Characterization of a voltage-dependent Na\(^+\) current in human esophageal smooth muscle

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Characterization of a voltage-dependent Na\(^+\) current in human esophageal smooth muscle. Am J Physiol Cell Physiol 283: C1045–C1055, 2002. First published May 29, 2002; 10.1152/ajpcell.00359.2001.—Smooth muscle contraction is critical to peristalsis in the human esophagus, yet the nature of the channels mediating excitation remains to be elucidated. The objective of this study was to characterize the inward currents in human esophageal smooth muscle cells (HESMCs). Esophageal tissue was isolated from patients undergoing surgery for cancer and grown in primary culture, and currents were recorded using patch-clamp electrophysiology. Depolarization elicited inward current activating positively to −40 mV and peaking at 0 mV and consisting of transient and sustained components. The transient current was half-activated at −16 mV and half-inactivated at −67 mV. The transient current was abolished by removal of bath Na\(^+\) or application of TTX (IC\(_{50} \sim 20 \text{nM}\)), whereas it persisted in the absence of bath Ca\(^{2+}\) or the presence of Cd\(^{2+}\). These data provide evidence that cultured HESMCs express voltage-dependent Na\(^+\) channels. RT-PCR revealed mRNA transcripts for Na\(_x\), the “atypical” Na\(^+\) channel isoform, as well as Na\(_{1.4}\). These studies provide the first evidence of Na\(_{1.4}\) in smooth muscle and contribute to a model of excitation in HESMCs.

Sodium channel; esophagus; smooth muscle; electrophysiology; Na\(_{1.4}\).

SMOOTH MUSCLE COMPRISES the muscularis externa in the distal third of the human esophagus and is critical for peristalsis (18). At the level of the individual smooth muscle cell (SMC), depolarization leads to the opening of voltage-dependent Ca\(^{2+}\) channels, and the subsequent rise in cytosolic Ca\(^{2+}\) initiates contraction (31). Ion channels, therefore, influence esophageal motility by controlling membrane potential. Little is known, however, about the nature and regulation of the channels mediating excitation in human esophageal smooth muscle.

Efforts to understand the mechanisms of excitation in human esophageal SMCs (HESMCs) have focused on identifying the ion channels and signaling pathways. Previous studies have identified several classes of K\(^+\) current (32). Delayed rectifier K\(^+\) (K\(_{\text{v}}\)) current serves to regulate resting tension, whereas Ca\(^{2+}\)-activated K\(^+\) (K\(_{\text{Ca}}\)) current limits depolarization during contraction. The excitatory inward currents in the human esophagus, however, have not been investigated. Studies of esophagus from other species have revealed an L-type Ca\(^{2+}\) current (3) and a Ca\(^{2+}\)-activated Cl\(^-\) current (34). The depolarization mediated by inward Cl\(^-\) current may lead to the activation of other voltage-dependent currents, thereby initiating action potentials.

Although the rising phase of the smooth muscle action potential was originally suggested to be due solely to Ca\(^{2+}\) current, it is now clear that Na\(^+\) currents also contribute to excitation in some, although not all, smooth muscles (23). Voltage-dependent Na\(^+\) currents have been recorded in a number of smooth muscle tissues, including arterial (10), lymphatic (19), uterine (36, 37), and vas deferens smooth muscle (6). In the gastrointestinal tract, Na\(^+\) current has been observed in the ileum (30), large intestine (35), and fundus (25). Zholos et al. (38) recently provided electrophysiological evidence of a TTX-sensitive Na\(^+\) current in human intestinal smooth muscle. The observed differences in activation and inactivation ranges of these currents have led to the suggestion that considerable heterogeneity may exist among smooth muscle Na\(^+\) channels (7). Thus it is critical to study channel properties in specific tissues.

Despite these reports of Na\(^+\) currents recorded in smooth muscle, little is known regarding the molecular identity of smooth muscle Na\(^+\) channels. Na\(^+\) channels are encoded by a single gene family (17), and the most recent nomenclature is based on the convention adopted for other voltage-gated channels. The first isoform shown to exist in smooth muscle was Na\(_x\) (22), formerly considered the “atypical” Na\(_2\) subfamily but subsequently reclassified (17). In humans, Na\(_x\) is found in heart and uterus (15), although because of the heterogenous composition of whole tissues, with multiple cell types present, it is unclear whether the channel is...
specifically expressed in SMCs. Notably, Na₅ has not been demonstrated to mediate voltage-dependent inward current, despite attempts to express it in heterologous expression systems, leading to the suggestion that it may be a pseudogene (4, 17). The skeletal muscle Na₅.1.4 (16) has not been previously identified in smooth muscle, whereas a recent report identified mRNA encoding SCNA5 (cardiac Na₅.1.5 channel) (14) in human jejunal muscle (20).

The overall objective of these studies was to characterize the inward currents in human esophageal smooth muscle. To address limited availability of fresh tissues, we have developed a culture cell system that has proven valuable for studying esophageal physiology (33). Immunocytochemistry experiments performed on these cultures reveal the presence of smooth muscle α-actin, but not markers for endothelial cells, neuronal cells, or interstitial cells of Cajal (33). Thus the culture system provides an opportunity to examine the molecular identity of smooth muscle ion channels without contamination from other cell types. We present evidence that cultured HESMCs exhibit a TTX-sensitive, voltage-dependent Na⁺ current and that this current contributes to cellular excitation. Furthermore, we have used RT-PCR to examine the molecular identity of smooth muscle Na⁺ channels and find evidence for multiple Na⁺ channel isoforms. In addition to the atypical Na₅ isoform, we report the presence of the Na₅.1.4 skeletal muscle isoform for the first time in any smooth muscle. These data suggest a putative function for Na⁺ channels in esophageal motility and have possible implications for our understanding of esophageal development.

METHODS

Tissue retrieval and cell culture. Retrieval of human esophageal tissue and subsequent cell culture were performed as previously described (33), in accordance with the guidelines of the University of Western Ontario Review Board for Research Involving Human Subjects. Tissues were obtained from a disease-free region of the distal esophagus from patients undergoing surgery for cancer. The circular muscle layer was dissected and dispersed by incubation with 1.7 mg/ml collagenase, 0.5 mg/ml elastase, and 1 mg/ml bovine serum albumin in Hanks’ balanced salt solution for 45 min at 37°C. Dispersed cells were grown in primary culture in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ in air. In some cases, dissected tissue samples were rapidly frozen and stored at −70°C for subsequent RNA extraction (see RNA extraction and RT-PCR).

Electrophysiology. For electrophysiological recording, cells were lifted from culture dishes using 0.25% trypsin-0.1 mM EDTA (GIBCO) and allowed to settle on the glass bottom of a perfusion chamber. The chamber had a volume of 1 ml and was placed on the stage of a Nikon inverted phase-contrast microscope. Patch-clamp recordings were performed at room temperature using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Experiments using K⁺ in the patch pipette (see Solutions) were performed using the nystatin perforated-patch technique. To block K⁺ currents and allow characterization of inward current, we used Cs⁺ electrode solution (see Solutions) and recorded in the whole cell configuration. Pipette resistance in solution ranged from 3 from 6 MΩ, with up to 80% series resistance compensation applied. In some traces, capacitive and leak currents were compensated off-line, as assessed by subthreshold pulses. Uncancelled capacitive transients have been blanked in the current traces.

Solutions. During electrophysiological recordings, cells were perfused with Ringer solution containing (in mM) 130 NaCl, 5 KCl, 20 HEPES, 10 glucose, 1 CaCl₂, and 1 MgCl₂ and adjusted to pH 7.4 with NaOH. K⁺ electrode solution contained (in mM) 140 KCl, 20 HEPES, 1 EGTA, 1 MgCl₂, and 0.4 CaCl₂ and was adjusted to pH 7.2 with KOH. Cs⁺ electrode solution contained (in mM) 117 CsCl, 13 NaCl, 1 EGTA, 20 HEPES, 1 MgCl₂, 10 tetraethylammonium chloride, and 0.4 CaCl₂ and was adjusted to pH 7.2 with CsOH.

RNA extraction and RT-PCR. HESMCs were grown for 10–15 days, and total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. For controls, RNA was also isolated from human brain and heart. We also obtained RNA from intact tissues of the proximal esophagus, consisting of striated muscle, and of the distal esophagus, consisting of smooth muscle. RNA integrity was verified by agarose gel electrophoresis using ethidium bromide staining. Total RNA (4 μg) was reverse transcribed using random hexamers and Superscript RT (Life Technologies, Gaithersburg, MD). Three micrograms of cDNA reaction mixture were used in each PCR. The PCR oligonucleotide primers used to amplify cDNA were designed on the basis of human sequences and are listed in Table 1. PCR was performed in a 50-μl reaction containing PCR buffer, 2 mM MgCl₂, 200 μM dNTPs, primer at 0.1 nM each, and 2 U of Taq DNA polymerase (Qiagen, Valencia, CA). PCR was carried out in a GeneAmp 2400 PCR thermal cycler (Perkin-Elmer, Norwalk, CT) for 28–33 cycles. Each cycle was timed for 1 min at 94°C, 1.5 min at 55°C, 2 min at 72°C, and a final 10-min extension at 72°C. PCR primers for β-actin were used to detect genomic DNA contamination. Amplified products (10 μl) were analyzed by electrophoresis on 1% agarose-TAE gels (10 mM Tris, pH 7.5, 5.7% glacial acetic acid, 0.5 mM EDTA) and visualized by ethidium bromide staining. PCR product identity was confirmed by direct sequencing (Robarts Research Institute Core Molecular Biology Facility, London, ON, Canada).

Drugs. Unless otherwise stated, all drugs were obtained from Sigma (St. Louis, MO) or Calbiochem (San Diego, CA). Drugs were prepared as concentrated stock solutions and diluted into bathing solution just before use. During electrophysiological recording, test solutions were applied focally by pressure ejection (Picospritzer II, General Valve, Fairfield, NJ) from a micropipette. Concentrations reported are those in the application pipette.

RESULTS

Characterization of ionic currents in HESMCs. Electrophysiological experiments were performed on a total of 152 HESMCs that had been derived from 21 patients and grown in primary culture for 7–15 days. We first investigated the outward K⁺ currents to establish that the electrophysiological properties were consistent with previous reports. For these studies, voltage-clamp recording was carried out using the nystatin perforated-patch configuration with electrode solution containing 140 mM K⁺ (see METHODS).
Depolarization from −60 mV to positive potentials elicited outward current that peaked and then declined to a steady state (Fig. 1A; \( n = 16 \)). The steady component exhibited greater noise at more positive potentials, suggesting the presence of at least two distinct conductances. The current-voltage relationship of the peak and sustained currents was plotted (Fig. 1B), with the sustained current amplitude taken as the average over 100 ms, 3 s after the beginning of the voltage step. The transient current showed outward rectification and delayed onset, consistent with a \( K_v \) current. We therefore examined the effect of the \( K_v \) channel blocker 4-aminopyridine (4-AP, 2–5 mM), which abolished the transient component but left the sustained current (Fig. 1C; \( n = 6 \)). This sustained, 4-AP-insensitive component also exhibited voltage dependence and was reversibly blocked by 2 mM tetraethylammonium (Fig. 1D; \( n = 6 \)), consistent with a \( K_{Ca} \) current. On the basis of similarities to the \( K_v \) currents previously characterized in HESMCs (21, 32), we refer

<table>
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<th>Protein Accession No.</th>
<th>Primer Pair Sequence</th>
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<td>5′-GAGATGAGCCGACCAGCATACA-3′ (S) 5′-ATCAGAAGAGGCGATTCCTGC-3′ (AS)</td>
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<tr>
<td>β-Actin X00351</td>
<td>5′-AGAAGATGGTCCAGAGCAATG-3′ (S) 5′-CTCGAAGCCATCTACACACGGA-3′ (AS)</td>
<td>314†</td>
<td>892−1133</td>
</tr>
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S, sense; AS, antisense. *β-Actin primer pair was designed to span 2 introns, with a combined size of 415 bp. †Given the similarity in expected PCR product size for Na+1.5, Na+ and the first β-actin primer pair, a second β-actin primer pair was designed to discriminate between products, spanning a single 206-bp intron.

Fig. 1. Outward delayed rectifier (\( K_v \)) and \( Ca^{2+} \)-activated \( K^+ \) (\( K_{Ca} \)) currents in human esophageal smooth muscle. Dashed lines, zero-current level. A: a series of superimposed traces illustrating outward current elicited after depolarization. Outward current peaked, then inactivated to a steady level, with steady current exhibiting greater noise at more positive potentials. B: amplitudes of peak current (\( I_p \)) and steady current (\( I_s \)) plotted against membrane potential (\( V_m \)) to generate a current-voltage relationship. Steady current was measured as the average over 100 ms, 3 s after beginning of voltage step. C: transient outward current was blocked by 5 mM tetraethylammonium (TEA), leaving the \( K_v \) current. D: sustained current was more prominent in some cells and was blocked by 2 mM tetraethylammonium (TEA), leaving the \( K_v \) current. E: current elicited by voltage ramps from −100 to 50 mV over a duration of 200 ms. ACh (10 μM) shifted voltage dependence of activation to more negative potentials, consistent with activation of \( K_{Ca} \). F: \( Ca^{2+} \) ionophore A-23187 (10 μM) reversibly activated the outward current.
to the transient and sustained outward currents as $K_v$ and $K_{Ca}$, respectively.

The relative amount of $K_{Ca}$ current under control conditions varied from cell to cell, likely indicating that it was subject to regulation. Indeed, cells that initially exhibited little outward current responded to the excitatory agonist ACh (10 μM) with a reversible increase in outward current. When voltage ramps from −100 to 50 mV, 200 ms in duration, were applied, ACh shifted the voltage dependence of activation to more negative potentials (Fig. 1E; $n = 7$). Consistent with this change being due to elevation of intracellular Ca$^{2+}$ concentration, the Ca$^{2+}$ ionophore A-23187 (10 μM) caused similar reversible activation of the current (Fig. 1F; $n = 6$). The properties and regulation of the $K_v$ and $K_{Ca}$ currents described here resemble those reported earlier (21, 32), further validating the use of this culture system.

**Depolarization elicits a mixture of inward and outward currents.** In addition to the outward $K_v$ currents, depolarization also elicited a transient, inward current (Fig. 2A), which became the focus of the present studies (4 of 6 cells studied with $K_v$ electrode solution and with a prepulse to −100 mV). The current was voltage dependent and activated and inactivated rapidly; thus shorter voltage steps were used to examine its properties. The inward current first became apparent with voltage jumps to −20 mV, peaked near 10 mV, and then declined in amplitude at more positive potentials (Fig. 2B). The outward current activated with depolarization as shown above, although the decline shown above was not evident on this rapid time scale. On the basis of the time course and kinetics of the inward current, we examined its sensitivity to the Na$^+$ channel blocker TTX. TTX (1 μM) selectively blocked the transient inward component, leaving outward $K^+$ currents (Fig. 2C). A small persistent, inward current was also evident at negative potentials, which may be an L-type Ca$^{2+}$ current (see below). The TTX-sensitive component was isolated by subtraction of current traces in the presence of blocker from control traces (Fig. 2D). These results suggest the presence of a voltage-dependent Na$^+$ current in HESMCs. We proceeded to characterize the inward current by investigating its voltage-dependent properties, ion permeation, and pharmacological regulation.

**Voltage-dependent properties of the inward current.** $K^+$ in the electrode solution was replaced with 117 mM Cs$^+$ and 13 mM Na$^+$ (see METHODS), thereby eliminating the outward $K^+$ currents and establishing a theoretical Na$^+$ equilibrium potential of +60 mV. Many of the experiments were performed in the whole cell configuration to minimize access resistance. This configuration was also used to minimize the contribution of L-type Ca$^{2+}$ current, which is subject to rundown, allowing us to examine the TTX-sensitive current in isolation. Cells were held at −60 mV and step depolarized to various potentials after a hyperpolarizing pre-pulse to maximize available current (Fig. 3A). The resulting inward current displayed the rapid activation and inactivation described earlier, and a persistent component was also evident at several potentials. For the purposes of these studies, we have defined these currents as the transient and sustained components, respectively. An examination of the current-voltage relationship for the peak inward current demonstrated activation commencing at approximately −40 mV and peak current occurring at −0 mV (Fig. 3B;

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Fig. 2. Depolarization elicits a mixture of inward and outward current. A: depolarizing voltage steps elicited an initial, transient inward current followed by an outward $K^+$ current. Currents were elicited at potentials ranging from −50 to 50 mV, with 10-mV increments. Capacitive and leak currents were subtracted off-line using currents elicited by subthreshold pulses. Transient current was rapidly activating and inactivating. A smaller, persistent inward current was also apparent at some negative potentials, characteristic of an L-type Ca$^{2+}$ current. B: current-voltage relationship of peak transient inward current (●) and maximum positive current (○). C: application of TTX abolished the transient inward current, leaving only outward and persistent inward currents. D: subtraction of currents in the presence of TTX revealed the transient inward current.
The voltage dependence of steady-state inactivation was determined using a two-step protocol with a 5-s conditioning pulse followed by a test pulse to 0 mV (Fig. 4A; n = 15). To quantify the voltage dependence of activation, conductance was calculated from the current-voltage relationship as the chord conductance at each point and normalized to peak conductance to produce an activation curve (Fig. 4B). When conductance was fitted with a Boltzmann relation, the half-maximal activation occurred at −16 mV and half-maximal inactivation occurred at −67 mV. The average activation and inactivation values are summarized in Fig. 4B and are consistent with a voltage-dependent Na⁺ current.

Na⁺ serves as charge carrier for the transient inward current. Given that the inward current appeared unaffected by rundown in the whole cell configuration, we considered it unlikely to be carried by Ca²⁺. Blockade of the current by TTX was suggestive of a Na⁺ current, although the involvement of other ions could not be excluded. We therefore used ion-substitution experiments to resolve the identity of the current. Currents were recorded in solution where Na⁺ was replaced with the large cation N-methyl-D-glucamine in an

Fig. 3. Transient inward current in human esophageal smooth muscle cells (HESMCs). Dashed lines, zero-current level. A: currents were recorded using Cs⁺ electrode solution to block K⁺ currents. Cells were held at −60 mV and depolarized to various potentials after a prepulse to −120 mV. Depolarization elicited a rapidly activating and inactivating current as voltages became more positive. Also apparent was a steady, sustained component. Recordings from this representative cell were made in the whole cell configuration with K⁺-free electrode solution. B: peak inward current from each depolarization, normalized as proportion of maximum current, plotted as a function of Vm. Values are means ± SE (n = 10). Current began to activate at −40 mV and peaked at 0 mV.

Fig. 4. Voltage-dependent properties of activation and inactivation. A: superimposed currents elicited by test pulses to 0 mV after 5-s conditioning prepulses at various potentials. Prepulse potentials are indicated at left of individual current traces. Current amplitude decreased at increasingly positive prepulse potentials, demonstrating voltage-dependent inactivation. B: summary of activation and inactivation properties. Normalized peak current (I/I_{max}) was plotted against prepulse potential to demonstrate voltage dependence of steady-state inactivation. Voltage dependence of activation was determined from current-voltage relationship as chord conductance (g/g_{max}) at each point. Values are means ± SE (n = 10). Half-maximal activation occurred at −16 mV; half-maximal blockade occurred at −67 mV.
Fig. 5. Removal of bath Na\(^+\) abolishes inward current. Dashed line, zero-current level. A: step depolarization to 0 mV after a prepulse to −90 mV elicited a rapid, inward, control current. Replacement of bath Na\(^+\) by equimolar N-methyl-D-glucamine abolished the inward current demonstrating that transient current was carried by Na\(^+\). B: time course of ion substitution experiment, demonstrating full reversibility of the effect. Peak inward current is plotted as a function of time, with Na\(^+\)-free solution perfused during the interval shown by horizontal bar.

equimolar fashion. Removal of bath Na\(^+\) reversibly abolished the inward current (Fig. 5; n = 5), indicating that Na\(^+\) is the charge carrier. Further experiments were performed to assess the contribution of Ca\(^{2+}\). Application of the L-type Ca\(^{2+}\) channel blocker nifedipine at 10 μM (Fig. 6A; n = 10) caused a slight reduction of the sustained component but failed to abolish the transient inward current. Similarly, the transient current persisted after withdrawal of bath Ca\(^{2+}\) in solution containing 130 mM Na\(^+\), again accompanied by a slight reduction of the persistent component (Fig. 6B; n = 3). These data suggest that although L-type Ca\(^{2+}\) channels may partially mediate the sustained current, Ca\(^{2+}\) does not contribute to the rapidly inactivating inward current. The transient current also remained in the presence of 1 mM Cd\(^{2+}\), which blocks voltage-dependent Ca\(^{2+}\) channels and TTX-resistant Na\(^+\) channels (Fig. 6C).

**TTX sensitivity of the Na\(^+\) channel.** The TTX sensitivity of the inward component shown in Fig. 2C provided evidence that the Na\(^+\) current was carried through specific Na\(^+\) channels. Given the considerable variation known to exist in the TTX sensitivity of Na\(^+\) channels, the concentration dependence of TTX blockade in HESMCs was established. Concentration dependence was examined by measuring percentage of peak current at 0 mV in various concentrations of TTX (Fig. 7A), with the average values showing half-maximal blockade at −20 nM (Fig. 7B; n = 4 at each concentration). At 1 μM, TTX completely abolished the inward current (Fig. 7C; n = 20). Interestingly, an examination of the difference curve under these conditions revealed that the transient and sustained components were TTX sensitive, suggesting that Na\(^+\) channels also partially account for the sustained current (Fig. 7D).

**Na\(^+\) channels contribute to the esophageal action potential.** The role of Na\(^+\) channels in promoting cellular excitation in many tissues is well established (2). We wished to determine the contribution of these channels to the action potential in HESMCs. Using K\(^+\) electrode solution and a series of depolarizing current steps, we recorded from cells in the current-clamp configuration. The magnitude of each current step was increased until action potentials became apparent, in addition to the passive charging of the membrane. The rapid phase of depolarization was inhibited by 1 μM TTX, with recovery on washout (Fig. 8; n = 5), demonstrating a role for Na\(^+\) channels in excitation.

**HESMCs express multiple Na\(^+\) channel isoforms.** To determine the molecular isoform of Na\(^+\) channels in HESMCs, we extracted mRNA as described in METHODS and used RT-PCR to probe for mRNA transcripts of several Na\(^+\) channel isoforms. Primers were designed for the coding regions of Na\(^{v}\)1.4 (skeletal muscle isoform), Na\(^{v}\)1.5 (cardiac muscle isoform), Na\(^{v}\)1.2 (neuronal isoform), and Na\(^{v}\) (heart and uterine tissue) and are listed in Table 1 with predicted product sizes. Tissues known to express each of these isoforms were used as positive controls. Striated muscle from the proximal esophagus was used as a control for Na\(^{v}\)1.4 (16), whereas human heart was used as a control for Na\(^{v}\)1.5 and Na\(^{v}\) (14, 15), and human brain was used as a control for Na\(^{v}\)1.2 (1). PCR was carried out in the absence or presence of cDNA from these respective tissues. The results are shown in Fig. 9. Primer sets for β-actin, designed to span one or more introns, were used to amplify the same cDNA. In all cases, a single transcript was detected, excluding the possibility of genomic DNA contamination in our samples. The identity of amplified products was confirmed by direct sequencing. These findings are summarized in Table 2.

Expression of mRNA for Na\(^{v}\)1.2, the neuronal isoform, was not detected in cultured cells or esophageal smooth muscle tissue (Fig. 9A). As a positive control, PCR amplification of mRNA isolated from human brain did yield a product of the expected size for Na\(^{v}\)1.2. Moreover, no message for Na\(^{v}\)1.2 was found in heart or striated muscle from proximal esophagus. These data confirm that the primers were specific for neuronal Na\(^+\) channels and indicate that Na\(^{v}\)1.2 is not present in esophageal muscle.

Na\(^{v}\)1.4 mRNA was expressed in cultured cells and esophageal smooth muscle tissue (Fig. 9B). As expected, message for Na\(^{v}\)1.4 also was detected in striated muscle of the proximal esophagus. Na\(^{v}\)1.4 mRNA was also present in human heart, but not in human brain. We also probed for the cardiac Na\(^{v}\)1.5 isoform, although our electrophysiological studies suggested
expected, it was also found in heart, although not in brain or striated esophageal muscle.

**DISCUSSION**

We have identified a voltage-dependent Na⁺ current in cultured esophageal smooth muscle cells. Using patch-clamp electrophysiology, we characterized this current on the basis of its biophysical properties and pharmacological sensitivity. Furthermore, we used RT-PCR to identify the specific Na⁺ channel subtypes. Esophageal smooth muscle tissue expresses the Naᵥ1.4 isoform, characteristic of skeletal muscle, and the Naₓ isoform, found in heart and uterus. To our knowledge, these studies represent the first report of Naᵥ1.4 expression in any smooth muscle. We propose that these channels contribute to esophageal excitation and may have implications for understanding esophageal muscle development.

Given the heterogeneity known to exist among smooth muscle Na⁺ currents (7), attention was paid to the biophysical and pharmacological properties of the current identified here. The rapid activation and inactivation kinetics, as well as the voltage activation range, are characteristic of the fast Na⁺ current found in many neuronal and muscle tissues (8, 17). However, because L-type Ca²⁺ currents have been reported in esophageal SMCs (3), we first considered the possibility that this current was carried by Ca²⁺. We did not anticipate a large contribution by Ca²⁺ to the inward current, inasmuch as Ca²⁺ current rundown is well established in the whole cell configuration. Although the sustained inward current was slightly reduced by application of nifedipine or withdrawal of bath Ca²⁺, the transient current persisted under those conditions. The abolition of the inward current in the absence of bath Na⁺ provides evidence that cultured HESMCs exhibit a voltage-dependent Na⁺ current. Because half-maximal inactivation occurs at −67 mV, it is likely that this current is largely inactivated in resting cells in vivo. Examination of the activation and inactivation curves reveals a small window current over a physiological range of membrane potentials. It remains to be established whether this window current contributes to esophageal excitability. Although these biophysical studies support the presence of an Na⁺ current, the pharmacological characterization provides evidence that the current is carried through specific Na⁺ channels. The TTX sensitivity of the current (IC₅₀ ~20 nM), combined with its insensitivity to Cd²⁺, indicates that cultured HESMCs express channels similar to those found in skeletal muscle and neurons, but not those found in cardiac muscle (2). This finding is consistent with the characteristics of some Na⁺ channels found in the smooth muscle of humans (28, 35, 38) and animals (6, 25, 30). In contrast, a recent report of voltage-dependent Na⁺ channels in human jejunal SMCs found them to be relatively resistant to blockade by TTX (20).

We observed that the sustained component of the current also displayed sensitivity to TTX, suggesting...
that the Na⁺ current consists of two components. Sustained Na⁺ currents have been identified in a number of cell types. In neurons, they enhance rhythmicity and facilitate repetitive firing of action potentials (5). Other studies have provided evidence that transient and sustained Na⁺ currents are mediated by the same population of Na⁺ channels (5, 24). Interestingly, Gage and co-workers (13) demonstrated that sustained Na⁺ currents also occur in mammalian skeletal muscle. Expression studies in oocytes showed that, much like neuronal Na⁺ channels, transient and sustained currents can be mediated by a single population of skeletal muscle Na⁺ channels (9). Therefore, although sustained currents have been observed in several smooth muscle systems (10, 28), their presence cannot be used to distinguish between neuronal and skeletal muscle channel isoforms.

Our culture system is useful for determining the molecular identity of the Na⁺ channels. Previous studies have used RT-PCR to identify mRNA for Na⁺ channels in tissues containing smooth muscle (15), but multiple cell types are present in whole tissues. Previous studies from this laboratory demonstrate that these cultured cells show positive staining for smooth muscle-specific α-actin and do not stain for markers of endothelial cells, neuroglial and Schwann cells, or interstitial cells of Cajal (33). We recognize that cells in culture can exhibit some properties that differ from their tissue of origin. We addressed this possibility in several ways. Only cells that had been maintained in

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**Fig. 7.** TTX blocks inward current in a concentration-dependent manner. Dashed lines, zero-current level. A: representative trace in which several concentrations of TTX were applied to a single cell. Current blockade occurs in a concentration-dependent manner. B: concentration dependence as a percentage of peak current at 0 mV persisting in various TTX concentrations ([TTX]). Values are means ± SE (n=4–20). Half-maximal inhibition occurred at ~20 nM. C: 1 μM TTX is sufficient to abolish transient inward current. D: subtracting the currents elicited in the control condition and in the presence of TTX yields only the TTX-sensitive current. Sustained inward current is also sensitive to TTX.

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**Fig. 8.** Na⁺ channels contribute to the human esophageal action potential. Recordings were performed in the current-clamp configuration to determine the contribution of Na⁺ channels to the action potential in HES-MCs. Action potentials were evoked by 50-pA depolarizing current steps applied at regular 5-s intervals. Control action potentials were inhibited by 1 μM TTX applied for the time indicated by horizontal bar. Recovery occurred on washout. Dashed line, zero-potential level.
primary culture were used, reducing the chance that an unrepresentative subpopulation might dominate the culture. It has been demonstrated previously that cultured and freshly dissociated HESMCs express all five muscarinic subtypes and respond to ACh with a rise in cytosolic Ca\(^{2+}\) (27, 29). In the present studies, we show that these cultured HESMCs exhibit two distinct K\(^{+}\) currents, each with biophysical and pharmacological properties characteristic of the Kv and KCa currents described in freshly isolated cells (32). In combination with the molecular and immunologic studies described earlier (33), this finding further supports the use of our culture system as a model for investigating esophageal physiology.

We used RT-PCR to show the presence of mRNA encoding voltage-dependent Na\(^{+}\) channels in fresh esophageal smooth muscle tissue. Our findings indicate that HESMCs express mRNA for the Nax isoform, previously the only isoform reported in cultured and whole esophageal smooth muscle (16, 22). However, previous attempts to demonstrate functional Na\(^{+}\) currents from Nax have not been successful (12, 17). On the basis of the structure of this channel, Akopian et al. (4) argued that Nax is unlikely to be a functional voltage-gated Na\(^{+}\) channel. Hence, we consider it unlikely that the Na\(^{+}\) currents recorded here are mediated by Nax. Our investigation focused on the two remaining muscle isoforms: the skeletal muscle Nav1.4 isoform and the cardiac Na v1.5 isoform.

The presence of mRNA encoding Na1.4 in cultured HESMCs and fresh esophageal tissue is consistent with the pharmacological sensitivity of the whole cell Na\(^{+}\) current we recorded. The cells being studied were from the smooth muscle portion of the esophagus, and we confirmed the presence of Na1.4 in intact muscle tissue from the distal esophagus, which contains smooth but not striated muscle (18). Accordingly, we also identified Na1.4 in striated muscle isolated from the proximal esophagus of humans. The electrophysiological evidence of a whole cell TTX-sensitive Na\(^{+}\) channel, combined with the expression of mRNA for Na1.4 in cultured HESMCs, suggests that the Na1.4 expressed in esophageal tissue is specifically present in
smooth muscle. Although we did not identify transcripts for Na$_V$1.5 in the cultured HESMCs, evidence for expression of this cardiac isoform was recently found in human jejunal muscle (20), consistent with some heterogeneity of channel expression in different muscles.

The presence of skeletal muscle Na$^+$ channels in esophageal smooth muscle presents possible implications in organ development. Several studies have demonstrated that muscle cells in the mammalian esophagus undergo a process known as transdifferentiation in the normal course of development (26). In the fetal mouse, individual esophageal SMCs are suggested to convert from the smooth to a skeletal muscle phenotype (26). This transition is accompanied by the appearance of cells expressing markers of both muscle phenotypes. In contrast to the adult mouse esophagus, the distal muscularis externa of humans is composed of smooth muscle (18). Thus our finding of Na$_V$1.4 expression in human esophageal smooth muscle indicates that these cells express characteristics of skeletal muscle, perhaps reflecting a fundamental developmental process.

Because Na$^+$ channels are involved in the rising phase of the action potential in several tissues (2), we suspected that the Na$^+$ current in HESMCs served to promote cellular excitation. We have provided evidence that Na$^+$ channels contribute to action potentials in these cells, similar to the effect seen in lymphatic SMCs (19). Earlier recordings of action potentials in human gastrointestinal smooth muscle revealed that Na$^+$ and Ca$^{2+}$ act as charge carriers to mediate action potentials (11), providing precedent for the findings here. Depolarization elicited by Na$^+$ channel activity could lead to the opening of voltage-dependent Ca$^{2+}$ channels and a subsequent rise in cytosolic Ca$^{2+}$.

In summary, these studies have identified inward Na$^+$ current in cultured human esophageal smooth muscle. We used RT-PCR to demonstrate the expression of mRNA encoding multiple Na$^+$ channel isoforms in HESMCs, including the first report of Na$_V$1.4 expression in smooth muscle. These data contribute to a developing model of excitation in HESMCs and may have implications in our understanding of esophageal development and motor function.

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