of thousands of deaths each year [1,2]. Other protozoan parasites causing widespread disease include Giardia intestinalis (giardiasis), Entamoeba histolytica (dysentery) and Trichomonas vaginalis (trichomoniasis). Current treatments for diseases caused by protozoa are often ineffective or poorly tolerated, and emergence of drug resistance is an imminent threat to their efficacy [3–5]. New therapeutic targets and drugs are therefore needed.

K+ channels are a diverse family of transmembrane proteins, which form K+-selective pores and mediate K+ flux across membranes [6,7]. K+ channels are essential components in a multitude of homeostatic and signalling pathways and are present in animal cells [6], plants [8,9], fungi [10,11] and many bacteria [7,12]. Only a handful of organisms appear to lack K+ channels completely, and most of these are bacteria that are obligate intracellular organelles such as mitochondria [23,24], nuclei [25–28], endosomes [29], endoplasmic reticulum [30], secretory vesicles [31,32] and intracellular vacuoles [33–35]. Physiological roles of K+ flux include setting or altering membrane potentials, effecting osmolyte homeostasis, altering enzyme activity, promoting mitogenesis or apoptosis, and facilitating transmembrane transport processes [6-9,12,36]. Pharmacological or genetic perturbation of K+ channel activity has profound effects on cell function in many organisms, suggesting that parasite homologues of these channels might represent novel drug targets. Consistent with this, disruption of K+ channel function in Plasmodium falciparum and Plasmodium berghei is lethal to these parasites [16,37].

Several subtypes of K+ channel exist, including voltage-gated (Kv), inward rectifier (Kir), two-pore (K2P), calcium-gated (KCa) and cyclic nucleotide-gated (KCNG) channels [6] (Figure 1A). These channels are all formed by a tetrameric arrangement of pore-forming domains, contributed to by each of four monomeric subunits in most channels, except K2P channels which exist as a dimer of subunits, with each subunit containing two domains that contribute to the pore (Figure 1A) [6,18]. The K+-conducting pore region is comprised of a tetrameric arrangement of re-entrant pore loops (P-loops), part of which forms the selectivity filter, together with the following pore-lining transmembrane domains (TMDs) from each subunit, which form the inner pore (Figures 1A and 1B). Diversity of K+ channels is increased by subunit heteromerization and by the association of auxiliary subunits, such as Kvβ, KCNE, KChIP, BKβ, and sulfonlurea (SUR) subunits, which alter the functional properties, trafficking, modulation and pharmacology of K+ channels [19–22].

In many cell types K+ channels are found mainly in the plasma membrane, but they are also found in the membranes of intracellular organelles such as mitochondria [23,24], nuclei [25–28], endosomes [29], endoplasmic reticulum [30], secretory vesicles [31,32] and intracellular vacuoles [33–35]. Physiological roles of K+ flux include setting or altering membrane potentials, effecting osmolyte homeostasis, altering enzyme activity, promoting mitogenesis or apoptosis, and facilitating transmembrane transport processes [6-9,12,36]. Pharmacological or genetic perturbation of K+ channel activity has profound effects on cell function in many organisms, suggesting that parasite homologues of these channels might represent novel drug targets. Consistent with this, disruption of K+ channel function in Plasmodium falciparum and Plasmodium berghei is lethal to these parasites [16,37].

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Recent advances in genomics have resulted in whole-genome sequencing of many pathogenic protozoa [1,38–56]. In this study we examine the genomes of pathogenic protozoa comprehensively, using diverse $K^+$ channel sequences from mammals, plants, fungi, bacteria and archaea, to search for the presence of predicted proteins that may fulfil roles as $K^+$ channels. We show that genes encoding homologues of $K^+$ channels exist in all pathogenic protozoa examined. Sequence divergence of putative protozoan channels from their human counterparts in regions that are known to be important for channel activation, ion conduction or drug binding may result in distinct pharmacological profiles. These parasite channels may therefore represent novel targets for anti-parasitic therapy.

**Results**

**Identification and classification of $K^+$ channel homologues**

The defining feature of $K^+$ channels is their selectivity for $K^+$ ions, which is conferred by residues within the selectivity filter region of the pore [57] (Figure 1). Diverse mammalian $K^+$ channels show sequence similarity in the selectivity filter region, with a core selectivity filter motif of $\text{XXGXXG}$, most commonly $\text{TXGYGD}$ [58]. $K^+$ selectivity is known to be tolerant of some sequence variation in this selectivity filter motif [59] as well as in the outer and inner pore regions, and such variation exists between channel subtypes [58]. For example, selectivity filter sequences of $K^+$-selective channels include $\text{TGYGF}$ (eg. $K_\text{v}2.1$, $K_\text{v}2.3$), $\text{TIGYG}$ (eg. $K_\text{v}2.2$), $\text{XXGFG}$ (eg. $K_\text{v}6.2$, ERG, EAG, mouse $K_{\text{Ca}1.1}$), and $\text{XXGLGD}$ (eg. some $K_{\text{Ca}}$) [58]. We therefore searched parasite genomes using diverse $K^+$ channel sequences from humans, plants, fungi, bacteria and archaea (see Methods), which together cover most known $K^+$-selective pore sequences. We identified predicted protein products in the genomes of pathogenic protozoa, which display significant sequence similarity to $K^+$ channels in the pore region, including the selectivity filter (Table 1 and Figure 2). These proteins also satisfy other criteria for defining them as putative $K^+$ channel homologues, such as the presence of multiple TMDs (see Methods). These homologues may therefore function as $K^+$-selective channels in protozoan parasites. Homologues were classified according to the family of human $K^+$ channel to which they showed greatest sequence similarity, and according to the presence of conserved functional domains (Figure 1A) such as putative voltage sensors, $\text{Ca}^{2+}$-...
Table 1. Identity of K+ channel homologues in pathogenic protozoa.

<table>
<thead>
<tr>
<th>Parasite</th>
<th>Kv or KCNα*</th>
<th>KCa</th>
<th>Kv</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>XP_001350669 (12) (Kv)</td>
<td>XP_001348796 (10)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Plasmodium knowlesi</em></td>
<td>XP_002262343 (13) (Kv)</td>
<td>XP_002260211 (8)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Plasmodium vivax</em></td>
<td>XP_001617360 (10) (Kv)</td>
<td>XP_001615733 (8)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>XP_002365940 (8) (Kv)</td>
<td>XP_002366551 (10)</td>
<td>XP_002369151 (4)</td>
</tr>
<tr>
<td><em>Cryptosporidium hominis</em></td>
<td>NF</td>
<td>XP_668687 (8)</td>
<td>XP_666498 (2)</td>
</tr>
<tr>
<td><em>Cryptosporidium muris</em></td>
<td>XP_002140632 (10)(Kv)</td>
<td>XP_002140632 (10)</td>
<td>XP_002140833 (2)</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>NF</td>
<td>XP_626777 (8)</td>
<td>XP_626299 (2)</td>
</tr>
<tr>
<td><em>Babesia bovis</em></td>
<td>NF</td>
<td>XP_001609692 (2)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Giardia intestinalis</em></td>
<td>NF</td>
<td>EFO63588 (9)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>NF</td>
<td>XP_655083 (6)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Leishmania major</em></td>
<td>NF</td>
<td>XP_001687475 (8)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Leishmania infantum</em></td>
<td>NF</td>
<td>XP_001462697 (7)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Leishmania braziliensis</em></td>
<td>NF</td>
<td>XP_001561516 (6)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>NF</td>
<td>EAN76555 (7)</td>
<td>NF</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>XP_821941 (8)</td>
<td>NF</td>
<td></td>
</tr>
<tr>
<td><em>Trichomonas vaginalis</em></td>
<td>XP_001324404 (6)*</td>
<td>NF</td>
<td></td>
</tr>
</tbody>
</table>

Protein accession numbers are shown and NF denotes that no homologues were found. Number of predicted TMDs is indicated in parentheses. K+ channel homologues are classified on the basis of closest similarity to a particular subtype of human K+ channel subunit and according to the presence of characteristic functional domains, such as a charged TMD4 (Kv), the presence of a cyclic nucleotide-binding domain CNBD (KCNα), or the presence of RCK domains (KCa). Where a protein showed similarity to more than one class of K+ channel, its accession number is shown in both relevant columns (eg. all Kv homologues are also KCa homologues). *KCNα homologues which contain a CNBD, but also a charged TMD4;
sensing regulator of conductance (RCK) domains of KCa channels [60–63], calmodulin (CaM)-binding domains (CaMBDs) [60,64], or cyclic nucleotide-binding domains (CNBDs) [65] (Table 1 and Figure 2). The P. falciparum proteins XP_001609692 and XP_001350669 are identical to the previously described PfKch2 and PfKch1 proteins respectively [14,16]. The S. bovis (XP_001610013) and C. hominis (XP_668687) proteins have been identified previously as orthologues of PfKch1 in P. berghei [16]. In addition to the K+ channel homologues shown, homologues of putative adenylyl cyclase/K+ channel fusion proteins [123,124] that contain GXG motifs after their TMD6 domains were also identified in P. falciparum (XP_001348216), P. knowlesi (XP_002260946), P. vivax (XP_00161004), T. gondii (XP_00368352) and XP_002370938 and XP_002367966, C. muris (XP_002140763), C. hominis (XP_668631) and C. parvum (XP_626352). Homologues of these proteins were absent in all other parasites examined.

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Table 1. Cont.

<table>
<thead>
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<th>K+ channel homologues</th>
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| The genomes of Plasmodium spp., T. gondii, Cryptosporidium muris and T. vaginalis contain genes encoding homologues of K+ channels, with TMD4 regions that contain a number of regularly spaced basic residues, similar to the S4 voltage sensor sequences of K+ channels [60] (Table 1, Figure 1 and Figure 3). The apicomplexan K+ homologues also show sequence similarity to the C-terminal tails of KCa1 channels, including the RCK domains that bind Ca2+ or other ions [61,62,86] (data not shown). This suggests that these parasite homologues may be dually modulated by voltage and ions such as Ca2+, Mg2+ or H+. In contrast, K+ homologues in T. vaginalis all contain conserved CNBDs in their C-terminal tails (see later), suggesting that they, like mammalian hyperpolarization-activated cyclic nucleotide-gated non-selective (HCN) channels [65], may be dually modulated by voltage and cyclic nucleotides such as cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP).

KCa channel homologues |
| The most common genes encoding K+ channel homologues in protozoan parasites are those encoding KCa4 channel homologues (Table 1 and Figure 2). This is consistent with the presence of complex Ca2+-signalling machinery in protozoa [2,9,88]. Known KCa3 channels form two main families. Proteins in one family are activated by Ca2+ (and in some cases by other ions such as Mg2+, H+ and Na+) via direct binding to domains within the channel, including the C-terminal RCK domains of KCa1 channels [60–63] (Figure 1A). Proteins in the second family, which includes KCa2 and KCa3 channels, are activated by Ca2+ via binding of their C-terminal tails to the accessory Ca2+-binding protein CaM [60,64] (Figure 1A). Many of the protozoan KCa4 channel homologues show sequence similarity to the C-terminal tails of KCa1 or KCa2/3 channels, including the RCK domains and CaMBDs (data not shown), suggesting that their activity may be regulated by Ca2+, or by other ions such as Mg2+, H+ or Na+. |
Figure 2. Multiple sequence alignment of protozoan K⁺ channel homologues with the pores of mammalian K⁺ channels. Predicted pore-lining TMD regions are underlined. The GXG motif of human K⁺ channels is shaded in grey. Total number of residues in each protein is indicated in parentheses to the right of each sequence. L. braz. denotes L. braziliensis, and G. int. denotes G. intestinalis. The proteins XP_001609692 and XP_001350669 encoded by the P. falciparum genome are identical to the previously described PfKch2 and PfKch1 proteins respectively [14,16]. The proteins XP_001610013 and XP_0066867 are identical to previously identified K⁺ channel homologues in B. bovis and C. cryptosporidium hominis respectively [16]. The proteins labelled GYX have GYRD, GYSD or GYSE-containing selectivity filter regions, suggesting a lack of K⁺ selectivity or function.

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We were most interested in the multiple KCa channel homologues present in Leishmania parasites and chose to interrogate these homologues further (Figure 4). Based on full-length alignments and phylogenetic relationships (Figure 4A), KCa homologues in Leishmania parasites could be grouped into KCa1-like proteins (which showed similarity to the discontinuous RCK domains of hKCa1.1, data not shown) and KCa2/3-like proteins which had greater similarity to KCa2/3 channels. The latter group contain homologues with canonical GYG-containing putative selectivity filter regions, as well as homologues with GYX-containing selectivity filters (Figure 4A and Figure 2). These KCa2/3-like proteins have consensus IQ-motifs in their C-terminal tails, shortly after the final TMD, which are predicted to form CaMBDs (shown in Figure 4B for the GYG-containing homologues). These predicted CaMBDs overlap closely with the well-characterized CaMBD of KCa2.2 (Figure 4B) and share sequence identity at several loci that are critical for binding of Ca2+ to human KCa2.2 (Figure 4B, open triangles) [64]. This suggests that these parasite homologues may form KCa channels whose activity is controlled by Ca2+ acting via bound CaM, similar to human KCa2.2 channels. The KCa homologues in Leishmania (as well as the other protozoa examined) show considerable sequence divergence from human KCa2.2 channels in the P-loop and selectivity filter, including at positions implicated in the binding of isoform-specific drugs and toxins to KCa2.2 channels (filled triangles in Figure 4B) [78]. These homologues also differ markedly from human KCa2 channels in the predicted TMD3-TMD4 linker region (data not shown), which is also an important determinant of drug binding in KCa2 channels [89]. This suggests that these parasite KCa homologues may exhibit unique pharmacological profiles.

KCNQ homologues. Several genes encoding K+ channel homologues with conserved CNBDs (identified by searches of the Conserved Domains Database, NCBI) are present in T. vaginalis, but are absent from all other protozoa examined (Table 1 and Figure 5). These putative K+ channel homologues exhibit sequence similarity to the CNBD of the well-characterized HCN2 channel that is activated by cAMP and cGMP, including some conserved residues involved in binding of cyclic nucleotides [65] (Figures 5A, 5B and 5C). Predicted secondary structure of the CNBD domain in HCN2 (Figure 5A) closely matched secondary structure in the crystal structure of HCN2 [65] (Figure 5B), suggesting that secondary structure of the parasite proteins could also be predicted accurately. Predicted secondary structure of the putative CNBDs of protozoan KCNG channel homologues in most cases closely matched that of HCN2 (Figures 5A and 5B), suggesting that the structure of these domains is also conserved in human HCN2 and T. vaginalis KCNG homologues. One exception was the C-terminal end of this domain in the GYX protein XP_001325751 (which as discussed earlier may not form functional or K+ selective channels), which lacked entirely the final alpha-helical stretch of HCN2 termed the C-helix, which is involved in binding and efficacy of cyclic nucleotides in HCN2 [90] (Figure 5A and Figure 5B). Hence most of the protozoan KCNG homologues identified here may contain conserved CNBDs that bind cyclic nucleotides. The cyclic nucleotide selectivity of binding and efficacy in these putative channels cannot at present be predicted on the basis of sequence alone, and will require experimental testing. Unlike the non-selective mammalian HCN and CNG channels, but similar to prokaryotic KCNG channels [7], these T. vaginalis proteins contain canonical K+ channel selectivity filter motifs (T/AAXGYGD), suggesting that they may be K+ selective. We also searched parasite genomes with sequences of the K+ permeable (but non-selective) mammalian cyclic nucleotide-gated non-selective cation (CNG) channels and hyperpolarization-activated cyclic nucleotide-gated non-selective cation (HCN) channels, but no additional homologues were found.

KIR channel homologues

Among the protozoan genomes examined, genes encoding KIR channel homologues are found only in the genomes of Cryptosporidium spp. and T. gondii (Table 1 and Figure 2). KIR channels are widespread among many organisms, and many subtypes exist that are differentially regulated by diverse stimuli including adenosine triphosphate (ATP), G-protein activation, phospholipids, and divalent cations [71]. As discussed earlier, the predicted pore region of the protozoan KIR channel homologues shows some differences to human KIR channels, which may confer unique characteristics on these homologues. The KIR homologue in T. gondii shows most similarity to human ATP-modulated K6.2 and G-protein activated K3.3 channels (data not shown), suggesting that cytosolic ATP or G-proteins within parasites may regulate this
homologue. KCa homologues in Cryptosporidium spp. were also most similar to human ATP-modulated KCa6, as well as KCa2 channels [data not shown], suggesting that these homologues might also be regulated by cytosolic ATP within parasites.

KCa channel homologues

We used the sequences of human and yeast KCa channels to search for predicted proteins with both sequence similarity to KCa channels [19, 91] and at least four predicted TMDs in two distinct regions, each with credible potential as selectivity filter regions. Using these criteria, we found no evidence for genes encoding homologues of KCa channels in the protozoan genomes examined. Although the KCa homologue in T. gondii contained four predicted TMDs in two regions, only the second pair of TMDs had an intervening sequence with similarity to canonical selectivity filters [data not shown].

Protozoan parasites lack homologues of K+ channel auxiliary subunits.

Many K+ channels are associated with auxiliary proteins that can change their biophysical properties, localization or regulation by cellular signalling pathways [19, 20]. These include the KCNE, KChIP and Kβ subunits that alter KC channel activity and localization [20, 21]. Other auxiliary subunits include the BKβ family of subunits that alter BK channel activity and pharmacology [20], and the sulfonylurea (SUR) subunits that are responsible for the nucleotide-diphosphate sensitivity and pharmacological profile of KC6 channel complexes [92, 93]. We searched the genomes of protozoan pathogens for genes encoding predicted proteins with similarity to these auxiliary subunits, but none were found. This suggests that the protozoan parasites examined here lack conventional auxiliary subunits of K+ channels.

Discussion

All protozoan genomes examined contain genes encoding K+ channel homologues (Table 1), suggesting that these putative channels have widespread and conserved physiological functions in these organisms. Many of these putative K+ channels are not yet annotated in available pathogen databases (http://eupathdb.org/ eupathdb) [94]. Genes encoding homologues of most of the major families of K+ channel (Kv, KCa, Kβ, and KUNG channels) are present in protozoan genomes, although genes encoding homologues of KCa channels appear to be absent. Experimental studies will be required to confirm the expression and function in parasites of these putative K+ channel homologues.

In mammalian cells, most plasma membrane K+ channels mediate K+ efflux, due to the common occurrence of large transmembrane K+ concentration gradients and relatively depolarized membrane potentials. Outward rectification or depolarization-induced activation of the channels themselves also contributes to selective efflux of K+ in many cases. In contrast, some plasma membrane K+ channels are capable of mediating K+ influx [95, 96], due to unusual K+ concentration gradients or membrane potentials. Inward rectification [71] and hyperpolarization-induced activation [97–99] also facilitate selective K+ influx in some cases. Protozoan parasites in many cases spend part of their lifecycle within cells and part in an extracellular environment. The K+ concentrations of these environments, a mammalian host differ by ~40-fold (4 mM extracellular K+ versus 155 mM intracellular K+) [100]. Depending on the lifecycle stage, the concentration gradient for K+ flux across the parasite plasma membrane may therefore differ considerably. In this context it is interesting that Plasmodium parasites induce alterations of the Na+/K+ ratio in host cells and thereby reduce intracellular K+ concentration [101–104]. Whether other parasites also exert similar effects on host cells is unknown. Whether influx of K+ occurs through K+ channel homologues in the plasma membrane of intracellularly located parasites, and whether this might be exploited therapeutically (see later) remains to be explored.

Protozoan KCa channel homologues may be regulated by transmembrane voltage. Most KCa channels are activated by depolarization [60], while a few are activated by hyperpolarization [98, 99]. However, known depolarization-activated and hyperpolarization-activated channels show similar voltage sensor sequences [105], making it difficult to determine the polarity of voltage dependence on the basis of sequence alone. Further experimental studies will therefore be required to define the properties of these homologues. The vast majority of mammalian KCa channels are present and functional in the plasma membrane, which experiences the most substantial changes in transmembrane potential. Protozoan KCa channel homologues therefore seem likely to reside within the plasma membrane of these organisms, although this will require experimental testing. Protozoan KCa channels may also be expressed in the membranes of their host cells. For example, in the case of P. falciparum, the PfKch1 channel is located in the plasma membrane of the host erythrocyte, while PfKch2 is mainly located in the parasite [15]. The presence of putative KCa channel homologues in protozoa suggests that these organisms may experience dynamic physiological changes in membrane potential. The plasma membrane potentials of some protozoa have been estimated. For example, the intraerythrocytic form of P. falciparum has an estimated membrane potential of ~95 mV, which is contributed to by K+ flux [106]. Similarly, the plasma membrane potential of the bloodstream form of T. brucei has been measured as ~82 mV and is mainly due to K+ flux [107]. In addition, the membrane potential of Leishmania donovani amastigotes has been measured as between ~90 and ~113 mV and is also contributed to by K+ flux [108]. However, whether the membrane potentials of protozoan parasites change between stages of the lifecycle or in response to environmental stimuli, and whether the KCa channel homologues identified here respond to such changes is unknown.

Genes encoding KCa channel homologues are present in all protozoa except T. vaginalis. The KCa2/3 channel homologues in many cases contain consensus CaMDBs [65], while the KCa1 homologues show similarity to the discontinuous Ca2+-binding RCK domains of KCa1 channels [60–63]. Searches of parasite genomes using the sequence of human CaM showed that genes
Figure 5. K⁺ channel homologues in *T. vaginalis* contain domains similar to mammalian cyclic nucleotide-binding domains. (A) Multiple sequence alignment of the C-terminal CNBD of human HCN2 (residues 516–668) with the putative CNBD-containing regions of protozoan KCNG homologues. The boundary between the C-linker and CNBD of HCN2, as well as the C-helix of the CNBD [65], are indicated. Residues of HCN2 that are shaded in yellow are those known to be directly involved in binding cNMP [65,90,148]. Asterisks below the alignment indicate absolutely conserved residues, while colons indicate conservation of physicochemical properties (ClustalW2). Predicted secondary structure was determined using SABLE (http://sable.cchmc.org) [144] and indicated by red underline (predicted alpha helical) or black underline (predicted beta-sheet). (B) Crystal structure of the CNBD of mouse HCN2 in complex with cAMP (a fragment of PDB accession number 1Q5O) [65]. Only the region encompassing the residues analogous to those of hHCN2 in the alignment in Figure 5A are shown (residues 490–641 of mHCN2, equivalent to residues 516–668 of hHCN2). Bound cAMP is shown in yellow, and side-chains of some key residues important for cAMP binding [90] are shown in red (E582, R591 and R632 of mouse HCN2, equivalent to E609, R618 and R659 respectively of hHCN2 – labelled with filled triangles in Figure 5A); (C) A representation of the coordination of cAMP by specific residues within the CNBD of mHCN2, made using LIGPLOT v4.5.3 [149]. Labels of mHCN2 residues interacting with cAMP that are conserved in parasite KCNG homologues are shown in magenta boxes.

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Potassium Channels in Pathogenic Protozoa

Genes encoding homologues of \( K^+ \) channel auxiliary subunits are absent in the genomes of the protozoa examined. This suggests that functional \( K^+ \) channel complexes in these organisms may lack the diversity of human \( K^+ \) channel complexes. In addition, since auxiliary subunits can dictate the pharmacology of native \( K^+ \) channel complexes in mammalian cells [20,92,93], the pharmacology of native \( K^+ \) channel complexes in parasites may differ substantially from those of humans. It is also possible that unique and as yet unidentified auxiliary subunits exist in protozoa, which are unrelated to currently known auxiliary subunits of \( K^+ \) channels in other organisms.

**\( K^+ \) channels in parasitic protozoa and their free-living relatives**

In contrast to the relatively few genes encoding \( K^+ \) channel subunit homologues in parasitic protozoa, the genome of their free-living ciliate relative *Paramecium tetraurelia* has several hundred genes encoding \( K^+ \) channel subunits [13]. Whether this dramatic difference arose due to the acquisition of a parasitic existence in some protozoa is unclear.

BLAST searches using the sequences of KcsA as well as human \( K_{1.2}, K_{2.1} \) and \( K_{Ca1.1} \) proteins suggest that the genome of the free-living flagellate *Monosiga brevicollis* encodes at least nine distinct \( K^+ \) channel subunit homologues (data not shown), compared with the smaller number in kinetoplastid parasites (eg. only two in *T. brucei*). Whether the fewer \( K^+ \) channel homologues in the flagellate parasites examined here is due to a parasitic existence is unclear.

**Protozoan \( K^+ \) channels as therapeutic targets**

\( K^+ \) channels are critical for cellular homeostasis and signal transduction, and pharmacological modulation of these channels can lead to marked changes in cell growth and viability [36,77,126,127]. Disrupting \( K^+ \) channel function in *P. falciparum* and *P. berghei* severely compromises survival of these parasites [16,37], suggesting that parasite \( K^+ \) channels may be attractive drug targets for treatment of parasitic disease. Interestingly, genetic disruption of \( K^+ \) channel function affects different life cycle stages in *P. falciparum* and *P. berghei*. In *P. falciparum* the asexual blood stage is affected [15], while in *P. berghei* the sexual mosquito stage is severely affected but the asexual stage is relatively unaffected [16]. While the human genome encodes more than 70 \( K^+ \) channel subunits [13], the genomes of pathogenic protozoa each contain only a small number of genes encoding homologues of \( K^+ \) channel subunits (Table 1). This striking lack of redundancy in the \( K^+ \) channel complement of protozoan parasites further suggests that these channels might be effective therapeutic drug targets.

Several drugs that inhibit \( K^+ \) channel activity are known to be toxic to protozoan parasites. In many cases the primary anti-parasitic mode of action of these drugs is likely to be on processes other than \( K^+ \) flux. However, \( K^+ \) channel-blocking drugs may alter the activity of the protozoan parasite \( K^+ \) channel homologues described in this study, perturb cellular \( K^+ \) homeostasis and contribute to the decreased survival of parasites. For example, chloroquine blocks \( K_{ir} \) channels [128] and is also toxic to various species of *Plasmodium* [129]. The anti-trypanosomal drug pentamidine also blocks \( K_{ir} \) channels [130]. The \( K_{Ca} \) channel antagonist glibenclamide [131] and the \( K^+ \) channel blocker amiodarone [132] are both known to be lethal to *Leishmania mexicana*. The \( K^+ \) channel blocker amantadine is toxic to *P. falciparum* [133]. In addition, bicusculine and tubocurarine, which block some \( K^+ \) channels in addition to other targets [134,135] are also toxic to *P. falciparum* [37]. Functional evidence will be required before any
causal links between inhibition of parasite K⁺ channel activity and anti-parasitic effects can be established.

Drugs that activate K⁺ channels may also be toxic to protozoan parasites. This has not been experimentally tested in protozoa, although it has been reported to play a role in the action of some drugs against parasitic nematodes [136]. Yeast also exhibit a loss of viability in response to a K⁺ channel-activating toxin which leads to excessive K⁺ flux [11]. Protozoan parasites within mammalian cells could be uniquely susceptible to drug-induced activation of parasite plasma membrane K⁺ channels, as the relatively high cytosolic K⁺ concentration of the host cell might allow K⁺ overload of the parasite, a scenario rarely encountered by mammalian cells due to the relatively low concentration of extracellular K⁺. Well-defined K⁺ channel openers exist which have minimal negative effects in humans, and indeed some of these are used as anti-nematode drugs [136], muscle relaxants, antiepileptics and analgesics [137,138]. It is possible that the effectiveness of this strategy would be reduced in parasites that lower the cytosolic K⁺ concentration of the host cell, such as Plasmodium spp. [101–104].

The well-characterized pharmacology of K⁺ channel, together with sequence differences between human and parasite proteins, suggests that specific block of parasite K⁺ channel homologues might be an achievable target. For example, determinants of isoform-specific block of KCa2 channels by drugs and toxins have been described in the outer pore region [78,139,140], where sequence differences exist between human and parasite homologues. This suggests avenues for development of drugs that specifically block parasite KCa channel homologues rather than human isoforms. Pharmacological blockers of other K⁺ channels also bind within the pore region and in some cases their binding affinity can be predicted with confidence from primary protein sequence alone. For example, TEA binds to the outer pore and a residue following the selectivity filter (GXGXXX) is a critical determinant of its binding affinity [75]. Channels with an aromatic residue at this position almost universally display a high-affinity interaction with TEA. Hence it is likely that the KCa channel homologues in Plasmodium spp. (XP_001348796, NP_001615733 and XP_001348796) as well as the homologue in E. histolytica (XP_655083), which all possess a phenylalanine or tyrosine residue at this locus, will display high affinity block by TEA. As discussed earlier, the lack of homologues of mammalian KCa channel auxiliary subunits in the protozoan parasites examined here also suggests a lack of diversity in native parasite K⁺ channel complexes and an opportunity for selective targeting of these parasite K⁺ channels by drugs.

This study presents the opportunity for cloning and functional characterization of K⁺ channels in pathogenic protozoa, and suggests that rational design of therapeutic strategies targeted against parasite K⁺ channels may be an attractive prospect. Future studies of parasite genomes and cellular signalling will lead to a deeper understanding of the presence and function of these channels in pathogenic parasites.

Materials and Methods

Genome analysis, sequence alignments and topology analysis

Analysis of genomes, sequence alignments and topology analysis were conducted as reported previously [2]. BLASTP and TBLASTN searches of protozoan genomes were carried out against the National Center for Biotechnology (NCBI) genomic protein databases. In multiple sequence alignments (ChastlW2) asterisks below the alignment indicate positions that have a single fully conserved residue, while colons below the alignment indicates positions that have residues with highly similar properties (scoring >0.5 in the Gonnet PAM 250 matrix, ChastlW2).

BLASTP analysis was carried out using the sequences of the following diverse human K⁺ channels (protein accession number in parentheses): K⁺/1.2 (NP_004965.1), K⁺/1.1 (NP_002089.2) and K⁺/1.1.1 (heRG1) (Q19089.1); K⁺/1.1 (ROMK1) (NP_002011.1), K⁺/2.1 (IKK1) (NP_000062.1), K⁺/3.1 (GIRK1) (NP_002235.1), K⁺/4.1 (P70508.1), K⁺/5.1 (Q9NPD1.1), K⁺/6.1 (K⁺TP4) (Q15421.1), K⁺/6.2 (NP_000516.3) and K⁺/7.1 (CAAB68719.1); K⁺/1.1 (TWIK1) (NP_002235.1), K⁺/2.1 (TREK1) (NP_001017425.2), K⁺/3.1 (TASK1) (NP_002237.1), K⁺/3.1 (THIK1) (NP_071337.2), K⁺/3.1 (TALK1) (NP_001128577.1) and K⁺/3.1 (TRESK2) (NP_062823.1); K⁺/3.1 (BK) (NP_001154824.1), K⁺/2.1 (SK1) (NP_002239.2), K⁺/2.2 (SK2) (NP_067627), K⁺/3.1 (IK/SK4) (NP_002241.1) and K⁺/4.1 (SLACK/K⁺/1) (NP_065873.2); Other K⁺ channel sequences were also used to search for parasitic homologues, including: Plasmodium falciparum PfKcH1 (XP_001350669.2) and PfKcH2 (XP_001348796.2) [15], bacterial KcsA (P003344), bacterial cyclic nucleotide-gated MloK1 (Q9SGN8.1), archaean depolarization-activated K₂AP (Q9YDF8.1), archaean hyperpolarization-activated MVP (Q57603.1), archaean Ca⁺⁺-activated MhK (O27564.1), and fungal TOK1 (CAAB68719.1). Plant K⁺ channel sequences were also used, including: the vacuolar outwardly rectifying, calcium-regulated vacuolar two-pore TPK1 channel (NP_200374.1); vacuolar KC03 (NP_001190480.1); the pollen plasma membrane TPK4 (NP_171752.1), the inward rectifier KAT1 (NP_199436.1), the outward rectifier SKOR (porcine K⁺/3.1 residue 271–340 to avoid ankyrin hits), and AKT1 (NP_180323.1). Sequences of human GNG4 (EAW93049; full length, and TMD residues 200–420), and CNGb1 (NP_001285), as well as human HCN2 (NP_001185.3; full length, and TMD residues 200–470) were also used to search for parasite homologues. In addition, the sequence of a novel putative adenylyl cyclase/K⁺ channel fusion protein in P. falciparum (PfAC1 or PfAC2; XP_001348216) was used to search for homologues in other parasites. Sequences of K⁺ channel auxiliary subunits that were used to search for parasite homologues include: human KCNE1 (NP_001121142.1), human Kβ1 (NP_751892.1), human KChIP1 (NP_001030009), human BKβ (NP_004128.1), human SUR1 (NP_000343) and human SUR2A (NP_005682). The sequences of both the isolated nucleotide-binding domains of SUR1 and SUR2A and the sequences outside these nucleotide-binding regions were also used to search for parasite homologues. Results of BLASTP analysis were confirmed using TBLASTN analysis in all cases. Default BLAST parameters for assessing statistical significance and for filtering were used in all cases (ie. an Expect threshold of 10, and SEG filtering).

Several procedures ensured that hits were probably K⁺ channel homologues. Firstly, the occurrence of multiple putative TMDs was confirmed using TOPCONS [141]. Secondly, reciprocal BLASTP searches (non-redundant protein database at NCBI) were undertaken, using identified parasite hits as bait, and only proteins that gave the original mammalian protein family as hits were analyzed further. Thirdly, conserved domains were identified using the Conserved Domains Database (NCBI). Lastly, only hits with regions of sequence similarity that encompassed the selectivity filter sequence of the K⁺ channel subunit used as bait were acknowledged. K⁺ channel parasite homologues were identified as proteins showing sequence similarity in both the pore and the CNBD regions. Where a hit showed similarity to more than one human K⁺ channel, the parasite protein was designated as a homologue of the human channel to which it showed greatest sequence similarity (ChastlW2) and which contained similar putative functional domains.
For phylogenetic analysis, multiple sequence alignments were constructed with MUSCLE v3.7 using default parameters. After use of GBLOCKS at low stringency to remove regions of low confidence, and removal of gaps, Maximum Likelihood analysis was undertaken using PhyML v3.0 (WAG substitution model; 4 confidence, and removal of gaps, Maximum Likelihood analysis use of GBLOCKS at low stringency to remove regions of low confidence). The phylogenetic tree is shown using TreeDyn [198.3]. MUSCLE, GBLOCKS, PhyML and TreeDyn are all functions of Phylogeny.fr [http://www.phylogeny.fr/] [142].

CaM-binding sites were identified using The Calmodulin Target Database search facility [http://calcium.uhnres.toronto.ca/ctdb] [143]. Secondary structure of proteins was predicted using SABLE [http://sable.cchmc.org] [144].

Author Contributions
Conceived and designed the experiments: DLP. Performed the experiments: DLP. Analyzed the data: DLP. Contributed reagents/materials/analysis tools: DLP. Wrote the paper: DLP NVM.

References


