Brief Communication

Voltage Dependence of Intracellular \([\text{Ca}^{2+}]_i\) Transients in Guinea Pig Ventricular Myocytes

Laura Barcenas-Rwz and W. Gil Wier

[Ca\(^{2+}\)]\(_i\) transients, elicited by voltage-clamp depolarization of single guinea pig cardiac ventricular cells, were observed through use of the fluorescent Ca\(^{2+}\) indicator, fura-2. Individual cells, loaded with fura-2 either by internal perfusion or by exposure to fura-2/AM, were studied with the use of an inverted microscope that was equipped with ultraviolet epifluorescence illumination, an intensified silicon intensifier target camera, and a photomultiplier tube. Variation of membrane voltage and exposure of cells to verapamil (a Ca\(^{2+}\) channel blocker) and ryanodine (which was assumed to abolish selectively the release of Ca\(^{2+}\) from the sarcoplasmic reticulum) were used to investigate the cellular processes that determine the [Ca\(^{2+}\)]\(_i\) transient. The principal results of the study are: 1) When appropriate methods are used, the properties of cytosolic fura-2 inside guinea pig cells are similar to those of fura-2 in solution, irrespective of the method of loading. 2) The amplitude (at 100 msec) of verapamil-sensitive fluorescence transients elicited by pulse depolarization (range \(-30\) to \(80\) mV) has a bell-shaped dependence on membrane voltage (maximum at \(10\) mV). 3) Rapid, ryanodine-sensitive and verapamil-sensitive "tail transients" are elicited on repolarization from membrane potentials greater than \(30\) mV; their amplitude increases as the amplitude of the preceding pulse increases. 4) The amplitude of slow fluorescence transients that are insensitive to verapamil and ryanodine increases continuously with membrane potential throughout the range \(-20\) to \(80\) mV. The voltage dependence and pharmacology of the rapid transients elicited by pulse depolarization or by repolarization are consistent with their having arisen from Ca\(^{2+}\) released from the sarcoplasmic reticulum, via Ca\(^{2+}\)-induced Ca\(^{2+}\) release. The [Ca\(^{2+}\)]\(_i\) transients remaining in the presence of ryanodine and verapamil may arise from Ca\(^{2+}\) entering via the sodium-calcium exchanger.

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Additional information on the voltage dependence of the intracellular [Ca\(^{2+}\)]\(_i\) transient is critical in the evaluation of theories of excitation-contraction coupling in mammalian cardiac muscle. Specifically, it is essential to know the sources of Ca\(^{2+}\) for the [Ca\(^{2+}\)]\(_i\) transient, and the dependence of these sources on membrane voltage and intracellular [Ca\(^{2+}\)]

Although a major theory of excitation-contraction coupling in cardiac muscle has been developed (that of Ca\(^{2+}\)-induced Ca\(^{2+}\) release [CICR]\(^{1-3}\), the applicability of this theory to intact cardiac muscle is unknown because it has been developed almost exclusively from experiments on skinned cardiac preparations, in which membrane voltage cannot have any role.

The use of fluorescent Ca\(^{2+}\) indicators to observe [Ca\(^{2+}\)]\(_i\) transients in voltage-clamped single cells\(^{4,5}\) has many advantages over previous methods\(^6\) for obtaining the required information. Control of membrane voltage and measurement of membrane currents is more reliable, extensive signal averaging of Ca\(^{2+}\) indicator signals is not necessary, subcellular heterogeneity of [Ca\(^{2+}\)]\(_i\) (or Ca\(^{2+}\) indicator) can be observed directly by imaging, and both the intracellular and extracellular environments can be controlled.

The voltage dependence of [Ca\(^{2+}\)]\(_i\) transients that has been observed in the present study, like the voltage dependence of contractions observed recently in voltage-clamped guinea pig cells\(^7\), supports the theory of CICR. In particular, the "tail transients" reported here are a clear demonstration of CICR in an intact cell under physiological conditions since they arise from a rapid, spatially homogeneous release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) that does not depend on depolarization. We also report verapamil-insensitive and ryanodine-insensitive fluorescence transients, the characteristics of which support certain concepts on the sodium-calcium exchanger in cardiac muscle\(^8\).

Materials and Methods

Preparation of Cells

Guinea pigs (250 g) were anesthetized with sodium pentobarbital (75 mg/kg i.p injection, Abbott Laboratories) and anticoagulated with heparin (1,000 U/300 mg, Elkins-Sinn, Inc.). Retrograde coronary perfusion

From the Department of Physiology, University of Maryland, School of Medicine, Baltimore, Md.

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W G W is an Established Investigator of the American Heart Association and its Maryland Affiliate.

Address for correspondence and reprints: Dr W Gil Wier, Department of Physiology, University of Maryland, School of Medicine, 660 West Redwood St., Baltimore, MD 21201.

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of the isolated heart was performed as follows. 5 minutes with a nominally calcium-free Tyrode's solution, 8 minutes with Tyrode's solution containing collagenase Type I (20 mg/25 ml, Sigma, lot 10SF-0682) and protease, Type XIV (7 mg/25 ml, Sigma, lot 54F-0545), and 5 minutes with Tyrode's solution containing 200 μM [Ca2+]i. The cells were dispersed from small pieces of the ventricles by mechanical agitation in the final solution above, filtered through a mesh, and maintained at 37°C in a Tyrode's solution containing (in mM): CaCl2 1.8 or 2.5, NaCl 135, KCl 4, MgCl2 1, Na2HPO4 0.33, dextrose 10, HEPES 10, and pH 7.3 with NaOH.

Fluorescence Recording

Individual cells were studied by a digital imaging fluorescence microscope technique described previously. Briefly, a liquid light guide, the end of which was masked to a pinhole and located at the second image plane within the ocular tube of the microscope, led fluorescence from a region of the cell approximately 7 μm in diameter to a photomultiplier tube (EMI 9635B, THORN EMI Electron Tubes, UK). Ultraviolet illumination (360 nm or 380 nm) of the cells was limited to the periods of interest by an electronically controlled shutter (Vincent Associates, Rochester, NY), the operation of which was synchronized with the voltage clamp pulses.

Voltage Clamp

Cells were voltage-clamped using the "whole-cell recording" technique. The membrane-holding potential was always −40 mV; the inward currents elicited by pulse depolarization from −40 mV were always abolished by the Ca2+ channel blocker, verapamil, at a concentration of 10 μM (Figures 2B and 5). Therefore, we conclude that the inward currents observed are produced by current flow through the "L" type Ca2+ channels that exist in these cells.

Loading of Cells With Fura-2

Fura-2/AM. The method used was that described by Poenie and co-workers, with minor modification. A mixture was made of 10 μl of 1 mM fura-2/AM (Molecular Probes, Junction City, Ore., lot 6H), K glutamate 120, KC1 20, MgCl2 1, Na2HPO4 0.33, dextrose 10, HEPES 10, and pH 7.3 with NaOH.

### Calibration

[Ca2+]i, is related to the ratio (R) of measured fluorescence signals (F), elicited at two excitation wavelengths, 1, and 1.2, according to the following equations:

\[
R = \frac{F_{1\text{max}} - F_{1\text{bg}}}{F_{2\text{max}} - F_{2\text{bg}}}
\]

\[
[Ca^{2+}]_i = K_d \times \frac{(R - R_{\min})}{(R_{\max} - R)}
\]

F1,max is the total measured fluorescence of the cell and F1,bg is the fluorescence background signal, including autofluorescence and noncellular fluorescence in the system. Kd is the dissociation constant of the complex, Ca-fura-2. Rmin and Rmax symbolize, respectively, the F1:F2 of the fluorescence signals of fura-2 (free of Ca2+) and Ca-fura-2 at the two excitation wavelengths. R is the ratio of fluorescence signals of fura-2 to Ca-fura-2, with excitation at 1. In general, 1 was 380 nm and 1, was 360 nm, a wavelength that is nearly isosbestic in our system.

### Methods for Determination of β, Rmin, Rmax, Fbg, and Kd

Rmin (1.1, 380 nm; 1, 360 nm) was determined by perfusing cells internally with a solution containing fura-2 (70 μM), no added Ca2+, and EGTA (10 mM) in an external solution free of Ca2+. Background fluorescence, (Fbg) which consisted in this case of fluorescence from fura-2 in the micropipette and the autofluorescence of the cell, was measured after establishing the giga-seal, but before "breaking in" to the cell. In two cells, the average value of Rmin was 2.85, nearly identical to that (2.95) found with the microscope in a thin layer of fura-2 (see ref. 10 for further details of methods).

To determine Rmax (1, 380 nm; 1, 360 nm), cells were loaded with fura-2 by exposure to fura-2/AM or by internal perfusion of a solution containing no added Ca2+ or EGTA, and fura-2 (70 μM). Fbg was determined as above, prior to perfusion, or as the mean Fbg of 5–10 unloaded (control) cells. Rmax was determined by mechanically damaging the membrane around the patch electrode, such that the cell leaked Ca2+ from the extracellular space, and [Ca2+]i, rose to a high level, as judged by the extreme shortening of the cell. Some cells retained their fura-2 during this procedure, and in such cells the smallest ratio that was recorded (corresponding, in this case, to Rmax) was 0.65. In thin solutions, Rmax was 0.60.

The similarity of Rmax and Rmin in cells and in solutions is inconsistent with the presence of partially desensitized intermediates or other Ca2+-insensitive fluorescent species. Thus, the fura-2/AM was converted completely to fura-2.

β (1, 360 nm) was found to be 1.14 by analysis of thin solutions, indicating, as expected, that 360 nm is nearly isosbestic. (It is difficult to measure β in cells.
because of the change in thickness of the cell that occurs as a result of contraction when [Ca\(^{2+}\)], is high.)

A disadvantage of the use of fura-2/AM is that the levels of background fluorescence signal (F\(_{bg}\)) cannot be determined in the particular cell being studied. Individual cells can have F\(_{bg}\) quite different from the mean, and this can lead to considerable uncertainty in the estimation of [Ca\(^{2+}\)]. In cells being voltage-clamped with a pipette containing fura-2 (salt), fluorescence from the pipette will contribute and should be accounted for in the measured F\(_{bg}\). In theory, such fluorescence could be measured after the gigaseal has been established but before breaking in to the cell. However, such measurements were not made routinely in all cells of this study. Therefore, we present only the fluorescence signals (F\(_{380\text{nm}}\)) without background subtraction.

The value of K\(_d\) determined by spectrofluorometric analysis of material released by digitonin lysis\(^\text{16}\) of suspensions of cells loaded by exposure to fura-2/AM was 156 nM, slightly higher than the value of 131 nM found for fura-2 (salt) that had been added to material released from unloaded cells. The latter value is almost identical to that (135 nM) originally found in cuvettes.\(^\text{13}\) However, a value of 200 nM was determined with a pipette containing fura-2 (salt), fluorescence from such fura-2 is reported to be punctate, rather than diffuse. The methods we used are reported specifically\(^\text{12}\) to reduce this problem and, indeed, no punctate fluorescence was detectable in images obtained with the ISIT camera. In confirmation, application of digitonin (which permeabilizes surface membrane selectively) to a single loaded cell (by "puffing" it from a microelectrode) resulted in a rapid and complete loss of fura-2 fluorescence from the cell. In 7 loaded cells, the mean fluorescence elicited by illumination with light at 360 nm (in kilo-cps) was 40.7 before digitonin lysis and 4.0 after digitonin lysis. In 5 unloaded cells from the same animal, these values were 3.4 and 0.8, respectively. Thus, approximately 10% of the fura-2 fluorescence remained after digitonin lysis.

In summary, all the data indicate that, when the methods reported by Poenie and colleagues\(^\text{12}\) are used, cells loaded by exposure to fura-2/AM contain mainly fully hydrolyzed fura-2 that is freely diffusible in the cytoplasm, as required for an indicator of cytoplasmic [Ca\(^{2+}\)].

**Results**

**Comparison of Cells Loaded Using Fura-2/AM and Cells Loaded by Internal Perfusion of Fura-2 (Salt)**

Since we used methods\(^\text{11}\) for producing reliable signals in cells loaded with fura-2 by exposure to fura-2/AM, it was of interest to compare results obtained in such cells with results obtained in cells loaded by internal perfusion with fura-2 (pentapotassium salt), as shown in Figure 1.

Figure 1A illustrates a typical signal from a cell that had been perfused with fura-2 (salt). In this case, the pipette was removed from the cell and, after resealing, the cell was subjected to external stimulation (at arrow). The background signals, F\(_{bg}\), are presented in this case because they were measured before injecting...
the cell with fura-2, and because the pipette had been
removed from the cell for measurement of the \([\text{Ca}^{2+}]\)
transient. The \([\text{Ca}^{2+}]\) transient calculated from this
record begins at slightly less than 100 nM and rises to a
peak of about 600 nM, a value that is at the low end of
the physiological range of \([\text{Ca}^{2+}]\) transients in ferret
papillary muscles. The following values were used for
the calculation: 1, 380 nM, \(1, 360 \text{ nm}, \beta 1.14, K_+\)
200 nM, \(R_{m} 60,\) and \(R_{m} 2.95.\)

Figure 1B illustrates typical recordings from a cell
loaded with fura-2 by exposure to fura-2/AM and sub-
jected to external stimulation (at arrow). The ampli-
cation of the fluorescence signals has been adjusted
such that the signals elicited by illumination at 360 nm
appear to be the same as in the cell of Figure 1A. The
mean levels of \(F_{bg}\) measured in 5 unloaded (control)
cells, were as indicated.

It can be seen that the fluorescence signals are the
same in relative amplitude and time course in the two
cells

\[\text{[Ca}^{2+}]\text{, Transients in Voltage-Clamped Cells}\]

Relation of fluorescence transient to clamp-
pulse potential. Figure 2A illustrates the dependence
of the fluorescence transient on clamp-pulse potential
under control conditions, over the range −20 to 80
mV, in a cell loaded with fura-2 (salt) via the patch
pipette. The records in Figure 2B were obtained in the
presence of 10 μM verapamil. The changes in fluo-
rescence with excitation at 380 nm are shown inverted
\((-\Delta F 380)\) to indicate increases in \([\text{Ca}^{2+}]\), as positive-
going deflections of the fluorescence record. The pulse
protocol is shown by the tracings at the top of each
column; each test pulse was preceded by a train of four
conditioning pulses (to 0 mV), the last of which is
shown. The membrane current and fluorescence tran-
sients elicited by the last conditioning pulse are shown
preceding those elicited by the test pulse. The re-
sponses to the last conditioning pulse are the same in
all traces. Therefore, we conclude that the cellular
processes involved in E-C coupling are in the same
state at the time of each test pulse.

Under control conditions (Figure 2A), as the test
pulse potential is made more positive, up to 20 mV, the
amplitude and rapidity of the change in fluorescence
increases. At −30 mV and −20 mV, the fluorescence
change continues to increase during the test pulse, at
10 mV, the peak (90%) is reached within 35 milliseconds
As the test pulse potential is increased over the range
20−80 mV, the amplitude and rapidity of the fluorescence
change declines. The voltage dependence of the fluo-
rescence transient, measured at 100 milliseconds (Figure 3A), is similar to that of the \(\text{Ca}^{2+}\) current in guinea pig cells (Figure 2), consistent with the theory of CICR. The possibility that these flu-
orescence transients decline at positive potentials be-
cause the SR is depleted of \(\text{Ca}^{2+}\) can be excluded,
because the cell was put into the same condition prior
to each test depolarization by the train of conditioning
pulses.

Fluorescence transients elicited by repolariza-
tion. At test pulses to potentials of 40 mV and greater,
fluorescence transients are elicited upon repolarization
(Figure 2A; two traces at bottom); these increase as the
test pulse potential is increased from 40 mV to 80 mV
(Figure 3B). The changes in fluorescence elicited by
repolarization from very positive potentials occur as
rapidly as any of those elicited by test depolarizations.

Fluorescence transients elicited by repolarization will
be called tail transients, because the evidence (see
"Discussion") supports the concept that they are in-
duced or "triggered" via CICR by "tails" of \(\text{Ca}^{2+}\) cur-
rent (see "Discussion"). The voltage dependence of the
tail transients is illustrated in Figure 3B.

Fluorescence transients in presence of verapamil.
Figure 2B illustrates the result that verapamil abolishes
the rapidly rising fluorescence transients elicited by
pulse depolarization, as well as the tail transients.

Fluorescence transients in presence of ryanodine.
Figure 4 illustrates the result that the rapidly rising
fluorescence transients elicited by pulse depolarization
are strongly reduced by ryanodine, a substance be-

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** A. Recordings of membrane current (upper traces)
and corresponding fluorescence transients (lower traces) under
control conditions. Cell was loaded directly with fura-2 (salt)
via voltage-clamp pipette. Fluorescence signals shown are
\(-\Delta F 380;\) thus increases in \([\text{Ca}^{2+}]\), are indicated by upward
deflections of traces. Top tracings, above each column, illustrate
voltage-clamp protocol schematically. Each test pulse
(second pulse) was preceded by train of 4 conditioning pulses,
the last pulse of which is shown. Voltages of conditioning and
test pulses are indicated to left of each tracing of current and
fluorescence. Each trace is result of averaging 4 signals. B.
Recordings from same cell after exposure to verapamil (10
μM) \([\text{Ca}^{2+}]_o\). 2.5 mM Capacity currents have been re-
touched to aid clarity.
Figure 3: Voltage dependence of fluorescence transients. Voltage protocol as in Figure 2. Insets indicate method of measurement. A. Fluorescence transients elicited by pulse depolarization. Circles and triangles represent data from 2 cells loaded with fura-2 by exposure to fura-2/AM. Diamonds represent data from cell loaded with fura-2 (salt) via electrode. Data has been "normalized" by dividing all measurements by that at 0 mV for each cell. B. Tail transients from same three cells as illustrated in A. For each cell, normalizing factor used was same as in A. Thus, relative amplitudes of data plotted in B reflect relative amplitudes of original signals.

The rapidly rising fluorescence transients elicited by pulse depolarization are almost certainly dominated by Ca\(^{2+}\) released from the SR, since they are reduced strongly by ryanodine, when Ca\(^{2+}\) current is present (Figure 4). The mechanism may be CICR since the voltage dependence is similar to that of the Ca\(^{2+}\) current.

The fluorescence transients elicited by repolarization (tail transients) (Figures 2 and 3B) may also arise from CICR. Repolarization from positive potentials believed to abolish release of Ca\(^{2+}\) from SR.\(^\text{31,22}\) As reported previously,\(^\text{23}\) Ca\(^{2+}\) currents are not abolished by ryanodine. The tail transients are abolished by ryanodine.

Fluorescence Transients in Ryanodine and Verapamil. The fluorescence transients that occur in the presence of verapamil and ryanodine (Figure 5) are distinctly different in time course and voltage dependence from those under control conditions. The peak amplitude increases with increasing pulse depolarization over the entire range of membrane potential examined (−30 to 80 mV), rises throughout the duration of the depolarizing pulse, and there is no tail transient upon repolarization from positive potentials.

Discussion

The patterns of voltage dependence of the fluorescence transients elicited by pulse depolarization (Figure 3A), repolarization (Figure 3B), and pulse depolarization in the presence of verapamil and ryanodine (Figure 5B) are all distinct from each other. These data strongly support certain concepts on the origins of these fluorescence transients as discussed below.
elicits a large, instantaneous, inward Ca\textsuperscript{2+} current (tail) during the time taken by Ca\textsuperscript{2+} channels to deactivate. Nevertheless, it is unlikely that the tail transients arise from Ca\textsuperscript{2+} current directly because ryanodine abolishes tail transients but does not abolish Ca\textsuperscript{2+} current\textsuperscript{22} (Figure 4). Thus, tail transients probably arise from Ca\textsuperscript{2+} released from the SR, and the mechanism must be CICR since the release occurs on repolarization not depolarization. The increase in the tail transient over the range of 40–80 mV could be accounted for by one or a combination of 3 possible mechanisms. 1) an increased trigger, i.e., increase in the tail of Ca\textsuperscript{2+} current, which would result from a decrease in the Ca\textsuperscript{2+}-dependent inactivation of Ca\textsuperscript{2+} current\textsuperscript{24} during the preceding pulse, 2) an increased availability in the SR of Ca\textsuperscript{2+} to be released; this would result from less having been released during the preceding pulse, or 3) by an increased availability of the release mechanism itself, as a result of a decrease in the putative Ca\textsuperscript{2+}-dependent inactivation of Ca\textsuperscript{2+} release\textsuperscript{2} that occurs during the pulse. These possibilities are supported, but not distinguished, by the observation that the tail transient appears and increases only as the fluorescence transient on which it is superimposed decreases. Observation of tail transients with digital imaging microscopy revealed that they are spatially uniform, distinct from the spontaneous, unphysiological, spatially inhomogeneous “waves” of [Ca\textsuperscript{2+}], also attributed to CICR, that can be observed in a subpopulation of isolated cardiac cells.\textsuperscript{10} Thus, we believe that these tail transients are a clear demonstration of CICR in an intact cell under physiological conditions; tail transients arise from a rapid, spatially homogeneous release of Ca\textsuperscript{2+} from the SR that does not depend on depolarization.

In the presence of ryanodine and verapamil, fluorescence transients elicited by pulse depolarization are unlikely to arise either from Ca\textsuperscript{2+} entry through surface membrane Ca\textsuperscript{2+} channels (Ca\textsuperscript{2+} channels blocked and wrong voltage dependence) or from Ca\textsuperscript{2+} release from the SR. Therefore, one possible candidate is Ca\textsuperscript{2+} entry via sodium-calcium exchange. The voltage dependence is similar to that observed for sodium-calcium exchange currents when a similar holding potential is used\textsuperscript{25,26}. In sodium-loaded frog atrial cells, outward membrane currents through the sodium-calcium exchanger (presumably reflecting Ca\textsuperscript{2+} influx) are pulse-like during depolarizing voltage clamp pulses\textsuperscript{25,26}. Provided that intracellular Ca\textsuperscript{2+} buffers and uptake systems are either far below saturation ([Ca\textsuperscript{2+}]\textsubscript{i} is far less than their K\textsubscript{m}'s) or unable completely to take up Ca\textsuperscript{2+}, as may be the case for the SR, the [Ca\textsuperscript{2+}],

![Figure 5A](https://example.com/figure5a.png)

**Figure 5A** Recordings of fluorescence transients in presence of verapamil (10 \mu M) and ryanodine (10 \mu M). Voltage protocol was same as that used in experiment illustrated in Figure 2. Each trace is result of averaging 4 signals. **Figure 5B** Voltage dependence of verapamil-insensitive and ryanodine-insensitive fluorescence transients. Circles and triangles represent data from 2 different cells, both loaded directly with fura-2 (salt) via clamp electrode. Data in this figure have been normalized by dividing all measurements by value at 40 mV [Ca\textsuperscript{2+}]\textsubscript{o}. 2.4 mM Capacity currents have been retouched to aid clarity.
transient that would result from such a current would be the integral of the current and would rise linearly throughout the pulse approximately as observed. There would be no tail transient since upon repolarization the sodium-calcium exchanger would produce Ca\(^{2+}\) efflux not Ca\(^{2+}\) influx. More experiments, however, are needed to identify unequivocally the source of Ca\(^{2+}\) for fluorescence transients in the presence of verapamil and ryanodine.

**Acknowledgments**

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L Barcenas-Ruiz and W G Wier

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