Effect of internal sodium and cellular calcium load on
temperature-dependence of the Indo-1 transient in guinea-pig ventricular
myocytes

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Received 30 October 1995; accepted 17 April 1996

Abstract

Objective: To investigate the effect of altering internal Na and cell Ca load on the temperature-dependence of the intracellular Ca
transient. Methods: Ventricular myocytes were isolated enzymatically from the guinea-pig heart. They were patch clamped and dialysed
internally with pipette solutions which contained either 0 or 10 or 20 mM Na. Intracellular Ca was monitored with Indo-1 and
experiments were carried out at 36°C. A standard level of Ca loading was established before each test pulse by applying a train of
conditioning pulses. The temperature-dependence of the Ca, (Indo-1) transient provided information about the mechanisms which trigger Ca
release from the sarcoplasmic reticulum (SR). Results: The temperature-dependence of L-type Ca current (I_{Ca,L}) was assessed in separate
experiments by dialysing myocytes with a Cs-based solution, I_{Ca,L} had a maximum amplitude at 0 mV, declined at more positive
potentials and there was little net inward I_{Ca,L} at +100 mV. The rapid initial phasic component of the Indo-1 transient was abolished by
ryanodine/thapsigargin; therefore, this component reflected the magnitude of SR Ca release. In cells dialysed with 10 mM Na, the
temperature-dependence of the Indo-1 transient was different from I_{Ca,L}. The Indo-1 transient became maximal at +20 mV, and the decline
of the Indo-1 transient at positive potentials was less steep than the decline of I_{Ca,L}. A large proportion of the phasic Indo-1 transient
could remain at positive potentials where there was no detectable I_{Ca,L}. Increasing dialysing Na from 10 to 20 mM led to a marked
change in temperature-dependence of the Indo-1 transient. With 20 mM Na, the temperature-dependence of the Indo-1 transient became maximal at +20 mV, and the decline
temperature-dependence of the Indo-1 transient at positive potentials was less steep than the decline of I_{Ca,L}. A large proportion of the phasic Indo-1 transient
could remain at positive potentials where there was no detectable I_{Ca,L}. Increasing dialysing Na from 10 to 20 mM led to a marked
change in temperature-dependence of the Indo-1 transient. For each dialysing [Na], the level of cellular and SR Ca content was altered by varying the potential of conditioning pulses
applied before each test pulse. There was no significant effect on temperature-dependence of the Indo-1 transient of either increasing or
reducing the cellular Ca content. Conclusion: These data are consistent with the hypothesis that the temperature-dependence of the Ca
transient results from the sum of the temperature-dependencies of the two main trigger mechanisms—I_{Ca,L} and reverse Na/Ca exchange.

Keywords: Calcium transient; Calcium, intracellular concentration; Calcium channel L-type; Guinea pig, ventricular myocytes; SR, calcium release; Na+/Ca2+ exchange

1. Introduction

The amplitude of intracellular Ca release which can be
evoked in heart muscle by depolarisations to different
potentials provides important information about the mechan-
isms involved in excitation-contraction coupling. Depo-
larisation of the membrane leads normally to a small Ca
entry into the cell, which ‘triggers’ the rapid release of a
much larger amount of Ca from the main intracellular Ca
store, the sarcoplasmic reticulum (SR). This triggering
mechanism, known generally as ‘Ca-induced Ca-release’
(CICR; e.g., [1–3]), allows the magnitude of SR Ca release
to be graded proportionally to the amount of trigger Ca
entry. The rapid release of Ca from the SR leads to a sharp
and transient rise in cytoplasmic Ca concentration (the
‘Ca transient’) which activates the myofilaments to pro-
duce a phasic contraction. Under conditions where a stan-

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PII S0008-6363(96)00100-9
the standard amount of Ca loading is established in the SR before each depolarising test pulse, the dependence of Ca transient (or phasic contraction) on membrane potential might be expected to reflect the voltage-dependence of the mechanisms which provide the trigger Ca entry.

However, previous studies of the voltage-dependence of either contraction (e.g., [4-7]) or the Ca transient [8-10] have produced results which do not agree. Possible reasons for this lack of agreement might be that studies have been carried out on different species (cat, guinea-pig, rat and rabbit myocytes), at different experimental temperatures, and with different concentrations of Na and Mg in the pipette solution which internally dialyses the cell (see [11]). Despite this variability, however, there are a number of general statements which might be made. If Ca entry via the L-type Ca channel (the Ca current, ICa,L) was the only trigger mechanism for SR Ca release, then it might be expected that SR release (and amplitude of the Ca transient) would have a similar voltage-dependence to ICa,L. A ‘bell-shaped’ voltage-dependence of the Ca transient, similar to that for ICa,L, has indeed been found in some previous studies [4,8,9]. This would suggest that (under the particular conditions of these studies), ICa,L might have been the only functioning trigger mechanism for SR Ca release. However, other studies have found that the Ca transient or phasic contraction has a different voltage-dependence from ICa,L [7,10,12]—especially at potentials more positive than +40 mV where the phasic contraction can persist, despite the fact that ICa,L is becoming progressively smaller. It would seem that under the conditions of these studies, ICa,L might not have been the sole trigger mechanism for SR release. It has been a general conclusion that in addition to ICa,L, Ca entry via reverse Na/Ca exchange might also be capable of triggering release of Ca from the SR (e.g., [11,13,14]).

The possible involvement of reverse Na/Ca exchange in triggering SR release leads to two predictions. The first is that voltage-dependence of the Ca transient might reflect the additive effect of the voltage-dependence of ICa,L with the voltage-dependence of reverse Na/Ca exchange. The second prediction is that since reverse Na/Ca exchange will be increased by a raised internal level of Na (e.g., [15,16]), it might be anticipated that voltage-dependence of the Ca transient might be modulated by the level of internal Na. The degree to which voltage-dependence of the Ca transient is modulated might reflect the proportion of Ca transient which can be triggered by reverse Na/Ca exchange.

The objective of this study was to investigate the effect of changing internal Na on the voltage-dependence of the Ca transient in guinea-pig myocytes. We have used Indo-1 to monitor the Ca transient (rather than measuring contraction) because contraction is known to be related to Ca in a highly non-linear fashion [17]. Therefore the Indo-1 transient is expected to provide a more direct indication of the magnitude of SR Ca release (e.g., [18]).

2. Methods

This investigation conformed with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985).

2.1. Cell isolation

Myocytes were isolated from the left ventricle of the hearts of guinea-pigs (300–500 g) using the following method. Animals were injected with pentobarbitone, the heart was removed quickly and the aorta mounted on a cannula for retrograde perfusion (e.g., [5]). The basic (nominally Ca-free) isolation solution (solution A) contained (in mmol-1-1): NaCl 130; HEPES 23; dextrose 21; taurine 20; creatine 5; MgCl2 5; NaH2PO4 1; Na pyruvate 5; titrated to a pH of 7.3 with 4 mmol·1-1 of NaOH. The heart was perfused first with solution A alone towards 12 min as heart weight increased) with an enzyme-containing solution consisting of solution A + 100 μM Ca with 1 mg·ml-1 collagenase (Type 2, Worthington) and 0.1 mg·ml-1 protease (Type XIV, Sigma). The enzyme was then washed out by perfusing solution A + 100 μM Ca for a further 5 min. The left ventricle was removed from the heart, chopped into small pieces and
then shaken for 6 min at 37°C in a glass conical flask containing 50 ml of solution A + 100 μM Ca. The cell suspension was filtered (200-μm mesh), sedimented in a 50-ml glass beaker for 4 min, and the supernatant then replaced with a higher Ca-containing solution; [Ca] was increased first to 500 μM and then to 1 mM. Cells were kept at room temperature in solution A + 1 mM Ca until use, they usually survived well and could be patch-clamped for up to 12 h after isolation.

2.2. Electrical recording

Cells were placed in a perspex chamber with a thin glass bottom, which was mounted on the stage of an inverted microscope (Nikon Diaphot). They were superfused at 36°C with Tyrode's solution containing (in mmol·l⁻¹): NaCl 138; CaCl₂ 2.7; KCl 4.4; MgCl₂ 1; HEPES 12; dextrose 11; titrated to a pH of 7.4 with 6 mmol·l⁻¹ of NaOH. Patch-pipettes (Corning 7052 glass, AM Systems Inc., 1.65 mm o.d., 1.2 mm i.d.) were pulled to resistances of 2.5 to 3 MΩ (Kopf vertical puller) and fire-polished to between 3 and 5 MΩ. The pipette filling solution contained (in mmol·l⁻¹): KCl 110; K₂ATP 5; HEPES 10; MgCl₂ 0; dextrose 5; titrated to a pH of 7.1 by adding 11 mmol·l⁻¹ KOH. The Na concentration of the pipette filling solution was set at 0, 10 or 20 mM by adding appropriate aliquots from an NaCl stock solution. In experiments to measure selectively the voltage-dependence of L-type Ca current (e.g., Fig. 2B), KCl in the standard pipette dialysis solution was replaced by CsCl to block contaminating outward K currents. Patch clamp recordings were made using an Axopatch 200 amplifier and a CV202 headstage (Axon Instruments Ltd). Compensations were made for cell capacitance and series resistance—80% of series resistance could normally be compensated. Membrane current and voltage signals were filtered at 5 kHz. The voltage clamp command waveform was generated by a Labmaster A/D board controlled by PCLAMP 6 software (Axon Instruments). After attaining whole cell recording conditions, each myocyte was left to dialyse with the pipette filling solution for 5 min before voltage protocols and data recording began.

2.3. Monitoring of intracellular Ca

Myocytes were loaded with Indo-1 [22] by incubating with 7.5 μM of the acetoxymethyl (AM) ester (Indo-1 AM; Molecular Probes) in normal Tyrode's solution for 25 min at room temperature. Cells were then left for 30 min in dye-free solution to allow intracellular de-esterification of Indo-1. It has been shown previously that these loading conditions appear to minimise intracellular compartmentation of Indo-1 [23,24]. We used Indo-1-AM loading for these experiments because, in order to use Indo-1 free acid loaded into the cell by dialysis from the patch pipette, it would have been necessary to use pipettes of resistance less than 2 MΩ. However, we found that myocytes dialysed with such low-resistance pipettes displayed a rapid run-down of contraction amplitude over the first 2 to 4 min of whole cell recording, presumably due to rapid and effective internal dialysis. To perform the experiments reported here, it was necessary to preserve normal excitation–contraction coupling for a longer period than this (typically up to 20 min). To do this, it was important that we used pipettes of resistance between 4 and 6 MΩ so that internal dialysis was minimised. It was not possible to load Indo-1 free acid into the cell effectively via these higher resistance pipettes, and thus we used the AM ester loading route for Indo-1.

To detect whether incorporation of Indo-1 might alter Ca buffering, we measured the shortening amplitude (normalised to resting cell length) and shortening time course of 15 myocytes stimulated externally at 0.5 Hz before dye loading, and then the shortening characteristics of a second batch of 15 cells (from the same heart) after Indo-1-AM loading. Before Indo-1 loading, cells shortened by 9.0 ± 1.4% of cell length, whereas cells loaded with Indo-1 shortened by 8.7 ± 0.8% of cell length, there was no significant difference (P > 0.05) between these values. There was also no significant difference in the time to peak contraction of control vs. Indo-1-loaded cells (129.2 ± 8.0 vs. 135.9 ± 7.5 ms; P > 0.05). However, the time to half relaxation was prolonged slightly in Indo-1-loaded cells (70.3 ± 4.5 vs. 112.7 ± 12.0 ms; P < 0.05). These data suggest that incorporation of Indo-1 using this AM loading procedure caused only a small increase in net Ca buffering (e.g., [25]).

The apparatus used for fluorescence measurements has been described previously [26]. Briefly, the system used a high-pressure 200-W mercury arc lamp (Oriel, Stratford, USA) as the excitation source; light first passed through a water filter to remove infra-red components, and then through a 350-nm narrow band pass filter (Corion, USA).
Each cell was illuminated via epifluorescence optics using a 380-nm dichroic mirror and a Fluor ×40 oil immersion objective lens (Nikon, Japan). Fluorescent light emitted by the cell was collected by the objective and passed to two photomultiplier tubes (Hamamatsu Corp, Bridgewater, USA) which measured emitted light at 410 and 500 nm. The light emission signal was restricted to that emitted from one cell by a mechanical diaphragm in the light emission path. The ratio of 410/500 emitted fluorescence was obtained on line by an analogue divider circuit and the

![Graph A](image)

![Graph B](image)
ratio signal was filtered at 300 Hz. The cell image was displayed on a TV monitor at the same time as monitoring fluorescence by illuminating the cell with red light (> 600 nm; Kodak Wratten filter) from the upper microscope light source. Light > 600 nm in the emission light path was directed to a CCD TV camera (Pulnix, Sunnyvale, USA) mounted on the microscope side port by a 600 long-pass dichroic mirror placed before the photomultiplier tubes.

In all the figures, the Indo-1 measurements have been expressed as the 410/500 Indo ratio, since it is accepted generally there are a number of uncertainties in accurately calibrating cells for Ca when loaded with the AM ester (e.g., [27,28]). Because of this issue, we designed the study in such a way that each experiment had its own internal control and did not depend on calibration of Indo-1 for Ca. Before a test pulse to each potential, a train of 6 conditioning pulses was applied to establish a standard level of cellular and SR Ca load (e.g., Fig. 1A). The amplitude and time course of the Indo-1 transient elicited by a test pulse to each potential was compared routinely with the Indo-1 transient elicited by the conditioning pulse which immediately preceded each test pulse. Background fluorescence was subtracted from all signals and auto-fluorescence was less than 1% of individual 410 and 500 nm signals.

2.4. Chemicals, data analysis, statistics

Ryanodine was purchased from Research Biochemicals and thapsigargin obtained from Sigma. Data were recorded using a 4-channel digital data recorder (Vetter) and processed ‘off-line’ using PCLAMP 6 software, or else data were displayed on a thermal array chart recorder with a frequency response up to 10 kHz. Data are expressed as mean ± s.e.m. values. To compare observations within the same group, a two-sample paired Student’s t-test was used. To compare observations from different groups with unequal sample size, a two-sample Student’s t-test was used in which sample variance was not assumed equal (Microsoft Excel version 5, statistical functions). The two-tail P-value was used to compare groups of data and a value less than 0.05 was taken as significant. In the figures, ‘*’ refers to a P-value of < 0.05 and ‘**’ to a P-value of < 0.01.

3. Results

The basic pulse protocol used for all the experiments in this paper is shown in Fig. 1A. The membrane potential of myocytes was held at −40 mV to inactivate fast Na current (I_{Na}) and T-type Ca current. To establish a standard level of cell and SR Ca loading before each test pulse, a train of six conditioning pulses (400 ms duration) from −40 to +10 mV was applied before each test pulse. The test pulse activated after each conditioning train was 800 ms in duration and was changed in successive runs from −20 to +100 mV, in 20-mV increments. The 800 ms duration test pulse allowed the ‘phasic’ and ‘tonic’ components of the Ca transient to be separated—an initial rapid ‘phasic’ increase of Ca will have decayed after 800 ms, whereas a slower ‘tonic’ increase of Ca will become progressively greater over 800 ms. The left panel in Fig. 1A shows the last 5 pulses in the conditioning train—each conditioning pulse elicited a clear inward current spike (largely due to L-type Ca current, I_{Ca,L} since this current is inhibited by the Ca channel blocker nifedipine). With each activation of I_{Ca,L}, there was a rapid phasic rise of the Indo-1 signal (i.e., a Ca transient). The test pulse after the conditioning train was applied to −20 mV; this elicited a small inward spike of I_{Ca,L} and a relatively small Indo-1 transient compared to the +10 mV conditioning pulse. The right panel of Fig. 1A shows a similar protocol, but this time with a test pulse to +60 mV applied after the conditioning train. The +60 mV pulse elicited a small inward spike of I_{Ca,L} with a large Indo-1 transient.

3.1. Components of the Indo-1 signal due to SR Ca release

There are 3 possible sources of Ca for the Indo-1 transient elicited by membrane depolarisation: (a) Ca release from the SR; (b) Ca entry via I_{Ca,X}; (c) Ca entry via reverse Na/Ca exchange. The experiment illustrated in Fig. 1B was performed to assess the role of SR Ca release under these conditions. The myocyte was dialysed with a pipette solution containing 20 mM Na (the highest internal Na level used in this study) to maximise any contribution to the Ca transient from Ca entry via reverse Na/Ca exchange. The left panels of Fig. 1B show the Indo-1 transient elicited by test pulses to different potentials under...
**A. Indo-1 Ratio**

- Membrane current (nA)
  - -20 mV
  - 0 mV
  - +20 mV

**B. Membrane current (nA)**

- -20 mV
- +10 mV
- +40 mV
- +80 mV

**C. Indo-1 transient**

- Ica amplitude 1.2 (relative to 1.0 last conditioning pulse)

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**Graph C**

- 10 mM Na⁺

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**Legend**

- *: Statistical significance
- **: Highly significant
the control situation. Each test pulse is shown with the +10 mV conditioning pulse that immediately preceded it. It can be seen that both the conditioning pulses and each test pulse elicited a rapid initial rise of the Indo-1 transient. As test pulse potential became more positive, there was a larger ‘tonic’ component of the Indo-1 signal, indicated by a larger maintained or progressive rise of the Indo-1 signal during the 800 ms test pulse. The right panels in Fig. 1B show records obtained from the same cell after 10 min exposure to 20 μM ryanodine and 2 μM thapsigargin, which together disable the SR by blocking Ca release and Ca uptake [30,31]. It is clear that the rapid rise of the Indo-1 signal with each depolarisation had been abolished by ryanodine and thapsigargin. However, as test potential became more positive, a small progressive rise of the Indo-1 signal could still be observed during the test pulse at the most positive potentials. Similar results were obtained in 4 cells. These results suggest that the rapid initial rise of the Indo-1 transient elicited by each depolarisation was due primarily to Ca release from the SR.

3.2. Voltage-dependence of the Indo-1 transient in myocytes dialysed with 10 mM internal Na

A level of internal Na close to 10 mM is within the normal physiological range (e.g., [32]). Fig. 2A shows typical recordings from a cell dialysed with pipette solution containing 10 mM Na. Each test pulse to the indicated potential is shown with the last conditioning pulse which immediately preceded it. The top left panel shows that a test pulse to −20 mV elicited little detectable I_{Ca,L} and a relatively small Indo-1 transient. A test pulse to +20 mV elicited a similar size I_{Ca,L} to that for the +10 mV conditioning pulse, and also a similar size Indo-1 transient. With a +60 mV test pulse there was only a small net inward I_{Ca,L}, and yet the initial rapid phase of the Indo-1 transient was of similar size to the conditioning pulse. The lower right panel shows a test pulse to +100 mV. There was no detectable net inward I_{Ca,L} with this pulse, and the Indo-1 transient still had an initial rapid rising phase, the amplitude of which was 56% of that elicited by the +10 mV conditioning pulse. Note also the ‘tail Indo-1 transient’ which occurred on repolarisation from +100 back to −40 mV. Tail transients on repolarisation have been observed in previous studies, and appear to be triggered by a brief Ca entry through the L-type Ca channel which remains open for a brief time after repolarisation [9,33].

The amplitude of the phasic Indo-1 transient was assessed by measuring the initial rapid upstroke phase of the transient at each test potential (to reflect the SR Ca release component of the transient), and expressing this as a fraction of the phasic component elicited by the preceding +10 mV conditioning pulse. This method of measuring the transient prevented any interfering effect from a small run-down in the Indo-1 signal during an entire pulse sequence.

Fig. 2B illustrates membrane currents recorded in a cell dialysed with a Cs-based pipette solution. Due to K channel currents which might overlap with I_{Ca,L} (e.g., [34]), the amplitude of I_{Ca,L} is not measured optimally in cells dialysed with a K-based pipette solution. Therefore, experiments were performed in 6 cells using a Cs-based pipette solution to block outward K currents (see Methods). Fig. 2B shows clearly that a small I_{Ca,L} was elicited by a pulse from −40 to −20 mV, I_{Ca,L} was maximal with a +10 mV pulse, and then declined at positive potentials. At +80 mV, little net inward I_{Ca,L} could be detected. The amplitude of I_{Ca,L} was measured as the difference between peak inward current at the start of the pulse, and steady current at the end of the pulse (e.g., [4,7]).

Fig. 2C compares the voltage-dependence of I_{Ca,L} with the voltage-dependence of the Indo-1 transient. The voltage-dependence of I_{Ca,L} in cells dialysed with a Cs-based solution (open squares) showed a typical ‘bell shape’, with I_{Ca,L} beginning to activate at −20 mV, reaching a maximum amplitude between 0 and +20 mV, and declining to small values at potentials greater than +60 mV. Although it was appropriate to use Cs-based pipette solution to assess I_{Ca,L}, recent evidence suggests that internal Cs might interfere with excitation-contraction coupling (e.g., [19,21]). Therefore all Indo-1 experiments were performed using K-based pipette solution. Fig. 2C shows in addition the mean voltage-dependence of the Indo-1 transient, using results from 9 different myocytes dialysed with K-based solution containing 10 mM Na. The voltage-dependence of the Indo-1 transient had a quite different shape from the voltage-dependence of I_{Ca,L}. Whereas a pulse to −20 mV elicited only 5.8 ± 1.1% of I_{Ca,L}, it elicited 48.5 ± 3.4% of the phasic component of the Indo-1 transient. The amplitude of the Indo-1 transient was maximal at +20 mV, and declined at more positive potentials. The amplitude of the transient at potentials of +40 mV and more positive was significantly smaller than the transient at +20 mV (P < 0.05 or < 0.01). However, the decline in Indo-1 transient at positive potentials was less steep than the decline in I_{Ca,L} over the same potential range. In cells dialysed with 10 mM Na, 61.5 ± 5.6% of the rapid phase of the transient could still be elicited at a test potential of +100 mV, and in contrast there was very little (or no detectable) net inward I_{Ca,L} at this potential.

3.3. The effect of altering cellular Ca load on voltage-dependence of the Indo-1 transient

An objective of this study was to determine the effect of altering cellular Ca load on the voltage-dependence of the Indo-1 transient, for each level of dialysing Na. The Ca loading status of myocytes was altered in 3 ways: (i) To decrease Ca load, conditioning pulses were applied from −40 to only −20 mV (instead of the normal +10 mV conditioning pulse potential). A pulse potential of −20 mV activates a small I_{Ca,L} (e.g., Fig. 2), so that pulse
trains applied to this potential are expected to reduce Ca entry and therefore cellular Ca load. (ii) To increase Ca load, conditioning pulse trains were applied from -40 to +50 mV. A pulse potential of +50 mV is anticipated to cause Ca entry on the Na/Ca exchange in addition to \( I_{Ca, L} \) and thus result in increased cellular Ca loading. (iii) A second method of increasing Ca load, especially useful in cells dialysed with Na-free solution, was to increase the frequency of pulses applied during the conditioning train. Eighteen +10 mV pulses were applied at a rate of 1 Hz (instead of 6 pulses at the normal 0.33 Hz rate) during the conditioning train. This protocol increased the frequency

\[
\begin{align*}
\text{A} & \quad \text{Membrane current (nA)} \\
\text{Indo-1 Ratio} & \quad \text{0} \\
\text{B} & \quad \text{Indo-1 transient (relative to last conditioning pulse)} \\
10 \text{mM Na}_i & \quad \text{0.2 to 1.2} \\
\text{Membrane potential (mV)} & \quad \text{-40 to 100}
\end{align*}
\]

Fig. 3. The effect of altering cellular Ca load. Panel A: Experimental traces to illustrate the pulse protocol used. Left panel: The standard conditioning train consisting of 400 ms duration pulses from -40 to +10 mV was applied. This was followed by an 800 ms duration test pulse to 0 mV. Right panel: In the same cell, each conditioning pulse in the train was applied from -40 to -20 mV, which elicited only a small \( I_{Ca,L} \). The conditioning train was followed by an 800 ms duration test pulse to 0 mV. Panel B: Effect of altering cellular Ca load on the voltage-dependence of the Indo-1 transient, for cells dialysed with a pipette solution containing 10mM Na. The pulses of the conditioning train were applied to from -40 to -20 mV to establish a low level of cellular Ca load. The standard conditioning train with pulses from -40 to +10 mV was used to establish an intermediate cellular Ca load. To establish a high Ca load, a conditioning train with pulses from -40 to +50 mV was used. The plot shows mean results from 9 different cells to which +10 mV conditioning pulses were applied. Mean results from 5 different cells to which -20 and +50 mV conditioning trains were applied are also shown for comparison. There was no significant difference between the voltage-dependence of the Indo-1 transient for each of the 3 levels of Ca load (\( P > 0.05 \) for each test potential).
A

Indo-1 Ratio

Membrane current (nA)

-20 mV

+20 mV

+60 mV

+100 mV

B

Indo-1 transient (relative to last conditioning pulse)

20 mM Na+

-20 mV cond pulse train

+10 mV cond pulse train

+50 mV cond pulse train

Membrane potential (mV)
of $I_{Ca,L}$ activation by 3-fold compared to control, and the increased time integral of Ca entry led to an increased cellular Ca loading before each test pulse.

An example record showing the effect of altering conditioning pulse potential is shown in Fig. 3A. The left panel shows the standard train of +10 mV conditioning pulses applied at 0.33 Hz, followed by a test pulse to 0 mV. The right panel, in the same cell, shows a train of -20 mV conditioning pulses, followed by an identical 0 mV test pulse. Although the Indo-1 transients elicited by -20 mV conditioning pulses were clearly smaller than for +10 mV pulses, it can be seen that both 0 mV test pulses still elicited a phasic Indo-1 transient.

Fig. 3B shows, for 5 cells dialysed with 10 mM Na, the effect of altering cellular Ca load to 3 different levels on the voltage-dependence of the Indo-1 transient. In each cell dialysed with 10 mM Na, for each Ca loading level, a maximal or near-maximal transient was elicited by a +20 mV pulse. Therefore, for each Ca loading level, the amplitude of the phasic transient elicited at each test potential was normalised to the transient elicited by a +20 mV test pulse. It is clear that altering the level of Ca load had little effect on the voltage-dependence of the Indo-1 transient. For each of the 3 levels of Ca loading (-20, +10 and +50 mV conditioning pulses), there was no significant difference between the normalised percentage of Indo-1 transient elicited at each test potential ($P > 0.05$ for all test potentials).

3.4. Voltage-dependence of the Indo-1 transient in cells dialysed with 20 mM Na

Typical Indo-1 transients recorded in a cell dialysed with 20 mM Na are displayed in Fig. 4A. An Indo-1 transient elicited by each indicated test potential is shown together with the preceding +10 mV conditioning pulse. In the upper left panel, a test pulse to -20 mV elicited a small $I_{Ca,L}$, and a small phasic Indo-1 transient. A test pulse to +20 mV elicited a near-maximal $I_{Ca,L}$ and a large phasic transient. In the lower left panel, a +60 mV test pulse elicited only a small net inward $I_{Ca,L}$, and it is notable there was a large phasic transient. Even with a +100 mV test pulse (lower right panel), for which current was net outward throughout the pulse, there was a large phasic transient. There were 3 other notable features of the transients in cells dialysed with 20 mM Na: (i) There was a prominent slow secondary rise of the Indo-1 signal which occurred after the initial phasic rise of the transient, which became larger and more rapid at increasingly positive potentials. (ii) Tail Indo-1 transients were not observed on repolarisation from positive potentials back to -40 mV. (iii) In 4 cells, test pulses were applied to potentials as far positive as +140 mV (the calculated Nernst potential for Ca, $E_{Ca}$, under these conditions). Although this should ensure there was no trigger Ca entry via the L-type Ca channel, we still observed large phasic Indo-1 transients with +140 mV depolarisations (Levi and Li, personal observations).

Fig. 4B shows the mean voltage-dependence of the Indo-1 transient for cells dialysed with 20 mM Na. In addition, it shows the effect of altering cellular Ca load on voltage-dependence of the transient. The data for mean voltage-dependence of the transient with +10 mV conditioning pulse trains (filled circles) came from 7 cells. Compared to the transient elicited by a +20 mV pulse, the transients elicited by pulses to less positive potentials (0 and -20 mV) were significantly smaller ($P < 0.05$ or $< 0.01$). However, there was no significant difference in the transient elicited by a +20 mV pulse, and the transients elicited by potentials between +40 and +100 mV ($P > 0.05$). Thus, the amplitude of the phasic transient was independent of membrane potential over the range +20 to +100 mV.

In 5 cells we were able to obtain runs using either -20 or +50 mV conditioning pulse trains to decrease or increase cellular Ca load, respectively. It is clear from Fig. 4B that altering the level of cellular Ca load had no significant effect on the voltage-dependence of the transient, in cells dialysed with 20 mM Na. For each conditioning train pulse potential used (-20, +10 and +50 mV) there was no significant difference in the voltage-dependence of Indo-1 transient ($P > 0.05$ for each level of test potential).

3.5. Voltage-dependence of the Indo-1 transient in cells dialysed with Na-free solution

A representative record illustrating Indo-1 transients elicited in a cell dialysed with Na-free pipette solution is shown in Fig. 5A. Membrane currents and Indo-1 transients elicited by each test pulse are shown together with the preceding +10 mV conditioning pulse. There was a
A

Indo-1 Ratio

Membrane current (nA)

B

Indo-1 transient (relative to last conditioning pulse)

Na-free dialysis

0.33Hz, +10mV cond pulses

1Hz, +10mV cond pulses

Membrane potential (mV)
small $I_{\text{Ca,L}}$ and Indo-1 transient at $-20$ mV; with a test pulse to $+60$ mV the amplitude of $I_{\text{Ca,L}}$ was small and 80% of phasic transient was still elicited. At a pulse potential of $+100$ mV, however, there was only a small phasic component of the transient, and a tail Indo-1 transient could be observed on repolarisation back from $+100$ to $-40$ mV.

The mean voltage-dependence of the transient for cells dialysed with Na-free solution is displayed in Fig. 5B. The data for the control situation (+10 mV conditioning pulses at a rate of 0.33 Hz) were averaged from 6 cells (indicated by solid circles). The amplitude of the phasic Indo-1 transient reached a maximum at $+20$ mV, and at more positive potentials the transient declined. The amplitude of the phasic component of the transient was significantly smaller for pulses between $+60$ and $+100$ mV than for a $+20$ mV pulse ($P < 0.05$, or < 0.01). A $+100$ mV test pulse elicited an Indo-1 transient, and the phasic component of this transient had a mean value of $44 \pm 3.6\%$ of the phasic transient elicited by the preceding $+10$ mV conditioning pulse.

The level of cellular Ca load might be anticipated to be relatively reduced in cells dialysed with Na-free solution compared to 10 mM (or 20 mM) Na dialysis; therefore we investigated the effect of increasing cellular Ca load on voltage-dependence of the transient. The level of cellular Ca load was increased by raising the frequency of $+10$ mV conditioning pulses from 0.33 to 1 Hz, to increase the time integral of Ca entry via $I_{\text{Ca,L}}$. There was no effect on voltage-dependence of the Indo-1 transient of increasing time integral of Ca entry via $I_{\text{Ca,L}}$. There was no effect on voltage-dependence of the phasic transient elicited at the same potential in cells dialysed with 10 mM Na.

Fig. 6 shows the mean voltage-dependence of the Indo-1 transient for cells dialysed with Na-free solution. Mean results are displayed from 6 different cells (obtained from cells dialysed with a Cs-based pipette solution) has been plotted on the same graph for comparison (open squares). It is clear that the level of dialysing Na has a significant effect on the voltage-dependence curve. Cells dialysed with Na-free solution showed a significant decline in phasic Indo-1 transient amplitude between $+20$ and $+100$ mV. Cells dialysed with 20 mM Na showed no decline in phasic Indo-1 transient amplitude between $+20$ and $+100$ mV. Symbols close to each data point indicate whether the Indo-1 transient at a particular potential was significantly different from the transient elicited at the same potential in cells dialysed with 10 mM Na solution. * $P < 0.05$; * * $P < 0.01$.

Three conclusions might be made from these results: (1) The voltage-dependence of the Indo-1 transient in guinea-
pig myocytes at 36°C can be modulated by the level of internal Na. (2) Altering the level of cellular Ca load, which is expected to be accompanied by a parallel change in SR Ca load, had little effect on the voltage-dependence of the Indo-1 transient, for each level of internal Na. (3) For all levels of dialysing Na, even for dialysis with Na-free solution, the voltage-dependence of the Indo-1 transient was different from the voltage-dependence of \( I_{\text{Ca,L}} \).

4.1. Mechanisms contributing to the Indo-1 transient activated by depolarisation

When the SR was disabled with ryanodine and thapsigargin, the phasic rise of the Indo-1 transient became almost completely abolished. This indicated that, under these experimental conditions, the phasic component of the Indo-1 transient was due primarily to SR Ca release. Although there was still a prominent \( I_{\text{Ca,L}} \) in the presence of ryanodine and thapsigargin, a rise of the Indo-1 signal could not be detected with each activation of \( I_{\text{Ca,L}} \). There are two possible explanations for this. First, Ca entry via \( I_{\text{Ca,L}} \) might be relatively small, and although adequate to cause a local accumulation at the SR to activate CICR, it causes little increase in whole cell \( C_{\text{a}} \) (e.g., [35,36]). A second possibility is that the Indo-1 system might not have been sensitive enough to detect a small \( C_{\text{a}} \) rise. However, this would seem unlikely since we were able to detect small increases of \( C_{\text{a}} \) which were caused by relatively small 10 or 20 mV depolarisations from the holding potential of \(-40 \text{ mV}\).

A third mechanism (in addition to SR Ca release and Ca entry via \( I_{\text{Ca,L}} \)) which might contribute to the \( C_{\text{a}} \) transient is Ca entry via reverse Na/Ca exchange. In the first few milliseconds after a step depolarisation (before whole-cell \( C_{\text{a}} \) has begun to rise), if membrane potential suddenly becomes more positive than the reversal potential of the exchange, then (in cells with a finite level of internal Na) this is expected to make the exchange work in reverse mode and so generate a Ca entry (e.g., [37,38]). Whilst such an initial Ca entry may cause a local accumulation at the SR to activate CICR (e.g., [12–14]), it might have little impact on whole-cell \( C_{\text{a}} \) and therefore not be detected by the Indo-1 signal. However, in the presence of ryanodine and thapsigargin, it was noticeable that 800 ms test depolarisations to the most positive potentials (+80 and +100 mV) caused a slow progressive rise of the Indo-1 signal during the pulse. These ‘tonic’ rises of \( C_{\text{a}} \) have been shown previously to be due to a continuous Ca entry via reverse Na/Ca exchange during depolarisation (e.g., [29]). Even in cells with a normally functioning SR, after the initial phasic component of the \( C_{\text{a}} \) transient, we recorded a slow tonic rise which was greatest in cells dialysed with 20 mM Na, and became larger and more rapid with greater depolarisation (compare Figs. 2 and 4).

4.2. The Indo-1 signal and intracellular Ca

It is a relevant issue for this study to consider how accurately the amplitude of the Indo-1 transient might reflect the magnitude of the cytoplasmic Ca transient. (a) We loaded myocytes with Indo-1 by using the AM ester. We chose to use this technique rather than use the free acid of Indo-1, since free acid loading necessitated the use of low-resistance patch pipettes, and we found that excitation–contraction coupling became rapidly and irreversibly affected when cells were dialysed with low-resistance pipettes. (b) It is an unavoidable consequence of Ca indicator loading that there will be an increase of \( C_{\text{a}} \) buffering. We observed a slower relaxation of contraction in Indo-1-loaded cells, and this is consistent with an increase in \( C_{\text{a}} \) buffering. However, this buffering effect did not appear to be large, since there was no significant change in the amplitude of contraction, or in time to peak contraction, in Indo-1 loaded cells. In addition, the voltage-dependence of the Indo-1 transient in cells dialysed with 10 mM Na had a similar shape to the voltage-dependence of phasic contraction reported in other studies [5,7,39]. Thus, it would appear that the increase in \( C_{\text{a}} \) buffering under these conditions did not have a major effect on voltage-dependence of the \( C_{\text{a}} \) transient. (c) A number of previous studies have used a similar Indo-1 loading procedure and identical fluorescence equipment to the one used in this study, and have provided much information about the calibration parameters of Indo-1-AM for \( C_{\text{a}} \). The standard equation relating the Indo-1 ratio to \( C_{\text{a}} \) is [22]:

\[
C_{\text{a}} = K_D \cdot \beta \cdot \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)}
\]

where \( K_D \) = the dissociation constant of Indo-1 for \( C_{\text{a}} \), \( \beta \) = the ratio of 500 nm fluorescence in zero \( C_{\text{a}} \) to that in saturating \( C_{\text{a}} \). \( R = \) Indo-1 ratio at a given \( C_{\text{a}} \), \( R_{\text{max}} = \) Indo-1 ratio in saturating \( C_{\text{a}} \), \( R_{\text{min}} = \) Indo-1 ratio in zero \( C_{\text{a}} \).

\( R_{\text{min}} \) for this study was between 0.18 and 0.3, \( R_{\text{max}} \) between 0.7 and 1.3, and \( \beta \) between 2.5 and 3. The changes in Indo-1 ratio during the \( C_{\text{a}} \) transient found in this study are very similar to those reported in previous investigations (e.g., [18,23–25,40]). There is some debate, however, between the different studies about the effect of the intracellular environment on the \( K_D \) of Indo-1 for \( C_{\text{a}} \). The \( K_D \) value used conventionally by many previous studies is that measured for Indo-1 in vitro ([Ca] 250 mM; e.g., [22]). However, the in vitro \( K_D \) of Indo-1 for \( C_{\text{a}} \) has also been reported as 400 nM [40] and more recently the in vivo \( K_D \) of Indo-1 for \( C_{\text{a}} \) has been measured as 844 nM using a careful calibration procedure [18]. Based on these results, we constructed two calibration curves for the rela-
4.3. Voltage-dependence of the hMo-1 transient in cells
dialysed with 10 mM Na

In cells with a normally functioning SR, and since SR Ca release appeared to be exclusively responsible for the phasic component of the Indo-1 transient, it would seem reasonable that the amplitude of the phasic transient might provide an index of the magnitude of SR Ca release. In cells dialysed with 10 mM Na, and using a train of +10 mV conditioning pulses to maintain SR Ca content between pulses, we found that the phasic Indo-1 transient had a shallow ‘inverted U-shape’ voltage-dependence. A maximal phasic transient, and therefore presumably a maximal SR Ca release, occurred with a pulse potential of +20 mV. With pulse potentials more negative than +20 mV, the magnitude of SR release declined, but notably it did not fall as steeply as $I_{Ca,L}$ amplitude declined. As potential became more positive than +20 mV, the magnitude of SR release also declined, but once again not as steeply as the amplitude of $I_{Ca,L}$ declined at positive potentials. At +100 mV, 61.5 ± 5.6% of the phasic component of the Indo-1 transient could be elicited; in contrast, at this potential there was no detectable net inward current via the L-type Ca channel.

When conditioning pulse trains are used to ensure that the cell has a constant SR load before each test pulse, the voltage-dependence of the transient elicited by test pulses might be expected to reflect the voltage-dependence of the mechanisms which are able to trigger CICR. On this basis, the first point to be made is that the voltage-dependence of the Indo-1 transient was different from the voltage-dependence of $I_{Ca,L}$; therefore, it would seem that $I_{Ca,L}$ might not be the only trigger mechanism for SR release in guinea-pig myocytes dialysed with 10 mM Na at 36°C. However, a complicating factor is that the voltage-dependence of net current which flows via the L-type Ca channel (including that measured in cells dialysed with Cs) may not reflect precisely the voltage-dependence of the trigger Ca flux which enters via the channel. The L-type Ca channel is known to have significant permeability to both K and Cs ions. Because of this, net channel current does not reverse at $E_{Ca}$ (+140 mV under these conditions) as might be expected from a channel which was selectively permeable to Ca ions. Instead, the net L-type Ca channel current becomes small and begins to reverse at less negative potentials (+60 to +80 mV; e.g., [41,42]) because inward flux of Ca ions is accompanied by a simultaneous outward flux of K (or Cs) ions. Therefore, at a potential of +60 mV, although little net Ca channel current can be recorded, it might be anticipated that there will remain a finite Ca entry via the channel, since this potential is less positive than $E_{Ca}$. At more positive potentials (e.g., +100 mV), net current via the L-type Ca channel may become slightly outward, reflecting the fact that at this potential K (or Cs) efflux via the channel is greater than Ca entry. However, since +100 mV is still less positive than $E_{Ca}$, the driving force for Ca entry is still inward, and so it can be predicted that there might still be an inward flux of Ca at +100 mV. Therefore, it is possible that Ca entry via $I_{Ca,L}$ may be responsible (at least partly) for triggering the residual phasic Indo-1 transient which remains at +100 mV.

For all levels of dialysing Na, at potentials more negative than 0 mV, there was also a disparity between the degree of Indo-1 transient and $I_{Ca,L}$. For instance, in cells dialysed with 10 mM Na, 48.5 ± 3.4% of the phasic transient could be elicited by a pulse to −20 mV, whereas only 5.8 ± 1.1% of maximal $I_{Ca,L}$ was activated. In addition, it would seem that reverse Na/Ca exchange does not appear to take part in triggering CICR over this negative potential range, since voltage-dependence of the Indo-1 transient over this range was not affected by altering dialysing Na between zero and 20 mM. Therefore, with potentials more negative than +20 mV, it would appear that a situation exists whereby a small fraction of Ca entry via $I_{Ca,L}$ is able to trigger a large fractional SR release. One possible explanation is that the gain of $I_{Ca,L}$-induced CICR might be voltage-dependent (e.g., [43]) and at negative potentials the mechanism might have a high gain, such that a small Ca entry via $I_{Ca,L}$ is able to trigger a large fractional SR release. It is possible that the mechanistic basis of this behaviour lies in the random stochastic be-
haviour and spatial localisation of both the L-type Ca channels and SR Ca release channels (e.g., [44,45]).

4.4. The effect of altering dialysing Na on voltage-dependence of the Indo-1 transient

Although previous studies have investigated the effect of dialysing Na on the voltage-dependence of contraction (measured as either myocyte shortening or tension; e.g., [7,12,39]) the present study is one of the first to assess the effect of Na\textsubscript{i} on voltage-dependence of the Ca\textsubscript{i} transient. Since the relation between cytoplasmic Ca and contraction is non-linear [17], it was anticipated that the Ca\textsubscript{i} (Indo-1) transient would provide a more precise index of the magnitude of CICR.

The voltage-dependence of the Indo-1 transient had a more shallow ‘bell shape’ than has been reported for voltage-dependence of contraction in guinea-pig myocytes [5,7,39]. Nevertheless, the effect of altering dialysing Na on voltage-dependence of the Indo-1 transient had some resemblance to that reported for contraction. The main effect of increasing dialysing Na was to elevate the voltage-dependence curve at potentials more positive than +40 mV. With Na-free dialysis, the phasic Indo-1 transient declined significantly at potentials more positive than +40 mV, whereas with 20 mM dialysing Na, there was no significant decline in the Indo-1 transient amplitude between +20 and +100 mV. This resulted in the amplitude of the phasic Indo-1 transient elicited at +100 mV becoming graded with the level of dialysing Na.

Since there was no effect of dialysing Na on the voltage-dependence of I\textsubscript{Ca,l}, there are two other main possibilities for the effect of dialysing Na on voltage-dependence of the Indo-1 transient: (a) It might be due primarily to a different extent of SR Ca loading with each level of dialysing Na. (However, this would appear less likely because altering the level of SR load caused no detectable effect on voltage-dependence of the transient—see later.) (b) The effect of dialysing Na on voltage-dependence of the Indo-1 transient might reflect a role for reverse Na/Ca exchange in triggering CICR. A number of recent studies would seem consistent with the possibility that, in addition to I\textsubscript{Ca,l}, Ca entry via reverse Na/Ca exchange at the start of a step depolarisation might trigger SR release [5,7,12–14,39]. As internal Na rises, this would tend to enhance any depolarisation-activated trigger Ca entry via reverse exchange and allow this mechanism to play a greater role in triggering SR release.

At least theoretically, if a myocyte could be completely depleted of internal Na (especially under the sarcolemma), then it might be expected that I\textsubscript{Ca,l} would become the sole trigger mechanism, and that the voltage-dependence of the Ca\textsubscript{i} transient would become similar to that for trigger Ca entry via I\textsubscript{Ca,l}. However, it is notable that even in myocytes dialysed with Na-free solution, the Indo-1 transient did not have the same voltage-dependence as I\textsubscript{Ca,l}. In part, this might be due to the fact that net I\textsubscript{Ca,l} does not reflect precisely the magnitude of trigger Ca flux via I\textsubscript{Ca,l}. It might also be due to the inability of Na-free dialysis to completely deplete sub-sarcolemmal Na, so that a small component of reverse Na/Ca exchange triggering might still remain.

4.5. The effect of altering cellular Ca load on voltage-dependence of the Indo-1 transient

A potential limitation of altering the dialysing Na level is that this might cause a change in the general Ca loading of the cell. This is expected to be accompanied by a corresponding change in SR Ca content, which might itself influence voltage-dependence of the Ca\textsubscript{i} transient. It was therefore of great importance to investigate directly the effect of changing SR Ca load on voltage-dependence of the Indo-1 transient. We found consistently that when cellular Ca load was varied by altering the potential of conditioning pulse trains, there was no detectable effect on voltage-dependence of the Indo-1 transient.

One mechanism which might be modulated by cellular Ca load is an effect of SR Ca content itself on CICR. Previous workers have put forward the idea that the level of Ca loading inside the SR might regulate the sensitivity of CICR to a given Ca entry trigger, but the evidence so far is contradictory. Some reports suggest that an increase of Ca inside the lumen of the SR Ca might have an inhibitory effect on CICR [46]; in contrast, other studies have suggested indirectly that raised intraluminal Ca might facilitate CICR [47,48]. If there was an effect of SR Ca content on the sensitivity of CICR to trigger Ca, then we might have expected to observe a resulting change in voltage-dependence of the Indo-1 transient. For instance, if increased SR Ca content led to an increased sensitivity of CICR, then it might have been predicted that pulses to negative and positive potentials (which give a submaximal trigger) would produce a larger fractional SR release and Ca\textsubscript{i} transient. However, we found no detectable effect of altering cellular Ca load on voltage-dependence of the Indo-1 transient. A final point is that since a direct alteration of cellular Ca load did not affect voltage-dependence of the transient, whereas altering the level of dialysing Na had a marked effect, this would seem to suggest that the effect of dialysing Na might not be via a change of cellular Ca load. Instead, the results appear more consistent with a direct effect of dialysing [Na] on the magnitude of trigger Ca entry via reverse Na/Ca exchange.

Acknowledgements

For the period of this study, A.J.L. was supported by a two-year Research Leave Fellowship from the Wellcome Trust. The study was also supported by an International Travel Award from the British Heart Foundation to AJL.
(No. FS 94030). A.J.L. was supported by project grants from the Wellcome Trust, British Heart Foundation, Royal Society and United Bristol Healthcare Trust. K.W.S. was supported by the National Heart, Lung and Blood Institute, by the Nora Eccles Treadwell Foundation and by the Richard A. and Nora Eccles Harrison Fund for Cardiovascular Research. We are grateful to Jules Hancox, John Bridge, Jenny Bates, Chris Howarth and John Mitcheson for excellent suggestions on the manuscript. A.J.L. would also like to thank Jackie Addison and Diane Tanner for helpful comments. Amy Morani helped in the cell isolation and setting up of some of the experiments, and Carolyn Lively and Gary Webster provided superb technical assistance.

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