Activation of contraction in cat ventricular myocytes: effects of low Cd\(^{2+}\) concentration and temperature

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Wasserstrom, J. Andrew, and Ana-Maria Vites. Activation of contraction in cat ventricular myocytes: effects of low Cd\(^{2+}\) concentration and temperature. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H488–H498, 1999.—The effects of Cd\(^{2+}\) (20 µM) and different bath temperatures were used to study the contributions of two separate triggering mechanisms, L-type Ca\(^{2+}\) current (I\(_{Ca}\)) and reverse mode Na\(^{+}/Ca\(^{2+}\) exchange, to excitation-contraction (E-C) coupling in cat ventricular myocytes. Ionic currents and cell shortening were studied with patch pipettes filled with K\(^{+}\)-containing internal solution and discontinuous ("switch") voltage clamp. Superfusion with Cd\(^{2+}\) blocked cell shortening that closely mirrored the block of I\(_{Ca}\); the voltage dependence of Cd\(^{2+}\)-induced reduction in contraction was bell-shaped, displaying minima at test potentials below −10 mV and above +50 mV and a maximum at about +20 mV. Cd\(^{2+}\)-insensitive cell shortening was blocked by ryanodine (10 µM) and Ni\(^{2+}\) (4–5 mM). When an action potential was used as the command waveform for the voltage clamp (action potential clamp), Cd\(^{2+}\) reduced contraction to −60 ± 7% of control cell shortening (n = 7). The remaining contraction was blocked by ryanodine and Ni\(^{2+}\). Superfusion with nifedipine (10 µM) caused nearly identical effects to Cd\(^{2+}\). The voltage dependence of contraction was sigmoidal at temperatures above 34°C but bell-shaped below 30°C. When Cd\(^{2+}\) was added to superfusate, contraction was abolished at 25°C (to 6 ± 3% of control) but reduced only modestly at 34°C (to 65 ± 13% of control, test potential +10 mV, n = 4, P < 0.01). These results indicate that 1) there is a component of contraction that is sensitive to I\(_{Ca}\) antagonists, and the block is equivalent with either organic or inorganic antagonists; 2) the contribution of Na\(^{+}/Ca\(^{2+}\) exchange to triggering of contraction under our experimental conditions is fairly linear throughout the entire voltage range tested; 3) the contribution of I\(_{Ca}\) is superimposed on this background component contributed by the Na\(^{+}/Ca\(^{2+}\) exchanger; and 4) triggering via the exchanger is temperature-dependent, providing a major contribution at physiological temperatures but failing at temperatures below 30°C in a nearly all-or-none fashion.

nifedipine; Na\(^{+}/Ca\(^{2+}\) exchange; calcium current; ryanodine; nickel

IT IS WIDELY ACCEPTED that the activation of cardiac contraction is the result of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the sarcoplasmic reticulum (SR) and that the source of the activator Ca\(^{2+}\) is entry via voltage-gated L-type Ca\(^{2+}\) current (I\(_{Ca}\)) (1, 4, 23). A great deal of work has been directed at defining the role and characteristics of Ca\(^{2+}\) entry via this pathway (see Ref. 35 for review). However, Leblanc and Hume (15) reported that activation of fast Na\(^{+}\) current could also activate contraction, even when I\(_{Ca}\) was blocked with 5 µM nisoldipine. These authors concluded that activation of contraction could also be accomplished by activation of Na\(^{+}/Ca\(^{2+}\) exchange in its Ca\(^{2+}\) influx or "reverse" mode. Around that time, several other intriguing reports (2, 3, 24) suggested that Ca\(^{2+}\) influx via the exchanger might occur under conditions of Ca\(^{2+}\) overload; however, the study by Leblanc and Hume (15) was the first report to indicate a role for reverse mode Na\(^{+}/Ca\(^{2+}\) exchange under conditions approaching the physiological. A number of recent reports (14, 17–20, 30, 31, 34) have since confirmed and extended this finding and have indicated that Na\(^{+}/Ca\(^{2+}\) exchange may play a significant role in triggering cardiac contractions at physiological temperatures and in K\(^{+}\)-containing solutions. The field remains highly controversial in light of additional reports (25, 27, 28) suggesting that rapid Ca\(^{2+}\)-induced Ca\(^{2+}\) release was the exclusive result of Ca\(^{2+}\) entry via Ca\(^{2+}\) current and not the Na\(^{+}/Ca\(^{2+}\) exchanger.

The purpose of the present study was to examine the contributions to excitation-contraction (E-C) coupling of the Na\(^{+}/Ca\(^{2+}\) exchanger and I\(_{Ca}\) by taking advantage of their differing sensitivities to temperature and to different classes of antagonists. In particular, we used a low concentration of Cd\(^{2+}\) (20 µM), which we found to be sufficient to block I\(_{Ca}\) but had only modest effects on other aspects of E-C coupling, to try to distinguish between the two mechanisms.

MATERIALS AND METHODS

Isolation of Cat Ventricular Myocytes

The methods used in this study have been described in detail in other publications (30, 31, 34). Cat myocytes were isolated with a modification of the methods of Silver et al. (26). Briefly, mongrel cats of either sex were anesthetized with pentobarbital (45 mg/kg ip), and the heart was removed through a mid-ternal incision. The heart was then mounted on a Langendorff perfusion apparatus and retrogradely perfused through the aorta and coronary arteries with Ca\(^{2+}\)-free modified Krebs-Henseleit buffer (KHb) for 5 min. The heart was then perfused with the same solution containing collagenase (0.08%; CLS III, Worthington Biochemical, Freehold, NJ) for 15–30 min at 37°C. The heart was cut down from the Langendorff apparatus and minced in the enzyme solution, and the chunks were incubated for an additional 5 min. The cells were filtered through a 200-µm nylon mesh and washed with enzyme-free KHb via several centrifugations and resuspended in a Hanks’ balanced salt medium containing 0.2% d-glucose and 16 µg/ml gentamicin sulfate. Typical cell yields were 80–90% with this technique.
Electrophysiological and Mechanical Recordings

We used the discontinuous single electrode ("switch") voltage clamp mode of the Axoclamp-2 amplifier (Axon Instruments, Foster City, CA) with 2- to 4-MΩ glass micropipettes filled with pipette solution. Voltage control was determined to be suitable if the settling time of the current in response to a voltage step from −65 mV to −95 mV was < 0.5 ms at a switching rate of 20 kHz. The advantage of the switch clamp is that it allows simultaneous measurement of both current and transmembrane potential, not the command voltage, within the limitations of an A-D sampling frequency of 5 kHz.

Several drops of cell suspension were placed in the experimental chamber (0.5 ml vol), which was mounted on the stage of an inverted microscope (Nikon Diaphot TMD, Nikon Optical, Tokyo, Japan). After 5–10 min it was allowed for the cells to stick to the glass chamber bottom, the chamber was perfused (1–2 ml/min) with Tyrode solution heated with a Peltier device to temperatures ranging from room (21–22°C) to 37 ± 1°C. Only normal-appearing, rod-shaped, Ca2+-tolerant myocytes with visible cross-striations were used for this study. The visual image was recorded via a video camera attached to the side-port of the microscope and displayed on a video monitor; the camera was rotated so that the cell image was aligned with the rasters of a video edge detector (Crescent Electronics, Salt Lake City, UT). The pipette was pressed gently against the cell surface, and suction was applied, which allowed a gigahm seal to form within 3–5 min. Subsequent application of a brief suction pulse ruptured the membrane patch, giving electrical and physical access to the cell interior. Voltage and current clamp protocols were then directed by pClamp 6 software (Axon Instruments) on a 486 PC. The analog signals from cell shortening and transmembrane potential and current were digitized at 5 kHz and stored in pClamp 6 files for later analysis. All voltage clamp protocols were preceded by 1–5 conditioning pulses to +100 mV for 100–700 ms (interpulse interval of 1.5 s) to ensure that the SR was sufficiently loaded with Ca2+ to produce ICaR and activation of cell shortening even in the maintained presence of Ca2+-channel blockers. The number of pulses was determined at the beginning of each experiment so that Ca2+ overload could be avoided (as indicated by the development of aftercontractions or transient inward current) and maximal contraction could be activated. A 1.5-s interval was interposed between conditioning pulses and test pulses, and a 3- to 5-s interval was imposed before the start of the next protocol. Solution exchange in the experimental chamber was completed within 2 min.

Action Potential Voltage Clamp

The action potential voltage clamp was accomplished by recording an action potential in current clamp mode (152 μs/sample) from a cat ventricular myocyte under the same conditions described in Electrophysiological and Mechanical Recordings at 37°C. Elimination of the stimulus artifact was accomplished by use of a brief current pulse (1 ms) and the start of acquisition immediately after the pulse. The digital-to-analog form of the action potential was then played back with pClamp 6 software. The same action potential waveform was used in all experiments to eliminate variability between native action potentials, especially after addition of interventions known to alter action potential configuration dramatically, such as Cd2+, nifedipine, and Ni2+.

Chemicals and Solutions

All chemicals and pharmacological agents were obtained from Sigma (St. Louis, MO). Nifedipine was dissolved in Tyrode solution at a concentration of 10 µM and stored in the dark for several hours before use. Modified Tyrode solution. Modified Tyrode solution contained (in mM): 140 NaCl, 5.4 KCl, 0.5 MgCl2, 2 HEPES, 0.4 NaH2PO4, 11 glucose, and 1.8 CaCl2 (pH 7.4 with NaOH).

PiPette solution. PiPette solution contained (in mM): 120 K-aspartate, 25 KCl, 0.5 MgCl2, 6 NaCl, 4 K2-ATP, 0.06 EGTA, and 20 HEPES (pH 7.2 with KOH at 37°C). The measured free intracellular Ca2+ concentration under these conditions was 82 nM with indo-1 fluorescence. Free intracellular Mg2+ concentration was not measured and is likely to be below physiological levels. However, as Litwin et al. (21) have recently indicated, high levels of Mg2+ added to patch electrode filling solutions interfere with E-C coupling, although the mechanism for this action is not yet known. For this reason, we chose to use filling solutions similar to the ones they used.

Measurements of ICa were made in identical solutions except that Cs+ was substituted for K+ and tetramethylammonium for Na+ in both internal and external solutions, with the exception of the 4 mM K2-ATP.

KHB solution. KHB solution contained (in mM): 130 NaCl, 4.8 KCl, 1.2 MgSO4, 1.2 NaHCO3, 25 d-glucose, and 2 CaCl2 (pH 7.4 when bubbled with 95% O2-5% CO2).

Data Analysis

Data are presented as means ± SE. Data were compared with a paired or unpaired Student's t-test or a one-way ANOVA (with secondary comparisons made with a Newman-Keuls test). Differences between sample means were considered significant if P < 0.05 unless indicated otherwise.

RESULTS

Efficacy of ICa, Block by Cd2+

It is well-known that high concentrations of Cd2+ (0.1–0.3 mM) block cardiac ICa (31). However, we have recently found that micromolar concentrations of Cd2+ directly affect the cardiac sarcoplasmic reticulum Ca2+-release channel (33), so we wanted to determine the lowest concentration of Cd2+ that blocked ICa under our experimental conditions. Figure 1A shows the effect of low concentrations (10–20 µM) of Cd2+ on ICa in Cs+-containing solutions at 37°C. Figure 1A shows a family of original current traces activated in response to test potentials (Vh) ranging from −30 to +50 mV from a holding potential (Vh) of −70 mV (left) and −40 mV (right). External Na+ was replaced with tetramethylammonium in this experiment and four other similar experiments. Inward ICa was activated at about −20 mV, increased in magnitude until a peak was achieved at +10 mV, and then declined to a minimum at +50 mV. ICa was greater at all Vh when Vh = −70 mV than at Vh = −40 mV, presumably because of a greater level of steady-state availability at the more negative voltage. After superfusion with 10 µM Cd2+, most but not all inward current was blocked at all Vh at both Vh. Increasing the Cd2+ concentration to 20 µM blocked virtually all remaining inward current at both Vh. The graph in Fig. 1B summarizes the results of five experiments of this type (20 µM Cd2+) at Vh of both −40 mV and −70 mV because these are the conditions of primary interest in the present study. Peak inward

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The effects of Cd$^{2+}$ on the voltage dependence of contraction at 25°C are summarized in Fig. 2. Under control conditions including normal internal and external K$^+$-containing solutions, the voltage dependence demonstrates the typical bell shape reported in numerous publications of results obtained at or near room temperature (1, 32). After superfusion with Cd$^{2+}$, contraction was abolished at all test voltages, indicating that contractions activated under these conditions are the result of CICR activated by I$_{Ca}$.

Effects of 20 µM Cd$^{2+}$ on Cell Shortening at Various $V_t$

The effects of Cd$^{2+}$ on cell shortening and ionic current under voltage clamp conditions are shown in Fig. 3. These and all subsequent experiments were performed in K$^+$-containing solutions at 37°C unless specified otherwise. Cell shortening was activated in response to a $V_t$ of +10 mV from a $V_h$ of −40 mV. Net ionic current displayed a rapid inward phase early during depolarization, which was followed by a secondary phase of inward current that declined and became more outward during maintained depolarization. This secondary inward current is thought to result from the removal of Ca$^{2+}$ from the cytoplasm via Na$^+$/Ca$^{2+}$ exchange immediately after its release from the SR because ryanodine suppresses this current (7, 9). After superfusion with Cd$^{2+}$, peak cell shortening was diminished by only ~20%. In contrast, nearly all rapid inward current was abolished by Cd$^{2+}$, leaving only the slowly rising and declining phase. Addition of Ni$^{2+}$ to the superfusate abolished the Cd$^{2+}$-insensitive cell shortening and the secondary “hump” of inward current.

Figure 3B shows the effects of Cd$^{2+}$ at a $V_t$ of +80 mV. A large and rapid contraction occurred in response to depolarization. In addition, there was a large component of outward current that activated rapidly and then inactivated within the first 100 ms of the depolarizing step. During superfusion with Cd$^{2+}$, cell shortening was diminished by only ~12%. Note that the activation time was slowed slightly in the presence of Cd$^{2+}$.

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**Fig. 1.** Block of L-type Ca$^{2+}$ current (I$_{Ca}$) by Cd$^{2+}$ (10–20 µM). A: family of original current recordings activated in response to voltage clamp steps from a holding potential ($V_h$) of −70 mV (left) and −40 mV (right) to test potentials ($V_t$) ranging from −30 to +50 mV. Each test step was preceded by 1 conditioning pulse to +100 mV for 100 ms (see MATERIALS AND METHODS). Top, traces recorded under control conditions; middle, traces recorded after 2 min of superfusion with 10 µM Cd$^{2+}$; bottom, traces recorded during exposure to 20 µM Cd$^{2+}$. External Na$^+$ was replaced with tetramethylammonium, and both internal and external K$^+$ was replaced with Cs$^+$. Current recordings have been leak corrected. B: results of 5 experiments under control conditions (open symbols) and during superfusion with Cd$^{2+}$ (filled symbols) at a $V_h$ of −40 mV (circles) and −70 mV (squares). I$_{Ca}$ was normalized (100%) to its peak value at +10 mV in control ($V_h$ = −40 mV).

Current was measured as the difference between peak inward current and steady-state current at the end of the test voltage step. This concentration of Cd$^{2+}$ blocked I$_{Ca}$ by at least 95%. Also, this concentration of Cd$^{2+}$ was equally effective in blocking I$_{Ca}$ activated from a $V_h$ = −70 mV.

**Effect of Cd$^{2+}$ on Contraction at 25°C**

Most previous reports demonstrating the effects of Ca$^{2+}$ channel antagonists on cell shortening were conducted at room temperature. We tested the effects of Cd$^{2+}$ on contraction to confirm the efficacy of Ca$^{2+}$ channel block on E-C coupling under these experimental conditions.
evoked at both \( V_t \). Ni\(^{2+}\) also blocked the secondary outward current that was associated with the contraction in the presence of Cd\(^{2+}\).

The effects of this concentration of Cd\(^{2+}\) on cell shortening over a wide range of \( V_t \) are summarized in Fig. 3C (top). In controls, cell shortening increased to a maximum at about +20 mV, above which contraction declined slightly but remained near maximal before rising again above +80 mV, giving an N-shaped voltage dependence of contraction quite different from the bell shape shown in Fig. 2 at room temperature (32). Cd\(^{2+}\) had little effect on cell shortening at \( V_t \) up to −10 mV. It significantly reduced shortening at potentials between −10 and +50 mV but had no effect on the magnitude of contraction at more positive potentials up to +100 mV. The fact that the curves under both experimental conditions converge at the extreme positive \( V_t \), even showing some evidence of an increase with Cd\(^{2+}\), suggests that loading of the SR with Ca\(^{2+}\) was nearly equivalent in the two conditions. Fig. 3C (bottom) presents the Cd\(^{2+}\)-sensitive cell shortening that demonstrates a voltage dependence that is nearly identical to \( I_{Ca} \) (Fig. 1).

These results demonstrate that this low concentration of Cd\(^{2+}\) (20 \( \mu \)M) produces a block of cell shortening in cat myocytes that reflects the potent blockade of \( I_{Ca} \). As a consequence, there is little effect on cell shortening at voltages where little Ca\(^{2+}\) entry occurs via \( I_{Ca} \), cell shortening is relatively unaffected by Cd\(^{2+}\) except at \( V_t \) between −10 and +50 mV. Even at these \( V_t \), the extent of block of cell shortening by Cd\(^{2+}\) is ~50% at a maximum (\( V_t = +20 \) to +30 mV), leaving ~50% or more that is Cd\(^{2+}\) insensitive. This result is not expected according to the traditional view that Ca\(^{2+}\) influx via \( I_{Ca} \) provides the exclusive trigger for E-C coupling.

Table 1. Effects of Ni\(^{2+}\) and ryanodine on Cd\(^{2+}\)-insensitive contractions

<table>
<thead>
<tr>
<th>Test Potential, mV</th>
<th>+10</th>
<th>+80</th>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>132 ± 20</td>
</tr>
<tr>
<td>Cd(^{2+}) (20 ( \mu )M)</td>
<td>64 ± 12*</td>
<td>99 ± 31</td>
</tr>
<tr>
<td>+Ni(^{2+}) (5 mM)</td>
<td>0†</td>
<td>0†</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>79 ± 15</td>
</tr>
<tr>
<td>Cd(^{2+}) (20 ( \mu )M)</td>
<td>62 ± 8*</td>
<td>154 ± 26*</td>
</tr>
<tr>
<td>+Ryanodine (10 ( \mu )M)</td>
<td>0†</td>
<td>1 ± 1†</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>105 ± 12</td>
</tr>
<tr>
<td>Nifedipine (20 ( \mu )M)</td>
<td>59 ± 10*</td>
<td>72 ± 10*</td>
</tr>
<tr>
<td>+Ni(^{2+}) (5 mM)</td>
<td>0†</td>
<td>0†</td>
</tr>
</tbody>
</table>

Data are percentage of corresponding value taken at +10 mV under control conditions; \( n = 5 \) for experiments 1 and 3; \( n = 4 \) for experiment 2. *\( P < 0.05 \) vs. control. †\( P < 0.05 \) vs. Cd\(^{2+}\) or nifedipine alone.
Evidence that Cd$^{2+}$-Insensitive Cell Shortening is Activated by SR Ca$^{2+}$ Release

Because the Na$^+$/Ca$^{2+}$ exchanger is capable of bringing sufficient amounts of Ca$^{2+}$ into the myocyte to activate myofilaments and contraction directly, it is important to demonstrate that the contractions that remain in the presence of Cd$^{2+}$ are the result of CICR from the SR. Figure 4 shows the results of an experiment similar to that shown in Fig. 3 where ionic current and cell shortening were activated at $V_t = +10$ mV from a $V_h = -40$ mV. Under control conditions (Fig. 4, left), an early inward current component was not evident in the net current recording but a substantial secondary inward current was apparent. A large contraction was activated in response to the step depolarization. During superfusion with Cd$^{2+}$ (Fig. 4, middle), the early inward current was diminished and contraction was reduced by ~25%. Addition of ryanodine (10 µM) completely eliminated the contraction and most of the secondary inward current that remained in the presence of Cd$^{2+}$.

The data from several experiments are summarized in Table 1. Contractions remaining in the presence of Cd$^{2+}$ at selected $V_t$ (+10 mV and +80 mV) were completely abolished by ryanodine. These results indicate that the contractions activated during blockade of I$_{Ca}$ with Cd$^{2+}$ were a consequence of the normal process of CICR from the SR and not a result of direct activation of the myofilaments by Ca$^{2+}$ entering via Na$^+$/Ca$^{2+}$ exchange.

Effects of Nifedipine on Cell Shortening in Cat Ventricular Myocytes

We also examined the effects of the I$_{Ca}$ antagonist nifedipine on cell shortening to determine if the effects of Cd$^{2+}$ were unique or were common to any agent that blocks I$_{Ca}$. Figure 5 (top) shows a summary of the effects of nifedipine over the same voltage range as that tested with Cd$^{2+}$. The shape of the voltage dependence is again N-shaped under control conditions. The effect of nifedipine was nearly identical to that of Cd$^{2+}$; there is little effect of nifedipine below 0 mV and above +30 mV, whereas there was significant reduction in shortening magnitude between those voltage ranges. Figure 5 (bottom) shows the nifedipine-sensitive component of shortening that, as in the presence of Cd$^{2+}$, is bell-shaped and is most likely a reflection of I$_{Ca}$ block by this concentration of nifedipine in cat myocytes under these experimental conditions (31). When five of these cells were subsequently superfused with Ni$^{2+}$ (4–5 mM),
nifedipine-insensitive contraction was eliminated by these interventions just as they were in the presence of Cd\(^{2+}\) (Table 1).

These results indicate that nifedipine blocks 50% of contraction or less at any V, despite nearly complete block of I\(_{Ca}\). As with Cd\(^{2+}\), the net result is that blockade of contraction with nifedipine (maximum of ~50% inhibition at +20 mV) mirrors its blocking effects on I\(_{Ca}\); however, there is clearly a major component of contraction that is nifedipine insensitive but that is Ni\(^{2+}\) sensitive, suggesting the involvement of reverse mode Na\(^+\)/Ca\(^{2+}\) exchange in the activation of CICR and contraction.

**Effects of Cd\(^{2+}\) and Nifedipine on Contractions Activated by the Action Potential Clamp**

Because the normal stimulus for activation of cell shortening in the intact heart is the action potential, we used a typical action potential, recorded from an isolated cat ventricular myocyte, as the command waveform for the voltage clamp to examine the effects of Cd\(^{2+}\) on cell shortening in response to the normal physiological stimulus.

Figure 6A shows the recorded transmembrane potential (top), ionic current (middle), and cell shortening (bottom) under control conditions. V\(_{h}\) was ~75 mV. As with all protocols, the action potential clamp was preceded by conditioning pulses to +100 mV (300 ms). The voltage waveform shows an early phase of voltage escape during activation of rapid inward Na\(^+\) current (I\(_{Na}\)) and then follows a typical cat ventricular action potential. The current trace displays an associated rapid inward current immediately after the outward capacity transient during rapid depolarization, which was then followed by a small outward current transient during phase 1, a fairly stable current during the plateau of the action potential, and a slowly increasing outward current as the rapid phase of repolarization was approached. A large contraction (15.7 µm) was activated by the action potential clamp. Superfusion with Cd\(^{2+}\) (Fig. 6B) caused a noticeable increase in the outward current transient that occurred during phase 1, which probably reflects the block of I\(_{Ca}\). The current during the plateau was again fairly constant and became net outward during repolarization. Contraction was decreased to ~73% of control (11.4 µm). Similar results were obtained in a total of seven cells that responded to Cd\(^{2+}\) with a reduction of shortening to 60 ± 7% (P < 0.01) of control. After superfusion with Ni\(^{2+}\) (Fig. 6C), all remaining contraction was abolished. A total of four cells was exposed to Ni\(^{2+}\) (4–5 mM), and three others were exposed to ryanodine; both agents completely blocked the Cd\(^{2+}\)-insensitive contraction.

The effects of nifedipine on ionic current and contraction in response to the action potential clamp are shown in Fig. 6, D–F. The control action potential clamp evoked a current response characterized by an inward fast I\(_{Na}\) followed by an inward current transient that declined and became progressively more outward as repolarization occurred. A large contraction (6.4 µm) was activated in response to the action potential stimulus. During superfusion with nifedipine, there were modest changes in the current waveform; most of the inward current immediately after I\(_{Na}\) was abolished with little change in outward current. Cell shortening was reduced to 67% of control (4.3 µm). Note that the rates of both activation and relaxation were slowed during superfusion with nifedipine. Addition of Ni\(^{2+}\) (4 mM) to the superfusate blocked contraction completely during the action potential and removed a slow secondary component of inward-directed current superimposed on the primarily outward current that remained in the presence of nifedipine.

The results of multiple experiments showed that cell shortening in response to the action potential clamp was reduced to 69 ± 9% of control by nifedipine (n = 15; P < 0.01 compared with control) and was completely abolished by Ni\(^{2+}\).

These observations suggest several important characteristics about activation of contraction by an action potential: 1) Cd\(^{2+}\) and nifedipine reduce contraction by <40% even though I\(_{Ca}\) was blocked almost completely and 2) the nifedipine- and Cd\(^{2+}\)-insensitive contraction was abolished by Ni\(^{2+}\), suggesting that its activation relies on reverse mode Na\(^+\)/Ca\(^{2+}\) exchange.
Effects of Temperature on Activation of Contraction

We have previously reported that the voltage dependence of cell shortening is bell-shaped at room temperature (~21°C) but is sigmoidal at 34°C in rat myocytes, confirming the observation in guinea pig myocytes reported by Vornanen et al. (32). We wanted to investigate the temperature dependence of this phenomenon further to define the temperature at which this bell-shaped relation is converted to a sigmoidal one under our experimental conditions.

Figure 7A shows the results obtained from a myocyte in which cell shortening was measured in response to test voltage steps to +10 and +80 mV at four different temperatures. At 37°C, a large and rapid twitch was activated in response to both test voltage steps. Nearly identical results were obtained at 34°C. However, when the temperature was reduced to 30°C, there was no contraction activated during a pulse to +80 mV, whereas a normal contraction was evoked at a V_t of +10 mV. These results were repeated when the temperature was decreased further to 21°C. A contraction was activated after repolarization at this temperature.

The results obtained from 5–7 cells are summarized in Fig. 7B. Only the results for 30°C and 34°C are presented because experiments conducted at 21°C gave nearly identical changes in peak shortening as at 30°C, whereas results obtained at 37°C were the same as at 34°C. The voltage dependence of cell shortening at 34°C (and above) described a constantly increasing relationship, whereas the curve was bell-shaped at 30°C (and below). Several experiments at 32°C showed that a normal contraction was activated up to +10 mV but that contractions at V_t greater than about +40 mV showed a slight decline with progressive depolarization (n = 3, data not shown). These results demonstrate that the voltage dependence of cell shortening is extremely sensitive to temperature; this relationship is sigmoidal at physiological temperatures but is bell-shaped below ~30°C.

Combined Use of Temperature- and Cd^{2+}-Sensitivity to Separate Mechanisms of Contraction

The final series of voltage clamp experiments sought to take advantage of the differing sensitivities of the two different trigger mechanisms to temperature and block by Cd^{2+} to distinguish between them. Figure 8 shows the results of an experiment in which the effects of Cd^{2+} were tested in the same myocyte at two different temperatures at V_t = +10 mV. Figure 8A and B, shows the current and contraction recordings obtained at 34°C and 25°C, respectively. Both current recordings show an early inward current transient, but only the higher temperature shows the slow secondary inward current transient that is thought to occur as a result of removal by Na^{+}/Ca^{2+} exchange of intracellular Ca^{2+} released from the SR. After superfusion with Cd^{2+} (Fig. 8C and D), the early inward current was abolished at both temperatures. At 34°C (Fig. 8C), the contraction was decreased to 70% of control by Cd^{2+} and its continued presence was reflected by the fact that the secondary inward current was still present. In contrast, both the contraction and the secondary inward current were abolished by Cd^{2+} at 25°C. In a total of four experiments, Cd^{2+} reduced contraction to 65 ± 13% of control at 34°C (P < 0.05 compared with control) but nearly abolished contraction entirely at 25°C (6 ± 3% of control, P < 0.01 compared with 34°C with Cd^{2+}).

These results provide a separation between these two mechanisms that are capable of triggering E-C coupling in cardiac cells. Because Na^{+}/Ca^{2+} exchange is suppressed at the low temperature, it seems to be unable to provide sufficient Ca^{2+} influx to activate contraction under these conditions; I_{ca} then provides the only Ca^{2+} influx capable of activating CICR and contraction at the lower temperatures. Consequently, Cd^{2+} blocks contraction completely at all V_t, even though there might still be some functional Ca^{2+} channels available. In contrast, the Na^{+}/Ca^{2+} exchanger is active enough at 34°C to provide sufficient Ca^{2+} influx to activate contraction even when I_{ca} is blocked by Cd^{2+}.

Fig. 7. Temperature dependence of cell shortening. A: contractions evoked by test voltage steps to +10 mV (left) and to +80 mV (right) from a V_h = -40 mV at 4 different experimental temperatures. B: results of voltage dependence of cell shortening at 2 selected experimental temperatures, 30°C (△) and 34°C (○). Cell shortening was normalized to value obtained at +10 mV for each temperature; n = 4–9 measurements for each point. *P < 0.05; **P < 0.01 vs. 30°C.
The effects of Cd\(^{2+}\) (20 µM) on an action potential and cell shortening (37°C) are shown in Fig. 9. In control, the action potential was ~275 ms in duration and the cell shortened by ~6 µm. After addition of Cd\(^{2+}\) to the superfusate, action potential duration was reduced overall with a particularly strong suppression in the plateau voltage range. Contraction was completely abolished. These results were very similar in all six cells exposed to this concentration of Cd\(^{2+}\) and are consistent with the reported effect of Cd\(^{2+}\) to block \(I_{\text{Ca}}\).

**DISCUSSION**

**Effects of Ca\(^{2+}\) Channel Blockade on E-C Coupling**

We found that either organic or inorganic Ca\(^{2+}\) channel antagonists caused block of contraction that closely follows the voltage dependence of \(I_{\text{Ca}}\), such that nearly all of the reduction in contraction developed between \(V_t\) of -10 and +50 mV. The fact that the Cd\(^{2+}\)/nifedipine-insensitive contraction is blocked by Ni\(^{2+}\), which is a known antagonist of the Na\(^{+}\)/Ca\(^{2+}\) exchanger (13, 36), suggests that the remaining contraction is activated by the exchanger. Our results provide a rough estimate for the Ni\(^{2+}\)-sensitive component of ~50% of total contraction magnitude for Cd\(^{2+}\) and about the same for nifedipine at \(V_t = +20\) mV under our experimental conditions. The bell-shaped, Cd\(^{2+}\)-nifedipine sensitive component is superimposed on a second, more linear background component that is responsible for nearly all of the contraction below -10 mV and above +50 mV, with variable proportions of the two components within the intermediate voltage range. It is probably no coincidence that the peak overshoot of the action potential in intact myocardial preparations is ordinarily between +20 and +40 mV; this is the voltage range where the contribution of \(I_{\text{Ca}}\) to triggering is maximal, thus providing the greatest margin of safety for either or both mechanisms to ensure adequate triggering. In fact, cell shortening activated by the action potential damp showed nearly identical reductions by either antagonist (~30-40%) as predicted from the voltage dependence of block.

The fact that both antagonists cause about the same negative inotropic effect on contractions activated by
the action potential voltage clamp also suggests that there is comparable block of \( I_{Ca} \) at \( V_n \) of both \(-40 \text{ mV} \) and \(-70 \text{ mV} \), which is consistent with our previous report of the blocking potency of nifedipine under the same experimental conditions in rat myocytes (31). Therefore, it is unlikely that the two agents produce differing amounts of block during the action potential clamp.

Finally, it is worth noting that there were virtually no differences between the effects of \( \text{Cd}^{2+} \) and those of nifedipine, with the exception of a small decrease in contraction at nearly all test voltages with the latter. This effect is most likely the result of a diminished ability of the SR to accumulate or release \( \text{Ca}^{2+} \) in the presence of nifedipine, which may be related to the known actions of other dihydropyridines to alter SR function in addition to that of the L-type \( \text{Ca}^{2+} \) channel (12, 22).

There have been several recent reports (17, 19, 32) describing only moderate effects of agents that block \( I_{Ca} \) on contraction in ventricular myocytes under voltage clamp conditions. Usually these studies have involved a brief exposure either to nifedipine (17, 19) or to \( \text{Cd}^{2+} \) (32) using a rapid solution switching device. The results have uniformly demonstrated that contraction is diminished by \(-30\%\) during rapid solution exchanges. It is also useful to superfuse the cell with these agents, thus ensuring a more complete blockade of \( I_{Ca} \) than can sometimes develop within the several seconds of exposure, especially with nifedipine, using a rapid switching device. Conversely, prolonged exposure has the drawback that secondary intracellular actions of these agents can interfere with the interpretation of those that are considered to be purely at the level of the sarcolemma. This is clearly observed in the slowing in relaxation observed after prolonged superfusion to both agents. Thus it was reassuring that nearly identical results were obtained with the two approaches.

Effects of Temperature on Contraction

The observation that there is a dramatic sensitivity to temperature of the voltage dependence of cell shortening is very important when comparing our results with other published reports. Nearly all of the information about SR \( \text{Ca}^{2+} \) release and intracellular \( \text{Ca}^{2+} \) transients has been derived from experiments performed at or below 30°C (1, 4, 6, 23). These reports have uniformly found a bell-shaped voltage dependence of contraction that was abolished by \( \text{Ca}^{2+} \) channel blockers. However, the current observations as well as several others (11, 17–19, 30–32, 34) indicate that activation of contraction can also be accomplished by reverse mode \( \text{Na}^{+}/\text{Ca}^{2+} \) exchange at physiological temperatures. The question of temperature dependence is especially important because of the relatively high temperature coefficient (\( Q_{10} \)) of the exchanger estimated at \( 3–4 \) (13). Given this high temperature sensitivity, it is not surprising that \( \text{Na}^{+}/\text{Ca}^{2+} \) exchange could serve as a trigger for contraction at \( 35–37°C \) and yet be unable to deliver an adequate trigger at lower temperatures. This all-or-none behavior would not be expected from any temperature-dependent effects on the triggering efficacy of \( I_{Ca} \) where graded results have been reported for years. The net result would be that only \( \text{Ca}^{2+} \) entering via \( I_{Ca} \) would be available to activate CICR and contraction at these low temperatures, as has been reported extensively and confirmed in the present experiments at \( 25°C \). The fact that other investigators (14, 15) have reported effective activation of \( \text{Na}^{+}/\text{Ca}^{2+} \) exchange-induced CICR and/or sigmoidal voltage dependencies of contraction at room temperature may be related to the fact that higher \( \text{Na}^{+} \) concentration was usually used in the internal solution (10–20 mM), which might promote activation of the exchanger even at the lower temperature.

Contribution of Several Triggering Mechanisms to Cardiac E-C Coupling

A central role for L-type \( \text{Ca}^{2+} \) channels in the activation of contraction is well-known (35). A possible contribution of the \( \text{Na}^{+}/\text{Ca}^{2+} \) exchanger to triggering was recognized under conditions in which intracellular \( \text{Na}^{+} \) was elevated, with a resulting intracellular \( \text{Ca}^{2+} \) overload (2, 3, 24). In addition, Sipido and co-workers (28) have recently examined the role of the exchanger in triggering E-C coupling under similar experimental conditions and concluded that triggering can occur as a result of reverse mode exchange but that it does so with a lower efficacy than \( I_{Ca} \). This conclusion is based on their observation of a delay in activation on the order of hundreds of milliseconds in the presence of \( \text{Ca}^{2+} \) channel blockers, a finding that we observed only on occasion. The differences in experimental findings and interpretation are probably the result of differences in the conditions under which the experiments were performed (particularly SR loading protocols and intracellular \( \text{Na}^{+} \) concentration), and additional experiments are required to sort out these interesting and important discrepancies. However, there have also been some recent suggestions that the exchanger might contribute to E-C coupling under physiological conditions (5, 11, 15). This has become an important issue because of the potential relevance not only in understanding the normal physiology of cardiac contraction but also because of the potential pathophysiological role that the \( \text{Na}^{+}/\text{Ca}^{2+} \) exchanger may have in the development of cardiac disease and, conversely, as a possible therapeutic target for development of new treatment strategies.

It has been difficult to determine the relative contributions of these two mechanisms, and possibly that of a third voltage-dependent mechanism (8), to cardiac E-C because of the artificial environment required for experimental investigation of this problem. However, there are several recent observations that have important implications for our understanding of contraction and the interpretation of much of the previously published information. These studies uniformly demonstrated that experimental conditions are extremely important in determining the results that were obtained. First, we and others (18, 34) have found that the sigmoidal shape of the shortening-voltage relationship observed with the normal \( K^{+} \) gradient is converted to a bell shape in
the presence of Cs+ in the internal solution, suggesting that this ionic substitution, which is extremely useful in measuring I_{Ca}, also affects E-C coupling. Second, there are now several reports (32, 34, and the present study) indicating that the contribution of the exchanger to contraction is highly sensitive to temperature. Third, it is important to ensure sufficient loading of the SR with Ca^{2+} because recent work (29) suggests that the extent of SR Ca^{2+} load determines the efficacy of the trigger for SR Ca^{2+} release. This is likely to underlie the fact that the contribution of the exchanger to triggering can easily be overlooked if the SR is not adequately loaded with Ca^{2+} (16). It is clear that any quantitative consideration of the contributions of the various triggering mechanisms must take into account the experimental conditions under which the study was performed. In this vein, it is important to note that a recent study by Grantham and Cannell (10) used an action potential clamp to quantify the relative contributions of I_{Ca} and reverse mode exchange and concluded that ~30% of trigger Ca^{2+} seems to derive from the latter. It should be noted, however, that these investigators used Cs+ internal solutions, so it is possible that this is an underestimation of the actual contribution of the exchanger that might occur under more physiological conditions.

One very interesting suggestion that might resolve the question of contributions of the different mechanisms to cardiac E-C coupling was made recently by Litwin et al. (21). These investigators propose that there is a nonlinear summation of triggering at the ryanodine receptor as Ca^{2+} entering both by I_{Ca} and by the exchanger causes a rightward shift along the open probability-Ca^{2+} concentration relationship. The result is an amplification of Ca^{2+} release with increasing Ca^{2+} concentration that is independent of how Ca^{2+} is delivered to the release channel. This interesting idea would explain how both mechanisms might act in concert to produce an extremely effective trigger with fine local control of Ca^{2+} delivery to the ryanodine receptor.

Limitations of the Study

An accurate distinction between the two mechanisms of triggering contraction relies on providing sufficient block of one without interfering with the second to study the latter. It is extremely difficult to block all Ca^{2+} channels in a cardiac cell, although this could probably be accomplished with 0.1–1 mM of either agent used in this study. However, there is evidence that there may be appreciable secondary actions that could interfere with E-C coupling directly. In fact, we have found that 10–30 µM Cd^{2+} completely blocks the activity of purified single cardiac Ca^{2+}-release channels measured in artificial lipid bilayers (33). It is also known that modification of I_{Ca} with BAY K 8644 causes alterations in SR Ca^{2+} release (12, 22). Thus the use of high concentrations of either inorganic or organic antagonists to ensure complete block of I_{Ca} may introduce additional complicating factors.

The question then remains, is nearly complete block of I_{Ca} (>95%) sufficient to allow the study of other mechanisms of E-C coupling? To interpret our results in light of conventional reliance of contraction and CICR on Ca^{2+} entry via I_{Ca}, 5% or less of I_{Ca} (Fig. 1B) would have to be responsible for 50–100% of contraction (Figs. 3C and 5). However, we found that contraction was virtually abolished by the same degree of I_{Ca} block after the contribution of the exchanger was removed at low temperature. Under these conditions, there is indeed a close association between I_{Ca} and cell shortening, such that elimination of Ca^{2+} influx via the sole remaining pathway, the voltage-gated Ca^{2+} channels, resulted in virtually complete block of contraction. In fact, according to this principle, it should be difficult to obtain a bell-shaped contraction-voltage relationship under any conditions where SR is well loaded; even a small I_{Ca} should then be sufficient to produce nearly full release if this idea is correct, which is clearly not what is observed. Thus, although it is difficult at this time to quantify the separate contributions of these two mechanisms to E-C coupling, the fact that some Ca^{2+} channels (<5%) might still be available should not necessarily be taken as evidence to invalidate the potential role of the voltage-dependent Na+/Ca^{2+} exchanger as a trigger for E-C coupling.

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