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Progress in Biophysics and Molecular Biology 92 (2006) 185–208
Progress in Biophysics & Molecular Biology
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Review

A structural interpretation of voltage-gated potassium channel inactivation

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Available online 8 November 2005

Abstract

After channel activation, and in some cases with sub-threshold depolarizing stimuli, Kv channels undergo a time-dependent loss of conductivity by a family of mechanisms termed inactivation. To date, all identified inactivation mechanisms underlying loss of conduction in Kv channels appear to be distinct from deactivation, i.e. closure of the voltage-operated activation gate by changes in transmembrane voltage. Instead, Kv channel inactivation entails entry of channels into a stable, non-conducting state, and thereby functionally reduces the availability of channels for opening. That is, if a channel has inactivated, some time must expire after repolarization of the membrane voltage to allow the channel to recover and become available to open again. Dramatic differences between Kv channel types in the time course of inactivation and recovery underlie various roles in regulating cellular excitability and repolarization of action potentials. Therefore, the range of inactivation mechanisms exhibited by different Kv channels provides important physiological means by which the duration of action potentials in many excitable tissues can be regulated at different frequencies and potentials. In this review, we provide a detailed discussion of recent work characterizing structural and functional aspects of Kv channel gating, and attempt to reconcile these recent results with classical experimental work carried out throughout the 1990s that identified and characterized the basic mechanisms and properties of Kv channel inactivation. We identify and discuss numerous gaps in our understanding of inactivation, and review them in the light of new structural insights into channel gating.

Contents

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doi:10.1016/j.pbiomolbio.2005.10.001

1. Introduction

1.1. K^+ channel structure in coarse and fine detail

The structures of several bacterial K^+ channels and isolated eukaryotic Kv channel domains have been determined at atomic resolution (Minor et al., 2000; Bixby et al., 1999; Kreusch et al., 1998; Doyle et al., 1998; Kuo et al., 2003; Zhou et al., 2001b; Jiang et al., 2002a, 2003b). However, it is instructive to consider the organization of the multiple domains of an intact Kv channel. Recent advances in single-molecule electron microscopy have provided interesting new insights into the overall organization of K^+ channels and their auxiliary subunits (Sokolova et al., 2001, 2003; Orlova et al., 2003). Single-molecule electron microscope images of the *Shaker* channel suggest that the tetrameric assembly of the pore-forming α subunits is arranged into two distinct domains connected by thin linkers 2–3 nm in length (Sokolova et al., 2001). This overall organization of the channel is now frequently described as the 'hanging gondola' model of channel structure, reflecting the modular division of the transmembrane and cytoplasmic domains of the channel. In the single-molecule EM structure, these two domains are clearly structurally distinct, and linkers between them form four lateral openings through which ions or blockers likely must pass to gain access to the channel pore. The electron density attributed to the transmembrane domains includes segments S1–S6 of the channel. A second smaller electron density (the gondola) is thought to be cytoplasmic. The bulk of this electron density can be accounted for by the highly conserved N-terminal T1 domain, although there is evidence that the C-terminus is also a component of the cytoplasmic channel module (Sokolova et al., 2003). This distinct modular arrangement of Kv channel structure has been observed in single electron microscope images of *Shaker* and mammalian Kv1 channels in complex with auxiliary β subunits (Sokolova et al., 2003; Orlova et al., 2003), and most recently in Kv4.2 channels in complex with KChIP2 (Kim et al., 2004).

The significance of a modular channel arrangement is unclear at present, although specific functions have been attributed to each channel module. The transmembrane domains of the channel comprise all of the structural elements required for voltage-dependent gating, particularly a gated K^+-selective pore (formed by the S5 and S6 helices, and their linker), and a coupled voltage-sensing structure (formed by the S1–S4 transmembrane helices). The cytoplasmic domains, in contrast, have a well-established role in mediating channel assembly and subfamily specificity, and channel association with auxiliary subunits (e.g. α subunits). In addition, there is also likely some interplay between the transmembrane and cytoplasmic domains, although the underlying mechanisms remain unclear. That is, while mutations in the cytoplasmic N- or C-termini generally do not abolish K^+ selectivity or voltage-dependent gating, there are many examples in which such mutations alter parameters of activation or inactivation. In this vein, the cytoplasmic module may be accurately described as a 'regulatory domain', as sites in both the N- and C-termini of certain channels also underlie the gating effects of various intracellular signaling molecules or auxiliary subunits (Huang et al., 1993, 1994; Drain et al., 1994; Holmes et al., 1996).

1.2. Detailed features of K^+ channel pores

In all K^+ channel structures determined to date, the pore-forming regions, and particularly the selectivity filter, exhibit remarkable structural similarity (Doyle et al., 1998; Jiang et al., 2002a; Kuo et al., 2003; Zhou et al., 2001b). The K^+ channel pore provides a hospitable permeation pathway for the dehydration, diffusion, and rehydration of potassium ions as they move between the extracellular and intracellular sides of the plasma membrane. The ion-conducting pore contains structural elements conferring ion selectivity, and also elements that can gate the pathway and regulate ion conduction through the channel.

Determination of the structure of the *Streptomyces lividans* K^+ channel KcsA at atomic resolution has proven to be a landmark achievement in the study of ion channels (Fig. 1A; Doyle et al., 1998; Zhou et al., 2001b). An image of two opposing KcsA subunits is illustrated in Fig. 1A. Each subunit of the KcsA channel consists of two transmembrane helices (M1 and M2), arranged with a four-fold axis of symmetry along the center of the permeation pathway, perpendicular to the plane of the plasma membrane. The M1 and M2 transmembrane helices exhibit relatively weak primary sequence identity with the corresponding S5 and S6

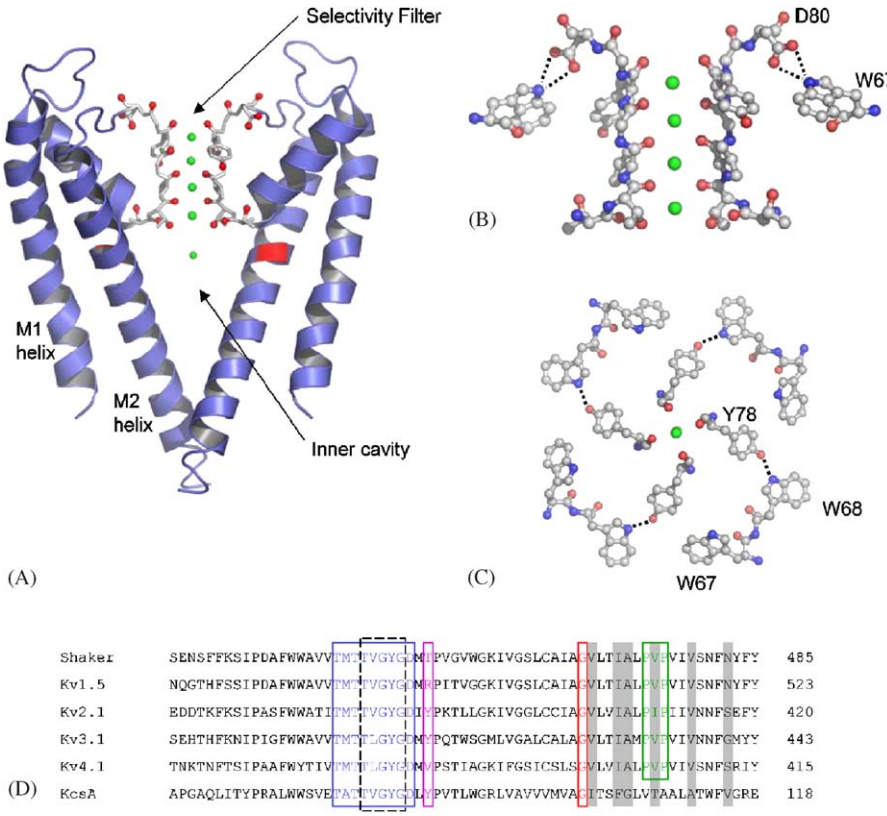

Fig. 1. Detailed features of the KcsA channel. (A) Side view of two opposing subunits of KcsA. Residues within the selectivity filter are illustrated in 'ball-and-stick' format, and magnified in panel (B). Potassium ions are represented as green spheres. Panels (B) and (C) also illustrate stabilizing interactions between residues in the selectivity filter and elsewhere in the channel. (B) Side chain carboxyl oxygen atoms of KcsA residue D80 (*Shaker* D447) are in very close proximity to the W67 (*Shaker* W434) side chain, and these two residues may form an intra-subunit hydrogen bond. (C) The side chain hydroxyl group of Y78 in KcsA (Y445 in *Shaker* GYG sequence) is stabilized by hydrogen bonding with W68 (*Shaker* W435) in an adjacent subunit, and van der Waals interactions with W67 (*Shaker* W434) within the same subunit. (D) Amino acid sequence alignment for KcsA, *Shaker*, and multiple mammalian Kv channel clones. Blue amino acids comprise the K^+ channel signature sequence, while those enclosed within the dashed line contribute backbone carbonyl oxygen atoms to the channel selectivity filter. The red box demarcates a conserved glycine termed the 'gating hinge', and the green box demarcates the Pro–X–Pro motif present in voltage-gated K^+ channels, but absent in KcsA and MthK. The purple box demarcates residues equivalent to *Shaker* T449. Gray shading indicates pore-lining amino acids in the S6 segment, which are predominantly hydrophobic in nature.

helices of voltage-gated K^+ channels, although there is much stronger sequence identity within the P region (see alignment in Fig. 1D). Furthermore, chimeric substitution of the S5–P–S6 region in *Shaker* with homologous sequence from KcsA allows the formation of functional K^+-selective and voltage-gated channels, suggesting that the KcsA structure provides a reasonable approximation of a Kv channel pore (Lu et al., 2001).

The P region of *Shaker* and its mammalian Kv1 homologues contain the TMTTVGYGD sequence, a subset of the TXXTXGYGD K^+ channel signature sequence, which forms the selectivity filter of the channel (Heginbotham et al., 1994). Amino acids forming the selectivity filter are highlighted in 'ball-and-stick' in Fig. 1A, and are magnified in Fig. 1B. This structural element of the channel allows selective permeation of K^+ ions, and excludes the other predominant physiological cation, Na^+. The selectivity filter is lined with backbone carbonyl oxygen atoms contributed by each channel subunit (Fig. 1B; Doyle et al., 1998), a structural arrangement of the protein backbone that depends critically on the flexibility of the glycine residues within the K^+ channel signature sequence (Valiyaveetil et al., 2004; MacKinnon, 2003). Selectivity for K^+ over Na^+ arises from close octahedral co-ordination of each K^+ ion in the filter by eight backbone carbonyl oxygen atoms, which closely match the dimensions of the hydration sphere of K^+ in solution (Zhou et al., 2001b). Thus, the selectivity filter provides a very hospitable milieu for K^+ ions in transit, resembling the hydrated conditions of bulk solution.

The geometry of the selectivity filter shown in Fig. 1B is optimal for co-ordination of K^+ ions, and is maintained by several critical interactions between residues in the K^+ channel signature sequence and residues elsewhere in the protein (Fig. 1B and C). Firstly, the planar arrangement of two adjacent tryptophan residues (W67 and W68 in KcsA, W434 and W435 in *Shaker*) forms an 'aromatic cuff' that interacts with the tyrosine residue in the K^+ channel selectivity filter (Fig. 1B and C). This residue (Y78 in KcsA, Y445 in *Shaker*) contributes a carbonyl oxygen near the extracellular entrance to the selectivity filter (Fig. 1B and C; Doyle et al., 1998). In the KcsA structure, the hydroxyl group of Y78 forms an intersubunit hydrogen bond with the side chain nitrogen of W68. In addition, significant intrasubunit van der Waals interactions are likely between Y78 and W67 (Fig. 1C; Doyle et al., 1998). Fig. 1B illustrates the relative positions of residues D80 and W67 in KcsA (*Shaker* residues D447 and W434, respectively), showing the close proximity of the carboxyl group of D80 with the side chain nitrogen atom in W67 (Doyle et al., 1998). A critical role for these amino acids is corroborated by numerous mutagenesis studies of Kv channels. In various cases, disruption of residues within the selectivity filter or the aromatic cuff has been shown to accelerate inactivation (in some instances sufficiently to render channels non-conducting), alter pore selectivity, and alter gating (Heginbotham et al., 1994; Perozo et al., 1993; Kirsch et al., 1995; Yang et al., 1997).

1.3. Conformational changes of the pore during activation

Like KcsA, the MthK channel (from *Methanobacterium thermoautotrophicum*) is formed by a rotationally symmetric tetramer of a 2 transmembrane domain subunit. Functional experiments have demonstrated that opening of the MthK channel is promoted by elevation of intracellular Ca^{2+}, and the determination of the MthK structure in the presence of Ca^{2+} has provided a plausible model for a gating mechanism of Kv channels (Jiang et al., 2002a). The KcsA and MthK structures exhibit a significant difference in the arrangement of the transmembrane M2 helices (Fig. 2). In the KcsA channel, the M2 helices form a constriction near the cytoplasmic side of the channel, referred to as the inner helix bundle, or bundle crossing (Doyle et al., 1998). In stark contrast, the M2 helices in MthK are wide open—no bundle crossing is apparent, and the aqueous central cavity of the channel is accessible to what would be the intracellular medium. These differences between the KcsA and MthK channel structures suggest a structural interpretation of channel opening, with the KcsA structure representing a closed/resting pore conformation, and the MthK structure representing an open pore (Jiang et al., 2002a, b; Doyle et al., 1998). Under conditions that promote channel opening, it has been suggested that the M2 helices (corresponding to the S6 helix of a Kv channel) undergo a conformational change dependent on the flexibility of a highly conserved glycine residue referred to as the 'gating hinge' (Gly 83 in MthK, red residues in Fig. 1D), in which the helices swing open and expose the internal cavity to the intracellular solution (Jiang et al., 2002b). The location of the 'gating hinge' is colored red in the KcsA and MthK structures in Figs. 1 and 2.

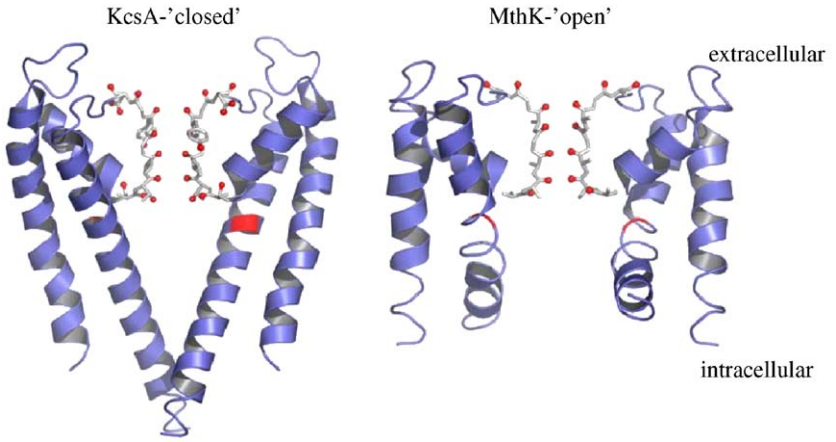

Fig. 2. KcsA and MthK structures. In the KcsA structure, the M2 helices form a constriction at the cytoplasmic entrance to the channel, called the inner helix bundle, or 'bundle crossing'. In the MthK structure, the M2 helices are splayed open around residue Gly83, the 'gating hinge', colored red in both structures. Motion of the M2 helix around the gating hinge has been suggested to underlie channel activation (Jiang et al., 2002b).

Can this gating model, based on the bacterial MthK and KcsA channels, be generalized to suggest a pore opening mechanism for Kv channels? In *Shaker* channels, accessibility studies of substituted cysteine residues in the channel pore clearly suggest the presence of a functional intracellular gate between residues 474 and 478, at a position corresponding to the KcsA bundle crossing (Liu et al., 1997; Del Camino and Yellen, 2001; Holmgren et al., 1997; Del Camino et al., 2000). In the closed state, pore-lining residues above and including *Shaker* residue 474 remain inaccessible to cations as small as Cd^{2+} or Ag^{+}, and to uncharged (MTSACE) or negatively charged (MTSES) thiol-reactive MTS reagents, suggesting the presence of a steric intracellular gate regulating ion flux. In addition, there is a conserved glycine residue analogous to MthK Gly 83 in the S6 segment of *Shaker* and other Kv channels (Fig. 1D, red residues). However, the presence of a conserved helix-breaking Pro–X–Pro motif in the S6 segment of Kv channels (Fig. 1D, green residues) suggests a kinked or bent S6 helix in both the open and closed channel conformations (Del Camino et al., 2000; Webster et al., 2004). This structural detail of the S6 helix differs from the α-helical arrangement of the M2 helices in the KcsA structure (Fig. 2), and below the 'gating hinge' in the MthK structure, since the Pro–X–Pro motif is absent in both KcsA and MthK, and the implications of these structural differences in our interpretation of activation gating have been reviewed recently in some detail (Swartz, 2004). Interestingly, there is no absolute requirement for the position or number of prolines in the S6 helix of Kv1.5 in order to maintain voltage-dependent gating (Labro et al., 2003), and it remains unclear whether the S6 proline residues in Kv channels provide an alternative hinge for channel opening, or whether they position S6 to allow coupling to voltage-sensing modules of the channel (Yellen, 2002).

Despite these important differences and uncertainties, both models espouse the general concept of a functional gate at the cytoplasmic entrance of the channel, formed by the S6 helices of Kv channels (Del Camino and Yellen, 2001; Jiang et al., 2002b). This picture of gating by the S6 helix is also generally consistent with the understanding of channel interactions with open-state channel blockers, such as quaternary ammonium ions. That is, the constriction formed at the intracellular entrance to the channel (by the M2 helices in the KcsA structure) occludes entry of quaternary ammonium ions into the permeation pathway. However, opening of the gate at the cytoplasmic entrance exposes the internal cavity of the channel, allowing the entry of organic cations. Rapid closure of the activation gate, as is possible in Kv channels, can also trap cations in the internal vestibule of the channel (Holmgren et al., 1997; Armstrong, 1971).

It should be noted that in other channel types, the establishment of a cytoplasmic gate analogous to the KcsA bundle crossing remains controversial. One prominent example is the cyclic-nucleotide gated (CNG) family of channels, in which accessibility of cysteine residues substituted within the pore to Cd^{2+} ions appears

to be essentially independent of whether the channel is open or closed, although the CNG pore can gate accessibility to MTS reagents (Flynn and Zagotta, 2001). While conformational rearrangements of S6 likely take place during CNG channel activation (Johnson Jr. and Zagotta, 2001), this observation argues against the existence of a tight steric gate on the cytosolic side of CNG channels that is able to gate the passage of permeant ions. Similar suggestions have been made for inwardly rectifying potassium (Kir) channels. For instance, some reports suggest that Ba^{2+} blockade of the ATP-sensitive K^+ channel (K_{ATP}) is also essentially state-independent (Proks et al., 2003), and similar suggestions have been made for the strong inward rectifier Kir2.1 (Xiao et al., 2003). However, others have clearly demonstrated trapping of intracellular organic blockers such as spermine by ATP-mediated channel closure of Kir 6.2 (Phillips and Nichols, 2003), analogous to the 'blocker-trapping' experiments described above for Kv channels, and have also demonstrated gated accessibility of MTS reagents to the inner cavity of K_{ATP} channels (Phillips et al., 2003).

In addition to the steric gate on the intracellular side of Kv channels, some evidence suggests that Kv channel activation may also involve conformational changes of the selectivity filter (Chapman et al., 1997). Most notable are single channel studies demonstrating altered ionic selectivity in subconductance levels that appear during activation and deactivation (Zheng and Sigworth, 1997, 1998). In particular, subconductance states of T442S mutant *Shaker* channels, or WT *Shaker* and T442S heteromultimers, exhibit enhanced permeability for K^+ over Rb^+ or NH_4^+ relative to the fully open state (Zheng and Sigworth, 1997, 1998). Others have also observed activation-dependent changes in accessibility of cysteine residues substituted in the pore helix of CNG channels (Sun et al., 1996; Liu and Siegelbaum, 2000), although this observation has not been confirmed in a Kv channel. Collectively, these observations suggest that Kv channel opening at the cytoplasmic bundle crossing may also be accompanied by conformational changes around the selectivity filter.

Although recent structural work has provided many novel insights into the underlying principles of ion permeation, and conformational changes underlying Kv channel activation, insights into detailed aspects of K^+ channel inactivation (and particularly 'slow', non-N-type mechanisms) have been less forthcoming. In the following sections, we review the basic functional aspects of various mechanisms of Kv channel inactivation, and relate these to the fine details of K^+ channel structure discussed thus far.

2. N-type inactivation

N-type inactivation is frequently described as the 'ball-and-chain' mechanism of inactivation, in which a sequence of amino acids (the inactivation domain) at the N-terminus of the channel occludes the intracellular channel pore and prevents ion permeation. The N-type inactivation mechanism underlies a very rapid inactivation process in *Shaker* channels, and several mammalian Kv channels including Kv1.4 and Kv3.4 (Hoshi et al., 1990; Rasmusson et al., 1995; Beck et al., 1998). In addition, the association of Kv1 channels with certain auxiliary Kvβ subunits can also confer rapid inactivation by an N-type mechanism (Sewing et al., 1996; Morales et al., 1995; Accili et al., 1997). Furthermore, a recent study has suggested that the N-terminus of Kv4 channels can confer a rapid inactivation phenotype, with properties consistent with N-type inactivation, in chimeric constructs with the transmembrane domains of Kv2.1 and Kv1.4, leading to the suggestion that an N-type mechanism accounts for the rapid component of the multi-exponential decay that is characteristic of Kv4 channels (Gebauer et al., 2004).

The 'ball-and-chain' model of inactivation is illustrated in cartoon form in Fig. 3 and has been confirmed by several lines of evidence. Most importantly, N-type inactivation is abolished by deletion or enzymatic removal of the N-terminus, and can be restored by subsequent application of exogenous peptides derived from the N-terminus of one of many N-type inactivating channels (Antz et al., 1999; Zagotta et al., 1990; Murrell-Lagnado and Aldrich, 1993b). A tetrameric K^+ channel can contain multiple inactivation domains, and these are thought to behave roughly independently (Gomez-Lagunas and Armstrong, 1995), although some reports have suggested a small degree of negative co-operativity between inactivation domains (Hashimoto et al., 2000). A single inactivation domain is sufficient to confer N-type inactivation (MacKinnon et al., 1993; Lee et al., 1996).

Another important characteristic of N-type inactivation is sensitivity to intracellular pore blockers, notably TEA^+ and its derivatives (Choi et al., 1991). This effect arises because the N-terminal inactivation domain and cytosolically applied QA ions compete for identical or nearby/overlapping binding sites, and so their pore

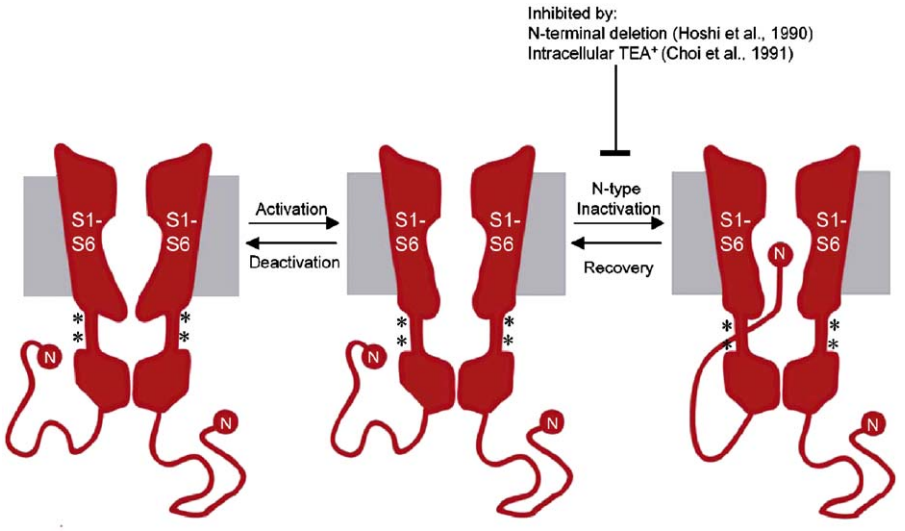

Fig. 3. Cartoon representation of N-type inactivation. Upon channel opening, the N-terminal inactivation domain is able to access its binding site within the inner cavity of the channel. To access this site, the N-terminal inactivation domain must pass through one of four lateral openings, or fenestrations, formed by T1–S1 linkers (Sokolova et al., 2001). Interactions between positively charged residues in the inactivation domain, and negatively charged acidic residues in the T1–S1 linker sequence (shown as asterisks) promote entry of the inactivation domain into the channel pore (Gulbis et al., 2000).

occupancy is mutually exclusive (Choi et al., 1991, 1993; Baukrowitz and Yellen, 1996a; Zhou et al., 2001a). Therefore, intracellular QA ions generally slow the process of N-type inactivation, whereas application of extracellular TEA^+ has no effect on this process (Choi et al., 1991). The apparent kinship between N-type inactivation and intracellular blockade by quaternary ammonium ions is also reflected in their similar effects on gating currents. The onset of either process results in a significant slowing of gating charge return upon repolarization, termed 'charge immobilization' (Roux et al., 1998). The prevailing structural interpretation of charge immobilization by N-type inactivation is that occupancy of the inner cavity by either a blocking ion or inactivation domain interferes with closure of the activation gate, and thereby delays the return of voltage-sensing elements to their resting conformation.

2.1. Structural determinants of N-type inactivation

Detailed studies of the efficacy of isolated N-terminal inactivation domains (inactivation peptides) from multiple channels, and several synthetic inactivation peptides, revealed fairly non-specific requirements for effective blockade of ionic currents. The basic requirements were a series of ~10 hydrophobic amino acids, followed by a series of amino acids with a net positive charge. Furthermore, modification of the charged amino acids generally altered the binding rate of the inactivation peptide, whereas mutations of the hydrophobic amino acids predominantly affect unbinding (Murrell-Lagnado and Aldrich, 1993a, b). These data led to speculation that hydrophobic residues within the inactivation domain interact directly with hydrophobic regions of the channel, while long-range electrostatic interactions between charged residues might effectively increase the local concentration of the inactivation domain and promote entry to its binding site (Murrell-Lagnado and Aldrich, 1993a, b; Aldrich, 2001). These results are also consistent with the identification of several cellular mechanisms of modulation of N-type inactivation that act by altering the net charge of the inactivation domain. For instance, protein kinase C phosphorylation of multiple serine residues in the Kv3.4 N-terminus dramatically attenuates the N-type inactivation normally observed in this channel (Beck et al., 1998; Antz et al., 1999). Similar effects have been attributed to protein kinase A phosphorylation

of serine 24 in the N-terminus of the Kvβ1.3 subunit (Kwak et al., 1999). Although some of these effects have been attributed to altered secondary structure of the N-terminal domain (Antz et al., 1999), in both instances the site of phosphorylation is distinct from the hydrophobic N-terminal residues currently thought to interact with the inner cavity of Kv channels (Zhou et al., 2001a). In what may be a complimentary mechanism, others have demonstrated that PKA phosphorylation of the C-terminus of *Shaker* stabilizes N-type inactivation (Drain et al., 1994). This effect may arise by the addition of negative charge near the inactivation domain receptor, in a location able to interact with positively charged residues in the *Shaker* N-terminus. Other examples of chemical modifications of the channel N-terminus impacting N-type inactivation include the inhibitory effect of Src tyrosine kinase phosphorylation of tyrosine 8 in the *Shaker* N-terminus (Encinar et al., 2002), and the stimulatory effects of intracellular acidification on Kv1.4 inactivation gating due to protonation of N-terminal histidine residue 16 (Padanilam et al., 2002).

How do these structural determinants of the N-terminal inactivation domain fit into current models of channel structure? Firstly, N-type inactivation is generally described as being dependent on channel opening, although some apparent exceptions have been identified (Murrell-Lagnado and Aldrich, 1993a, b; Ayer Jr. and Sigworth, 1997). This view of the inactivation peptide as an open channel blocker that binds to the intracellular cavity of the channel (Demo and Yellen, 1991) is consistent with the presence of a cytoplasmic gate formed by the S6 helices in K^+ channels. As suggested by the KcsA and MthK structures (Doyle et al., 1998; Jiang et al., 2002b), and scanning cysteine accessibility studies (Liu et al., 1997; Del Camino et al., 2000), channel opening exposes the intracellular cavity that can readily accommodate large intracellular agents such as the N-terminal inactivation domain, or bulky QA compounds (Holmgren et al., 1997). Pore-lining residues in KcsA and other K^+ channels are predominantly hydrophobic, and therefore may form a binding partner for the hydrophobic residues in the N-terminal inactivation domain (Zhou et al., 2001a). In addition, several studies clearly suggest that the inactivation domain must be located at the immediate N-terminus of the channel to effectively confer N-type inactivation, as inactivation domains do not exhibit significant blockade of current if buried within the N-terminal sequence (Wissmann et al., 2003; Hollerer-Beitz et al., 1999; Kondoh et al., 1997). This is consistent with results of a thermodynamic mutant cycle analysis between the Kvβ1 N-terminus and the inner pore of Kv1.4, which suggested that the N-terminal inactivation domain enters the pore as an extended peptide with minimal secondary structure (Gulbis et al., 2000; Zhou et al., 2001a). Finally, a role for positively charged residues in the inactivation domain has emerged from work demonstrating the likely presence of complementary acidic residues in the linkers between the T1 domain and the S1 transmembrane segment (Fig. 3; Gulbis et al., 2000). As described, these linkers form four fenestrations that likely constitute an obligatory pathway for the N-terminus to reach the intracellular mouth of the channel pore (Gulbis et al., 2000; Aldrich, 2001; Sokolova et al., 2001). Interactions between N-terminal basic residues and acidic residues in the T1–S1 linker have been suggested to promote entry and thereby effectively stabilize binding of the inactivation domain (shown in cartoon form in Fig. 3; Gulbis et al., 2000).

Interestingly, this structural picture is generally consistent with a variation of N-type inactivation identified among the genes encoding β subunits of BK channels. Co-expression of the BK channel with the β2 subunit confers a rapid inactivation phenotype that depends on the N-terminal residues of the β2 subunit (Xia et al., 2003). Unlike the Kvβ subunits, which are soluble cytoplasmic proteins that interact with the cytoplasmic Kv channel T1 domain, the BK β subunits are transmembrane proteins with two transmembrane domains and cytoplasmic N- and C-termini (Lingle et al., 2001; Xia et al., 2000). However, by analogy to Kvβ-subunit-mediated inactivation of Kv channels, the BK β2 subunit mediates its effects by entry of the immediate N-terminal amino acids into the cavity, likely with little or no ordered structure. As for N-type inactivation of Kv channels, this process also appears to depend on hydrophobic interactions within the inner cavity of the BK channel.

2.2. Regulation of N-type inactivation, new questions and future directions

The preceding description of N-type inactivation is relatively straightforward, and the understanding of this process has likely been greatly assisted by its many similarities to blockade by compounds in the inner cavity of Kv channels. However, recent work centered upon the regulation of N-type inactivation has revealed many complexities and variations, suggesting that our understanding of this process is incomplete. Roeper and

colleagues have identified a domain in the N-terminus of Kv1.6 channels that appears to preclude N-type inactivation during co-expression with Kvβ1.1, or in heteromultimers with Kv1.4 (Roeper et al., 1998). Furthermore, chimeric transfer of this domain to the Kv1.5 channel also confers resistance to N-type inactivation, leading this domain to be named the 'N-type Inactivation Preventing' (NIP) domain. This sequence does not appear to prevent co-assembly with auxiliary β subunits, and so it may act by either neutralizing the N-terminal inactivation domain or impeding access to its receptor site. In addition, the actions of the NIP domain exhibit some degree of specificity, as this domain does not inhibit N-type inactivation of Kv1.6 co-expressed with Kvβ3, suggesting that many complexities of this process are unresolved (Bahring et al., 2004). Interestingly, some kinetic effects of KChIPs, auxiliary subunits of Kv4 channels, may also be mediated by immobilization of the immediate N-terminus of the Kv4 channel, as co-expression of KChIPs and Kv4 channels appears to abolish the fastest kinetic component of Kv4 inactivation (similar to short N-terminally deleted Kv4 channels). Furthermore, crystal structures of the Kv4 T1 domain reveal a large hydrophobic pocket that has been proposed to accommodate and possibly sequester the hydrophobic Kv4 N-terminus (Scannevin et al., 2004).

A further generally unresolved issue in studies of N-type inactivation is the role of secondary/tertiary structure of the N-terminal inactivation domain. While NMR structures of various N-terminal inactivation domains have revealed significant structural heterogeneity, it is unclear what role this plays in inactivation or recovery, in light of studies mentioned previously that suggest that the inactivation domain enters the inner cavity of the channel as an extended peptide with little or no secondary structure (Antz et al., 1997, 1999; Wissmann et al., 2003). One possibility is that variable structures of the inactivation domain may determine association with regulatory elements before the onset of inactivation, such as the NIP domain or anionic phospholipids. Also in this context, NMR studies of the Kv1.4 N-terminus have revealed the presence of two inactivation domains, confirming several previous electrophysiological studies (Wissmann et al., 2003; Kondoh et al., 1997; Hollerer-Beitz et al., 1999). Deletion of either inactivation domain in the Kv1.4 N-terminus exhibits unique effects on inactivation or recovery, so both domains appear to be required for normal inactivation behavior of the channel (Wissmann et al., 2003). It has been suggested that one inactivation domain may serve a 'pre-docking' function, which primes the channel for rapid N-type inactivation, although the exact mechanisms of action of the Kv1.4 N-terminal domains remain unclear.

An additional novel mechanism of regulation of N-type inactivation by membrane phospholipids has also been described. In particular, enrichment of the plasma membrane with anionic phospholipids (e.g. PIP2) can inhibit N-type inactivation in Kv1.4 or in Kv1.1 co-expressed with a Kvβ subunit, effectively converting 'A-type' currents into classical delayed rectifiers (Oliver et al., 2004). This mechanism appears to be mediated by levels of PIP2 within a physiological range, and may therefore provide an important means to regulate cellular excitability and the properties of action potentials. Importantly, the mechanism of inhibition by PIP2 appears to depend upon neutralization/sequestration of the N-terminal inactivation domain, rather than occlusion of its binding site, since blockade by exogenous inactivation peptides derived from the N-terminus of Kv1.4 is unaffected by the addition of PIP2 to the excised patch (Oliver et al., 2004).

3. C-type inactivation

Early studies of *Shaker* channel inactivation clearly demonstrated that elimination of fast N-type inactivation by deletion of the *Shaker* N-terminal inactivation domain revealed a residual slow inactivation process (Hoshi et al., 1990). This slower inactivation was termed C-type inactivation, because its rate depended upon the identity of C-terminal *Shaker* splice variants, and particularly upon the identity of *Shaker* residue 463 (Hoshi et al., 1991). However, as described below, it is now thought that C-type inactivation primarily involves conformational changes around the selectivity filter and extracellular entrance to the channel. The C-type inactivation mechanism in *Shaker* can be clearly distinguished from N-type inactivation, because C-type inactivation is not abolished by deletion of N-terminal residues, nor is it substantially inhibited by intracellular TEA^+ (Choi et al., 1991). C-type inactivation is inhibited by elevated concentrations of extracellular K^+ (Hoshi et al., 1990; Lopez-Barneo et al., 1993), and this observation has been confirmed in several mammalian *Shaker* homologues (Fedida et al., 1999; Rasmusson et al., 1995). Also, in channels with pore residues permissive for TEA^+ binding, extracellular TEA^+ is also able to inhibit C-type inactivation

(Grissmer and Cahalan, 1989; Molina et al., 1997; Choi et al., 1991). Collectively, these observations have been interpreted as a 'foot in the door' mechanism, in which occupancy of an ion binding site on the extracellular side of the channel by K^+ or TEA^+ slows or prevents the conformational changes required for C-type inactivation (Rasmusson et al., 1998). As mentioned, this effect contrasts significantly with the N-type mechanism, which is insensitive to extracellular K^+ or TEA^+ (Choi et al., 1991).

C-type inactivation in *Shaker* channels can also be dramatically altered by mutations in the extracellular pore region (Lopez-Barneo et al., 1993). Mutagenesis studies of the extracellular mouth of the *Shaker* pore identified an important role for residue T449 (Fig. 1D, purple box) in the regulation of the inactivation rate in *Shaker*. Substitution of certain residues (arginine, lysine, glutamate, alanine) at this site dramatically accelerated the inactivation rate (Lopez-Barneo et al., 1993). In contrast, substitution at this site with valine or tyrosine dramatically inhibited inactivation. These effects contrast with the N-type inactivation mechanism, as studies in multiple channels have demonstrated that mutations at *Shaker* T449 or its equivalent residue in other Kv1 channels does not significantly affect the time course of N-type inactivation (Rasmusson et al., 1995; Baukrowitz and Yellen, 1995).

Importantly, the effects of mutations at T449 (or equivalent residues) as determined for *Shaker* are not universal among Kv1 channels. As an example, Kv1.4 and Kv1.5 possess a lysine and an arginine at this site, respectively, and both exhibit much slower rates of C-type inactivation than the T449R or T449 K *Shaker* mutants (Rasmusson et al., 1995; Fedida et al., 1999). Also, mutation of this residue to either a valine or a tyrosine has small effects on the C-type inactivation rates in Kv1.4 or Kv1.5 (Fedida et al., 1999; Rasmusson et al., 1995). Furthermore, in Kv1.2 a valine is present at the *Shaker* T449 equivalent position, yet at physiological pH this channel exhibits inactivation rates comparable to those observed in Kv1.5 (Russell et al., 1994; Steidl and Yool, 1999). Thus, among the mammalian Kv1 channels, the identity of the *Shaker* T449 equivalent residue exerts weaker effects on the time course of C-type inactivation than in the *Shaker* channel itself, and may not be a universally good predictor of the C-type inactivation rate.

A final critical distinction between C-type and N-type inactivation arises from their respective functional stoichiometry. As described previously, multiple N-terminal inactivation domains in a K^+ channel behave essentially independently, and only one is necessary to confer N-type inactivation (MacKinnon et al., 1993; Lee et al., 1996). In contrast, experiments in both *Shaker* and Kv1.3 suggest that the C-type inactivation transition is a co-operative transition of all four-channel subunits. Using chimeras or co-expression of channels with different rates of C-type inactivation (*Shaker* A463 or V463, and equivalent mutations in Kv1.3), several groups demonstrated that all four subunits of a channel contribute to the activation energy of the C-type inactivation transition (Panyi et al., 1995; Ogielska et al., 1995). This demonstration of co-operativity has also been confirmed with mutations at other locations in the *Shaker* channel (Ogielska et al., 1995; Larsson and Elinder, 2000). These findings suggest that C-type inactivation does not arise from independent gates in individual subunits (as for the N-type mechanism), but rather a co-operative rearrangement of all four subunits of the channel.

3.1. Regulation of C-type inactivation

Unlike the N-type mechanism of inactivation, very few mechanisms of cellular/physiological regulation of C-type inactivation have been described (one exception is the interplay between N- and C-type inactivation, discussed in detail in upcoming sections). One frequently described mechanism of regulation of K^+ channel availability is the effects of extracellular K^+. In a number of K^+ channel types, increasing the extracellular K^+ concentration increases the observed current magnitude—a potentiation effect that is opposite to that expected based on driving force effects. In several instances, as in the Kv1.4 and hERG channels, this effect can be extremely profound and has been tied to an inactivation process, although the relevance of K^+ potentiation in a biological process has not been specifically addressed. The Kv1.4 and Kv1.5 channels also exhibit a unique and remarkable interplay between extracellular pH and K^+ concentration (Kehl et al., 2002; Claydon et al., 2004; Li et al., 2003). These channels are both inhibited by extracellular acidification, due to protonation of a histidine residue in the outer pore (H463 in Kv1.5 and H508 in Kv1.4), and increasing the extracellular K^+ concentration relieves this inhibition (Kehl et al., 2002). This antagonism between extracellular K^+ and H^+ ions also appears to be related to channel inactivation and changes in channel

availability, and may play a role in regulating K^+ channel availability during periods of anoxia or hyperexcitability where pH and extracellular K^+ concentrations are known to change. There are very few descriptions of regulation of C-type inactivation by phosphorylation, and the reported effects are generally far more subtle than those reported for the N-type inactivation process. One example is a receptor-linked tyrosine kinase phosphorylation of Kv1.3 channels that results in a slight acceleration in the time course of C-type inactivation (Bowlby et al., 1997). However, the exact molecular determinants of this effect remain unknown, and identification of specific phosphorylation sites mediating C-type inactivation of Kv1.3 has proven difficult and complex.

3.2. Synergistic interactions of N- and C-type inactivation

An important conclusion arising from N-terminal deletion studies of *Shaker* channel inactivation is that multiple mechanisms of inactivation can co-exist and interact in Kv channels, and studies examining these interactions have contributed significantly to our understanding of C-type inactivation. Characterization of both *Shaker* and Kv1.4 has demonstrated that despite causing a much more rapid inactivation rate, recovery from inactivation is unaffected by the presence of the N-type inactivation domain (Rasmusson et al., 1995; Baukrowitz and Yellen, 1995), showing that it is the C-type mechanism that governs the time course of recovery in channels with both N- and C-type inactivation. Additionally, Baukrowitz and Yellen (1995) made the important observation that use-dependent reductions of peak current in *Shaker* could be abolished either by removal of N-type inactivation (through N-terminal truncation of the channel), or by slowing of C-type inactivation (by the T449V point mutation in the extracellular pore). They reasoned that use-dependent current reduction in *Shaker* arose from an interaction between the N- and C-type mechanisms, and suggested that N-type inactivation actually promotes and accelerates the onset of the C-type inactivation process. A further critical observation was that use-dependent current reduction in *Shaker* was strongly dependent on the concentration of extracellular K^+. This observation was extended to demonstrate that quaternary ammonium ions, which are intracellular open pore blockers of K^+ channels, also promoted C-type inactivation when applied intracellularly, in a manner highly dependent on extracellular K^+ concentration (Baukrowitz and Yellen, 1995, 1996b).

These experiments culminated with the proposal that C-type inactivation depends on the occupancy of a highly K^+-selective 'control site' located near the extracellular mouth of the channel, and likely within the selectivity filter of the channel (Baukrowitz and Yellen, 1995). It was hypothesized that certain permeant ions including K^+ or Rb^+, but not Na^+, can occupy this site and stabilize the open state, preventing C-type inactivation (Baukrowitz and Yellen, 1995; Yellen, 1998). In the simplest conception of this model, blockade of outward K^+ permeation by N-type inactivation allows K^+ ions to evacuate the K^+ channel pore and 'control site', and prevents outward-flowing K^+ ions from replenishing this binding site. If extracellular K^+ concentration is high, prolonged evacuation of K^+ from the pore is unlikely, and so the onset of C-type inactivation is delayed or eliminated. In contrast, in minimal concentrations of extracellular K^+, ions are absent from the control site for longer durations, and C-type inactivation is accelerated (Baukrowitz and Yellen, 1996b). Delineation of this mechanism of interaction between the N- and C-type mechanisms of inactivation provided a critical foundation for the current understanding of the C-type inactivation process, with the realization that C-type inactivation is fundamentally affected by ion occupancy of the pore (Baukrowitz and Yellen, 1995, 1996b).

3.3. Allosteric coupling of N- and C-type mechanisms

An 'allosteric' mechanism of coupling between N- and C-type inactivation has also been suggested, in which direct structural interactions between the N-terminal inactivation domain (or intracellular blockers such as quinidine) and the inner pore of the channel govern the onset of C-type inactivation, and outweigh the effects of K^+ depletion (Rasmusson et al., 1998; Bett and Rasmusson, 2004; Wang et al., 2003). It is now recognized that various quaternary ammonium derivatives can exhibit a spectrum of allosteric effects on C-type inactivation. Furthermore, 4-AP appears to antagonize C-type inactivation (and prevent charge immobilization) in *Shaker* and mammalian Kv1 channels despite its prolonged dwell time in the pore and blockade of K^+

flux (Russell et al., 1994; Rasmusson et al., 1998; Castle et al., 1994; Fedida et al., 1996), although this effect may arise by a destabilization of channel opening, rather than destabilization of C-type inactivation itself (Choquet and Korn, 1992; McCormack et al., 1994). Presumably, this variability arises from detailed differences in the structural interactions between various blockers and the inner Kv channel pore. At various times, it has been proposed that binding of the N-terminal inactivation domain immobilizes the voltage sensors for as long as the N-terminus remains bound to the channel, and that this immobilization of the voltage sensors in their activated conformation promotes C-type inactivation (Rasmusson et al., 1998). More recently, it has been proposed that lipophilic interactions between the Kv channel inner cavity and the inactivation domain causes a reorientation of the S6 transmembrane domain into a conformation that resembles or promotes C-type inactivation (Bett and Rasmusson, 2004). While allosteric interactions between the pore and the N-type inactivation domain or other intracellular blockers seem likely (Baukrowitz et al., 1996a, b; Rasmusson et al., 1998), structural interpretations of this process remain vague, and have not yet provided detailed insights into the mechanism of C-type inactivation.

3.4. Role of the selectivity filter in C-type inactivation

The role of the selectivity filter in C-type inactivation has been highlighted and refined by studies of Na^+ permeation through Kv channels during channel inactivation. In particular, when K^+ ions are absent from recording solutions, and replaced with Na^+ ions, *Shaker* inactivation is significantly accelerated, and its inactivated state retains a significant permeability to Na^+ ions. This is apparent as a Na^+ current that persists after the onset of inactivation (Starkus et al., 1997). These findings, and similar results in other channels, have many important implications regarding the conformational changes associated with C-type inactivation (Starkus et al., 1997; Wang et al., 2000a, b). Firstly, the rapid rate of inactivation observed with Na^+ as the permeant ion suggests that Na^+ does not inhibit C-type inactivation with nearly the potency of K^+, consistent with the results of Baukrowitz and Yellen (1995). This may arise either because Na^+ does not interfere with (or may promote) closure of the C-type inactivation gate, or because Na^+ has a relatively weak affinity and/or brief dwell time at the ion binding site regulating C-type inactivation (Ogielska and Aldrich, 1999; Starkus et al., 1997). In addition, the persistent Na^+ current through inactivated channels suggested that C-type inactivation does not entail a complete closure/collapse of the permeation pathway, but involves a conformational change at or near the selectivity filter that remains permissive for Na^+ permeation (Yellen, 1998).

Careful examination of the effects of extracellular K^+ ions on the Na^+ permeation properties of Kv channels has yielded further insight into the role of the selectivity filter in C-type inactivation (Kiss and Korn, 1998). In general, extracellular K^+ exerts two very significant effects on Na^+ permeation. Firstly, addition of micromolar concentrations of extracellular K^+ results in a reduction of Na^+ currents through the channel. This is a reflection of competition for binding sites within the selectivity filter. Tightly binding K^+ ions are able to exclude more weakly binding Na^+ ions, and thereby impede Na^+ permeation through the channel (Korn and Ikeda, 1995; Kiss et al., 1998). Secondly, addition of extracellular K^+ results in slower inactivation of macroscopic currents (Kiss et al., 1998). Importantly, Kiss and Korn observed that the K^+ concentration dependence of these two effects were superimposable, and highly K^+ selective, as both effects exhibited a K_d for K^+ of roughly 30 μM in the presence of 145 mM extracellular Na^+. These experiments, conducted in a chimeric channel consisting of the Kv1.3 pore fused into the Kv2.1 channel (Gross et al., 1994), suggested that a high-affinity K^+ binding site(s) underlying selectivity for K^+ over Na^+ (e.g. within the selectivity filter) also underlies K^+ inhibition of C-type inactivation (Kiss et al., 1998). Similar results from Kv1.5 also support this conclusion, although addition of small concentrations of extracellular K^+ actually results in mild potentiation of Na^+ through Kv1.5, masking any blockade of Na^+ current by K^+ competition in the pore. However, small concentrations of K^+ are again sufficient to inhibit C-type inactivation, even in the presence of much larger concentrations of Na^+ ions (Wang et al., 2000b).

Additional support for the role of the selectivity filter arises from apparent changes in ionic selectivity during inactivation (Kiss et al., 1999). In particular, prolonged depolarizations can generate significant changes in the reversal potential of macroscopic ionic currents through Kv channels, as they shift from an open state that is highly K^+ selective to an inactivated state that conducts Na^+ ions with greater facility

(Kiss et al., 1999). Under appropriate ionic conditions, it is possible to observe complete reversal of the direction of ionic current during a single depolarization, due to relative changes in the K^+/Na^+ permeabilities during inactivation. Although most of these experiments were performed using Kv2.1 as a model channel, Kiss et al. (1999) reported similar results for a *Shaker* mutant (A463C), and the Kv1.3/Kv2.1 chimeric construct mentioned above (Gross et al., 1994), suggesting that conformational changes of the selectivity filter underlying C-type inactivation may be conserved in multiple Kv channel subtypes (Kiss et al., 1999). Importantly, interpretation of these results has been met with some criticism, as significant intracellular K^+ depletion or accumulation during whole-cell recordings have also been shown to alter the reversal potential (Frazier et al., 2000). Nevertheless, similar changes in selectivity have been reported in C-type inactivated *Shaker* channels, using the inside-out patch clamp configuration, which is likely much less prone to artifacts resulting from ion accumulation or depletion (Starkus et al., 1997, 1998).

In *Shaker*, it should be noted that the K^+ concentrations required for inhibition of C-type inactivation are somewhat larger than the concentrations required for blockade of Na^+ current. In *Shaker* A463C channels, 50 μM extracellular K^+ can significantly block Na^+ permeation, without slowing the time course of inactivation (Ogielska and Aldrich, 1998, 1999). This diverges somewhat from the reports described earlier, in which the K^+ affinities for Na^+ current blockade and inhibition of C-type inactivation were identical (Kiss et al., 1998), but the basis for this difference is unresolved. In contrast to the 30–50 μM value reported by Kiss et al. (1998), Ogielska and Aldrich (1998) report a K^+ affinity for inhibition of C-type inactivation closer to 2 mM, which is similar to the earlier estimate of Baukrowitz and Yellen in *Shaker* (Baukrowitz and Yellen, 1995). This observation implies that K^+ may occupy a very-high-affinity binding site (presumably within the selectivity filter) without inhibiting C-type inactivation, prompting the suggestion that conformational changes of the selectivity filter underlying C-type inactivation may be extremely localized (Ogielska and Aldrich, 1999).

Other experimental data also supports the notion that C-type inactivation involves a highly localized change in the conformation of the selectivity filter. Firstly, channel blockade by Ba^{2+} does not impede C-type inactivation, and it has been shown that C-type inactivation can actually trap Ba^{2+} within the *Shaker* channel pore (Harris et al., 1998). Since the high-affinity Ba^{2+} binding site is thought to overlap with a K^+ binding site at the cytoplasmic end of the selectivity filter (Jiang and Mackinnon, 2000), this data suggests that C-type inactivation may require only a very localized constriction of the selectivity filter. Also, C-type inactivated channels, while impermeant to K^+, seem to maintain some elements of K^+ selectivity. As evidence for this, sub-millimolar concentrations of K^+, particularly in the intracellular medium, are able to inhibit Na^+ permeation through C-type inactivated *Shaker* channels (Starkus et al., 1997), suggesting that the C-type inactivated pore retains the ability to bind at least one K^+ ion with high affinity over Na^+. These observations suggest that constriction of the filter during C-type inactivation may occur in only a restricted segment, possibly near the extracellular side of the filter (Harris et al., 1998; Ogielska and Aldrich, 1999; Yellen, 1998).

X-ray structures of the KcsA channel solved in different ionic conditions have also demonstrated multiple conformations of the selectivity filter. Of particular interest to this discussion of C-type inactivation is the 'low potassium' structure of KcsA, solved with Na^+ as the predominant cation. In the presence of low concentrations of K^+, the KcsA selectivity filter exhibits a distorted structure, with the backbone carbonyls no longer directed towards the central axis of the pore (Zhou et al., 2001b). It has been suggested that this partially collapsed structure of the selectivity filter may resemble the C-type inactivated K^+ channel pore, although there is no definitive data correlating C-type inactivation with the low K^+ structure (Yellen, 2001, 2002). Nevertheless, this structure suggests that the selectivity filter is a dynamic, non-rigid element of the channel, with a detailed structure determined by the balance of stabilizing interactions with other residues in the channel and the occupant ions.

3.5. Conformational changes underlying C-type inactivation

Other experimental approaches have also added to our understanding of conformational changes at the extracellular mouth of the channel during C-type inactivation. Yellen et al. (1994) demonstrated that substitution of a cysteine for *Shaker* residue T449, at the extracellular entrance to the selectivity filter, allowed modulation of slow inactivation by the addition of extracellular divalent cations including Zn^{2+} and Cd^{2+}.

The onset of C-type inactivation resulted in a dramatic increase in the affinity for metal binding in the T449C *Shaker* mutant, and therefore stabilization of inactivation in this mutant in the presence of extracellular Cd^{2+} (Yellen et al., 1994). These results clearly suggested a conformational rearrangement around the T449 residue during C-type inactivation. In an extension of this study, Liu et al. (1996) examined changes in accessibility of several substituted cysteine residues in the extracellular pore mouth during various gating states, and demonstrated a dramatically accelerated rate of cysteine modification by thiol-reactive MTS reagents of *Shaker* pore residues 448–450, after channels had undergone C-type inactivation. This modification rate was relatively insensitive to activation and deactivation transitions of the channel, and these results further suggested a conformational rearrangement of the outer mouth of the K^+ channel pore during inactivation (Liu et al., 1996). Also consistent with conformational changes of the extracellular pore mouth, is the demonstration that the rate of chloramine T oxidation of *Shaker* residue M448 depends upon the rate of C-type inactivation, as determined by residues substituted at position 449 (Schlief et al., 1996).

Others have also examined the time dependence of conformational changes around the extracellular entrance to the channel pore by tagging introduced cysteine residues with fluorescent reporter dyes. Conformational changes that alter environmental quenching of the dye affect the fluorescence emission, and these changes can be tracked with various optical methods. Fluorescent dyes introduced immediately above the selectivity filter at residue 449 (Cha and Bezanilla, 1997), and in several other residues throughout the extracellular mouth of the channel (Loots and Isacoff, 1998, 2000; Gandhi et al., 2000) exhibit changes in emission upon depolarization, with kinetics that approximate the time course of C-type inactivation in these mutants.

Conformational changes at the intracellular side of the channel remain poorly understood and have been less widely studied, especially among Kv channels. However, conformational changes of this region may be of considerable interest in the context of state-dependent drug interactions in the inner channel pore. This is particularly true in the hERG channel, where conformational changes of the S6 helix during C-type inactivation have been suggested to underlie high-affinity methanesulfonanilide drug binding (Mitcheson et al., 2000; Chen et al., 2002). In addition, one study has demonstrated that elevation of intracellular tonicity results in acceleration of C-type inactivation in Kv1.4, suggesting that C-type inactivation is accompanied by a significant constriction of the inner cavity (Jiang et al., 2003a). If Kv channels do indeed undergo a constriction of the inner cavity during C-type inactivation, it seems unlikely that this is sufficient to occlude $K+$ permeation or constitute a C-type inactivation 'gate', as recent work has demonstrated that the inner cavity of Kv1.5 remains accessible to quaternary ammonium blockers, and to the N-terminal inactivation domain of Kv1.4, after the onset of C-type inactivation (Kurata et al., 2004).

3.6. A structural conception of conformational changes during C-type inactivation

Work by Larsson and Elinder (2000) has delineated the importance of *Shaker* residue E418 in the regulation of C-type inactivation. They demonstrated that disulfide linkage between cysteine residues substituted at E418 (in the *Shaker* S5 segment) and V451 (in the P region) stabilized C-type inactivation. In contrast, disulfide linkage of E418C to G452C appeared to slow inactivation and favor the open state of the channel. These observations have led to speculation of possible conformational changes of the outer pore during C-type inactivation, illustrated in detail in Fig. 4A and B. Based on the KcsA structure, it is likely that in an intact *Shaker* channel the side chain of residue E418 forms a hydrogen bond with the backbone carbonyl atoms of residue V451 and/or G452. Carbon atoms from *Shaker* residues E418, V451, and G452 are colored white in Fig. 4A and B, illustrating the close proximity of these residues. It is predicted that breakage of this intra-subunit hydrogen bond is an important step during C-type inactivation, permitting a rotation of residues 448–452, with the side chain of V451 turning towards the E418 residue: this conformational state would resemble a disulfide bonded conformation of E418C and V451C (Larsson and Elinder, 2000).

Conformational rearrangement of the outer pore associated with disulfide linkage of cysteines substituted at residues 418 and 451 may be transmitted to the selectivity filter of the channel as follows. Residue P450 lies wedged between the W434 and W435 residues (aromatic cuff residues) from adjacent channel subunits (green residues in Fig. 4B), and it is known that the P450 side chain undergoes significant changes in accessibility during inactivation (Liu et al., 1996). Rotation of residues 448–452 would alter the position of residue P450

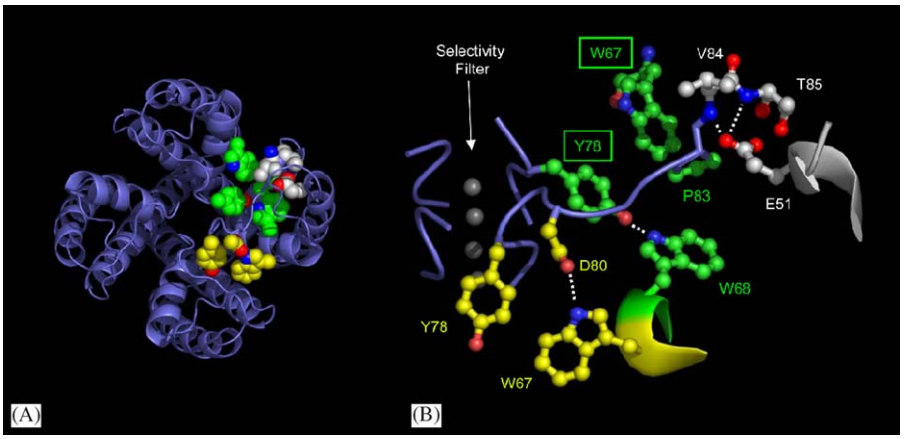

Fig. 4. Detailed molecular model of extracellular residues involved in C-type inactivation. (A), (B) The KcsA structure has been used to highlight residues proposed to be involved in C-type inactivation of *Shaker* channels (Larsson and Elinder, 2000). For clarity of discussion, carbon atoms in discrete groups of interacting residues have been color coded (either green, yellow, or white). It is proposed that C-type inactivation requires breaking of hydrogen bonds between E51 (*Shaker* E418) and the backbone nitrogen atoms of V84 and T85 (*Shaker* V451 and T452, white residues). This may precipitate a conformational change in the extracellular pore leading to destabilization of the selectivity filter. Residue P83 (*Shaker* P450) is wedged between residues in the aromatic cuff (green residues). Movement of *Shaker* P450 may allow the aromatic cuff to constrict slightly, leading to conformational changes within the selectivity filter. Alternatively, movement of residue D80 (*Shaker* D447) may also affect a network of stabilizing interactions around the selectivity filter (yellow residues). In panel (B), all residues depicted are from a single subunit, except those with boxed labels, which arise from an adjacent subunit.

and may allow the aromatic cuff residues to approach one another and constrict the selectivity filter (Larsson and Elinder, 2000). A second possible influence on the selectivity filter may arise from slight changes in the position of residue D447 (KcsA residue D80), which also likely stabilizes the selectivity filter structure through interactions with W435 in the aromatic cuff (Fig. 1C, yellow residues in Fig. 4A and B; Kirsch et al., 1995). This interpretation provides a plausible structural model of the rearrangements occurring at the outer pore mouth during inactivation, and the proposed rotation of residues 448–452 is consistent with the previously demonstrated changes in the accessibility of *Shaker* residues 448–450 during inactivation (Liu et al., 1996). Interestingly, many of these residues (particularly those in the aromatic cuff, and Y445 in the selectivity filter, see Fig. 1B and C) are involved in both intra- and inter-subunit contacts, which may underlie a structural basis for co-operativity in C-type inactivation.

3.7. *Voltage-sensor interactions with the pore during C-type inactivation*

There is evidence that the voltage sensor is intimately involved in C-type inactivation, potentially as a trigger for the process, and also likely undergoes some form of conformational rearrangement in concert with C-type inactivation. As previously described, fluorescent probes introduced at residue T424C and other sites in the outer pore of *Shaker* channels exhibit a slow change in fluorescence intensity that matches the time course of macroscopic inactivation (Gandhi et al., 2000; Loots and Isacoff, 1998, 2000). Importantly, after the onset of inactivation (but not when channels are open), T424C conjugated fluorophores appear to detect rapid molecular motions of the voltage sensor. Complementary observations were made for the fluorescence signal observed from tags conjugated to the extracellular end of the S4 transmembrane helix (A359C). That is, two components were observed from the A359C conjugated fluorophore upon depolarization: a fast component attributed to rapid motion of the voltage sensor, and a slow component with kinetics equal to the inactivation rate (Loots and Isacoff, 1998). A subsequent scan of residues in the S5 segment demonstrated that fluorophores conjugated to the *Shaker* channel pore, particularly at residue 419, can detect conformational changes of the voltage sensor (S4) (Loots and Isacoff, 2000). Together, these observations strongly suggest

that localized regions of the pore and S4 segment may lie in close proximity in an intact channel. This idea has also been suggested by many recent studies designed to examine the location of S4 relative to the pore. Specific evidence includes the demonstration of disulfide bond formation between cysteine residues substituted in the extracellular ends of the *Shaker* S4 and S5 segments, and the suggestion that residue E418 may act as a surface charge and modulate voltage sensor movement in *Shaker* (Elinder et al., 2001a, b; Elinder and Århem, 1999; Laine et al., 2003; Gandhi et al., 2000, 2003). Consequently, it has been suggested by several groups that movement of the voltage sensor into its activated conformation alters voltage sensor–pore interactions. The ability of fluorophores conjugated to *Shaker* residue 419 to detect movement of the S4 segment has led to the suggestion that interactions in this region might promote breakage of hydrogen bonds around the adjacent E418 residue (Loots and Isacoff, 2000), precipitating the conformational twisting of residues 448–452, and C-type inactivation, as described previously (Fig. 4B).

Functional changes in voltage sensor behavior are also observed during C-type inactivation, made apparent through characterization of the effects of inactivation on gating charge movement. In particular, it has been demonstrated that the onset of C-type inactivation causes apparent 'immobilization' of gating charge movement (Olcese et al., 1997, 2001; Chen et al., 1997). For example, depolarizations to 0 mV from a holding potential of –60 mV (where channels are at rest) displace substantially more gating charge than repolarizations to –60 mV from a holding potential of 0 mV (where channels have C-type inactivated). This asymmetry reflects a hyperpolarizing shift of the voltage dependence of gating charge movement in channels that have undergone C-type inactivation (Olcese et al., 1997), and has also been confirmed with fluorescence measurements of conformational changes of the voltage sensor (Loots and Isacoff, 1998). The shift is probably better described as a hysteresis of the voltage dependence of gating charge movement, and suggests that the energetics of voltage-sensor movement are altered significantly after the onset of slow inactivation (Olcese et al., 1997).

Gating current studies in Kv1.5 have provided the important insight that gating charge immobilization can be profoundly decelerated or prevented by permeating cations or the channel blocker 4-aminopyridine (but not quaternary ammonium ions(Fedida et al., 1996; Wang and Fedida, 2001; Chen et al., 1997). For instance, inclusion of weakly permeant Cs^+ ions in the intracellular recording solutions accelerates off gating currents observed upon repolarization, and thereby abolishes any apparent shift of the Q–V relationship. These observations correlate with previously described studies of the effects of permeant ion binding within the pore on C-type inactivation (Kiss et al., 1998; Wang et al., 2000b). That is, these experiments suggest that high-affinity binding of permeant ions within the selectivity filter is able to stabilize the open pore structure and somehow prevent the charge immobilization that accompanies C-type inactivation (Chen et al., 1997).

A further interesting observation, although lacking a clear interpretation at present, is that charge immobilization functionally reduces the number of charge systems involved in gating charge movement. That is, the gating charge–voltage relationship measured from negative holding potentials is described by a two-component Boltzmann function, which is thought to reflect the movement of two distinct charge systems ($Q1$ and $Q2$) during activation (Stefani et al., 1994; Bezanilla et al., 1994). However, the gating charge–voltage relationship measured from positive holding potentials (favoring charge immobilization), essentially collapses onto a single Boltzmann function, with a $V_{1/2}$ equal to the *Shaker* $Q1$ charge system but encompassing a total gating charge equivalent to $Q1$ and $Q2$ (Olcese et al., 1997).

3.8. 'P-type' inactivation

The term 'P-type' inactivation has been employed in several contexts in the literature describing K^+ channel inactivation. 'P-type' inactivation was originally introduced to describe the effects of mutations in the P region of Kv2.1 (e.g. I396L) that altered channel inactivation and notably resulted in potentiation of macroscopic currents by extracellular TEA^+ (De Biasi et al., 1993). At this time, C-type inactivation had been identified primarily by mutations in the S6 transmembrane helix of *Shaker*, and thus the term 'P-type' was intended to distinguish a mechanism of inactivation mediated by the P region/selectivity filter. However, as described in preceding sections, C-type inactivation of *Shaker* is now generally viewed as a conformational change involving the selectivity filter and outer pore of the channel. In this regard, the potentiating effects of TEA in the Kv2.1 I396L mutant channel could be explained by relief of a C-type inactivation process from resting channel states.

Most recently, the term 'P-type' inactivation has been employed to draw a more subtle distinction with C-type inactivation, with respect to the extent of gating charge immobilization. Based on voltage-clamp fluorimetry data, Loots and Isacoff (1998) have suggested that slow inactivation of *Shaker* channels (i.e. N-type inactivation removed) comprises two sequential processes: an initial constriction/closure of the channel pore resulting in current decay, followed by a much slower conformational change that stabilizes the voltage sensor and pore and results in gating charge immobilization. They revisited the term 'P-type' inactivation to refer to the initial pore closure, and proposed that 'C-type' refer to the stabilized inactivated conformation of the channel, after the onset of gating charge immobilization. This distinction between P-type and C-type inactivation has also arisen in studies of the 'non-conducting' W434F *Shaker* mutant, in which channels appear non-conducting due to a permanent (or extremely rapid) inactivation process but do not exhibit permanent immobilization of gating charge (Yang et al., 1997). Importantly, it remains unclear whether these sequential inactivation processes alter the functional behavior of a channel. However, there are hints that prolonged depolarization of Kv1.5 alters the kinetics of recovery from inactivation, which would be consistent with a time-dependent stabilization of channel inactivation (Rich and Snyders, 1998).

While the current use of 'P-type' inactivation implies a difference between the onset of macroscopic inactivation vs. gating charge immobilization, the available data on most Kv channel types is clearly insufficient to draw this distinction. To do so requires methodology allowing simultaneous measurement of voltage sensor movement and macroscopic inactivation, as exemplified by the voltage-clamp fluorimetry techniques that have been primarily applied to the study of *Shaker* channels (Gandhi et al., 2000; Mannuzzu et al., 1996; Cha and Bezanilla, 1997, 1998). Also, although the distinction provided by the 'P-type' terminology may offer a more comprehensive description of the events involved in $K+$ channel inactivation, the current meaning of 'P-type' denotes a process (e.g. decay of macroscopic current) that has been traditionally referred to as C-type inactivation. In many cases, where there is uncertainty regarding the relative extent of pore inactivation and charge immobilization, these terms are now sometimes conflated into 'P/C-type' inactivation to avoid confusion.

4. 'U-type' inactivation, an example of divergence from the classical Shaker inactivation phenotype

Essentially by default, inactivation mechanisms that persist after N-terminal deletion have frequently been categorized as 'C-type' mechanisms. However, as detailed studies of inactivation have been expanded to include multiple Kv channel types, it has become apparent that the properties characterized for C-type inactivation in *Shaker* channels frequently fail to generalize to many Kv channels. This heterogeneity of inactivation properties between Kv channel types includes important differences in the state- and voltage dependence of inactivation, as well as regulation by extracellular cations, and inactivation of the mammalian Kv2.1 channel (*drk1*), provides an important and well-characterized example of divergence from properties described for *Shaker* channels. Particularly, the inactivation–voltage relationship of Kv2.1 exhibits a very prominent 'U-shape', with more rapid and profound inactivation observed at intermediate voltages (e.g. 0 mV) than at higher voltages (e.g. +60 mV) (Klemic et al., 1998, 2001). This apparent voltage dependence of inactivation contrasts with previous descriptions of C-type inactivation in *Shaker* and its mammalian Kv1 homologues, which generally exhibit relatively voltage-independent inactivation at voltages that saturate channel open probability (Kurata et al., 2001; Hoshi et al., 1991; Fedida et al., 1999). Mechanistically, this inactivation behavior of Kv2.1 has been attributed to faster inactivation from intermediate (partially activated) states in the activation pathway vs. the open state. Such models predict faster macroscopic inactivation at voltages near or below the $V_{1/2}$ of channel activation, that favor occupancy of partially activated closed states. Inactivation is less favored at higher voltages that favor occupancy of the more slowly inactivating open state. Importantly, this model predicts that the apparent voltage dependence of macroscopic inactivation of Kv2.1 does not arise from an intrinsic voltage dependence of channel inactivation, but rather from allosteric coupling between inactivation and voltage-dependent transitions involved in channel activation. In contrast, C-type inactivation of *Shaker* is thought to be strongly coupled to channel opening, with inactivation occuring fastest from the open state (Olcese et al., 1997; Klemic et al., 1998).

Other important features also distinguish the inactivation process in Kv2.1 from that classically described in *Shaker*. Surprisingly, Kv2.1 inactivation is accelerated in elevated concentrations of extracellular K^+ or

TEA^+, while these treatments slow C-type inactivation in *Shaker*. Clearly, these observations are inconsistent with the 'foot in the door' interpretation of quaternary ammonium or permeant ion effects on *Shaker* inactivation, although an alternative structural explanation for these data is not obvious, and has not been suggested in the literature. Similar properties have been observed in other channel types, and in some instances, the term 'U-type' inactivation has been used to collectively describe these features of inactivation (a U-shaped inactivation–voltage relationship, and accelerated inactivation in elevated extracellular K^+ or TEA^+) (Klemic et al., 2001). Structure–function studies have provided little insight into the structural basis for 'U-type' inactivation, although several manipulations of channel structure within the N-terminal T1 domain of the *Shaker* homologue Kv1.5 have been shown to impart properties of 'U-type' inactivation. While full-length Kv1.5 channels exhibit inactivation properties generally consistent with C-type inactivation in *Shaker*, N-terminal deletions that disrupt the T1 domain result in a U-shaped inactivation–voltage relationship and a significant increase in the voltage dependence of recovery from inactivation (Kurata et al., 2002). In addition, the inactivation properties of Kv2.1 can be altered by co-expression with modulatory α subunits, and these effects have been tied to either an N-terminal regulatory domain, or to the identity of a specific residue (P410) in the inner cavity of Kv2.1 (Kerschensteiner and Stocker, 1999; Kerschensteiner et al., 2003).

4.1. A broad view of heterogeneous K+ channel inactivation phenotypes

Many other channel types also exhibit inactivation properties that diverge from the properties originally described in *Shaker*. In rapidly inactivating Kv4 channels, elevation of extracellular K^+ concentration promotes inactivation and inhibits recovery (Jerng and Covarrubias, 1997; Jerng et al., 1999; Bahring et al., 2001). Similarly, inactivation of Kv3.1 channels (and Kv2.1 described above) appears to accelerate with higher extracellular K^+ concentrations. Several mutations have been identified that appear to shift the K^+-dependence of inactivation of various Kv channels. The Kv1.4 V561A mutant, for instance, exhibits accelerated inactivation in elevated extracellular K^+ concentrations (Li et al., 2003; Bett and Rasmusson, 2004), whereas WT Kv1.4 inactivation (as for *Shaker*) is inhibited by extracellular K^+ (Rasmusson et al., 1995). The inactivation process in hERG has been described as 'C-type' because it is inhibited by extracellular TEA^+, but unlike *Shaker*, hERG inactivation exhibits considerable voltage dependence (Smith et al., 1996; Zhang et al., 2003).

Various Kv channel types also exhibit a range of state dependencies of inactivation. As mentioned, C-type inactivation of *Shaker* is generally considered to be coupled to channel opening and exhibits little or no voltage dependence. In some instances, as for Kv2.1 (described above) and Kv3.1, a predominant closed-state inactivation process results in an apparent voltage dependence of inactivation, and thus a prominent difference from the inactivation phenotype in *Shaker* (Klemic et al., 1998, 2001). In the Kv4 channel family, a rapid and absorbing closed-state inactivation process accounts for much of the overall time course of inactivation, although inactivation of these channels exhibits little apparent voltage dependence.

Overall, the heterogeneity in the state dependence, voltage dependence, and sensitivity of inactivation to extracellular cations raises the difficult issue of whether classification of multiple non-N-type inactivation mechanisms is merited. At present, the bulk of our understanding of the conformational changes of non-N-type mechanisms of inactivation has arisen from studies of C-type inactivation in *Shaker*, but whether similar conformational changes underlie inactivation in other channels is unclear. Given that various inactivation properties, including state dependence and the response to extracellular cations, can be altered by minor alterations of the primary sequence (even in regions structurally distinct from the pore), it seems plausible that reported differences in classically characterized inactivation properties may not reflect dramatically different gating processes. Indeed, some reports have suggested that inactivation is accompanied by similar changes in selectivity in various channel types that exhibit significantly different regulation by cations, including Kv2.1, *Shaker*, *Shaker* A463C, and a Kv1.3/Kv2.1 chimeric channel (Gross et al., 1994; Kiss et al., 1999; Starkus et al., 1997). Thus, it remains unclear whether the range of inactivation phenotypes observed in Kv channels truly reflects multiple distinct inactivation mechanisms, or simply highlights our incomplete understanding of the regulation of a generalized mechanism of inactivation mediated by the selectivity filter. In this vein, recent work in both *Shaker* and Kv2.1 has revisited the topic of regulation of C-type inactivation by extracellular TEA^+, demonstrating that extracellular TEA^+ does not prevent MTSET

modification of cysteine residues substituted at *Shaker* residue T449, but interferes with modification of cysteines substituted at a more peripheral location in the outer vestibule or 'turret' of the channel (Andalib et al., 2004). While the 'foot-in-the-door' interpretation of the effects of TEA^+ on C-type inactivation in *Shaker* suggest a direct steric interference of TEA^+ binding, these results raise the possibility that TEA^+ may exert effects on C-type inactivation from a more distant site. Perhaps more importantly, these results suggest that the understanding of channel interactions with various cations is incomplete, and raises the possibility that differences in coupling of TEA^+ binding to inactivation may underlie the varied effects of TEA^+ on inactivation in different Kv channel types. A satisfactory resolution of these issues will likely require continued studies directed towards understanding the conformational changes in multiple channels, with varying sensitivity to K^+, TEA^+, or other 'classical' modifiers of C-type inactivation.

A final important consideration is the mechanisms underlying the variable state dependence of Kv channel inactivation. N-type inactivation of Kv channels exhibits a well-defined state dependence, as channels must open before the N-terminal inactivation domain can bind. The structural correlate of this state dependence is well understood to be the steric gate formed by the cytoplasmic ends of the S6 transmembrane helices, which occlude entry of the inactivation domain into the inner cavity of closed channels (Liu et al., 1997; Del Camino and Yellen, 2001; Zhou et al., 2001a; Aldrich, 2001). In contrast, very little is known regarding the structural basis for differences in the state-dependencies of non-N-type inactivation mechanisms in Kv channels (Kurata et al., 2001, 2002; Jerng et al., 1999; Bahring et al., 2001; Klemic et al., 1998, 2001).

In most recently published kinetic models of Kv channel inactivation, there is a common implication that slow inactivation is somehow coupled to movement of voltage sensors into their activated conformation (Klemic et al., 2001; Olcese et al., 1997, 2001; Kurata et al., 2001; Marom and Abbott, 1994). The structural model of Larsson and Elinder for the role of E418 in *Shaker* inactivation (Larsson and Elinder, 2000), together with the demonstration that the S4 segment and residues near E418 approach one another very closely (Elinder et al., 2001a, b; Laine et al., 2003; Gandhi et al., 2000, 2003), provides a hypothetical structural mechanism by which conformational changes of the voltage sensor can influence the conformation of the outer pore (Loots and Isacoff, 1998, 2000). While the possibility that activation of voltage sensors somehow promotes C-type inactivation seems likely, this does not provide an obvious explanation for the most basic distinction of state dependence during inactivation, which is the relative ease with which channels inactivate from the open state vs. closed states. Clearly, in some Kv channel types the conformational changes accompanying opening of the pore must affect the propensity of the pore to inactivate. Changes in ionic selectivity during activation/deactivation suggest that activation includes rearrangements around the selectivity filter, and these may differentially impact the ability to C-type inactivate in different channel types (Zheng and Sigworth, 1997, 1998). A second possibility is that subtle differences in the interactions between the extracellular pore and the extracellular end of S4 may influence C-type inactivation by promoting conformational changes of the extracellular pore (Loots and Isacoff, 2000).

5. Summary

Inactivation of Kv channels can occur by multiple mechanisms that vary in their time- and state dependence, and sensitivity to permeant ions and blockers. N-type inactivation arises through blockade of the channel by an inactivation domain tethered to the N-terminus of the channel. C-type inactivation is clearly a very complex, and more poorly understood mechanism of channel closure. The structural basis for the C-type inactivation 'gate' appears to be a localized constriction of the selectivity filter. However, this constriction is likely accompanied by conformational changes throughout the channel, including multiple residues in the extracellular channel mouth, residues affecting movement of the voltage sensor, and potentially even residues in the inner pore of the channel.

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