TTX-sensitive voltage-gated Na$^+$ channels are expressed in mesenteric artery smooth muscle cells

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The presence and properties of voltage-gated Na$^+$ channels were studied using whole cell patch-clamp recording. SMCs from mouse and rat mesenteric arteries were enzymatically dissociated using two dissociation protocols with different enzyme combinations. Na$^+$ and Ca$^{2+}$ channel currents were present in myocytes isolated with collagenase and elastase. In contrast, Na$^+$ currents were not detected, but Ca$^{2+}$ currents were present in cells isolated with papain and collagenase. Ca$^{2+}$ currents were blocked by nifedipine. The Na$^+$ current was insensitive to nifedipine, sensitive to changes in the extracellular Na$^+$ concentration, and blocked by tetrodotoxin with an IC$_{50}$ at 4.3 nM. The Na$^+$ conductance was half maximally activated at $-16$ mV, and steady-state inactivation was half-maximal at $-53$ mV. These values are similar to those reported in various SMC types. In the presence of 1 μM batrachotoxin, the Na$^+$ conductance-voltage relationship was shifted by 27 mV in the hyperpolarizing direction, inactivation was almost completely eliminated, and the deactivation rate was decreased. The present study indicates that TTX-sensitive, voltage-gated Na$^+$ currents are present in mesenteric artery SMCs from the rat and mouse mesenteric artery. The presence of these channels in freshly isolated SMCs depends critically on the enzymatic dissociation conditions. This could resolve controversy about the presence of Na$^+$ channels in arterial smooth muscle.

TTX-sensitive voltage-gated Na$^+$ channels are expressed in mesenteric artery smooth muscle cells. Am J Physiol Heart Circ Physiol 289: H137–H145, 2005; doi:10.1152/ajpheart.01156.2004.—The presence and properties of voltage-gated Na$^+$ channels in mesenteric artery smooth muscle cells (SMCs) were studied using whole cell patch-clamp recording. SMCs from mouse and rat mesenteric arteries were enzymatically dissociated using two dissociation protocols with different enzyme combinations. Na$^+$ and Ca$^{2+}$ channel currents were present in myocytes isolated with collagenase and elastase. In contrast, Na$^+$ currents were not detected, but Ca$^{2+}$ currents were present in cells isolated with papain and collagenase. Ca$^{2+}$ currents were blocked by nifedipine. The Na$^+$ current was insensitive to nifedipine, sensitive to changes in the extracellular Na$^+$ concentration, and blocked by tetrodotoxin with an IC$_{50}$ at 4.3 nM. The Na$^+$ conductance was half maximally activated at $-16$ mV, and steady-state inactivation was half-maximal at $-53$ mV. These values are similar to those reported in various SMC types. In the presence of 1 μM batrachotoxin, the Na$^+$ conductance-voltage relationship was shifted by 27 mV in the hyperpolarizing direction, inactivation was almost completely eliminated, and the deactivation rate was decreased. The present study indicates that TTX-sensitive, voltage-gated Na$^+$ currents are present in mesenteric artery SMCs from the rat and mouse mesenteric artery. The presence of these channels in freshly isolated SMCs depends critically on the enzymatic dissociation conditions. This could resolve controversy about the presence of Na$^+$ channels in arterial smooth muscle.

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A suspension of single cells was obtained after either dissociation protocol by gently triturating the tissue with a fire-polished Pasteur pipette in low-Ca\(^{2+}\) PSS for protocol 1 and using a Ca\(^{2+}\)-free dissociation solution for protocol 2. Dispersed cells obtained with both protocols were used immediately or stored at 4°C and used within 4 h. For an experiment, four to five drops of cell suspension were placed on a coverslip and allowed to sit for 10 min at 4°C to permit the cells to adhere. The coverslips were placed in a perfusion chamber mounted on an inverted phase-contrast microscope (Nikon Diaphot; Tokyo, Japan). The cells were superfused at 2 ml/min with external solution. Only cells with elongated morphology were studied.

**Electrophysiological Recording**

Membrane currents were recorded from freshly dissociated SMCs in the whole cell patch-clamp configuration using an Axopatch 200 patch-clamp amplifier (Axon Instruments; Union City, CA). Fire-polished micropipettes (1–3 MΩ) resistance were manufactured from borosilicate capillary tubing (Garner Glass; Claremont, CA) using a micropipette puller (model P-97, Sutter Instruments; Novato, CA). An Ag-AgCl reference electrode was connected to the bath using an agar salt bridge containing 1 M KCl. The offset voltage was zeroed using a 902LPF low-pass Bessel filter (Frequency Devices; Haverhill, MA), sampled at 500 kHz (unless otherwise stated), filtered at 5 kHz with an amplifier and digitized using a multichannel interface. Data were analyzed using the pCLAMP software (version 9.0, Axon Instruments). I_\text{Na} peak amplitudes were measured relative to the current level before the pulse. Data are expressed as means ± SE.

The TTX dose-response data were fit to an equation of the form

\[
Y = \frac{100}{1 + \frac{IC_{50}}{[\text{TTX}]}}
\]

where Y is the response (relative i_\text{Na} amplitude), [TTX] is the TTX concentration, and IC_{50} is the [TTX] that reduces the response to 50% of control.

The data for the voltage dependence of Na\(^+\) channel activation or inactivation were fit with a Boltzmann equation of the form

\[
Y = \frac{1}{1 + \exp \left( \frac{V_M - V_{1/2}}{k} \right)}
\]

For inactivation, Y is the fraction of channels not inactivated at any V_M (in mV), and constant s equals 1. For activation, Y is the fraction of channels activated at V_M and s equals −1. V_{1/2} is the V_M at which the conductance is half-maximal, and k is a constant.

**RESULTS**

**Cell Morphology**

Two dissociation protocols, using different combinations of enzymes, were tested in order to find a method that produced viable cells for whole cell patch clamping. Typical rat or mouse SMCs obtained with each protocol are shown in Fig. 1. Mouse cells dissociated with protocol 2 (Fig. 1B) were long, slender, and fusiform. Those dissociated with protocol 1 (Fig. 1A) were somewhat shorter and fatter but still elongated. Similar results were obtained with the rat mesenteric artery (Fig. 1, C and D). With either protocol, SMCs from rat or mouse mesenteric arteries maintained the same morphology described above when they were superfused with external solution containing 1.8 mM Ca\(^{2+}\).

**Inward Currents in Murine Mesenteric SMCs**

Inward currents were initially recorded with 10 mM Ba\(^{2+}\) in the external solution to increase the amplitude of L-type Ca\(^{2+}\) channel currents. When single SMCs dissociated from the mouse or rat mesenteric artery with protocol 1 were voltage clamped at 0 mV, a current such as that shown in Fig. 2A was recorded. The inward current consists of two distinct components: an initial fast transient inward current superimposed upon a slowly activating, noninactivating component. Inward currents were recorded from about 95% of the rat or mouse cells isolated with protocol 1; they all had the pattern illustrated in Fig. 2A. Surprisingly, in either rat or mouse cells dissociated with protocol 2, the fast, transient inward current component was absent (Fig. 2B). The fast current was never observed in 26 cells (from 5 different arteries) isolated with protocol 2.

Cells that express both voltage-gated Na\(^+\) and Ca\(^{2+}\) channels generate inward currents with a time course similar to that shown in Fig. 2A (19, 23). Because arterial SMCs are known to have L-type Ca\(^{2+}\) channels, the effect of the L-type Ca\(^{2+}\) channel blocker nifedipine (10 μM) was tested. As shown in Fig. 2C, nifedipine blocked the slowly activating component but not the fast component in cells dissociated with protocol 1. This implies that L-type Ca\(^{2+}\) channels are responsible for the slowly activating component. In cells dissociated with protocol 2.
2, nifedipine almost completely blocked all the inward current in all cells studied, indicating that only L-type Ca\textsuperscript{2+} channels were present. The fast component remaining in the presence of nifedipine in cells dissociated with protocol 1 kinetically resembles VGNC current (13). It reached a peak in 0.44 ± 0.02 ms and decayed to zero during the next 2 ms at 0 mV. To identify further the nifedipine-insensitive fast component of the current, we studied its dependence on Na\textsuperscript{+} and its sensitivity to TTX.

**Sodium Dependence of the Fast Current**

If the fast component is carried by Na\textsuperscript{+}, its reversal potential should change with the sodium equilibrium potential (\(E_{Na}\)). To change \(E_{Na}\), the external Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]\textsubscript{o}) was decreased using equimolar substitution with the impermeant cation NMDG. Inward currents recorded during 5-ms steps to 0 mV in the presence of 155, 105, or 55 mM [Na\textsuperscript{+}]\textsubscript{o} are superimposed in Fig. 3A. Lowering [Na\textsuperscript{+}]\textsubscript{o} decreased the...
ical Nernst equilibrium potentials for Na respectively. These reversal potentials are close to the theoret-

in cells superfused with 155, 105, or 55 mM NaCl was 68.0 (n = 2.1), 57.8 ± 2.5 (n = 5), and 38.4 ± 2.1 (n = 10) mV, respectively. Thus Na is the main charge carrier during the fast, transient current, and this current will be referred to as I.

TTX Sensitivity of I

Specific subtypes of VGNCs differ in their sensitivity to block by TTX (37). The superimposed current traces shown in Fig. 4A show the effect of increasing the concentration of TTX from 1 to 10 nM on I in mesenteric artery SMCs. The addition of 1 nM TTX decreased the amplitude of the inward current by about 23%, and inward current was almost abol-

is hished at a TTX concentration of 10 nM. Removal of TTX from the bath solution restored I completely within a few minutes (not shown). The relationship between TTX concentration and I amplitude, averaged for three to five cells from six different arteries, is shown in Fig. 4B. The smooth curve is the fit of Eq. 1 to the data, with an IC50 at 4.3 nM. Thus the VGNCs in mesenteric artery SMCs have a high affinity for TTX.

Steady-State Activation and Inactivation

VGNCs activate and then inactivate as VM becomes progressively more positive. A plot of the normalized Na conductance (gNa) as a function of VM shows the voltage dependence of activation (Fig. 5). gNa was calculated from the following equation:

\[ g_{Na} = \frac{I_{Na}}{V_M - V_{rev}} \]

where \( I_{Na} \) is the peak magnitude of the current generated by a 5-ms step to \( V_M \) and \( V_{rev} \) is the \( I_{Na} \) reversal potential. The \( g_{Na} \) vs. \( V_M \) curve indicates that VGNCs begin to open when \( V_M \) is positive to -40 mV.

The voltage dependence of VGNC inactivation was determined using a double-pulse protocol. The \( V_M \) was initially stepped to a level in the range of -120 to -20 mV for 2 s (“the prepulse”) to inactivate the channels. The prepulse was fol-

Fig. 3. Fast inward current depends on extracellular Na concentration ([Na+]o). A: inward currents were generated by 5-ms steps to 0 mV from a holding potential of -70 mV. Superimposed are 3 currents recorded from the same cell in the absence of TTX (control) and after the addition of 1 or 10 nM TTX. The voltage dependence of the fast component is shown in Fig. 3B. The current activates at 40 mV, reaches a maximum at about 0 mV, and reverses at 68 mV in 155 mM [Na+]o. The reversal potential of the fast component was shifted in the negative direction as [Na+]o was lowered, and the magnitude of the shift is consistent with the change in \( E_{Na} \). The \( I_{Na} \) reversal potential in cells superfused with 155, 105, or 55 mM NaCl was 68.0 ± 0.8 (n = 11), 57.8 ± 2.5 (n = 5), and 38.4 ± 2.1 (n = 10) mV, respectively. These reversal potentials are close to the theoretical Nernst equilibrium potentials for Na under these conditions: 60, 51, and 33.4 mV, respectively. Thus Na is the main charge carrier during the fast, transient current, and this current will be referred to as I.

Fig. 4. Na current (I) is blocked with high affinity by TTX. A: currents were generated by 5-ms steps to 0 mV. Superimposed are currents from the same cell in the absence of TTX (control) and after the addition of 1 or 10 nM TTX. The current amplitude decreases in a concentration-dependent manner. Dashed lines represent the zero-current level. B: dose-response relationship for TTX inhibition of I. The peak magnitude of I was measured during a 5-ms step to 0 mV. The amplitude of I expressed as a percentage of the control I recorded in the absence of TTX, is plotted against TTX concentration. Data points are means ± SE; n = 3–6. The continuous curve was obtained by fitting the data to Eq. 1, with IC50 = 4.3 nM.
lowed by a 5-ms test pulse to 0 mV, and the \(I_{\text{Na}}\) during the test pulse was used as a measure of the channels that were not inactivated. A plot of the normalized test pulse current vs. \(V_\text{M}\) shows the steady-state voltage dependence of inactivation (Fig. 5). VGNCs begin to inactivate at about \(-80\) mV and become fully inactivated at about \(-20\) mV. The overlap between the activation and inactivation curves in Fig. 5 suggests the possibility of \(\text{Na}^+\) channel “window current,” i.e., there is a narrow range of membrane potentials, from about \(-40\) to \(-20\) mV, where some VGNCs could be open in the steady state.

Effects of BTX on \(I_{\text{Na}}\) in Mesenteric Artery SMCs

A number of lipid-soluble toxins, such as BTX and veratridine, bind to receptors on VGNCs and alter channel gating such that the channels open at a more negative \(V_\text{M}\) and they do not inactivate (36). If these toxins open VGNCs in arterial SMCs in a similar way, the channels could be opened at the normal resting potential to investigate if they play any role in arterial SMC function. Because BTX binds preferentially to the open conformation of VGNCs (35), BTX binding was facilitated by applying a train of voltage-clamp steps (to \(+40\) mV for 5 ms) at a frequency of 20 Hz for 5 min.

\(\text{Na}^+\) channel gating was modified by 1 \(\mu\text{M}\) BTX in three significant ways. First, BTX shifted the activation of the \(\text{Na}^+\) channels in the hyperpolarizing direction (Fig. 6). After BTX modification, inward current was clearly present at \(-50\) mV (Fig. 6A, right), a potential at which no current was present in control cells (Fig. 6A, left). In BTX, \(I_{\text{Na}}\) reached its maximum value at about \(-30\) mV, a potential at which current was barely detectable under control conditions in the absence of BTX. The peak amplitudes of both the control and BTX-modified currents are plotted as functions of \(V_\text{M}\) in Fig. 6B. The control \(I_{\text{Na}}\) began to activate at \(-30\) mV, and the BTX-modified \(I_{\text{Na}}\) became evident at \(-50\) mV. To analyze further the voltage shift for the activation of modified channels, \(g_{\text{Na}}\) was calculated for both normal and modified channels; \(g_{\text{Na}}\) is plotted as a function of \(V_\text{M}\) in Fig. 7. The \(g_{\text{Na}}\) as \(V_\text{M}\) curve was shifted about \(27\) mV in the hyperpolarizing direction by BTX. The midpoint of the \(g_{\text{Na}}\) as \(V_\text{M}\) relationship shifted from \(-10.7\) mV to \(-37.9\) mV in the presence of BTX.

A second effect of BTX was to decrease \(\text{Na}^+\) channel inactivation. In control SMCs, \(I_{\text{Na}}\) inactivates when \(V_\text{M}\) is depolarized (Fig. 6A, left). BTX almost completely abolished this fast inactivation of \(I_{\text{Na}}\) at all potentials (Fig. 6A, right).

A third effect of BTX was to slow \(\text{Na}^+\) channel deactivation kinetics significantly. Under control conditions, \(\text{Na}^+\) channels deactivate rapidly: at \(-70\) mV, the deactivation time constant \(\tau_d\) was \(0.18 \pm 0.02\) ms \((n = 11)\). After BTX modification of the channels, \(\tau_d\) at \(-70\) mV was \(1.56 \pm 0.40\) ms \((n = 4\); Fig. 8).

DISCUSSION

\(I_{\text{Na}}\) Dependence on Isolation Protocol

This report reveals that mouse SMCs from the mesenteric artery dissociated with protocol 1 exhibit both \(I_{\text{Na}}\) and \(\text{Ca}^{2+}\) current. In contrast, cells dissociated with protocol 2 exhibit only \(\text{Ca}^{2+}\) currents, as shown by the ability of nifedipine to block almost completely the inward current in these cells. The precise cause(s) of this discrepancy is unknown but does not seem to be correlated with the different saline solutions used in protocols 1 and 2. When collagenase and elastase were used with the saline from protocol 2, \(I_{\text{Na}}\) as well as \(\text{Ca}^{2+}\) current also were observed (data not shown). This suggests that the results are more likely related to differences in the proteolytic actions of the enzymes employed for dissociation.

We observed differences in the morphology of the cells obtained with protocols 1 and 2. SMCs dissociated with protocol 2 (Fig. 1, B and D) were long, slender, and fusiform, and those dissociated with protocol 1 were shorter and fatter (Fig. 1, A and C). A possible explanation for these morphological differences is that in protocol 2 the cell dispersion was performed in \(\text{Ca}^{2+}\)-free solution where the cells were fully relaxed. In contrast, in protocol 1, the \(\text{Ca}^{2+}\) concentration in the dispersion solution was 0.05 mM; at this concentration, during dispersion, a small amount of \(\text{Ca}^{2+}\) could enter the cells, thereby causing partial contractions. Despite these morphological differences, we obtained similar \(\text{Ca}^{2+}\) currents in cells isolated with both protocols, indicating that the cells were not drastically damaged by either enzyme cocktail. The fact that only \(I_{\text{Na}}\) was absent in cells dissociated with protocol 2 suggests that \(\text{Na}^+\) channels could be more sensitive to certain enzymatic conditions than other voltage-gated channels. This could explain the controversial data about the absence of \(I_{\text{Na}}\) in the mesenteric artery (9, 27) and maybe in other types of SMCs. Our results suggest that some dissociation protocols may have subtle detrimental effects on the cells that may not normally be recognized. This may clearly alter the cell physiology and influence data interpretation.

\(I_{\text{Na}}\) Characteristics

The present investigation is, to our knowledge, the first report of the presence of voltage-gated \(I_{\text{Na}}\) in mesenteric artery SMCs under voltage-clamp conditions. \(V_{1/2}\) is approximately \(-16\) mV, and the half-inactivation voltage is approximately \(-53\) mV. These values are more positive than those reported
for cardiac (4, 20, 32), and nerve cells (13) but similar to or slightly more positive than those reported in various vascular SMC types (18, 25, 29). The reversal potential of $I_{Na}$ was 38.4 mV (Fig. 3B) in cells dialyzed with 15 mM NaCl and superfused with 55 mM NaCl, close to the theoretical $E_{Na}$ calculated with the Nernst equation under these conditions (+33.4 mV). Increasing the $[Na^+]_o$ to 105 and 155 mM increased the reversal potential to 57.8 and 68.0 mV (Fig. 3B), respectively. This was 8 mV more positive than the Nernst reversal potential calculated for these conditions (+51 and +60 mV, respectively). This small discrepancy between experimental and theoretical reversal potentials could be due to an unsubtracted component of the capacitative current. This component may interfere with the accurate measurement of $I_{Na}$ especially at high command potentials where the channel kinetics are very fast.

**TTX Sensitivity**

The marine guanidinium toxin TTX has played a crucial role in the study of VGNCs. Na$^+$ channels in excitable tissues have been categorized as TTX sensitive or insensitive. TTX-sensitive channels, found in nerve and adult skeletal muscle, are blocked by nanomolar concentrations of the toxin with an apparent $K_d \sim 1–10$ nM (10). In contrast, TTX-insensitive Na$^+$ channels in cardiac muscle require a much higher concentration of TTX for block ($\sim 10^{-5}$ M) (22). In our experiments, the
**Functional Role of \( I_{Na} \)**

The primary function of VGNCs in most cells is to depolarize the membrane and generate the upstroke of the action potential. Intracellular microelectrodes have been used in the mesenteric artery to demonstrate the presence of action potentials (12, 21, 38). In all cases, the action potentials appear to be generated by \( Ca^{2+} \) current: 1) action potentials induced by \( K^+ \) channel blockade with tetraethylammonium ion were insensitive to changes in \( [Na^+]_o \) (12); 2) action potential amplitude was increased by raising extracellular \( Ca^{2+} \) concentration, and the action potentials were blocked by the \( L \)-type \( Ca^{2+} \) channel blockers verapamil, Mn\(^{2+} \) (12), and nifedipine (21); and 3) action potentials were not affected by TTX (12, 21, 38).

A physiological role of \( I_{Na} \) in vascular SMCs has been suggested, however, by experiments on aortas and coronary arteries. Veratridine and BTX cause a gradual contraction in rat aorta rings (33). This suggests that activation of \( Na^+ \) channels in SMCs induces a contraction that is probably mediated by \( Ca^{2+} \) influx through voltage-gated \( Ca^{2+} \) channels. Recently, Boccara et al. (5) demonstrated the presence of a TTX-sensitive \( I_{Na} \) in primary cultured human coronary myocytes using the patch-clamp technique. In addition, they measured \([Na^+]_{cyt}\) with sodium-binding benzofuran isophthalate and \([Ca^{2+}]_{cyt}\) with fura-2. They reported that 1) veratridine increases \([Na^+]_{cyt}\) and 2) veratridine, toxin V from *Anemonea sacculata*, and \( \beta \)-bromoacetamide all increase \([Ca^{2+}]_{cyt}\). These effects were blocked by TTX or by the absence of extracellular \( Na^+ \) and \( Ca^{2+} \). The \( Ca^{2+} \) channel blocker nicardipine partially blocked the effect of veratridine, and replacement of external \( Na^+ \) with Li\(^+\) blocked the residual component. They concluded that \( I_{Na} \) regulates \([Ca^{2+}]_{cyt}\) via voltage-gated \( Ca^{2+} \) channels and, to a lesser extent, via the

**BTX Effects**

BTX, a steroidal alkaloid toxin extracted from the skin of the Colombian frog *Phyllobates terribilis* (2), is one of the most potent and specific activators of \( Na^+ \) channels. BTX shifts the voltage dependence of activation to more negative potentials, disables the inactivation process, and alters the channel conductance and selectivity. As a result, \( Na^+ \) channels open persistently and irreversibly in the presence of BTX, even at the resting \( V_M \) (7, 14).

Shinjoh et al. (33) report that BTX induces endothelium-independent contractions in the isolated rat aorta, suggesting that the influx of \( Na^+ \) depolarized the plasma membrane and thereby activated voltage-gated \( Ca^{2+} \) channels to cause contraction. To our knowledge, however, there are no reports of the effects of BTX on \( I_{Na} \) under voltage-clamp conditions in vascular SMCs. In this study, we report that 1 \( \mu \)M BTX profoundly changes \( Na^+ \) channel behavior in mesenteric artery SMCs. The conductance-voltage relationship was shifted by 27 mV in the hyperpolarizing direction, and inactivation was almost completely abolished. Together, the results obtained with TTX and BTX clearly reveal the presence of “classic” VGNCs in mesenteric artery SMCs.
NCXs. These studies suggest an important role for $I_{Na}$ in the excitation-contraction coupling processes in vascular SMCs. Na$^+$ channels could play a similar role in mesenteric artery SMCs because the voltage dependence of $I_{Na}$ activation and steady-state inactivation indicates a “window” current between −40 and −20 mV (Fig. 5), where some Na$^+$ channels would be open in the steady state.

Molecular Species of VGNCs in Vascular Smooth Muscle

$I_{Na}$ has been identified in several types of vascular SMCs. Nevertheless, the presence of specific Na$^+$ channel genes in vascular tissue has only recently been determined. Jo et al. (18) showed abundant expression of SCN9A in cultured cells isolated from the human bronchus, main pulmonary artery, and coronary artery. The Na$^+$ channel types expressed in the mesenteric artery remain unknown. Our results demonstrate that VGNCs in mesenteric artery SMCs bind TTX with high affinity. Therefore, the low TTX-affinity SCN5 channels normally expressed in cardiac myocytes do not appear to be involved in mesenteric artery SMCs.

In conclusion, the present data reveal the presence of VGNCs in rat and mouse mesenteric artery SMCs. The current is insensitive to nifedipine, sensitive to changes in [Na$^+$]o, and blocked by TTX (100 nM); its gating is modified by BTX. $I_{Na}$ characteristics were similar to those described in other vascular SMCs. An especially noteworthy observation is that the TTX-sensitive Na$^+$ channels appear to be very sensitive to the conditions used to dissociate the SMCs.

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