No Apparent Requirement for Neuronal Sodium Channels in Excitation–Contraction Coupling in Rat Ventricular Myocytes

Fabien Brette, Clive H. Orchard

Abstract—The majority of Na channels in the heart are composed of the tetrodotoxin (TTX)-resistant (K_D, 2 to 6 μmol/L) “cardiac” NaV1.5 isoform; however, TTX-sensitive (K_D, 1 to 25 nmol/L) “neuronal” Na channel isoforms have recently been detected in several cardiac preparations.

In mammalian cardiac muscle, release of Ca from the sarcoplasmic reticulum (SR) is the key event linking membrane depolarization and mechanical activity during excitation–contraction (EC) coupling. Ca influx via L-type Ca channels is the major source of trigger Ca, which activates ryanodine receptors in the membrane of the adjacent SR by a process known as Ca-induced Ca release. Ca-induced Ca release may also be triggered by Ca entry via reverse Na/Ca exchange, albeit with lower efficacy.

Voltage-gated Na channels play an important role in EC coupling by causing the rapid upstroke of the action potential and the propagation of excitation from cell to cell. Ten genes encoding the α (pore-forming) subunit of the Na channel have been cloned from different mammalian tissues. Until recently, it appeared that the main pore-forming subunit in mammalian cardiac tissue was the NaV1.5 isoform. However, although NaV1.5 is highly expressed at the mRNA level in the mammalian heart, other α subunit isoforms have also been detected (NaV1.1, NaV1.2, NaV1.3, NaV1.4, NaV1.6, collectively called neuronal in this study). These isoforms are preferentially located in the central nervous system and adult skeletal muscle (see Goldin for review) and are inhibited by nanomolar concentrations of tetrodotoxin (TTX) (K_D, 1 to 25 nmol/L), in contrast to NaV1.5 (K_D, 2 to 6 μmol/L).

In ventricular myocytes, the subcellular localization of Na channel isoforms is unclear. Some immunocytochemistry data show that these neuronal Na channel isoforms are present in murine5,9–13 and canine4 cardiac myocytes. This is supported by electrophysiological recordings showing that TTX-sensitive Na current (I_Na) represents between 5% and 30% of total I_Na in diverse cardiac preparations.

In this study, we investigated the possible contribution of TTX-sensitive I_Na to EC coupling, using 200 nmol/L TTX to selectively block TTX-sensitive I_Na. TTX decreased the rate of depolarization of the action potential by 10% but did not delay the rise of systolic Ca in the center of the cell (transverse confocal line scan), suggesting that TTX-sensitive I_Na does not play a role in synchronizing Ca release at the t-tubules; the amplitude of the Ca transient and contraction were also unchanged by 200 nmol/L TTX. The quantity of charge entering via I_Ca elicited by control or TTX action potential waveforms was similar, suggesting that the trigger for Ca release is not altered by blocking TTX-sensitive I_Na. We conclude that neuronal I_Na is concentrated at the t-tubules, but there is no evidence of a requirement for these channels in normal excitation–contraction coupling in ventricular myocytes.

Key Words: cardiac myocytes • sodium channels • excitation–contraction coupling • electrophysiology
and Orchard 16 for review), this subcellular localization of neuronal Na channels raises the possibility that these channels contribute to t-tubule excitability and hence EC coupling. Indeed, it has been shown recently that block of these neuronal Na channels by a low concentration of TTX (100 and 200 nmol/L) decreases left ventricular contractility in a whole guinea pig heart preparation.13 This unexpected observation has been interpreted as suggesting a distinct role for the neuronal Na channels localized at the t-tubules, in linking depolarization of the surface sarcolemma with EC coupling in ventricular myocytes.13 This hypothesis has important functional implications. However, direct evidence at the single cell level is lacking.

In the present study, we used acute detubulation, which enables us to determine the functional localization of currents,16 in conjunction with the patch clamp technique, to determine the functional localization of the different INa isoforms in rat ventricular myocytes. We found that TTX-sensitive INa is concentrated at the t-tubules. We therefore explored a possible functional role of TTX-sensitive INa by investigating the effect of low concentrations of TTX on key steps of EC coupling in isolated rat ventricular myocytes.

Materials and Methods

Isolation and Detubulation of Rat Ventricular Myocytes

Myocytes were isolated from the ventricles of Wistar rat hearts using a standard enzymatic dissociation protocol. Detubulation was induced by osmotic shock as described previously.17 All experiments were performed at room temperature (~23°C).

Electrophysiological Recording

Membrane potential and currents were recorded using the whole-cell configuration of the patch clamp technique; settings and properties were as described previously.5 INa was measured in low extracellular sodium ([Na+]o) solution (20 mmol/L) to reduce INa and improve voltage control. Low-resistance (1.6±0.1 MΩ; n=49) patch pipettes were used and cell capacitance and series resistance (2.9±0.2 MΩ; n=49) compensated by 85% to 90%. Activation curves were fitted using the Boltzmann equation, a=1/(1+exp((Vm−V1/2)/k)), where a is the activation variable, Vm is the membrane potential, V1/2 is the potential at the current is half activated and k is the slope factor. Action potentials were recorded as previously described.19 The rate of rise of the action potential (dV/dt) was calculated by differentiation using Origin software. In some experiments, the action potential and cell shortening were recorded simultaneously. ICa was measured using Na- and K-free external and internal solutions to avoid contamination by overlapping ionic currents and to allow us to use a physiological holding potential.19 ICa was elicited by action potential waveforms. Ca influx measurements were converted to changes in [Ca] as previously described.19

Ca Imaging

Ca imaging was performed using a laser scanning inverted confocal microscope; settings and properties were as described previously.20 Line-scan images (8-bit) are presented as the original signal. Traces showing the time course of fluorescence are presented as a ratio of fluorescence/background fluorescence (F/FR). The rate of rise of Ca transients recorded from the subsarcolemmal space (SS) and cell center (CC) was calculated by differentiation of F/FR using Origin software.

Cell Shortening Recording

Cell length was monitored using an edge detection system as previously described,17 and the change in cell length during stimulation was used as an index of contractility.

Statistics

Data are presented as mean±SEM. The estimate of standard error (ESE) and 95% confidence interval (CI) are given for parameters derived from Hill fitting (using Origin software). Paired or unpaired t tests (2-tailed) were performed as appropriate. P<0.05 was taken as significant.

An expanded Material and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Presence of TTX-Resistant and TTX-Sensitive INa in Rat Ventricular Myocytes

To establish the presence of TTX-sensitive INa, we determined the concentration dependence of TTX block of INa at test potentials of −60 mV and −10 mV from a holding potential of −120 mV. At −60 mV, TTX-resistant Na channels (NaH1.5) are activated, whereas TTX-sensitive Na channels are closed: they are activated at more depolarized potentials.5,10,21 At −10 mV, all channels are fully activated. Thus if cells contain TTX-sensitive and TTX-resistant Na channels, the dose-response curves should be different at the 2 test potentials with a deviation at nanomolar concentrations of TTX.5,10 Original recordings of control INa and block by TTX, at the 2 potentials are shown in Figure 1A. Figure 1B shows the dose-response curves; at TTX concentrations of 10 and 100 nmol/L, a significantly larger fraction of current was blocked at −10 mV, compared with −60 mV. This differential effect of TTX is not attributable to voltage-dependent block of NaH1.5 because heterologously expressed NaH1.5 exhibits the same block at both potentials.5 These results suggest that 2 components of INa, TTX sensitive and TTX resistant, are present in rat ventricular myocytes. The dose-response curve at −60 mV is well fitted with a Hill curve (with a Hill coefficient of 1), yielding a K0 of 628 nmol/L (black line, Figure 1B). This value is lower than most reports of the TTX sensitivity of NaH1.5, which are generally in the micromolar range (2 to 6 μmol/L).4 However, it is known that TTX affinity depends on [Na]o.22,23 Our K0 is similar to previous reports using low [Na]o in cardiac ventricular myocytes5,13 and after correction for the effect of [Na]o,22 is in agreement with the micromolar range reported previously. The dose-response curve at −10 mV is well fitted with a double Hill curve (gray line, Figure 1B); if it is assumed that the K0 of TTX-resistant current is 628 nmol/L (and the Hill coefficients of both TTX-resistant and TTX-sensitive currents are 1), this gives a K0 for the TTX-sensitive current of 8.8 nmol/L (ESE, 6.4; CI, 27) and the fraction of TTX-resistant and TTX-sensitive currents as 0.86 and 0.14 (ESE, 0.03; CI, 0.11), respectively. The K0 of the TTX-sensitive current is consistent with published data (1 to 25 mmol/L),4 even after correction for the effect of [Na]o (which has a similar effect on cardiac and neuronal Na channels).22 The fraction of TTX-sensitive INa (14±3%) is in the range of other cardiac preparations (5% to 30%).5,6,10,13
Characterization of TTX-Resistant and TTX-Sensitive $I_{\text{Na}}$ in Rat Ventricular Myocytes

$I_{\text{Na}}$ subtypes were characterized by their differential TTX sensitivity: 100 nmol/L TTX is expected to block 92% of TTX-sensitive $I_{\text{Na}}$, with little effect on TTX-resistant $I_{\text{Na}}$ (16% block, assuming the $K_D$ given above). Figure 2A shows $I_{\text{Na}}$ recorded at different potentials under control conditions, in the presence of 100 nmol/L TTX and the current blocked by 100 nmol/L TTX (obtained by subtraction, TTX-sensitive $I_{\text{Na}}$). Figure 2B shows current–voltage relationships for 8 myocytes (Figure 2B) and activation curves constructed and fitted using a Boltzmann equation (Figure 2C) (see Materials and Methods). TTX-sensitive $I_{\text{Na}}$ was activated at more positive potentials than TTX-resistant $I_{\text{Na}}$: the $V_{1/2}$ and $k$ values are $-43 \pm 1$ and 8.5$\pm1.7$ mV and $-55 \pm 2$ and 7.0$\pm1.2$ mV, respectively ($P < 0.05$; Figure 2C).

Kinetic analysis showed that the time to peak and inactivation of $I_{\text{Na}}$ accelerate with increasing amplitude of the test pulse, with TTX-sensitive $I_{\text{Na}}$ activating and inactivating more rapidly than TTX-resistant $I_{\text{Na}}$ (Figure 2D and 2E). Thus, 2 distinct currents with different activation and inactivation can be isolated by their sensitivity to TTX. Such differences in activation and inactivation properties are hallmarks of TTX-sensitive $I_{\text{Na}}$. These data therefore support the idea that TTX-sensitive $I_{\text{Na}}$ and TTX-resistant $I_{\text{Na}}$ are carried by different Na channel isoforms in rat ventricular myocytes.

Presence of TTX-Resistant and TTX-Sensitive $I_{\text{Na}}$ at the Cell Surface of Rat Ventricular Myocytes

We next investigated the presence of TTX-sensitive $I_{\text{Na}}$ at the cell surface only by recording $I_{\text{Na}}$ in detubulated myocytes. Cell capacitance was significantly decreased following detubulation (by 31%; Table). However, $I_{\text{Na}}$ density was not significantly different between control and detubulated myocyte (Table).

We determined the block of $I_{\text{Na}}$ by TTX in detubulated myocytes using a similar approach as for control myocytes (Figure 3A). Figure 3B shows that the dose-response curve at $-60$ mV is well fitted by a Hill curve with a $K_D$ of 614 nmol/L (black line), a value similar to that obtained for control myocytes. However, assuming a $K_D$ of 614 nmol/L for TTX-resistant $I_{\text{Na}}$, the dose-response curve at $-10$ mV is not well fitted with a double Hill curve, probably because a smaller fraction of TTX-sensitive $I_{\text{Na}}$ was present, and hence blocked at TTX concentrations of 10 and 100 nmol/L, compared with control myocytes. This occurs when TTX-sensitive $I_{\text{Na}}$ represents 5% in the preparation. However, if we assume a $K_D$ of 614 nmol/L for TTX-resistant and 8.8 nmol/L for TTX-sensitive $I_{\text{Na}}$ (the values for control myocytes), the data are well fitted by a double Hill yielding a fraction of TTX-resistant and sensitive currents of 0.96 and 0.04, respectively ($r^2 = 0.9954$; Figure 3B). Thus, TTX-sensitive $I_{\text{Na}}$ represents a maximum of 4% of total $I_{\text{Na}}$ in detubulated myocytes compared with 14±3% in control myocytes (see below). This suggests that TTX-sensitive $I_{\text{Na}}$ is located predominantly in the t-tubules rather than on the sarcolemma.

Distribution of $I_{\text{Na}}$ Isoforms in the Surface Sarcolemma and T-Tubules

Using these data, we calculated the distribution of currents (Table) as previously described. We used total $I_{\text{Na}}$ density at $-10$ mV, where both TTX-sensitive and TTX-resistant $I_{\text{Na}}$ are fully activated (Figure 2C). TTX-sensitive $I_{\text{Na}}$ density was calculated assuming that it represents up to 14% and 4% of total $I_{\text{Na}}$ at the total sarcolemma and cell surface membrane, respectively (ie, data from Figures 1B and 3B). $I_{\text{Na}}$ densities in the t-tubules were calculated as the difference in whole-cell current between control and detubulated myocytes. These currents were divided by the difference in membrane capacitance between control and detubulated cells to derive the current density in the t-tubules. In rat ventricular myocytes, 70% of total $I_{\text{Na}}$ is present at the surface sarcolemma and 30% in the t-tubules, so that total $I_{\text{Na}}$ density is not different at the surface sarcolemma and the t-tubules (Table). However, 78% of the TTX-resistant $I_{\text{Na}}$ is located at the surface membrane, where it is 1.6 times more concentrated than in the t-tubules.
(Table). In contrast, 80% of the TTX-sensitive INa is located in the t-tubules, where it is ~9 times more concentrated than at the surface membrane (Table). To derive the proportion of current generated by specific isoforms at each subcellular location, we corrected the current density to account for the partial block of INa by Co (by ~30% for TTX-resistant and ~15% for TTX-sensitive INa; see online data supplement). Assuming similar block by Co at the t-tubule and surface membranes, this correction does not alter quantification of subcellular localization (above). However, the corrected values indicate that TTX-sensitive INa accounts for ~11% of total INa in rat ventricular myocytes. At the surface membrane, TTX-resistant INa generates nearly all INa (~97%). In contrast, at the t-tubules TTX-resistant INa generates ~69% and TTX-sensitive INa represents ~31% of INa. This suggests that TTX-sensitive INa may play a role in EC coupling, which occurs predominantly at the t-tubules in rat myocytes. To explore this possibility, we investigated the effect of a low concentration of TTX (200 nmol/L as in other recent work on cardiac preparations6,10,11,13) on key steps in EC coupling.

Effect of a Low Concentration of TTX on the Action Potential

We first determined the effect of a low concentration of TTX on the action potential, which initiates EC coupling.1 Action potentials recorded from a representative myocyte before, during, and after application of 200 nmol/L TTX are shown in Figure 4A. As summarized in Figure 4B, the dV/dt (inset Figure 4A) and action

**Figure 2.** Characteristics of TTX-resistant and TTX-sensitive INa in control ventricular myocytes. A, Superimposed recordings of INa elicited by +10 mV step voltage-clamp pulses (from −80 to +30 mV from a holding potential of −120 mV) in a myocyte in the absence (left) and presence (middle) of 100 nmol/L TTX, with the current blocked by 100 nmol/L TTX (obtained by subtraction; right). B, Current–voltage relationships for total INa, TTX-resistant INa (in the presence of 100 nmol/L TTX), and TTX-sensitive INa (blocked by 100 nmol/L TTX). Activation curves (C), time to peak (D), and T50 inactivation (E) for TTX-resistant INa and TTX-sensitive INa. Mean±SEM from 8 cells.
potential amplitude, which are indicators of Na channel activity, are significantly decreased (by \(17 \pm 100 \mu M, 17 \pm 100 \mu M, 17 \pm 100 \mu M\), respectively, \(n=12, P<0.05\)) in the presence of 200 nmol/L TTX (Figure 4B and 4C). In contrast, action potential duration was not altered (Figure 4D). These data suggest that TTX-sensitive \(I_{Na}\) influences the shape of action potential. We next investigated whether these changes in the action potential alter synchronization of Ca release at the t-tubules.

Ca Transients Are Not Altered by a Low Concentration of TTX
To investigate the possible role of TTX-sensitive \(I_{Na}\) in SR Ca release, we recorded transverse confocal line-scan images of Ca transients in myocytes loaded with fluo-3 and field stimulated at 0.5 Hz. If TTX-sensitive \(I_{Na}\) plays a role in t-tubule excitability and function, SR Ca release at the t-tubules should be altered by 200 nmol/L TTX. Thus, we investigated the characteristics of Ca transients in the SS and CC regions in the absence and presence of TTX. Myocytes bathed in Tyrode solution exhibited temporally and spatially uniform Ca release (Figure 5A, left); the amplitude and rate of rise of the Ca transient (d[Ca]/dt) was the same in the SS and CC (Figure 5B and 5C). This spatial and temporal uniformity of SR Ca release is attributable to the t-tubules.\(^{20,25}\) Surprisingly, myocytes bathed in 200 nmol/L TTX also had uniform SR Ca release (Figure 5A, right); the amplitude and d[Ca]/dt of the Ca transient in SS and CC were similar to those observed in the absence of TTX (Figure 5B and 5C). As a result, the peak systolic Ca (average fluorescence along the line scan) and d[Ca]/dt of the global Ca transient were the same in the absence and presence of 200 nmol/L TTX (Figure 5B and 5C). These results suggest that despite block of TTX-sensitive \(I_{Na}\), SR Ca release at the t-tubules is unaltered. To investigate this finding further, we recorded cell shortening, as an index of cell contraction.

Cell Shortening Is Not Altered by Low Concentration of TTX
Cell shortening was first recorded during current-clamp experiments. Application of 10 \(\mu M\) TTX abolished cell contraction (Figure 6A). In contrast, application of 200 nmol/L TTX had no significant effect on cell contraction (Figure 6B, \(n=5\)). Because cell shortening was small during these experiments (presumably because of the presence of 0.5 mmol/L EGTA in the pipette solution), we also recorded shortening in field-stimulated experiments. Myocytes bathed
in Tyrode and Tyrode plus 200 nmol/L TTX had similar amplitude and time to peak of cell shortening (Figure 6C, n = 11 and 12, respectively). These results support the idea that blocking TTX-sensitive INa does not alter SR Ca release and, hence, contraction. We next examined the consequences of the changes in the action potential, induced by blocking TTX-sensitive INa, on nICa (the major trigger for SR Ca release; see the introduction) by recording ICa during action potential command waveforms.

ICa Is Not Altered by Change in Action Potential Following Blocking of TTX-Sensitive INa

Figure 7A shows representative recordings of ICa elicited by a control action potential waveform (left) and by an action potential waveform recorded in the presence of 200 nmol/L TTX (right). The time to peak ICa was not significantly different when using the 2 voltage waveforms (Figure 7B, n = 8), although a small but significant increase in the amplitude of ICa was observed when using the waveform recorded in TTX (Figure 7C). However, the amount of Ca entering via ICa during the time to peak, which can be doubled to give the effective Ca trigger for SR Ca release,26 and the amount of Ca entering during the action potential, were not significantly different between the 2 voltage waveforms (Figure 7D). These data suggest that the change in action potential characteristics following block of TTX-sensitive INa does not alter ICa, which triggers SR Ca release.

Discussion

The objective of the present study was to determine the functional subcellular localization of Na channel isoforms and whether neuronal Na channel isoforms participate in EC coupling of ventricular cardiac myocytes at the single cell level.

Experimental Approach

The method used to detubulate rat ventricular myocytes has been described and validated previously.17,20 This method
enables direct determination of the distribution of ion channel function between the surface and t-tubule membranes. Notably, this procedure has no effect on ionic currents measured in atrial myocytes, which lack t-tubules. Using this technique, we have shown previously that \( I_{Ca} \), \( Na/Ca \) exchange, and \( Na-K \) pump currents are located predominantly in the t-tubules, in agreement with our previous work. The present study shows marked differential localization of \( I_{Na} \) isoforms in rat ventricular myocytes. This may, as hypothesized by Maier et al., suggest specific physiological roles for \( I_{Na} \) (excitability and conduction between cells) and \( I_{Na} \) (t-tubule excitation). However, our experiments show no change in the amplitude or time course of the Ca transient and contraction in isolated ventricular myocytes (Figures 5 and 6) during application of 200 nmol/L TTX. We found that \( d[Ca]/dt \) near the cell surface (SS) and at the t-tubules (CC) is similar in control and TTX-treated cells. Computer simulation has shown that a decrease in EC coupling efficiency is expected to alter \( d[Ca]/dt \); our data therefore suggest no difference in the SR Ca release process at the 2 sites (Figure 5) and that despite inhibition of \( I_{Na} \) by \( \approx31\% \) in the t-tubules, the remaining \( I_{Na} \) is able to depolarize the t-tubules sufficiently to activate \( I_{Ca} \) to trigger SR Ca release. We also found that the changes in action potential characteristics induced by blocking TTX-sensitive \( I_{Na} \) (mainly a small decrease in \( dV/dt \); Figure 4) did not alter the characteristics of \( I_{Ca} \) (Figure 7). The amount of Ca entering the cell via \( I_{Ca} \) during the time to peak, which can...
be doubled to give the effective Ca trigger for SR Ca release\textsuperscript{26} was the same when using the 2 waveforms. This estimate gives a value very similar to the integral of the first 20 ms of $I_{Ca}$, which was suggested by Fabiato\textsuperscript{29} to provide the trigger for SR Ca release. Thus, despite the change in the action potential, it seems likely that the trigger for SR Ca release provided by $I_{Ca}$ is unaltered, consistent with studies showing that the initial part of action potential repolarization (phase 1) is crucial in the efficiency of $I_{Ca}$ to trigger SR Ca release.\textsuperscript{30} Our results therefore contrast with those of Maier et al,\textsuperscript{13} which show that low [TTX] decreases ventricular contractility in a whole heart preparation; it is possible that this effect was caused by block of TTX-sensitive $I_{Na}$ in other regions of the heart, rather than ventricular myocytes. Indeed, it has been shown recently that the contribution of TTX-sensitive $I_{Na}$ is greater in Purkinje fibers than in ventricle (\textsim 22\%).\textsuperscript{6}

To summarize, the present study shows that TTX-sensitive $I_{Na}$ accounts for a small fraction of total $I_{Na}$ in rat ventricular myocytes and is concentrated in the t-tubules. However, our data show that at the single cell level, low concentrations of TTX have no effect on EC coupling, providing no evidence of a requirement for neuronal Na channels in EC coupling in ventricular myocytes. It remains possible that neuronal Na channels present in ventricular myocytes act as a safety mechanism, which can be recruited under pathological conditions when the resting membrane potential is depolarized, because of the electrophysiological properties of these currents.

Acknowledgments

This work was supported by the Wellcome Trust. F.B. is a Wellcome Trust Fellow.

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_Circ Res._ published online February 16, 2006;
_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Isolation of rat ventricular myocytes

Male Wistar rats (250-300 g) were killed humanely by cervical dislocation following stunning, and the heart rapidly removed, in accordance with the UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986. The heart was mounted on a Langendorff apparatus and perfused retrogradely with a HEPES-based isolation solution containing (in mmol/L): 130 NaCl, 5.4 KCl, 0.4 NaH₂PO₄, 1.4 MgCl₂, 0.75 CaCl₂, 10 HEPES, 10 glucose, 20 taurine, and 10 creatine (pH 7.3 with NaOH). When the coronary circulation had cleared of blood, perfusion was continued with Ca-free isolation solution (in which CaCl₂ was replaced with 0.1 mmol/L EGTA) for 4 min, followed by perfusion for a further 16-18 min with isolation solution containing 0.8 mg/ml collagenase (type I; Worthington Biochemical, Lakewood, NJ), and 0.08 mg/ml protease (type XIV; Sigma, St. Louis, MO). The ventricles were then excised from the heart, minced, and the cells resuspended in isolation solution containing 0.075 mmol/L and then 0.75 mmol/L Ca to reduce Ca paradox.¹

Cells were next suspended in the experimental physiological salt solution (Tyrode) containing (in mmol/L): 135 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 10 glucose, set to pH 7.4 with NaOH. All experiments were performed at room temperature (~23°C).

Detubulation of rat ventricular myocytes

Detubulation was induced by osmotic shock as described previously.² Briefly, 1.5 mol/L formamide was added to the cell suspension for 15-20 min, before returning the cells to the standard solution. Detubulation occurs because of the osmotic shock produced by formamide withdrawal.
Electrophysiological recording

Myocytes were studied in a chamber mounted on the stage of an inverted microscope (Nikon Diaphot, Japan). Membrane potential and currents were recorded using the whole-cell configuration of the patch clamp technique. An Axopatch 200B (Axon Instruments, CA) amplifier was used, controlled by a Pentium PC connected via a Digidata 1322A A/D converter (Axon Instruments, CA), which was also used for data acquisition and analysis using pClamp software (Axon Instruments, CA). Signals were filtered at 2-10 kHz using an 8-pole Bessel low pass filter before digitization at 10-20 kHz and storage. In voltage clamp experiments, cell membrane capacitance was measured by integrating the capacitance current recorded during a 10 mV hyperpolarizing pulse from –80 mV.

$I_{Na}$ was measured in low [Na] external solution to reduce the size of $I_{Na}$ and therefore improve voltage control. A local perfusion system delivered the external solution which contained (in mmol/L): NaCl 20, CsCl 110, 4AP 5, MgCl$_2$ 0.5, HEPES 10, Glucose 10, CoCl$_2$ 2, pH set to 7.4 using CsOH. Low resistance (1.6 ± 0.1 MΩ, n=49) patch pipettes were used and the pipette solution contained (in mmol/L): CsCl 110, NaCl 10, MgCl$_2$ 0.5, Mg-ATP 5, EGTA 1, HEPES 10, GTPTris 0.4, set to pH 7.2 with CsOH. These solutions avoid contamination by overlapping ionic currents. However Co, which was used to block Ca current, also preferentially blocks TTX-resistant $I_{Na}$, compared to TTX-sensitive $I_{Na}$ (~30 and ~15% respectively at 2 mmol/L). We will, therefore, slightly overestimate the fraction of TTX-sensitive current obtained by fitting the TTX dose-response curve. To derive the proportion of current generated by each isoform at each sub-cellular location we therefore corrected the value of current density to account for the partial block of $I_{Na}$ by Co. Assuming similar block by Co at the t-tubule and surface membranes, this correction does not alter quantification of sub-cellular localization. Cell capacitance and series resistance (2.9 ± 0.2 MΩ, n=49) were compensated by 85-90%. Residual linear leak and capacitance were subtracted using a P/4 protocol. Current-voltage relationships were determined using test
pulses to voltages between –80 mV and +40 mV in 10 mV increments, from a holding potential of -120 mV, applied at a frequency of 0.2 Hz. $I_{Na}$ was also elicited by a test pulse to –60 mV or –10 mV from a holding potential of –120 mV at a frequency of 0.25 Hz. Activation curves were fitted by the Boltzmann equation, $a = 1/(1+\exp((V_m-V_{1/2})/k))$, where $a$ is the activation variable, $V_m$ is the membrane potential, $V_{1/2}$ is the potential at which the current is half activated and $k$ is the slope factor.

Action potentials were evoked by 2.5 ms current steps. Trains of pulses were applied at 0.33 Hz. Patch pipette resistance was typically 2-4 MΩ when filled with intracellular solution (in mmol/L): 130 K-glutamate, 9 KCl, 10 NaCl, 0.5 MgCl$_2$, 5 Mg-ATP, 0.5 EGTA, 10 HEPES, 0.4 GTPTris, set to pH 7.2 with KOH. The rate of rise of the action potential (dV/dt in V/s) was calculated by differentiation of the action potential using Origin software. Action potential amplitude was measured as the difference between resting membrane potential and the peak of the overshoot. Action potential duration was measured at 20, 50 and 90% repolarization (APD$_{20}$, APD$_{50}$, APD$_{90}$). In some experiments, action potential and cell shortening were recorded simultaneously.

$I_{Ca}$ was measured using Na- and K-free external and internal solutions to avoid contamination by overlapping ionic currents, and to allow us to use a physiological holding potential. A local perfusion system delivered the external solution which contained (in mmol/L): 5 4AP, 130 TEACl, 0.5 MgCl$_2$, 10 HEPES, 10 Glucose, 1 CaCl$_2$, pH set to 7.4 using TEAOH. Patch pipette resistance was typically 1.5-2.5 MΩ when filled with intracellular solution (in mmol/L): 110 CsCl, 20 TEACl, 0.5 MgCl$_2$, 5 Mg-ATP, 5 EGTA, 10 HEPES, 0.4 GTPTris, set to pH 7.2 with CsOH. $I_{Ca}$ was elicited by action potential waveforms. Trains of depolarizing pulses were applied at 0.1 Hz. $I_{Ca}$ was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse. Currents are expressed as current density (pA/pF). Time to peak $I_{Ca}$ was measured from the start of the depolarizing pulse. $I_{Ca}$ was also analyzed by integrating $I_{Ca}$ during the test
pulse to obtain total Ca influx during the pulse. Ca entry is expressed as cytosolic Ca concentration using estimates of surface to volume ratios for cardiac myocytes.\textsuperscript{7}

**Ca imaging**

Ca imaging was performed using a laser scanning inverted confocal microscope (LSM Pascal, Zeiss). Myocytes were loaded with 10 µmol/L Fluo-3 AM (Molecular Probes) for 30 min and then returned to either Tyrode solution or Tyrode solution plus 200 nmol/L TTX. Fluo-3 was excited at 488 nm and emitted fluorescence collected at wavelengths > 505 nm. The confocal slit aperture was set so that the confocal plane was < 1 µm with a \times 40 oil-immersion objective lens (Plan-Neofluar, n.a.=1.2, Zeiss). Transverse line scans were acquired at 2 ms intervals. 8 bit linescan images are presented as the original signal. Traces showing the time course of fluorescence are presented as a ratio of fluorescence/background fluorescence (F/F\textsubscript{0}). Image analysis was performed off-line using Zeiss LSM 5 Image Examiner Software V2.81 (Zeiss, Germany). The rate of rise of Ca transients recorded from the sub-sarcolemmal space (SS) and cell center (CC) was calculated by differentiation of F/F\textsubscript{0} using Origin software. Cells were electrically stimulated at 0.5 Hz via extracellular electrodes.

**Cell shortening recording**

Isolated cells were placed in an experimental chamber on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). Myocytes were illuminated with red light (>610 nm) to generate an image of the cell detected by a camera mounted on the microscope and displayed on a monitor. Cell length was monitored using an edge detection system (Crescent Electronics) as previously described,\textsuperscript{2} and the change in cell length during stimulation was used as an index of contractility. In field simulation experiments (figure 6C), cells were stimulated by external platinum electrodes at a frequency of 0.33 Hz.
Chemicals

All solutions were prepared using ultrapure water supplied by a Milli-Q system (Millipore, UK). All solution constituents were reagent grade and purchased from Sigma (St. Louis, MO).

Statistics

Data are presented as mean ± S.E.M. Paired or unpaired t-tests (two-tailed) were performed as appropriate. \( P<0.05 \) was taken as significant.

REFERENCES


