Cs⁺ inhibits spontaneous Ca²⁺ release from sarcoplasmic reticulum of skinned cardiac myocytes

MAKOTO KAWAI, MUNIR HUSSAIN, AND CLIVE H. ORCHARD
Department of Physiology, University of Leeds, Leeds LS2 9NQ, United Kingdom

Kawai, Makoto, Munir Hussain, and Clive H. Orchard. Cs⁺ inhibits spontaneous Ca²⁺ release from sarcoplasmic reticulum of skinned cardiac myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H422–H430, 1998.—The effect of Cs⁺ on the function of the cardiac sarcoplasmic reticulum (SR) function has been investigated in skinned cardiac myocytes. Isolated rat ventricular myocytes were permeabilized using saponin and then perfused with a solution containing 150 nmol/l Ca²⁺ and 10 µmol/l fura 2. Fura 2 fluorescence from the skinned cell was monitored to assess SR Ca²⁺ release. The frequency of spontaneous Ca²⁺ release from the SR decreased when K⁺ in the bathing solution was completely replaced with Cs⁺. Cs⁺ had little effect on the amplitude of spontaneous but prolonged both the rise time and decay time. The SR Ca²⁺ content, assessed by application of caffeine, was reduced in the Cs⁺ solution. Cyclopiazonic acid produced effects similar to those of Cs⁺. Extracellular Cs⁺ (20 mmol/l) increased the amplitude of the Ca²⁺ transient and the SR Ca²⁺ content in intact field-stimulated cells but had little effect on the Ca²⁺ transient when the amplitude and duration of depolarization were kept constant using voltage clamp. These data suggest that Cs⁺ slows Ca²⁺ movement across the SR membrane, possibly by blocking the SR K⁺ channel, but has additional effects in intact cells that overcome its inhibitory effects on the SR.

POTASSIUM IONS (K⁺) are important for the normal electrical activity of cardiac muscle; the transsarcolemmal K⁺ gradient underlies the resting membrane potential and is necessary for the repolarization of the cell membrane at the end of the action potential. The role of intracellular K⁺ in excitation-contraction (E-C) coupling is less clear, but there are K⁺ channels in the cardiac sarcoplasmic reticulum (SR) membrane similar to those in the sarcolemma (13, 22). It has been suggested that K⁺ crosses the SR membrane via these channels to maintain charge balance during Ca²⁺ release and uptake by the SR (1, 21, 22). This idea is supported by measurements of ion concentrations in the terminal cisternae (TC) of skeletal muscle SR that showed that the K⁺ content of the TC increases significantly during tetanic contraction (28).

Cs⁺ is known to block K⁺ channels in the squid giant axon (2) and in single heart cells (17). Cs⁺ also blocks the K⁺ channel in cardiac SR (13, 27) and skeletal SR (5). Blocking the SR K⁺ channel with Cs⁺ leads to inhibition of Ca²⁺ release (1) and uptake (8) in skeletal SR. Despite these effects of Cs⁺, K⁺ is frequently replaced by Cs⁺ in studies of E-C coupling in single heart cells to block sarcoplasmal K⁺ currents (17). However, such replacement appears to alter the size and voltage dependence of the Ca²⁺ transient (12, 14, 15, 18), although the mechanism is unclear. In this study, the effect of replacing K⁺ with Cs⁺ on cardiac SR function has been investigated in chemically skinned ventricular myocytes. Preliminary results have been published in abstract form (16).

MATERIALS AND METHODS

Ventricular myocyte isolation. Ventricular myocytes were isolated from adult female Wistar rat hearts as described previously (11). Briefly, the animal was stunned and then killed by cervical dislocation. The heart was quickly removed and mounted on a Langendorff apparatus and retrogradely perfused with a HEPES (Sigma, St. Louis, MO)-Tyrode solution (see Solutions and chemicals and Table 1) containing 0.75 mmol/l Ca²⁺ for 2–3 min. This solution was then replaced by a Ca²⁺-free solution (see Table 1), and after 4 min the Ca²⁺-free solution was replaced by an enzyme solution (see Table 1) containing 1 mg/ml collagenase (type I; Worthington, Freehold, NJ), 0.1 mg/ml protease (type XIV; Sigma), and 50 µmol/l Ca²⁺ for 6 min. At the end of this perfusion, both ventricles were removed from the heart and cut longitudinally, and the opened ventricles were incubated with the enzyme solution containing 1% bovine serum albumin (Sigma) for 5 min. The cells were then filtered through gauze and collected by centrifugation at 400 rpm for 30 s. Isolated cells were resuspended in HEPES-Tyrode solution until use. This procedure was repeated at 5-min intervals to obtain several batches of cells. These experiments were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986.

Measuring spontaneous Ca²⁺ release using skinned cells. Isolated ventricular myocytes in the HEPES-Tyrode solution were centrifuged and then resuspended in Ca²⁺-free relaxing solution (see Table 2) containing 1.5 mg/ml collagenase and 10 µmol/l fura 2 (pentapotassium salt; Molecular Probes, Eugene, OR) at a rate of 8 µl/s. The single skinned cell under study was held on the bottom of the experimental bath by a glass micropipette. Spontaneous release of Ca²⁺ from the SR was monitored using fura 2 fluorescence at 510 nm during alternate (every 2 s) excitation by 340-nm and 380-nm light from a xenon lamp (100 W; Nikon). The fluorescence excited by these two wavelengths was collected by the objective lens (>40 oil immersion Fluor 40, N.A. 1.30; Nikon) and detected by a photomultiplier (Cairn Research, Kent, UK). The ratio of fura 2 fluorescence at 510 nm during excitation at 340 nm to that during excitation at 380 nm (340 nm/380 nm signal) was determined using an analog divided circuit and used as a measure of Ca²⁺ within the preparation. Shuttles in the light path of

H422 0363-6135/98 $5.00 Copyright © 1998 the American Physiological Society
the emitted fluorescence were narrowed to almost the same dimensions as the image of the skinned cell to exclude fluorescence from sources other than the cell under study. The fluorescence signals and the fluorescence ratio were stored on videotape [on an SR-330MS video recorder (Victor, Tokyo, Japan) via a DR-890 digitizing unit (Neuro Data Instruments, New York, NY)] and on a microcomputer hard disk (model 486DX2/66, Dan Technology, London, UK) for later off-line analysis. The microcomputer used a 1401 plus analog-to-digital (A/D) interface and SIGAVG software (Cambridge Electronic Design, Cambridge, UK) for data storage and analysis. The signals were also displayed on a six-channel chart recorder (model 2600S; Gould, Cleveland, Ohio).

The Ca^{2+} content of the SR was assessed by monitoring Ca^{2+} released in response to pressure ejection of 20 mmol/l caffeine (Sigma) from a pipette placed close to, but upstream from, the cell being studied.

Measuring Ca^{2+} transients in intact cells. The effect of Cs^{+} on intact cardiac myocytes was also investigated by monitoring Ca^{2+} transients and caffeine-induced Ca^{2+} release with the use of fura 2 (cells loaded by 15 min incubation with 3 µmol/l fura 2-AM; Molecular Probes) and almost with the use of fura 2 (cells loaded by 15 min incubation as described for skinned cells). The relaxing solution had the same composition as the HEPES-Tyrode solution. The control solution was used for permeabilizing (skinning) ventricular myocytes, contained 0.05 mmol/l EGTA and 0 Ca^{2+}. The control solution, used during the experiments, contained 150 mmol/l Ca^{2+} and 10 µmol/l fura 2 but was otherwise the same in composition as the relaxing solution. The control solution was prepared by mixing the relaxing solution with the Ca-EGTA solution in proportions necessary to obtain the desired free [Ca^{2+}], which was calculated by solving simultaneous equations using the binding constants listed by Martell and Smith.

### Table 1. Cell isolating solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>K^+</th>
<th>Na^+</th>
<th>Mg^2+</th>
<th>Ca^2+</th>
<th>EGTA</th>
<th>Cl^-</th>
<th>H_2PO_4^-</th>
<th>HEPES</th>
<th>Creatine</th>
<th>Glucose</th>
<th>Taurine</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-Tyrode</td>
<td>5.4</td>
<td>130.4</td>
<td>1.4</td>
<td>0.75</td>
<td>0</td>
<td>139.7</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Ca^{2+} free</td>
<td>5.4</td>
<td>130.4</td>
<td>1.4</td>
<td>0</td>
<td>0.1</td>
<td>138.2</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Enzyme</td>
<td>5.4</td>
<td>130.4</td>
<td>1.4</td>
<td>0.05</td>
<td>0</td>
<td>138.3</td>
<td>0.4</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>20</td>
</tr>
</tbody>
</table>

Values are concentrations of solution contents in mmol/l. The enzyme solution also contained 1 mg/ml collagenase and 0.1 mg/ml protease. pH of all solutions was adjusted to 7.3 at room temperature with NaOH.

### Table 2. Experimental solutions (skinned cells)

<table>
<thead>
<tr>
<th>Solution</th>
<th>K</th>
<th>Cs</th>
<th>Free Mg^2+</th>
<th>Free Ca^{2+}</th>
<th>EGTA</th>
<th>ATP</th>
<th>NaATP</th>
<th>Fura 2</th>
<th>CPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxing</td>
<td>149</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.05</td>
<td>4.0</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ca-EGTA</td>
<td>149</td>
<td>0</td>
<td>1</td>
<td>1.79</td>
<td>0.05</td>
<td>4.0</td>
<td>5.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>149</td>
<td>0</td>
<td>1</td>
<td>0.15</td>
<td>0.05</td>
<td>4.0</td>
<td>5.2</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Cs^{+}</td>
<td>0</td>
<td>150</td>
<td>1</td>
<td>0.15</td>
<td>0.05</td>
<td>4.0</td>
<td>5.0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>CPA</td>
<td>149</td>
<td>0</td>
<td>1</td>
<td>0.15</td>
<td>0.05</td>
<td>4.0</td>
<td>5.2</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>EGTA calibration</td>
<td>139</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>10</td>
<td>4.0</td>
<td>5.2</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Ca calibration</td>
<td>139</td>
<td>0</td>
<td>1</td>
<td>25.12</td>
<td>10</td>
<td>4.0</td>
<td>5.2</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are concentrations of solution contents in mmol/l, except for free Ca^{2+}, fura 2, and cyclopiazonic acid (CPA), which are given in µmol/l. All solutions contained (in mmol/l) 20 NaNO_3 to block Ca^{2+} uptake by mitochondria, 10 Na_2CrP, and 20 PIPES (ionic strength, 0.2 mmol/l; temperature, 20°C; pH adjusted to 7.1 with KOH or CsOH as appropriate). Na^+ was added (in mmol/l) as Na_2ATP, 10 Na_2CrP (disodium salt of phosphocreatine), and 20 NaNO_3. Total Cl^- concentration was similar in the control, Cs^{+}, and CPA solutions used during experiments with skinned cells.
(20). Finally, for K+ replacement experiments, 150 mmol/l Cs+ was used instead of 149 mmol/l K+, and pH was adjusted to 7.1 with CsOH (Sigma).

The solutions for the experiments on intact cells contained 1 mmol/l Ca2+, pH 7.4 (see Table 3), to which CsCl (Sigma) was added to produce a final concentration of 20 mmol/l. The pipette solution used for the voltage-clamp experiments contained (in mmol/l) 120 K-glutamate, 20 KCl, 10 NaCl, 4 MgATP, and 10 HEPES, buffered to pH 7.3 with KOH.

Cyclopiazonic acid (CPA; Sigma) was dissolved in DMSO (Sigma) to make a 20 mmol/l stock solution and was added to give a final concentration of 10 or 50 µmol/l (see Table 2). The control solution contained the same concentration of DMSO as the 10 µmol/l CPA solution, and the percentages of added DMSO were 0.05% (vol/vol) at 10 µmol/l and 0.25% (vol/vol) at 50 µmol/l/CPA.

All other chemicals were reagent grade. All experiments were carried out at room temperature (22–24°C).

Calibration of [Ca2+]i. Calibration of [Ca2+]i in the experimental solutions was carried out using solutions containing various [Ca2+]i: 10 µmol/l fura 2, and 10 mmol/l EGTA. The solutions containing various [Ca2+]i were prepared by mixing two complementary solutions: EGTA-calibration solution and Ca-calibration solution. The apparent dissociation constant of EGTA for Ca2+ was 267 nmol/l (for pH 7.1, 1 mmol/l Mg2+, and 20°C). The compositions of the calibration solutions are shown in Table 2.

Statistical analysis. Data are presented as means ± SE, and statistical analysis was performed using paired t-tests. Statistical significance was taken as P < 0.05.

RESULTS

Effects of Cs+ on spontaneous SR Ca2+ release in skinned cells. Figure 1A shows the effect of replacing control (K+-based) solution with Cs+-based solution on spontaneous Ca2+ release in a skinned cardiac myocyte, showing that Cs+ markedly slowed the frequency of spontaneous release and that this effect was reversible on returning to the control solution. Cs+ also significantly decreased the amplitude of spontaneous Ca2+ release, but the decrease in frequency was greater than the decrease in amplitude: frequency decreased from 15.81 ± 0.89 min−1 in the K+-solution to 7.45 ± 0.41 min−1 in the Cs+-solution (P < 0.001, n = 16), and amplitude decreased from 0.134 ± 0.004 fura 2 ratio units in the K+-solution to 0.124 ± 0.004 fura 2 ratio units in the Cs+-solution (P < 0.05, n = 16). Figure 1B shows faster time-base records of spontaneous Ca2+ release (left) and caffeine-induced Ca2+ release (right) in a skinned cardiac myocyte perfused with control (K+-based) solution. Figure 1C shows records from the same cell perfused with the Cs+-based solution, showing that Cs+ also appeared to prolong the duration of spontaneous Ca2+ release. This prolongation is shown more clearly in Fig. 2A, which shows normalized and superimposed records of spontaneous release monitored in the K+-solution and the Cs+-solution. The time course of spontaneous Ca2+ release was assessed using the time taken for Ca2+ to increase from 25% of its peak value to its peak value (TP75) and the decay time from its peak to 25% of peak value (DT75). Figure 2B shows that Cs+ significantly prolonged both the rise time and decay time of spontaneous Ca2+ release: TP75 increased from 0.277 ± 0.012 s in K+-solution to 0.374 ± 0.016 s in Cs+-solution (P < 0.001, n = 16), and DT75 increased from 0.579 ± 0.024 s in the K+-solution to 0.649 ± 0.026 s in the Cs+-solution (P < 0.01, n = 16). These data suggest, therefore, that Cs+ has marked effects on SR function, prolonging the time course and decreasing the frequency of spontaneous Ca2+ release. One possible explanation for these effects is that Cs+ is slowing Ca2+ flux across the SR membrane. This could prolong the time course of each spontaneous Ca2+ release. In addition, if luminal Ca2+ is an important determinant of the frequency of spontaneous Ca2+ release, then the changes in the frequency of release are likely to have been caused by the effects of Cs+ on the viscosity of the luminal Ca2+.

Table 3. Experimental solutions (intact cells)

<table>
<thead>
<tr>
<th>Solution</th>
<th>K+</th>
<th>Cs+</th>
<th>Na+</th>
<th>Mg2+</th>
<th>Ca2+</th>
<th>Cl−</th>
<th>HPO42−</th>
<th>SO42−</th>
<th>Buffer</th>
<th>Acetate</th>
<th>Glucose</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES-control</td>
<td>5</td>
<td>0</td>
<td>135</td>
<td>1</td>
<td>1</td>
<td>118</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>20</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>HEPES-Cs+</td>
<td>5</td>
<td>20</td>
<td>135</td>
<td>1</td>
<td>1</td>
<td>118</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>20</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Bicarbonate-Tyrode</td>
<td>5</td>
<td>0</td>
<td>135</td>
<td>1</td>
<td>1</td>
<td>102</td>
<td>1</td>
<td>1</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Bicarbonate-Ca2+-Tyrode</td>
<td>5</td>
<td>20</td>
<td>135</td>
<td>1</td>
<td>1</td>
<td>102</td>
<td>1</td>
<td>1</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

Values are concentrations of solution contents in mmol/l, except for insulin, which is given in U/l. pH of HEPES-based solutions was adjusted to 7.4 at room temperature with NaOH, and HCO3−-buffered solutions were equilibrated with 5% CO2-95% O2 (pH 7.35). HCO3−-buffered solutions were used for voltage-clamp experiments. However, the response of field-stimulated cells to Cs+ was the same in HEPES- and HCO3−-buffered solutions (not shown); thus buffer type had no apparent effect on response to Cs+. 

Fig. 1. Effects of Cs+ on spontaneous and caffeine-induced Ca2+ release from a skinned cardiac myocyte. A: representative slow time-base chart recording of fura 2 fluorescence from a skinned rat ventricular myocyte showing the effect of spontaneous Ca2+ release on returning to the control solution. Cs+ also appears to prolong the duration of spontaneous Ca2+ release. B and C: typical faster time-base recordings of spontaneous Ca2+ release (left) and caffeine-induced Ca2+ release (right) from a representative myocyte in K+- and Cs+-based solutions, respectively. Caffeine solution was applied at the time indicated by arrows. [Ca2+]i, Ca2+ concentration.
The presence of Cs⁺ was found to decrease frequency (see Discussion), because it would decrease the rate of spontaneous release before the caffeine-induced release could be synchronized to the spontaneous release, the time between the last spontaneous release before the caffeine-induced release and the caffeine-induced release itself was measured in the K⁺ and Cs⁺ solutions. Figure 4B shows that this loading time was significantly longer in the presence of Cs⁺, presumably because of the decrease in the rate of spontaneous release; the loading time increased from 1.305 ± 0.148 to 3.082 ± 0.360 s (P < 0.01, n = 10). This would be expected to increase SR Ca²⁺ content. The observation that the SR Ca²⁺ load was decreased in the presence of Cs⁺ (Fig. 4A) is compatible with the idea that Cs⁺ slows the rate of Ca²⁺ uptake into the SR, thus decreasing SR content.

Effects of CPA on skinned cells. This series of experiments was designed to test the hypothesis that the effects of Cs⁺ could be due, at least in part, to slowed Ca²⁺ uptake by the SR. If this hypothesis is correct, it might be expected that the effects of Cs⁺ could be mimicked by drugs such as CPA that inhibit SR Ca²⁺ uptake by inhibiting the SR Ca²⁺-ATPase.

Figure 5 shows original chart records of spontaneous Ca²⁺ release in a skinned myocyte in control solution and Cs⁺ solutions. Figure 4B also shows that the time between the caffeine-induced release and the first subsequent spontaneous release was significantly prolonged from 7.555 ± 1.585 s in K⁺ to 14.355 ± 1.075 s in the presence of Cs⁺ (P < 0.05, n = 10). This is also consistent with the hypothesis that Cs⁺ slows Ca²⁺ uptake by the SR so that a longer time is required following the application of caffeine to the SR Ca²⁺ content to increase sufficiently to generate a spontaneous Ca²⁺ release.
A and in the presence of 10 µmol/l (B) and 50 µmol/l (C) CPA. These traces show that CPA decreased the frequency of spontaneous Ca\(^{2+}\) release from 10.27 ± 1.75 min\(^{-1}\) in control to 2.41 ± 0.33 and 0.68 ± 0.06 min\(^{-1}\) in 10 and 50 µmol/l CPA, respectively (P < 0.01 and P < 0.001, respectively, n = 8). In addition, the amplitude of spontaneous Ca\(^{2+}\) release was slightly decreased to 89.8 ± 3.6% of control in 10 µmol/l CPA and to 93.8 ± 3.5% in 50 µmol/l CPA (both P < 0.05, n = 8; Fig. 6B). CPA also prolonged the decay time of the spontaneous Ca\(^{2+}\) release (DT\(_{75}\) in Fig. 6C) from 0.655 ± 0.045 to 1.307 ± 0.077 s in 10 µmol/l CPA and to 3.037 ± 0.295 s in 50 µmol/l CPA, suggesting that the Ca\(^{2+}\) uptake rate of the SR was reduced. The rise time of Ca\(^{2+}\) release (TP\(_{75}\) in Fig. 6C) was also slightly prolonged by CPA: TP\(_{75}\) increased from 0.325 ± 0.03 s in control to 0.483 ± 0.04 s in 10 µmol/l CPA and to 0.506 ± 0.03 s in 50 µmol/l CPA (P < 0.01 and P < 0.001, respectively, n = 8). Thus the effect of CPA on skinned cells was to decrease both the frequency and amplitude of spontaneous Ca\(^{2+}\) release and to prolong both the rise time and decay time of such release, although the prolongation was more pronounced for the decay than for the rise time. These effects, therefore, are similar to those produced by Cs\(^{+}\).

Fig. 5. Effects of cyclopiazonic acid (CPA) on spontaneous Ca\(^{2+}\) release. Representative chart recordings of spontaneous Ca\(^{2+}\) release in a skinned rat ventricular myocyte in control conditions (A) and in presence of 10 (B) and 50 (C) µmol/l CPA. D: superimposed normalized fast time-base recordings of spontaneous Ca\(^{2+}\) release in control conditions and in presence of 10 and 50 µmol/l CPA.

Effects of Cs\(^{+}\) on intact cells. To test whether Cs\(^{+}\) could also alter Ca\(^{2+}\) release in intact myocytes, the effects of extracellular Cs\(^{+}\) were investigated in field-stimulated and voltage-clamped ventricular myocytes. Figure 7A shows a typical slow time-base fluorescence record from an intact cell that was being field stimulated before, during, and after the addition of 20 mmol/l CsCl to the bathing solution. Cs\(^{+}\) caused a reversible increase in both systolic and diastolic [Ca\(^{2+}\)] (Fig. 7A).

Fig. 6. Mean effects of CPA on spontaneous Ca\(^{2+}\) release. Effects of 10 and 50 µmol/l CPA on frequency (A), amplitude (B), and time course (C) of spontaneous Ca\(^{2+}\) release. Time course was assessed using TP\(_{75}\) (C) and DT\(_{75}\) (C). Data are means ± SE; n = 8. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control.

Fig. 7. Effects of Cs\(^{+}\) on the Ca\(^{2+}\) transient and caffeine-induced Ca\(^{2+}\) release in field-stimulated intact cells. A: slow time-base chart recording showing Ca\(^{2+}\) transients and caffeine-induced Ca\(^{2+}\) release (indicated by arrows) in control and Cs\(^{+}\)-containing (20 mmol/l) solutions in a representative myocyte. B: superimposed Ca\(^{2+}\) transients recorded in control solution and in presence of Cs\(^{+}\). C: representative averaged (n = 15) Ca\(^{2+}\) transients from A before, during, and after exposure to Cs\(^{+}\).
the intact cells the amplitude of the Ca$^{2+}$ transient (Fig. 7, A–C) and caffeine-induced Ca$^{2+}$ release (Fig. 7A) were both slightly increased in the presence of Cs$^+$. The Ca$^{2+}$ transient was $109.0 \pm 3.4\%$ of control ($P < 0.05$, $n = 11$), and the caffeine-induced Ca$^{2+}$ release was $109.5 \pm 2.7\%$ of control ($P < 0.01$, $n = 11$). The rise and decay times of the Ca$^{2+}$ transient were not significantly affected by Cs$^+$ ($n = 11$; see Fig. 7B for normalized averages of Ca$^{2+}$ transients). Thus the effects of Cs$^+$ on the intact cell are different from those expected for inhibition of the SR. These results suggest that Cs$^+$ has additional effects in the intact cell that overcome the inhibitory effects on the SR. To test whether these additional effects could be due to changes in the configuration of the action potential, 20 mmol/l extracellular Cs$^+$ was added to cells in which the duration and amplitude of depolarization were kept constant by voltage clamp using the perforated patch-clamp technique (see MATERIALS AND METHODS for details).

Figure 8, A and B, shows original chart recordings of membrane current (top) and Ca$^{2+}$ transients (bottom) from a representative cell in which membrane potential was controlled before and during the application of Cs$^+$. Under these conditions Cs$^+$ had no significant effect on the amplitude of $I_{\text{Ca}}$ or the Ca$^{2+}$ transient but caused an inward shift of holding current that is compatible with inhibition of outward current. The amplitude of the Ca$^{2+}$ transient was $1.50 \pm 0.53$ and $1.56 \pm 0.16$ fura 2 ratio units in the absence and presence of 20 mmol/l Cs$^+$, respectively ($n = 6$, not significant (NS)). Figure 8C shows representative fast time-base recordings of averaged $I_{\text{Ca}}$ and Ca$^{2+}$ transients in the absence and presence of Cs$^+$, showing that Cs$^+$ also had no significant effect on the time course of these variables. The time to peak of the Ca$^{2+}$ transient was $55.7 \pm 4.7$ ms during control and $54.6 \pm 5.3$ ms in Cs$^+$ (NS). The decay of the Ca$^{2+}$ transient was also unaffected by Cs$^+$. These data suggest that the changes in the Ca$^{2+}$ transient produced by Cs$^+$ in field-stimulated cells were due to changes in the resting potential and/or action potential.

**DISCUSSION**

Spontaneous Ca$^{2+}$ release from SR. The present paper describes a method that enables Ca$^{2+}$ release from the SR to be monitored in a single skinned myocyte. This method has been used to investigate the effect of Cs$^+$ on spontaneous Ca$^{2+}$ release and SR Ca$^{2+}$ content, assessed using caffeine. Previous studies of SR Ca$^{2+}$ release have used SR Ca$^{2+}$ channels incorporated in planar phospholipid bilayers (see, e.g., Ref. 26), mechanically skinned single cells (10), and chemically skinned (29) or intact (24) multicellular preparations of cardiac muscle. The present technique has the advantage that it enables the function of the SR to be monitored in situ while the composition of the solution bathing the SR in a single cell is controlled, thus overcoming the problems of an unknown solution composition around the SR, inhomogeneity between cells, and diffusion delays, but without the technical difficulties associated with mechanical skinning. Although it is possible that SR function is altered by skinnng, the solutions were designed to mimic the normal intracellular environment, and previous studies using such cells have provided useful information about SR function. In addition, the data obtained in the present study are consistent with previous observations of the effects of Cs$^+$ on intact cells (see inhibitory effects of Cs$^+$ on spontaneous Ca$^{2+}$ release), supporting the idea that the Sr of the skinned cells is responding to Cs$^+$ in the same way as in intact cells.

The amplitude of the rise of [Ca$^{2+}$] observed during SR Ca$^{2+}$ release in a skinned cell will depend on the volume of solution into which the Ca$^{2+}$ is released and the rate of flow of this solution; for example, increasing perfusion rate will truncate the observed rise of Ca$^{2+}$. This makes absolute calibration difficult. However, because these variables will be constant in a particular cell, it is possible to study changes in [Ca$^{2+}$]. Diffusion and washout of Ca$^{2+}$ following release will also influence the rate of rise and decline of the observed Ca$^{2+}$ release. An increase in the rate of solution flow, for example, will result in a more rapid decline of [Ca$^{2+}$] as Ca$^{2+}$ is washed out. Thus the observed rate of decline of Ca$^{2+}$, which is due to both reserquestration of Ca$^{2+}$ by the SR (see below) and washout by the perfusing solution, is the fastest decline of Ca$^{2+}$ that could be attributed to SR-pump activity alone. However, the variables that will affect diffusion and washout of Ca$^{2+}$ will remain constant in a particular cell. Thus it seems likely that changes in time course will reflect changes in cell function rather than technical artifacts. The observation that the SR Ca$^{2+}$ ATPase inhibitor CPA slows the rate of decline of spontaneous Ca$^{2+}$ release (Fig. 6) is also consistent with the suggestion that
changes in SR function can influence the time course of the observed changes in [Ca\(^{2+}\)].

The characteristics of the spontaneous Ca\(^{2+}\) release observed in this preparation are consistent with those reported previously for spontaneous Ca\(^{2+}\) release from the SR of cardiac cells. The fraction of the SR Ca\(^{2+}\) content released during each spontaneous release was estimated by comparing the area of a spontaneous release with that of a caffeine-induced release. This gave a value of 14.5 ± 2.1%, similar to the value of 13.6% reported by Díaz et al. (6) for the intact cell. In addition, the frequency of spontaneous release increased as the Ca\(^{2+}\) in the bathing solution increased: frequency was 5.78 ± 0.63 min\(^{-1}\) with 100 nmol/l [Ca\(^{2+}\)], 8.83 ± 0.69 min\(^{-1}\) with 140 nmol/l [Ca\(^{2+}\)], 13.25 ± 1.04 min\(^{-1}\) with 180 nmol/l [Ca\(^{2+}\)], and 17.79 ± 1.60 min\(^{-1}\) with 240 nmol/l [Ca\(^{2+}\)] (each P < 0.05, n = 10). These values are within the range reported previously in intact preparations (3, 4, 6, 10).

It has long been known that increasing the [Ca\(^{2+}\)] around the SR of skinned cardiac cells leads to cyclic Ca\(^{2+}\) release from the SR (10) and that the frequency of such release increases with bathing [Ca\(^{2+}\)] (10). It is less clear whether such spontaneous release depends on the [Ca\(^{2+}\)] around the SR triggering Ca\(^{2+}\)-induced Ca\(^{2+}\) release or on the [Ca\(^{2+}\)] within the lumen of the SR (7, 25). However, because Ca\(^{2+}\)-induced Ca\(^{2+}\) release and spontaneous Ca\(^{2+}\) release have different characteristics, and because the latter can be observed in conditions that inhibit the former (9), it has been suggested that spontaneous Ca\(^{2+}\) release is not due to Ca\(^{2+}\)-induced Ca\(^{2+}\) release (7, 9). The present data support this suggestion. First, because the frequency of spontaneous release decreases in the presence of Cs\(^{+}\), even though bathing [Ca\(^{2+}\)] is constant, it is suggested that some factor other than bathing [Ca\(^{2+}\)] determines the frequency of release. Second, because the amplitude of release is relatively constant, it is suggested that luminal [Ca\(^{2+}\)] has to reach a certain critical threshold before Ca\(^{2+}\) release occurs (7). Recent reports that an increase in the [Ca\(^{2+}\)] at the luminal face of the ryanodine receptor increases the open probability of this channel (19) suggest a possible mechanism whereby an increase in luminal [Ca\(^{2+}\)] leads to spontaneous Ca\(^{2+}\) release. It is possible that the released Ca\(^{2+}\) then acts on the cytoplasmic face of the ryanodine receptor to increase open probability further and trigger further release, leading to a rapid and large Ca\(^{2+}\) release before stochastic attrition results in closure of the release channels.

Inhibitory effect of Cs\(^{+}\) on spontaneous Ca\(^{2+}\) release. It has previously been reported that intracellular Cs\(^{+}\) reduces Ca\(^{2+}\) transient amplitude by ~40–50% in guinea pig myocytes (12, 15) and reduces “phasic” contraction amplitude by 40–45% in rabbit myocytes (18). In the present study, replacement of K\(^{+}\) in the bathing solution by Cs\(^{+}\) decreased both the frequency and amplitude of spontaneous Ca\(^{2+}\) release and prolonged the rise time and decay time. The SR Ca\(^{2+}\) content, estimated by the application of caffeine, decreased slightly, and the time before the next spontaneous Ca\(^{2+}\) release (recovery time) was prolonged in the Cs\(^{+}\) solution. Similar but less marked effects were observed when only 50% of K\(^{+}\) was replaced with Cs\(^{+}\).

Because the bathing [Ca\(^{2+}\)] was constant, it appears likely that changes in luminal, rather than “cytoplasmic,”[Ca\(^{2+}\)] underlie the observed changes in spontaneous Ca\(^{2+}\) release in the presence of Cs\(^{+}\) (see Spontaneous Ca\(^{2+}\) release from SR). One possible mechanism whereby Cs\(^{+}\) could alter luminal Ca\(^{2+}\) is by slowing the rate of Ca\(^{2+}\) flux across the SR membrane; this could explain the present data as follows. 1) Slower Ca\(^{2+}\) uptake by the SR would prolong the time required for luminal [Ca\(^{2+}\)] to reach a critical level required for spontaneous Ca\(^{2+}\) release to occur, thus slowing the frequency of spontaneous Ca\(^{2+}\) release, with little change in amplitude (Fig. 1). 2) Slower SR Ca\(^{2+}\) uptake would also be expected to decrease SR Ca\(^{2+}\) content, assessed using caffeine (Fig. 4). The observed decrease in amplitude would presumably have been greater if the loading time had not also been increased in the presence of Cs\(^{+}\) (Fig. 4; see RESULTS). 3) Slowed SR Ca\(^{2+}\) uptake would also account for the increased time between depletion of the SR using caffeine and the first subsequent spontaneous Ca\(^{2+}\) release (Fig. 4), because the SR would take longer to refill sufficiently to generate another spontaneous release. 4) Slower movement of Ca\(^{2+}\) across the SR membrane could also account for the slower rise and fall of the spontaneous Ca\(^{2+}\) release (Fig. 2).

In support of this idea, the SR Ca\(^{2+}\)-ATPase inhibitor CPA mimicked many of the effects of Cs\(^{+}\), decreasing the frequency of spontaneous release with little change in amplitude and slowing the time course of the spontaneous Ca\(^{2+}\) release (Figs. 5 and 6). Although our hypothesis could explain why CPA might be expected to decrease the frequency of spontaneous release with little effect on amplitude (by increasing the time required for luminal Ca\(^{2+}\) to reach the critical level required for spontaneous release to occur) and to prolong the declining phase of the spontaneous Ca\(^{2+}\) release (by slowing reuptake into the SR), it is less clear why CPA should prolong the rising phase of the Ca\(^{2+}\) release. It is unlikely that this change is caused by a change in SR Ca\(^{2+}\) content, because the amplitude of the Ca\(^{2+}\) release is almost the same as in the absence of CPA. This suggests, therefore, that Ca\(^{2+}\) uptake occurring during the release phase can alter the time course of release. However, it appears unlikely that the effects of Cs\(^{+}\) on the rising phase of the Ca\(^{2+}\) release can be completely explained by this mechanism, because Cs\(^{+}\) has smaller effects than CPA on the frequency and rate of decline of spontaneous Ca\(^{2+}\) releases (suggesting a smaller effect on rate of Ca\(^{2+}\) uptake) but a larger effect than CPA on the rise of the spontaneous Ca\(^{2+}\) release (compare Figs. 3 and 6). This suggests, therefore, that Cs\(^{+}\) has effects on Ca\(^{2+}\) release in addition to its effects on Ca\(^{2+}\) uptake.

The hypothesis that Cs\(^{+}\) slows Ca\(^{2+}\) uptake and release by the SR could also explain earlier observations that, in cells dialyzed with Cs\(^{+}\)-containing solutions, the size of the Ca\(^{2+}\) transient and, hence, contraction, appears to decrease: slower SR Ca\(^{2+}\) uptake would
tend to decrease the amount of Ca\textsuperscript{2+} sequestered by the SR and, hence, SR Ca\textsuperscript{2+} content so that during regular stimulation the amount of Ca\textsuperscript{2+} available for release in response to a stimulus would be decreased. Thus the amount of Ca\textsuperscript{2+} released and, hence, the size of the Ca\textsuperscript{2+} transient would be decreased.

Unexpectedly, however, in field-stimulated intact cells, Cs\textsuperscript{+} increased the amplitude of the Ca\textsuperscript{2+} transient. There are a number of possible explanations for this result. First, Cs\textsuperscript{+} was applied at a relatively low concentration and extracellularly. Thus it is not clear that sufficient Cs\textsuperscript{+} entered the cell to inhibit SR function significantly. Second, K\textsuperscript{+}-channel blockade by Cs\textsuperscript{+} would be expected to prolong the action potential and, hence, increase the Ca\textsuperscript{2+} content of the cell, which might offset the inhibitory effects of Cs\textsuperscript{+} on the SR. Finally, the inhibition of outward current by Cs\textsuperscript{+} observed in voltage-clamped cells (Fig. 8) would cause depolarization of the resting membrane potential in intact cells. This could be expected to produce an increase in resting [Ca\textsuperscript{2+}], as observed in intact cells in the presence of Cs\textsuperscript{+} (Fig. 7), that would increase the Ca\textsuperscript{2+} available for uptake by the SR Ca\textsuperscript{2+} ATPase and, hence, increase the SR Ca\textsuperscript{2+} content (11). This would offset the direct inhibitory effects of Cs\textsuperscript{+} on the SR.

In support of the hypothesis that changes in the resting potential and/or action potential underlie the observed changes in the Ca\textsuperscript{2+} transient in the intact cell the data in Fig. 8 show that such changes were not observed when the Ca\textsuperscript{2+} transient in the intact cell is the data in Fig. 8 show that such changes were not observed when the resting potential and the duration and amplitude of depolarization were kept constant. Because I\textsubscript{Ca} is not altered by Cs\textsuperscript{+} (Fig. 8), it is unlikely that changes in I\textsubscript{Ca} modulate the Ca\textsuperscript{2+} transient during exposure to Cs\textsuperscript{+}. These data, therefore, are consistent with the idea that the addition of 20 mmol/l Cs\textsuperscript{+} to the bathing solution alters the resting and/or action potential and that it is these changes that alter the Ca\textsuperscript{2+} transient, possibly because under these conditions, unlike those in the skinned or dialyzed cell, intracellular Cs\textsuperscript{+} does not increase sufficiently to inhibit SR function significantly.

Possible mechanisms for action of Cs\textsuperscript{+} on SR. Cs\textsuperscript{+} is well known as an inhibitor of K\textsuperscript{+} channels (see Introduction), which are also present within the SR membrane. Although the role of these channels is unknown, it has been suggested that K\textsuperscript{+} moves in the opposite direction to Ca\textsuperscript{2+} across the SR membrane during uptake and release of Ca\textsuperscript{2+} by the SR to avoid the development of large charge imbalances that could inhibit Ca\textsuperscript{2+} movement. The present data are consistent with this suggestion. The blockade of the SR K\textsuperscript{+}-channels by Cs\textsuperscript{+} (27) may result in the development of a potential across the SR membrane during Ca\textsuperscript{2+} uptake or release that would tend to inhibit further Ca\textsuperscript{2+} movement.

Although other ions may also cross the SR membrane to help compensate for the charge movement associated with Ca\textsuperscript{2+} movement, the blockade of K\textsuperscript{+} flux will slow the rate at which charge movement can be compensated, thus slowing the rate of Ca\textsuperscript{2+} movement.

In summary, the present data suggest that Cs\textsuperscript{+} slows the flux of Ca\textsuperscript{2+} across the SR membrane, possibly by blocking the SR K\textsuperscript{+} channel, thus inhibiting compensation for the charge movement associated with Ca\textsuperscript{2+} uptake and release. In the intact cell this could decrease the Ca\textsuperscript{2+} content of the SR and, hence, the amount of Ca\textsuperscript{2+} released in response to stimulation. Although changes in the electrical activity of the cell may help to compensate for the direct inhibitory effects of Cs\textsuperscript{+} on the SR in the intact cell, it seems likely that the high concentrations of intracellular Cs\textsuperscript{+} achieved during whole cell clamp and the subsequent dialysis of the cytoplasm can inhibit the SR sufficiently to decrease SR Ca\textsuperscript{2+} content and, hence, the size of the Ca\textsuperscript{2+} transient (12, 15). This may have important consequences for studies of E-C coupling in which Cs\textsuperscript{+} has been used to block sarcoremmal K\textsuperscript{+} currents. The present data also suggest that SR luminal [Ca\textsuperscript{2+}] is an important determinant of spontaneous Ca\textsuperscript{2+} release and that such release occurs when luminal [Ca\textsuperscript{2+}] reaches a critical level (7).

We thank Dr. Derek Steele and Dr. Simon Harrison for useful discussion.

This work was supported by the Japan Heart Foundation and the British Heart Foundation.

Address for reprint requests: M. Kawai, Dept. of Physiol., Univ. of Leeds, LS2 9NQ, UK.

Received 4 December 1997; accepted in final form 30 April 1998.

REFERENCES
12. Han, S., A. Schiefer, and G. Isenberg. Ca\textsuperscript{2+} load of guinea-pig ventricular myocytes determines efficacy of brief Ca\textsuperscript{2+} currents as trigger for Ca\textsuperscript{2+} release. J. Physiol. (Lond.) 480: 411–421, 1994.


22. Meissner, G., and D. McKinley. Permeability of canine cardiac sarcoplasmic reticulum vesicles to K\textsuperscript{+}, Na\textsuperscript{+}, H\textsuperscript{+}, and Cl\textsuperscript{−}. J. Biol. Chem. 257: 7704–7711, 1982.


