Functional evidence for Na\(^+\)-activated K\(^+\) channels in circular smooth muscle of the opossum lower esophageal sphincter

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Zhang Y, Paterson WG. Functional evidence for Na\(^+\)-activated K\(^+\) channels in circular smooth muscle of the opossum lower esophageal sphincter. *Am J Physiol Gastrointest Liver Physiol* 292: G1600–G1606, 2007; doi:10.1152/ajpgi.00561.2005.—Na\(^+\) reduction induces contraction of opossum lower esophageal sphincter (LES) circular smooth muscle strips in vitro; however, the mechanism(s) by which this occurs is unknown. The purpose of the present study was to investigate the electrophysiological effects of low Na\(^+\) on opossum LES circular smooth muscle. In the presence of atropine, quanethidine, nifedipine, and substance P, conventional intracellular electrodes recorded a resting membrane potential (RMP) of $-37.5 \pm 0.9$ mV ($n = 4$). Decreasing [Na\(^+\)] from 144.1 to 26.1 mM by substitution of equimolar NaCl with choline Cl depolarized the RMP by 7.1 $\pm$ 1.1 mV. Whole cell patch-clamp recordings revealed outward K\(^+\) currents that began to activate at $-60$ mV using 400-ms stepped test pulses ($-120$ to $+100$ mV) with increments of 20 mV from holding potential of $-80$ mV. Reduction of [Na\(^+\)] in the bath solution inhibited K\(^+\) currents in a concentration-dependent manner. Single channels with conductance of 49–60 pS were recorded using cell-attached patch-clamp configurations. The channel open probability was significantly decreased by substitution of bath Na\(^+\) with equimolar choline. A 10-fold increase of [K\(^+\) ] in the pipette shifted the reversal potential of the single channels to the positive by $-50$ mV. These data suggest that Na\(^+\)-activated K\(^+\) channels exist in the circular smooth muscle of the opossum LES.

sodium-activated potassium channels; intracellular recording; patch-clamp recording

IT HAS BEEN REPORTED that removal of extracellular Na\(^+\) produces contraction of several different smooth muscles, which is accompanied by either membrane depolarization or hyperpolarization (1, 7, 26, 30). Contraction induced by external Na\(^+\) reduction that is associated with hyperpolarization can be explained by activation of a reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchange mechanism (7). However, the Na\(^+\)/Ca\(^{2+}\) exchange mechanism does not apply to contraction accompanied by membrane depolarization. More recently, it has been shown that reduction of Na\(^+\) inhibits K\(^+\) channels in cardiac muscle cells and neurons (9, 19, 28, 29), suggesting that Na\(^+\)-activated K\(^+\) (K\(_{Na}\)) channels are present in the tissues studied. To date, K\(_{Na}\) channels have not been described in gastrointestinal smooth muscles.

We (25) have previously demonstrated that Na\(^+\) reduction induces contraction of opossum lower esophageal sphincter (LES) circular smooth muscle strips in vitro. Furthermore, preliminary data have suggested that Na\(^+\) removal depolarized resting membrane potential (RMP), raising the possibility that the contraction induced by low Na\(^+\) may be in part due to inhibition of K\(_{Na}\) channels. The goals of the present study, therefore, were to investigate the effects of low Na\(^+\) on electrical activity of opossum LES smooth muscle and to determine whether these effects involve inhibition of K\(^+\) channels.

METHODS

Tissue preparation. Protocols were approved by the Animal Care Committee of Queen’s University. Opossums (*Didelphis virginiana*) of either sex, weighing between 2.5 and 5 kg, were used. Preparations of left LES strips for intracellular recordings and acutely dispersed single cells for patch-clamp recordings were prepared as previously described (35–37).

Intracellular recordings. Conventional intracellular microelectrode recordings were performed on opossum left LES circular smooth muscle perfused at a flow rate of 2.5 ml/min with preassaged (95% O\(_2\)-5% CO\(_2\)) Krebs solution containing atropine (3 $\mu$M), quanethidine (3 $\mu$M), nifedipine (1 $\mu$M), and substance P (1 $\mu$M) at 35°C. An agar bridge was employed to minimize junction potentials. Patch-clamp recordings. Experiments were conducted at room temperature (22–23°C) using a 3 M KCl agar bridge to minimize junction potentials. Atropine (3 $\mu$M) was included in all bath solutions to prevent the activation of muscarinic receptors when Na\(^+\) was replaced by choline. Current recordings were obtained only in relaxed cells by whole cell, cell-attached patch-clamp configurations using an Axopatch 200B amplifier coupled to a Pentium computer running pCLAMP 8.0 software through an analog-to-digital and digital-to-analog converter (Axon Instruments). Recordings were filtered at 1,000 Hz through a low-pass, four-pole Bessel filter (Frequency Device) and digitized at 2 kHz. Pipettes were pulled from borosilicate capillary glass (0.8–1.10 $\times$ 100 mm, Kimble Glass) and had a resistance of 3–10 M$\Omega$ after being filled with high-K\(^+\) solution containing 120 mM free Ca\(^{2+}\) (buffered by EGTA). Free [Ca\(^{2+}\)] was calculated by Eqcal software (Biosoft). Reduction of [Na\(^+\)] in the bath solution was achieved by substituting Na\(^+\) with either equimolar choline or N-methyl-D-glucamine (NMDG). Although atropine was in the bath, NMDG was also used to fully exclude the possibility that the effects seen following choline substitution were due to activation of muscarinic receptors.

Cell-attached configurations are shown in Fig. 1. The RMP of single cells was partially nullled to approximately $-20$ mV by raising bath [K\(^+\)] to 65 mM. As the calculated junction potential was $<1.6$ mV, it was ignored. In single channel experiments, the open probability ($P_o$) of channels was determined by fitting sums of Gaussian functions to all-point histograms generated by Fetchan 6.0 using a least-squares fitting program in pStat 6.0. $P_o$ was calculated by summing the proportion of each Gaussian component multiplied by its corresponding channel level and dividing this total by the maximal numbers of channels that were open simultaneously in that patch at the most depolarized potential, which was taken as an indication of the total number of channels (N) in that patch. However, it should be

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noted that this assumption likely underestimates the number of channels in the patch, especially if $P_o$ is low and no overlapping openings are observed. Therefore, the derived parameter should be read as “apparent or observable” $P_o$ (32). Single channel current amplitude was measured from all-point histograms.

Solutions and drugs. The modified Krebs solution contained (in mM) 118.07 NaCl, 25.00 NaHCO3, 11.10 D-glucose, 4.69 KCl, 2.52 CaCl2, 1.00 MgSO4, and 1.01 NaH2PO4. The modified Hank’s solution contained (in mM) 120.00 NaCl, 15.50 NaHCO3, 10.00 D-glucose, 5.36 KCl, 1.00 CaCl2, 1.00 MgSO4, 0.44 NaH2PO4, and 0.34 Na2HPO4. The physiological bath solution contained (in mM) 150.00 NaCl, 2.50 KCl, 5.50 D-glucose, 10.00 HEPES, 1.00 CaCl2, and 1.00 MgSO4, and pH was adjusted to 7.40 with 1 N NaOH. The high-K+/H11001 pipette solution for whole cell recordings contained (in mM) 150.00 KCl, 10.00 D-glucose, 10.00 HEPES, 1.00 MgSO4, 1.00 CaCl2, and variable concentration of EGTA to adjust free [Ca2+]. The composition of pipette and bath solutions for cell-attached recordings is shown in Fig. 1, B and C. The 0 mM Na+ bath solution was made by substitution of NaCl with 87.5 mM choline chloride or NMDG chloride. Choline chloride, NMDG base, tetraethylammonium (TEA)
chloride, and nifedipine were purchased from Sigma-Aldrich (Burlington, ON, Canada), and EGTA was from GIBCO. Nifedipine was dissolved in anhydrous ethanol as a stock solution (1 mM).

Statistical analysis. Data are shown as means ± SE; n represents the number of animals. Pre- and postdrug comparisons were made using a paired Student’s t-test. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of extracellular Na⁺ reduction on properties of RMP. Similar to our previous report, in the presence of atropine (3 μM), quinethidine (3 μM), and substance P (1 μM), LES circular smooth muscle RMP averaged −37.5 ± 0.9 mV (n = 4) and was characterized by RMP fluctuations of 1–4 mV. Reducing [Na⁺] in the bath from 144.1 to 26.1 mM by the substitution of equimolar NaCl with choline Cl depolarized RMP, which reached a maximum in 1–1.5 min and then remained in a steady state (Fig. 2A). This RMP depolarization returned to control values within 20–30 min of returning [Na⁺].

Spontaneous action potentials were usually superimposed on the membrane depolarization phase. The effects of TEA, a large-conductance Ca²⁺-activated K⁺ channel blocker, on the low-Na⁺-induced membrane potential depolarization was tested (Fig. 2, B and C). In the presence of nifedipine (1 μM), TEA (2 mM) produced a slight membrane depolarization (~3 mV). However, the RMP depolarization induced by low Na⁺ was not affected by the preapplication of TEA. These results are consistent with those of a study (11) in cultured brain stem neurons from chicks suggesting that TEA-sensitive Ca²⁺-activated K⁺ channels were not involved in low-Na⁺-induced membrane depolarization.

K⁺ channel currents recorded in the whole cell configuration. K⁺ currents (Iₖ) were recorded by patch clamping relaxed single smooth muscle cells dispersed from LES circular muscle. The Nernst reversal potential for K⁺ (Eₖ) was set at −103 mV, as pipette and bath solutions contained 150 and 2.5 mM K⁺, respectively. Iₖ was evoked by stepped test potentials of 400 ms from −120 to +100 mV with a holding potential of −80 mV and increments of 20 mV (Figs. 3 and 4, insets).

Iₖ started to be activated at −60 to −40 mV (Fig. 4A,a). Reduction of bath Na⁺ from 150 to 0 mM by substitution with either choline or NMDG significantly inhibited Iₖ (Figs. 3A and 4B). Time-course experiments demonstrated that this inhibition peaked within 3–4 min and then remained in a steady state (Fig. 3B). KNa channel current [Iₖ(Na)] was obtained by subtracting the current recorded after reduction of bath Na⁺ from that in control solution. Iₖ(Na) was characterized by fast activation that peaked within 50 ms upon the onset of the stepped test potentials. There was little inactivation above +80 mV of the test potential (Fig. 3A,a) and no inactivation at potentials of −80 mV or below (Fig. 4A,c). Activation curves of Iₖ(Na) (Fig. 4B,a and b) were obtained by measuring Iₖ(Na) at the peak (within 50 ms) and at 400 ms (P-400) and plotting Iₖ(Na) against test potentials (Fig. 4A,c). However, Iₖ(Na) did not show any inward rectification at the tested negative poten-

![Fig. 4. Effects of Na⁺ reduction on whole cell Iₖ. inset; voltage-clamp protocol. A,a and b: currents under control conditions (extracellular [Na⁺] = 150 mM; a) and 5 min after external Na⁺ removal (extracellular [Na⁺] = 0 mM; b). A,c: Na⁺-sensitive currents calculated by subtraction of A,b currents from A,a currents. B,a and b: statistical analysis of Na⁺-sensitive Iₖ measured at peak [•]: 150 mM choline, n = 6; ○: −150 mM N-methyl-D-glucamine (NMDG), n = 4; and P-400 (△: 150 mM choline; ▲: 150 mM NMDG). B,c: concentration response of Na⁺-sensitive currents obtained from Fig. 2A,b 5 min after Na⁺ reduction (substitution of choline for Na⁺). Curves were fitted by Hill’s equation, which yielded EC50 of [Na⁺] = 80.8 ± 2.5 and 70.6 ± 4.5 mM with slopes of 1.1 and 2.2 at P-400 and the peak of Iₖ (n = 6), respectively. The slopes of 1.8 and 2.2 at P-400 and the peak of Iₖ suggests that one binding site of K⁺ channel needs 2 Na⁺.

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physiological solution containing 2.5 mM K$^+$ was tested by varying bath [Na$^+$] in which Na$^+$ was replaced by equimolar choline or NMDG (Fig. 4B, d and b). Concentration-response curves of $I_{K(\text{Na})}$ were acquired by plotting $I_{K(\text{Na})}$ against external [Na$^+$]. A curve fit by the Hill’s function yielded an EC50 for [Na$^+$]/[K$^+$] addition of EGTA. Under such conditions of asymmetrical K$^+$ gradients across the membrane, amplitudes of steady-state single channel currents in the cell-attached configuration were obtained by curve fitting all-point histograms (Fig. 5A). By curve fitting the plot of single channel amplitudes against clamped membrane potentials, single channel conductance was calculated to be 49.3 ± 3.6 pS (n = 4) with a reversal potential of −94 mV (Fig. 5C). Increasing [K$^+$] to 25 mM in the pipette solution produced single channel conductance of 60.3 ± 2.9 pS (n = 5) with a reversal potential of −49 mV, suggesting that channel currents were carried by K$^+$ (Fig. 5, B and C). However, channel currents showed significant inward rectification at membrane potentials of −60 and −80 mV, when the pipettes were filled with solution containing 25 mM K$^+$ (Fig. 5B, f and g, and C).

The results shown in Fig. 6 demonstrate the dependence of single K$^+$ channel $P_o$ on Na$^+$. In these experiments, recording pipettes were filled with solution containing 150 mM Na$^+$ and 25 mM K$^+$, and single cells were perfused by solutions containing 87.5 mM Na$^+$ and 65 mM K$^+$. Free [Ca$^{2+}$] was 100 nM in both solutions. The cell-attached configuration recorded spontaneous single K$^+$ channel activity (Fig. 6A). $P_o$ of single K$^+$ channels generated by fitting an all-point histogram (Fig. 6B, unfilled) were 0.48 ± 0.08, 0.41 ± 0.06, 0.45 ± 0.09, and 0.70 ± 0.09 at the patch-clamped membrane potentials of −20, 0, +20, and +40 mV, respectively. After the replacement of Na$^+$ by choline (87.5 mM) in the bath solution, single K$^+$ channel $P_o$ were markedly decreased to 0.16 ± 0.15, 0.23 ± 0.09, 0.26 ± 0.09, and 0.27 ± 0.09, respectively (Fig. 6, B)

**Fig. 5.** Steady-state single channel activity in the cell-attached configuration. A and B: single channel activity at different clamped $V_m$ (a–g) with [K$^+$] in the pipette solution ($[K^+]_{\text{p}i} = 2.5$ and 25 mM). C: single channel closed state; $n_{o,s}$ number of single channel open levels. Note that the single channels in Bf and g displayed inward rectification. C: plot of single channel currents against clamped $V_m$. Linear regression yielded single channel conductances (g) of 49.3 ± 3.6 (n = 4) and 60.3 ± 2.9 pS (n = 5) with $[K^+]_{\text{p}i} = 2.5$ and 25 mM, respectively. However, a 10-fold change of [K$^+$]$_{\text{p}i}$ (from 2.5 to 25 mM) shifted reversal potential from −94 to −49 mV, which is close to that predicted by the Nernst equation, suggesting the K$^+$ channel identity.
and C). These data strongly suggest the dependency of these K\(^{+}\) channels on Na\(^{+}\), which is independent of voltage.

**DISCUSSION**

The present study demonstrates that in LES circular smooth muscle, reduction of external [Na\(^{+}\)] induces membrane depolarization, inhibits K\(^{+}\) currents in a concentration-dependent manner, and inhibits the \(P_\text{o}\) of single K\(^{+}\) channels with conductances of 49–60 pS. These data provide physiological evidence for the presence of K\(_{\text{Na}}\) channels in gastrointestinal smooth muscle.

It is well known that cell membrane ion channels are regulated by intracellular factors, including Ca\(^{2+}\), ATP, and cyclic nucleotides, in a variety of species and tissues (27). Therefore, it is perhaps not surprising that Na\(^{+}\) can play a similar role. The phenomena of K\(^{+}\) channel activity being increased by intracellular Na\(^{+}\) in a concentration-dependent manner was first reported in guinea pig ventricular single cells (19) and has been subsequently demonstrated in a variety of neuronal cells (3, 8, 11–13, 15). This kind of K\(^{+}\) channel was called the K\(_{\text{Na}}\) channel. The present study adds intestinal smooth muscle to the diversity of tissues in which the K\(_{\text{Na}}\) channel is functionally expressed.

The single-channel conductance of the K\(_{\text{Na}}\) channel seems to be diverse, depending on the different species and tissues studied. Multiple subconductance states are a conspicuous feature of K\(_{\text{Na}}\) channels in previous studies. For instance, K\(_{\text{Na}}\) conductance was reported as 105 pS in chick midbrain neurons (11), 170 pS in chick sensory ganglion neurons (15, 16), 170–200 pS in rat olfactory bulb neurons (12, 13), 142 pS in rat dorsal root ganglion neurons (6), 88 pS in peripheral myelinated axons of Xenopus laevis (20), and 210 pS in guinea pig ventricular myocytes (19). Moreover, the K\(_{\text{Na}}\) conductance of recombinant channels expressed in Xenopus oocytes is \(~95\) pS (28, 29). In the present study, the K\(_{\text{Na}}\) conductance was 49–60 pS with variable asymmetrical K\(^{+}\) gradients across the membrane. Several kinds of channels, including Na\(^{+}\), Ca\(^{2+}\), and several K\(^{+}\) channels, have a property of voltage dependence. However, the voltage dependence of the K\(_{\text{Na}}\) channel is controversial. Its \(P_\text{o}\) increased only slightly with depolarization in sensory neurons (12–15) but markedly in frog embryo spinal neurons (8). Opposing results were also reported in central nervous system neurons (10, 11) and cardiac myocytes (19).

The present study is consistent with the K\(_{\text{Na}}\) channel in LES smooth muscle being voltage independent (Fig. 6). This makes it unlikely that this K\(_{\text{Na}}\) channel is carried by either a large- or small-conductance channel, as both are voltage-dependent. Furthermore, the large-conductance channel blocker TEA had no effect on the RMP depolarization induced by low Na\(^{+}\). We did not test the effect of small-conductance channel blockers because the conductance of small-conductance channels is well below that of the \(I_{\text{K(Na)}}\) we recorded in the present experiments (32).

Depending on the cell type and study methodology, the sensitivity of K\(_{\text{Na}}\) channels to intracellular Na\(^{+}\) is also greatly variable, with EC\(_{50}\) ranging from 7.3 to 80 mM, with Hill slopes of 2–7 (5, 9, 28). The present study showed EC\(_{50}\) of extracellular [Na\(^{+}\)] of \(~70\) and 80 mM with Hill slopes of 2.2 and 1.8 at the peak and P-400, respectively, of the K\(^{+}\) currents (Fig. 4), although the exact intracellular [Na\(^{+}\)] was unknown. In neurons, several lines of evidence support a close localization of K\(_{\text{Na}}\) and voltage-dependent Na\(^{+}\) channels, suggesting that local accumulation of intracellular Na\(^{+}\) following a single action potential could be high enough to rapidly activate K\(_{\text{Na}}\) channels (5, 21, 24). The activation of K\(_{\text{Na}}\) channels, in turn, results in afterhyperpolarization. It has been reported that intracellular [Na\(^{+}\)] is \(~7.4\) mM in smooth muscle cells (1). In ureter smooth muscle, intracellular [Na\(^{+}\)] subsequently corresponds closely with alteration of extracellular [Na\(^{+}\)] (1).

The signal transduction pathways regulating K\(_{\text{Na}}\) channel activation in smooth muscle have not been fully characterized. Several lines of evidence suggest that the \(\beta\gamma\)-subunit of G...
proteins and phosphatidylinositol 4,5-bisphosphate (PIP2) are involved in the activation of K\textsubscript{Na} channels (17, 22). In cardiac myocytes, muscarinic K\textsuperscript{+} channels (K\textsubscript{ACH} channels), which are members of the inwardly rectifying K\textsuperscript{+} channel family, have been reported to be influenced by internal Na\textsuperscript{+} gating to control excitability of cells (29). ACh released upon vagal stimulation binds M\textsubscript{2} receptors to activate K\textsubscript{ACH} channels in pacemaker and ventricular myocytes, resulting in a decrease in heart rate and contractility. K\textsubscript{ACH} channels are activated by the \textbeta\textgamma-subunit of G proteins or intracellular Na\textsuperscript{+}, depending on the presence of PIP2, which is synthesized via the hydrolysis of ATP (17, 22, 23, 28, 29). Whether K\textsubscript{Na} channels utilize similar pathways is unclear. The fact that our I\textsubscript{K(Na)} was recorded in the presence of atropin suggests that it is not mediated by a K\textsubscript{ACH} channel. Inside-out patch-clamping experiments were attempted in four opossums (data not shown). However, once inside-out patches were excised, K\textsubscript{Na} channels lost activity, suggesting an intracellular second messenger is required for their activation (28, 29).

The molecular identity of the K\textsubscript{Na} channel has been characterized in recent studies (4, 33). The K\textsubscript{Na} channel is encoded by genes of either the Slo family (Slack) (4) or Slick (33). Slack was originally reported to encode large- and intermediate-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels (2, 18). Slo and Slick are closely related to each other and located on human chromosome 1, and the proteins they encode are highly homologous (5). Both K\textsubscript{Na} channel proteins are characterized by a large COOH-terminal region that consists of at least four domains, namely, an ATP-binding domain for metabolic regulation, a PDZ-binding domain (31), and two “regulator of K\textsuperscript{+} conductance” (RCK) binding domains. RCK domains are likely to be sites that bind Na\textsuperscript{+} for channel gating. The NH\textsubscript{2}-terminal of Slick is half the size of Slack. Slack and Slick share similar channel properties including sequence, single channel conductance, and activation by cooperative cytoplasmic Na\textsuperscript{+} and Cl\textsuperscript{−}. Slick is directly inhibited by intracellular ATP, similar to a classical ATP-sensitive K\textsuperscript{+} channel. We attempted to identify messages for Slick and Slack in opossum LES muscle using PCR and commercially available probes (data not shown) but were unsuccessful. However, no evidence for Slick and Slack messages was found when opossum brain (data not shown) but were unsuccessful. However, no evidence for Slick and Slack messages was found when opossum brain or spinal neurons of the chick embryo. Neurosci Lett 149: 133–136, 1993.


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