Regulation of Ca$^{2+}$ Homeostasis by Atypical Na$^{+}$ Currents in Cultured Human Coronary Myocytes

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Abstract—Primary cultured human coronary myocytes (HCMs) derived from ischemic human hearts express an atypical voltage-gated tetrodotoxin (TTX)-sensitive sodium current ($I_{\text{Na}}$). The whole-cell patch-clamp technique was used to study the properties of $I_{\text{Na}}$ in HCMs. The variations of intracellular calcium ([Ca$^{2+}$]) and sodium ([Na$^{+}$]) were monitored in non–voltage-clamped cells loaded with Fura-2 or benzofuran isophthalate, respectively, using microspectrofluorimetry. The activation and steady-state inactivation properties of $I_{\text{Na}}$ determined a “window” current between −50 and −10 mV suggestive of a steady-state Na$^{+}$ influx at the cell resting membrane potential. Consistent with this hypothesis, the resting [Na$^{+}$] was decreased by TTX (1 μmol/L). In contrast, it was increased by Na$^{+}$ channel agonists that also promoted a large rise in [Ca$^{2+}$]. Veratridine (10 μmol/L), toxin V from Anemonia sulcata (0.1 μmol/L), and N-bromoacetamide (300 μmol/L) increased [Ca$^{2+}$], by 7- to 15-fold. This increase was prevented by prior application of TTX or lidocaine (10 μmol/L) and by the use of Na$^{+}$-free or Ca$^{2+}$-free external solutions. The Ca$^{2+}$-channel antagonist nicardipine (5 μmol/L) blocked the effect of veratridine on [Ca$^{2+}$], only partially. The residual component disappeared when external Na$^{+}$ was replaced by Li$^{+}$ known to block the Na$^{+}$/Ca$^{2+}$ exchanger. The resting [Ca$^{2+}$] was decreased by TTX in some cells. In conclusion, $I_{\text{Na}}$ regulates [Ca$^{2+}$], in primary cultured HCMs. This regulation, effective at baseline, involves a tonic control of Ca$^{2+}$ influx via depolarization-gated Ca$^{2+}$ channels and, to a lesser extent, via a Na$^{+}$/Ca$^{2+}$ exchanger working in the reverse mode. (Circ Res. 1999;85:606-613.)

Key Words: vascular smooth muscle ■ persistent Na$^{+}$ current ■ [Ca$^{2+}$] ■ Ca$^{2+}$ channel ■ Na$^{+}$/Ca$^{2+}$ exchanger

Ca$^{2+}$ ions play a central role in the regulation of arterial smooth muscle tone.1,2 The changes in the cytosolic free calcium concentration ([Ca$^{2+}$]) mediate the effect of various vasoconstrictor and vasodilator agents, including hormones, neurotransmitters, and drugs that bind to specific receptors. Both direct mobilization of intracellular Ca$^{2+}$ and transmembrane Ca$^{2+}$ entry can activate contraction of smooth muscle cells.2,3 The relationship between smooth muscle membrane potential and arterial tone is quite steep.3 Membrane depolarization results in activation of voltage-gated Ca$^{2+}$ channels, thereby leading to Ca$^{2+}$ influx. In contrast, membrane hyperpolarization through activation of potassium (K$^{+}$) channels is an effective mechanism to inactivate Ca$^{2+}$ channels and thus dilate arteries.3

Voltage-gated Na$^{+}$ channels are generally responsible for the fast depolarizing phase of the action potential in most electrically excitable tissues. In response to moderate depolarization, these channels open for a few milliseconds and, thereby, generate a rapid and massive influx of Na$^{+}$ ions promoting the large depolarization that gates other voltage-activated ion channels. Na$^{+}$ channels are present in a wide variety of cells, including neurons and heart and skeletal myocytes.4–6 In contrast, Na$^{+}$ channels have only recently been detected in arterial smooth muscle cells.7–11 We have recently identified a tetrodotoxin (TTX)-sensitive $I_{\text{Na}}$ in primary cultured human coronary myocytes (HCMs) derived from end-stage failing hearts of transplanted patients with an ischemic cardiopathy.10 This current exhibits quite unusual properties, including the presence of a sustained component resulting from very slow inactivation. The major aim of the present work was to investigate whether, and how, this current plays a role in the regulation of Ca$^{2+}$ homeostasis in HCMs.

Materials and Methods

Myocyte Isolation and Culture

Tissue samples were obtained from the left descending coronary artery of 6 male patients (age range, 44 to 58 years) undergoing heart transplantation. Those patients had end-stage heart failure (New York Heart Association, classes III and IV) caused by an ischemic disease. Cells were enzymatically isolated and grown in culture as...
described previously. Briefly, arteries were collected aseptically after cardiectomy, and myocytes were then dispersed in Hanks’ solution containing 0.6 mg/mL collagenase (Worthington) and 1 mg/mL elastase (Boehringer Mannheim) for at least 50 minutes at 37°C. Next, the cells were centrifuged (100g, 7 minutes); resuspended in Ham’s F-10 (Eurobio) supplemented with 10% human serum (Institut Jacques Boy SA), 2 mM/L glutamine, and antibiotics (Eurobio); and plated (10,000 cells per mL) in 35-mm disposable Petri dishes (Falcon) that were placed at 37°C in an air/CO2 incubator. The standard medium was changed every day during the first week. Thereafter, the cells were transferred to maintenance medium (DMEM and Ham’s F-10; 1:1 vol/vol; Eurobio) containing human serum (5%) and 5% FBS (Myocline Super Plus; Life Technology, Cergy Pontoise, France), which was changed every 2 to 3 days. Confluence was obtained 15 to 20 days after plating.

Electrophysiological Recordings
The whole-cell patch-clamp technique was performed at room temperature (20°C to 22°C). For whole-cell recordings, pipettes (borosilicate glass, Sutter) were filled with the following (in mM/L): CsCl 130, EGTA 10, HEPES 25, Mg-ATP 3, Na-GTP 0.5, glucose 10, succinic acid 5, and aspartic acid 5, with pH adjusted to 7.3 with Cs2SO4. The bathing solution contained the following (in mM/L): NaCl 140, MgCl2 2, CaCl2 0.02, HEPES 10, 4-aminopyridine 5, and glucose 10, pH adjusted to 7.3 with CH3SO4H. Junction potential was zeroed before seal formation. Voltage errors resulting from the residual, uncompensated series resistance (≤1 MΩ) were estimated to be ≥2 mV (IgK<2 nA). Leak and capacitive currents were subtracted using a 5-subpulse method. Sampling frequencies ranged from 0.16 to 2 kHz, and current signals were filtered at 3 to 5 kHz before digitization and storage. The holding potential (HP; -100 mV), test potentials, and rate of stimulation (0.1 Hz) were controlled with an IBM personal computer connected to the electrophysiological equipment (Axopatch 200A amplifier; Axon Instruments). Data acquisition and analysis were performed using the pCLAMP software (version 6.02).

Solutions for [Ca2+] and [Na+], Measurement Experiments
Standard normal Locke buffer was used. The buffer contained the following (in mM/L): NaCl 140, KCl 5, KH2PO4 1.2, MgSO4 1.2, CaCl2 1.8, glucose 10, and HEPES 10, with pH 7.2 adjusted with NaOH. A Na+-free solution was prepared by replacing Na+ with N-methyl-D-glucamine chloride. The high K+ solution contained the following (in mM/L): NaCl 90, KCl 50, KH2PO4 1.2, MgSO4 1.2, CaCl2 1.8, glucose 10, and HEPES-NaOH 10, pH 7.2. The osmolarity of all of the solutions ranged between 298 and 303 mosm/L.

Dye Loading and Measurement of [Ca2+] and [Na+]

The [Ca2+] in single cells was measured as described previously. Briefly, the cells were loaded by incubation with 2.5 μmol/L Ca2+-sensitive dye fura-2-acetoxymethyl ester (AM) (dissolved in DMSO) plus 0.02% Pluronic F-127, a surfactant polyol (dissolved in water; Molecular Probes Inc), in Locke buffer. Dye loading was performed as described previously.

The [Na+] was measured in individual cells that had been loaded with the Na+ indicator benzofuran isophthalate (SBFI, 5 μmol/L; Sigma). To load with SBFI-AM, cells were incubated in Locke buffer containing 5 μmol/L SBFI-AM, 0.02% wt/vol Pluronic F-127 for 60 minutes at 37°C in a humidified air atmosphere. After loading, coverslips were rinsed with Locke buffer and mounted in a recording chamber. All recordings were made at room temperature (21°C to 23°C) using fast fluorescence photometry equipment previously described for [Ca2+] measurements. For [Na+], measurements, fluorescence ratios obtained from background-subtracted fluorescence signal at each wavelength were converted to [Na+], using the following equation described by Grynkiewicz et al:

\[ \frac{[Na^+]}{[Na^+]_0} = \frac{F(340) - F(380)}{F(340) - F(380)} \]

where \( K \) is a constant describing the apparent affinity of the dye for Na+ and is related to the dissociation constant (KD) of SBFI (18 mM/L). Rmin and Rmax are the fluorescence ratios measured in the normal absence and in the presence of saturating amounts of ion, respectively. K, Rmin, and Rmax were obtained from standard curves. Because the spectral characteristics of SBFI in the cytosol are different from those of SBFI in solution, the [Na+] standard curve was constructed from fluorescence ratios obtained in situ on exposing cells loaded with SBFI-AM to perfusion solutions containing known concentrations of Na+ (0, 50, 100, and 140 mM/L) and 5 μmol/L gramicidin D.13 In our experimental conditions, when measuring the fluorescence ratio for these concentrations of Na+, the relationship between the fluorescence ratio and [Na+], was linear and the levels of [Na+], happened to fall within the most linear part of the equation. Experiments were performed after a resting period of 15 minutes from the end of the incubation. Transient variations of [Na+] and [Ca2+] were measured at their maximal amplitude.

Drugs
TTX, lidocaine, N-bromosacacetamide, toxin V from the sea anemone Anemonia sulcata (a generous gift of Dr H. Schwarz and Prof M. Lazdunski, Nice, France), and nicardipine (Sandoz) were prepared as stock solutions (1 mM/L for TTX in 0.1% acetic acid, 0.1 mM/L each lidocaine and N-bromosacacetamide in double-distilled H2O, and 1 mM/L nicardipine in dry DMSO), stored at −20°C, and subsequently diluted at the desired working concentrations in test solutions. The lipid-soluble plant alkaloid toxin veratridine (Sigma) was prepared contemporaneously (0.01 mM/L stock solutions in 0.1N HCl). The control and test solutions were applied using a multiple capillary perfusion system (200 μm inner diameter tubing, flow rate 100-150 μL/min) placed to the proximity of each cell tested (<0.5 mm). Each capillary was fed by a reservoir 50 cm above the bath. After each application, the cells were washed with Locke buffer. Incubations (5 minutes) with inhibitory substances were carried out in a 500-μL bath containing inhibitors diluted in Locke buffer.

Analyses
The results were analyzed using the Student t test. Unless otherwise stated, all inhibitors used here showed a “significant” effect at P<0.01. The results are expressed as mean±SEM.

Results

Window Current and Slow Inactivating Component

Iw in HCMs activates at voltages positive to ~50 mV and peaks between ~10 and 0 mV. This is illustrated in Figure 1A. Another surprising feature is the presence of a slow inactivating current component with relatively large amplitude (up to 30% of total peak amplitude in some cells), which is totally blocked by 1 μmol/L TTX (Figure 1B). The steady-state inactivation curve of Iw, determined by applying various conditioning potentials for 5 seconds before the test depolarization, shows that a significant fraction of Iw can still activate from depolarized membrane potentials (Figure 1C). The membrane potential at which 50% of the channels are still available for opening (V1/2) is around ~45 mV (Figure 1D). Furthermore, the overlap between the conductance and inactivation curves determines a “window” between ~50 mV
Figure 1. Electrophysiological properties of the macroscopic whole-cell i_{Na}. A, Family of i_{Na} evoked at various test potentials from HP -100 mV. B, Complete suppression of i_{Na} by 1 μmol/L TTX (same cell as in panel A). C, Family of i_{Na} evoked at -10 mV from different conditioning potentials applied for 5 seconds (to ensure complete inactivation) before the test depolarization (see protocol [bottom of panel C]; same cell as in panel A). D, Voltage-dependent steady-state availability (h; □, ■) and normalized conductance-voltage relationship (g_{Na}/g_{Na,max}; ○, ●) of the i_{Na} peak amplitude (i_{Na,peak}; ■, ●) and of the slowly inactivating (sustained) component of i_{Na} (i_{Na,sus}, which was measured after 200-ms depolarization; □, ○). g_{Na} was calculated from the relation g_{Na}=i_{Na}/E_{m}-E_{Na}, with E_{Na} considered as close to +60 mV (E_{m} is the membrane potential). The original data were taken from panel A. Curves were best fitted assuming a Boltzmann distribution using the equation h_{m}=g_{max}[1+exp(V-V_{o})]/k, where V_{0.5} is the voltage at which h_{m} is half maximal and k the slope factor for the conductance increase. We determined that V_{0.5}=-25 mV (k=4.9) for i_{Na,peak} and V_{0.5}=-26 mV (k=5.0) for i_{Na,sus}. The h_{m} curves were determined from panel C. i_{Na} was measured for each prepulse, normalized, and plotted as a function of the conditioning voltages. The fitted curves were obtained using the equation h_{m}=1/[1+exp(V-V_{o})]/k, where V_{0.5} is the voltage at which h_{m} is half inactivated and k the slope factor for voltage dependence. We determined that V_{0.5}=-44 mV (k=7.7) for i_{Na,peak} and V_{0.5}=-35 mV (k=7.0) for i_{Na,sus}. E, Example of sustained i_{Na} recorded for a long depolarization at -40 mV (same cell as in panel A). Cell capacitance was 100 pF.

Figure 2. Effects of Na\(^{+}\) channel agonists on i_{Na}. A, Effect of 10 μmol/L veratridine (VT) in absence and presence of 1 μmol/L TTX. B, Effect of 100 nmol/L toxin V from A sulcata (As-V) in absence and presence of 1 μmol/L TTX. C, Effect of 300 μmol/L N-bromocacetamide (NBA). In all cells, i_{Na} was evoked at -10 mV from HP -100 mV.

Effect of Na\(^{+}\) Channel Agonists
To increase Na\(^{+}\) channel activity in non–voltage-clamped cells, HCMs were subjected to pharmacological agents known to be potent Na\(^{+}\) channel agonists. Although i_{Na} in HCMs has particular properties, it can be blocked or enhanced by various specific agents and toxins\(^{10}\) that bind on receptor sites of the vertebrate Na\(^{+}\) channel family. The water-soluble heterocyclic guanidine TTX binds to site 1 and block i_{Na} in HCMs (Figure 1B). Toxin binding on the other receptor sites generally increases Na\(^{+}\) influx, although there are substantial mechanistic differences among the effects of various activators on i_{Na}. This was also the case in HCMs for veratridine, which binds to site 2, and for toxin V from A sulcata, which binds to site 3.\(^{4,5,19}\) The effect of veratridine is complex, as illustrated in Figure 2A. Veratridine (10 μmol/L) decreased the peak amplitude of i_{Na} by suppressing completely the fast inactivating component in all cells tested (n=12). However, it promoted at the same time the appearance of both a large sustained component during the test depolarization and a large, slowly deactivating tail relationship (Figure 1A), i.e., at potentials far higher than those determining the window current, indicating that a slow inactivating current is also generated by particular gating properties of the channels. Therefore, in addition to the overlap in the voltage-dependent activation/inactivation properties, the slow decay kinetics of i_{Na} constitute an intrinsic mechanism to promote massive Na\(^{+}\) influx into the cells. Interestingly, the sustained component of i_{Na} (i_{Na,sus}) was less sensitive to voltage-dependent inactivation than the peak current (i_{Na,peak}; Figure 1D), implying that its amplitude also contributes to determine the amplitude of the steady-state window current (eg, at -30 mV; Figure 1D).

and -10 mV (Figure 1D), suggesting that a fraction of the Na\(^{+}\) channels are open and generate a steady-state Na\(^{+}\) influx in this range of potentials. At the minimum voltage required for activation (~40 mV), a substantial noninactivating Na\(^{+}\) influx was indeed observed (Figure 1E). However, a persistent component was also observed at all potentials of the I-V
current at repolarization. The effect of toxin V from *A sulcata* (100 nmol/L) was different. This toxin increased consistently the peak amplitude of $I_{Na}$ (33±10%; n=6) and slowed significantly its decay, with no major effect on current deactivation (Figure 2B). However, the agonistic effect of both toxins was blocked by addition of 1 μmol/L of TTX (Figure 2A and 2B) in all cells tested (n=4 for each).

Veratridine and toxin V from *A sulcata* had no significant effect on the activation threshold of $I_{Na}$, in none of the four cells tested. In addition to these two natural toxins, we also used *N*-bromoacetamide, a chemical known to prolong the open time of $Na^+$ channels and to slow the kinetics of macroscopic $I_{Na}$. In HCMs, we found that *N*-bromoacetamide (300 μmol/L) had no significant effect on current amplitude (−5±8% decrease; n=4) but prevented the inactivation of $I_{Na}$ with no or only minor effect on the deactivating tail current. *N*-Bromoacetamide had no effect on the activation threshold of $I_{Na}$ (data not shown). Therefore, we expected that, despite clear mechanistic differences in their effects on $Na^+$ channels gating in HCMs, veratridine, toxin V from *A sulcata* and *N*-bromoacetamide could be used to increase $Na^+$ influx through drug-modified channels into non-voltage-clamped myocytes.

### Na\(^+\) Channel-Dependent Increase of [Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)

We investigated whether the modulation of Na\(^+\) channel activity results in modulation of [Ca\(^{2+}\)], in resting (non-voltage-clamped) HCMs. To increase Na\(^+\) channel activity, the cells were subjected to veratridine (10 μmol/L). First, we investigated the effect of veratridine on [Na\(^+\)]\(_i\) in SBFI-loaded cells (see Materials and Methods). In control cells, the mean fluorescence intensity ratio was 1.81±0.26 (n=4), which corresponds to 8.6±0.2 mmol/L [Na\(^+\)]. The resting value remained stable for up to 1 hour under superfusion of standard Locke solution. Brief applications of veratridine induced large increases of [Na\(^+\)]\(_i\). (Figure 3A and 3B). On average, the resting [Na\(^+\)]\(_i\) was augmented to 25.9±2.9 mmol/L [Na\(^+\)]\(_i\) (≈3-fold increase), suggesting that veratridine could be used to increase Na\(^+\) channel activity.

The next experiments were performed in fura-2–loaded cells (see Materials and Methods). First, we found that the addition of the depolarizing agent K\(^+\) (KCl 50 mmol/L) induced a transient increase of [Ca\(^{2+}\)], from 41±7 to 44±70 nmol/L (data not shown) in all cells tested (n=8), suggesting the involvement of depolarization-activated Ca\(^{2+}\) channels. Then we assessed the effects of Na\(^+\) channel antagonists and agonists without the help of high extracellular K\(^+\). Figure 4A through 4C shows that *N*-bromoacetamide, veratridine, and toxin V from *A sulcata* induced large rises in [Ca\(^{2+}\)]. Figure 4A shows the effects of *N*-bromoacetamide at 30 and 300 μmol/L. On average, *N*-bromoacetamide (300 μmol/L) increased [Ca\(^{2+}\)], from 47±4 to 368±40 nmol/L (n=5) (Figure 4Aa and 4Ab), veratridine (10 μmol/L) increased [Ca\(^{2+}\)], from 78±6 to 611±44 nmol/L (n=12) (Figure 4Ba and 4Bb), and toxin V from *A sulcata* induced large increases in [Ca\(^{2+}\)], from 59±5 to 588±42 nmol/L (n=11) (Figure 4Ca and 4Cb). The increase of [Ca\(^{2+}\)] was greater in amplitude with veratridine and toxin V from *A sulcata* than with *N*-bromoacetamide. Biphasic responses (even oscillatory activity) were sometimes observed, possibly reflecting release of Ca\(^{2+}\) from intracellular stores. In total, >80% of the cells responded to Na\(^+\) channel agonists.

The rise in [Ca\(^{2+}\)] was strictly dependent on the presence of external Na\(^+\), consistent with our previous observation that $I_{Na}$ is abolished in Na\(^-\)free conditions. The increase in [Ca\(^{2+}\)] induced by veratridine and toxin V from *A sulcata* was indeed abolished when extracellular Na\(^+\) was substituted with the nonpermeating N-methyl-D-glucamine ion (Figure 5Aa). On average, veratridine, which increased [Ca\(^{2+}\)], from 64±9 to 639±36 nmol/L (n=4) in the presence of external Na\(^+\), had no effect after preincubation of the cells in the Na\(^-\)free conditions.
solution (Figure 5Ab). The [Ca\(^{2+}\)] remained stable at 73±12 nmol/L. Figure 5Ba shows that the rise in [Ca\(^{2+}\)] induced by toxin V from A sulcata was also abolished when extracellular Ca\(^{2+}\) was removed. On average, toxin V from A sulcata, which increased [Ca\(^{2+}\)] from 66±9 to 467±88 nmol/L (n=5) in control conditions, had no significant effect after external Ca\(^{2+}\) was removed (Figure 5Ba). The [Ca\(^{2+}\)] was 86±6 nmol/L (n=5) in free Ca\(^{2+}\) conditions (Figure 5Bb). Taken together, these results suggest that transmembrane influxes of both Na\(^{+}\) and Ca\(^{2+}\) ions are required to observe any rise in [Ca\(^{2+}\)] after activation by veratridine and by toxin V from A sulcata.

The effects of veratridine and toxin V from A sulcata were prevented in the presence of 1 μmol/L TTX (Figure 6A and 6B) and of 10 μmol/L lidocaine (Figure 6C). The effects of agonists were recovered on washout of the antagonists (eg, see Figure 6A). These experiments confirmed that the effects induced by the agonists reflect a genuine increase of Na\(^{+}\) channel activity. Furthermore, using the whole-cell technique (see Materials and Methods), we found that veratridine and toxin V from A sulcata were unable to promote any current in all of 6 cells with no detectable basal macroscopic \(I_{Na}\), suggesting that these agonists do not activate a silent Na\(^{+}\) channel (as has been reported for veratridine in rat aorta), but rather modulate the activity of channels active at baseline. It was also unlikely that veratridine and toxin V cause any Ca\(^{2+}\) influx, eg, by increasing the permeability of Na\(^{+}\) channels for Ca\(^{2+}\) or by direct activation of Ca\(^{2+}\) channels (\(I_{Na}\) is decreased by external Ca\(^{2+}\); S.R., J.F.Q., unpublished results, 1997).

**Involvement of Ca\(^{2+}\) Channels and Na\(^{+}\)/Ca\(^{2+}\) Exchanger**

We assessed whether the rise in [Ca\(^{2+}\)], induced by veratridine or toxin V from A sulcata involved depolarization-activated Ca\(^{2+}\) channels. The rise in [Ca\(^{2+}\)], observed after K\(^{+}\) depolarization was abolished by a saturating concentration of nicardipine (5 μmol/L; data not shown), which blocks all of the L-type Ca\(^{2+}\) current in these cells. Veratridine, which increased [Ca\(^{2+}\)], from 70±6 to 782±71 nmol/L (n=5) in control conditions, had a much smaller effect in the presence of nicardipine (177±16 nmol/L) (Figure 7Aa and 7Ab), suggesting that voltage-gated Ca\(^{2+}\) channels are the main pathway involved in the response. Nevertheless, a residual

![Figure 5.](image)

**Figure 5.** The rise in [Ca\(^{2+}\)], induced by Na\(^{+}\) channel agonists in fura-2–loaded HCMs depends on both extracellular Na\(^{+}\) and Ca\(^{2+}\). Aa, Increase of [Ca\(^{2+}\)] induced by 10 μmol/L veratridine; after wash, the cell was preincubated with Na\(^{+}\)-free Locke buffer and then exposed to veratridine again. Ab, averaged effect. Ba, Increase of [Ca\(^{2+}\)] induced by 0.1 μmol/L toxin V from A sulcata (ASv); after wash, the cell was preincubated with Ca\(^{2+}\)-free buffer and then challenged again with toxin V from A. sulcata. Bb, Averaged effect. A and B, Ctrl indicates control; ***P<0.001.

![Figure 6.](image)

**Figure 6.** Effects of TTX and lidocaine on the Na\(^{+}\) channel–dependent rise of [Ca\(^{2+}\)] in fura-2–loaded HCMs. Cells were preincubated for 5 minutes with the antagonist before the agonist was applied again. A, Effect of 1 μmol/L TTX on the [Ca\(^{2+}\)] increase induced by veratridine (VT, 10 μmol/L). Note that after wash of TTX, the [Ca\(^{2+}\)] response to veratridine was restored. B, Rise in [Ca\(^{2+}\)] promoted by 100 nmol/L toxin V from A sulcata (ASv) and blocked by 1 μmol/L TTX. C, Rise in [Ca\(^{2+}\)] promoted by 100 nmol/L toxin V from A sulcata and blocked by 1 μmol/L lidocaine (Lido). The antagonistic effects of TTX and toxin V from A sulcata were observed consistently in each of 10 cells.
nicardipine-insensitive rise in [Ca\(^{2+}\)], could still be observed consistently (Figure 7AA and 7AB). But, when extracellular Na\(^+\) was replaced by Li\(^+\) on an equimolar basis (Na\(^+\)-free medium), this residual component was abolished (Figure 7ABa). Similar results were observed in all cells tested (n=4). Because Li\(^+\) permeates through Na\(^+\) channels but is not taken by the Na\(^+\)/Ca\(^{2+}\) exchanger, these data suggest that the dihydropyridine-insensitive Ca\(^{2+}\) influx is provided by the exchanger working in reverse mode. Consistent with this observation, we found that, although Li\(^+\) and Na\(^+\) have similar permeating properties through the Na\(^+\) channels and, presumably, equivalent depolarizing effects, veratridine increased [Ca\(^{2+}\)], from 34±7 to only 389±34 nmol/L (n=4) when Li\(^+\) was used as the permeating ion (in absence of Ca\(^{2+}\) channel blocker) (Figure 7BBb). Therefore, the rise was significantly lower than when Na\(^+\) was used as the permeating ion (611±44 nmol/L; n=12), confirming that the dihydropyridine-sensitive pathway is not the only route for Ca\(^{2+}\) entry. This latter result was also consistent with the participation of the Na\(^+\)/Ca\(^{2+}\) exchanger in the Ca\(^{2+}\) influx activated after Na\(^+\) channel activation.

Na\(^+\) Channel-Dependent Decrease of Resting [Na\(^+\)] and [Ca\(^{2+}\)]

The fact that veratridine induces a rise in [Na\(^+\)], suggests that the Na\(^+\) channels are active at baseline, given that this substance (and toxin V from A sulcata as well) would not work on closed channels. Indeed, the electrophysiological characterization is consistent with some Na\(^+\) channels being open in HCMs with membrane potentials between −50 and −10 mV (Figure 1D). We further assessed the existence of such a steady-state Na\(^+\) influx in resting (non–voltage-clamped) SBF1-loaded cells. Brief applications of 1 μmol/L TTX induced a decrease in the fluorescence ratio (Figure 8AA) from 1.93±0.14 to 0.58±0.06, corresponding to a decrease in [Na\(^+\)], from 10.6±0.8 to 3.2±0.3 mmol/L (n=4). This decrease occurred within seconds after application of TTX and lasted as long as the blocker was applied. We next addressed the question of whether such a steady-state Na\(^+\) influx could eventually control the resting [Ca\(^{2+}\)]. Figure 8B illustrates the effect of TTX on the resting [Ca\(^{2+}\)], as observed in 3 different fura-2–loaded cells. No significant effect was observed in 5 other cells tested. The cells that responded had a high resting [Ca\(^{2+}\)], level (>200 nmol/L), whereas the other had a resting [Ca\(^{2+}\)], level <100 nmol/L (data not shown).

Discussion

We recently reported the presence of voltage-activated I\(_{Na}\) in cultured HMCs. By particular voltage- and time-dependent properties (compared with most types of I\(_{Na}\)), I\(_{Na}\) here is likely to exert a unique type of regulation on the resting [Ca\(^{2+}\)]. In the present work, we show that I\(_{Na}\) actually regulates [Na\(^+\)], and [Ca\(^{2+}\)]. This type of regulation seems well adapted to vascular physiology.

First, we demonstrated that an enhancement of Na\(^+\) channel activity using various agonists (N-bromoacetamide, vera-
tridine, and toxin V from A sulcata) increases [Ca\(^{2+}\)]\(_i\) in the vast majority of HCMs. Clear evidence, such as prevention of the increase in [Ca\(^{2+}\)]\(_i\) by external Na\(^+\) depletion and by TTX or lidocaine, showed that this rise reflects a genuine effect on Na\(^+\) channels.

Second, we showed that the rise in [Ca\(^{2+}\)]\(_i\), induced by application of Na\(^+\) channel agonists occurs mainly as a consequence of the opening of voltage-activated Ca\(^{2+}\) channels. The presence of Ca\(^{2+}\) channels in the HCMs,\(^{12}\) the requirement of extracellular Ca\(^{2+}\) to observe the effects of I\(_{Na}\) on [Ca\(^{2+}\)]\(_i\), and the fact that the rise in [Ca\(^{2+}\)]\(_i\) is largely antagonized by nicardipine all suggest that voltage-gated Ca\(^{2+}\) channels are a major route for the transmembrane Ca\(^{2+}\) influx and a key step between Na\(^+\) channel activation and elevation of [Ca\(^{2+}\)]\(_i\). Therefore, our proposed cascade is the following: (1) the Na\(^+\) channel agonists enhance Na\(^+\) channel activity and, thereby, generate a Na\(^+\) influx, and (2) this Na\(^+\) influx, in turn, promotes the membrane depolarization that gates the Ca\(^{2+}\) channels and thus produces the Ca\(^{2+}\) influx. Although the cultured HCMs express both T- and L-type Ca\(^{2+}\) channels,\(^{12}\) the effects described here concern only L-type channels, because T-type Ca\(^{2+}\) channels are completely inactivated at the voltages required to open the Na\(^+\) channels (>−50 mV). Third, part of the transmembrane Ca\(^{2+}\) influx that contributes to the rise in [Ca\(^{2+}\)]\(_i\), induced by Na\(^+\) channel agonists is not mediated by Ca\(^{2+}\) channels. This contribution, which is smaller than that of Ca\(^{2+}\) channels, is independent of the depolarizing effect induced by Na\(^+\) (or Li\(^+\)) influx through Na\(^+\) channels on membrane potential. It seems rather related to the nature of the permeating ion, because it is not observed when Li\(^+\) is used as the depolarizing permeating ion. Consistently, the nicardipine-insensitive rise in [Ca\(^{2+}\)]\(_i\) is suppressed when external Na\(^+\) is replaced by Li\(^+\), which blocks the Na\(^+\)/Ca\(^{2+}\) exchanger. Therefore, enhanced activity of the Na\(^+\)/Ca\(^{2+}\) exchanger working in the reverse mode (entry of Ca\(^{2+}\) against Na\(^+\) extrusion) provides an additional route for transmembrane Ca\(^{2+}\) influx after activation of Na\(^+\) channels in HCMs.

Fourth, we showed that, because of their particular electrophysiological properties, Na\(^+\) channels may play a role in the control of the resting [Na\(^+\)], and, thereby, of the basal [Ca\(^{2+}\)]\(_i\), in HCMs. Two factors contribute to the development of a steady-state Na\(^+\) influx. One is the sustained current predicted by the Hodgkin-Huxley analysis that occurs as a result of the voltage dependence of I\(_{Na}\) activation and inactivation overlap. The other is the presence of slowly inactivating Na\(^+\) currents, which could be observed even at positive potentials, raising the possibility that these currents are generated by an unusual subtype of Na\(^+\) channels. However, whether the transient and the sustained component are related to one or two channels is not completely clear.

The fact that Na\(^+\) channel activators increase [Ca\(^{2+}\)]\(_i\) as a result of the opening of voltage-gated Ca\(^{2+}\) channels could be considered as relatively implicit, although this occurs at potentials relevant to vascular physiology. It could also be argued that the use of exogenous substances leads to unphysiological increases in [Na\(^+\)]. We actually used these agonists to identify clearly the mechanisms involved downstream.

Another important result from these experiments is that some Na\(^+\) channels stay open at baseline potentials. Indeed, N-bromosuccinimide, veratridine, and toxin V from A sulcata, the primary effect of which is to cause Na\(^+\) channels to open more easily and/or to stay open longer than normal,\(^{1,5}\) induced large rises in [Ca\(^{2+}\)]\(_i\), without the help of the usual depolarizing agent, K\(^+\). Consistently, we observed a TTX-induced decrease of [Na\(^+\)], confirming the contribution of a basal steady-state Na\(^+\) influx to the resting [Na\(^+\)], in non–voltage-clamped cells. Therefore, the regenerative mechanism probably involved in the effect of agonists would lead to the following sequence of events: (1) the Na\(^+\) channel agonist prolongs the open time of channels already open and may also help opening some others; (2) this leads to increased Na\(^+\) influx; and (3) as channels become activated, the resulting depolarization recruits additional Na\(^+\) channels, which, ultimately, leads to activation of voltage-gated Ca\(^{2+}\) channels.

The prolonged activity of the HCM Na\(^+\) channels during sustained depolarization, their availability for opening from relatively depolarized membrane potentials, and the existence of a substantial window current at a wide range of potentials, are likely to confer a potential physiological role to these Na\(^+\) channels in the vascular myocytes. In a variety of neurons, such noninactivating I\(_{Na}\) act physiologically to amplify synaptic potentials and set repetitive action potentials to enhance endogenous rhythmicity.\(^{21–23}\) In cardiac cells, it is involved in the regulation of action potential duration and resting membrane potential.\(^{26–29}\) In HCMs, I\(_{Na}\) may contribute to a tonic control of Ca\(^{2+}\) channel activity, possibly counterbalancing membrane hyperpolarization through activation of K\(^+\) channels, and as a result of [Ca\(^{2+}\)]. Interestingly, we detected a TTX-sensitive decrease in [Ca\(^{2+}\)]\(_i\) in some cells with a high resting [Ca\(^{2+}\)], which may occur because of the overlap of the window currents generated by both Na\(^+\) channels and L-type Ca\(^{2+}\) channels. Because I\(_{Ca}\) starts to activate at ∼−30 mV,\(^{12}\) this tonic control of Ca\(^{2+}\) channel activity can probably be observed only in depolarized cells. The steady-state Na\(^+\) influx may therefore ensure a fine graded regulation of the steady-state Ca\(^{2+}\) influx. It is certainly an effective mechanism to induce and maintain cell depolarization, to turn on Ca\(^{2+}\) channel activity and promote sustained Ca\(^{2+}\) influx and, in addition, to maintain elevated [Na\(^+\)].

Because I\(_{Na}\) was observed in primary cultured cells, it is difficult to speculate on any physiological function in vivo at the moment. Direct extrapolation from in vitro observations to in vivo physiology must be considered with caution, specially because expression of I\(_{Na}\) seems to be related to cell dedifferentiation.\(^{40}\) Nevertheless, it is conceptually interesting to note that the repertoire of ion currents of HCMs is changing (eg, T-type Ca\(^{2+}\) currents) during phenotypic modulation of the cells in vitro.\(^{52}\) Interestingly, vascular smooth muscle cells in culture undergo many changes resembling those occurring in diseased vessels. Indeed, dedifferentiation and proliferative disorders play a major part in coronary artery diseases, including atherosclerosis, neointimal formation after endothelial injury, restenosis after angioplasty, and also hypertension. Therefore, the possibility that I\(_{Na}\) is expressed under certain pathophysiological circumstances and helps regulate the basal arterial tonus, or other Ca\(^{2+}\)– or Na\(^+\)–dependent function(s), is worth considering and will be explored in the near future.
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