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REVIEW

Intracellular regions of potassium channels: Kv2.1 and heag

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Abstract Intracellular regions of voltage-gated potassium channels often comprise the largest part of the channel protein, and yet the functional role of these regions is not fully understood. For the Kv2.1 channel, although there are differences in activation kinetics between rat and human channels, there are, for instance, no differences in movement of the S4 region between the two channels, and indeed our mutagenesis studies have identified interacting residues in both the N- and C -terminal intracellular regions that are responsible for these functional effects. Furthermore, using FRET with fluorescent-tagged Kv2.1 channels, we have shown movement of the C-termini relative to the N-termini during activation. Such interactions and movements of the intracellular regions of the channel appear to form part of the channel gating machinery. Heag1 and heag2 channels also display differing activation properties, despite their considerable homology. By a chimeric approach, we have shown that these differences in activation kinetics are determined by multiple interacting regions in the N-terminus and membrane-spanning regions. Furthermore, alanine mutations of many residues in the C-terminal cyclic nucleotide binding domain affect activation kinetics. The data again suggest interacting regions between N- and C- termini that participate in the conformational changes during channel activation. Using a mass-spectrometry approach, we have identified α-tubulin and a heat shock protein as binding to the C-terminus of the heag2 channel, and α-tubulin itself has functional effects on channel activation kinetics. Clearly, the intracellular regions of these ion channels (and most likely many other ion channels too) are important regions in determining channel function.

Keywords Ion channels · Potassium channels · Voltage-dependent channels · Electrophysiology

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Introduction

Voltage-activated potassium channels comprise a large family of ion channels, of considerable importance in cell function, as sites for drug action, and as sites for inherited disorders (Bracey and Wray 2006). The role of membrane-spanning regions of these tetrameric potassium channels has been well-studied. There are six transmembrane regions, S1–S6, with a P region between S5 and S6 (Fig. 1a) (Wray 2000, 2004). The channel pore is formed by the S5 and S6 regions, with the selectivity filter formed by the P region near the outer mouth of the pore. Upon activation, the positively charged S4 region moves outwards, and the negatively charged S2 and S3 regions are also involved. Several X-ray structures of regions of voltage-activated potassium channels have now been published (Doyle et al. 1998; Gulbis et al. 2000; Jiang et al. 2003; Long et al. 2005, 2007).

The N- and C-terminal regions of voltage-gated potassium channels are located intracellularly. In many channels, these regions comprise the largest part of the channel protein, and the functional roles of these regions are increasingly coming under scrutiny (Wray 2004). For instance, for the Kv2 channels and eag channels (members of the ether-a-go-go family), the intracellular regions are particularly large, and comprise about 75% of the channel protein. In this report, we review recent progress in our laboratory in

understanding some aspects of the roles of the intracellular regions of Kv2.1 and heag channels.

Kv2.1 channels

The human and rat forms of the Kv2.1 potassium channel have identical membrane-spanning regions, but there are differences between the rat and human channel in the intracellular regions, particularly in the C-terminal region. These two forms of the channel display differing activation kinetics (Ju et al. 2003), implying a role for the intracellular regions in channel gating. Indeed these differences do not appear to arise from differences in movements of the S4 region, as shown by our experiments with cysteine mutations of S4 (Fig. 1b, c) (Bracey et al. 2005a). During repetitive stimulation, applications of parachloromercuribenzensulfonate (PCMBS) to channels with S4 cysteine mutations gave similar extent and time course of block by PCMBS, whether for rat or human forms of the Kv2.1 channel. Furthermore, during activation, the extent of movement outwards of the S4 region was identical for both rat and human channels. Thus PCMBS had no effect on residue M298 in both cases, indicating outward movement of S4 outward as far as residue 297 in both the rat and human Kv2.1 channel (in fact similar to the extent of movement for the Shaker channel, Fig. 1d, Yusaf et al. 1996). Therefore, bearing in mind the identical membrane-spanning regions between rat and human Kv2.1 channel, the data indicate that, although S4 movement obviously drives the overall process of channel opening, modulation of activation kinetics occurs because of the intracellular regions, rather than by modulation of S4 movements.

The N-terminal region of Kv2.1 includes a stretch of amino acids comprising the "T1" domain. This region hangs below the channel as a tetramer ("hanging gondola"), as indeed shown by a crystal structure of Kv1.2 with its T1 domain (Long et al. 2005). The T1 domain binds to the auxiliary beta subunit in some channels (though not Kv2.1), and it is also concerned with control of subunit assembly within families (Zerangue et al. 2000; Liu et al. 2001; Minor 2001; Strang et al. 2001). Mutations in the T1 domain can affect activation and deactivation properties, so that this domain must also participate in conformational changes involved in gating. Recently a single particle electron microscope structure has been obtained for the Kv2.1 channel (Adair et al. 2008). The outline of the entire structure of the Kv2.1 channel, obtained from the single particle analysis, together with our homology model for the Kv2.1 T1 domain (Ju et al. 2003), is shown in Fig. 2e. It is clear that there are large spaces surrounding the T1 region that are occupied by the C-terminal regions. However, to date there are no structural data for the C terminus itself.

In our earlier work, we showed that the N and C termini of this channel interact, and that both regions are concerned with modulation of channel function (Ju et al. 2003). For this, we used a chimeric approach between rat and human Kv2.1 channels with expression in oocytes followed by electrophysiological analysis. Using this approach, which was also supported by biochemical experiments, we were able to show that the C terminus wraps around the T1 domain and interacts with it. In particular residues 67 and

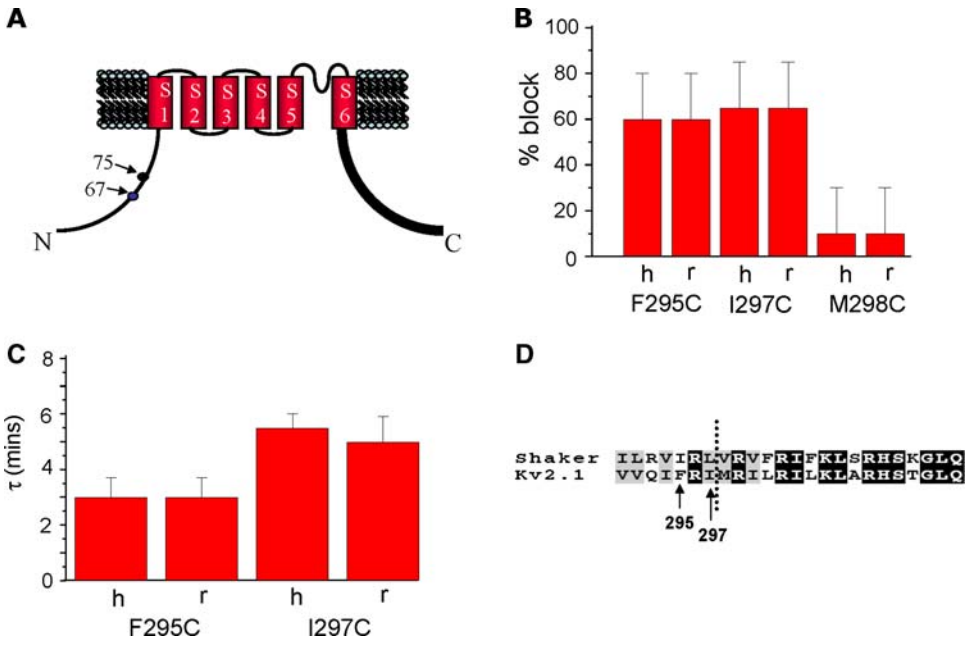

Fig. 1 Kv2.1 channel and S4 movement. A schematic diagram of a voltage gated channel (Kv2.1) is shown (**a**). The figure also shows results for application of PCMBS (100 μM) to rat (r) and human (h) cysteine mutations F295C, I297C and M298C in the S4 region of the Kv2.1 potassium channel. The percent block (**b**) and time constant (τ) of block (**c**) by PCMBS are shown during repetitive stimulation from −80 mV holding potential to +40 mV (n = 6–12). An alignment of Kv2.1 with Shaker S4 is shown (**d**); in both cases the S4 region moves outward during depolarisation, exposing residues extracellularly as far as to the left of the *dotted line*

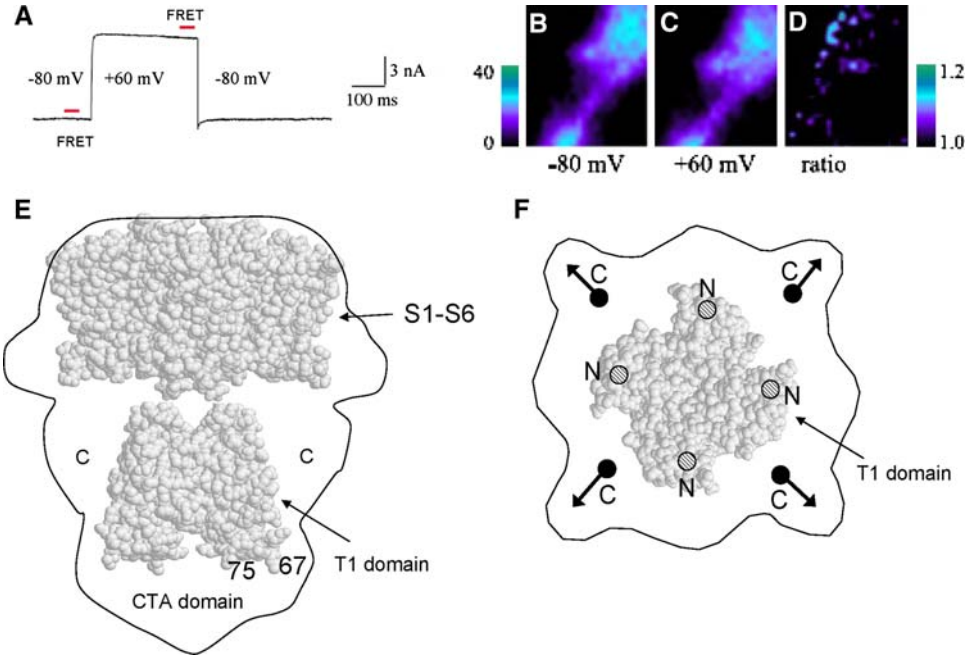

Fig. 2 Movement of the C termini of the Kv2.1 channel during depolarisation. The Kv2.1 channel was labelled with fluorescent tags YFP at the N terminus and CFP at the C terminal. Depolarising steps were made to activate Kv2.1 currents (**a**), and images of FRET between the fluorophores are shown before (**b**) and during depolarisation to +60 mV (**c**). The ratio of images is shown (**f**), indicating a change in FRET at the membrane during depolarisation. The relative positions of the membrane spanning domain and the T1 domain are shown for Kv2.1 (**e**), using our homology model; also shown is the outline of the entire structure of the Kv2.1 channel, obtained from EM single particle analysis (adapted from Adair et al. 2008). The position of the homology model within the EM structure is merely a suggestion, guided by eye. Residues 67 and 75 in the N terminus are also shown. The movement of the C termini deduced from FRET measurement is shown viewed from the bottom of the protein (**f**), drawn to scale with the T1 domain homology model and EM structure outline also indicated. The *absolute* positions of the N and C termini are not known, but the figure indicates approximately to scale the direction and extent of estimated outward movement (*arrows*) of the C termini *relative* to the N termini, as obtained from FRET measurements during depolarisation

75 in the T1 domain appear to interact with residues within the tail of the C-terminus [residues 741–853, forming the "C-terminal activation (CTA) domain"]. Important residues within the CTA domain appear to be located in two stretches (749–795 and 796–838). Of interest here is the fact that residues 67 and 75 in the T1 domain are located at the bottom of the T1 domain (Fig. 2e), so not only does the C-terminal domain extend to this point, but also, interestingly, amino acids so far away from the membrane-spanning region can also affect channel activation properties. Clearly there can be no direct interaction of this part of the protein with the membrane-spanning part of the channel; the only interaction could be via allostery and conformational change. Thus it seems likely that, when the channel is activated, conformational changes occur within the whole of the channel structure, not just within the membrane-spanning parts of the protein.

To test directly whether conformational changes do indeed occur in the N- and C- termini of Kv2.1, we used an approach based on FRET, with fluorescent tags placed on the N- and C-termini themselves (Kobrinsky et al. 2006). We applied voltage steps to cells expressing these constructs, and recorded changes in FRET while applying depolarising pulses and simultaneously recording associated channel currents (Fig. 2a). With a yellow fluorescent protein (YFP) tag at the N-terminus and a cyan fluorescent protein (CFP) at the C-terminus of each Kv2.1 subunit, we were able to show a change in FRET signal upon depolarisation of the cell (Fig. 2b–d). Thus there is indeed movement of the N- and C- termini relative to each other. Similarly we could observe a change in FRET upon depolarisation for experiments with equimolecular mixtures of single labelled subunits (YFP at the N-terminus of one subunit, CFP at the C-terminus of the other). Detailed analysis (Kobrinsky et al. 2006) suggested that changes in FRET, and hence depolarisation-induced movements between N- and C-termini, occurred both within a subunit and between subunits.

To investigate relative movements between the N-termini themselves, we co-expressed N-tagged YFP-Kv2.1 with N-tagged CFP-Kv2.1. However, upon depolarisation, we found no change in FRET in this case, suggesting that there is no relative movement between the termini themselves. Thus, either the N-termini do not move, or they move together as if part of a rigid body. It was not possible to test for movement between the C-termini themselves, because no FRET was obtained between C-terminal

labelled tags, suggesting that the C-termini may be rather far apart and more distant from the centre of the tetramer than the N termini. We also looked for FRET changes during depolarisation between fluorescently labelled Kv2.1 channel and fluorescent tags immobilised nearby on another membrane protein (a domain from phospholipase C). For this arrangement, again we observed no change in FRET during depolarisation, suggesting that movements of the channel termini occur in a plane parallel to the membrane.

Taking the data together, these results from extensive FRET analysis suggested a model where depolarisation induces movements of the termini such that the C- termini move outwards, away from the N-termini, as shown in Fig. 2f. Movement of the C-termini is appreciable. Although there are uncertainties in predictions of distances from FRET measurements, primarily because of uncertainties in orientation of the dipoles, the data suggest that the C-termini may move outward by some 10Å or more.

It seems likely that this relative movement between N- and C- termini may underlie conformational changes during channel activation that are responsible for interactions between N- and C- terminal regions that affect activation properties of the channel. Such interactions and movements of the intracellular regions may well form part of the channel gating machinery. These conformational changes of the intracellular regions could be communicated to the membrane-spanning part via the linkers to S1 and S6, as well as by direct interaction of the upper part of the intracellular region with the S2/S3 and S4/S5 loops, as also suggested by Scholle et al. 2004.

Heag channels

The ether-a-go-go family of channels comprises a family of two eag channels (eag1 and eag2), three elk channels and three erg channels. The last member to be cloned in this family, eag2, was obtained in rat (Saganich et al. 1999; Ludwig et al. 2000) and in human (Ju and Wray 2002; Schonherr et al. 2002). Structurally, this family of channels all contain a "PAS" (Per-Arnt-Sim) domain in the N-terminal region, and a cyclic nucleotide-binding domain, cNBD, in the C-terminal region (Fig. 3a). The X-ray crystal structure of the PAS domain has been obtained for the herg channel (Cabral et al. 1998), but so far no structure has been obtained for cNBD in the ether-a-go-go family. However, the X-ray structure of the cNBD in a related channel (hyperpolarisation-activated channel, HCN) has been obtained (Zagotta et al. 2003). Using these structures for herg and HCN, we have constructed a homology model for these regions in the heag channel (Ju and Wray 2006) (Fig. 3b).

The heag1 and heag2 channels are highly homologous, and yet they show differences in steady-state activation and activation kinetics (Fig. 3c, d) (Ju and Wray 2002). To investigate the structural components underlying these functional differences between these channels, we utilised a

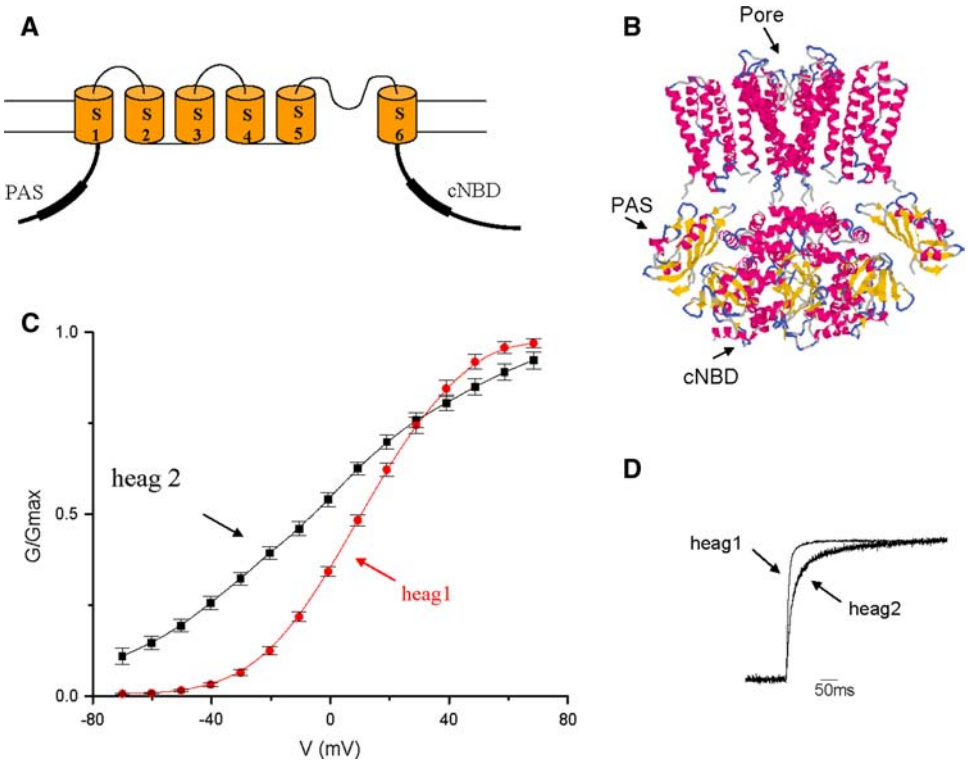

Fig. 3 Heag channel properties. The figure shows the location of the PAS and cNBD domains in the heag channel (**a**), and also in our homology model of the heag2 channel (**b**). In this model, the position of the cNBD lies centrally below the membrane spanning region, as for the HCN channel (Zagotta et al. 2003), but the position of the PAS domain is entirely unknown (a suggested possible position is indicated; only two PAS domains are shown for clarity). Also the model does not show any of the C terminus sequence after the cNBD, since no predictions are available. Comparisons between the properties of heag1 and heag2 channels are shown for conductance-voltage (G-V) curves (**c**) and for the time course of currents (normalised to the same maximum) following a voltage step from −80 mV to 0 mV (**d**)

chimeric approach, studying the functional effects of swapping regions of the protein between heag1 and heag2 channels, again with expression in oocytes (Ju and Wray 2006). The results for activation times, $t_{20–80\%}$ (time to rise from 20% to 80% amplitude), are shown in Fig. 4 for several chimeras between heag1 and heag2. The data for chimeras 1 and 2 show that replacing either the N-terminal region or the membrane-spanning region of heag2 with the corresponding region of heag1 confers activation times characteristic of heag1, indicating the role of the N-terminal and membrane-spanning regions in determining activation kinetics of these channels. Results for chimeras 3, 4 and 5 show that swapping parts of the N- terminal domain of heag1 into heag2 conferred activation properties of heag1, indicating functional roles for the extreme N-terminus region as well as the PAS domain (more specifically the N-terminal proximal part of the PAS domain). Replacing just the S1 or P-S6 regions of heag2 with heag1 had an intermediate effect on the activation time (chimeras 6 and 9), indicating partial contributions of S1 and P-S6 regions in determining activation kinetics. Replacement of S2, S3 and S5 regions (the S4 region is identical between the two channels) had no effect (chimeras 7 and 8), suggesting that these regions do not contribute to the differences in activation time between heag1 and heag2 channels. So, in summary, multiple regions of the channel protein are involved in determining the activation kinetics of the heag channels: in the intracellular region at the extreme N-terminus and the proximal PAS domain, in the membrane-spanning region at the S1 and P-S6 regions.

Also of interest for heag channels is the "Cole-Moore shift", a phenomenon particularly displayed by heag channels. Activation from hyperpolarised holding potentials causes the transiting through several closed states, the result of which is a slower activation time for steps from hyperpolarised holding potentials. This is particularly evident for the initial sigmoidal time course in the case of heag1 (there is less effect of hyperpolarisation on the initial time course of heag2). For this initial component of the time course, our chimeric approach again indicated multiple interacting regions of the N-terminus and the membrane-spanning region as being involved (Ju and Wray 2006).

Most probably, interactions occur between the N-terminal region and the membrane-spanning region; the S4/S5 linker has previously been suggested as a possible site of interaction (Terlau et al. 1997; Wang et al. 1998; Chen et al. 1999; Sanguinetti and Xu 1999). The amino acids of the S4/S5 linker are identical between heag1 and heag2 except for one amino acid (the hydrophobic residues I in heag1, L in heag2). However, when we mutated this residue, the mutation did not affect activation kinetics, suggesting that this residue, and indeed the whole S4/S5 linker are not the main site of interaction with the intracellular components (Ju and Wray 2006). Most likely, the N-terminal region also interacts with other sites of the membrane-spanning part of the channel, such as S1, the S2/S3 loop, and S6.

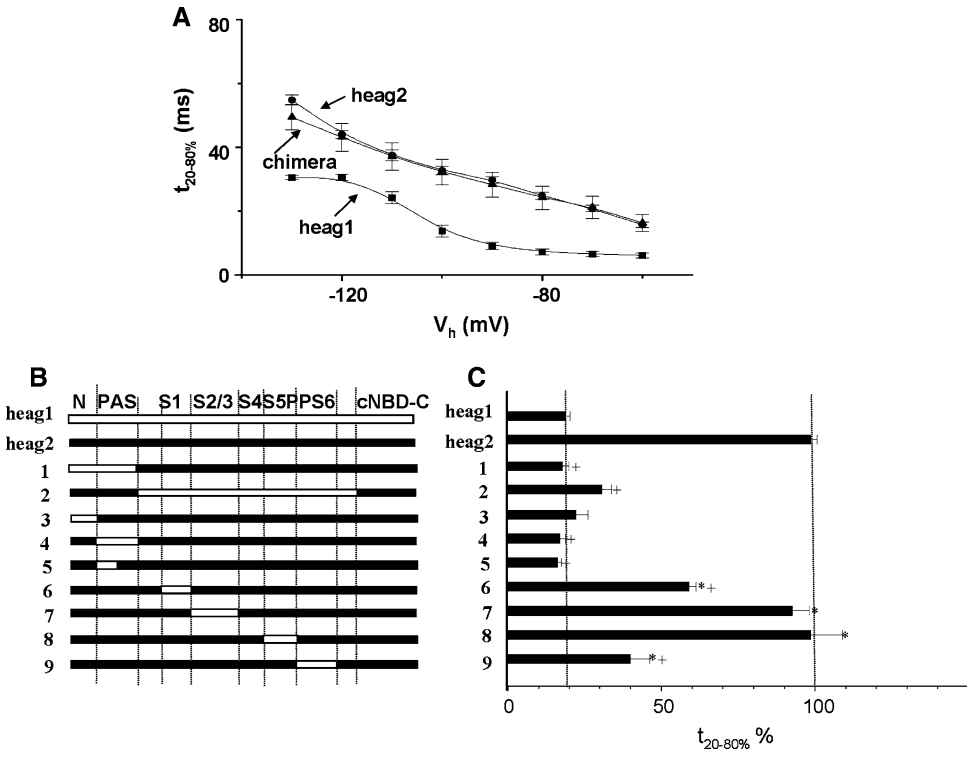

Fig. 4 Effects of molecular regions of heag channels on activation kinetics. Activation times are different for heag1 and heag2 channels, and also have a different dependence on holding potential (**a**). The example chimera shown in **a** is for heag2 with the PAS-S1 linker replaced by heag1, indicating no involvement of the linker in activation kinetics. Part **b** shows the chimeras that were made between heag1 and heag2 channels, and the corresponding activation times (time from 20 to 80% maximum current, $t_{20–80\%}$ for steps from −80 mV holding potential to 0 mV) are shown in **c**

Steady-state activation properties also differ between heag1 and heag2 channels (Fig. 3c). The conductance/voltage curve for heag2 is shifted to the left, and is less steep, as compared with heag1. Our chimeric approach (Ju and Wray 2006) indicated that the N- and C-terminal regions are not involved in determining differences in steady-state activation between the two channels; swapping the entire membrane-spanning region between the two channels indicated that only the membrane-spanning part is involved. However, our attempts to narrow down the important relevant parts by separately replacing the S1–S3 region or the S5–S6 region of heag1 with heag2 gave channels with Boltzmann parameters closest to heag1, which is more difficult to interpret. A likely explanation is the functional involvement of multiple regions in the membrane-spanning part.

Surprisingly, although there are many differences in amino acids between heag1 and heag2 channels in the long C-terminal region, this latter region did not contribute to any differences in functional properties of heag1 and heag2 channels. However, using our homology model for the cNBD, it appears that most of the residues that are different between heag1 and heag2 lie inside the cNBD or beneath it, rather than on the surfaces of the cNBD at the sides. Therefore if the main interacting surfaces of the cNBD with the rest of the heag channel structure are with the top and the sides of the cNBD, it is perhaps not so surprising that there is indeed little role for the C terminus in determining functional differences between heag1 and heag2 channels. Obviously, the residues in the cNBD that are conserved between heag1 and heag2 are not changed in chimeras between the two channels and so, while not contributing to differences in properties between the two channels, may indeed have a common functional role for both channels. Therefore, to test for possible functional roles of conserved residues in the cNBD, we mutated these residues to alanine, and then tested for any effects on activation kinetics (Ju et al. 2006). We indeed found that many of the cNBD residues we mutated to alanine caused marked increases in activation time for the heag1 channel, clearly indicating a role for the C terminus in the activation of the channel. Furthermore, and most interestingly, these residues lie on a band on the surface of the cNBD structure (Fig. 5). This is very suggestive of an interaction region in the cNBD with some other element of the channel structure—perhaps with the PAS domain itself. Thus, to summarise all our data, all regions of the heag channels, i.e. the N and C terminal regions as well as the membrane-spanning regions, participate in the processes involved in channel activation. Just as for Kv2.1, channel activation probably involves a global conformational change in the whole channel protein. Most of our experiments on intracellular domain interactions and conformational changes were carried out using oocytes; therefore physiological intracellular solutions were present so that the results we report may be expected to be those occurring under normal physiological conditions for the channels.

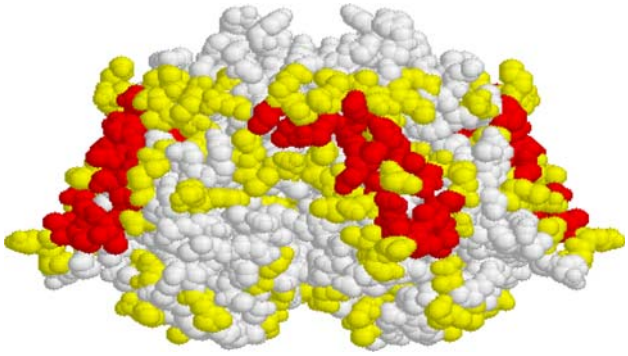

Fig. 5 Regions of the cNBD affecting channel function. The figure shows our homology model for the cNBD domain of the heag1 channel. Residues were mutated to alanine and effects on activation time of the channel measured. Many of the residues (*shown in red*) caused a slowing of channel activation time, and these residues lie in a band across the protein. Residues that caused no effect when mutated to alanine are *shown in yellow*

Another role for the intracellular regions of the heag channels may be as binding sites for various intracellular binding partner proteins. To investigate this, we made GST fusion proteins with various intracellular regions of the heag2 channel, and used these to identify binding partners with human brain proteins (Fig. 6a) (Bracey et al. 2005b). After mixing the fusion proteins with brain proteins and purifying with glutathione, we identified binding proteins by tryptic digest followed by mass spectrometry. In this way, we found binding of α- and β-tubulin, heat shock cognate protein (Hsc71), and myelin basic protein. We were also able to show binding to heag2 of α-tubulin and a related heat shock protein (Hsp70) using immunoprecipitation, though not myelin basic protein (the latter most likely due to non-specific binding in our mass-spectrometry assay).

Interestingly, when α-tubulin was co-expressed with the heag2 channel, activation times were markedly increased (Fig. 6b) (Bracey and Wray 2007). Furthermore, and mimicking this effect, the microtubule-disrupting drug, colchicine, also increased activation times in cells expressing heag2, while the microtubule-stabilising drug, paclitaxel, had the reverse effect (in cells co-expressing heag2 and α-tubulin). The data may be understood when it is considered that co-expression of tubulin and application of colchicine both increase levels of free tubulin (and vice versa for paclitaxel), so that it seems likely that it is free tubulin that binds to the heag2 channel and has a functional effect on its activation kinetics. However, our data do not show whether tubulin binds directly to the channel or whether another intermediary protein is involved. Both microtubules and other ether-a-go-go potassium channels are involved in cell proliferation, and our data therefore suggest the possibility

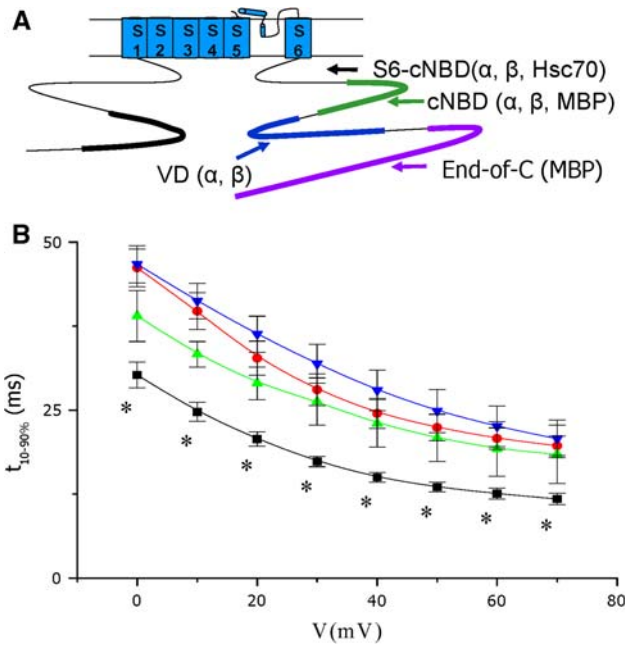

Fig. 6 Binding partners of the heag2 channel. Proteins identified by mass spectrometry as binding to fragments of the intracellular C terminus of the heag2 channel are indicated (**a**) (α α-tubulin, β β-tubulin, *Hsc70* heat shock 70 cognate protein, *MBP* myelin basic protein). The effects of α-tubulin and colchicine on activation times ($t_{10–90\%}$) are indicated (*filled square* heag2, $n = 21$, *filled triangle* heag2 + α-tubulin, $n = 30$, *filled circle* heag2 and colchicine, $n = 12$, *filled inverted triangle* heag2 + α-tubulin and colchicine, $n = 10$. In these experiments, where stated α-tubulin RNA was co-injected into oocytes with heag2 RNA; oocytes were incubated where stated in colchicine (50 μM, applied in the bathing solution for 24 h following heag2 RNA injection)

that the heag2 channel may also have a physiological role in cell proliferation via interaction with microtubules, perhaps via the intermediary of free tubulin.

Although heat shock 70 proteins (Hsc71 and Hsp70) were identified in our binding studies, neither protein affected heag2 currents in co-expression studies. Even so, it is likely that the heat shock proteins play a role in heag2 cellular trafficking, bearing in mind the well-known role of heat shock proteins as molecular chaperones involved in protein folding. Indeed, Hsc71 appears to be involved in trafficking of another member of the ether-a-go-go family, the herg channel (Ficker et al. 2003).

In summary, it can be seen that for both the Kv2.1 and heag channels, the intracellular regions are of considerable importance in ion channel function. Furthermore, intracellular regions probably play an important role in a range of channel types. Future work on these intracellular N- and C- terminal regions may well continue to elaborate the fundamental importance of these regions in ion channel function.

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