The Barium Site in a Potassium Channel by X-Ray Crystallography

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abstract X-ray diffraction data were collected from frozen crystals (100°K) of the KcsA K$^+$ channel equilibrated with solutions containing barium chloride. Difference electron density maps ($F_{barium} - F_{native}$, 5.0 Å resolution) show that Ba^{2+} resides at a single location within the selectivity filter. The Ba^{2+} blocking site corresponds to the internal aspect (adjacent to the central cavity) of the "inner ion" position where an alkali metal cation is found in the absence of the blocking Ba^{2+} ion. The location of Ba^{2+} with respect to Rb$^+$ ions in the pore is in good agreement with the findings on the functional interaction of Ba^{2+} with K$^+$ (and Rb$^+$) in Ca^{2+}-activated K$^+$ channels (Neyton, J., and C. Miller. 1988. *J. Gen. Physiol.* 92:549–567). Taken together, these structural and functional data imply that at physiological ion concentrations a third ion may interact with two ions in the selectivity filter, perhaps by entering from one side and displacing an ion on the opposite side.

key words: potassium channel • barium • ion channel • ion selectivity • ion conduction

INTRODUCTION

To a K$^+$ channel, the alkaline earth metal Ba^{2+} is like a divalent K$^+$ ion. Barium's size allows it to fit into the selectivity filter, but its charge apparently causes it to bind too tightly. When it is bound in the filter, Ba^{2+} prevents the rapid flow of K$^+$.

The K$^+$ channel inhibitory properties of Ba^{2+} have been analyzed in detail (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Benham et al., 1985; Miller, 1987, Neyton and Miller, 1988a,b; Zhou et al., 1996; Harris et al., 1998; Vergara et al., 1999). Neyton and Miller (1988a,b) showed through single channel studies of Ca^{2+}-activated K$^+$ channels that Ba^{2+} inhibition is sensitive to the presence of K$^+$ ions in the pore. They observed that the mean blocking dwell time of Ba^{2+} depended on the K$^+$ concentration on both sides of the membrane. Low external (extracellular side of the membrane) K$^+$ ion concentrations (0.01 mM) tended to prevent Ba^{2+} from exiting to the external side, while higher concentrations (500 mM) actually caused Ba^{2+} to dissociate more rapidly to the internal side (intracellular side of the membrane) as if it could be pushed through. These phenomena were attributed to the presence of two K$^+$ ion sites located between the blocking Ba^{2+} ion and the external solution. The presence of K$^+$ at the first of these

sites, termed the external lock-in site, would obstruct barium's outward movement. Occupancy of both sites by K$^+$ (the site closest to Ba^{2+} was termed the enhancement site) would destabilize Ba^{2+} and speed its exit to the inside. By evaluating a series of different ions, the lock-in and enhancement sites were shown to be highly selective for alkali metal ions that permeate K$^+$ channels such as K$^+$ and Rb$^+$, but not Na$^+$. An additional monovalent ion site, termed the internal lock-in site, was hypothesized to lie between the position of Ba^{2+} and the internal entryway; this site became occupied with an affinity constant around 10 mM. In contrast to the sites lying external to Ba^{2+}, the internal lock-in site was not very selective for K$^+$ when compared with Na$^+$.

The membrane voltage dependence of lock-in and enhancement effects exerted by K$^+$ on Ba^{2+} provided an estimate of the electrical distance that K$^+$ must travel through the channel to reach a specific site. Ions in the pore presumably move in a somewhat concerted fashion, making the electrical distance a complicated quantity without a simple structural interpretation. Nevertheless, electrical distances for the K$^+$ sites were informative. The external lock-in and enhancement sites appeared to reside ~15 and 50%, respectively, of the way across the membrane electric potential difference relative to the outside, and the internal lock-in site appeared to reside ~70% of the way (i.e., 30% of the electrical distance from the internal solution). These experiments supported the picture in Fig. 1, right. Following the Neyton and Miller (1988a,b) analysis of Ca^{2+}-activated K$^+$ channels, similar results were obtained by Vergara et al. (1999) and by Harris et al.

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(1998) in their studies of the voltage-dependent *Shaker* K^+ channel.

X-ray structure determination of the KcsA K^+ channel showed that it contains multiple permeant ions in its pore (Doyle et al., 1998). Given the high degree of amino acid sequence conservation in the pore-forming regions of the Ca^{2+}-activated K^+ channel, the *Shaker* K^+ channel, and the KcsA K^+ channel, we expect their three-dimensional structures to be conserved. In this study, we determined the position of Ba^{2+} in the pore of the KcsA K^+ channel. The results allow a comparison of the ionic configuration deduced by structural (x-ray crystallographic) and functional (electrical analysis of single channels) methods.

METHODS

The KcsA K^+ channel was purified and crystallized as described (Doyle et al., 1998). The Ba^{2+}-containing crystals were prepared by first washing crystals in a solution of 50% PEG400, 200 mM $CaCl_2$, 150 mM NaCl, 5.0 mM LDAO, 100 mM HEPES, pH 7.5, and then soaking them in an otherwise identical solution containing 100 mM $CaCl_2$ and 100 mM $BaCl_2$ for 1 h. Crystals were frozen in a cold nitrogen stream ($\sim$100°K) and stored in liquid nitrogen–cooled propane. Data were collected at the Cornell High Energy Synchrotron Source, A1 station, and processed with DENZO and SCALEPACK (Otwinowski, 1993; Otwinowski and Minor, 1997). The electron density of Ba^{2+} in the channel was calculated from the Fourier transformation of $(F_{barium} - F_{native})$ $e^{-i\phi}$, where F_{barium} and F_{native} are the observed structure factors for the Ba^{2+}-containing and native crystals, respectively, and ϕ is the experimental (MIR, solvent-flattened, averaged) phase. Due to crystal damage during the soaking procedure, data were useful only to 5.0-Å resolution.

RESULTS

Crystals of the KcsA K^+ channel were equilibrated in solutions containing 100 mM $BaCl_2$. Ionic strength was kept constant by replacing $CaCl_2$ present in the crystal growth solution with $BaCl_2$. At the same time, 150 mM KCl was replaced by an equal amount of NaCl. It was necessary to lower the K^+ concentration to observe Ba^{2+} in the pore. We assume that K^+, at high concentrations, displaces Ba^{2+} from the pore.

The location of Ba^{2+} in the pore is well defined in a difference Fourier map $(F_{barium} - F_{native})$ calculated at 5.0-Å resolution (Fig. 1, left and center, green mesh). Electron density for Ba^{2+} is compared with that for Rb^+ ions determined in a separate experiment (red mesh). The Rb^+-difference Fourier map, calculated at 3.8-Å resolution, shows two ions in the selectivity filter and one in the cavity at the membrane center, below the selectivity filter (Doyle et al., 1998). The two ions in the selectivity filter are called the outer and inner ions. The outer ion is near the extracellular entryway and the inner ion is closer to the cavity and can be at either of two positions. The Ba^{2+} ion very clearly binds at the location of the inner ion closest to the central cavity.
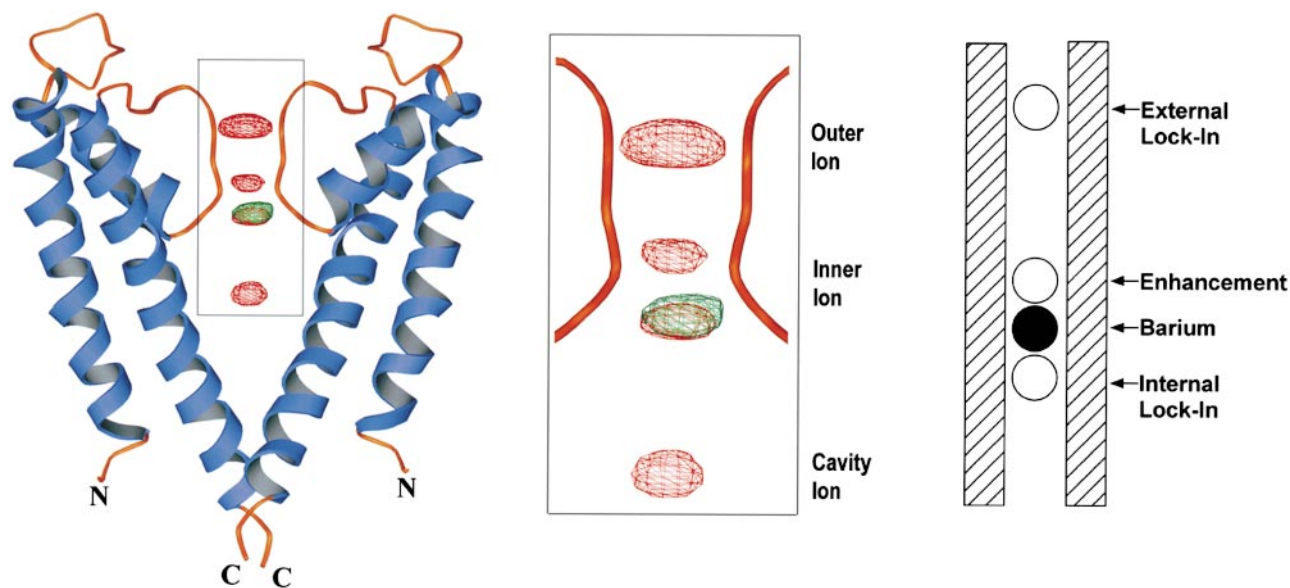

Figure 1. Visualization of barium in the KcsA K^+ channel by x-ray crystallography and in a Ca^{2+}-activated K^+ channel by analysis of single channel function. (Left) A ribbon diagram showing two subunits of the KcsA K^+ channel (blue and orange) with difference electron density showing the positions of Rb^+ (red mesh) and Ba^{2+} (green mesh) in the pore. (Middle) Magnified view of the boxed region on the left. The Rb^+ difference maps were calculated at 3.8-Å resolution and contoured at 9.0 σ. Three ion positions are labeled outer, inner, and cavity ion. The inner ion shows two peaks reflecting alternative positions. The Ba^{2+} difference map was calculated at 5.0-Å resolution and contoured at 10.0 σ; Ba^{2+} is located at the innermost position of the inner ion. (Right) Diagram adapted from Neyton and Miller (1988) depicting the relative locations of Ba^{2+} and K^+ or Rb^+ sites (external lock-in, enhancement, and internal lock-in) as determined by examination of single-channel records. The ions are positioned according to their electrical distance across the membrane potential difference. The figure was prepared using Ribbons (Carson, 1997).

DISCUSSION

The consistency of structural data on the KcsA K+ channel with functional data on Ca^{2+}-activated K+ channels is quite remarkable. The external lock-in effect implied the existence of a first ion binding site external to Ba^{2+}. This site had to be highly selective for permeant alkali metal cations and very near the extracellular solution, because external lock-in is a weakly voltage-dependent process. The outer ion in the KcsA selectivity filter fulfills these criteria. The external enhancement effect implied a second site, also external to Ba^{2+} and highly selective for permeant ions. The stronger voltage dependence of its apparent occupancy suggested that the enhancement site is deeper in, closer to the Ba^{2+} ion. The effective K_d for achieving the enhancement configuration of two K+ ions external to Ba^{2+} is nearly 500 mM, indicating that it is a relatively unstable configuration. In the structure, the outermost peak of the inner ion is too close to Ba^{2+} to be the precise site of the enhancement ion. However, the structure of the selectivity filter provides several potential ion binding sites external to Ba^{2+}.

The internal lock-in effect required a site internal to Ba^{2+} that, in contrast to the external sites, exhibits little ion selectivity. The cavity ion in the crystal structure, being fully hydrated and not in direct contact with protein functional groups, fulfills the requirements of the internal lock-in site completely. Even the relatively weak voltage dependence of internal lock-in is consistent with the idea that a large fraction of the membrane voltage falls across the narrow selectivity filter (Doyle et al., 1998).

Given the high affinities of the external and internal lock-in sites, it seems likely that they would contain a K+ most of the time at physiological ion concentrations. Therefore, the picture of the pore with three ions (two in the selectivity filter and one in the cavity), derived from the crystal structure, is probably not very different from a snapshot of what the conducting channel looks like in the membrane. The external enhancement effect is interesting because it suggests that a fourth ion enters the pore (that is, a third ion in the selectivity filter) to push the queue along (and Ba^{2+} out). The affinity for the enhancement site is low, and corresponds reasonably well to the concentration range over which the conductance increases as K+ concentration is raised (0.3–1.0 M, depending on the K+ channel). This enhancement configuration would not have been abundant in the crystal bathed in only 150 mM KCl (or RbCl). Further experiments are needed to test this possibility.

The idea that K+ channels have multiple ions in their pore is as old as the ion channel field itself (Hodgkin and Keynes, 1955). The mind's eye picture of multiple ions in a queue earned K+ channels the name "long-pore channels" because it seemed that multiple ions

with a large separation between them would require a long pore. When mutational analysis of K+ channels identified a short pore-region sequence, and even shorter signature sequence forming the selectivity filter, the result seemed contradictory (Heginbotham et al., 1994). How could so few amino acids form a long enough pore to accommodate the queue of ions? The K+ channel structure answers this question. And ironically, in the answer, we see that the long pore is not very long at all: three ions are compressed into a distance of roughly 15 Å—about half the membrane thickness. The close spacing is probably not happenstance; it allows for strong interactions between ions in the pore. Ionic interactions, mediated by electrostatics and perhaps by the structure of the selectivity filter itself, offer a solution to the apparent paradox of simultaneous high turnover and high selectivity in K+ channels.

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