A Limited Access Compartment between the Pore Domain and Cytosolic Domain of the BK Channel

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Cytosolic N-terminal segments of many K⁺ channel subunits mediate rapid blockade of ion permeation by physical occlusion of the ion-conducting pore. For some channels with large cytosolic structures, access to the channel pore by inactivation domains may occur through lateral entry pathways or “side portals” that separate the pore domain and associated cytosolic structures covering the axis of the permeation pathway. However, the extent to which side portals control access of molecules to the channel or influence channel gating is unknown. Here we use removal of inactivation by trypsin as a tool to examine basic residue accessibility in both the N terminus of the native auxiliary β2 subunit of Ca²⁺-activated, BK-type K⁺ channels and β2 subunits with artificial inactivating N termini. The results show that, for BK channels, side portals define a protected space that precedes the channel permeation pathway and excludes small proteins such as trypsin but allows inactivation domains to enter. When channels are closed, inactivation domains readily pass through side portals, with a central antechamber preceding the permeation pathway occupied by an inactivation domain approximately half of the time under resting conditions. The restricted volume of the pathway through side portals is likely to influence kinetic properties of inactivation mechanisms, blockade by large pharmacological probes, and accessibility of modulatory factors to surfaces of the channel within the protected space.

Key words: BK channels; inactivation mechanisms; channel structure; auxiliary β subunits; patch clamp; trypsin accessibility

Introduction

Rapid inactivation of voltage-dependent K⁺ (Kv) channels (Zhou et al., 2001) and large-conductance, Ca²⁺-activated K⁺ channels (BK) (Xia et al., 2003) involves insertion of a cytosolic hydrophobic N-terminal peptide segment into a position in the ion permeation pathway that obstructs ion flux. Such hydrophobic inactivation domains (IDs) can be either intrinsic to the N terminus of the pore-forming α subunits (Hoshi et al., 1990; Ruppersberg et al., 1991) or arise from N termini of associated auxiliary β subunits (Rettig et al., 1994; Rasmusson et al., 1997; Wallner et al., 1999; Xia et al., 1999). For many channels, a large cytosolic domain hanging from the membrane-embedded pore module almost certainly requires that IDs gain access to the channel through lateral entry pathways, termed side portals, that separate the pore domain from the cytosolic structure (Gulbis et al., 2000; Koberz et al., 2000; Sokolova et al., 2001; Zhou et al., 2001). Consideration of the open-state structure of Ca²⁺-activated bacterial methanobacterium thermoautotrophicum (MthK) K⁺ channel (Jiang et al., 2002a) suggests that side portals may place significant constraints on access to the pore (see Fig. 1A). The role, if any, of side portals in defining access of molecules to the channel or in channel gating is unknown.

BK channels contain a large cytosolic domain appended to the pore-forming elements of the channel (Butler et al., 1993; Xia et al., 2004). Given that the cytosolic domain of BK channels shares homology with the cytosolic domain of the MthK channel (Jiang et al., 2002a), similarities in side portal architecture and the linkage between pore and cytosolic domain may exist. Inactivation of BK channels is mediated by N-terminal segments of auxiliary β subunits (Wallner et al., 1999; Xia et al., 1999, 2000, 2003). Previous work using artificial N termini in which polymeric amino acid chains of different length tether a hydrophobic inactivation motif to a β2 subunit has defined a minimal length of the N terminus that is necessary to permit inactivation (Xia et al., 2003). The short length of inactivation-competent N termini coupled with the large size of the BK cytosolic domains imply that IDs access their blocking site by passage through side portals. To examine constraints that side portals may play in defining accessibility on IDs or other molecules to the BK channel pore, here we examined the ability of trypsin to attack basic residues (i.e., arginine and lysine) in both the native β2 N terminus and the artificial N termini appended to the β2 subunit. By exploiting the fact that inactivation results in protection from digestion by trypsin, the results show that side portals, by excluding entry of trypsin, define a protected antechamber at the cytoplasmic end of the pore. Furthermore, when channels are in resting states, the results require that passage of IDs through side portals occurs readily, but only a single ID can occupy the antechamber at a time. Thus, for BK channels, side portal access and the dimensions of the...
antechamber may strongly influence several important functional properties of the channel.

**Materials and Methods**

**Oocyte removal and culture.** Stage IV Xenopus laevis oocytes were harvested and used for cRNA injection as described previously (Xia et al., 1999, 2002). mSl1 α and hβ2 cRNA were prepared at ~1 μg/μl, and, after initial dilution of the α cRNA to 1:20 by volume, the injection solution was prepared at cRNA ratios of 1:2 (α/β2). Currents were recorded within 3–5 d.

**Constructs and mutations.** The mSl1 construct (GenBank accession number NP_034740) was identical to that in previous work (Xia et al., 1999, 2002) (L. Salkoff; Washington University School of Medicine, St. Louis, MO). Wild-type human β2 subunit (GenBank accession number NP_852006) was as described previously (Xia et al., 1999). Some of the mutated hβ2 constructs have been mentioned previously (Xia et al., 2003), whereas preparation of new hβ2 N-terminal mutations followed previously published procedures (Xia et al., 2003).

**Electrophysiology.** Currents were recorded in the inside-out configuration (Hamill et al., 1981) using an Axopatch 200 amplifier (Molecular Devices, Sunnyvale, CA) and the Clampex program (Molecular Devices). Patch-clamp recording pipettes were made from boro-silicate capillary tubes, had resistances of 1–3 MΩ, and were coated with Sylgard (Sylgard 184; Dow Corning, Midland, MI) before final remelting of the tip with a microforge.

Gigaohm seals were formed in frog Ringer’s solution (in mM: 115 NaCl, 2.5 KCl, 1.8 CaCl2, and 10 HEPES, pH 7.4). The standard pipette/extracellular solution contained the following (in mM): 140 K-methanesulfonate, 20 KOH, 10 HEPES, and 2 MgCl2, pH 7.0. Solutions bathing the cytosolic membrane contained the following (in mM): 140 potassium methanesulfonate, 20 KOH, 10 HEPES (H+), and either 5 EGTA (for nominally 0 Ca2+) or 5 N-(2-hydroxyethyl)ethylene diaminetetraacetic acid (with Ca2+ added to make 10 μM free Ca2+). The latter solution was calibrated with a Ca2+-sensitive electrode using commercial Ca2+ calibration solutions (World Precision Instruments, Sarasota, FL). Excised patches were bathed in continuously flowing streams from a multibarrel local application system. Experiments were at ~22–25°C. Salts were obtained from Sigma (St. Louis, MO).

**Trypsin application methods.** Trypsin for all illustrated experiments was from porcine pancreas (type IX-S, catalog #T0303, chymotrypsin <1 U/mg protein; Sigma). Three different lots of T0303 were used. We also tested bovine pancreatic trypsin specifically treated with N-tosyl-l-phenylalanylchloromethylethylketone (TPCK) to reduce chymotrypsin activity (Sigma catalog #T8802; chymotrypsin <0.1 N-benzoyl-l-tyrosine ethyl ester U/mg protein). No obvious differences between T0303 and T8802 were observed. A β2 N terminus in which all basic residues were neutralized to Q (β2–10Q) exhibited similar resistance to digestion by either T0303 and the TPCK-treated T8802. In contrast, porcine pancreatic trypsin (catalog #22715; United States Biochemicals, Cleveland, OH) resulted in rapid removal of inactivation of β2–10Q, indicative of chymotrypsin contamination.

Control experiments (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) established that 0.1 mg/ml is on a linear portion of the relationship between the rate of trypsin-mediated removal of inactivation and [trypsin]. Furthermore, we were concerned that autodigestion by trypsin after initial preparation of a trypsin solution might alter the initial effective trypsin concentration among experiments. To minimize this concern, trypsin solutions were maintained on ice as long as possible before addition to the syringe reservoirs. Furthermore, in contrast to previous studies in which 0.5 mg/ml trypsin was used to remove inactivation (Solaro and Lingle, 1992; Xia et al., 1999), we used 0.1 mg/ml to minimize the concentration-dependent autodigestion process. By measuring the rates of removal of inactivation produced by nominal solutions of 0.1 mg/ml trypsin that were incubated for different periods of time at room temperature, we also established that, even for a 2 h period of autodigestion, the change in effective digestion rate is only ~10–15% (supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

Peak and steady-state BK current were monitored by 160 or 200 ms test steps to +150 mV after a 30–50 ms preconditioning step to −140 mV in the presence of 10 μM Ca2+. Between sets of test steps to monitor BK current, trypsin was applied for timed intervals while the patch was maintained at 0 mV in 0 μM Ca2+. For test of state dependence of trypsin accessibility, trypsin was applied during various test conditions [(1) 0 Ca2+, 0 mM; (2) 0 Ca2+, −80 mV; (3) 10 μM Ca2+, 0 mM]. Although the duration of each application of trypsin was precisely controlled, we cannot be sure of the precise time course of wash-in and washout of trypsin from the patch. For most experiments, trypsin was washed out from the patch for 5–10 s before voltage protocols to test BK current status. However, in experiments comparing use dependence of trypsin accessibility, care was taken to ensure that each patch was maintained in the particular test condition for at least 15 s after trypsin washout before test steps to monitor BK status were initiated. In all cases, voltage steps to monitor the status of BK current were applied only in the absence of trypsin and between successive trypsin applications. Trypsin application times represent the cumulative time of trypsin application.

**Data analysis.** Analysis of current recordings was accomplished with either Clampfit (Molecular Devices) or programs written in this laboratory.

**Rates of trypsin removal of inactivation** were fit with the following function (Ding et al., 1998; Wang et al., 2002):

\[
I(t)/I_{\text{max}} = (1 - \exp(-t/\tau))^{n},
\]

where \(I_{\text{max}}\) is the maximal outward current observed after removal of inactivation, \(\tau\) is the time constant of the removal process, and \(n\) is a power factor. This function postulates that a single ID is sufficient for inactivation, but that domains per channel must be cleaved by trypsin to remove inactivation (Ding et al., 1998; Wang et al., 2002). Empirically optimal fits to recovery time courses among different patches and data-sets were obtained with \(n = 1.8–3.0\). For initial comparisons of differences in \(\tau\) among constructs, we constrained \(n = 2\) to minimize the effect of random errors in \(n\) on estimates of \(\tau\). However, values of \(\tau\) with \(n\) unconstrained are also reported. We also measured \(\tau\) and \(n\) from individual patches and determined the mean and SD for those parameters for all patches for a particular construct. Overall, the mean \(\tau\) estimated from the set of individual patches agreed well with that estimated from fitting the averaged data. Not unexpectedly, the mean \(n\) estimated from individual patches resulted in slightly larger estimates of \(n\) than estimated from fitting the averaged data but consistent with the general conclusions regarding the power term given in the text. Error limits on fitted estimates reflect the 90% confidence limit on that parameter.

**Modeling of the trypsin-mediated removal of inactivation.** To model the trypsin-mediated removal of inactivation, the macroscopic simulation capabilities of the QUB software suite was used (State University of New York–Buffalo, Buffalo, NY). Parameters for all steps in the models were based on previous studies of BK activation and inactivation. The steady-state dependence of inactivation and inactivation on Ca2+ and voltage has been reasonably well defined for α + β2 channels (Ding and Lingle, 2002). Because both rates of inactivation and digestion by trypsin are slow relative to any transitions between closed (C) and open (O) states, the exact rates of closed–open gating transitions are not critical. Values for the channel opening rate, \(k_{o}\), and channel closing rate, \(k_{c}\), were chosen to yield open probabilities \((P_{o})\) expected for our experimental conditions (see Table 3) and absolute rates were chosen to be in accordance with previous measurements of current activation and deactivation (Zhang et al., 2001; Horrigan and Aldrich, 2002). The microscopic rate of trypsin digestion of a single ID (\(k_{d}\)) was set at 0.04 to yield a predicted time course for digestion by trypsin of \(\tau_{d} = 25.0\) s with a cooperativity factor of 4 (see Fig. 4E). The rates for onset and recovery from inactivation are based on previous information about the inactivation rates and steady-state open probability of α + β2 channels (Xia et al., 1999; Wang et al., 2002; Benzinger et al., 2006), for which the \(P_{o}\) for α + β2 channels at 10 μM Ca2+ is ~0.05 at −80 mV, ~0.8 at 0 mV, and ~0.95 at +60 mV (Xia et al., 1999). We assume that intrinsic inactivation rate, \(k_{i}\), is negligibly voltage dependent, being ~12.5 s−1 (Xia et al., 1999; Wang et al., 2002). Steady-state inactivation data at 10 μM Ca2+ suggest that the probability of being inactivated \((P_{i})\) is ~0.2 at −80 mV and ~0.98 at 0 mV (Ding and...
Table 1. Sequences of the human β2 N terminus and mutated N termini

<table>
<thead>
<tr>
<th>Construct</th>
<th>1</th>
<th>11</th>
<th>21</th>
<th>31</th>
<th>41</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2</td>
<td>FWTSGR5T</td>
<td>SSYHDEKRKN</td>
<td>IYQKQDDHDL</td>
<td>LDKRKTVDAL</td>
<td>KAGEDRAILL</td>
</tr>
<tr>
<td>6Q</td>
<td>FWTSGR5T</td>
<td>SSYHDEKQDN</td>
<td>IYQKQDDHDL</td>
<td>LDKRKTVDAL</td>
<td>KAGEDRAILL</td>
</tr>
<tr>
<td>10Q</td>
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<td>IYQKQDDHDL</td>
<td>LDKRKTVDAL</td>
<td>KAGEDRAILL</td>
</tr>
<tr>
<td>β2–6QR8R</td>
<td>FWTSGR5T</td>
<td>SSYHDEKQDN</td>
<td>IYQKQDDHDL</td>
<td>LDKRKTVDAL</td>
<td>KAGEDRAILL</td>
</tr>
<tr>
<td>FW–KK2Q</td>
<td>FW</td>
<td>KQQQKQQQQQ</td>
<td>KQQQKQQQQQ</td>
<td>KQQQKQQQQQ</td>
<td>KQQQKQQQQQ</td>
</tr>
<tr>
<td>FW–2MK27Q</td>
<td>FW</td>
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<td>QQKQQQQQQQ</td>
<td>QQKQQQQQQQ</td>
<td>QQKQQQQQQQ</td>
</tr>
</tbody>
</table>

The beginning of the first transmembrane segment is underlined. The bottom three are examples of artificial N termini.

| Table 2. Time constants (τd) of trypsin-mediated removal of inactivation |
|-----------------------------|-----------------------------|-----------------------------|
| Construct | τd (90% confidence limit) |
| β2 | 38.0 ± 1.5 |
| 6Q–R8R | 35.1 ± 2.0 |
| 10Q–R8R | 3.2 ± 0.3 |
| 6Q–R19R | 2.9 ± 0.2 |
| 10Q–R19R | 2.5 ± 0.2 |
| 6Q–R8R,6Q–R19R | 3.1 ± 0.2 |
| 6Q–R14R | 2.7 ± 0.2 |
| 10Q–R14R | 2.4 ± 0.2 |
| 6Q–K18K | 2.3 ± 0.2 |
| 6Q–K24K | 2.2 ± 0.2 |
| 6Q–R26R | 2.1 ± 0.2 |
| 10Q–R26R | 2.0 ± 0.2 |
| 6Q–R8R,R19R | 2.6 ± 0.2 |

In column 2, τd is determined from fitting Equation 1 to cumulative recovery data with n constrained to 2.0. The 90% confidence limit on the fitted estimate of τd reflects the 90% confidence limit on the fitted estimate of n. Column 3 provides values of τd with n unconstrained, and column 4 provides the estimate for n. For all constructs, three to seven patches were used to define the time course of digestion by trypsin.

Results

Trypsin removes inactivation of wild-type β2 subunits

The β2 subunit, like other BK β subunits (Knaus et al., 1994; Orio et al., 2002), contains two transmembrane segments (TM1 and TM2), an extracellular loop connecting TM1 and TM2, an extracellular loop connecting TM1 and TM2, and both a cytosolic N terminus and a cytosolic C terminus (Fig. 1). The β2 N terminus contains 45 residues with a total of 10 basic residues (Table 1). Residue 46, denoting the boundary between TM1 and the cytosolic N terminus, is also basic but not considered here. Trypsin at 0.1 mg/ml gradually removes inactivation of α + β2 currents (Fig. 1C). The time course of removal of inactivation (Fig. 1D) was fit with an equation assuming that complete removal of inactivation requires digestion of multiple IDs per channel (Wang et al., 2002) [(1/I(t)/Imax = (1 − exp(−t/τd))𝑛)] (Eq. 1, see Materials and Methods). When n = 4, the time constant (τd) derived from fitting Equation 1 approximates the microscopic rate of digestion of a single ID of the four per channel. For this set of patches, τd for α + β2 channels was 38.0 ± 1.5 s, assuming a power factor, n, of 2.0 (Table 2). With no constraint on n, τd = 35.1 ± 4.2 s and n = 2.23 ± 0.39.

in the supplemental data (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Simulations using this full model are consistent with the partial models evaluated in the text.

Structural models. Comparison of the structures of β2 (1JO6) (Bentrop et al., 2001), MhK (1LNQ) (Jiang et al., 2002a), and porcine pancreatic trypsin A-chain (1AVW) (Song and Suh, 1998) was done with VMD and PyMOL (DeLano, 2002).

Lingle, 2002). We have therefore adjusted the intrinsic rate of recovery from inactivation (kᵢ) to yield a Pᵢ consistent with previous observations (Wang et al., 2002). A full model accounting for protection against trypsin digestion both by inactivation and during resting conditions is given.
residues still present in The blue line is the predicted time course based on the sum of the rates of digestion for the four basic trypsin. Currents were activated as in 1, with values summarized in Table 1. inactivation for constructs 6Q–R14R, 6Q–K18K, 6Q–K24K, and 6Q–R26R (Fig. 2). Solid lines through the points represent the best fit of Equation 1, assuming recovery to $n = 0.9$ with $n = 2$. G, For N termini with a poly-Q linker, the rate of trypsin-mediated digestion of inactivation domains depends on the spacing between the paired basic residues and TM1. Each point represents the reciprocal of the fitted $r_0$ based on sets of at least four patches for each construct. The red line indicates the wild-type $\beta 2$ digestion rate. H, The digestion rates for individual basic residues in the wild-type $\beta 2$ N terminus are plotted as in 6. Blue lines indicate segments thought to be $\alpha$ helical, probably resulting in protection against digestion.

Mapping trypsin cleavage sites in the $\beta 2$ N terminus

We first define the trypsin accessibility of basic residues in the native $\beta 2$ N terminus. A mutated $\beta 2$ N terminus in which the first six basic residues are changed to Q (Table 1, 6Q) still supports inactivation and is almost completely insensitive to 0.1 mg/ml trypsin (Fig. 2A, B). Similarly, a 10Q construct (Table 1) is resistant to digestion by trypsin (Fig. 2B). Thus, the four basic residues closest to TM1 are protected, and trypsin sensitivity of the

Figure 2. Differential sensitivity to trypsin of basic residues in the $\beta 2$ N terminus. A, An N terminus with the first six basic residues neutralized to glutamine ($\beta 2$–6Q) is resistant to digestion by trypsin. Currents were activated by voltage steps to $+150$ mV with $10 \mu M Ca_{2+}$. Application of $0.1$ mg/ml trypsin for up to $900$ s had no effect on inactivation. B, The time course of removal of inactivation for wild-type $\beta 2$ subunits is compared with $\beta 2$–6Q and $\beta 2$–10Q constructs. For B, D, and F, each point represents the mean and SE for four to six patches. C, Trypsin readily removes inactivation in an $\alpha$ helical, probably resulting in protection against digestion.
wild-type β2 subunit results from attack on all or some of residues R8, R14, K18, R19, K24, or R26.

Using a library of mutated β2 constructs (supplemental Table 1, available at www.jneurosci.org as supplemental material), we found that mutated N termini containing only a single target basic residue exhibited marked differential sensitivity to trypsin (Fig. 2). Constructs 6Q–R8R (Table 1, only R8 remains charged) ($\tau_d = 43.9 \pm 1.4$ s) and 6Q–R19R ($\tau_d = 110.6 \pm 4.2$ s) exhibited a trypsin sensitivity comparable with the wild-type N terminus ($\tau_d = 38.0 \pm 1.5$ s) (Fig. 2C,D). However, 6Q–R14R, 6Q–K18R, 6Q–R24R, and 6Q–K26K were relatively resistant to digestion by trypsin (Fig. 2E,F; Table 2) but were more sensitive to trypsin than the 6Q construct. Comparable results were obtained when only a single basic residue was preserved within the 10Q background. Thus, preferential attack on residues R8 and R19 appears primarily responsible for removal of inactivation in native wild-type β2 subunits. In support of this, in the β2–R8QR19Q construct, containing eight basic residues but lacking R8 and R19, the rate of trypsin removal of inactivation ($\tau_d = 196.6 \pm 12.0$ s) was approximately five-fold slower than for the wild-type β2 N terminus (Fig. 2G). Thus, the simultaneous presence of R14, K18, K24, and R26 results in trypsin sensitivity less than in constructs containing either R8 or R19 alone.

Several factors suggest that it is unlikely that mutationally caused structural changes account for the differential sensitivities we observe. First, the similarity of $\tau_d$ for cleavage of particular basic residues in either the 6Q or 10Q backgrounds suggests that mutation at positions 33, 34, 35, and 41 does not alter trypsin accessibility of other basic residues. Second, the observed $\tau_d$ of trypsin cleavage of β2–R8QR19Q (196.6 s) is in close agreement with that calculated (238.1 s) based on the sum of the individual rates of the other four trypsin-sensitive residues. This additivity suggests that individual mutations to glutamine do not significantly alter global structure. Similarly, the sum of the measured cleavage rates of 6Q–R8R and 6Q–R19R results in a predicted $\tau_d$ (31.4 s) (Table 2) similar to that of the wild-type $\tau_d$ (38.0 and 26.8 s for two sets of patches) (Fig. 2H). This additivity suggests that accessibility of R8 and R19 is not markedly altered in the 6Q (or 10Q) constructs.

### Accessibility of lysine residues to digestion by trypsin in artificial N termini

The differential trypsin digestibility of basic residues in the β2 N terminus may arise from either specific structural characteristics of the N terminus or restrictions on access of the ~20 kDa trypsin molecule to small volumes. We therefore examined the position dependence of trypsin sensitivity of basic residues inserted into artificial N termini. The critical inactivation motif, MFIW, was connected to TM1 by a linear chain of polyglutamines (poly-Q) (Xia et al., 2003) with two consecutive lysines inserted at different positions in the chain (Table 1) (supplemental Table 1, available at www.jneurosci.org as supple-
Table 3. Parameters for simulation of removal of inactivation by trypsin

<table>
<thead>
<tr>
<th>Condition</th>
<th>$k_0$ (s$^{-1}$)</th>
<th>$k_1$ (s$^{-1}$)</th>
<th>$P_0$</th>
<th>$k_1^*$ (s$^{-1}$)</th>
<th>$P_1$</th>
<th>$\tau_i$ (s)</th>
<th>$n$</th>
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<tr>
<td>0 Ca$^{2+}$, 0 mV</td>
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<td>5000</td>
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<td>50</td>
<td>0.2</td>
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<td>5</td>
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<td>12.5</td>
<td>0.2</td>
<td>0.98</td>
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Inactivated ID can be cleaved

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<th>$k_1$ (s$^{-1}$)</th>
<th>$P_0$</th>
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<th>$P_1$</th>
<th>$\tau_i$ (s)</th>
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<tr>
<td>0 Ca$^{2+}$, 0 mV</td>
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<td>1</td>
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<td>10 Ca$^{2+}$, −80 mV</td>
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<td>0.05</td>
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<td>50</td>
<td>0.2</td>
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<td>10 Ca$^{2+}$, 0 mV</td>
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</table>

Parameters for different simulations are given in columns 2–8, whereas values obtained from fitting Equation 1 to the simulated time course are given in columns 9–10. Bold conditions in column 1 correspond to simulations displayed on Figure 6.

During inactivation, the N termini presumably undergo a relatively large conformational movement, perhaps conferring state-dependent changes in trypsin accessibility. We biased the channel population between either a resting condition or an inactivated condition by manipulation of holding potential, cytosolic Ca$^{2+}$ and 0 mV is slowed relative to removal of inactivation at 0 Ca$^{2+}$ and 0 mV or 10 μM Ca$^{2+}$ and −80 mV (Fig. 4A). These results suggest that residues R8 and R19 are more resistant to attack by trypsin when the channels are inactivated. Similar to β2, 10 μM Ca$^{2+}$ at 0 mV also produced a slowing of $\tau_{iG}$ for 6Q–R8R (Fig. 4B) and 6Q–R19R (Fig. 4C). The protection of residue R8 may not seem surprising, because it may occupy a position relatively protected within the ion permeation pathway. However, the protection of residue R19 suggests that even this portion of the N terminus undergoes marked structural or positional changes during inactivation. The possibility that the trypsin sensitivity of other residues changes during inactivation is excluded by the fact that the protection observed for the native β2 N terminus is similar to that observed for 6Q–R8R and 6Q–R19R. If R14, K18, K24, or R28 were significantly more sensitive to attack by trypsin, we would expect that the native β2 N terminus should not show a marked state-dependent protection.

When inactivated, inactivation domains may be completely resistant to digestion by trypsin

The half-time for removal of inactivation changes >10-fold between channels primarily in resting states compared with channels in inactivated states. Because binding of a single ID causes inactivation (Wang et al., 2002), under inactivating conditions, the four IDs per channel are in equilibrium among activated, pre-inactivated, and inactivated states. Perhaps three of the IDs still remain trypsin accessible, although one particle is protected. To test this idea, we evaluated a physically plausible model of inactivation and digestion by trypsin (Fig. 4D). We assume that trypsin-mediated digestion of each ID ($k_d$) occurs independently and does not change between resting closed states and open states. However, when an ID produces inactivation, it is completely protected from digestion by trypsin.

Using sets of gating parameters for channel activation and inactivation defined from other experimental work (supplemental data, available at www.jneurosci.org as supplemental material), this digestion process was simulated. The appearance of channels in states with completely digested N termini then defines the time course of removal of inactivation by trypsin. Using Equation 1, the empirical $\tau_{iG}$ and $n$ for the simulated digestion time course was then determined (Table 3). A condition that favors inactivation (0 mV, 10 μM Ca$^{2+}$) resulted in a slowing of the trypsin-mediated removal of inactivation with an increase in $\tau_{iG}$ and reduction in $n$, approaching a theoretical limit of 1.0 (Fig. 4E). Based on parameters that approximate the three conditions we studied (Table 3) (supplemental information, available at www.jneurosci.org as supplemental material), the simulated...
digestion time course at 0 mV and 0 Ca\(^{2+}\) is similar to that at 80 mV and 10 \(\mu\)M Ca\(^{2+}\). A marked shift in digestion time course is predicted (Fig. 4E; for values, see Table 3). Based on reasonable parameter estimates for activation and inactivation rates, this model provides a good approximation of the experimental observations.

Before accepting the conclusion that an inactivated ID is completely protected from digestion by trypsin, we modified the model to assume a finite rate of digestion of an inactivated ID. Specifically, \(k_{t*}\), the intrinsic rate of digestion of an inactivated ID, was set to values 10-fold (0.04 s\(^{-1}\)), 20-fold (0.02 s\(^{-1}\)), and 100-fold (0.004 s\(^{-1}\)) slower than the digestion rate of an ID in non-inactivated conformations (Fig. 4F). Even with \(k_{t*} = k_{t}/10\), the predicted time course of removal of inactivation is much faster than what is observed experimentally. Thus, the intrinsic rate of digestion of inactivated IDs is probably substantially more than an order of magnitude slower than for resting IDs. We conclude that residues R8 and R19, when in the inactivated configuration, are completely resistant to digestion by trypsin.

The state dependence of R19 is somewhat surprising given its distance from the MFIW inactivation motif, because R19 seems unlikely to occupy a position deep within the pore. Two
This suggests that occupancy of the antechamber, independent of inactivation, might influence the power term under resting conditions. Specifically, suppose that IDs transiently move in and out through the side portals, such that for some fraction of time basic residues are protected from attack by trypsin, although not binding with high affinity within the antechamber. Two different formulations of this idea are given in Figure 6A. For the full model, all four IDs can independently move in and out of the side portals (with equilibrium constant $P$), becoming protected within the antechamber. In a reduced model denoted by the dotted rectangle (Fig. 6A), only a single ID can occupy the antechamber at a time. Using these models, we varied $P$ to examine the impact of fractional occupancy [for a single ID, fractional occupancy $= P/(1 + P)$] within the putative antechamber on the predicted time course of digestion for channels in resting states. When four IDs can simultaneously occupy the antechamber, greater fractional occupancy slows the digestion time course (Fig. 6B). However, as occupancy of the antechamber increases, an $\sim 100$-fold slowing of $\tau_d$ is associated with no change in $n$ (Fig. 6C). In contrast, when only a single ID can occupy the antechamber at a time, increases in fractional occupancy both slow the predicted digestion time course (Fig. 6D) and reduce the power factor (Fig. 6E), with $n$ varying between 1.0 and 4.0 over a 100-fold change in $\tau_d$. Thus, a physically plausible model in which IDs can transiently move in and out of the protected space within the antechamber can result in power factors of $2\sim3$ under resting conditions, but only in the case that only a single ID can occupy the antechamber at a time.

If this correctly explains the experimentally observed digestion time courses, it allows determination of several aspects of the behavior of the $\beta_2$ N terminus. For empirically observed power terms in the range of $2\sim3$, the fold slowing of the observed $\tau_d$ relative to the microscopic time constant of digestion of a single ID by trypsin is in the range of $3\sim6$ (Fig. 6E). With $\tau_d$ of $\sim 30$ s, this would suggest a microscopic time constant for digestion of a single $\beta_2$ ID of $\sim 5\sim10$ s (a rate of $0.1\sim0.2$ s$^{-1}$). Furthermore, the analysis indicates that a single ID occupies the antechamber $\sim 15\sim30$% of the time, whereas overall the antechamber is occupied by one of the four IDs approximately half the time. How might these results relate to other inactivating N termini? We predict that the equilibrium occupancy of the antechamber may reflect specific interactions of a given N terminus with features of the antechamber or the ease of moving in and out of the antechamber. As a consequence, under resting conditions and independent of the specific distribution of basic residues, alternative N termini may exhibit sensitivity to trypsin that differs somewhat from the $\beta_2$ sensitivity, dependent on the fractional occupancy within the antechamber.
Discussion

We used the classical method of defining residue accessibility in native proteins through examination of trypsin digestion rates. Using the status of the BK channel inactivation mechanism as an indicator of the digestion process, quantitative comparisons of differential accessibility of basic residues were made and state-dependent changes associated with the inactivation process could be monitored. We found that the leading 16 residues of inactivated artificial N termini are protected from attack by trypsin. This corresponds exactly to the minimal length of residues in an artificial N terminus necessary to produce inactivation (Xia et al., 2003). The results therefore empirically define a protected volume at the cytosolic face of the channel but distinct from the permeation pathway, which we term an antechamber. This functional demonstration of a protected space corresponds well with evidence that, for many ion channels (Gulbis et al., 2000; Kobertz et al., 2000; Jiang et al., 2002a), cytosolic domains hanging from the membrane-embedded pore domain may require that permanent ions and other molecules access the permeation pathway by movement through so-called side portals. Here we discuss two aspects of our results: first, the implications concerning the native structure of the β2 N terminus and, second, implications concerning the dimensions and significance of the antechamber.

For channels in resting states, relative differences in digestion rates of particular basic residues will primarily reflect intrinsic structural characteristics of the β2 N terminus. In this regard, the present results are, in part, consistent with nuclear magnetic resonance (NMR) structures previously reported for the isolated 45 amino acid N-terminal β2 peptide (Bentrop et al., 2001). The isolated peptide in solution adopts a large family of structures, with two α-helical segments, residues 10–17 and 20–30. Peptide secondary structure will provide some protection against enzymatic digestion (Hubbard and Beynon, 2001), although α helices are not as disfavored as β sheets for proteinase attack. An α-helical structure over residues 10–17 and 20–30 may account for protection against digestion of R14, K24, and R26, whereas R8 positioned on the flexible initial segment of the N terminus is readily attacked. Residues on loops or turns are generally primary nick sites consistent with the sensitivity of position R19 but apparently inconsistent with the relative resistance of K18. Similarly, the resistance of residues K33, R34, K35, and K41 to trypsin does not agree simply with the NMR results and suggests that, in the native environment, these residues are part of a structure, perhaps through interaction with the α subunit, that confers complete protection against digestion by trypsin.

Evaluation of the state dependence of trypsin digestion shows that the β2 N terminus can enter a restricted space from which trypsin is completely excluded. Specifically, when the channel is inactivated, basic residues within the first 16 positions of an extended artificial N terminus are protected from digestion by trypsin. Interestingly, this is exactly the same chain length that is the minimum length necessary for an inactivation-competent N terminus. This 16 residue chain length can be used to estimate a specific physical distance. NMR results indicate that polyglutamine chains predominantly adopt a random coil conformation in solution (Masino et al., 2002). The end-to-end root mean square length of a random coil of 16 residues is given approximately by \( \sqrt{130N} \) (Creighton, 1993) yielding a length of \( \sim 45.6 \) Å. However, during the protection experiment, the 16 residue chain is tethered within the pore. Because the chain is flexible and will occasionally be nearly fully extended, to be completely resistant to attack by trypsin, it is likely that the basic residues must be fully

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**Figure 6.** Under resting conditions, inactivation domains can pass in and out through the portals, but only one inactivation domain can occupy the antechamber at a time. A, The model summarizes two possible conditions involving movement of inactivation domains in and out of an antechamber from which trypsin is excluded. All states are assumed to be closed, but inactivation domains can exist either in accessible (red) or protected (black) conditions. The full model corresponds to a situation in which all four inactivation domains can independently move in and out of the antechamber, and the subscheme delimited by the dotted rectangle defines the case in which only one inactivation domain can bind within the antechamber at a time. B, For the model in which all four IDs can independently occupy the protected space within the antechamber, the predicted digestion time courses were determined for different effective fractional occupancies, in which effective fractional occupancy was defined as \( P(1 + P) \). Higher values of \( P \) shift the digestion time course to the right. C, The time courses in B were fit with Equation 1 to yield \( \tau_d \) and \( n \). These values are plotted here in (n, open circles; fold prolongation of \( \tau_d \) (filled circles) as a function of the fraction of time an individual ID will spend inside the antechamber. For this model, the power factor under resting conditions is always \( \sim 4 \). D, Predicted digestion time courses are shown for the model in which only a single ID can bind with the antechamber at a time. Increased fractional occupancy by a given ID results in a rightward shift in the recovery curves and a change in slope. E, The power factors (open circles) and fold prolongation of \( \tau_d \) (filled circles) are shown as a function of fractional occupancy for the single occupancy model. The dotted lines denote the range of fractional occupancies associated with power factors between \( \sim 2.0 \) and 3.0, corresponding to the range of experimentally observed power factors (2–3). This suggests that, for closed channels, an individual ID binds to a site within the antechamber \( \sim 10–30\% \) of the time. Over this range of fractional occupancies, the experimentally observed \( \tau_d \) time course is \( \sim 2.5-6 \) fold slower than the true microscopic digestion time constant.
protected even when in that fully extended configuration. This suggests that the protected length defined by the 16 residues may more closely reflect a fully extended conformation, which is ~60.8 Å. Conservatively then, trypsin is unable to approach closer than 45–60 Å from the axis of the permeation pathway.

What structural features of the BK channel might account for this restricted space? The MthK and BK channels seem likely to share a generally similar structural arrangement (Jiang et al., 2002a,b) in regards to the cytosolic structure and its connection to the S6 pore helix. Each MthK subunit contains within its cytosolic domain a specific structural motif termed the RCK domain (regulator of conductance for potassium) (Jiang et al., 2002a,b) with clear homology to a similar domain in BK channels. Therefore, the open conformation of the MthK K⁺ channel can be used to make a general estimate of the volume defined by the cytosolic ends of the S6 inner helices and the beginning of the cytosolic structure. Together, the four residues at the end of S6 and four residues at the beginning of the RCK domains define a truncated square pyramid (Fig. 7A), which we term the inner antechamber. For MthK, the height of the inner antechamber is ~19 Å, whereas the distances from the axis of the pore to the top and bottom ends of the linkers are ~13 and 26 Å. Based on the length estimates of the peptide chain over which basic residues are protected from digestion by trypsin, the protected volume extends for a distance almost twofold beyond the inner antechamber. We think it unlikely that, in open BK channels, the cytosolic end of the S6 helix could be sufficiently displaced relative to the open MthK channel to account for this. It seems more likely that the region protected from trypsin access begins well outside the dimensions defined by the inner antechamber. The simplest explanation is that the lateral margins of the protected space are defined, not by positions of the S6-to-RCK linkers, but simply represent the margins of the region of close apposition of the pore domain and the cytosolic domain (Fig. 7B). For comparison, from the Kv1.2 crystal structure (Long et al., 2005), the lateral radius from the axis of the pore to the outer membrane helices is ~50 Å. Thus, it would not be surprising that the β2 TM segments would be positioned just outside the most outer α-subunit membrane helices at a distance of 45–60 Å from the permeation pathway axis.

Based on ~19 Å distance between the pore domain and cytosolic domains estimated from the MthK structure, exclusion of trypsin is not surprising and is consistent with the size of the A chain of trypsin (Fig. 7D). Given the vulnerability of ion channels to blockade by a variety of charged and uncharged moieties, it is tempting to speculate that these constraints on lateral access to the pore might help to reduce extraneous block or rectification that might arise from components of the intracellular soup. Of particular interest, a number of other cellular enzymes including soluble protein kinases and By G-protein dimers would be excluded from entering this restricted space.

Do changes in the side portal pathways occur during gating? Our results require that, under resting conditions, IDs can transiently pass in and out through the side portals, but only one ID
can bind within the central antechamber at a time. Although the static pictures derived from crystal structures of channels with appended cytosolic material suggest that side portals are always open (Gulbis et al., 2000; Jiang et al., 2002a), there has been no specific experimental result that addresses this issue. Although our results cannot distinguish small differences in the ease of access of IDs to the antechamber between closed and open states, certainly occupancy of the antechamber by the β2 N terminus can occur readily under resting conditions. Thus, for BK channels, side portals in BK channels may be essentially static elements.

Our results indicate that, under resting conditions, there is a position of occupancy within the antechamber that can accommodate a single β2 ID. That only a single ID is allowed suggests that this site is within the central antechamber. Given the physical dimensions of the inner antechamber defined from the MthK structure (Figs. 1 B, 7C,D) (Jiang et al., 2002a), it is certainly not surprising that only a single β2 N terminus can occupy the antechamber at a time.

The geometrical constraints imposed by side portals and an antechamber of finite dimension on access of molecules lead to several interesting speculations. First, for channels with antechambers, peptide domains that occupy the antechamber would be protected from enzymatic modifications such as phosphorylation reactions. Second, it is possible that some kinds of blocking effects on ion channels might involve obstruction of passage through side portals. Third, occupancy of the antechamber by an ID may help define kinetic characteristics of inactivation mechanisms. Thus, in channels with bulky cytosolic domains, the characteristics of side portals and antechambers leading to the permeation pathway may play a significant role in defining mechanisms of how such channels are regulated.

References