Multilevel regulation and BK channels
At first glance, many aspects of physiology appear to function like a Rube Goldberg machine, using convoluted, over-engineered components to perform simple tasks. Then, we remember that any given physiological system must operate within strict bounds despite exposure to a broad range of environments and behavioral scenarios. This perspective allows elegance and power to emerge from the inherent complexity of overlapping regulatory pathways and feedback loops and explains how proteins subject to multilevel regulation can operate in diverse tissues.

The large-conductance Ca\textsuperscript{2+} and voltage-activated K\textsuperscript{+} channel, known alternatively as the BK, BK\textsubscript{Ca}, MaxiK, K\textsubscript{Ca}1.1, or Slo 1 channel, is an example of a protein machine that is subject to control by multilevel regulation. Although the BK channel is encoded by a single gene (\textit{Kcnma1}), the resultant proteins are first diversified by extensive pre-mRNA splicing of exons (Butler et al., 1993; Fodor and Aldrich, 2009). Next, in common with other membrane proteins, the density and residence time of BK channels at the plasma membrane are controlled by the activity of trafficking pathways. The subcellular localization of BK channels is also important. In excitable cells, BK channels often dwell close to voltage-activated Ca\textsuperscript{2+} (Ca\textsubscript{i}) channels and so gain exposure to the micromolar surges in Ca\textsuperscript{2+} required to shift the activation curve from supra-physiological potentials (>200 mV) into the physiological voltage range during depolarization (Fakler and Adelman, 2008). The biophysical attributes of the resultant pore-forming \( \alpha \) subunits are further regulated, both by partnerships with various accessory \( \beta \) and \( \gamma \) subunits and by the activity of a slew of posttranslational modification pathways, including phosphorylation and palmitoylation (Reinhart et al., 1991; Shipston, 2013). This impressive arsenal of regulatory sentinels fine-tunes the operation of BK channels to support disparate physiological roles in multiple tissues.

In excitable cells, BK channel currents contribute to the repolarization of action potentials and, in particular, the fast phase of afterhyperpolarizations (Adams et al., 1982; Lancaster and Nicoll, 1987; Storm, 1987). In this way, the activity of BK channels impacts a plethora of physiological functions, including vascular tone (Nelson et al., 1995), vasoregulation (Brenner et al., 2000b), bladder function (Meredith et al., 2004), hearing (Pyott et al., 2007), and circadian rhythms (Meredith et al., 2006). Deletion of \textit{Kcnma1} leads to disorders of hyperexcitability, prompting the notion that BK channel agonists have therapeutic potential for treating a range of disorders (Nardi and Olesen, 2008; Gesnner et al., 2012).

One of the most intriguing roles ascribed to BK channels is to influence diurnal oscillations in the electrical activity of pacemaker neurons in the suprachiasmatic nucleus (SCN) (Meredith et al., 2006). In mammals, the SCN is located in the hypothalamus and acts as a master clock to synchronize circadian rhythms throughout the body. Central clock neurons are notable for the ability to produce robust cell-autonomous, self-sustaining oscillations in action potential frequency that peak at 5–10 Hz during the day and diminish at night (<3 Hz) (Schwartz et al., 1987; Yamaguchi et al., 2003; Belle et al., 2009). The core process responsible for circadian rhythms is a transcriptional–translational auto-feedback loop (TTL) that generates and destroys clock gene products with close to 24-h periodicity (Hardin et al., 1990; Reppert and Weaver, 2002). This remarkable mechanism operates even when the machinery is isolated from zeitgebers, environmental stimuli that act as time cues. Some progress has been made in understanding how TTls are converted into the ion channel choreography responsible for each phase of the electrical cycle in SCN neurons (Colwell, 2011). As in other neurons, channels that pass hyperpolarizing currents are key regulators of action potential firing frequency. Among the active principles, rhythmic expression of BK channels peaks at night and contributes to the nocturnal quiescence of SCN neurons (Cloues and Sather, 2003; Meredith et al., 2006; Pitts et al., 2006). Studies with iberiotoxin, a scorpion toxin that blocks the conduction pore of BK channels, showed that although BK channels contribute as much as 40% of the current that mediates nocturnal afterhyperpolarizations in clock neurons, they have much less impact during the day (Cloues and Sather, 2003). Indeed, forced overexpression of BK channels is required to disrupt daytime electrical rhythms in the SCN of transgenic mice (Montgomery et al., 2013).
The activity of BK channels in clock neurons is colored by a dynamic excitatory landscape reflecting the varying expression patterns of multiple proteins and the time of day that the cells are studied. For example, the activity of all BK channel variants is tuned by the expression profile of CaV channels. L-type CaV channels mediate much of the Ca2+ influx in SCN neurons and, along with P/Q and T-type CaV channels, are more highly expressed during the day (Panda et al., 2002; Nahm et al., 2005). At midday, when intrinsic electrical activity peaks, the resting cytosolic Ca2+ level in SCN neurons is ~150 nM, approximately double what has been measured during times of lower activity (Colwell, 2000). In addition to shifting the activation curve of BK channels, intracellular Ca2+ levels are critically important for global regulation of TTLs and circadian clock oscillations throughout the natural kingdom, from plants to Aplysia and up to the mammals (Colwell et al., 1994; Johnson et al., 1995; Ikeda et al., 2000; Harrisingh et al., 2007). Given that excitatory drive and Ca2+ influx are both elevated during the day, it is reasonable to question if the decreased density of BK transcripts is sufficient to explain why the activity of the residual large-conductance K+ channels appears to be inconsequential to the daytime activity of clock neurons? In this issue of the JGP, Shelley et al. provide further insight into this question and reveal how multilevel regulation of BK channels might impact the symphony of SCN biophysics.

**Novel BK channel variants from SCN neurons**
The study describes two BK variants cloned from mouse SCN. BK0 and BKSRKR are identical except for the inclusion of four amino acids in the intracellular C-terminal domain of BKSRKR at exon splice site 1 (Shelley et al., 2013). Amid the many splice options described for BK channels, BK0 and BKSRKR both include the “MANG” alternative translation initiation N-terminal sequence; both have no insert at splice site 2 (the stress axis–regulated exon site [STREX]); both include the 27–amino acid alternative exon at splice site 3 (Ha et al., 2000); and both have the VYR C terminus at splice site 4. Intriguingly, compared with control transcript for BK channels, the relative expression of BKSRKR transcripts in the SCN was increased by ~75% during the day, a time when the activity of clock neurons is augmented but the role of BK channels is diminished. This result indicates that TTL control of BKSRKR is temporally distinct from other BK channel transcripts and predicts that BK SRKR channel activity will be subject to increased excitatory drives in SCN neurons.

**Figure 1.** A cartoon illustrating how multiple regulatory factors might impact the activity of BK0 and BKSRKR channels in clock neurons. (Top) BK channels are formed by four α subunits, each of which has seven-transmembrane domains, characterized by an external N terminus and an intracellular C-terminal domain that is subject to extensive pre-mRNA editing. BKSRKR transcripts are more prevalent during the day and differ from BK0 by the inclusion of four C-terminal residues (SRKR) that allow phosphorylation at S642 (red). Most BK channel types are more highly expressed at night, lack the SRKR motif, and are not phosphorylated at S642. (Bottom) Possible activation curves for BK0 and BKSRKR channels in clock neurons in the presence of 100 µM of internal Ca2+ (cyan), with the β4 subunit (magenta) or the dephosphorylating agent alkaline phosphatase (Alk P; black). The green boxes represent resting and peak membrane potentials observed in SCN neurons during the night (left) or during the day (right). Assuming phosphorylation at S642 and assembly with β4, the expected right-shift in the activation profile of BKSRKR channels will reduce K+ currents (magenta) despite the increased daytime excitability of clock neurons. Other neurophysiological and biophysical factors not considered here also contribute to the activity of native BK channels. The BK activation curves and the depolarization range of SCN neurons are based on data presented by the Meredith group in this issue of the Journal (Shelley et al., 2013) and in prior studies (Montgomery and Meredith, 2012).
To assess the impact of the SRKKR motif on BK channel function, BK0 and BKSRKR were heterologously expressed in HEK293 cells and studied in inside-out, excised patches with varying levels of Ca2+ in the perfusate. Although at least 10 µM Ca2+ was required to bring the midpoint of voltage-dependent activation ($V_{1/2}$) of both BK0 and BKSRKR channels into the physiological voltage range, the G-V relationships remained right-shifted when compared with mouse mbr5 channels, a benchmark clone of BK. These relative shifts were apparent at 0, 1, and 100 µM Ca2+, but not at 10 µM Ca2+, a pattern hypothesized to reflect differences in the operation of the high affinity “Ca2+ bowl” caused by inclusion of the 27-amino acid alternate exon at splice site three. $V_{1/2}$ values for BKSRKR channels were further right-shifted in the presence of 1 or 100 µM of internal Ca2+ and exhibited slowed activation and increased deactivation kinetics. Such biophysical changes are consistent with a model in which BKSRKR channels pass relatively lower currents at steady state despite the increased daytime excitatory drive of clock neurons (Fig. 1). Of note, BK channels containing the SRKR motif have been cloned previously from human brain and from chick and turtle cochlear (Tseng-Crank et al., 1994; Rosenblatt et al., 1997; Jones et al., 1999). The SRKR motif was observed to have less functional impact in these early reports, likely reflecting the influence of other alternate exons incorporated into the clones.

Exposing the channels to alkaline phosphatase ameliorated the biophysical differences observed between BKSRKR and BK0 with 1 or 100 µM Ca2+. Further investigation revealed that the distinct phenotype of BKSRKR channels resulted from phosphorylation at an unexpected residue. The functionally relevant site was not S642 in the SRKR motif; rather, the effects were a consequence of phosphorylation at S642, a residue present in both BKSRKR and BK0. Thus, the G-V relationship, the $V_{1/2}$, and the activation and deactivation time constants of BKSRKR-S642A channels closely resemble those measured from BK0 channels, arguing that phosphorylation of S642 in BK channels discriminates the biophysical attributes of the variants and is only permitted when the SRKR motif is incorporated at the first exon splice site.

To further characterize the biophysical attributes of BK0 and BKSRKR channels, the Meredith group embarked on two further studies. Channel activity was studied in the presence of coexpressed β4, the predominant BK accessory subunit in the SCN (Brenner et al., 2000a; Montgomery and Meredith, 2012), and in response to action potential waveforms rather than by square-pulse protocols. In addition to slowing gating kinetics, assembly with β4 imparts complex changes to the Ca2+ response of BK channels, decreasing sensitivity at low Ca2+ concentrations and increasing sensitivity at high concentrations, with little impact at moderate concentrations of Ca2+ (~10 µM) (Brenner et al., 2000a).

Coexpression of β4 slowed both the activation and deactivation kinetics of BK0 and BKSRKR channels, augmenting the difference observed between the variants at 100 µM Ca2+; deactivation rates were decreased by as much as fourfold at 10 µM Ca2+. A marked rightward shift in the G-V relationship was also observed in both channels, with changes in the $V_{1/2}$ of 30 mV for BK0 and as much as 84 mV for BKSRKR channels. Thus, coexpression of the β4 subunit augmented the functional differences between the variants, suggesting that BKSRKR β4-channel complexes would pass less current in SCN neurons (Fig. 1).

To study the impact of physiologically relevant excitatory stimuli, whole-cell BK0 and BKSRKR channel currents were evoked in HEK293 cells in response to action potential waveforms recorded from SCN neurons during the peak (day) and trough (night) of intrinsic electrical activity. Daytime waveforms are distinguished by a greater peak depolarization, shorter half-width, and a more negative resting level and firing threshold (Montgomery and Meredith, 2012). In the presence of 50 µM Ca2+, and in the absence of the β4 subunit, BK channel currents followed the depolarizing phase of both waveforms, passing larger peak currents with decreased half-width when stimulated by the daytime action potential. When compared with BK0, BKSRKR Channels passed smaller currents with decreased charge transfer and current half-width in response to either waveform, most likely reflecting the differences in gating kinetics observed between the variants rather than differences in behavior at steady state. However, action potential waveform-evoked currents from cells expressing BKSRKR-S642A channels more closely resembled BK0 than BKSRKR, suggesting that SRKKR-dependent phosphorylation of S642 could play a role in influencing the activity of SCN neurons.

Coexpression of β4 significantly increased the differences between BK0 and BKSRKR channels measured in response to nighttime action potentials, from 2.5- to threefold. In contrast, coexpression of β4 did not significantly alter the 2.8-fold difference in peak current when BK0 and BKSRKR channels were activated by the daytime waveform. Thus, assembly with β4 in the SCN is expected to have less impact on the biophysical attributes of native BK channel complexes during the day, when levels of BKSRKR channel transcripts peak, although its impact on subunit trafficking and surface levels of BKSRKR channels in neurons remains an open question.

What comes next?

In the SCN, BK channels are more prevalent at night when clock neurons are less active. Writing in this issue of the JGP, the Meredith group describes BKSRKR, a variant cloned from mouse SCN that is more highly expressed during the day, when BK channels have little impact on excitability. The data presented argue that except for temporal regulation of pre-mRNA spliced
transcript number, multilevel regulatory factors will act to reduce the magnitude of BKSRKR channel currents in clock neurons. Thus, assembly with coexpressed β4 subunits and SKRR motif-dependent phosphorylation at S642 reduce currents by modulating gating kinetics and responses to Ca2+ (Fig. 1). A model emerges wherein the increased expression of BKSRKR transcripts diminishes the contribution of BK channel currents to the daytime activity of SCN neurons, permitting increased excitability.

The strength of this model relies on many aspects of the native system that are as yet unclear but represent tantalizing questions moving forward. These include understanding the mechanisms that selectively generate BKSRKR transcripts and discriminate daytime from nighttime expression. BKSRKR is not unique in this regard; TTL pathways have been described previously for RNA-binding proteins that act to preferentially produce specific transcript variants (Lareau et al., 2004). Similarly, the activity of BKSRKR channels will depend on the temporal expression profiles of many other SCN proteins including the β4 subunit and the kinases and phosphatases that act at S642. Also unknown is the subunit composition of native channels. Heteromultimeric of BK α subunits is yet another opportunity for cells to modulate excitability by diversifying channel function. This could be particularly relevant when channels contain a mixture of BKSRKR and other α subunits, as combining components with different phosphorylation status is known to differentially govern both activation and current magnitude (Tian et al., 2004).

Multilevel regulation of BK channels offers a dizzying array of competing and complementary regulatory permutations by which cells can tune excitability in a dynamic manner. This is especially true in clock neurons, where fearless exploration into the fascinating realm of chronobiology is revealing the utility of the many tricks available to control the activity of these enigmatic channels. As the Meredith group (Shelley et al., 2013) and others continue to break new ground, we find that, as is often the case with complex physiological systems, the more we come to understand, the more there is for us to learn—and therein lies the fun!

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