$I_{Ca(TTX)}$ Channels Are Distinct from Those Generating the Classical Cardiac Na^+ Current

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ABSTRACT The Na^+ current component $I_{Ca(TTX)}$ is functionally distinct from the main body of Na^+ current, I_{Na}. It was proposed that $I_{Ca(TTX)}$ channels are I_{Na} channels that were altered by bathing media containing Ca^{2+}, but no, or very little, Na^+. It is known that Na^+-free conditions are not required to demonstrate $I_{Ca(TTX)}$. We show here that Ca^{2+} is also not required. Whole-cell, tetrodotoxin-blockable currents from fresh adult rat ventricular cells in 65 mm Cs^+ and no Ca^{2+} were compared to those in 3 mM Ca^{2+} and no Cs^+ (i.e., $I_{Ca(TTX)}$). $I_{Ca(TTX)}$ parameters were shifted to more positive voltages than those for Cs^+. The Cs^+ conductance-voltage curve slope factor (mean, −4.68 mV; range, −3.63 to −5.72 mV, eight cells) is indistinguishable from that reported for $I_{Ca(TTX)}$ (mean, −4.49 mV; range, −3.95 to −5.49 mV). Cs^+ current and $I_{Ca(TTX)}$ time courses were superimposable after accounting for the voltage shift. Inactivation time constants as functions of potential for the Cs^+ current and $I_{Ca(TTX)}$ also superimposed after voltage shifting, as did the inactivation curves. Neither of the proposed conditions for conversion of I_{Na} into $I_{Ca(TTX)}$ channels is required to demonstrate $I_{Ca(TTX)}$. Moreover, we find that cardiac Na^+ (H1) channels expressed heterologously in HEK 293 cells are not converted to $I_{Ca(TTX)}$ channels by Na^+-free, Ca^{2+}-containing bathing media. The gating properties of the Na^+ current through H1 and those of Ca^{2+} current through H1 are identical. All observations are consistent with two non-interconvertable Na^+ channel populations: a larger that expresses little Ca^{2+} permeability and a smaller that is appreciably Ca^{2+}-permeable.

INTRODUCTION

We described a new Na^+ current component, $I_{Ca(TTX)}$, in rat ventricular myocytes that is functionally distinct from all other characterized Na^+ current components (Aggarwal et al., 1997). As compared with the main body of classical cardiac Na^+ current, I_{Na}, the channels generating $I_{Ca(TTX)}$ express (1) clearly different kinetics, with $I_{Ca(TTX)}$ activating and inactivating more slowly; (2) different voltage dependencies for activation and inactivation, with those for $I_{Ca(TTX)}$ both shifted in the negative direction and less steeply dependent on potential; and (3) different permeability properties, with $I_{Ca(TTX)}$ channels displaying substantial permeability for Ca^{2+} in contrast to I_{Na} channels which express very little. $I_{Ca(TTX)}$ channels are Na^+ channels as they are permeable to Na^+ even in the presence of appreciable concentrations of external Ca^{2+} and express the pharmacological profile of Na^+ rather than Ca^{2+} channels. $I_{Ca(TTX)}$ has been reported in a number of preparations (squid giant axons, Meves and Vogel, 1973; rat hippocampal CA1 cells, Akaike and Takahashi, 1992; guinea pig ventricular cells, Cole et al., 1997; Heubach et al., 2000; rat ventricular cells, Aggarwal et al., 1997; rat atrial cells, Chen-Izu, Shorofsky, Goldman and Balke, unpublished observations) and in both human atrial (Lemaire et al., 1995) and ventricular cells (Gaughan et al., 1999).

$I_{Ca(TTX)}$ has generally been attributed to a population of channels that are distinct from those generating the main body of classical Na^+ current, constituting either a post-translationally modified subset of the classical Na^+ channels or a distinct Na^+ channel isoform. In contrast to this interpretation, however, Cole et al. (1997) proposed that the $I_{Ca(TTX)}$ they described in guinea pig ventricular myocytes is generated by classical cardiac Na^+ channels whose gating and permeation properties have been reversibly altered by exposure to certain bathing media. The conditions specified for the proposed conversion of I_{Na} into $I_{Ca(TTX)}$ channels are: (1) the absence or near absence of external Na^+ (Na^+_o), and (2) the presence of external Ca^{2+} (Ca^{2+}_o). Cole et al. (1997) suggested that under these conditions Ca^{2+} ions would permeate classical Na^+ channels, and that this Ca^{2+} ion permeation would produce an alteration of Na^+ channel gating properties. We refer to this as the channel conversion proposal.

It is important to distinguish whether $I_{Ca(TTX)}$ is generated by a distinct population of channels that are always present or by the alteration of the properties of some channel population. Therefore, we examined the predictions of the channel conversion proposal to see if they are consistent with the experimental behavior of $I_{Ca(TTX)}$. If $I_{Ca(TTX)}$ is attributable to the conversion of classical Na^+ channels as proposed by Cole et al. (1997), then it must only be seen in little or no Na^+_o and in the presence of Ca^{2+}_o. We note that results already available in the literature clearly establish that the absence or near absence of Na^+_o is not required for the demonstration of $I_{Ca(TTX)}$. $I_{Ca(TTX)}$ is seen in the pres-

Received for publication 9 February 2001 and in final form 19 July 2001.

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0006-3495/01/11/2647/13 $2.00

ence of substantial concentrations of Na^+_o (Aggarwal et al., 1997; Akaike and Takahashi, 1992; Meves and Vogel, 1973). We show here that $I_{Ca(TTX)}$ can also be recorded in the complete absence of Ca^{2+} or any other permeant divalent ion. Neither of the conditions specified for the proposed conversion of I_{Na} into $I_{Ca(TTX)}$ channels are, in fact, required for the demonstration of $I_{Ca(TTX)}$. In addition, we were unable to demonstrate conversion of cardiac Na^+ channels expressed heterologously into $I_{Ca(TTX)}$ channels (as were Guatimosim et al., 2001, although they came to different conclusions; see Discussion). In no case could the gating characteristics of the current through H1 channels be converted to those of $I_{Ca(TTX)}$ by reducing or eliminating Na^+_o in the presence of Ca^{2+}_o.

Taken together, these observations are not consistent with the proposal that classical Na^+ channels are converted to Ca^{2+}-permeable channels by the experimental conditions. Rather, the data suggest that two distinct populations of Na^+ channels exist: a larger population that expresses little Ca^{2+} permeability and a smaller population that expresses substantial Ca^{2+} permeability.

A preliminary report of some of these results has been made (Chen-Izu et al., 2001).

MATERIALS AND METHODS

Ventricular cell preparation

Two- to 4-month-old Sprague-Dawley or Wistar rats (200–300 g) were anesthetized with sodium pentobarbitol (170 mg kg^{-1} injected intraperitoneally). The hearts were removed from the animals via a midline thoracotomy, and single ventricular cells were obtained by an enzymatic dispersion technique described in detail previously (Balke and Wier, 1992). Isolated cells were harvested and stored in a modified Tyrode solution containing (in mM) NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 0.5; HEPES, 10; and glucose, 10 (pH adjusted to 7.25 with NaOH). The cells were studied within 10 h of isolation.

Culture of stably transfected HEK 293 cells

HEK 293 cells stably transfected with the gene encoding for the human heart Na^+ channel hH1, as described in Kambouris et al. (2000), were kindly provided by Dr. E. Marbán. Cells were cultured in DMEM (Cell-Gro; Mediatech, Washington, DC) supplemented with 10% FBS, 1X penicillin-streptomycin, at pH 7.4, and 400 μg/mL geneticin sulfate (G 418; Life Technologies, Rockville, MD) at 37°C in a 5% CO_2-humidified incubator. Cells were maintained in selection media.

Solutions

For experiments on rat ventricular cells, the bathing solution contained either (in mM): tetraethylammonium chloride (TEA-Cl), 145; $MgCl_2$, 1; $CaCl_2$, 3; HEPES, 10; and glucose, 10 (pH adjusted to 7.3 with TEA-OH) for recording Ca^{2+} currents; or TEA-Cl, 75; $MgCl_2$, 1; CsCl, 65; HEPES, 10; glucose, 10 (pH adjusted to 7.3 with CsOH) for recording Cs^+ currents. A few experiments were also done in a bathing solution containing (in mM): TEA-Cl, 110; MgCl, 20; HEPES, 10; and glucose, 10 (pH adjusted to 7.3 with TEA-OH). The pipette (internal) solution used in all ventricular cell experiments contained (mM): TEA-OH, 70; CsOH, 70; glutamic acid, 140; HEPES, 10; EGTA, 5; $MgCl_2$, 0.33; Mg-ATP, 4 (pH adjusted to 7.2 with CsOH; ~25 mM added). Twenty micromolar of $LaCl_3$ was used in all ventricular cell experiments to suppress L-type Ca^{2+} currents.

For experiments on HEK 293 cells, the bathing solution contained (in mM): TEA-Cl, 140; $MgCl_2$, 1; HEPES, 10; glucose, 10 (pH adjusted to 7.3 with TEA-OH) and either with or without 1 mM NaCl and/or 3mM $CaCl_2$ as indicated. The pipette solution contained (in mM): TEA-Cl, 20; glutamic acid, 120; CsOH, 120; $MgCl_2$, 0.33; Mg-ATP, 4; HEPES, 10; and EGTA, 5 (pH adjusted to 7.3 with CsOH).

Electrical recording

Membrane currents were recorded using the whole-cell configuration of the patch clamp technique. Small aliquots of either ventricular myocytes or HEK 293 cells were placed in a perfusion chamber mounted on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan). The chamber was designed to permit rapid exchange of the bathing medium. Cells were continuously superfused (rate of 2–3 ml·min^{-1}) with modified Tyrode solution during seal formation and the establishment of whole-cell recording conditions. The bathing solution was then switched to one of the external recording solutions described above. Data acquisition began at least 5 min after break-in. All experiments were performed at room temperature (21–23°C).

Glass suction pipettes were fabricated from borosilicate capillary glass using a Flaming-Brown type micropipette puller (model P-87; Sutter Instrument Co., Novato, CA). When filled with pipette solution, electrodes had resistances of 1.5–2.5 MΩ. Potential differences between the bath and pipette solutions were nulled before seal formation. After formation of a seal (>5 GΩ), a suction pulse was applied to rupture the membrane and allow access to the cell interior.

Whole-cell currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). Voltage clamp protocols were generated and currents recorded using a DigiData 1200B analog to digital converter (Axon Instruments) under computer control using pCLAMP 8 software (Axon Instruments). Currents were filtered at 5 kHz using a 4-pole Bessel filter and digitized at 10 kHz. Series resistance compensation was used throughout. Capacitative currents were determined by application of a 30 mV hyperpolarizing voltage step from a holding potential of −100 mV. The area under the capacity transient was integrated and used to calculate cell capacitance for each experiment. Mean ventricular cell capacitance was 79 ± 22 pF (mean ± S.D.; 53 cells). All kinetic analysis was performed on currents isolated using tetrodotoxin (TTX) subtraction. The holding potential was −100 mV for all experiments.

RESULTS

Observations on rat ventricular cells

The aim of these experiments is to determine whether the permeation of Ca^{2+} or any other divalent ion is, in fact, a necessary condition for the existence of $I_{Ca(TTX)}$ channels as required by the channel conversion proposal. Therefore, we asked whether $I_{Ca(TTX)}$ could be recorded in the absence of permeant divalent ions. An effective way to answer this question is to identify a monovalent cation which is substantially permeant through $I_{Ca(TTX)}$ channels, but to which classical Na^+ channels are only sparingly permeable. In this way, any $I_{Ca(TTX)}$ should be directly observable in the current records. In the presence of only ions to which classical Na^+ channels are appre-

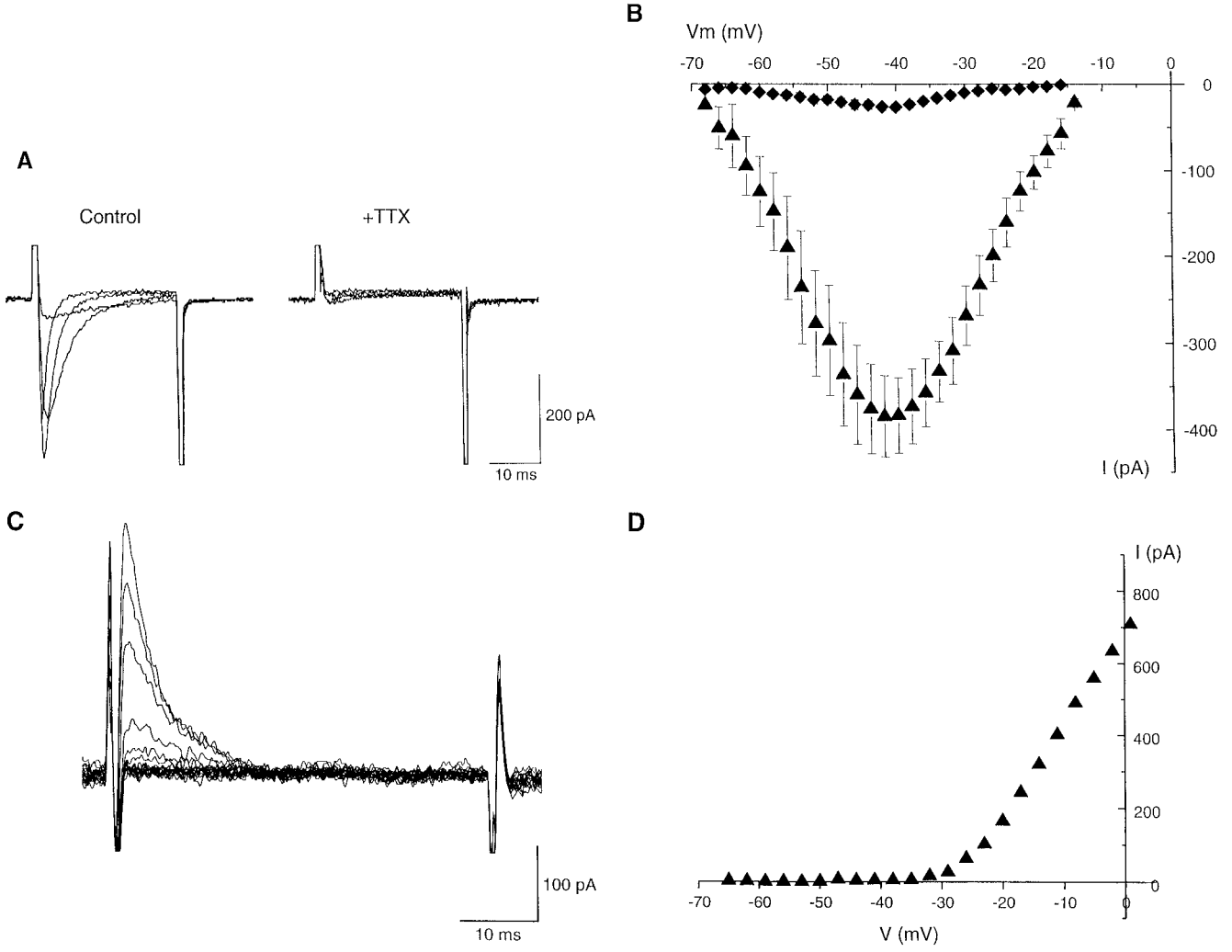

FIGURE 1 Current records and current-voltage relations from rat ventricular cells. *(A)* Whole-cell patch clamp records from a cell in 65 mM Cs^+_o and no Ca^{2+}_o. The left panel presents currents recorded on steps from a holding potential of −100 mV to −66, −56, −46, and −36 mV. The right panel presents current records from this same cell at these same potentials, but now in the presence of 10 μM TTX. *(B)* Collected peak current-voltage values for inward Cs^+ currents. All conditions as for *A*. ▲ indicate means and error bars indicate S.D. for values obtained in the absence of TTX. ♦ and error bars indicate corresponding values in the presence of 10 μM TTX. Means of 11 cells. *(C)* TTX-subtracted current records obtained in the absence of both Cs^+_o and Ca^{2+}_o. Currents recorded on steps from the holding potential of −100 mV to −65 to −17 mV in 3 mV increments. The bathing medium contained 20 mM Mg^{2+}. No inward current is seen, but robust outward Cs^+ currents are recorded. *(D)* Peak current-voltage relation for the cell of *(C)*. Clear outward current is seen positive to approximately −30 mV.

ciably permeable, $I_{Ca(TTX)}$ might be difficult to detect because of the substantially higher density of Na^+ as compared with $I_{Ca(TTX)}$ channels. Therefore, we examined the properties of the current carried by Cs^+. The clear outward $I_{Ca(TTX)}$ current reported by Aggarwal et al. (1997; their Fig. 5) was most likely carried by Cs^+ under their experimental conditions. However, the main body of classical cardiac Na^+ channels expresses little Cs^+ permeability (Sheets et al., 1987; Kurata et al., 1999) as do cardiac Na^+ (H1) channels expressed heterologously (Chen et al., 1997; Townsend et al., 1997; Grant et al., 1999; Nuss and Marbán, 1999; Guatimosim et al., 2001).

Rat ventricular myocytes express a TTX-blockable Cs^+ current

In the presence of 65 mM Cs^+_o, but absence of Ca^{2+}_o, clear inward currents are seen (Fig. 1 *A*, *left*). These inward currents are blocked entirely, or nearly so, by 10 μM TTX (Fig. 1 *A*, *right*). Collected results are presented in Fig. 1 *B*. The filled triangles indicate the peak current voltage relation recorded in 65 mM Cs^+_o and no Ca^{2+}_o (mean of 11 cells), and the filled diamonds indicate the corresponding values in the presence of 10 μM TTX. All of the rest of the results in this paper were obtained on currents isolated using TTX subtraction.

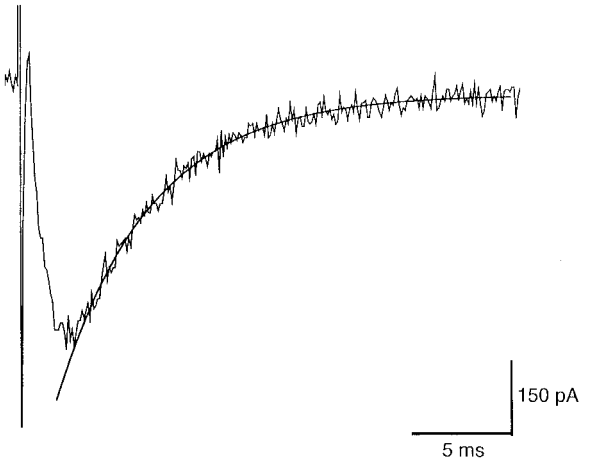

FIGURE 2 The TTX-subtracted Cs^+ current displays only a single inactivation relaxation. A TTX-subtracted current record from a rat ventricular cell on a step from −100 mV to −56 mV is shown. The superimposed smooth curve is a best-fit single exponential description of the inactivation time course (τ_h of 4.97 ms). An additional inactivation component with reliable parameters could not be defined. Bathing solution contained 65 mM Cs^+_o and no Ca^{2+}_o.

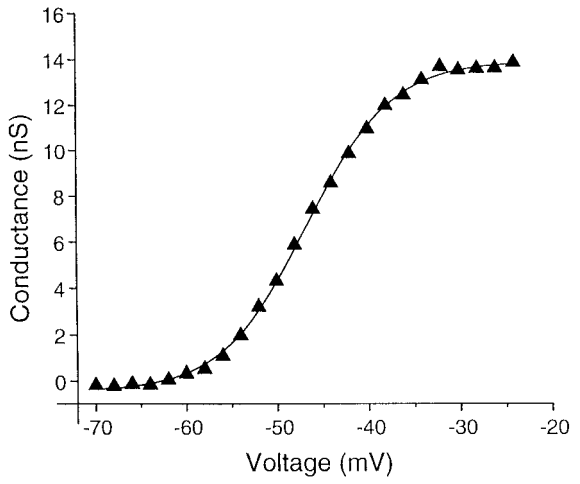

FIGURE 3 Peak conductance-voltage relation for a rat ventricular cell bathed in 65 mM Cs^+_o and no Ca^{2+}_o. Peak TTX-subtracted current values were converted to conductance (see text) and plotted as a function of potential (▲). The smooth curve is a best-fit single Boltzmann function (see text) with a $V_{1/2} = -46.6$ mV, $k = -4.71$ mV, and $g_{max} = 13.95$ nS. An additional Boltzmann component with reliable parameters could not be defined.

The TTX-blockable current seen under these conditions is carried by Cs^+. Fig. 1 *C* shows TTX-subtracted currents recorded in the absence of both Cs^+_o and Ca^{2+}_o. The bathing medium contained 20 mM Mg^{2+} (and TEA). The pipette solution always contained 95 mm Cs^+. Currents recorded on steps from a holding potential of −100 mV to potentials of −65 mV to −17 mV in 3-mV increments are shown. No inward current is seen under those conditions. Positive to approximately −30 mV, clear outward currents are seen. Virtually all of the TTX-blockable current must be attributed to Cs^+. The peak current voltage relation for this experiment is illustrated in Fig. 1 *D*. The TTX-blockable Cs^+ current is substantial.

The TTX-blockable Cs^+ current is generated by a single population of channels

Fig. 2 presents an inward Cs^+ current record obtained using TTX subtraction. A single exponential with a time constant, τ_h, of 4.97 ms has been fitted to the decay of the current (*smooth curve*). Attempts to detect an additional inactivation relaxation could not define an additional exponential component with reliable parameters. Inactivation of the TTX-blockable Cs^+ current proceeds as a single exponential, suggesting that it is generated by a single population of channels.

Accordingly, the activation curve for the TTX-blockable Cs^+ current is also well described by only a single Boltzmann function. The filled triangles in Fig. 3 indicate Cs^+ peak conductance values. Currents were first isolated using TTX subtraction then converted to conductance using the expression

$$I_i = g(V - V_{rev}),$$

where I_i is peak current, g is peak conductance, V is potential, and V_{rev} is the null potential. The smooth curve of Fig. 3 is a best fit single Boltzmann function given by

$$g(V) = g_{max}/(1 + \exp((V - V_{1/2})/k)),$$

with g_{max} (the maximum conductance) of 13.95 nS, $V_{1/2}$ (the potential at which $g(V) = 1/2g_{max}$) of −46.6 mV and k (a slope factor) of −4.71 mV. As was true for the inactivation time course, attempts to detect an additional component could not define an additional Boltzmann function with reliable parameters. Similar results were obtained in seven additional cells. These results indicate that the TTX-blockable Cs^+ current is generated by a single population of channels. In the next section, we show that the single population is $I_{Ca(TTX)}$.

The TTX-blockable Cs^+ current is generated by $I_{Ca(TTX)}$ channels

Aggarwal et al. (1997) found that virtually all of the TTX-blockable Ca^{2+} current they observed in rat ventricular cells was generated by $I_{Ca(TTX)}$ channels. In the presence of both Na^+_o and Ca^{2+}_o, inactivation of the total TTX-blockable current developed as the sum of two exponentials, one arising from classical Na^+ channels and the other from $I_{Ca(TTX)}$ channels. However, in the presence of Ca^{2+}_o alone, only the slower ($I_{Ca(TTX)}$) inactivation relaxation was seen. Similarly, in the presence of both Na^+_o and Ca^{2+}_o, the activation, $g(V)$, curve for the total TTX-blockable conductance consisted of the sum of two distinct Boltzmann functions, whereas only one of these Boltzmann functions was

seen in the presence of Ca^{2+}_o only. The TTX-blockable Ca^{2+} current is generated substantially by a single population of channels with gating properties that are clearly distinct from those of the classical I_{Na}, i.e., by $I_{Ca(TTX)}$ channels. Hence, to establish that the TTX-blockable Cs^+ current arises from $I_{Ca(TTX)}$ channels, it is only necessary to show that the TTX-blockable Cs^+ current has the gating properties of the TTX-blockable Ca^{2+} current.

The value of the slope factor, k, fitted to the Cs^+ $g(V)$ data of Fig. 3 agrees closely with the values reported for $I_{Ca(TTX)}$. Aggarwal et al. (1997) reported a mean k of -4.49 mV with a range of -3.95 to -5.49 mV. In eight cells with Cs^+ as the current carrier, we obtained a mean k value of -4.68 mV with a range of -3.63 to -5.72 mV in close agreement. The slope of the Cs^+ activation curve is indistinguishable from that of the $I_{Ca(TTX)}$ activation curve. The $V_{1/2}$ values, however, cannot be directly compared because of the change in surface potential produced on adding or removing Ca^{2+}_o. Note that the slope factor of the activation curve for the classical cardiac Na^+ channels was found to be clearly different from that of $I_{Ca(TTX)}$ (mean of -2.31 mV in 1 mM Na_o and 3 mM Ca_o; Aggarwal et al., 1997).

Fig. 4 (*left column*) presents a series of TTX-subtracted current records, obtained from the same cell recorded at the indicated potentials. These records were obtained in the presence of 65 mM Cs^+_o and no Ca^{2+}_o. The middle column presents TTX-subtracted records from the same cell, but now in the presence of 3 mM Ca^{2+}_o and no Cs^+_o. As indicated, the Ca^{2+}_o and Cs^+_o records for each row were not recorded at the same potentials. Those recorded in Ca^{2+}_o are all shifted to potentials 10 mV more positive than the corresponding records obtained in Cs^+_o because of the difference in surface potentials. The right column presents these two sets of records superimposed with the records in Ca^{2+}_o scaled so that their peaks match those in Cs^+_o. The current time courses in Ca^{2+}_o and Cs^+_o are the same. Because the current in Ca^{2+}_o is $I_{Ca(TTX)}$, the current in Cs^+_o must also be generated chiefly by $I_{Ca(TTX)}$ channels. If classical cardiac Na^+ channels expressed an appreciable Cs^+ permeability, then the current time courses in Cs^+ and Ca^{2+} could not be the same because the kinetics of I_{Na} and $I_{Ca(TTX)}$ are clearly different over this potential range (Aggarwal et al., 1997).

Fig. 5 *A* presents collected τ_h values as a function of potential. The filled triangles indicate τ_h values obtained in the presence of 65 mM Cs^+_o and no Ca^{2+}_o (means of eight cells), and the open circles indicate the values obtained in the presence of 3 mM Ca^{2+}_o and no Cs^+_o (means of six cells). In Fig. 5 *B*, the $\tau_h(V)$ obtained in Ca^{2+}_o has been shifted 8.5 mV to the left along the voltage axis. The two $\tau_h(V)$ functions superimpose, differing only by the change in surface potential produced by raising Ca^{2+}_o. Again, these results indicate that the TTX-blockable Cs^+ current is generated by $I_{Ca(TTX)}$ channels.

Fig. 6 *A* presents two inactivation curves, both from the same cell. Potential was stepped from a holding potential of -100 mV to various conditioning pulse levels. After 800 ms, at the conditioning level, potential was first returned to the holding potential for 2 ms, then, stepped to either -50 mV (filled triangles) or -30 mV (open circles), and the peak current during this test step was determined. Peak currents, normalized to the value seen at -110 mV, are shown as a function of conditioning potential. Filled triangles indicate the values, obtained in 65 mM Cs^+_o and no Ca^{2+}_o, and the open circles indicate those obtained in 3 mM Ca^{2+}_o and no Cs^+_o. The smooth curves are best fits to the expression

$$I/I_{110}(V) = (1 + \exp((V - V')/k'))^{-1},$$

where V' is the potential at which $I/I_{110} = ½$, and k' is a slope factor.

The V' values are clearly different in the two bathing media, with that in Ca^{2+}_o (-72.9 mV) shifted to the right along the voltage axis compared with that in Cs^+_o (-80.3 mV). However, the two k' values are essentially the same, 5.09 mV in Ca^{2+}_o and 4.65 mV in Cs^+_o. The two steady-state inactivation curves are the same with that in Ca^{2+}_o just translated along the voltage axis because of the change in surface potential. This is shown in Fig. 6 *B*, which presents the two steady-state inactivation curves now with that in Ca^{2+}_o, shifted 8.5 mV to the left along the voltage axis. Similar results were obtained in four additional cells.

Summary of results on rat ventricular cells

$I_{Ca(TTX)}$ channels are present, functional, and conducting even in the complete absence of Ca^{2+} or any other permeant divalent cation. These findings are not consistent with the channel conversion proposal. $I_{Ca(TTX)}$ channels are not called into existence by the permeation of divalent ions. They are present even when the current is carried entirely by monovalent ions. These results also demonstrate that the absence or near absence of all alkali metal ions is not required for the existence of $I_{Ca(TTX)}$ channels. Robust $I_{Ca(TTX)}$ is seen in the presence of 65 mM external and 95 mM internal Cs^+. Although Cs^+ is not Na^+, K^+, another alkali metal ion, can be substituted for Na^+ with no effect on Na^+ channel gating properties (Chandler and Meves, 1965).

These data also demonstrate that the Cs^+ permeability of classical cardiac Na^+ channels is very much less than that of $I_{Ca(TTX)}$ channels. If the Cs^+ permeability were comparable for these two channel types, then the TTX-blockable Cs^+ current should have largely or entirely displayed the gating properties of the classical I_{Na} rather than those of $I_{Ca(TTX)}$, because of the much higher density of classical Na^+ channels. This result is consistent with the reports of little Cs^+ permeability for H1 expressed heterologously (Chen et al.,

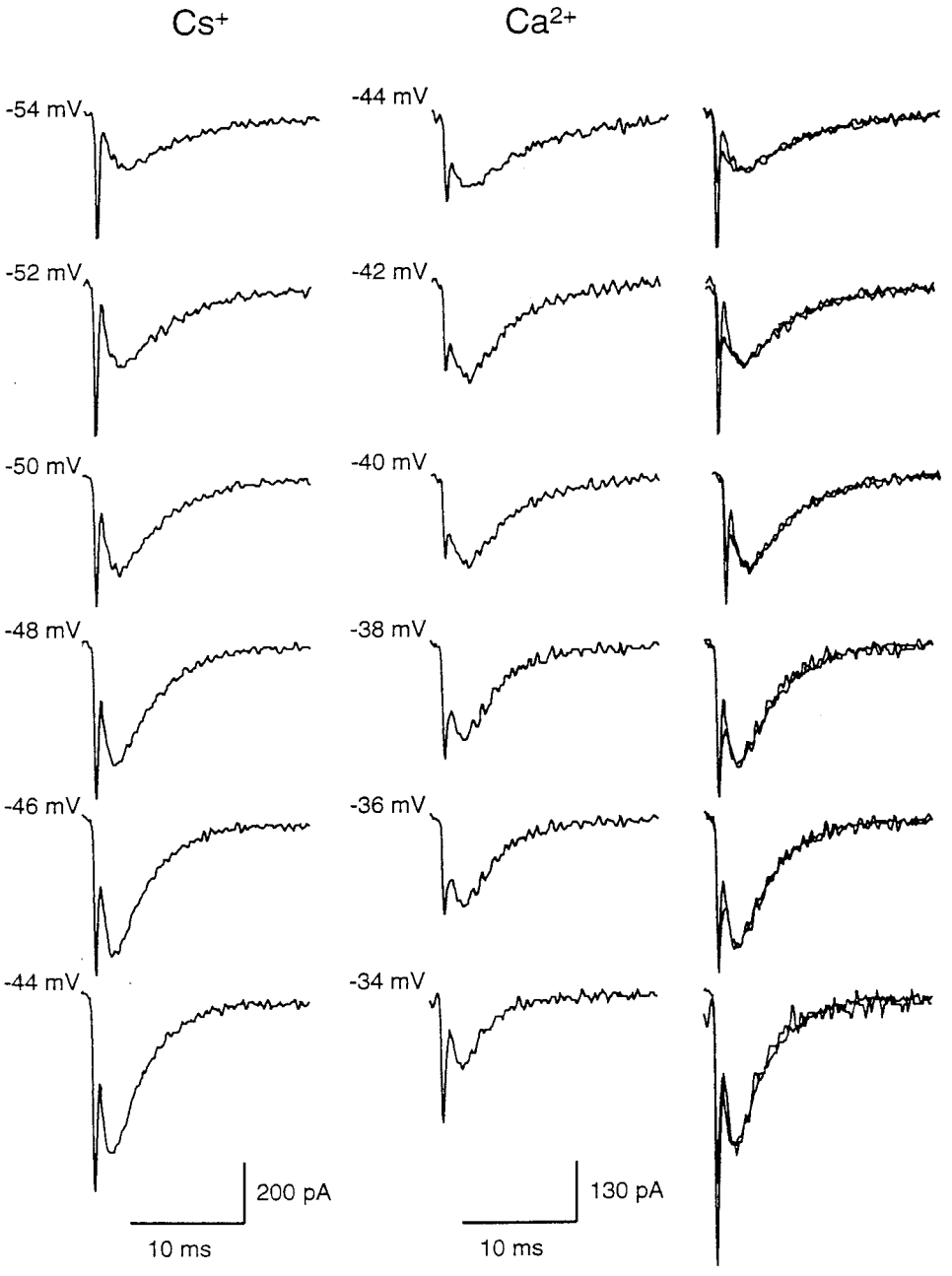

FIGURE 4 Current time course in Cs^+_o and in Ca^{2+}_o in a rat ventricular cell. The left column presents TTX-subtracted currents, all from the same cell recorded on a step from the holding potential of −100 mV to the indicated potentials. Bath contained 65 mM Cs^+ and no Ca^{2+}. The middle column presents TTX-subtracted current records from this same cell now in 3 mM Ca^{2+}_o and no Cs^+_o. Holding potential was again −100 mV but, as indicated, the potentials stepped to are all 10 mV more positive than the corresponding Cs^+ current records for each row. The right column again presents these records now superimposed. The records in Ca^{2+}_o have all been scaled so that their peak values are the same as those for the corresponding records in Cs^+_o. The current time courses are essentially identical in the two bathing media. Scale: 200 pA (*left column*), 130 pA (*middle column*), 10 ms.

1997; Townsend et al., 1997; Grant et al., 1999; Nuss and Marbán, 1999; Guatimosim et al., 2001) and constitutes another new finding of this paper: specifically, that the permeation properties of $I_{Ca(TTX)}$ channels differ from those of classical Na^+ channels in including a substantially higher permeability for Cs^+ as well as a substantially higher permeability for Ca^{2+}. With regard to permeation, $I_{Ca(TTX)}$ channels cannot be viewed simply as classical Na^+ channels through which some Ca^{2+} permeates in the absence of Na^+; rather, they have a distinct selectivity, including that for monovalent ions.

The results presented above and other available information (see Discussion) indicate that the predictions of the channel conversion proposal are not consistent with the

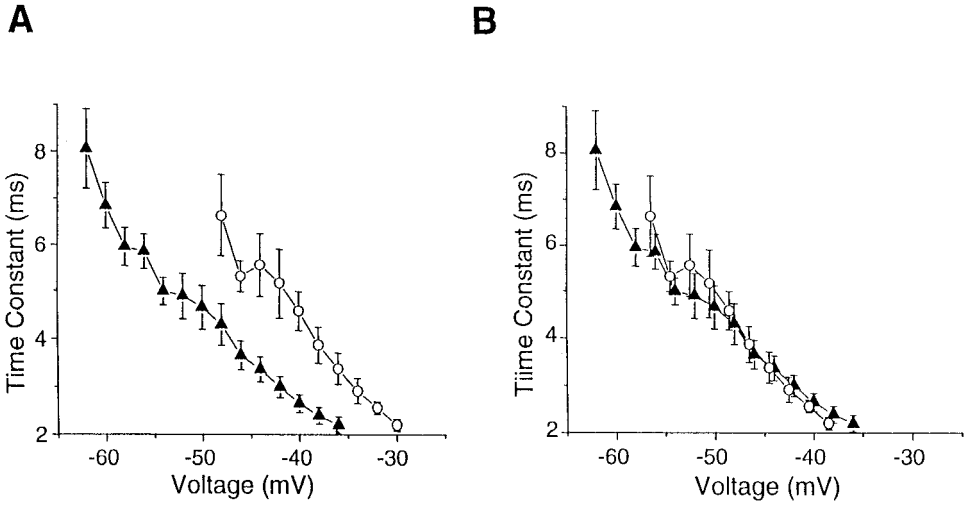

FIGURE 5 $\tau_h(V)$ in Cs^+_o and in Ca^{2+}_o in rat ventricular cells. *(A)* Time constant of inactivation, τ_h, as a function of potential, $\tau_h(V)$, in 65 mM Cs^+_o and no Ca^{2+}_o (▲) and in 3 mM Ca^{2+}_o and no Cs^+ (○). In each case, symbols indicate mean values and error bars indicate standard deviation (S.D.). Means of eight cells in Cs^+_o and six cells in Ca^{2+}_o. *(B)* The same data shown in *(A)*, but now with the $\tau_h(V)$ in Ca^{2+}_o shifted 8.5 mV to the left along the voltage axis. The two $\tau_h(V)$ functions are seen to superimpose.

experimental behavior of $I_{Ca(TTX)}$. In the next section, we show that Na^+ channels, expressed heterologously, are not converted to $I_{Ca(TTX)}$ channels.

Observations on HEK 293 cells stably transfected with H1

The conversion proposal also predicts that the cardiac Na^+ channel, H1, expressed heterologously, ought to display altered gating properties when exposed to Na^+_o-free, Ca^{2+}_o-containing bathing media. We therefore compared the gating properties of the current carried by Na^+ with that carried by Ca^{2+} through H1 channels stably transfected in HEK 293 cells.

Fig. 7 presents TTX-subtracted current records from a single transfected HEK 293 cell, recorded at the indicated potentials. The left column shows records obtained in the presence of 1 mM Na^+_o but in the complete absence of Ca^{2+}_o. They reflect just Na^+ current through normal, unconverted Na^+ channels. The middle column presents cur-

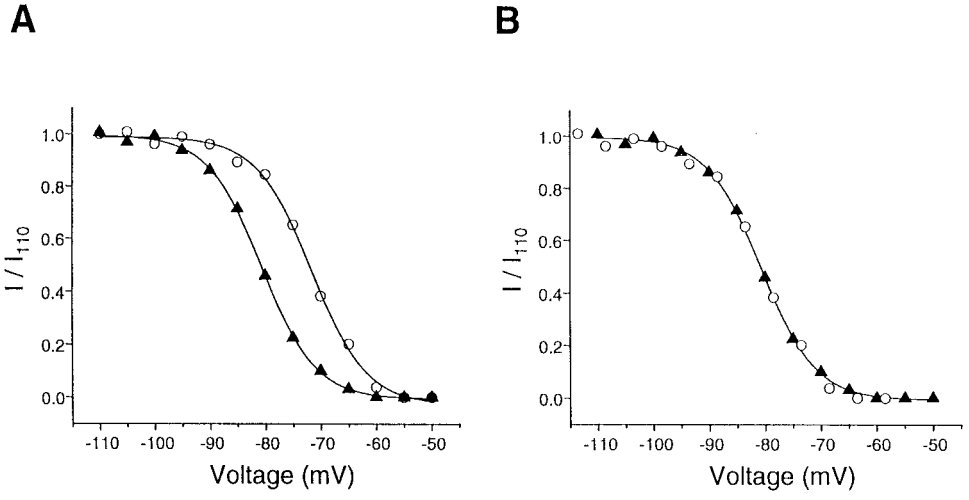

FIGURE 6 Inactivation curves in Cs^+_o and in Ca^{2+}_o in a rat ventricular cell. *(A)* Peak (TTX-subtracted) current values from a single cell during a test step to −50 mV (▲) or −30 mV (○) are shown. Each test step was preceded by an 800-ms conditioning step to one of the indicated potentials and a 2 ms return to the −100-mV holding potential. Peak currents are normalized to the value at −110 mV and shown as a function of conditioning potential. Determinations were in 65 mM Cs^+_o and no Ca^{2+}_o (▲) or 3 mM Ca^{2+}_o and no Cs^+_o (○). Smooth curves are best-fit single Boltzmann functions (see text) with V of −80.3 mV and k of 4.65 mV (▲) and V of −72.9 mV and k of 5.09 mV (○). *(B)* The same data shown in *A*, but now with the inactivation curve in Ca^{2+}_o shifted 8.5 mV to the left along the voltage axis. The two inactivation curves superimpose.

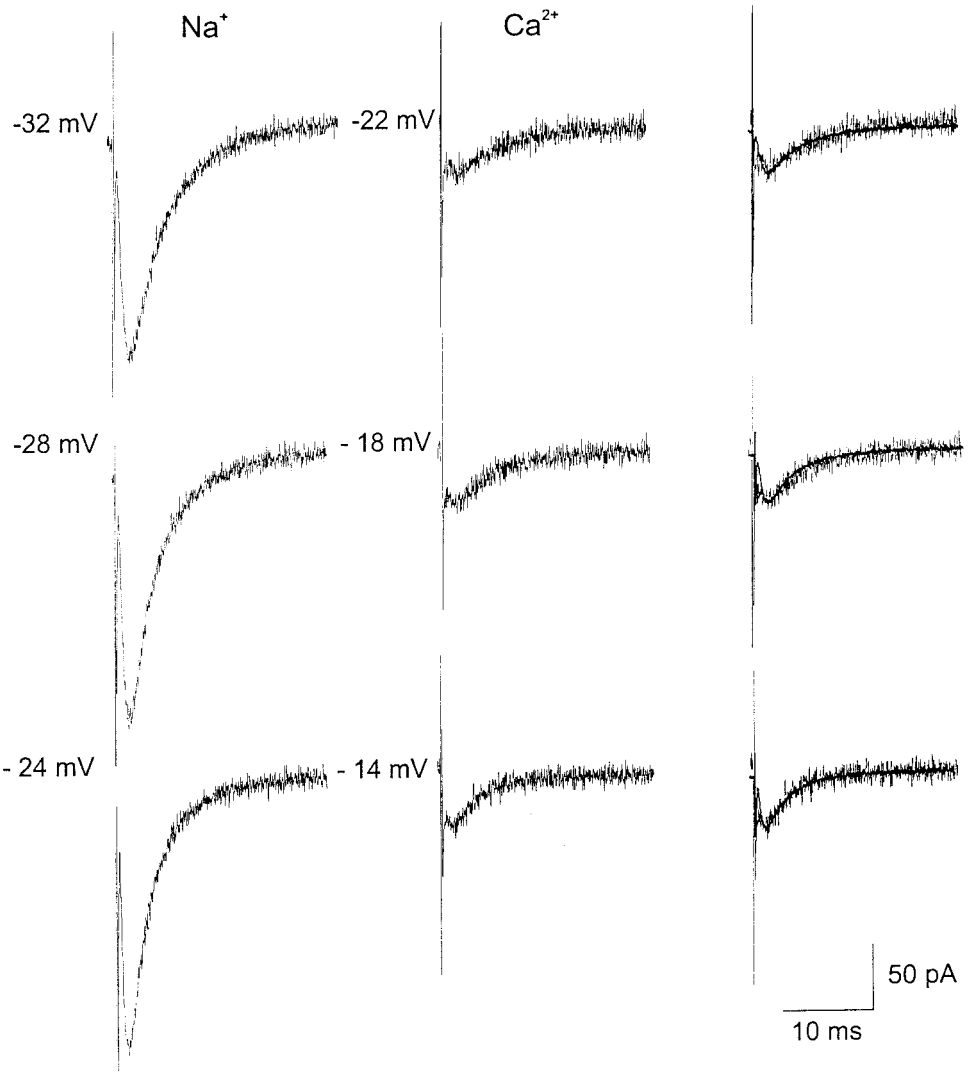

FIGURE 7 Whole-cell patch clamp current records from an HEK 293 cell stably transfected with the cardiac Na^+ channel, H1. The left column presents TTX-subtracted currents, all from the same cell, recorded on steps from the holding potential of −100 mV to the indicated potentials. Bath contained 1 mM Na^+ and no Ca^{2+}. The middle column presents TTX-subtracted current records from this same cell now in 3 mM $Ca^{2+}{}_o$ and no $Na^+{}_o$. As indicated, the potentials stepped to are all 10 mV more positive than the corresponding Na^+ current records for each row. The right column again presents these records, now superimposed. The records in $Na^+{}_o$ have all been scaled down so that their peaks match those of the corresponding records in $Ca^{2+}{}_o$. The current time courses are essentially identical in the two bathing media. Channel conversion did not occur. Scale: 50 pA, 10 ms.

rents in the presence of 3 mM $Ca^{2+}{}_o$, but in the complete absence of $Na^+{}_o$, that is under the experimental conditions for channel conversion. These records ought to reflect $I_{Ca(TTX)}$ if conversion does, in fact, occur. As indicated, the $Ca^{2+}{}_o$ and $Na^+{}_o$ records for each row were not recorded at the same potentials. Those in $Ca^{2+}{}_o$ are all shifted to potentials 10 mV more positive than the corresponding $Na^+{}_o$ records because of the difference in surface potentials.

The right column presents these two sets of records superimposed with the records in $Na^+{}_o$ scaled down so that their peaks match those in $Ca^{2+}{}_o$. The current time courses are identical at each voltage. Similar results were obtained in two additional cells. The small, TTX-blockable Ca^{2+} current through H1 channels is not $I_{Ca(TTX)}$ because it displays the gating properties of I_{Na}. It is a small Ca^{2+} permeation through normal, unconverted Na^+ channels. Channel conversion did not occur. Guatimosim et al., (2001), also working on HEK 293 transfected with H1, obtained identical results. Guatimosim et al. (2001) did not interpret their results as we have here, as they did not allow for the very different surface potentials produced by the different bathing media they used (see Discussion).

Both I_{Na} and $I_{Ca(TTX)}$ can be recorded simultaneously in native cells (Meves and Vogel, 1973; Akaike and Takahashi, 1992; Aggarwal et al., 1997). If H1 could be shown to simultaneously express two functionally distinct current

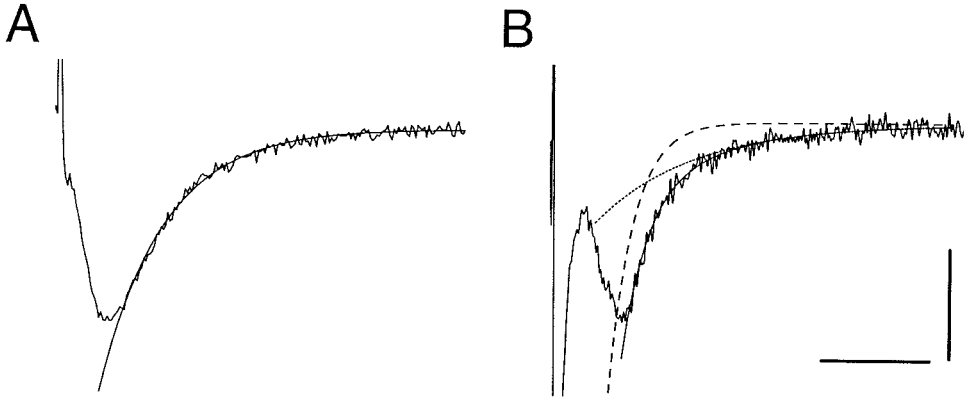

FIGURE 8 (*A*) A TTX-subtracted current record from a transfected HEK 293 cell on a step from −100mV to −40 mV. Bath contained 1 mM Na^+_o and 3 mM Ca^{2+}_o. The superimposed smooth curve is a best-fit single exponential description of the inactivation time course (τ_h = 3.02 ms). An additional inactivation component with reliable parameters could not be defined. (*B*) A TTX-subtracted current record from a rat ventricular myocyte on a step from −100 mV to −46 mV. Bath again contained 1 mM Na^+_o and 3 mM Ca^{2+}_o. In ventricular cells, under these conditions, inactivation develops as the sum of two relaxations (*smooth curve*) with τ_h values of 1.15 ms (*dashed curve*) and 4.58 ms (*dotted curve*). Scale: 170 pA (*A*), 400 pA (*B*), 5 ms.

components, then clear support for conversion would be provided. To test this possibility, we examined currents through H1 in the presence of both 1mM Na^+_o and 3mM Ca^{2+}_o, conditions under which ventricular cells express both I_{Na} and $I_{Ca(TTX)}$ (Aggarwal et al., 1997).

Fig. 8 *A* presents a TTX-subtracted current record obtained from a transfected HEK 293 cell in the presence of 1 mM Na_o and 3 mM Ca_o. A single exponential with a τ_h of 3.02 ms has been fitted to the decay of the current (*smooth curve*). Attempts to detect an additional inactivation relaxation could not define an additional exponential component with reliable parameters. Similar results were obtained in five additional cells. H1 channels express only a single kinetic component under conditions where ventricular myocytes display two distinct components. Fig. 8 *B* presents just such a TTX-subtracted current record obtained from a ventricular myocyte also in the presence of 1 mM Na^+_o and 3 mM Ca^{2+}_o. This record is from a cell studied by Aggarwal et al. (1997). Inactivation develops as two clear kinetic components. The smooth curve is a two exponential fit to the current decay with τ_h values of 1.15 ms (*dashed curve*) and 4.58 ms (*dotted curve*).

The single current component through H1 is carried largely by Na^+. Fig. 9 presents TTX-subtracted current records, all from the same transfected HEK 293 cell, recorded at the indicated potentials. The records in the left column were obtained in 1 mM Na^+_o plus 3 mM Ca^{2+}_o. The middle column presents records in the presence of 3 mM Ca^{2+}_o, but no Na^+_o. The current carried by Ca^{2+} in Fig. 9 ranges from ~13% to ~17.5% of that seen in the presence of Na^+_o. Note that the fraction of the inward current carried by Na^+ in the presence of both ions is likely to be larger than these values indicate. Ca^{2+}_o blocks classical Na^+ channels in a voltage-dependent manner (Yamamoto et al., 1984), and Na^+ and Ca^{2+} ions compete for access to the channel.

The right column of Fig. 9 presents the currents in Na^+_o plus Ca^{2+}_o superimposed with currents in Ca^{2+}_o only. Currents in Na^+_o plus Ca^{2+}_o have been scaled down so that their peaks match those in Ca^{2+}_o only. The current time courses are the same at each voltage. Again, the small Ca^{2+} current through H1 has identical gating properties as those of I_{Na} and cannot be identified as $I_{Ca(TTX)}$. Removing Na^+_o does not alter the gating properties of cardiac Na^+ channels, and conversion does not occur.

DISCUSSION

This paper asks whether $I_{Ca(TTX)}$ can be reasonably attributed to classical Na^+ channels whose properties have been reversibly altered by exposure to certain bathing media. The available evidence bearing on the question is summarized below. It is shown that the predictions of the channel conversion proposal cannot be experimentally verified and that there are experimental results not obviously reconcilable with conversion as proposed. The available evidence is, however, fully consistent with the idea that $I_{Ca(TTX)}$ is generated by a distinct population of channels that are always present.

$I_{Ca(TTX)}$ is seen in the presence of appreciable Na^+_o and in the absence of Ca^{2+}_o or any other permeant divalent ions

The two requirements of the conversion proposal are that $I_{Ca(TTX)}$ can be observed only in the absence or near absence of extracellular Na^+ and only in the presence of a permeant divalent ion. Neither of these conditions are required to observe $I_{Ca(TTX)}$. $I_{Ca(TTX)}$ has been seen in rat ventricular cells in the presence of 1 mM Na^+_o (Aggarwal et al., 1997), in rat hippocampal cells in the presence of 10 mM Na^+_o

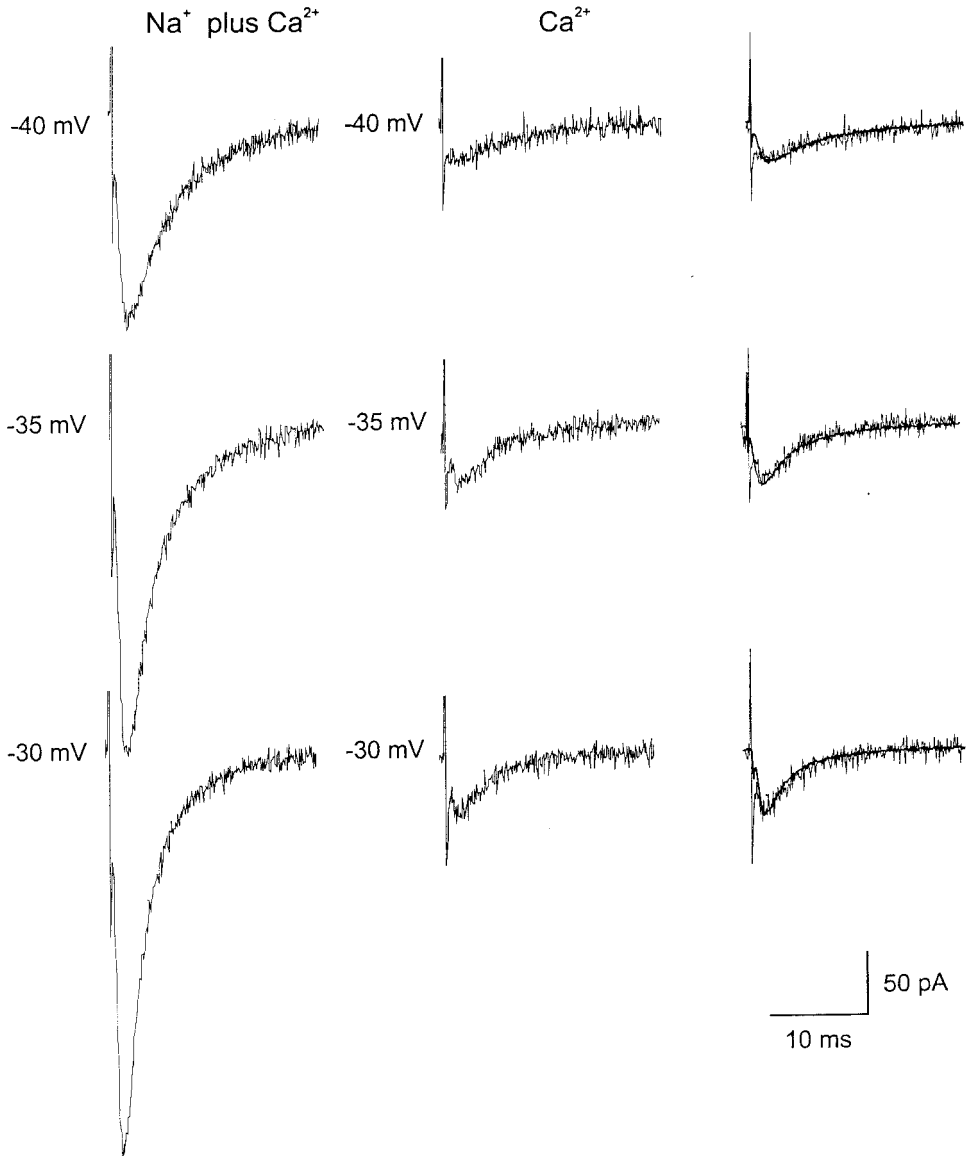

FIGURE 9 Current records from a transfected HEK 293 cell. The left column presents TTX-subtracted currents, all from the same cell, recorded on steps from the −100 mV holding potential to the indicated potentials. Bath contained 1 mM Na^+_o plus 3 mM Ca^{2+}_o. The middle column presents TTX-subtracted current records from this same cell at the same potentials still in 3 mM Ca^{2+}_o, but now with no Na^+_o. The right column presents these records superimposed. Records in Na^+_o plus Ca^{2+}_o have been scaled down so that their peaks match these in Ca^{2+}_o only. The current time courses superimpose. Removing Na^+_o in the presence of Ca^{2+}_o does not alter H1 gating kinetics. Scale: 50 pA, 10 ms.

(Akaike and Takahashi, 1992), and in squid giant axons in the presence of 50 mM Na^+_o (Meves and Vogel, 1973). We have shown here that $I_{Ca(TTX)}$ channels are present and conducting in the absence of permeant divalent ions. Consistent with these results, Brown et al. (1981) recorded I_{Na} in rat ventricular myocytes in the presence of 50 mM or more Na^+_o but the absence of Ca^{2+}. With these large Na^+_o concentrations, Brown et al. (1981) were able to resolve two I_{Na} inactivation relaxations. Their fast and slow $\tau_h(V)$ functions are in rough agreement with those reported by Aggarwal et al. (1997) for I_{Na} and $I_{Ca(TTX)}$ respectively. Hence, neither of the conditions specified for conversion of I_{Na} into $I_{Ca(TTX)}$ channels are, in fact, required for the demonstration of $I_{Ca(TTX)}$.

Adding Na^+_o to Ca^{2+}_o increases $I_{Ca(TTX)}$ rather than eliminating or decreasing it

In squid giant axons, adding Na^+_o to Ca^{2+} containing bathing media adds an additional, faster current component without either eliminating $I_{Ca(TTX)}$, reducing it, or altering its kinetics (Meves and Vogel, 1973). The same results were reported for $I_{Ca(TTX)}$ in rat hippocampal CA1 cells (Akaike

and Takahashi, 1992). In rat ventricular myocytes, adding Na^+_o to Ca^{2+} containing bathing media also adds a second, faster inactivation relaxation to that seen in Ca^{2+}_o only. The τ_h as a function of potential in Ca^{2+}_o only is unchanged upon addition of Na^+_o (Aggarwal et al., 1997). Again, in rat ventricular cells, Aggarwal et al. (1997) found that the activation, $g(V)$, curve for the TTX-blockable current seen in Ca^{2+}_o only (i.e., $I_{Ca(TTX)}$) was well described by a single Boltzmann function, but required two Boltzmann functions when Na^+_o was added to Ca^{2+}_o. One of the two Boltzmann functions seen in Ca^{2+}_o plus Na^+_o displayed the same midpoint and slope as that seen in Ca^{2+}_o only (indicating that the activation curve for $I_{Ca(TTX)}$ was the same) whereas the second displayed distinctly different characteristics. These observations are all as expected if I_{Na} and $I_{Ca(TTX)}$ are generated by distinct channel populations.

$I_{Ca(TTX)}$ increases on adding Na^+_o to Ca^{2+}_o containing bathing media in rat hippocampal CA1 cells (Akaike and Takahashi, 1992) and squid giant axons (Meves and Vogel, 1973). In rat ventricular cells, the $I_{Ca(TTX)}$ activation curve seen in Ca^{2+}_o only can also be identified in the presence of Na^+_o plus Ca^{2+}_o (Aggarwal et al., 1997). On adding Na^+_o, the amplitude of the $I_{Ca(TTX)}$ activation curve (value of the maximum conductance) increased with no significant change in either the midpoint or slope factor. These observations are at variance with the predictions of the conversion proposal. The re-introduction of Na^+_o ought to decrease $I_{Ca(TTX)}$ rather than increase it as $I_{Ca(TTX)}$ channels should be reconverted back to classical Na^+ channels. It might be argued that a particular set of experimental conditions could be found in which the reduced number of $I_{Ca(TTX)}$ channels produced by re-adding Na^+_o would be offset by the increased current carried by Na^+ through the remaining $I_{Ca(TTX)}$ channels. However, it does not seem possible to account for all the reports of increased $I_{Ca(TTX)}$ on re-introduction of Na_o in this way, because of the very diverse experimental conditions used. $I_{Ca(TTX)}$ increased whether the Na_o re-introduced was at 1 mM (Aggarwal et al., 1997), 10 mM (Akaike and Takahashi, 1992), or 50 mM (Meves and Vogel, 1973). In no case, under any experimental conditions, has the re-introduction of Na^+ been shown to reduce the density of $I_{Ca(TTX)}$ channels as required by the conversion proposal.

Heterologously expressed H1 channels are not converted to $I_{Ca(TTX)}$ channels

There seems to be broad agreement that heterologously expressed H1 channels display little Ca^{2+} permeability. In a variety of expression systems, no inward current was detected through H1 Na^+ channels in the complete absence of Na^+_o but presence of 1–10 mM Ca^{2+}_o (Chen et al., 1997; Townsend et al., 1997; Grant et al., 1999; Nuss and Marbán, 1999). Results were the same whether just the α or both α and β_1 subunits were expressed. Guatimosim et al. (2001) reported a small TTX-blockable inward current through heterologously expressed H1 (α subunit alone or with α, β_1 and β_2) in 8 mM Ca^{2+}_o and no Na^+_o. We also detect a small Ca^{2+} current through H1 channels (Figs. 7 and 9).

The low Ca^{2+} permeability of H1 channels contrasts sharply with that of $I_{Ca(TTX)}$ channels which display substantial Ca^{2+} permeability. In rat ventricular cells, nearly all TTX-blockable Ca^{2+} current is generated by $I_{Ca(TTX)}$ channels with no detectable component generated by classical Na^+ channels, even though classical Na^+ channels are present in appreciably higher density (Aggarwal et al., 1997). Hence, $I_{Ca(TTX)}$ channels express a much higher Ca^{2+} permeability then do classical Na^+ channels. The observations of little Ca^{2+} permeability for H1 noted above were all made under conditions where channel conversion should have occurred if it were going to. However, there are no reports of substantial Ca^{2+} currents through H1 channels, suggesting that conversion did not occur.

Correspondingly, attempts to directly demonstrate conversion of H1 channels have all been unsuccessful. The gating properties of Ca^{2+} current through H1 channels are identical to those of Na^+ current through H1 channels, and H1 channel gating properties are not altered by exposure to Na^+_o-free, Ca^{2+} containing bathing media (Figs. 7 and 9; Guatimosim et al., 2001). Moreover, in a number of native cell types, under a variety of conditions, two functionally distinct Na^+ current components, I_{Na} and $I_{Ca(TTX)}$, are seen simultaneously. In contrast, H1 channels have never been shown to simultaneously express two different current components, even under conditions where they are seen in ventricular myocytes (Fig. 8). These cardiac Na^+ channels do not undergo conversion when exposed to the conditions specified to produce it.

Guatimosim et al. (2001) did not interpret their results as described above. They compared τ_h values from H1 channels in 8 mM Ca^{2+}_o and no Na^+_o with those in either 2 or 8 mM Na^+_o and no Ca^{2+}_o. τ_h values in these two solutions were different when compared at the same potentials. Guatimosim et al. (2001) interpreted this difference as indicating an actual change in gating properties. However, the difference in τ_h values compared at the same potentials arises because surface potentials in the presence of 8 mM Ca^{2+}_o will differ appreciably from those in Ca^{2+}_o-free media, and gating parameters in the presence of Ca^{2+}_o will appear to be shifted in the positive direction along the voltage axis (Figs. 4–7). If Guatimosim et al.'s (2001) nonlinear τ_h (V) in Ca^{2+}_o only is translated 13 mV to the left along the voltage axis, it is found to superimpose exactly with their τ_h (V) in Na^+_o only (their Fig. 8 *H*). Kinetic properties in the two bathing media are, in fact, exactly the same, in agreement with the results presented here.

Part of the reason that we and Guatimosim et al. (2001) came to such different conclusions is that Guatimosim et al. (2001) refer to any TTX-blockable Ca^{2+} current as $I_{Ca(TTX)}$ even if nothing has been done to identify it as such. In their

study, an attempt was made to identify the nature of the TTX-blockable Ca^{2+} current in transfected HEK 293 cells in only one instance: the comparison of τ_h (V) in Na^+_o only and that in Ca^{2+}_o only. As noted, when differences in surface potentials are allowed for, these very data unequivocally indicate that H1 did not convert to $I_{Ca(TTX)}$. This also accounts for why Guatimosim et al. (2001) can only detect what they call $I_{Ca(TTX)}$ in HEK 293 cells in the complete absence of Na^+_o. In their experiments, Na^+ and Ca^{2+} are permeating the same channels with the same properties.

Thus, in no case has it been possible to show that H1 cardiac Na^+ channels, expressed heterologously, convert to $I_{Ca(TTX)}$ channels. Even with no or very little Na^+_o but in the presence of Ca_o (the conditions specified for channel conversion), H1 channels display the properties of Na^+ rather than $I_{Ca(TTX)}$ channels.

Single-channel studies indicate that there is more than one kind of cardiac Na^+ channel

The alternative to channel conversion is that $I_{Ca(TTX)}$ is generated by a distinct set of channels that are always present. In this case, more than one kind of Na^+ channel should be demonstrable in cells that express $I_{Ca(TTX)}$. Single-channel studies have shown that more than one type of Na^+ channel is, indeed, expressed in rat ventricular myocytes and other cardiac cells (Cachelin et al., 1983; Ten Eick et al., 1984; Kunze et al., 1985; Scanley and Fozzard, 1987; Ju et al., 1994; Zilberter et al., 1994), consistent with distinct channel populations. In all cases, both channel types are seen in the presence of substantial Na^+_o.

Issues concerning the evidence on which the conversion proposal was based

The central observation on which the channel conversion proposal was originally based was the apparent slowing of inactivation kinetics for some $I_{Ca(TTX)}$ channels seen on re-adding Na^+_o to Ca^{2+}-containing solutions (Cole et al., 1997). The other $I_{Ca(TTX)}$ channels were assumed to reconvert back to classical Na^+ channels. Cole et al. (1997) argued that if I_{Na} and $I_{Ca(TTX)}$ are generated by distinct, non-interconvertable channel populations, then adding Na^+_o to Ca^{2+}_o would be expected simply to add an additional, faster current component rather than alter $I_{Ca(TTX)}$ kinetics. This exact result has been reported by others (Meves and Vogel, 1973; Akaike and Takahashi, 1992; Aggarwal et al., 1997). This apparent slowing of $I_{Ca(TTX)}$ kinetics might be because of two technical issues.

First, Cole et al. (1997) did not use TTX to isolate $I_{Ca(TTX)}$. Their presumptive $I_{Ca(TTX)}$ seems to be contaminated with a Ca^{2+} current. Cole et al.(1997) reported that $I_{Ca(TTX)}$ inactivation in guinea pig ventricular myocytes typically developed as two exponential components. However, $I_{Ca(TTX)}$ inactivation in all other preparations (squid giant axons, Meves and Vogel, 1973; rat hippocampal CA1 cells, Akaike and Takahashi, 1992; human atrial cells, Lemaire et al., 1995; rat ventricular myocytes, Aggarwal et al., 1997) develops as a single exponential component with a τ_h comparable with the fast component of Cole et al. (1997). Heubach et al. (2000) reported that $I_{Ca(TTX)}$ inactivation in guinea pig ventricular cells also developed as a single, fast exponential. The slow inactivation relaxation of Cole et al. (1997) has not been reported by others.

$I_{Ca(TTX)}$ has been reported to be insensitive to the Ca^{2+} channel blocker, Ni^{2+} (Lemaire et al., 1995; Aggarwal et al., 1997), and to other Ca^{2+} channel blockers also (Akaike and Takahashi, 1992; Lemaire et al., 1995; Aggarwal et al., 1997). In contrast, Cole et al. (1997) reported that Ni^{2+}, at concentrations found to be ineffective in other preparations, selectively reduced the amplitude of their slow $I_{Ca(TTX)}$ relaxation with no other effects on the $I_{Ca(TTX)}$ time course. This selective block of only the slow relaxation suggested that it might arise from contamination of their records with a Ca^{2+} current. Ni^{2+} most likely did not affect $I_{Ca(TTX)}$ in guinea pig ventricular cells. It just reduced the amplitude of the contaminating Ca^{2+} current (Mitchell et al., 1983).

Second, Cole et al. (1997) reported that the slow relaxation slowed further on re-adding Na^+_o to Ca^{2+}-containing solutions. However, they selected for this analysis only cells in which just a single inactivation relaxation was resolved in Ca^{2+}_o only. Hence, the inactivation time constant they determined in these experiments was the weighted sum of a faster component from $I_{Ca(TTX)}$ and a slower from the contaminating Ca^{2+} current, and so faster than that of the Ca^{2+} current alone. On re-adding Na^+_o, the amplitude of $I_{Ca(TTX)}$ increases considerably because of the Na^+ permeability of these channels. Because of this increase, the inactivation of $I_{Ca(TTX)}$ is no longer confounded with that of the Ca^{2+} current. The slow "$I_{Ca(TTX)}$" inactivation relaxation now consists of just the contaminating Ca^{2+} current and so seems slower. This slow relaxation did not actually change. It is simply now observable separated from the faster $I_{Ca(TTX)}$ inactivation time course. The slowing of inactivation is only apparent, and provides no evidence in support of the channel conversion proposal.

Summary of findings

Our data demonstrate that $I_{Ca(TTX)}$ can be observed in the absence of a permeating divalent ion and that the cardiac sodium channel (H1) expressed in HEK 293 cells can not be converted to $I_{Ca(TTX)}$ by altering the experimental conditions. In addition, there is ample evidence in the literature that $I_{Ca(TTX)}$ can be observed even in the presence of appreciable Na_o. Thus, the conversion proposal is not consistent with the available experimental observations.

We note that $I_{Ca(TTX)}$ is completely unrelated to the proposal of slip-mode conductance of classical cardiac Na^+

channels in which exposure to certain pharmacological agents is said to increase the Ca^{2+} permeability of these channels (Santana et al., 1998). In the presence of these agents, Na^+-channel gating properties remain those of the classical cardiac Na^+ channels. Moreover, $I_{Ca(TTX)}$ is seen in the complete absence of these agents. The existence of $I_{Ca(TTX)}$ provides no evidence for slip-mode conductance nor is it obviously connected in any way with that proposal.

$I_{Ca(TTX)}$ is simply accounted for if it generated by a distinct population of channels, not interconvertable with I_{Na} channels. We conclude that in many cell types there exist two populations of Na^+ channels: a larger population that expresses little Ca^{2+} permeability and a smaller population that expresses substantial permeability to Ca^{2+}.

We thank Dr. E. Marbán for kindly providing stably transfected HEK 293 cells, Dr. R. Horn for his insightful comments on an earlier version of this manuscript, and Mr. Byron K. Norton and Mr. Gabe Sinclair for their valuable technical assistance. This work was supported by National Institutes of Health Grants HL50435 (C.W.B.) and HL60748 (W.G.W.), a Department of Veterans Affairs Merit Review Award (S.R.S), and a grant-in-aid from the American Heart Association (Mid-Atlantic Affiliate; L.G.). C.W.B. is an Established Investigator of the American Heart Association (National Center). Y.C-I. is supported, in part, by the Interdisciplinary Training Program in Muscle Biology, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine.

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