An old probe sheds new light on BK channel pore structure

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In many scientific areas, including drug discovery, the greatest challenge is picking the best targets and questions to pursue. To develop improved treatments using target-based therapeutics, a better understanding of the roles of specific channel types in normal and pathophysiological function is needed. Development of pharmacological probes provides a proven approach for target validation in which selective channel modulators can be developed as needed and then tested in animal models and human tissues. This approach is limited by availability of suitable ion channel modulators, which must display appropriate potency, selectivity, and physiochemical and pharmacokinetic properties to enable meaningful pharmacological experiments. An understanding of the mechanism of action of a probe molecule is also vital for interpreting its effects at the cellular, organ, and in vivo levels. A new paper by Zhou and Lingle (2014) provides a detailed mechanistic study of paxilline block of high conductance calcium-activated potassium channels (referred to as BK, maxi-K, or *slo*1 channels). Zhou and Lingle find that paxilline, a secondary fungal metabolite produced by *Penicillium paxilli*, blocks BK channels by an unusual closed-state mechanism. This information provides a framework to interpret the effects of this compound at the cellular and tissue levels, as well as insights into the gating mechanism of these channels.

A brief history of paxilline

Paxilline is a member of a group of indole diterpene natural products that are potent and selective blockers of BK channels (Knaus et al., 1994). These BK channel inhibitors were identified in a biochemical screen using iodinated charybdotoxin (^{125}I-ChTx), a scorpion toxin peptide that blocks BK channels by binding at the extracellular pore entrance and occluding potassium flux (MacKinnon and Miller, 1988; Giangiacomo et al., 2008). At the time the indole diterpene inhibitors were identified, binding assays afforded one of the few viable higher throughput methods for screening ion channels. Knaus et al. (1994) described eight structurally related indole diterpenes, which all inhibited BK channels in vascular smooth muscle at low nanomolar or sub-nanomolar concentrations. Initial experiments with these indole diterpenes showed high selectivity for inhibition of BK channels compared with other ion channels, and these agents remain among the most potent and selective BK channel inhibitors described. Paxilline and some related indole diterpenes are commercially available, enabling widespread use of these compounds as pharmacological probes of BK channel function.

In the initial description, paxilline and verruculogen stimulated ^{125}I-ChTx binding and reduced toxin dissociation rates, whereas the other indole diterpene BK blockers inhibited binding, suggesting an allosteric interaction between ChTX and these compounds. Paxilline stimulated toxin binding in membrane fragments and intact cells with an estimated EC_{50} in the 100-nM range, which is higher than the concentrations needed for BK channel block in electrophysiological experiments (Knaus et al., 1994; Schmalhofer et al., 2005).

Identification of paxilline as a research tool for BK channel studies was based on a series of studies by microbiologists and synthetic and natural product chemists that described a family of neurotoxic indole diterpenes. Investigations of the biosynthetic pathway of paxilline (Scott et al., 2013) and chemical syntheses of related indole diterpenes (Smith et al., 2003) were motivated by the unusual structural features of these molecules and their importance in agriculture. Ryegrass staggers is a livestock disease characterized by tremors and ataxia, which is caused by ingestion of grass infected by endocytic fungi that produce various indole diterpene neurotoxins, primarily lolitrem B (Gallagher et al., 1981). Imlach et al. (2008) used mice lacking BK channels to demonstrate that the neurological effects of lolitrem B and paxilline are mediated by block of BK channels. Combining the conclusions of these molecular, behavioral, and field studies indicates that brain-penetrant BK channel blockers may produce adverse effects on motor function.

Anatomy of paxilline blockade

BK channels are formed by tetramers of α subunits (they may also include β subunits), which each include

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seven transmembrane domains that contain the pore and voltage sensors, and an intracellular region composed of two RCK domains that are involved in calcium sensing (Rothberg, 2012; Hoshi et al., 2013). Schmalhofer et al. (2005) expressed truncated constructs containing the transmembrane domains but lacking the RCK domains in TsA-201 human renal epithelial cells, and showed that although these constructs did not form functional channels, they did form tetrameric complexes that were competent to bind either labeled ChTX or iberiotoxin (IbTX; a related scorpion toxin peptide that also blocks BK channels), and that this binding interaction was stimulated by paxilline. In a detailed mapping study, Zhou et al. (2010) identified a glycine residue in the S6 transmembrane domain that is critical for paxilline block, using electrophysiological analysis of chimeric and mutated BK channels. These localization studies point to a binding site for paxilline that is closely associated with the pore and fit nicely with the present finding of Zhou and Lingle (2014) that paxilline binding is strongly dependent on channel open probability (Po) and does not affect gating charge movements.

The kinetic attack

Zhou and Lingle (2014) recognized that, despite its use as a research tool to potently and specifically block BK currents, paxilline's mechanism of interaction with the channel has remained largely unknown. The first clue to a possible mechanism was based on previous observations that paxilline blockade was relieved slightly at elevated Ca^{2+} (Sanchez and McManus, 1996; Imlach et al., 2011). What Zhou and Lingle discovered, however, was that relief did not arise from competition between Ca^{2+} and paxilline; rather, paxilline blockade was reduced when the channel open probability was higher. Higher BK channel open probability can coincide with higher Ca^{2+} concentrations, but it can also increase with depolarization. The observation that blockade was inversely correlated with Po, rather than with Ca^{2+} concentration, was thus consistent with previous observations, and hinted that blocker affinity may depend on a particular conformation of the pore. This "blockable" conformation of the pore would be defined by its preferential occupancy at lower Po than higher Po.

Zhou and Lingle estimated the IC_{50} for paxilline as a function of Po and found that, quantitatively, paxilline binds to fully open BK channels with remarkably low apparent affinity. Mean IC_{50} values for channels with Po of ∼0.1 were around 10 nM, whereas they increased to >1 µM as Po increased to >0.9. Next, they tested a series of simple kinetic blocking models and found that paxilline blockade was best described by a simple four-state allosteric mechanism in which paxilline can bind to either the closed or open state, but has a strong (∼500-fold) preference for the closed state. This mechanism is distinct from one in which paxilline can bind only to the closed state. Zhou and Lingle quantitatively showed that the four-state model accounts for the small but measureable currents likely caused by paxilline-bound open channels that occur at high open probability and high paxilline concentrations, whereas mechanisms allowing only closed-state block predict nearly complete block under these conditions.

A physical picture of blockade

Whereas the functional mechanism of paxilline blockade is much clearer now than before, we are now tasked with gaining a better understanding of its physical correlate. Zhou and Lingle (2014) addressed the physical mechanism by testing whether paxilline block is slowed by other pore blockers like sucrose and bbTBA (*N*-(4-[benzoyl]benzyl)-*N,N,N*-tributylammonium). These molecules do slow the blocking kinetics, consistent with the idea that they impede access to a blocking site within the pore.

Paxilline does not, however, hinder chemical modification of a cysteine mutant, A313C, with the sulfhydryl-reactive MTS compound, MTSET, in closed channels. The position of this substituted cysteine, presumably within the vestibule of the BK pore, provides a key constraint on working hypotheses of the physical mechanism. If paxilline cannot impede MTS modification of C313, then paxilline must either occupy a site deeper in the pore than the position of C313, or (as presented by the authors) there must be a pathway that permits access and modification of C313 when paxilline is bound.

There is substantial evidence that, in the closed state, small molecules (including relatively bulky quaternary ammonium blockers like bbTBA) have unimpeded access to the BK channel vestibule region, consistent with the idea that the canonical "trap-door" mechanism of K channel gating does not apply (Li and Aldrich, 2004; Wilkens and Aldrich, 2006). If we assume that one paxilline molecule can bind within the pore and stabilize the nonconducting state, it is perhaps not difficult to imagine that this binding is destabilized when the channel is activated and conducting (as expected for an allosteric mechanism). This view of the closed state provides a key rationale for ongoing structural studies of this class of channels.

Finally, can the new information from Zhou and Lingle (2014) help us understand the allosteric interactions between paxilline binding to a site in the inner cavity of the pore and ChTX (and IbTX) binding to a site at the external entrance to the pore? Increased open probability causes a modest enhancement in ChTX binding through a sevenfold increase in the association rate for binding (MacKinnon and Miller, 1988). Increased open probability produces an opposite effect (a nearly 1,000-fold increase in the IC_{50}) for paxilline block of BK channels. Paxilline allosterically destabilizes ChTX and IbTX binding to BK channels in membrane vesicles

(Knaus et al., 1994) and in cells (Schmalhofer et al., 2005) under which conditions channel open probability is expected to be low. Further declines in the open–closed equilibrium in the presence of paxilline would not be expected to enhance toxin binding, suggesting that changes in closed-state occupancy are not the link between paxilline and toxin binding to BK channels. Functional mapping studies of toxin–BK channel complexes have identified interaction regions at the entrance to the ion conduction pathway, where ion selectivity is determined, and at the adjacent turret regions (Park and Miller, 1992; Giangiacomo et al., 2008). Perhaps further studies may show whether paxilline modifies one or more of these regions as part of its inhibitory effect.

Pharmacological relevance

Paxilline fulfills many requirements for a useful pharmacological probe; it is potent, apparently selective, widely available, and active in in vivo models. The findings of Zhou and Lingle (2014) that paxilline preferentially blocks closed channels suggest additional considerations when interpreting paxilline effects in cells and tissues. Because BK channels are typically open only at micromolar or higher calcium concentrations, or at very depolarized potentials, the low average open probability of BK channels in most cellular contexts renders the channels sensitive to paxilline block. Paxilline would then be expected to block BK channels during periods of transient activation as those that occur during action potentials or calcium sparks in smooth muscle (Hill-Eubanks et al., 2011). However, paxilline may be a less effective probe for BK channel activity during ischemia or pathological conditions with increased intracellular calcium concentrations. These are likely to be important considerations in the future development of BK-specific modulators for treatment of disease.

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