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The role of L-type Ca^{2+} current and Na^{+} current-stimulated Na/Ca exchange in triggering SR calcium release in guinea-pig cardiac ventricular myocytes

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Abstract

Objective: This study examines the relative ability of sodium current (I_{Na})-stimulated reverse mode Na/Ca exchange and the L-type calcium current (I_{Ca}) to trigger calcium-induced calcium release (CICR) in guinea-pig ventricular myocytes. **Methods:** Cytosolic Ca^{2+} transients were recorded from enzymatically dissociated guinea-pig ventricular myocytes using Indo-1. Macroscopic membrane currents were simultaneously recorded using the whole-cell patch-clamp technique. **Results:** At room temperature (22–25°C) Ca^{2+} transients were associated with the activation of I_{Na} , I_{Ca} or I_{Na} plus I_{Ca} in combination. However, after I_{Ca} was blocked by verapamil (10 μM), no Ca^{2+} transient could be evoked by the activation of I_{Na} alone at either -40 or $+5$ mV. Similar results were obtained with 5 and 8 mM intracellular sodium, and when the temperature of the bathing solution was raised to 35°C and cAMP (10 μM) added to the pipette solution. **Conclusions:** From consideration of the relative magnitudes of the Ca^{2+} influx via I_{Ca} and Na/Ca exchange and thermodynamic considerations, we suggest that I_{Ca} is the major source of ‘trigger’ calcium for CICR (and cardiac contraction) under normal conditions. Although the Na/Ca exchanger was incapable of triggering CICR under the conditions of these experiments, we suggest that it may become more important when cytosolic Ca^{2+} is elevated, a condition which will also lead to decrease the amplitude of I_{Ca} . © 1997 Elsevier Science B.V.

Keywords: Calcium channel, L-type; Calcium transient; Sarcoplasmic reticulum; Indo-1; $\text{Na}^{+}/\text{Ca}^{2+}$ exchange; Excitation–contraction coupling; Guinea pig, ventricular myocytes

1. Introduction

Calcium-induced calcium release (CICR) from the sarcoplasmic reticulum (SR) underpins excitation–contraction coupling in cardiac muscle [1–3] and it has been shown that a simulated calcium current (I_{Ca}) may supply sufficient calcium to ‘trigger’ CICR in a skinned cell preparation [4]. The obligatory role of I_{Ca} in triggering CICR has, however, been questioned, in light of evidence supporting a role for the sodium–calcium (Na/Ca) exchanger (for review, see Ref. [5]).

The Na/Ca exchanger plays a major role in cardiac calcium homeostasis by extruding calcium from the cyto-

plasm at rest [6–8]. However, Na/Ca exchange is voltage-dependent, with a reversal potential determined by the sodium (Na) and calcium electro-chemical gradients [6,7,9–12] and depolarisation of the cell during the action potential should reverse the direction of exchange to produce calcium influx (see [13] for calculations). Direct support for this idea has come from experiments examining the effects of Na/Ca exchanger modulation on action potential time course [14] and ‘slow inward currents’ [15]. When the exchanger is operating in such a ‘reverse’ mode, the resulting calcium influx may trigger CICR [16–18].

Na/Ca exchange-triggered SR calcium release was first demonstrated in a ‘calcium-overloaded’ cardiac preparation [16]. Exchanger-triggered SR release has also been demonstrated during depolarisation to $+100$ mV [19,20],

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and at less positive potentials in myocytes perfused with a high (20 mM) internal Na^+ solution [21]. Under more physiological conditions, SR calcium release has been evoked by reverse-mode Na/Ca exchange when I_{Ca} was blocked [5,18,22,23]. It has also been proposed that the Na/Ca exchange may supply a larger fraction of the trigger calcium for CICR than I_{Ca} [5,18]. Additional support for this proposal has come from observations of sodium-current (I_{Na})-triggered CICR, which was explained by I_{Na} increasing the [Na] at the cytoplasmic surface of the exchanger which, in turn, accelerated calcium influx via the exchanger to a point where CICR was activated [17,22,24].

Although the above evidence supports the idea that the exchanger can trigger CICR under some conditions, a major role for this mechanism has been questioned [19,25,26]. In view of the potential importance of I_{Na} -stimulated reverse-mode Na/Ca exchange in triggering SR calcium release we have repeated some recent experiments [22] to re-examine the ability of I_{Na} -stimulated Na/Ca exchange to trigger CICR at low (5–8 mM) internal Na^+ levels. A preliminary account of some of these experiments has been reported previously [27].

2. Methods

2.1. Cell dissociation

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23 1985). The isolation procedure was adapted from that reported elsewhere [28]. Isolated hearts from adult guinea-pigs were initially perfused for 5 min (at 37°C) with a nominally calcium-free solution (composition in mM): NaCl, 120; KCl, 5.4; HEPES, 10; pyruvate, 5; glucose, 20; taurine, 20 (pH 7.05 with NaOH). This basic solution was then switched to one containing 4 U · ml⁻¹ protease (Sigma type XXIV) and 100 μM CaCl_2 for 3 min, after which the protease in the perfusate was replaced with collagenase (Worthington type II, 1 mg · ml⁻¹) for a further 5–10 min. On completion of the enzyme treatment, the ventricles were cut free and placed in a Petri dish filled with warmed (35°C) enzyme-free isolation solution containing 100 μM CaCl_2 . The tissue was chopped into small pieces and triturated using a wide-bore pipette. The resulting cell suspension was filtered through gauze, centrifuged briefly and the pellet resuspended. The cells were stored at room temperature (22–25°C) until used.

2.2. Electrophysiology

Cells were transferred to the experimental chamber (200 μl volume), mounted on the stage of a Nikon Diaphot microscope (Nikon Instruments, Japan), and superfused (2

ml/min) with a physiological solution (composition in mM/l): NaCl, 135; KCl, 5.4; MgCl_2 , 1; CaCl_2 , 2.0; Glucose, 10; Na-HEPES, 10; NaH_2PO_4 , Na pyruvate, 1; CsCl, 20 (pH 7.4 with NaOH). Experiments were performed at room temperature (22–25°C), or at 35°C as indicated. I_{Na} and I_{Ca} were recorded in the whole-cell voltage-clamp configuration [29] using an Axopatch 1B patch-clamp amplifier (Axon Instruments Inc., USA). Patch pipettes (1–2.5 MΩ) were pulled from fibre-filled borosilicate glass. The pipette filling solution was (composition in mM/l): CsOH, 100; aspartate, 100; CsCl, 30; NaCl, 5; HEPES, 10; Mg-ATP, 5 (pH 7.2 with CsOH). Contaminating potassium currents were minimised by the presence of Cs^+ in both pipette and bath solutions.

Voltage-clamp command potentials were generated via pCLAMP data acquisition software (Axon instruments Inc., USA), driving a TL-1 analogue to digital converter (Axon Instruments Inc., USA). All electrical records were digitised and recorded on videotape, or collected and stored in a computer using the pCLAMP data acquisition software. A dual sampling rate was used to increase the sampling frequency at the beginning of the record and allow accurate measurement of the peak I_{Na} , I_{Ca} and the rising phase of the calcium transient.

2.3. Measurement of intracellular calcium

The Ca^{2+} indicator Indo-1 (25 μM) was added to the pipette solution, and injected into the cell by brief pulses of positive pressure. A low concentration of the dye was used to minimise calcium buffering [30] at the expense of some reduction in the signal-to-noise ratio. An epifluorescence illumination wavelength of 360 nm was used, derived from a xenon arc lamp. Emitted light was recorded at 410 and 510 nm wavelengths. Cell autofluorescence was measured after seal formation (before entering the whole-cell configuration) and subtracted from all data. Changes in fluorescence are presented as the 410 nm/510 nm ratio.

2.4. Conditioning protocols

In this investigation we used two different conditioning protocols in order to maintain a defined calcium-loaded state of the sarcoplasmic reticulum:

(1) When I_{Ca} was active, 10 conditioning pulses were applied (250 ms, 0.75 Hz) from a holding potential (VH) of –90 to +5 mV to load the SR. After these conditioning pulses, a 10 s rest period was allowed at post-conditioning potential of –90 mV. Test pulses of –40 mV and +5 mV were then used to elicit I_{Na} alone or a combination of I_{Na} and I_{Ca} , respectively. I_{Ca} was ‘selectively’ activated by inactivating I_{Na} with a post-conditioning potential of –40 mV and then applying a test pulse to +5 mV.

(2) When I_{Ca} was blocked, reverse-mode Na/Ca exchange was used to maintain the SR Ca^{2+} load, by apply-

ing depolarising steps from -40 to $+60$ mV (250 ms, 0.75 Hz). After these pulses, a 10 s rest was allowed at -40 mV. Since the efficacy of verapamil block of I_{Ca} was reduced at potentials negative to -40 mV, a brief (50 ms) pre-pulse to -90 mV had to be given to remove I_{Na} inactivation without affecting the block of I_{Ca} .

2.5. Chemicals

Indo-1 was obtained from Molecular Probes Inc. (Eugene, USA). Verapamil, c-AMP and all other chemicals were obtained from SIGMA Chemical Co. Ltd. (Poole, Dorset, England).

3. Results

3.1. I_{Na} - and I_{Ca} -induced calcium transients

Fig. 1A shows the activation of calcium current (I_{Ca} , upper panel) at $+5$ mV from a post-conditioning potential of -40 mV at room temperature (22 – 25°C). The peak amplitude of I_{Ca} was 7.51 pA/pf and was followed, upon repolarisation, by an inward tail current typical of that produced by ‘forward mode’ Na/Ca exchange [31]. The lower panel shows the concomitant Ca^{2+} transient triggered by I_{Ca} , which was characterised by a rapid rising phase, a sustained plateau and an exponential decline after repolarisation. A test pulse to -40 mV from a post-conditioning potential of -90 mV resulted in the rapid activa-

tion and inactivation of a large ‘Na current’ (I_{Na}) (Fig. 1B). The lower panel shows the I_{Na} -evoked Ca^{2+} transient. It is notable that this Ca^{2+} transient was smaller than that activated by I_{Ca} and declined during the test pulse. Fig. 1C shows the combined activation of I_{Na} and I_{Ca} (upper panel) at $+5$ mV from a post-conditioning potential of -90 mV. The peak amplitude of the inward current was twice that obtained by activation of I_{Na} at -40 mV, but the evoked calcium transient (lower panel) was of comparable amplitude and time course to that evoked by I_{Ca} alone (panel A). Similar results were obtained in 14 other cells. These results suggest that SR calcium release is principally activated by I_{Ca} and that I_{Na} -stimulated reverse-mode Na/Ca exchange does not further increase SR calcium release.

3.2. I_{Na} - and I_{Ca} -induced transients are verapamil-sensitive

In the presence of verapamil ($10\ \mu\text{M}$), I_{Ca} was blocked by repeated depolarization using the conditioning protocol (see Section 2). Fig. 2A shows that with I_{Ca} blocked in this way: (1) a test pulse from -40 mV to $+5$ mV failed to activate any discernable inward current; (2) there was no Na/Ca tail current upon repolarisation (upper panel); (3) no measurable calcium transient was evoked (lower panel). It is therefore apparent that verapamil completely abolished I_{Ca} -induced SR calcium release. Fig. 2B shows that with I_{Ca} blocked, ‘selective’ activation of I_{Na} by stepping from -90 to -40 mV did not activate SR

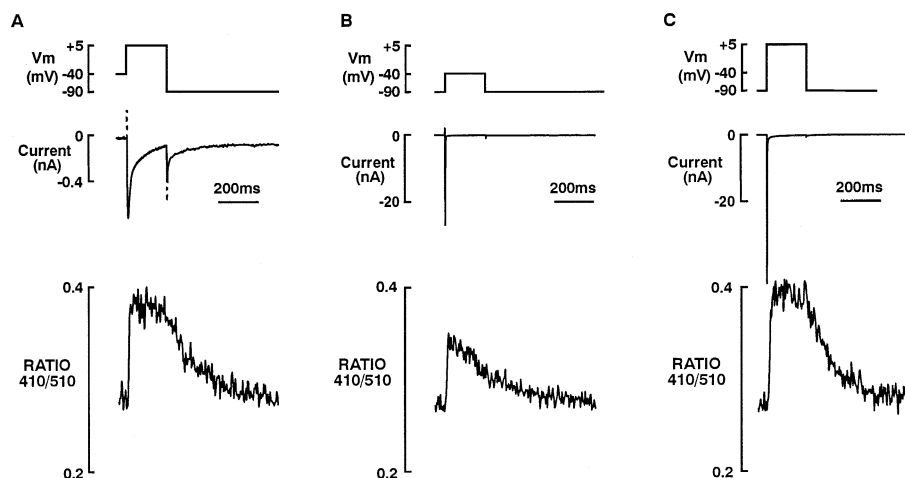


Fig. 1. Activation of I_{Ca} , I_{Na} and combined activation of I_{Na} and I_{Ca} evoke a Ca^{2+} transient in ventricular myocytes. In order to provide a defined Ca^{2+} -loaded state of the SR, a conditioning protocol was applied prior to the test pulse in A, B and C (see Section 2, protocol 1). (A) Activation of I_{Ca} alone (upper panel) by a test pulse to $+5$ mV after a 10 s rest at a post-conditioning potential of -40 mV (to inactivate I_{Na}). Lower panel shows the I_{Ca} -evoked Ca^{2+} transient. (B) activation of I_{Na} alone (upper panel) by a test pulse to -40 mV after a 10 s rest at a post-conditioning potential of -90 mV. Lower panel shows the I_{Na} -evoked Ca^{2+} transient. (C) Combined activation of I_{Na} and I_{Ca} (upper panel) by a test pulse to $+5$ mV after a 10 s rest at a post-conditioning potential of -90 mV. Lower panel shows the Ca^{2+} transient evoked by the combination of I_{Na} and I_{Ca} . The transients in this and all subsequent figures are shown as the 410/510 nm fluorescence ratio of Indo-1. Panels A, B and C were recorded from the same cell at room temperature (22 – 25°C).

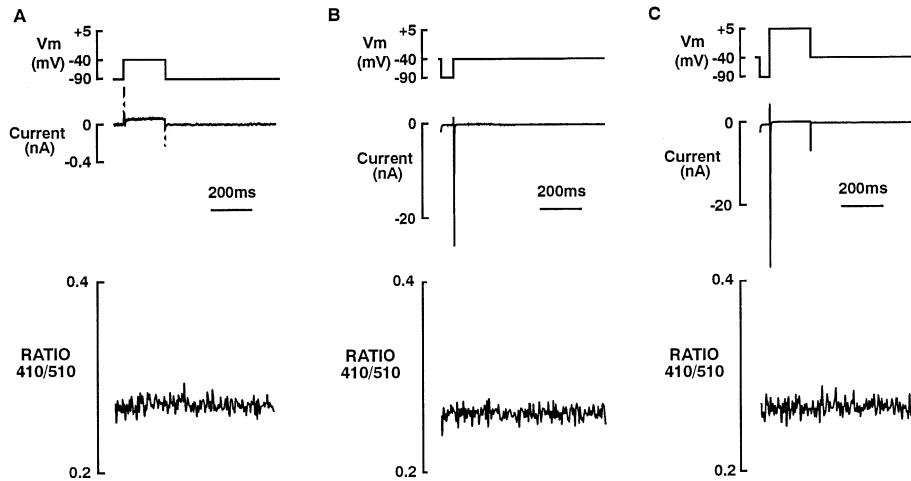


Fig. 2. Verapamil sensitivity of I_{Ca} - and I_{Na} -evoked Ca^{2+} transients. With I_{Ca} blocked by addition of verapamil ($10 \mu M$) to the bath solution, the SR was loaded by repeated activation of reverse mode Na/Ca exchange (see Section 2, protocol 2). (A) No inward current (upper record) or Ca^{2+} transient (lower record) was evoked by a test pulse to $+5$ mV after a 10 s rest at a post-conditioning potential of -40 mV. (B) Activation of I_{Na} (recovered by a 50 ms prepulse to -90 mV) by a test pulse to $+5$ mV after a 10 s rest at a post-conditioning potential of -40 mV (upper panel). The lower panel shows that I_{Na} failed to evoke a Ca^{2+} transient when I_{Ca} was blocked. (C) Stepping from -90 to $+5$ mV also fails to evoke a calcium transient in the presence of verapamil. All panels were recorded at room temperature (22 – $25^\circ C$) from the same cell as in Fig. 1.

calcium release (lower panel). As might be expected, no calcium transient was observed when the membrane potential was stepped from -90 to $+5$ mV (Fig. 2C). Similar results were obtained in every cell examined ($n = 14$), and suggest that the small Ca^{2+} transient evoked by 'selective' activation of I_{Na} (Fig. 1B), could be explained by threshold activation of I_{Ca} rather than by I_{Na} per se. These findings differ from those of the previous study [22] since the I_{Na} -evoked Ca^{2+} transient was verapamil-sensitive and smaller than the I_{Ca} -evoked transient. We therefore exam-

ined the extent to which these contrasting results might be accounted for by some inevitable loss in voltage control during I_{Na} [19,26].

When I_{Na} is activated at -40 mV, voltage escape could cause the membrane potential to overshoot the desired command potential and thereby enter the activation range of I_{Ca} . Fig. 3A shows that, in the absence of verapamil, activation of I_{Na} at -50 mV (upper panel) can produce an (apparent) inward current of 216 pA/pF, which is of comparable magnitude to that reported in other

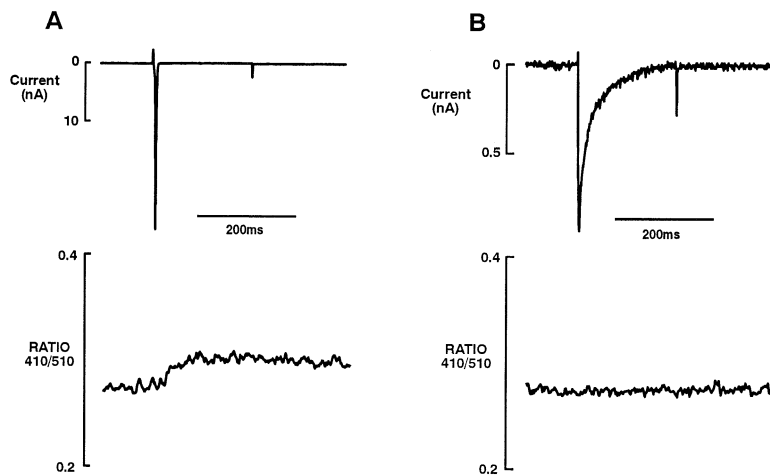


Fig. 3. Voltage escape and the I_{Na} -evoked Ca^{2+} transient. At a holding potential of -90 mV the SR was loaded by activation of I_{Ca} as described in Fig. 1. (A) Activation of I_{Na} at -50 mV after a 10 s rest at a post-conditioning potential of -90 mV (upper panel), and the I_{Na} evoked Ca^{2+} transient (lower panel). The series resistance was 5.81 M Ω , and was uncompensated. (B) Activation of I_{Na} at -50 mV from the same cell as in A (upper panel), after the series resistance was reduced by applying pulses of positive pressure to the pipette, and the remainder reduced by series resistance compensation (leaving 1.8 M Ω uncompensated). The magnitude of I_{Na} was substantially reduced under these conditions and failed to evoke a Ca^{2+} transient (lower panel). Records were obtained at room temperature (22 – $25^\circ C$).

studies [22,24]. However, in the same cell, when the series resistance (R_s) was reduced (from 5.8 to 1.8 M Ω by application of positive pressure pulses to the pipette and electronic compensation), the time course of I_{Na} was quite different ($n = 3$). As shown in Fig. 3B, the peak current was reduced to 11 pA/pf and inactivated (non-exponentially) with a half-time of 33 ms, in reasonable agreement with results obtained using an oil gap voltage-clamp method [32]. (Mitsuiye and Noma [32] reported that I_{Na} inactivated with two exponential time constants of 10 and 55 ms at -40 mV. At -50 mV slightly slower time constants would be expected—as observed here). These results suggest that, at least under some conditions, voltage escape may allow sufficient activation of I_{Ca} to cause SR Ca^{2+} release (and contraction). In connection with this point, it is notable that the small Ca^{2+} transient observed before R_s compensation was abolished when R_s was reduced (lower panels). In any case, the inability to clamp I_{Na} (as shown by the very rapid inactivation of I_{Na} at -40 mV) would always be problematic for such experiments.

3.3. Effects of increasing the SR Ca^{2+} content and temperature

Although conditioning protocols were employed to ensure a defined SR Ca^{2+} load, it is possible that increasing the SR Ca^{2+} content might enable a smaller trigger influx (via Na/Ca) exchange to activate SR Ca^{2+} release [2,4,24,33–35]. In addition, Ca^{2+} transport by the Na/Ca exchanger is very temperature-sensitive ($Q_{10} = 3–4$ [36]; see also [19]). We therefore examined the ability of I_{Na} to

evoke SR Ca^{2+} release in the presence of 10 μ M cAMP (which stimulates SR Ca^{2+} uptake) and at 35°C.

The addition of cAMP to the pipette solution at an experimental temperature of 35°C produced somewhat different results. Fig. 4 shows Ca^{2+} transients (lower panel) induced by activation of I_{Ca} alone at +5 mV (panel A) I_{Na} alone at -40 mV (panel B) and combined activation of I_{Na} and I_{Ca} at +5 mV (panel C). The Ca^{2+} transient evoked by I_{Ca} (panel A) rose rapidly to a peak and then declined to a plateau level during the test pulse. In contrast to results shown above, the activation of I_{Na} alone at -40 mV evoked a Ca^{2+} transient of equivalent amplitude to that evoked by I_{Ca} , but which decayed almost to baseline levels within the duration of the test pulse (Fig. 4B). Furthermore, combined activation of I_{Na} and I_{Ca} evoked a much larger Ca^{2+} transient (Fig. 4C) than did I_{Ca} (Fig. 4A) although, in each case, the Ca^{2+} transients declined to a similar plateau level during the test pulse. In fact, the Ca^{2+} transient evoked by combined activation of I_{Na} and I_{Ca} (Fig. 4C) appeared to be the sum of the Ca^{2+} transients evoked by the selective activation of I_{Ca} and I_{Na} . Apart from the different experimental conditions, these findings are in general agreement with those reported previously [22], except that the Ca^{2+} transient evoked by the combination of I_{Na} and I_{Ca} decayed rapidly during the test pulse, and there was no detectable difference in the kinetics of the rising phase of the Ca^{2+} transient evoked by either protocol (Fig. 4D).

The greater magnitude of the Ca^{2+} transient evoked by the combined activation of I_{Na} and I_{Ca} could be due to several factors: (1) the magnitude of I_{Ca} activated from

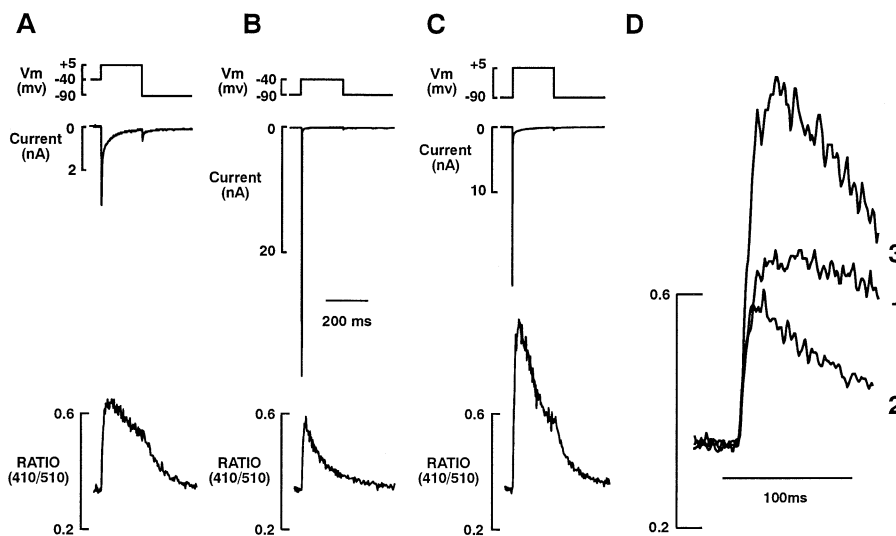


Fig. 4. At a holding potential of -90 mV, the SR was loaded by repeated activation of I_{Ca} , as described in Fig. 1. (A) Activation of I_{Ca} alone (upper panel) by a test pulse to +5 mV after a 10 s rest at a post-conditioning potential of -40 mV (to inactivate I_{Na}). Lower panel shows the I_{Ca} -evoked Ca^{2+} transient. (B) Activation of I_{Na} alone (upper panel) by a test pulse to -40 mV after a 10 s rest at a post-conditioning potential of -90 mV. Lower panel shows the I_{Na} -evoked Ca^{2+} transient. (C) Combined activation of I_{Na} and I_{Ca} (upper panel) by a test pulse to +5 mV after a 10 s rest at a post-conditioning potential of -90 mV. Lower panel shows the Ca^{2+} transient evoked by the combined activation of I_{Na} and I_{Ca} . (D) Comparison of the rise time kinetics of the Ca^{2+} transients evoked by activation of (1) I_{Ca} , (2) I_{Na} and a (3) combination of I_{Na} and I_{Ca} . The records in panels A, B, C and D were recorded from the same cell at 35°C. The pipette solution included cAMP (10 μ M).

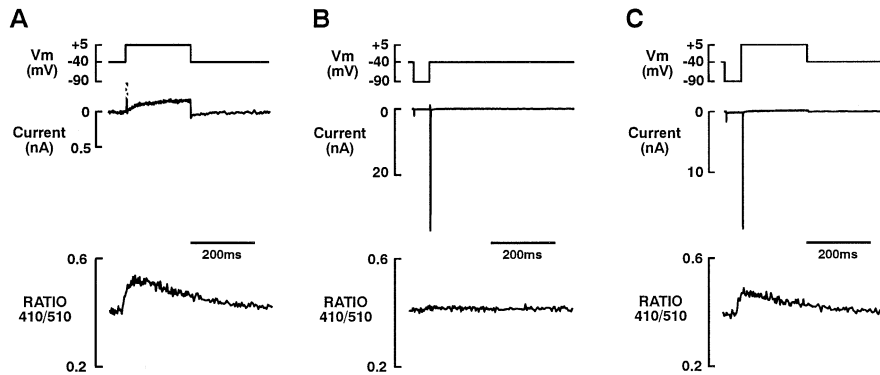


Fig. 5. With a holding potential of -40 mV, I_{Ca} was blocked by addition of verapamil ($10 \mu\text{M}$) to the bath solution at 35°C ; the pipette solution contained $10 \mu\text{M}$ cAMP. The SR was loaded by repeated activation of reverse-mode Na/Ca exchange as described in Fig. 2. (A) A small residual inward current (upper panel) and a small, concomitant Ca^{2+} transient (lower panel) were evoked by a test pulse to $+5$ mV after 10 s rest at a post-conditioning potential of -40 mV. (B) Activation of I_{Na} (recovered during a 50 ms prepulse to -90 mV, after a 10 s rest at a post-conditioning potential of -40 mV) by a test pulse to -40 mV (upper panel). Lower panel shows that when activated at -40 mV, I_{Na} failed to evoke a Ca^{2+} transient when I_{Ca} was blocked. (C) Activation of I_{Na} (recovered by a 50 ms prepulse to -90 mV) by a test pulse to $+5$ mV after a 10 s rest at a post-conditioning potential of -40 mV (upper panel). Lower panel shows that, when I_{Ca} was blocked, activation of I_{Na} at $+5$ mV failed to evoke a Ca^{2+} transient greater than that evoked in the absence of I_{Na} (A, lower panel).

-90 mV is greater than when activated from -40 mV [15,37]; (2) voltage escape during the combined activation of I_{Na} and I_{Ca} would lead to a more rapid depolarisation of the membrane and therefore a greater rate of Ca^{2+} channel activation; (3) the larger Ca^{2+} transient could be the product of two different Ca^{2+} influx pathways, one being I_{Ca} and the other being activated by I_{Na} independently of I_{Ca} (i.e., reverse-mode Na/Ca exchange).

Fig. 5 shows that when I_{Ca} was inhibited by verapamil ($10 \mu\text{M}$), there was a profound reduction in the amplitude of the Ca^{2+} transient. Under these conditions, a test pulse from -40 to $+5$ mV activated a small residual I_{Ca} which evoked a small Ca^{2+} transient (Fig. 5A). Although a small Ca^{2+} transient was also observed in response to the activation of both I_{Na} and I_{Ca} by a test pulse from -90 to $+5$ mV (Fig. 5C), it was of comparable amplitude to that observed with the selective activation of the residual I_{Ca} (Fig. 5A). Since there was no transient observed during activation of I_{Na} alone at -40 mV (Fig. 5B), the I_{Na} -evoked Ca^{2+} release was verapamil-sensitive and explainable by a small unblocked I_{Ca} , rather than being due to I_{Na} per se. This result is in direct conflict with the results of a previous study [22], although it is in general agreement with the conclusions of Sipido et al. [25].

4. Discussion

In the experiments reported here, we have failed to demonstrate that I_{Na} -stimulated reverse-mode Na/Ca exchange can trigger SR Ca^{2+} release, despite reproducing the experimental conditions used in a previous study [22] which suggested that I_{Na} activation at -40 mV could evoke a larger SR Ca^{2+} release than I_{Ca} . Our failure to observe Na/Ca exchange-triggered SR release immediately suggests that the Na/Ca exchanger is less able to

trigger SR calcium release than I_{Ca} at 5 mM internal $[\text{Na}^+]$.

It is possible that the method used to assess SR Ca^{2+} release may have some bearing on our failure to detect any I_{Na} -stimulated SR release. In the previous study [22], a confocal microscope was used to measure local $[\text{Ca}^{2+}]_i$ changes whereas we used conventional wide-field microscopy. Since the local light levels are very high in confocal microscopy, it is possible that there may be some local relief of nifedipine block (as nifedipine is light-sensitive) which would not be detected in the whole cell current record. However, this complication by itself does not explain why Li substitution blocked the I_{Na} -evoked transient. Simple interpretation of the Li-substitution experiments may be complicated by a Li-induced increase in resting $[\text{Ca}^{2+}]_i$ (e.g., [6,7]) which may inhibit I_{Ca} , and thereby decrease spurious calcium channel activation during the voltage escape that accompanies activation of I_{Na} .

4.1. I_{Na} escape

A major problem in studying the role of I_{Na} in activating contraction is the difficulty of obtaining adequate voltage control during the activation of the large and fast I_{Na} . Indeed, Bouchard et al. [26] showed that, without series resistance compensation, a series resistance of $6.7 \text{ M}\Omega$ results in a serious loss of voltage control and suggested that many of the previous reports of I_{Na} -activated Ca^{2+} transients could be explained by the membrane potential escaping to a point where I_{Ca} was activated. This view is supported by our findings (Fig. 3) and those of others [19,25,26]. Adequate voltage control over I_{Na} will always be problematic for these types of experiments and the current records that we (and others) have obtained at -40 mV do not reflect the true time course of I_{Na} . Nevertheless, a lack of voltage control does not, by itself, explain all the observations of Lipp and Niggli [22] or

Levesque et al. [24] since a loss of voltage control should have occurred during lithium exposure also.

4.2. Thermodynamics of Na/Ca exchange during E-C coupling

Calcium entry on the exchanger is determined by the membrane potential (E_m) and the sodium (E_{Na}) and calcium (E_{Ca}) electrochemical gradients (e.g., [6,9,10]). As an equation:

$$\text{Driving force} = E_m - 3E_{Na} + 2E_{Ca} = E_m - E_{Na/Ca}$$

where ($E_{Na/Ca}$) is the exchanger equilibrium potential. It has been suggested that normal E-C coupling results from a 100–250-fold increase in $[Ca^{2+}]_i$ in the space between the SR and T-tubule membranes (the diadic cleft) [38,39] which implies that for normal resting levels of $[Ca^{2+}]_i$, the local $[Ca^{2+}]_i$ that activates SR calcium release must be $\sim 6\text{--}15 \mu\text{M}$.

Fig. 6A shows the relationship between $E_{Na/Ca}$ and $[Na]_i$ for the exchanger to achieve a local $[Ca^{2+}]_i$ of 6–15 μM (shaded region). At 10 mM $[Na]_i$, the membrane potential would have to be $> +60$ mV for the exchanger to produce such a trigger $[Ca^{2+}]_i$ level while at 20 mM $[Na]_i$, the trigger $[Ca^{2+}]_i$ could be reached at membrane potentials $> +10$ mV—predictions in agreement with the results of Sham et al. [19] and Nuss and Houser [21], respectively. Between -50 and -40 mV (the test potentials used by Lipp and Niggli [22] and in this study) $[Na]_i$ would have to be > 35 mM to achieve a 6–15 μM trigger $[Ca^{2+}]_i$. Therefore, if $[Na]_i$ is clamped to ~ 5 mM by the patch pipette, thermodynamics show that it is impossible to achieve a normal trigger $[Ca^{2+}]_i$ level via the Na/Ca exchanger. However, it has been suggested that I_{Na} may locally increase $[Na]_i$ to enable Na/Ca exchange to achieve such trigger $[Ca^{2+}]_i$ levels [40].

To examine this point, the diadic cleft was modeled as a thin disk, 15 nm thick (the space between the SR and T-tubule membranes) and 150 nm in diameter with a single Na channel in its center (only one channel was included because the Na current density suggests that there are only ~ 5 Na channels per μm^2). The Na diffusion coefficients were reduced to 50, 21, 14, 7 and 5% of those observed in free solution to allow for the possibility of restricted diffusion. As shown in Fig. 6B, activation of a Na channel with a 4 pA single channel current would result in an appreciable increase in the local $[Na]_i$. However, the required local increase in $[Na]_i$ (38 mM) is only achieved in the immediate vicinity (i.e., within 4 nm) of the Na channel even with highly restricted diffusion. In other words, the Na/Ca exchangers would have to be: (1) tightly packed around Na channels for the local accumulation of $[Na]_i$ to be sufficient to achieve a trigger $[Ca^{2+}]_i$ level of 6 μM as well as (2) Na diffusion being highly restricted. In addition, these increases in $[Na]_i$ only persist during the open time of the sodium channel, so the exchanger would also have to be kinetically capable of

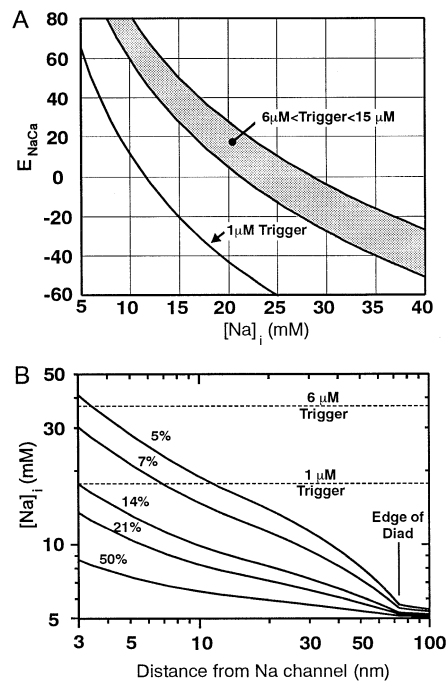


Fig. 6. (A) Relationship between the reversal potential for Na/Ca exchange and internal Na to achieve various trigger calcium levels. To meet or exceed a trigger level between 6 and 15 μM at -40 mV the exchanger would have to operate in the shaded area of the graph showing that internal Na would have to be > 35 mM. If the trigger calcium level is only 1 μM , internal Na would have to be > 18 mM. (B) Calculations of the Na levels that will occur across the diad during the activation of a 4 pA Na channel situated in the centre of the diadic space (modelled as a circular region 150 nm in diameter and 15 nm high) at steady state. Curves correspond to diffusion coefficients that are 50, 21, 14, 7 and 5% of those in free solution (corresponding to diffusion coefficients of 7×10^{-6} , 3×10^{-6} , and 2×10^{-6} , 1×10^{-6} and 7×10^{-7} $\text{cm}^2 \cdot \text{s}^{-1}$, respectively) are shown (note that both axes have logarithmic scales). The levels of intracellular Na that would be required to achieve trigger levels of 1 and 6 μM at -40 mV are shown. Note that such trigger levels can only be achieved by the exchanger if the diffusion of Na is restricted and if the exchanger is very close to the Na channel (i.e., within about 10 nm).

achieving thermodynamic equilibrium very rapidly (since 10 nm from the channel $[Na]_i$ takes only 8 μs to fall to 6.5 mM after channel closure). These considerations suggest that it is unlikely that the exchanger could achieve a trigger $[Ca^{2+}]_i$ level of 6 μM at -50 mV.

Nevertheless, Lipp and Niggli [22] were able to evoke SR calcium release, so we are forced to conclude that some other factors must have increased the ability of the exchanger to achieve the required trigger $[Ca^{2+}]_i$ level and/or the trigger $[Ca^{2+}]_i$ level must have been reduced. Under conditions of calcium overload, propagating waves of SR calcium release occur (e.g., Refs. [33,41]), implying that the required trigger calcium level could be reduced to $\sim 1 \mu\text{M}$ in calcium overload. To achieve this level of trigger $[Ca^{2+}]_i$ at -40 mV with the exchanger, internal Na would have to be about 18 mM (see Fig. 6A), which is more compatible with the levels of which are likely to occur during Na channel activation (cf. Fig. 6B). Thus the

results of Lipp and Niggli [22] may be partly due to SR calcium release being much more sensitive to the local trigger calcium level than in the experiments reported here. In addition, voltage escape during I_{Na} (see above) would make the trigger calcium level more attainable. However, our inability to record an I_{Na} -evoked $[Ca^{2+}]_i$ transient when SR load was increased (with cAMP) suggests that further factors are involved (see below).

4.3. Rates of calcium influx via the exchanger and I_{Ca}

The maximum I_{Ca} we have observed is 25 pA/pF, which is equivalent to an outward exchanger current of 12.5 pA/pf. This is 5 times larger than the exchanger current density reported by Kimura et al. [11] in whose experiments exchanger activity was increased by using 30 mM $[Na]_i$ at 35°C and a test potential of +10 mV. However, if the exchanger is deregulated by proteolysis, the exchanger current can be increased to ~ 30 pA/pF [42]. Therefore the exchanger *can* provide a calcium influx comparable to that of I_{Ca} , albeit under experimental conditions that increase outward exchanger current by deregulation and/or at very high $[Na]_i$. Such high levels of exchanger activity should not normally occur during depolarisation in the physiological range, even when accompanied by the activation of I_{Na} (see above). It may be argued that at the peak of the action potential (+40 mV) calcium influx via I_{Ca} will be reduced while the Na/Ca exchange-mediated influx will increase. However, the voltage dependence of the exchanger is quite shallow (changing e-fold in ~ 70 mV) and the stimulation of reverse-mode Na/Ca exchange by voltage should be more than offset by the concomitant decrease I_{Na} (which will change e-fold in ~ 27 mV) leading to a reduction in local Na accumulation. This view is supported by some recent estimates of exchanger current density during the action potential; at the time of peak I_{Ca} , the exchanger current density was less than 0.55 pA/pF whereas I_{Ca} was 3.9 pA/pF [44].

It should also be noted that any residual unblocked I_{Ca} can give rise to a calcium transient (e.g., Fig. 5) and that it may not always be possible to detect such a small current against larger background currents (in addition, the residual I_{Ca} is not readily seen in the low-gain current record of Fig. 5B). This problem will always be present at some level, since no channel blocker can produce complete block except at infinite concentration (from mass action). Therefore, it would be necessary to show that the amplitude of the unblocked I_{Ca} is not sufficient to explain the observed calcium transient before one could be sure of the importance (or existence) of an I_{Ca} -independent release pathway.

4.4. The role of SR Ca^{2+} content

Levesque et al. [24] showed that the ability of I_{Na} to induce SR Ca^{2+} release appeared to depend on the amount of Ca^{2+} in the SR. The relationship between SR Ca^{2+} release and L-type Ca^{2+} current amplitude has been noted

to be variable among studies [43], and such differences could be partly explained by alterations in SR calcium content affecting the sensitivity of CICR [35]. Additional support for this idea comes from the observation that when the SR load is increased by a conditioning train, the Ca^{2+} release evoked by a submaximal I_{Ca} trigger (2 ms depolarisation to 0 mV) increases from 25 to 54% of the maximum [34]. It is therefore possible that the increase in SR Ca^{2+} content during conditioning trains increases the sensitivity of CICR to the point where even a small Ca^{2+} influx via the Na/Ca exchanger can produce calcium release. Unfortunately, there is no simple way to set the exact level of SR Ca^{2+} loading in different experiments. Nevertheless, this possibility provides an additional explanation for the different results obtained in this study compared to those described earlier [17,22,24].

From all of the above considerations, we suggest that the exchanger-mediated calcium influx is unlikely to be as large as that due to I_{Ca} under normal physiological conditions [44]. Our failure to demonstrate I_{Na} -induced calcium release should *not* be taken to imply that the exchanger *cannot* evoke SR calcium release, but rather that the relative magnitude of I_{Na} -induced calcium release depends heavily on many other factors such as the SR calcium content, temperature and internal $[Na]$ levels as well as the accumulation of Na during I_{Na} and the extent of exchanger proteolysis produced by the experiment. Since these factors are not necessarily well controlled during experiments, one may expect wide variations in the reported ability of the exchanger to trigger SR calcium release. In contrast, I_{Ca} -induced SR calcium release is always observed, even under depolarised conditions (e.g., [34,44]). Nevertheless, the exchanger could become more important in triggering SR calcium release under conditions that lead to a large increase in $[Ca^{2+}]_i$ as there will be: (1) a decrease in the magnitude of I_{Ca} (due to calcium-dependent inactivation); (2) an increase in the SR calcium content which may decrease the amplitude of the calcium influx needed to trigger release; and (3) an increase in internal $[Na]$ due to the exchanger increasing calcium extrusion at rest which will promote calcium entry during the action potential [1,16,33,34]. Under such conditions, it is also possible that calcium-dependent proteases may lead to exchanger deregulation, which will increase the rate of calcium influx via the exchanger. Such a shift in the dependence of E–C coupling from the calcium current to the exchanger could provide a mechanism to help maintain myocardial contraction under pathological conditions. In connection with this point, it is notable that the first observation of exchanger-mediated calcium release was obtained in a calcium-overloaded preparation [16].

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