AuPS/ASB Meeting - Canberra 2005

Free Communications 2: Ion channels

Wednesday 28 September 2005

Chair: Peter Barry

Investigating the mechanism of proton transfer through the bacterial ClC transporter

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The ClC chloride channel family is a ubiquitous, yet highly unique family of ion channels, involved in a diverse range of physiological functions. Accardi & Miller (2004) showed that the bacterial ClC channel, ClC-ec1, is not a simple chloride channel, but a chloride / proton exchange transporter, exchanging two chloride ions for every one proton. More recent experimental studies have shown that two eukaryotic members of the family, ClC-4 and ClC-5, are also chloride / proton exchange transporters (Picollo & Pusch, 2005; Scheel *et al.*, 2005). Computational investigations have provided a detailed description of the mechanism of chloride permeation through several ClC isoforms (Cohen & Schulten, 2004; Corry *et al.*, 2004). However, there is very little information describing the transport of protons through ClC-ec1, or the location of the proton translocation pathway. It is known, however, that Glu148, Ser107 and Tyr455 are involved in the translocation pathways of both chloride and protons (Accardi & Miller, 2004).

Here we use computational techniques to probe ClC-ec1 to determine the most energetically favourable translocation pathway for protons. First we ran multiple searches using the HOLE program (Smart *et al.*, 1993) to identify every continuous pathway through the protein with a radius greater than 0.6Å. Our results converged on four possible pathways through each protein dimer. We then used a Poisson-Boltzmann calculation to determine which of these pathways was energetically favourable for protons. Our investigations reveal a narrow fissure through each dimer, 0.75 Å in radius, close to the dimer interface. The protein surrounding these fissures is relatively rich in polar and ionizable amino acids, creating an environment favourable for protons. In support of the experimental evidence, we find that Glu148, Ser107 and Tyr455 are pore-lining residues of our proposed proton translocation pathway, as well as the chloride translocation pathway.

Electrostatic calculations of the unoccupied ClC-ec1 transporter show that our proposed proton translocation pathway contains an electrostatic potential barrier to proton permeation, in the intracellular region of the pathway, effectively barring proton permeation. However, when two chloride ions occupy the chloride pathway, the potential energy barrier in the proton translocation pathway is converted to an electrostatic potential energy well of approximately 18 kT, deep enough to hold one proton in a stable configuration. This occupancy pattern, confirmed by Brownian dynamics simulations, supports the experimentally predicted exchange rate of one proton for every two chloride ions (Accardi & Miller, 2004).

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An electrostatic basis for valence selectivity in cationic channels

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It is well known that the charge distribution surrounding the pore is responsible for ion selectivity in cation channels. Negative charges lining the pore region present an energy well for cations and a barrier of a similar magnitude for anions, thereby selecting one species and excluding the other. Less well known is how these channels select between cations with differing magnitudes of charge. Here we have constructed models of the KcsA, sodium and calcium channels and compared the permeation of monovalent and divalent ions to understand the basis of this valence selectivity.

For both the KcsA and sodium channels, monovalent ions readily pass through the channel. However, as soon as a divalent ion enters the selectivity filter it binds strongly, rarely leaving and thereby almost completely preventing the permeation of monovalent ions. This effect is equally important for inward and outward currents in the KcsA channel, but more pronounced for the inward current in the sodium channel. In contrast, calcium channels conduct monovalent ions in the absence of any divalent ions, but when a mixture of monovalent and divalent ions is present, they allow only the divalent ions to pass.

This phenomenon of selectivity between monovalent and divalent ions can be attributed to electrostatics. We have used simulations of Brownian dynamics and electrostatic calculations to show that in all three channel types, the distribution of charges in the protein creates an energy well that attracts many ions into the channel, making conduction a three ion process for sodium and calcium channels and a four ion process for the KcsA channel. But for the case of divalent ions, the energy well in the KcsA and sodium channels is very deep. Once a divalent ion has entered it finds it difficult to leave, even with the aid of a repulsive kick from other ions in the channel. Thus divalent ion block of the channel causes the currents to plummet. On the other hand, for the calcium channel a second divalent ion entering the channel presents enough repulsion to push a resident divalent ion out of the channel, but a monovalent ion does not. Thus, the calcium channel is able to select divalent ions in a divalent-monovalent ion mixture.

A current source and a cation conductance are components of an electrical circuit connected across the plasma membrane of the malaria parasite *Plasmodium falciparum*

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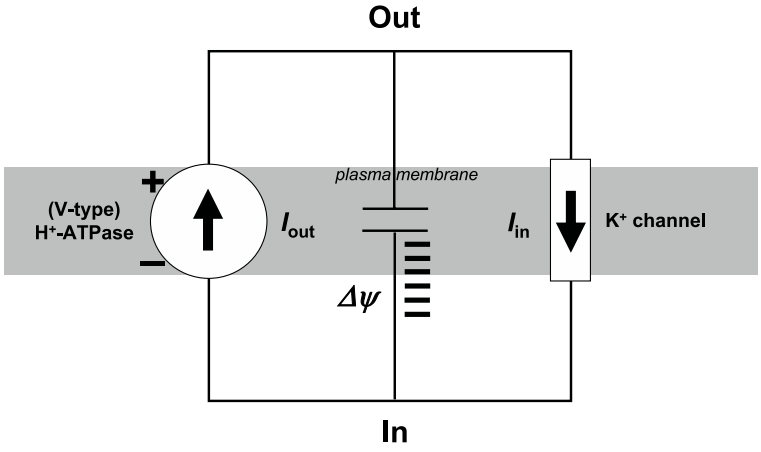
Like most cells, the intraerythrocytic malaria parasite *Plasmodium falciparum* requires a high intracellular concentration of K^+ (~135 mM) for normal development. Using $^{86}Rb^+$ and the potential-sensitive compound ^3H-TPP^+, we have shown that the parasite's mechanism of K^+ uptake is electrophoretic, mediated by a pathway with characteristics of a K^+ channel. The driving force, the parasite's membrane potential, $\Delta\psi$, originates from the extrusion of H^+ by a (V-type) H^+-ATPase on the plasma membrane. However, we have also shown that $\Delta\psi$ is modulated (partially offset) by extracellular K^+, indicating an interdependence between K^+ influx and $\Delta\psi$.

Investigations into the kinetics of K^+ uptake have shown that between 5 mM – 130 mM K^+, the influx of K^+ remains constant, despite there being a reduction in $\Delta\psi$ with increasing concentrations of extracellular K^+.

These phenomena may be reconciled by considering the H^+-ATPase as an 'ideal' current source, and the K^+ channel as a 'variable' conductance, the latter a function of the extracellular concentration of K^+ (see figure). In this electrical model, the inward current carried by K^+ influx through the K^+ channel, 'I_{in}', is equal to the outward current carried by the (*net*) export of H^+ via the H^+-ATPase, 'I_{out}' (*i.e.* $I_{in} = I_{out}$). As the K^+ conductance of the membrane is varied by altering the extracellular concentration of K^+, the offset to $\Delta\psi$ caused by the influx of K^+ also varies, so that the equality $I_{in} = I_{out}$ remains satisfied.



Requirement for steady state $\Delta\psi$: $I_{in} = I_{out}$

During its growth phase, the accumulation of K^+ by the parasite is achieved in the context of a >10-fold decrease in the concentration of K^+ (from ~140 mM) within the host red cell (itself a result of the parasite manipulating the permeability of the host cell membrane). The mechanism we describe is able to explain the parasite's ability to generate a stable influx of K^+, neither overwhelmed by, nor starved of, K^+, as the concentration of K^+ within the red cell undergoes a dramatic reduction.

Largely on the basis of sequence homology to the canonical selectivity filter of homotetrameric K^+ channels, two putative K^+ channel genes have been identified in the *Plasmodium falciparum* genome database. Hydropathy profiles suggest that both channels have additional transmembrane domains over and above the 6 characteristic of voltage-gated K^+ channels, a feature shared by several members of the 'slo' K^+ channel family. The function of these domains is unknown. Both channels are unusual for their great size (the larger has ~2000 residues per subunit), and have large hydrophilic domains which are predicted to reside cytosolically, the functions of which are also unknown. The larger protein has an 'S4' segment containing 3 regularly spaced arginines, in a pattern consistent with a (perhaps degenerate?) voltage sensor of a voltage-gated K^+ channel. Immunofluorescence studies demonstrate localisation of this protein to be predominantly at the host cell membrane, suggesting that it is not the K^+ uptake pathway in the parasite membrane discussed above, but perhaps plays a role in the alteration of the ionic makeup of the host cell cytosol by the parasite. No data currently exists for the location of the smaller protein. These putative K^+ channels are the subject of continuing investigations.

Role of protein flexibility in gramicidin A channel permeability

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Proteins have a flexible structure, and their atoms exhibit considerable fluctuations under normal operating conditions. However, apart from some enzyme reactions involving ligand binding, our understanding of the role of flexibility in protein function remains mostly incomplete. Here we investigate this question in the realm of membrane proteins that form ion channels. Specifically, we consider ion permeation in the gramicidin A channel (GA), and study how the energetics of ion conduction changes as the channel structure is progressively changed from fixed NMR structure to a completely flexible one as obtained from molecular dynamics (MD) simulations. For each channel structure, the potential of mean force for a permeating potassium ion is determined from MD simulations. Using the same MD data for completely flexible gramicidin A, we also calculate the average densities and fluctuations of the GA atoms and investigate the correlations between these fluctuations and the motion of a permeating ion. Our results show conclusively that peptide flexibility plays an important role in ion permeation in the gramicidin A channel, and hence it cannot be modeled using continuum electrostatics with an average, fixed structure.

Ca^{2+} influx through store-operated Ca^{2+} channel in mouse sinoatrial node

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In both excitable and non-excitable cells, the depletion of intracellular Ca^{2+} stores causes a flux of Ca^{2+} into the cells and refills the Ca^{2+} store to its original level. The inward Ca^{2+} flux resulting from depletion of Ca^{2+} store is through the store-operated cation channels (SOCCs). There is growing evidence that SOCCs play an important role in muscle cell signalling (for review see Gailly, 2002).

In previous studies, we found that intracellular Ca^{2+} stores are involved in cardiac pacemaking (for review see Ju & Allen, 2001). To examine if store–operated Ca^{2+} entry is present in cardiac pacemaker tissue and its possible role in regulating heart rate, sinoatrial node (SAN) tissue was dissected from mouse right atria of the heart and loaded with the Ca^{2+} indicator indo-1 AM. In the presence of extracellular Ca^{2+} ($[Ca^{2+}]_o$), cyclopiazonic acid (CPA), a selective sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, significantly increased resting $[Ca^{2+}]_i$ and gradually reduced the amplitude of $[Ca^{2+}]_i$ transients. Incubating SAN in Ca^{2+} free solution caused a substantial decline in resting $[Ca^{2+}]_i$ and stopped pacemaker activity. Reintroduction of Ca^{2+} (1.8 mM) to the perfusate in the presence of CPA evoked a striking increase in resting $[Ca^{2+}]_i$, a characteristic of SOCC activity. The Ca^{2+} influx in response to reintroduction of $[Ca^{2+}]_o$ was 7.1 ± 3.2 fold greater in the presence of CPA than in its absence ($p < 0.03$, $n = 11$), which suggested that the Ca^{2+} influx was dependent on the SR store depletion. It is known that the Na^+-Ca^{2+} exchanger exists in cardiac pacemaker tissue. After a period of incubation in zero Ca^{2+} solution, the reintroduction of Ca^{2+} could also activate the reverse mode of Na^+-Ca^{2+} exchanger and increase Ca^{2+} influx. To test this possibility, we applied Na^+-Ca^{2+} exchanger inhibitor KBR -7943. We found that in the presence of KBR -7943, there was still a significant rise of $[Ca^{2+}]_i$ in response to the depletion of SR the Ca^{2+} store. Moreover, gadolinium (100 μM), a known SOCC inhibitor, significantly inhibited 72 ± 8% of Ca^{2+} influx in the present of CPA ($P< 0.01$, $n = 4$).

Recent studies have suggested that SOCCs might be related to the transient receptor potential canonical (TRPC) gene family. We examined SAN mRNA expression of the seven known mammalian TRPC isoforms by RT-PCR. mRNA for TRPC1, 2, 3, 4, 6 and 7 was detected in SAN, whereas that for the TRPC5 was not. These results suggest that cardiac pacemaker tissue exhibits store-operated Ca^{2+} activity which may be due to expression of TRPCs in these cells.

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Supported by NH&MRC

A hydrogen peroxide insult causes sustained alteration in the sensitivity of the L-type Ca^{2+} channel to β-adrenergic receptor stimulation in ventricular myocytes

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We have shown previously that mitochondrial-derived hydrogen peroxide (H_2O_2) regulates the function of the L-type Ca^{2+} channel. A decrease in mitochondrial-derived H_2O_2 is associated with an increase in the sensitivity of the channel to the beta-adrenergic receptor agonist isoproterenol (Iso) and exposing myocytes to H_2O_2 attenuates the response. Here we examine the effect of a hydrogen peroxide insult on the function of the L-type Ca^{2+} channel. Ventricular myocytes were isolated from hearts excised from anaesthetised guinea-pigs as approved by the Animal Ethics Committee of The University of Western Australia and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC). The cells were exposed to 30 μM H_2O_2 for 5 min followed by 10U/ml catalase for 5 min to degrade the H_2O_2, and then the response of the channel to Iso was examined. In the absence of a peroxide insult, 10 nM Iso elicited a current that was 72.1±8.0% of the current elicited by 1 μM Iso (a maximally stimulating concentration of the agonist) within the same cell (n=6). However, after exposure of cells to peroxide 10 nM Iso elicited a current that was just 18.6 ± 10.0 % of the response elicited by 1 μM Iso within the same cell (n=6; $P<0.05$) suggesting that the peroxide insult significantly decreased the sensitivity of the channel to Iso. More importantly this effect persisted for several hours after the peroxide insult. We examined whether the effect was a result of enhanced production of reactive oxygen species by the cell. Cellular production of superoxide was measured using the fluorescent indicator dihydroethidium. Exposing cells to 30 μM H_2O_2 for 5 min followed by 10U/ml catalase for 5 min caused a 61.1±14.0% increase in superoxide production (n=13; $P<0.05$) compared to controls exposed to catalase only (n=8). The increase in superoxide was completely attenuated when cells were exposed to the mitochondrial inhibitor myxothiazol (6-10 nM; n=14; $P<0.05$). The NAD(P)H oxidase inhibitor apocynin (300 μM, n=5) did not alter the increase in superoxide associated with a peroxide insult nor did 100 μM of the xanthine oxidase inhibitor, allopurinol (n=5). We propose that a hydrogen peroxide insult causes a further increase in hydrogen peroxide production from the mitochondria and the increase in peroxide results in a sustained decrease in sensitivity of the channel to β-adrenergic receptor stimulation.