Characteristics of single large-conductance Ca\(^{2+}\)-activated K\(^+\) channels and their regulation of action potentials and excitability in parasympathetic cardiac motoneurons in the nucleus ambiguus

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Lin M, Hatcher JT, Wurster RD, Chen Q-H, Cheng ZJ. Characteristics of single large-conductance Ca\(^{2+}\)-activated K\(^+\) channels and their regulation of action potentials and excitability in parasympathetic cardiac motoneurons in the nucleus ambiguus. Am J Physiol Cell Physiol 306: C152–C166, 2014. First published November 6, 2013; doi:10.1152/ajpcell.00423.2012.—Large-conductance Ca\(^{2+}\)-activated K\(^+\) channels (BK) regulate action potential (AP) properties and excitability in many central neurons. However, the properties and functional roles of BK channels in parasympathetic cardiac motoneurons (PCMNs) in the nucleus ambiguus (NA) have not yet been well characterized. In this study, the tracer X-rhodamine-5 (and 6)-isothiocyanate (XRITC) was injected into the pericardial sac to retrogradely label PCMNs in FVB mice at postnatal 7–9 days. Two days later, XRITC-labeled PCMNs in brain stem slices were identified. Using excised patch single-channel recordings, we identified voltage-gated and Ca\(^{2+}\)-dependent BK channels in PCMNs. The majority of BK channels exhibited persistent channel opening during voltage holding. These BK channels had a conductance of 237 pS and a 50% opening probability at +27.9 mV, the channel open time constant was 3.37 ms at +20 mV, and dwell time increased exponentially as the membrane potential depolarized. At the +20-mV holding potential, the \([\text{Ca}^{2+}]_0 \) was 15.2 \(\mu\text{M} \) with a \(P_{0.5} \) of 0.4. Occasionally, some BK channels showed a transient channel opening and fast inactivation. Using whole cell voltage clamp, we found that BK channel mediated outward currents and afterhyperpolarization currents \(I_{\text{AHP}} \). Using whole cell current clamp, we found that application of BK channel blockeriberiotoxin (IBTX) increased spike half-width and suppressed fast afterhyperpolarization \(I_{\text{AHP}} \) amplitude following single APs. In addition, IBTX application increased spike half-width and reduced the spike frequency-dependent AP broadening in trains and spike frequency adaptation \(SFA \). Furthermore, BK channel blockade decreased spike frequency. Collectively, these results demonstrate that PCMNs have BK channels that significantly regulate AP repolarization, \(I_{\text{AHP}}, SFA \), and spike frequency. We conclude that activation of BK channels underlies one of the mechanisms for facilitation of PCMN excitability.

nucleus ambiguous; parasympathetic cardiac motoneurons; vagal; BK channels; repolarization; afterhyperpolarization; firing frequency; adaptation

PARASYMPATHETIC PREGANGLIONIC cardiac motoneurons (PCMNs) in the nucleus ambiguus (NA) regulate cardiac functions (negative control of chronotropic, dromotropic, and inotropic actions) (40). Previously, we have demonstrated that lesions of the NA abolish the baroreflex control of heart rate in rats (8). Therefore, the NA plays a key role in arterial baroreflex control of heart rate. Impairment of arterial baroreflex function has been used as an indicator of potential life-threatening arrhythmia and heart failure (13, 34). Despite this, little is known concerning the cellular mechanisms responsible for the alteration of membrane properties and neuronal activity of PCMN, which may underlie the dysfunction of arterial baroreflex in disease states. Therefore, it is very important to study the membrane properties and excitability of PCMN in normal animals. These studies will be used as a foundation for the investigation of disease-related changes in PCMN. In the literature, both small conductance and large conductance Ca\(^{2+}\)-activated K\(^+\) channels (SK and BK channels, respectively) were reported to mediate the firing properties of action potentials (APs) and excitability of central neurons (14–16, 59, 67). Previously, we have characterized the functional roles of SK channels in regulating firing properties of APs and excitability of PCMN (36). However, the membrane properties and the functional roles of BK channels of PCMN have not yet been fully determined.

The BK channels (Maxi-K or slow) are both voltage gated and intracellular Ca\(^{2+}\)-dependent (5, 33, 56). The BK channels have a single-channel conductance of >100 pS and are sensitive to the specific blockers IBTX (7, 19) or paxilline (57). When activated by membrane depolarization and Ca\(^{2+}\) during APs, BK channels allow the efflux of K\(^+\) out of the cell, thus repolarizing and hyperpolarizing the membrane potential. This turns off voltage-dependent Ca\(^{2+}\) channels and thus reduces the influx of Ca\(^{2+}\) into the cell. These negative feedback mechanisms allow BK channels to play key roles in regulating firing properties, such as repetitive firing of neurons (67), spike broadening during repetitive firing (59), and spike-frequency adaptation (26). There has also been evidence that BK channels can moderate neurotransmitter release and provide some protection against neuronal death (35, 51, 52, 68).

In the central nervous system, BK channels mainly contribute to AP repolarization and, to a lesser extent, to the fast \(I_{\text{AHP}} \) following APs (16, 42, 48, 50, 61, 62). Activation of the BK channels was previously reported to reduce the frequency of APs and limit epileptiform bursts in rat hippocampal CA1 pyramidal neurons (3, 32). More recently, however, an opposite effect of BK channels on neuronal excitability was found in mouse dentate granule neurons that lack the \(\beta_4\) subunit of BK channels. Blockade of BK channels lacking the \(\beta_4\) subunit widened the APs and reduced the excitability (6). Granule cells from \(\beta_4\)-knockout mice showed a gain-of-
function for BK channels that sharpened APs and supported higher firing rates, which may lead to temporal lobe epilepsy. Altogether, these data indicate that activation of BK channels either exert a negative or “brake” effect on the firing rate (spike frequency) of neurons or facilitate AP firing of neurons. In an early study on PCMNs, blockade of BK channels did not show significant effects on excitability (46). In contrast, we recently found that blockade of BK channels in PCMNs slightly but significantly decreased firing rate in response to low-intensity current injection (38). As indicated by Gu et al. (26), large current injection more effectively activated BK channels, which had powerful effects in facilitating high-frequency firing and causing early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. Therefore, the physiological role of BK channels in regulating membrane properties and excitability of PCMNs appears controversial. In this study, we first identified single BK channels and assessed their voltage and Ca2+ dependence in excised patches and then examined the effects of BK channels on the membrane properties and excitability of single APs and spike trains of PCMNs in whole cell patches. In addition, we studied BK outward currents and afterhyperpolarization currents (I_{AHP}), which may provide an underlying mechanism for AP firing properties and the control of excitability in PCMNs.

MATERIALS AND METHODS

Female (n = 12) and male FVB (or FVB/N in full name) (n = 4) mice were obtained from Jax Laboratory at age of 2–3 mo. Sixty-eight neonatal mice were used for patch-clamp recordings from brain slice preparations in experimental groups, and each group included 8–10 neurons (see RESULTS). Each neuron was only used for one test. Mice were maintained on a 12-h light/dark cycle and received food and water ad libitum. All animals were then maintained in the transgenic animal facility. When females became pregnant, they were transferred into individual cages. The procedures were approved by the University of Central Florida Animal Care and Use Committee and followed the guidelines established by National Institutes of Health.

Fluorescent Labeling of PCMNs and Brainstem Slice Preparation for Patch-Clamp Recordings

FVB neonatal mice (postnatal days 7–9) were anesthetized with 3% isoflurane (Abbott Laboratories, North Chicago, IL). When the animals were not responsive to tail and toe pinch, a right thoracotomy was performed and the fluorescent tracer X-rhodamine-5 (and 6)-isothiocyanate (XRITC, 2%, 4 µl; Molecular Probes, Eugene, OR) was injected into the pericardial sac at the base of the heart to retrogradely label cardiac motoneurons in the NA (25, 37). Previously, XRITC has been used to label NA neurons for patch-clamp study of PCMNs (25, 46, 71). After at least a 48-h recovery period, neonatal mice were deeply anesthetized with 4% isoflurane. The brainstem including PCMNs were sliced into serial sections (250 µm) using a vibrating blade microslicer (DTK-1000; Kyoto, Japan), and the brain slices were maintained in an interface chamber (36–38).

In brainstem slices, PCMNs were identified in the NA by the presence of XRITC. These slices were viewed with infrared illumination and differential interference optics (DIC; Carl Zeiss, Göttingen, Germany) under fluorescent illumination with a near-infrared sensitive, cooled charged-coupled device camera (AxioCom MRm; Carl Zeiss, Göttingen, Germany). XRITC-labeled PCMNs were identified by superimposing the fluorescent and DIC images (Fig. 1).

Single-channel recordings. Single-channel currents were recorded in both inside-out and outside-out patch configurations. For inside-out patches (27), patch pipettes were made using fire-polished borosilicate glass electrodes (1.5-mm outer diameter; World Precision Instruments, Sarasota, FL) on a Flaming/Brown puller (Sutter Instruments, Novato, CA) to a final resistance of 3–4 MΩ and filled with a solution containing the following (in mM): 130 K-glucolate, 10 KCl, 1 MgCl2, 10 HEPES, and 16 dexamethasone, adjusted to pH 7.4 with KOH. At room temperature (20–24°C), inside-out patches were obtained by withdrawing patch pipettes from varicosities after formation of a gigaohm seal. Following excision, the cytoplasmic face was bathed in an artificial intracellular solution containing the following (in mM): 140 K-glucolate, 2 MgCl2, 15 HEPES, 1 ATPNa2, and 5 EDTA, pH adjusted to 7.3 with KOH. CaCl2 was added to give the required free Ca2+ concentration calculated using the MAXCHELATOR program (Winmaxchelator software; Dr. Chris Patton, Stanford University, Pacific Grove, CA; http://www.stanford.edu/~cpatton/webmaxcS.htm).

In some experiments CaCl2 was added for a free Ca2+ concentration of 10 μM. To study the Ca2+ dependence of BK channels, CaCl2 was added to the artificial intracellular solution with Ca2+ concentrations adjusted from 0.5 to 100 μM. For the Ca2+-free solution, the bath solution contained no CaCl2 and was supplemented with 10 mM EGTA, giving a calculated free Ca2+ concentration of <1 nM. For outside-out patch recording, the patch was excised from the whole cell configuration at room temperature. The pipette was filled with a standard internal solution containing the following (in mM): 110 K-glucolate, 10 KCl, 5 NaCl, 2 MgCl2, 10 HEPES, 0.5 EGTA, 1 ATP, 0.2 GTP, and 0.1 leupeptin, with pH adjusted to 7.3 with KOH. The bath solution was identical to the HEPES solution for the whole cell voltage clamp recording (see below).

To identify single BK channels in inside-out or outside-out patches, the following criteria were used: 1) outward currents, 2) amplitudes

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Fig. 1. Identification of tracer X-rhodamine-5 (and 6)-isothiocyanate (XRITC)-labeled parasympathetic cardiac motoneurons (PCMNs) in the nucleus ambiguus (NA) in brainstem slices. A: differential interference optics imaging of PCMNs. B: fluorescent imaging of PCMNs shows XRITC-labeled PCMNs in the NA that were labeled by injection of the fluorescent tracer XRITC (2% solution, 4–8 µl; Molecular Probes) into the pericardial sac 2 days before experiment. C: merged image of A and B. Arrow indicates a recorded PCMN. Scale bar = 40 µm.
>10 pA at 60 mV, 3) voltage and/or Ca2+ dependence, and/or 4) IBTX or paxilline sensitivity. In all cases of single-channel recording, the current was low-pass filtered at 1 kHz and acquired at a sampling rate of 10 kHz using a Digidata 1300 analog-to-digital converter (Axon Instruments, Union City, CA) and the Clampex acquisition program of pCLAMP (version 9.2; Axon Instruments). Data were obtained in 1- to 3-min intervals while the patches were held at −65 mV. To determine the relationship between voltage and the channel steady-state open probability (Ps), events lists were made from data files using the half-amplitude threshold criterion of the Fetchan program of PCLAMP (10). The Ps was analyzed and the data were fit to the Boltzmann equation \[ P_s = \frac{1}{\sqrt{\pi}} \frac{1}{\sqrt{1 + e^{\frac{V_m - V_{1/2}}{\Delta V}}}^n} \]

where \( P_s \) is the open probability, \( V_m \) is the membrane potential, \( V_{1/2} \) is the half-maximal activation voltage, and \( \Delta V \) is the slope factor.

The relationship between intracellular Ca2+ concentration ([Ca2+]i) and the channel steady-state Ps was fitted according to a Hill equation: \[ P_s = \frac{P_{max}[Ca^{2+}]_{i}^{n}}{[Ca^{2+}]_{i}^{n} + K_{n}^{n}} \]

where \( P_{max} \) is the maximum open probability, \( n \) is the slope factor, and the \( n \)th root of \( K \) gives an estimate of the midpoint of the activation curve. The slope factor provides an estimate of the number of Ca2+ ions involved in maximally activating the channel (44). Thus the required [Ca2+]i to achieve the midpoint of activation (Ps50), designated as [Ca2+]i50, has been a widely used parameter to measure the calcium sensitivity of BK channels. To generate all-point histograms of the BK current events, single-channel current amplitudes were measured in an unbiased manner by using the Fetchan analysis program. Data were filtered to a final cut-off frequency of 500 Hz using the Fetchan digital Gaussian filter. Open times of BK channels were measured with half-amplitude threshold analysis. A single exponential function was used to fit the open time distribution curves, and the open time constant (\( \tau \)) was obtained. Current dwell times were binned using a log scale bin size, plotted on linear bin histograms of the BK current events, single-channel current amplitudes were measured in an unbiased manner by using the Fetchan analysis program.

Whole cell clamp recording. For whole cell voltage-clamp recordings, electrodes were heat-polished to a final tip resistance of 2–3 MΩ and filled with the same internal solution as the outside-out patch recording pipette described above. Within the recording chamber, slices were stably held in position using a nylon net stretched over a flattened U-shaped platinum wire and were continuously superfused at the room temperature (22–25°C) with artificial cerebrospinal fluid that contained the following (in mM): 126 NaCl, 2.5 KCl, 2 CaCl2, 26 NaHCO3, 1.25 NaH2PO4, 2 MgSO4, and 10 dextrose, was equilibrated with 95% O2-5% CO2, and had the pH adjusted to 7.4. Patch electrodes used for rupture patch recordings were fabricated from borosilicate glass with a Flaming/Brown horizontal puller. Electrodes were heat-polished to a final tip resistance of 3–5 MΩ and filled with intracellular solution containing the following (in mM) 120 potassium-glucuronate, 20 KCl, 2 MgCl2, 10 HEPES, 0.1 EGTA, 2 ATP, and 0.25 GTP, with pH adjusted to 7.3 with KOH.

To generate a single AP, a 10-ms depolarizing current pulse of sufficient intensity (40–100 pA) from the holding potential −65 mV to trigger a single AP was applied. We used a 10-ms duration because this stimulus duration has been commonly used to evoke single APs in other central neurons (11, 17, 36). Within minutes after good recording conditions were established (i.e., resting membrane potential was less than −60 mV and AP peak amplitude was >70 mV), AP half-width was measured as the spike width at the half-maximal voltage using Clampfit 9.2. To standardize AHP recordings, neurons were depolarized to a holding potential of −65 mV by direct current (DC) application through the recording electrode. AHP was recorded after applying a 10-ms depolarizing current pulse of sufficient amplitude (40–100 pA) to generate a single AP (11). The AHP was measured by subtracting the peak amplitude of the hyperpolarizing deflection after the spike from the threshold for spike initiation. AHP amplitudes were measured at 10, 50, and 100 ms.

To investigate the AP firing properties of PCMs, current injection steps were applied from 100 to 340 pA in 20 pA increments. Instantaneous firing rate was measured as the inverse of interspike intervals. Input resistance was determined by Ohm’s law using a linear regression within the linear range (±10 mV from the resting potential) of the voltage-current (I-V) relationship that had been established by plotting the steady-state voltage change in response to a series of depolarizing and hyperpolarizing current injections. To determine the voltage threshold of an AP, AP trains were evoked by 1.5-, 0.2 pA/msec ramp current injections from a holding potential of −65 mV, and the threshold was determined at the membrane potential once the first AP started to fire.

Data acquisition was controlled using the ClampEx program in the PClamp 9 software package. The series resistance was in the range of 5–10 MΩ (typically 5 MΩ) and was compensated 60% on-line. Membrane potential measurements were not corrected for the liquid junction potential (~15 mV). Leak currents were subtracted using a standard P/4 protocol. Before seals (5 GΩ) were made on cells, offset potentials were nulled. Capacitance subtraction was used in all recordings. Signals were recorded using a MultiClamp 700B patch-clamp amplifier (Axon Instruments, Foster City, CA). Responses were low-pass filtered at 5 kHz and digitized at 50 kHz with a 16-bit analog-to-digital data acquisition systems (Digidata 1322A; Axon Instruments).

Drugs and Chemicals

All channel blockers used in the present study were purchased from Sigma-Aldrich (St Louis, MO). Blockers contained in the micropipette solutions were delivered by focal ejection to the vicinity of the patches or soma (50–100 μm) from upstream of the perfusion (2 ml/min) using a pressure injector (MP8000; MDI, South Plainfield, NJ; 5 min, 5–15 psi).

Statistics

Data were presented as means ± SE. A t-test, one-way, or two-way ANOVA with repeated measures followed by a Tukey-Kramer post hoc test was used. P < 0.05 was considered as significant.
RESULTS

Figure 1 shows that the injection of the tracer XRITC into the pericardial sac retrogradely labeled NA PCMNs. All recorded neurons were strongly labeled by XRITC (25, 36, 37).

Identification of BK Channels

To identify single BK channels and characterize their properties, BK channels were first identified and examined in outside-out (n = 10) and inside-out (n = 8) excised patches. In outside-out patches, BK channels were usually opened to a unitary current level, had a large conductance, and were mostly in the open state at +40 mV (Fig. 2A, top trace). These single-channel currents were almost completely blocked by the specific BK channel blocker paxilline (10 μM; Fig. 2A, middle trace) and these single-channel currents recovered after paxilline washout (Fig. 2A, bottom trace). In inside-out patches, we identified single BK channels as well. In most recordings, we only observed unitary current levels (Fig. 2A, B). Occasionally, infrequent subconductance states were also observed in some traces (Fig. 2B, top trace). Outward currents were diminished in the Ca\(^{2+}\)-free solution, but they recovered after application of an external solution containing 10 μM free Ca\(^{2+}\) (Fig. 2B, bottom trace).

Voltage Dependence of BK Channels and Effects of [Ca\(^{2+}\)]

We examined the voltage dependence of BK channels in inside-out patches. At and below −20 mV, the BK channels were mostly in a closed state and showed only brief openings (inward currents; Fig. 3A). At +20 mV, a macroscopic outward single-level current was seen and its amplitude increased with further depolarization, reversing around 0 mV as would be expected for a potassium-selective channel in symmetrical potassium ([K]\(_i\) = [K]\(_o\), 140 mM; see MATERIALS AND METHODS). Depolarized potentials increased, the current amplitude increased. The current amplitude-voltage relationship was well fitted (Fig. 3C), giving a slope conductance of 237 pS, which is consistent with the conductance value of BK channels in other neurons and smooth muscle (44). The open probability (P\(_o\)) of BK channels exponentially increased as the depolarization increased (Fig. 3D). Activation was rapid, and there was no inactivation within 2–3 min of voltage steps.

We examined Ca\(^{2+}\) dependence of BK channels in inside-out patches held at +20 mV in bath solutions ranging from 0.5 to 100 μM Ca\(^{2+}\) (Fig. 3B). At this membrane potential, P\(_o\) increased as [Ca\(^{2+}\)] increased (Fig. 3E). Some channel openings were observed even at a cytoplasmic calcium level as low as 0.5 μM. BK channel open probability was P\(_o\) = 0.199 ± 0.034 at 0.5 μM Ca\(^{2+}\) and P\(_o\) = 0.488 ± 0.096 at 100 μM Ca\(^{2+}\). [Ca\(^{2+}\)]\(_{0.5}\) was 15.2 ± 1.0 μM at which the estimated P\(_{0.5}\) was ~0.4. Overall, BK channel activity depends both on membrane potentials and intracellular Ca\(^{2+}\) levels.

BK Channel Kinetics, Activation, and Inactivation

To characterize the distribution of current amplitude, opening time constant, and dwell time of BK channels, outside-out patches were used. The extracellular face was perfused with HEPES containing 2 mM Ca\(^{2+}\). The cytoplasmic face was exposed to an intracellular solution with 0 mM Ca\(^{2+}\) within the pipette. Figure 4A shows unitary BK currents recorded at holding potentials of +20 and +60 mV. All-points histograms of BK current amplitude at +20 mV (Fig. 4B) and +60 mV (Fig. 4C) had two peaks in each distribution. The amplitudes of the larger unitary current peaks were 9.45 ± 0.88 pA and 15.05 ± 0.48 pA at +20 mV and at +60 mV (n = 8), respectively. Current-voltage curves were fitted with a Boltzmann function.
Fig. 3. Voltage dependence and Ca\(^{2+}\) concentration dependence of BK channel currents (unitary upward current deflections) in inside-out patches from PCMN. 

A: representative currents from a single BK channel at different holding potentials in the presence of 10 μM free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). 

B: representative BK channel currents recorded from an inside-out patch held at +20 mV. Cytoplasmic face was perfused with intracellular solution from 0.5 to 100 μM Ca\(^{2+}\). C: current vs. voltage (I-V) curve; n = 10. D: BK channel open probability (P\(_o\)) was plotted as a function of the membrane potential (V\(_m\)); n = 10. E: P\(_o\) vs. Ca\(^{2+}\) concentration curve; n = 9.

(Fig. 4D), yielding values for half-maximal activation (V\(_{0.5} = 16.2 ± 0.7\) mV) and slope factor (κ = 24.8 ± 1.2). Open-time distributions were constructed from idealized data ignoring transitions of <0.5 ms duration and could be fitted well using a single exponential function as shown in Fig. 4E (+20 mV) and Fig. 4F (+60 mV). The mean time constants were not statistically different at +20 mV and +60 mV (P > 0.10, n = 15). In the 120-mM intracellular K\(^+\) condition, the dwell time of the channel opening was fitted using a single exponential function and increased exponentially as the membrane potential increased (Fig. 4G). The all-points histograms, open-time, and dwell time were obtained from those patches that contained only one BK channel based on the fact that maximum current amplitudes were unitary at high P\(_o\).

In inside-out patches we observed that some BK channels displayed fast inactivation. These BK channels opened, remained open for a few hundred milliseconds, and closed (inactivated) for the remainder of the period of continuous 2- to 3-min holding membrane potentials at +20, +40, and +60 mV (Fig. 5, A\(_1\), B\(_1\), and C\(_1\)). Following inactivation of such a transient opening, these channels could not be reactivated by further increasing depolarization membrane potential up to +80 mV. However, it could be reactivated by another depolarization potential (e.g., +40 mV) after the holding membrane potential completely returned to the baseline of −65 mV (not shown here). In comparison, the BK channels in the majority of patches exhibited persistent opening (i.e., high P\(_o\) and a significant dwell time) over a period of 2–3 min in which holding membrane potentials were maintained (Fig. 5, A\(_2\), B\(_2\), and C\(_2\)).

**BK Channel-Mediated Outward Currents and Afterhyperpolarization Current (Whole Cell Patch)**

To study BK channel-mediated currents, we used TTX (0.5 μM) to block Na\(^+\) and 4-AP (1 mM) to reduce voltage-dependent K\(^+\) currents but not BK currents (24). In the following experiments, TTX and 4-AP were routinely added in the bath solution. Under these conditions, outward currents and I\(_{AHP}\) were obtained.

A family of outward currents were evoked by 250-ms voltage steps ranging from −70 to +40 mV in 10-mV increments from a holding potential of −70 mV in the control bath solution. The outward currents were composed of a rapidly inactivating transient component and a slowly inactivating
persistent component (Fig. 6, $A_1$ and $B_1$). To dissociate the BK currents from outward currents, two specific BK channel blockers were employed. Application of 100 nM (saturation dose) of the BK channel blocker IBTX reduced outward currents (Fig. 6$A_2$). IBTX-sensitive outward currents, including the transient and persistent components, were obtained by subtracting the suppressed currents after IBTX application from the currents evoked in the control solution (Fig. 6$A_3$).

Likewise, the paxilline-sensitive currents (Fig. 6$B_3$) were obtained by subtracting the suppressed currents after 10/8262M (saturation dose) paxilline (Fig. 6$B_2$) from the outward currents before application (Fig. 6$B_1$). Since the cell size of PCMNs did not vary much (conductance: 67.3 ± 0.7 pF, $n = 64$), we plotted $I$–$V$ curve without normalizing BK current using conductance. Paxilline-sensitive outward currents were similar to IBTX-sensitive outward currents (Fig. 6$C$; $P > 0.10$, IBTX: $n = 9$, paxilline: $n = 10$).

To examine the whether BK channels mediate afterhyperpolarization current ($I_{AHP}$), $I_{AHP}$ was evoked by a 100-ms depolarizing voltage pulse from a holding potential of −70 to +10 mV followed immediately by a 1.5-s, 50-mV voltage pulse (Fig. 6, $D_1$ and $E_1$, black trace). Application of 100 nM IBTX (red) and 10/8262M paxilline (blue) reduced the amplitude of $I_{AHP}$ (Fig. 6, $D_1$ and $E_1$). By subtracting the currents before and after IBTX or paxilline, IBTX- and paxilline-sensitive $I_{AHP}$ were obtained (Fig. 6, $D_2$ and $E_2$). The mean peak amplitude of $I_{AHP}$ were 40.7 ± 15.2 pA (IBTX sensitive) and 29.1 ± 13.7 pA (paxilline sensitive), respectively (Fig. 6$F$). They were not significantly different ($P > 0.10$; IBTX: $n = 8$, paxilline: $n = 9$). Thus BK channels significantly contribute to outward currents and $I_{AHP}$.

**BK Channels Regulated AP Repolarization and AHP in Single APs**

To examine the contribution of BK channels to the AP properties, we first examined the effects of BK channel blockers on single spike repolarization. A single AP was evoked by
injecting a 10-ms depolarizing current pulse from the holding potential $-65$ mV with an intensity sufficient to generate a single AP before (Fig. 7A, black trace) and after blockers [Fig. 7A, red (IBTX) and blue (paxilline) traces]. Spike widths were measured at the half-maximal voltage. The mean half-widths were significantly increased after IBTX and paxilline, respectively (Fig. 7B; $P < 0.05$, $n = 9$ group).

To investigate whether BK channel-mediated currents contribute to AHP, AP was elicited using the same protocol as above before (Fig. 7C, black trace) and after blockers [Fig. 7C, red (IBTX) and blue (paxilline) traces]. Application of IBTX reduced the amplitude of AHP by $11.3\%$ from $-20.3 \pm 0.6$ to $-18.0 \pm 0.7$ mV at 10 ms relative to the beginning of AP (Fig. 7D; $P < 0.05$; $n = 9$ group). Application of paxilline reduced the amplitude by $4.8\%$, from $-20.9 \pm 0.3$ to $-19.9 \pm 0.2$ mV (Fig. 7E; $P < 0.05$; $n = 8$ group). Blockade of BK channels did not reduce the AHP at either 50 or at 100 ms (Fig. 7, D and E).

**Frequency Dependence of AP Repolarization in Spike Trains**

To characterize AP repolarization in spike trains, APs were evoked using current injections of different intensities. To standardize AP analysis, APs were evoked from a holding membrane potential of $-65$ mV and the current intensity was adjusted to trigger 4- to 20-Hz spike trains. The representative 1st APs in spike trains were shown in Fig. 8A. The half-widths of the first APs progressively increased as the firing frequency of the trains increased ($P < 0.05$; $n = 8$ group) and the first AP broadening was more pronounced at higher train frequencies (15 and 20 Hz; Fig. 8B). Within a train, the AP half-width was significantly increased as the spike order number increased (Fig. 8, C and D; $P < 0.05$; $n = 8$ group). Compared with the same spike order number across trains, the AP half-width was broader in higher frequency spike trains (frequency-dependent broadening).

**BK Channels Regulate AP Repolarization and Frequency-Dependent Broadening in Spike Trains**

To examine whether BK channels regulate AP repolarization and the frequency-dependent broadening, 10-, 15-, and 20-Hz spike trains were generated. The first two APs in 15- and 20-Hz trains are shown in Fig. 9A. Blockade of BK channels slowed the repolarization of APs and increased the interspike interval between first and second spikes. In trains, half-width had frequency dependency. Application of IBTX (100 nM; red trace) and paxilline (10 $\mu$M, blue trace) significantly increased the half-widths of individual APs within the trains (Fig. 9B; black trace, $P < 0.05$; $n = 8$ group). This indicates that BK channels regulate the AP repolarization for each individual AP within the train. To examine whether BK channels regulate the frequency-dependent broadening, we normalized the effect of BK channel blockade on the half-width relative to the first AP.
half-width, i.e., the half-width of a higher order AP was divided by the half-width of the first AP (Fig. 9C). As shown in Fig. 9C, the normalized half-width-spike number curves in control and BK channels blocked groups (IBTX and Paxil- 
line) were significantly different in 15- and 20-Hz trains 
(P < 0.05, n = 8/group) but only slightly different in the 
10-Hz train. Thus BK channels significantly contribute to 
the frequency-dependent broadening in 15- and 20-Hz trains 
but did not to the frequency-dependent broadening in lower 
frequency trains.

**Contribution of BK Channels to Spike Frequency and Spike Frequency Adaptation**

To evaluate the effect of BK channels on excitability, spike trains were evoked by 1-s depolarizing currents (100–340 pA in 20-pA increments) from a holding potential of −65 mV in control and after application of IBTX. Figure 10A shows original recordings before and after IBTX. Under control conditions, spike frequency significantly increased as injected currents increased (Fig. 10C, black trace). Application of IBTX to the somatic surface significantly reduced the spike firing rate compared with control (Fig. 10C; P < 0.05; n = 8/group), indicating that activation of BK channels significantly facilitates excitability. Figure 10B shows five to seven early spikes and the effect of IBTX on the early firing of a 1-s spike train that was evoked by a depolarizing current pulse (220 pA) before (black trace) and after IBTX (red trace). Application of IBTX (100 nM) slowed the repolarization and lengthened afterhyperpolarization of APs and increased interspike inter- 
vals [Fig. 10B; red trace; spike frequency adaption (SFA)], thus reducing the instantaneous firing frequency (Fig. 10D). It should be emphasized that IBTX significantly suppressed SFA as shown in Fig. 10D. To further analyze SFA, spike instantaneous frequencies were normalized relative to the second spike instantaneous frequency. Normalized spike instantaneous frequencies significantly decreased at the third spike and re-
remained unchanged afterwards in IBTX, while in control normalized spike instantaneous frequencies continued to decrease as the spike number increased up to the seventh spike (Fig. 10). IBTX did not significantly change the cell input resistance nor the DC current required to hold the background potential at \(-65\) mV (data not shown).

**BK Channels Did Not Affect Membrane Potential Threshold and Depolarizing Input Resistance**

Spike trains were elicited by a 1.5-s depolarizing ramp current from 0 to 300 pA from a holding potential of \(-65\) mV in control and during IBTX (Fig. 11, A and B). Similar to Fig. 7, instantaneous firing rate and half-width were progressively increased during depolarizing ramp current injections in the control. IBTX significantly reduced the instantaneous firing rate, increased half-width, and suppressed the frequency dependency of spike broadening (data not shown). However, IBTX had no significant effect on the first AP threshold (Fig. 11C) and the depolarizing input resistance (\(R_{\text{input}}\); Fig. 11D; \(P > 0.10; n = 8\) /group).

**DISCUSSION**

Using excised patch recordings, we first identified two types of BK channels in PCMN. The majority of them were persistent, meaning that they did not show inactivation during the voltage holding over 2–3 min (\(P_o\) was high during this period), whereas some other BK channels were transient, showing fast inactivation after a few 100 ms opening and stayed inactive during the rest of voltage holding (\(P_o\) was 0 during this period). Since the majority of the BK channels that we encountered were persistent, we further tested their voltage dependency and Ca\(^{2+}\) dependency as well as characterized their opening and closing kinetics. BK channels of PCMN had a conductance of 237 pS. BK channel activity exhibited a voltage and Ca\(^{2+}\) concentration dependence and could be reversibly blocked by specific BK blockers IBTX and paxilline. These characteristics are consistent with a variety of other cell types (see Ref. 24 for a review). Using whole cell voltage clamp, we found that there were transient and persistent components in IBTX-sensitive and paxilline-sensitive outward currents which were voltage dependent. Using whole cell current clamp, we found that blockade of BK channels markedly broadened the spike half-width in both single and train spikes, reduced the spike train frequency-dependent broadening, and decreased the amplitude of fAHP. In addition, BK channel blockers largely reduced the firing frequency and suppressed early spike-frequency adaptation. Taken together, these data indicate that BK channels in PCMN regulate the AP properties and facilitate AP firing.
possibly by regulating their repolarization, fAHP, and early spike-frequency adaptation.

**Physiological Properties of BK Channels of PCMNs**

BK channels are the only member of the voltage-dependent potassium channel family that is activated by both voltage and Ca\(^{2+}\) (44). This allows a particularly unique function of BK channels in the integration of voltage and Ca\(^{2+}\) signals and modulation of membrane excitability in a variety of cell types (21). In most previous studies, BK channels were shown to exhibit sustained activation (e.g., 29, 69). Likewise, Pérez et al. (49) recently found that BK channels from canine cardiac ganglia did not exhibit intrinsic inactivation. In our study, we also found fast inactivation in some BK channels in PCMNs in addition to the majority of sustained BK channels. For persistent BK channels, they had a conductance of 237 pS, which is comparable to those values for BK channels as reported in other neurons (18, 23, 49, 53, 60). In these PCMsNs, BK channels were highly voltage and intracellular Ca\(^{2+}\) dependent, and the channel open dwell time increased exponentially as membrane potential depolarized. The relationship between BK channel activity and intracellular Ca\(^{2+}\) was previously studied (30, 31, 47, 66). Similar to most of these studies, we found that intracellular Ca\(^{2+}\) concentrations \(\geq 0.5 \mu\text{M}\) were required to significantly induce unitary BK current and reached maximal activation at \(\sim 50 \mu\text{M}\).

For transient BK channels, fast inactivation of PCMNks occurred within several hundred milliseconds in response to continuous voltage holding and stayed inactivated. In previous studies (29, 43), fast inactivation of BK channels of hippocampal pyramidal neurons were observed and these channels showed fast inactivation within \(\sim 100\text{ ms}\). Using whole cell voltage-clamp recording, we found that IBTX and paxilline-sensitive currents showed both transient and persistent components, indicating that BK channels are capable of inactivation. Whether fast inactivation of transient and persistent BK currents identified in single channels contribute to the transient outward component and persistent BK channels, respectively, is an interesting issue. Alternatively, initial fast activation of all BK currents may quickly reduce Ca\(^{2+}\) influx, thereby reducing BK outward currents and resulting in a transient peak in outward currents.

**BK Channels Contribute to AP Repolarization and fAHP**

BK channels are involved in AP repolarization in other types of neurons (1, 32, 58, 63). Consistent with these reports, we showed that BK channels contribute to AP repolarization in PCMNks. After blockade of BK channels, the half-widths of individual APs in both single APs and in spike trains were increased. In addition, the broadening of each individual APs within a train was spike frequency dependent and reduced after blockade of BK channels. The contribution of BK channels to

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Fig. 8. Frequency dependence of AP broadening in spike trains. A: superimposed traces of the representative 1st AP from 4-, 7-, 10-, and 15-Hz spike trains. B: half-width of 1st AP was spike-train frequency dependent. C: representative 1st, 2nd, and 5th APs for 10- and 15-Hz trains. D: AP half-width for individual spikes within the 4- to 20-Hz trains.
spike frequency-dependent broadening during repolarization has been found in other neurons. BK channel-mediated spike broadening in other neurons reaches a plateau after the second or third spike, possibly due to the repolarizing currents that are fully inactivated within the first few spikes (15, 22, 59). In contrast, PCMNs exhibited a progressive cumulative broadening pattern up to the 20th spike in a 20-Hz spike train. Previously, it has been shown that fast-inactivating voltage-gated K\textsuperscript{+} channel-mediated currents (A-type K\textsuperscript{+} current: I\textsubscript{A}) contribute to repolarization of APs (4, 61) and inactivation of I\textsubscript{A} eliminated frequency-dependent AP broadening in Aplysia neurons (41). Since BK outward currents showed a rapid inactivation (transient peaks), BK channels are well suited to contribute to spike frequency-dependent broadening (55).

Activation of BK channels also contributes to the fAHP in some cell types (14, 15, 54, 60). Similarly in PCMNs, blockade of BK channels reduced fAHP, but it did not affect medium (m)AHP and slow (s)AHP in single APs. This confirmed our previous observation (38). In contrast, SK channels regulate both fAHP and mAHP but not sAHP (36). Of note: blockade of BK channels with IBTX noticeably increased AHP in trains. This might contradict our finding that blockade of BK channels had reduced fAHP in single APs. Actually, blockade of BK channels with IBTX could slow repolarization and delay inactivation of Ca\textsuperscript{2+} channels. This resulted in an increase of intracellular free Ca\textsuperscript{2+} and, in turn, activation of SK channels. As we showed previously (36), activation of SK channels may increase fAHP and mAHP which may contribute to the increase of mAHP in trains after blockade of BK channels as seen in Figs. 9A and 10B.

**BK Channels Facilitated Neuronal Excitability and Suppressed SFA in PCMNs**

In neurons, K\textsuperscript{+} channels are commonly believed to be inhibitory, and activation of K\textsuperscript{+} channels reduces excitability (28, 63). Accordingly, the activation of BK channels was frequently reported to reduce the excitability (3, 32, 70). In the present study, however, we found that BK channel blocker IBTX inhibited the firing rate in PCMNs. This indicates that the role of activation of BK channels in PCMNs is actually to increase excitability, rather than to inhibit it. Our data are
Fig. 10. Contribution of BK channels to spike frequency and spike frequency adaptation (SFA). A: representative spike trains were evoked by 1-s 220 pA depolarizing current injection in the absence (black trace) and presence of IBTX (red trace). B: average firing rates in response to current injections from 100 to 340 pA were significantly reduced after application of IBTX (†P < 0.05). C: superimposed initial 5–7 representative spikes from the trains evoked by 1-s 220 pA depolarizing current injection in the absence (black trace) and presence of IBTX (red trace). Apparently, IBTX increased the interspike interval and lengthened AHP in trains. D: instantaneous frequency as the inverse of interspike intervals. IBTX significantly decreased instantaneous frequency (†P < 0.05, n = 8/group). In addition, instantaneous frequency declined in the first a few spikes as the spike number increased, indicating an early SFA in both control and IBTX groups. E: normalized instantaneous spike frequency (NISF) relative to the 2nd spike. *P < 0.01, later spikes vs. the 2nd spike in control and IBTX; #P < 0.05, IBTX vs. control; n = 8/group.

Consistent with a more recent finding by Gu et al. (26) who found that BK channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. This facilitation was considered to be due to enhanced Na\(^+\) channel inactivation and the delayed rectifier K\(^-\) channel activation (26). Interestingly, paxilline blockade of BK channels lacking the β4-unit also decreased the number of APs in response to current injection in dentate gyrus neurons (6), indicating that BK channels lacking the β4-unit facilitate excitability. Furthermore, Du et al. (12) used a mutation of the α-subunit of the BK channel that enhanced BK channels, leading to increased excitability by inducing rapid repolarization of APs. More recently, Pérez et al. (49) reported that the BK current also increased excitability in isolated canine intracardiac neurons. Thus far, the mechanisms for inhibitory or excitatory roles of BK channels are still not fully understood. Apparently, BK channels may have different roles in regulating AP properties and excitability in different neurons and even under different conditions.

In PCMs, the instantaneous firing rate in the spike trains evoked by current injection was reduced dramatically during the first several initial spikes. This indicates that SFA occurred in PCMs. Since the AP half-width increased significantly during the first several spikes, it is possible that the increased half-width may contribute to SFA. After blockade of BK channels, SFA was seen only within the first two spikes. This indicates that BK channels significantly contribute to SFA, which is consistent with Gu et al. (26) who found that BK channels cause early SFA in CA1 hippocampal pyramidal cells. Since SFA did not completely disappear after blockade, we assume that other channels may contribute to SFA as well. As reported, voltage-gated Na\(^+\) and delayed-rectifier K\(^+\) currents may contribute to SFA in other neurons (45).

**BK and SK Channels: a Comparison**

Another Ca\(^{2+}\)-activated K\(^+\) channel, the SK channel, also regulates AP properties and excitability in many central neu-
rons (2). In contrast to BK channels, SK channels are activated solely by increases in intracellular Ca²⁺. Previously, we have characterized the functional roles of SK channels of PCMNs (36). As a comparison, BK channels contribute to AP repolarization and fAHP, whereas SK channels contribute to AP repolarization, fAHP, and mAHP in single APs. SK channel blockade increased the spike frequency and abolished spike-frequency dependence of AHP in trains but did not alter SFA. Also, SK channel blockade increased the depolarizing resistance and decreased the AP threshold. In contrast, BK channel blockade decreased the spike frequency, frequency dependence of AP repolarization, and SFA but did not change the depolarizing resistance and AP threshold. These data suggest that BK channels and SK channels play different roles in regulating PCMN excitability: while activation of SK channels provides a negative control for PCMN excitability, possibly by mediating repolarization, fAHP, mAHP, and AP threshold, activation of BK channels facilitates PCMN excitability possibly by regulating repolarization, fAHP and SFA.

Perspectives and Significance

BK channels play a key neuroprotective role against ischemic brain damage. Liao et al. (35) showed middle cerebral artery occlusion in mice lacking BK channels (loss-of-function) produced a significantly larger infarct volume and led to more severe neurological deficits and higher postischemic mortality than control wild type mice. Previously, we have demonstrated that PCMNs in the NA play a key role in regulating cardiac functions and that chronic intermittent hypoxia (CIH; a model for sleep apnea) may reduce PCMN control over the heart rate and induce cell loss (8, 9, 72, 73, 74). However, how CIH causes impaired function and cell death in the PCMs of the NA has not yet been elucidated. It has been reported that CIH markedly decreased open probability of BK channels in the CA1 pyramidal neurons which may account for the hippocampal injury in patients with sleep apnea (64). Further, Tjong et al. (65) demonstrated that administration of melatonin ameliorates CIH-induced deficits in the constitutive NO production and impaired BK channel activity, which may provide a potential therapeutic treatment of neuronal injury in sleep apnea patients. Since BK channels are not only voltage- and Ca²⁺-dependent, but are also sensitive to oxygen levels (20, 39), we speculate that CIH may impair BK channels in PCMs, which may lead to excessive Ca²⁺ influx, inducing cell death in the NA (72–74). In this study, we have systematically examined the functional role of BK channels in regulating excitability and AP properties of PCMs, which will underlie a foundation for the future investigation for disease-induced changes of PCMs.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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