Reduction of Calcium Inactivation of Sarcoplasmic Reticulum Calcium Release by Fura-2 in Voltage-clamped Cut Twitch Fibers from Frog Muscle

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ABSTRACT Cut fibers from *Rana temporaria* and *Rana pipiens* (striation spacing, 3.9–4.2 μm) were mounted in a double Vaseline-gap chamber and studied at 14°C. The Ca indicator purpurate-3,3′diacetic acid (PDAA) was introduced into the end pools and allowed to diffuse into the optical recording site. When the concentration at the site exceeded 2 mM, step depolarizations to 10 mV were applied and the [Ca] transient measured with PDAA was used to estimate Ca release from the sarcoplasmic reticulum (SR) (Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1983. *Journal of Physiology.* 344:625–666). With depolarization, the rate of SR Ca release increased to an early peak and then rapidly decreased several-fold to a quasi-steady level. The total amount of Ca released from the SR at the time of peak rate of release appeared to be independent of SR Ca content, consistent with the idea that a single activated channel might pass, on average, a fixed number of ions, independent of the magnitude of the single channel flux. A possible explanation of this property is given in terms of locally induced Ca inactivation of Ca release. The solution in the end pools was then changed to one with PDAA plus fura-2. SR Ca release was estimated from the [Ca] transient, as before, and from the Δ[Cafura-2] signal. On average, 2–3 mM fura-2 increased the quasi-steady level of the rate of SR Ca release by factors of 6.6 and 3.8, respectively, in three fibers from *Rana temporaria* and three fibers from *Rana pipiens*. The peak rate of release was increased in five of the six fibers but to a lesser extent than the quasi-steady level. In all fibers, the amplitude of the free [Ca] transient was markedly reduced. These increases in the rate of SR Ca release are consistent with the idea that Ca inactivation of Ca release develops during a step depolarization to 10 mV and that 2–3 mM fura-2 is able to reduce this inactivation by complexing Ca and thereby reducing free [Ca]. Once the concentration of fura-2 becomes sufficiently large, a further increase reduces the rate of SR Ca release. On average, 5–6 mM fura-2 increased the quasi-steady rate of release, compared with 0 mM fura-2, by 6.5 and 2.9, respec-

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/93/08/0333/38 $2.00
Volume 102 August 1993 333–370

tively, in four fibers from *Rana temporaria* and three from *Rana pipiens*. The factors for 7–8 mM fura-2 were 3.6 and 2.0, respectively, in three fibers from *Rana temporaria* and three from *Rana pipiens*. This reduction of the rate of SR Ca release at large concentrations of fura-2 may be due to a reduction of Ca-induced Ca release (Jacquemond, V., L. Csernoch, M. G., Klein, and M. F. Schneider. 1991. *Biophysical Journal.* 60:867–873) or to an effect of a large concentration of fura-2 not related to Ca buffering.

INTRODUCTION

The preceding article (Pape, Jong, Chandler, and Baylor, 1993) described the effect of (Ca-free) fura-2 on sarcoplasmic reticulum (SR) Ca release elicited by a single action potential. As the resting concentration of fura-2 at the optical recording site was increased from 0 to 0.5–2 mM, the amplitude and half-width of the free [Ca] transient decreased while both the amount of Ca released from the SR and the peak rate of release were found to increase. This result, obtained on cut fibers from *Rana temporaria,* is qualitatively similar to that obtained previously in intact fibers from the same species of frog by Baylor and Hollingworth (1988) and Hollingworth, Harkins, Kurebayashi, Konishi, and Baylor (1992). These authors attributed the action of fura-2 to its ability to complex Ca and reduce the amplitude of the myoplasmic free [Ca] transient, which leads to a reduction of Ca-dependent inactivation of SR Ca release (hereafter called Ca inactivation of Ca release).

In a related series of experiments, Jacquemond, Csernoch, Klein, and Schneider (1991) injected either a mixture of BAPTA and fura-2 or fura-2 alone into cut fibers from *Rana pipiens* and found a depression of both the free [Ca] transient and the peak rate of SR Ca release elicited by a step depolarization to 10 or 20 mV. A concentration of 1.5–2 mM fura-2 (2.2–2.8 mM total fura-2) or 3.8 mM total BAPTA, on average, eliminated the early transient component of release and left the quasi-steady level unchanged. These authors concluded that the early transient component of SR Ca release is Ca induced, since it was eliminated by fura-2.

These two different effects of fura-2 on SR Ca release appear to be contradictory. It is important, however, to realize that they were obtained with different methods of stimulation. With action potential stimulation, SR Ca release is influenced by events that occur during both depolarization and repolarization, whereas with voltage-clamp depolarization it is influenced by events during depolarization alone. Since the action potential experiments uniformly showed that 0.5–2 mM fura-2 increased SR Ca release, it seemed important to try to confirm the suppression of SR Ca release that was observed with a similar concentration of fura-2 in voltage-clamped fibers by Jacquemond et al. (1991). This article describes these results, obtained under experimental conditions similar to those used by Jacquemond et al. (1991).

Some of the results have been presented to the Biophysical Society (Jong, Pape, Chandler, and Baylor, 1993).

METHODS

The experimental and analytical procedures are the same as those in the preceding article (Pape et al., 1993) except that SR Ca release was elicited by voltage-clamp pulses to 10 mV

from a holding potential of −90 mV (Chandler and Hui, 1990). Fibers from both *Rana temporaria* (Charles D. Sullivan, Inc., Nashville, TN) and *Rana pipiens* (J. M. Hazen, Alburg, VT) were used. The experiments with *Rana temporaria* were carried out in March and June, 1992 and those with *Rana pipiens* in October, 1992. The solutions were similar to those used by Jacquemond et al. (1991). The central pool solution contained 117 mM TEA-methanesulfonate, 1.8 mM $CaCl_2$, 10 mM TEA-3-(*N*-morpholino)propanesulfonic acid (TEA-MOPS), and 1 μM tetrodotoxin (pH 7.1). The internal solution used in the end pools was similar to that used in the action potential experiments (Pape et al., 1993) except that Cs replaced K and Na, with [Na] + [K] < 1 mM. Another difference between the methods used in this article and those used in the preceding article is that the eight-pole Bessel filters for the electrical and optical signals were set to 1 kHz (instead of 2 kHz) and the digital Gaussian filter used for the indicator-related optical signals was set to 0.15 kHz (instead of 0.5 kHz). PDAA and fura-2 were used in all experiments. The striation spacing of the fibers at the optical recording site was 3.9–4.2 μm and the temperature in the central pool was 14°C.

RESULTS

Effect of Fura-2 on SR Ca Release in Fibers from Rana temporaria during a Voltage Pulse to 10 mV

Fig. 1 shows four panels of traces that were obtained with a nearly constant concentration of PDAA and different concentrations of fura-2 at the optical recording site. The records in Fig. 1 *A* were taken with 2.374 mM PDAA and no fura-2, 48 min after 2.796 mM PDAA without fura-2 had been introduced into the end pool solutions. The top trace shows the voltage associated with a 50-ms pulse to 10 mV. The second trace shows the Δ[Ca] signal estimated from the PDAA-related $\Delta A(570)$ signal. The value of Δ[Ca] progressively increased during the pulse and reached a maximum of 112 μM just after the end of the pulse.

SR Ca release was calculated from the Δ[Ca] trace with model 1 (Table I in Pape et al., 1993). The third and fourth traces in Fig. 1 *A* show $\Delta[Ca_T]$ (the estimated Ca released from the SR, expressed in terms of myoplasmic concentration) and $d\Delta[Ca_T]/dt$ (the rate of release), respectively. According to the calculation, the SR released sufficient Ca into the myoplasm to increase its total concentration by 746 μM, given by the final level of $\Delta[Ca_T]$. $d\Delta[Ca_T]/dt$ reached a peak value of 64 μM/ms about 5 ms after the depolarization; it then rapidly decayed to a quasi-steady level of ~ 11 μM/ms, estimated from its mean value 15–30 ms after the depolarization. This early decay is probably due to Ca inactivation of Ca release (Baylor, Chandler, and Marshall, 1983; Schneider and Simon, 1988; Simon, Klein, and Schneider, 1991). During the last 20 ms of the pulse, $d\Delta[Ca_T]/dt$ decreased gradually. This later decay is also consistent with Ca inactivation of Ca release, since the Δ[Ca] signal continued to increase gradually during this period.

The Δ[Ca] and $d\Delta[Ca_T]/dt$ signals in Fig. 1 *A* are qualitatively similar to those obtained by Jacquemond et al. (1991) before the injection of BAPTA or fura-2. The main difference between our records and theirs is that their records show an increase in Δ[Ca] of only 4–9 μM after a 50-ms depolarization to 10 or 20 mV, whereas the Δ[Ca] signal in Fig. 1 *A* shows an increase of 112 μM. Part of the reason for the 10–30-fold difference can be attributed to our use of PDAA, rather than antipyrylazo III, to estimate Δ[Ca]. As mentioned in the preceding article (Pape et al., 1993),

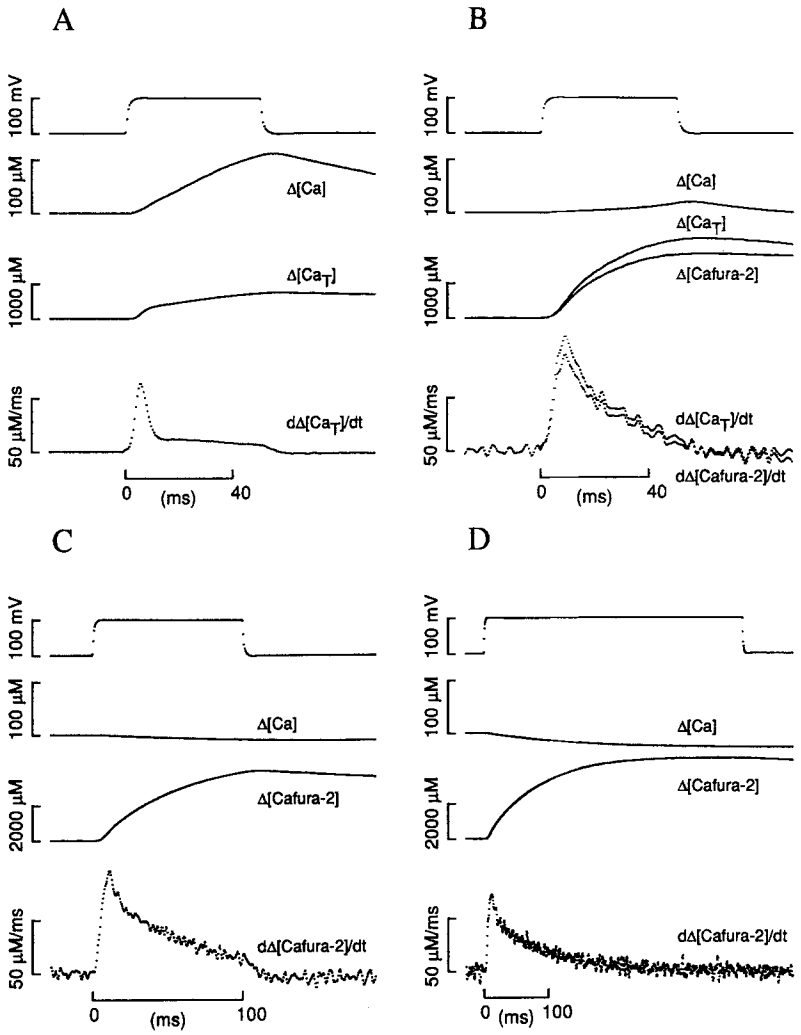

FIGURE 1. Effect of resting [fura-2] on Ca signals associated with step depolarizations to 10 mV. The format is similar to Fig. 10 in Pape et al. (1993). In *A* and *B*, $\Delta[Ca_T]$ was calculated from Δ[Ca] with model 1, Table I in Pape et al. (1993); in *B*, Δ[Cafura-2] was also added to $\Delta[Ca_T]$. In *C* and *D*, the $\Delta[Ca_T]$ signal could not be reliably calculated from the Δ[Ca] signal; consequently, only the Δ[Cafura-2] and $d\Delta[\text{Cafura-2}]/dt$ signals are shown. (*A*) Step duration, 50 ms; [PDAA] = 2.374 mM and [fura-2] = 0 mM. (*B*) Step duration, 50 ms; [PDAA] = 2.603 mM and [fura-2] = 1.825 mM. (*C*) Step duration, 100 ms; [PDAA] = 2.484 mM and [fura-2] = 4.512 mM. (*D*) Step duration, 400 ms; [PDAA] = 2.448 mM and [fura-2] = 6.977 mM. The Δ[Cafura-2] traces in *C* and *D* are plotted at a different gain than the $\Delta[Ca_T]$ and Δ[Cafura-2] traces in *A* and *B*. Different time bases are used in the panels. In this and subsequent figures, the prestimulus baseline of the PDAA-related $\Delta A(570)$ signal was set equal to its mean value to reduce the noise in the Δ[Ca], $\Delta[Ca_T]$, and $d\Delta[Ca_T]/dt$ traces. Fiber reference, 618921 (*Rana temporaria*); sarcomere spacing, 4.2 µm; temperature, 14°C. Range of values, beginning to end of experiment: fiber diameter, 85–71 µm; holding current, −21 to −31 nA. 2.796 mM PDAA was initially introduced into the end pools 21 min after saponin treatment of the end pool segments; 2.390 mM PDAA plus 8 mM [fura-2] without added Ca was introduced 53 min later. The traces in *A–D* were taken 69, 99, 119, and 149 min after saponin treatment.

antipyrylazo III appears to underestimate Ca transients in skeletal muscle fibers by a factor of 4–5.

Jacquemond et al. (1991) studied SR Ca release after the injection of fura-2 or a mixture of fura-2 and BAPTA. In the two experiments in which only fura-2 was used, the value of [fura-2_T] at the optical site after injection was 2.2–2.8 mM; fura-2_T denotes the total concentration of indicator, i.e. [fura-2_T] = [fura-2] + [Cafura-2]. If the indicator injected into the fiber was complexed with Ca to the same extent as that in the injection pipette, the corresponding concentration of fura-2 at the optical site was 1.5–2 mM. In 16 other experiments, the mean value of [$BAPTA_T$] after injection was 3.8 mM.

Jacquemond et al. (1991) found that the early transient increase in $d\Delta[Ca_T]/dt$ observed before injection almost completely disappeared with either 1.5–2 mM fura-2 or 3.8 mM $BAPTA_T$; only the quasi-steady level of the signal remained. Their interpretation of this striking result was that Ca-induced Ca release was responsible for the early transient increase in $d\Delta[Ca_T]/dt$ and that this increase was suppressed by the ability of fura-2 or BAPTA to complex Ca and thus reduce Δ[Ca].

Fig. 1, *B–D*, shows the effects of millimolar concentrations of fura-2 on the Ca signals in one of our fibers. After the traces in Fig. 1 *A* were obtained, the end pool solution was exchanged for one that contained 8 mM fura-2 without added Ca (in addition to PDAA). The traces in Fig. 1 *B* were obtained 30 min after those in Fig. 1 *A*, when the concentration of fura-2 at the optical site had reached 1.825 mM. The Δ[Ca] signal was greatly reduced by fura-2. During the first half of the pulse, Δ[Ca] showed a small increase; thereafter, it increased more rapidly as fura-2 and, presumably, the intrinsic myoplasmic Ca buffers became saturated with the Ca released from the SR.

The next (superimposed) traces in Fig. 1 *B* show $\Delta[Ca_T]$ and Δ[Cafura-2]. Fura-2 increased the maximal value of $\Delta[Ca_T]$ threefold, from 746 μM in Fig. 1 *A* to 2,269 μM in Fig. 1 *B*. The Δ[Cafura-2] signal accounts for $\sim$0.8 of the $\Delta[Ca_T]$ signal.

The bottom two superimposed traces in Fig. 1 *B* show the $d\Delta[Ca_T]/dt$ and $d\Delta$[Cafura-2]/dt signals. The peak value of the $\Delta[Ca_T]/dt$ signal was 106 μM/ms, which is about two-thirds greater than that in Fig. 1 *A*, 64 μM/ms. The peak value of the $d\Delta$[Cafura-2]/dt signal was 88 μM/ms.

Fig. 1, *C* and *D*, shows traces obtained with 4.512 mM (*C*) and 6.977 mM (*D*) fura-2 at the optical site. The Δ[Ca] signals did not show an early increase during depolarization, only a gradual decrease, which is probably artifactual. Although the origin of this signal is unknown, its time course and sign are similar to that of the late negative phase observed with tetramethylmurexide (Maylie, Irving, Sizto, Boyarski, and Chandler, 1987*a*). Without a positive component in the Δ[Ca] signal, there is no reliable way to estimate the amount of Ca bound by troponin and parvalbumin. It seems unlikely that it was very significant, however, since in Fig. 1 *B* the Δ[Cafura-2] signal is similar to the $\Delta[Ca_T]$ signal, and in Fig. 1, *C* and *D*, the Δ[Cafura-2] signals are expected to represent even better approximations of $\Delta[Ca_T]$ because the concentration of fura-2 at the optical site was larger. The peak values of the Δ[Cafura-2] and $d\Delta$[Cafura-2]/dt signals were, respectively, 3,948 μM and 95 μM/ms in Fig. 1 *C* and 4,531 μM and 73 μM/ms in Fig. 1 *D*.

Fig. 2 *A* shows a comparison of the SR Ca release signals in Fig. 1 during the first

50 ms of the depolarization. The top trace shows voltage. Each of the next three pairs of traces shows the $d\Delta[Ca_T]/dt$ signal from Fig. 1 *A* obtained without fura-2 (continuous trace labeled *a*) superimposed with the $d\Delta[Ca_T]/dt$ signal from Fig. 1 *B* (*b*) or the $d\Delta$[Cafura-2]/dt signal from Fig. 1 *C* (*c*) or Fig. 1 *D* (*d*).

Fig. 2 *B* is similar to Fig. 2 *A* except that the rate of SR Ca release (in units of micromolar per millisecond) has been corrected for SR Ca depletion to give the fractional rate of release (in units of percent per millisecond). The correction procedure is similar to that used by Jacquemond et al. (1991), although the method for estimation of the SR Ca content is different (see Discussion). The concentration of the readily releasable Ca inside the SR (expressed in terms of myoplasmic concentra-

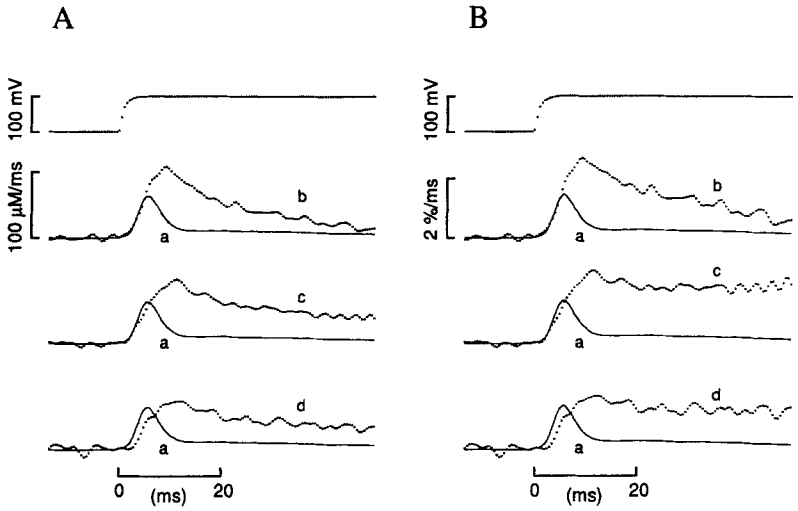

FIGURE 2. Effect of resting [fura-2] on the $d\Delta[Ca_T]/dt$ and $d\Delta$[Cafura-2]/dt signals. (*A*) The top trace shows the voltage. The next three pairs of traces show $d\Delta[Ca_T]/dt$ from Fig. 1 *A* (*continuous traces*, labeled *a*, calculated with model 1) superimposed with $d\Delta[Ca_T]/dt$ from Fig. 1 *B* (*b*) and $d\Delta$[Cafura-2]/dt from Fig. 1 *C* (*c*) and Fig. 1 *D* (*d*). (*B*) Similar to *A* except that the traces have been corrected for SR Ca depletion, as described in the text, and expressed in units of percent per millisecond. The concentration (referred to myoplasm) of the readily releasable Ca inside the SR was taken to be 4,689 μM for traces *a–c* and 4,557 μM for trace *d*.

tion) was taken from Fig. 3 *B* (see below) to be 4,689 μM for traces *a–c* and 4,557 μM for trace *d*. Each $d\Delta[Ca_T]/dt$ trace in Fig. 2 *B* was obtained from the corresponding trace in Fig. 2 *A* after multiplication at each moment in time by the quantity $100/(4{,}689\ \mu M - \Delta[Ca_T])$ (traces *a–c*) or $100/(4{,}557\ \mu M - \Delta[Ca_T])$ (trace *d*).

In the first pair of traces in Fig. 2 *B*, *a* and *b* virtually superimpose during the first few milliseconds of their rising phases. This suggests that the activation process during this period was similar in the two runs, although the amplitude of the Δ[Ca] signal in *b* during this period was only 0.15 times that in *a* (Fig. 1, *A* and *B*). About 4 ms after depolarization, traces *a* and *b* began to diverge. Trace *a* reached a peak at 5 ms and then decreased, whereas trace *b* continued to increase and did not reach a

peak until 8 ms after the voltage step. The decrease in trace *a* after its peak is consistent with Ca inactivation of Ca release. The continued increase in trace *b* is consistent with continued activation of SR Ca release and with a reduction of Ca inactivation of release by the Ca-buffering capacity of fura-2. The decrease in trace *b*, after its peak, is probably due to Ca inactivation of Ca release produced by the gradual increase in Δ[Ca] that occurred (Fig. 1 *B*). This kind of decrease is less pronounced in traces *c* and *d*, taken with resting [fura-2] = 4.512 and 6.977 mM, respectively. The likely reason is that the larger concentrations of fura-2 were able to further reduce Ca inactivation of Ca release by further reducing the Δ[Ca] signal. Traces *c* and *d* also show that SR Ca release became fully activated ~10 ms after the beginning of the depolarization.

The effect of fura-2 illustrated in Fig. 2 *B* is markedly different from that described by Jacquemond et al. (1991). Traces *a* and *b* show that 1.8 mM fura-2 increased both the peak and quasi-steady values of the rate of SR Ca release. In contrast, Jacquemond et al. (1991) found that 1.5–2 mM fura-2 completely eliminated the early transient component of release with little effect on the quasi-steady level. If we had obtained a similar result, trace *b* would have increased monotonically during the first 10 ms after depolarization and then superimposed trace *a*.

Fig. 3 *A* shows the peak values of $d\Delta[Ca_T]/dt$ (filled circles) and $d\Delta[\text{Cafura-2}]/dt$ (open circles), uncorrected for SR Ca depletion, plotted as a function of resting [fura-2], from the experiment in Figs. 1 and 2. The peak value of $d\Delta[Ca_T]/dt$ increased by about two-thirds as the value of resting [fura-2] increased from 0 to 1.8 mM. At larger concentrations of fura-2, the $d\Delta[Ca_T]/dt$ signal was approximately equal to the $d\Delta[\text{Cafura-2}]/dt$ signal. The $d\Delta[\text{Cafura-2}]/dt$ signal had a maximal value of 97 μM/ms at 3.286 mM fura-2. It then progressively decreased as resting [fura-2] increased, with a final value of 52 μM/ms at 8.668 mM fura-2.

The filled circles in Fig. 3 *B* show the peak level of Δ[Cafura-2] plotted as a function of the resting concentration of fura-2. The nine right-most points (the points without ×'s over them) were obtained with 400-ms pulses that were sufficiently long to deplete the SR of its readily releasable Ca (see description of Fig. 3 *C* below). Thus, they represent an estimate of the Ca content of the SR before the voltage step (expressed in terms of myoplasmic concentration). The first two points (4.512 and 5.516 mM fura-2), however, were obtained with 100- and 200-ms pulses, respectively, which were too brief for all the Ca to leave the SR. In each of these two runs, the final part of the Δ[Cafura-2] trace was fitted with a decreasing exponential function plus a constant. The constant, plotted as ×, was taken as an estimate of the SR Ca content, 4,550 μM with 4.512 mM fura-2 and 4,718 μM with 5.516 mM fura-2. The peak value of Δ[Cafura-2] obtained with the first 400-ms depolarization, with 5.943 mM fura-2, was 4,654 μM. This value progressively decreased as resting [fura-2] increased and was 4,088 μM with 8.668 mM fura-2.

In Fig. 3 *B*, the SR Ca content was in the range of 4,531–4,718 μM as the resting concentration of fura-2 increased from 4.5 to 7 mM. Since no estimate of Ca content is available for resting [fura-2] < 4.5 mM, we have assumed that it was the same as that with [fura-2] = 4.5 mM (strictly speaking, 4.512 mM). The essential assumptions are that the SR Ca content had reached a steady value when the records in Fig. 1 *A* were obtained, 64–67 min after introducing the experimental solutions into the

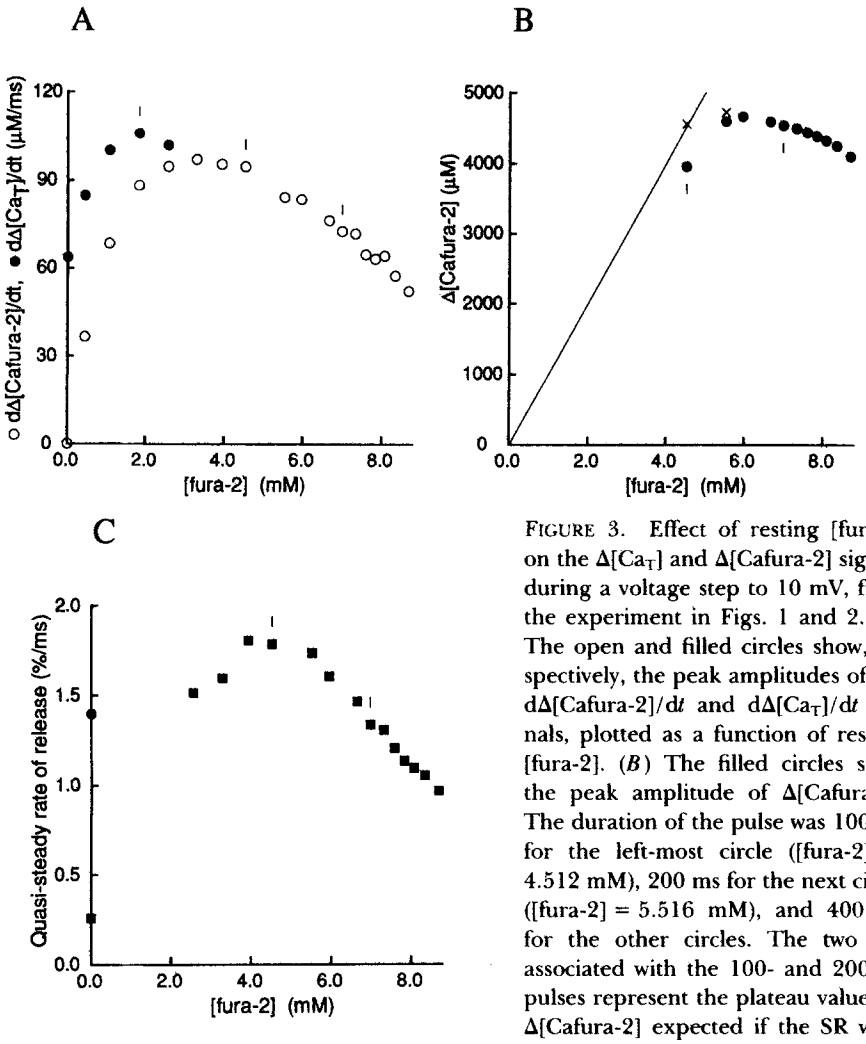

FIGURE 3. Effect of resting [fura-2] on the $\Delta[Ca_T]$ and Δ[Cafura-2] signals during a voltage step to 10 mV, from the experiment in Figs. 1 and 2. (*A*) The open and filled circles show, respectively, the peak amplitudes of the $d\Delta$[Cafura-2]/dt and $d\Delta[Ca_T]/dt$ signals, plotted as a function of resting [fura-2]. (*B*) The filled circles show the peak amplitude of Δ[Cafura-2]. The duration of the pulse was 100 ms for the left-most circle ([fura-2] = 4.512 mM), 200 ms for the next circle ([fura-2] = 5.516 mM), and 400 ms for the other circles. The two ×'s associated with the 100- and 200-ms pulses represent the plateau values of Δ[Cafura-2] expected if the SR were completely depleted of Ca. The value of each × is given by the constant obtained from a least-squares fit of a decreasing exponential function plus a constant to the final part of the Δ[Cafura-2] trace during the pulse. The straight line is plotted according to Δ[Cafura-2] = resting [fura-2]. (*C*) The filled circle and square at [fura-2] = 0 mM show, respectively, the peak and quasi-steady values of $d\Delta[Ca_T]/dt$ from trace *a* in Fig. 2 *B*; the quasi-steady value represents the average 15–30 ms after the depolarization. The filled squares at [fura-2] $\geq$ 2.561 mM show the quasi-steady values of $d\Delta$[Cafura-2]/dt, corrected for SR Ca depletion and expressed as a fractional rate of release; these represent the average values 30–50 ms (2.561 mM fura-2) and 30–100 ms (> 2.561 mM) after the depolarization. Ticks mark the points associated with the traces in Fig. 1, *B–D*, and traces *b–d* in Fig. 2; there is no tick at [fura-2] = 1.825 mM in *B* and *C*.

central pool and end pools, and that this value remained constant during the next 50 min, until resting [fura-2] = 4.5 mM. The first assumption seems reasonable since the SR Ca content would be expected to reach a steady state within an hour after changing internal and external solutions. The second assumption also seems reasonable since there is no obvious way for substantial amounts of Ca to enter or leave the optical site during the 50-min period that separated the traces taken with resting [fura-2] = 0 and 4.5 mM. Cafura-2 would not be able to diffuse from the end pools to the optical site and provide a source of Ca to be accumulated by the SR, since Ca was not added to the end pool solutions. In addition, the movement of Ca into the fiber from the external solution during depolarization would be expected to be minimal, since the fiber was depolarized only once every 5 min; each depolarization was estimated from the measurement of electrical current to increase total myoplasmic [Ca] by < 15 μM (not shown). Therefore, the only significant movements of Ca into or out of the optical site are the resting Ca influx from the external solution, Ca extrusion into the external solution, and diffusion of Ca or Ca complexes from the optical site to the end pools. Since the SR content remained relatively constant for resting [fura-2] = 4.5–7 mM, there is no reason to expect that any of these Ca movements produced a significant change in SR content as [fura-2] increased from 0 to 4.5 mM. Consequently, in Figs. 2 *B*, 3 *C*, 7, and 8, the SR Ca content for resting [fura-2] $\leq$4.512 mM was taken to be 4,689 μM, given by the mean value of the × at 5.516 mM fura-2 and the filled circle at 5.943 mM fura-2.

Fig. 3 *C* shows the quasi-steady rate of SR Ca release, expressed as the fractional rate of release, plotted as a function of the resting concentration of fura-2. The filled circle and square at [fura-2] = 0 mM show, respectively, the peak and quasi-steady values of $d\Delta[Ca_T]/dt$ obtained from trace *a* in Fig. 2 *B*. At concentrations of fura-2 $\geq$2.561 mM, the $d\Delta$[Cafura-2]/dt signal reached a quasi-steady level, as observed in traces *c* and *d* in Fig. 2 *B*. The quasi-steady levels, plotted as filled squares in Fig. 3 *C*, are considerably larger with fura-2 than without it. Between 2.561 and 6.549 mM fura-2, they are even larger than the peak value without fura-2.

As the resting concentration of fura-2 increased above 4 mM, the quasi-steady level of $d\Delta$[Cafura-2]/dt progressively decreased. With the largest concentration of fura-2 that was used, 8.668 mM, it was 0.96 %/ms. If the time course of SR Ca release were exponential, this fractional rate of release would correspond to a time constant of 104 ms. Consequently, a 400-ms pulse would be expected to deplete the SR of all but ~2% of its readily releasable Ca. Thus, in Fig. 3 *B* the estimates of SR Ca content that were obtained with 400-ms pulses (all but the two left-most points) are expected to be reliable.

The initial time course of the $d\Delta$[Cafura-2]/dt signals used for Fig. 3 *C* became progressively delayed as resting [fura-2] increased from 3.286 to 8.668 mM (cf. traces *c* and *d* in Fig. 2 *B*); the time to half-peak of the signal, after that of the depolarization, increased from ~4 to 6–7 ms. Thus, large concentrations of fura-2 also delay the SR Ca release signal.

Fig. 4, *A* and *B*, is similar in format to Fig. 11, *C* and *D*, in Pape et al. (1993). Fig. 4 *A* shows the value of Δ[Ca] at the time of the peak of the $d\Delta[Ca_T]/dt$ signal (filled circles) or the $d\Delta$[Cafura-2]/dt signal (open circles), plotted as a function of resting [fura-2]. Fig. 4 *B* shows a similar plot of the value of the integral of Δ[Ca], with the

period of integration extending from the start of the depolarization to the time of the peak of the $d\Delta[Ca_T]/dt$ (filled circles) or $d\Delta[Cafura\text{-}2]/dt$ (open circles) signal. As the concentration of fura-2 increased from 0 to 2–3 mM, the peak and quasi-steady levels of the rate of SR Ca release increased (Fig. 3, *A* and *C*), whereas the value of the Δ[Ca] signal (Fig. 4 *A*) and its integral (Fig. 4 *B*) decreased to nearly zero. At fura-2 concentrations ≥ 3 mM, the Δ[Ca] signal became unreliable (see Fig. 1).

The experiment illustrated in Figs. 1–4 shows that an increase in resting [fura-2] from 0 to 4 mM increased both the peak (Fig. 3 *A*) and quasi-steady (Fig. 3 *C*) levels of the rate of SR Ca release elicited by a step depolarization to 10 mV. The simplest interpretation of these increases is that fura-2 reduced the Δ[Ca] transient (Figs. 1 and 4) and that this, in turn, reduced Ca inactivation of Ca release.

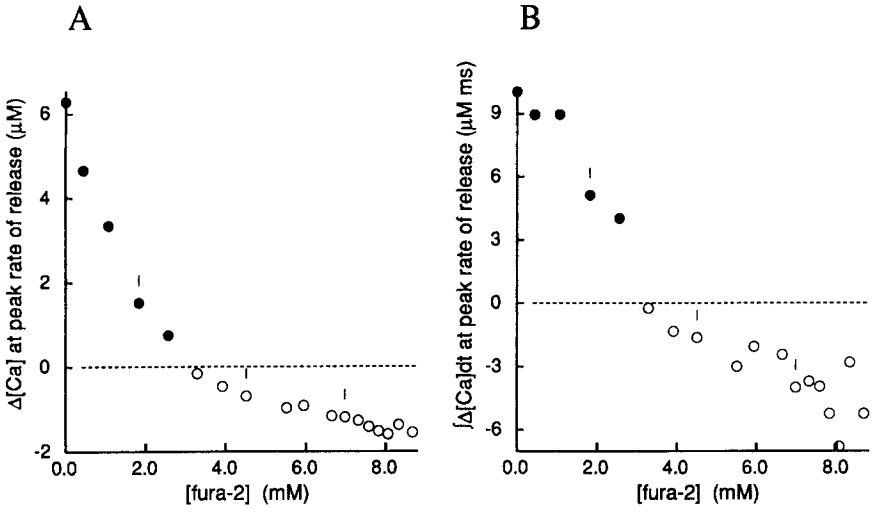

FIGURE 4. Effect of [fura-2] on the amplitude of the Δ[Ca] signal, from the experiment in Figs. 1–3. (*A*) The amplitude of myoplasmic free Δ[Ca] at the time when $d\Delta[Ca_T]/dt$ (*filled circles*) or $d\Delta[Cafura\text{-}2]/dt$ (*open circles*) had reached its peak value is plotted as a function of resting [fura-2]. *B* is similar to *A* except that the integral of the Δ[Ca] signal is shown. The integration time extended from the time of depolarization to the time when $d\Delta[Ca_T]/dt$ or $d\Delta[Cafura\text{-}2]/dt$ had reached its peak value. The significance of the ticks is given in the legend of Fig. 3.

A further increase in resting [fura-2] from 4 to 8.7 mM delayed the onset of the rate of SR Ca release and decreased both its peak and quasi-steady values almost twofold (although the quasi-steady rate with 8.668 mM fura-2 was still greater, by a factor of 3.7, than that estimated with 0 mM). These changes may be due to a reduction of Ca-induced Ca release, as suggested by Jacquemond et al. (1991), or to some other effect of the large concentration of fura-2.

Results similar to those in Figs. 1–4 were obtained in the three other experiments carried out on fibers from *Rana temporaria*. These will be discussed below, in connection with Tables I–III.

Effect of Fura-2 on SR Ca Release in Fibers from Rana pipiens during a Voltage Pulse to 10 mV

The action of fura-2 on the rate of SR Ca release in Figs. 1–4 is similar to that observed in our other experiments with fibers from *Rana temporaria* and very different from that reported by Jacquemond et al. (1991) on fibers from *Rana pipiens.* To find out whether the difference is due to the species of frog, three experiments were carried out with *Rana pipiens,* also cold-adapted to 5°C. Fig. 5 shows the results of one of these experiments.

Fig. 5 *A* shows a comparison of the time course of the rate of SR Ca release at different resting concentrations of fura-2, similar to that shown in Fig. 2 *B*. Traces *a* and *b* represent $d\Delta[Ca_T]/dt$ and *c* and *d* represent $d\Delta[\text{Cafura-2}]/dt$, all corrected for SR Ca depletion and expressed in terms of fractional rate of SR Ca release. The resting concentrations of fura-2 were similar to those used in Fig. 2 *B:* resting [fura-2] = 0 mM in *a,* 1.509 mM in *b,* 4.926 mM in *c,* and 6.899 mM in *d.* The effect of fura-2 on the rate of SR Ca release in Fig. 5 *A* (*Rana pipiens*) is similar to that shown in Fig. 2 *B* (*Rana temporaria*). In this experiment, the peak amplitude of the Δ[Ca] signal without fura-2 was 69 μM (not shown).

Fig. 5 *B* shows peak values of $d\Delta[Ca_T]/dt$ (filled circles) and $d\Delta[\text{Cafura-2}]/dt$ (open circles), uncorrected for depletion, plotted as a function of the resting concentration of fura-2. The results are qualitatively similar to those in Fig. 3 *A,* although the progressive decrease in $d\Delta[\text{Cafura-2}]/dt$ at resting [fura-2] above 2–3 mM was more marked in Fig. 5 *B* than in Fig. 3 *A*. Another difference in the two experiments is that the SR Ca content of the fiber in Fig. 5 (Fig. 5 *D*) was about half that of the fiber in Figs. 1–4.

Fig. 5 *C* shows the quasi-steady rate of SR Ca release, plotted against resting [fura-2] with the same format used in Fig. 3 *C*. The filled squares show the quasi-steady values of $d\Delta[Ca_T]/dt$ ([fura-2] = 0 mM, from trace *a* in Fig. 5 *A*) and $d\Delta[\text{Cafura-2}]/dt$ (resting [fura-2] ≥ 2.754 mM), expressed as fractional rates of release. The filled circle at [fura-2] = 0 mM shows the peak value of $d\Delta[Ca_T]/dt$ from trace *a* in Fig. 5 *A*. This value was exceeded by the quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ at resting [fura-2] = 2.754 mM. As resting [fura-2] was increased above 2.754 mM, the quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ decreased, and this reduction is more marked in Fig. 5 *C* than in Fig. 3 *C*. Even so, >8 mM fura-2 was required in the experiment in Fig. 5 *C* to reduce the quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ to that of $d\Delta[Ca_T]/dt$ estimated with 0 mM fura-2. Thus, the fiber from *Rana pipiens* used for Fig. 5 gave results that were very different from those reported by Jacquemond et al. (1991), who found that 1.5–2 mM fura-2 eliminated the transient component of SR Ca release and left the quasi-steady level unchanged.

Fig. 5 *D* shows the peak level of Δ[Cafura-2], the estimated Ca content of the SR (expressed in terms of myoplasmic concentration), plotted as a function of the resting concentration of fura-2. The format is the same as that used for Fig. 3 *B*. Between 3.926 and 6.899 mM fura-2, the value of Δ[Cafura-2] (filled circles) was nearly constant, 2,584–2,649 μM. As resting [fura-2] increased above 7 mM, the value of Δ[Cafura-2] progressively decreased and, at 9.353 mM, was 1,771 μM. The values of the ×'s, obtained from exponential fits similar to those used for Fig. 3 *B,* are <5%

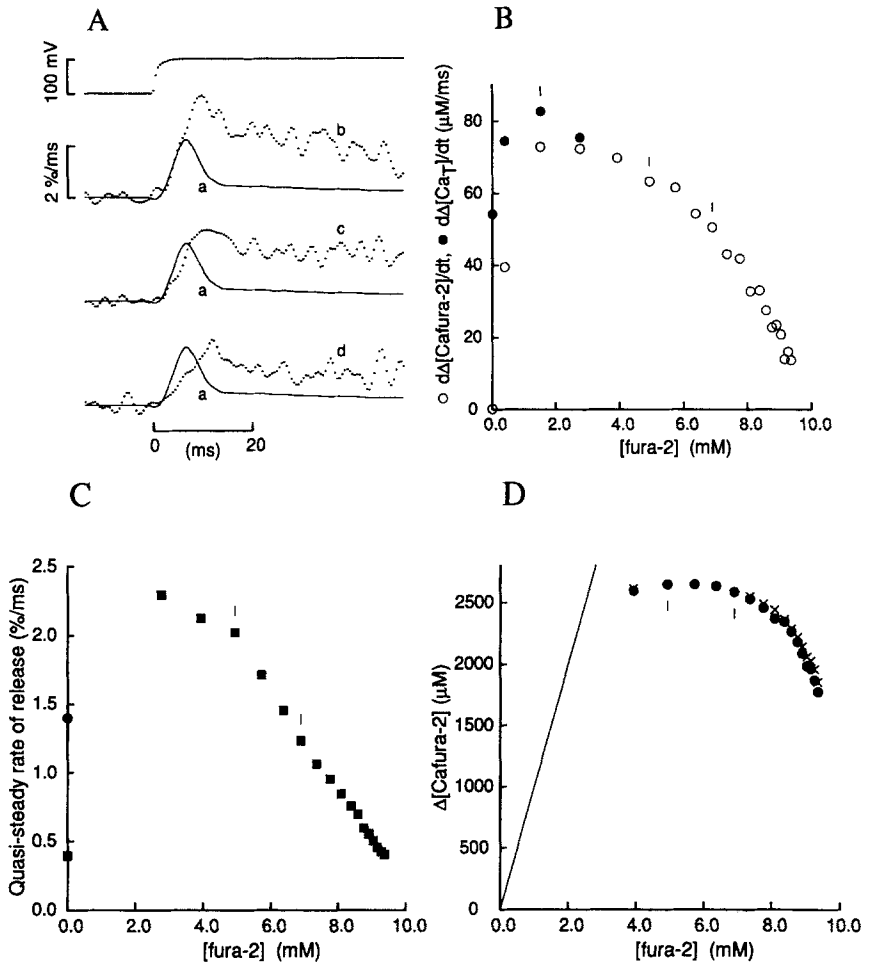

FIGURE 5. Effect of [fura-2] on the rate of SR Ca release during a step depolarization to 10 mV in a fiber from *Rana pipiens*. (*A*) Format similar to Fig. 2 *B*. Traces *a* and *b* show $d\Delta[Ca_T]/dt$ calculated with model 1 and *c* and *d* show $d\Delta[\text{Cafura-2}]/dt$, all corrected for SR Ca depletion and expressed in terms of fractional rate of release. The SR Ca content in *c* and *d* was taken from the final value of Δ[Cafura-2], 2,647 and 2,584 μM, respectively; the Ca content in *a* and *b* was assumed to be 2,600 μM. [PDAA] = 2.701 mM (*a*), 3.024 mM (*b*), 2.909 mM (*c*), and 2.801 mM (*d*); [fura-2] = 0 mM (*a*), 1.509 mM (*b*), 4.926 mM (*c*), and 6.899 mM (*d*). (*B*) Format similar to Fig. 3 *A*. The open and filled circles show the peak amplitudes of $d\Delta[\text{Cafura-2}]/dt$ and $d\Delta[Ca_T]/dt$, respectively, plotted as a function of resting [fura-2]. (*C*) Format similar to Fig. 3 *C*. The filled circle and square at [fura-2] = 0 mM show, respectively, the peak and quasi-steady values of $d\Delta[Ca_T]/dt$ from trace *a* in panel *A*. The filled squares at [fura-2] ≥ 2.754 mM show the quasi-steady values of $d\Delta[\text{Cafura-2}]/dt$, expressed in terms of fractional change. The quasi-steady values represent the average values 15–30 ms ([fura-2] = 0 mM), 18–50 ms ([fura-2] = 2.754 mM), and 30–100 ms ([fura-2] > 2.754 mM) after the depolarization. (*D*) Format similar to Fig. 3 *B*. The filled circles show the peak amplitude of Δ[Cafura-2] and the line indicates the relation Δ[Cafura-2] = resting [fura-2]. The pulse duration was 200 ms ([fura-2] = 3.926 mM), 400 ms (seven points, [fura-2] = 4.926–8.090 mM), 600 ms (five points,

larger than the corresponding values of the open circles, indicating that the pulse durations were sufficiently long for the SR to release almost all of its readily releasable Ca. Thus, in this experiment, the Ca content of the SR appeared to decrease as the concentration of fura-2 was increased above 7 mM.

Effect of Fura-2 on SR Ca Release in a Fiber from Rana pipiens That Had a Small SR Ca Content

One fiber from *Rana pipiens* responded somewhat differently to fura-2 than the fibers described in Figs. 1–5. Fig. 6 shows results from this fiber, presented with the same format used for Fig. 5. Unlike the results of our other experiments, 0.5–2 mM fura-2 did not increase the peak rate of SR Ca release above the value estimated with [fura-2] = 0 mM (traces *a* and *b* in Fig. 6 *A* and filled circles in Fig. 6 *B*). The peak rate of release progressively decreased as fura-2 diffused into the optical site and reached a relatively stable level once resting [fura-2] ≥ 3 mM (filled and open circles in Fig. 6 *B*).

The quasi-steady value of $d\Delta$[Cafura-2]/dt increased almost fourfold when the resting concentration of fura-2 increased from 0 to 0.944 mM (traces *a* and *b* in Fig. 6 *A* and filled squares at 0 and 0.944 mM fura-2 in Fig. 6 *C*). It then decreased to 1.5 times the [fura-2] = 0 mM value when the next measurement was made 5 min later, with 1.703 mM fura-2 at the optical site (trace *c* in Fig. 6 *A* and filled square at 1.703 mM fura-2 in Fig. 6 *C*). At larger concentrations of fura-2, the quasi-steady value of $d\Delta$[Cafura-2]/dt showed a small progressive decrease (filled squares in Fig. 6 *C*).

Fig. 6 *D* shows the estimated Ca content of the SR (Δ[Cafura-2]), plotted as a function of the resting concentration of fura-2, with the same format as used for Figs. 3 *B* and 5 *D*. The first two points were obtained with a 50-ms (1.703 mM fura-2) and 100-ms (2.593 mM fura-2) pulse. Since these pulse durations were too short to deplete the SR of all its readily releasable Ca, it was necessary to estimate the SR Ca content from exponential fits of the final time course of Δ[Cafura-2], as described in connection with Fig. 3 *B*. The values, plotted as ×'s, are essentially the same as the peak value of Δ[Cafura-2] measured with a 400-ms pulse in the presence of 3.487 mM resting fura-2 (third filled circle from the left). In this experiment, the Ca content of the SR appeared to be relatively constant, 702–707 μM, with 1.703–3.487 mM resting fura-2. At fura-2 concentrations >4 mM, the Ca content progressively increased and, at 7.676 mM fura-2, was 1.6 times the initial value. Such an increase

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[fura-2] = 8.380–9.046 mM), or 800 ms (three points, [fura-2] = 9.151–9.353 mM). The ×'s give the values of Δ[Cafura-2] that would be expected if the SR released all its Ca, as estimated from exponential fits described in connection with Fig. 3 *B*. In *B*, ticks mark the points associated with traces *b–d* in panel *A;* in *C* and *D*, they mark the points associated with traces *c* and *d*. Fiber reference, O20922 (*Rana pipiens*); sarcomere spacing, 4.1 μm; temperature, 14°C. Range of values, beginning to end of experiment: fiber diameter, 87–82 μm; holding current, −21 to −39 nA. 3 mM PDAA (nominal concentration) was initially introduced into the end pools 18 min after saponin treatment of the end pool segments; PDAA plus 8 mM [fura-2] without added Ca was introduced 48 min later. The traces in *A* were taken 44 min (*a*), 62 min (*b*), 77 min (*c*), and 92 min (*d*) after the saponin treatment.

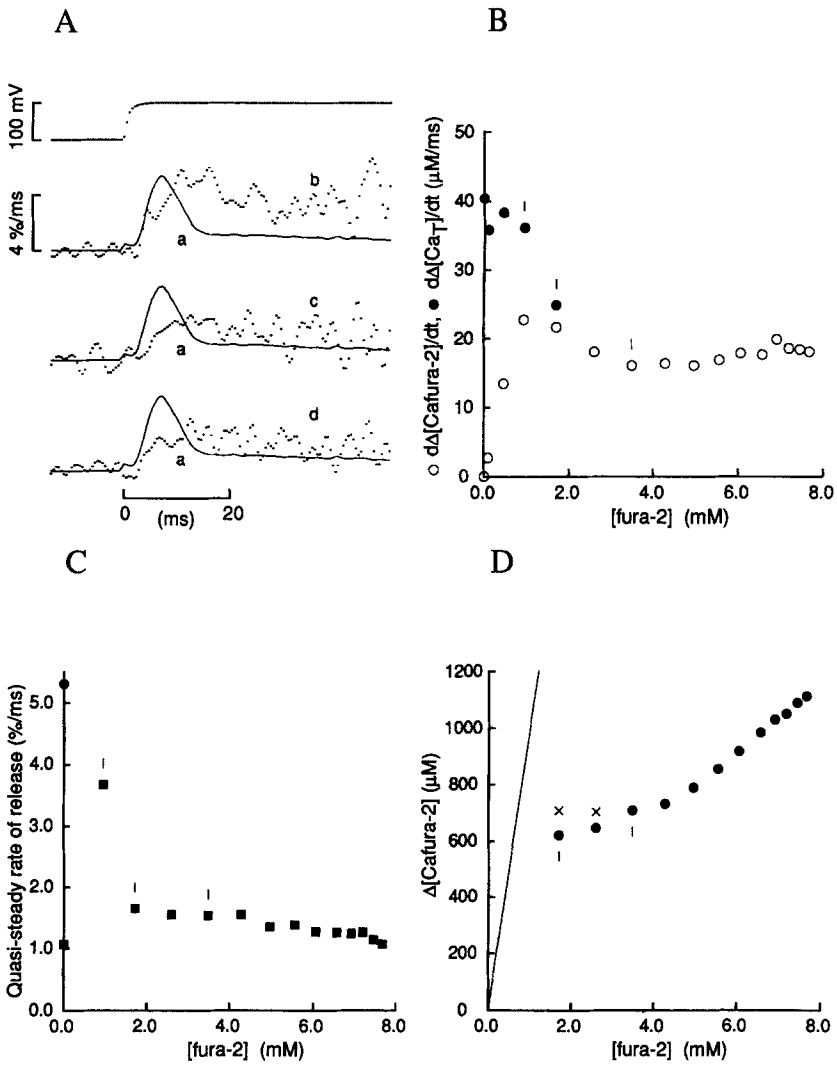

FIGURE 6. Effect of [fura-2] on the rate of SR Ca release during a step depolarization to 10 mV in a fiber from *Rana pipiens* that had a small SR Ca content. Format similar to Fig. 5. (*A*) Traces *a* and *b* show $d\Delta[Ca_T]/dt$ calculated with model 1 and *c* and *d* show $d\Delta[Cafura\text{-}2]/dt$. [PDAA] = 2.415 mM (*a*), 2.831 mM (*b*), 2.803 mM (*c*), and 2.682 mM (*d*); [fura-2] = 0 mM (*a*), 0.944 mM (*b*), 1.703 mM (*c*), and 3.487 mM (*d*). The SR Ca content was estimated from the value of Δ[Cafura-2] in *d* to be 707 μM. The maximal value of $\Delta[Ca_T]$ associated with trace *b* was 785 μM (trace not shown) so that the SR Ca content for traces *a–c* was rather arbitrarily taken to be 900 μM. (*B*) The open and filled circles show the peak amplitudes of $d\Delta[Cafura\text{-}2]/dt$ and $d\Delta[Ca_T]/dt$, respectively, plotted as a function of resting [fura-2]. (*C*) The filled circle and square at [fura-2] = 0 mM show, respectively, the peak and quasi-steady values of $d\Delta[Ca_T]/dt$ from trace *a* in panel *A*. The filled squares at [fura-2] ≥ 1.703 mM show the quasi-steady values of $d\Delta[Cafura\text{-}2]/dt$. The quasi-steady values represent the average values 15–30 ms ([fura-2] =

was atypical and was not observed in the other experiments (Figs. 3 *B* and 5 *D* and column 7 in Table III).

In our seven voltage-clamp experiments, four with *Rana temporaria* and three with *Rana pipiens,* the fura-2 response in Fig. 6, *A–C,* was closest to that reported by Jacquemond et al. (1991). Nonetheless, this fiber was not exactly like any of their fibers. First, in this fiber fura-2 was able to substantially increase the quasi-steady rate of SR Ca release, although the concentration range for the effect was narrow. Second, the quasi-steady rate of release with 0 mM fura-2 was more than three times larger in this fiber, 1.06 %/ms, than that in Fig. 4 of Jacquemond et al. (1991), 0.32 %/ms. Third, the SR Ca content of this fiber was unusually small (Fig. 6 *D*); with a resting concentration of fura-2 <4 mM, it was ≤ 0.3 times the SR Ca content of any of our other fibers (Table III, column 7) and smaller than any of the values given by Jacquemond et al. (1991). The peak amplitude of the Δ[Ca] transient with resting [fura-2] = 0 mM was also small in this fiber, 34 μM (not shown). This value is smaller than that recorded from any of our other fibers in the absence of fura-2 and, in particular, from the two other fibers from *Rana pipiens,* 72 μM (fiber O19921) and 69 μM (fiber O20922). Because of the small SR Ca content and small Δ[Ca] signal, it seems possible that this fiber was damaged or otherwise abnormal; consequently, the results from this fiber should probably be treated with caution.

SR Ca Release Estimated with Three Different Methods in Fibers from Rana temporaria and Rana pipiens

Model 2 (Table I in Pape et al., 1993), from Jacquemond et al. (1991), was also used to estimate the rate of SR Ca release from the myoplasmic free [Ca] signal. The main differences between the assumptions in this model and those in model 1 concern the binding of Ca to the Ca-regulatory sites on troponin and to the SR Ca pump. In model 1, the Ca dissociation constant (K_D) of the troponin binding sites is assumed to be 2 μM and the binding of Ca to the SR Ca pump is neglected. In model 2, troponin's K_D = 7.7 μM and the binding of Ca to the SR pump is represented by 200 μM high affinity sites that equilibrate instantaneously with Ca with K_D = 1 μM.

Fig. 7 *A* shows SR Ca release signals calculated from model 2 with the unscaled Δ[Ca] trace (model 2:Δ[Ca]) and then corrected for SR Ca depletion to give the fractional rate of release. The Δ[Ca] and Δ[Cafura-2] signals were the same as those used for Fig. 2 *B*. Trace *a* shows two early peaks. The first, and larger, peak contains a significant contribution from Ca binding to the SR Ca pump sites, which, as

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0 mM) and 30–100 ms ([fura-2] $\geq$ 1.703 mM) after the depolarization. (*D*) The pulse duration was 50 ms ([fura-2] = 1.703 mM), 100 ms ([fura-2] = 2.593 mM), 400 ms (eight points, [fura-2] = 3.487–7.196 mM), or 600 ms (two points, [fura-2] = 7.457–7.676 mM). In *B* and *C*, ticks mark the points associated with traces *b–d* in panel *A;* in *D*, they mark the points associated with traces *c* and *d*. Fiber reference, O20921 (*Rana pipiens*); sarcomere spacing, 4.1 μm; temperature, 14°C. Range of values, beginning to end of experiment: fiber diameter, 77–65 μm; holding current, −17 to −35 nA. 3 mM PDAA (nominal concentration) was initially introduced into the end pools 23 min after saponin treatment of the end pool segments; PDAA plus 8 mM [fura-2] and no added Ca was introduced 56 min later. The traces in *A* were taken 48 min (*a*), 75 min (*b*), 80 min (*c*), and 90 min (*d*) after the saponin treatment.

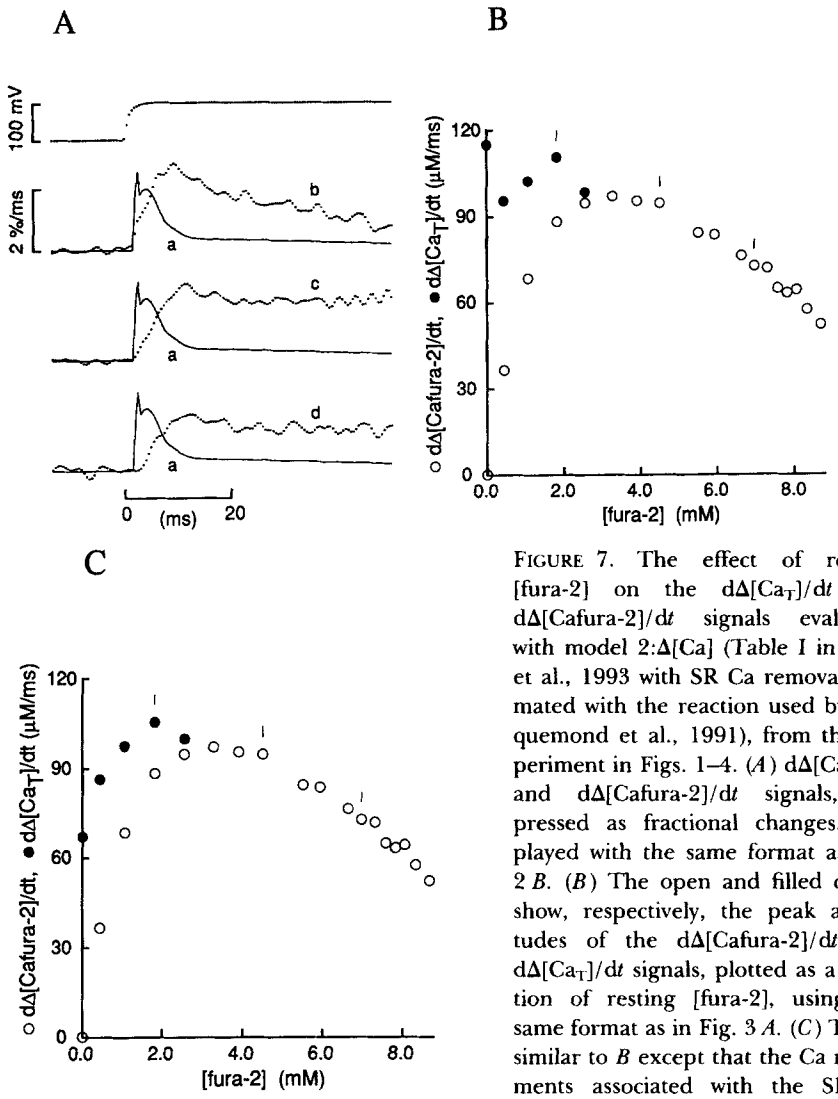

FIGURE 7. The effect of resting [fura-2] on the $d\Delta[Ca_T]/dt$ and $d\Delta[Cafura\text{-}2]/dt$ signals evaluated with model 2:Δ[Ca] (Table I in Pape et al., 1993 with SR Ca removal estimated with the reaction used by Jacquemond et al., 1991), from the experiment in Figs. 1–4. (*A*) $d\Delta[Ca_T]/dt$ and $d\Delta[Cafura\text{-}2]/dt$ signals, expressed as fractional changes, displayed with the same format as Fig. 2 *B*. (*B*) The open and filled circles show, respectively, the peak amplitudes of the $d\Delta[Cafura\text{-}2]/dt$ and $d\Delta[Ca_T]/dt$ signals, plotted as a function of resting [fura-2], using the same format as in Fig. 3 *A*. (*C*) This is similar to *B* except that the Ca movements associated with the SR Ca pump are represented by the 11-step reaction cycle proposed by Fernandez-Belda et al. (1984) instead of the reaction proposed by Jacquemond et al. (1991); see text for additional information. Ticks mark the points associated with traces *b–d* in panel *A*.

mentioned above, are assumed to react with Ca instantaneously and with high affinity, K_D = 1 μM. The second peak contains a large contribution from Ca binding to the troponin sites, which are assumed to react with a short delay and with a lower affinity, K_D = 7.7 μM. Rate of release signals with two peaks were also calculated with model 2:Δ[Ca] from Δ[Ca] signals elicited by action potential stimulation (mentioned but not shown in Pape et al., 1993). Two peaks were never observed in the estimates of the rate of SR Ca release obtained by Jacquemond et al. (1991), probably because

the amplitude of the Δ[Ca] signals that they measured with antipyrylazo III was 10–30 times less than that measured by us with PDAA, as mentioned above.

Trace *b* in Fig. 7 *A* clearly deviates from trace *a* during the early rising phase, unlike the similarity of the two traces in Fig. 2 *B*. Trace *b* lies at or below trace *a* during the first 5 ms of depolarization; it then crosses *a* as *a* decreases and lies above it thereafter. Traces *c* and *d*, which are the same as the corresponding traces in Fig. 2 *B*, show somewhat similar behavior.

Fig. 7 *B* shows the effect of resting fura-2 concentration on the peak values of $d\Delta[Ca_T]/dt$ (filled circles) and $d\Delta[Cafura\text{-}2]/dt$ (open circles). The format of the figure and the values of the open circles are the same as in Fig. 3 *A*. The main difference between the filled circles in Figs. 3 *A* and 7 *B* is the value of the point at [fura-2] = 0 mM; it is 64 μM/ms in Fig. 3 *A* and 115 μM/ms in Fig. 7 *B*. Since the corresponding values of the other filled circles are similar in Figs. 3 *A* and 7 *B*, the value at 0 mM determines whether the effect of the smallest fura-2 concentration that was used, 0.438 mM, was to increase $d\Delta[Ca_T]/dt$, as shown in Fig. 3 *A*, or to decrease it, as shown in Fig. 7 *B*.

One of the assumptions in model 2 is that the sites on the SR Ca pump bind Ca instantaneously and with a higher affinity than the Ca-regulatory sites on troponin. Consequently, during the early part of the free [Ca] transient used to calculate trace *a* in Fig. 7 *A*, the pump bound more Ca than troponin and did so earlier, thereby producing two peaks in the calculated rate of SR Ca release. Since it seems unlikely that the actual release waveform has two peaks, we wanted to assess the effect of using rate constants, rather than instantaneous equilibrium conditions, to describe the binding of Ca to the SR Ca pump. The use of rate constants should delay the complexation of Ca by the pump so that the two peaks might merge into a single peak.

An 11-step reaction cycle has been proposed by Fernandez-Belda, Kurzmack, and Inesi (1984) to describe the binding of Ca by the SR Ca pump and its subsequent translocation into the SR. The time course of Ca removal from the myoplasm can be calculated with this reaction cycle from the time course of myoplasmic free [Ca] and the values of the rate constants in the reaction cycle. This was done by Pape, Konishi, Hollingworth, and Baylor (1990) for Ca removal after a single action potential and the results are shown in their Fig. 7 *A*. Their calculations show that SR Ca release is complete 5–10 ms after stimulation and that, at this time, only ~0.1 of the released Ca has been bound and/or translocated by the SR Ca pump. Consequently, the amount of Ca removed by the SR Ca pump is expected to make little contribution to Ca movements during the first few milleseconds after an action potential.

Calculations similar to those carried out by Pape et al. (1990) were carried out with the Δ[Ca] signals in the experiment illustrated in Figs. 1–4 and 7. The time course of $d\Delta[Ca_T]/dt$ was calculated with model 2:Δ[Ca] with the exception that Ca removal by the SR Ca pump was estimated with the reaction cycle proposed by Fernandez-Belda et al. (1984) rather than the reaction used by Jacquemond et al. (1991). None of the time courses (not shown) had a double peak as shown in trace *a* in Fig. 7 *A*. The filled circles in Fig. 7 *C* show the peak amplitudes of the $d\Delta[Ca_T]/dt$ signals calculated with this modified version of model 2:Δ[Ca]. The values of the points and their depen-

dence on resting [fura-2] are extremely similar to those calculated with model 1 and shown in Fig. 3 *A*. The open circles in Fig. 7 *C* are the same as those in Fig. 7 *B*.

Fig. 8 is similar to Fig. 7 except that the calculations with model 2 were done with the Δ[Ca] signal scaled by the factor 0.25 (model 2:0.25Δ[Ca]). The use of the modified pump calculations (Fig. 8 *C*, analogous to Fig. 7 *C*) accentuates slightly the

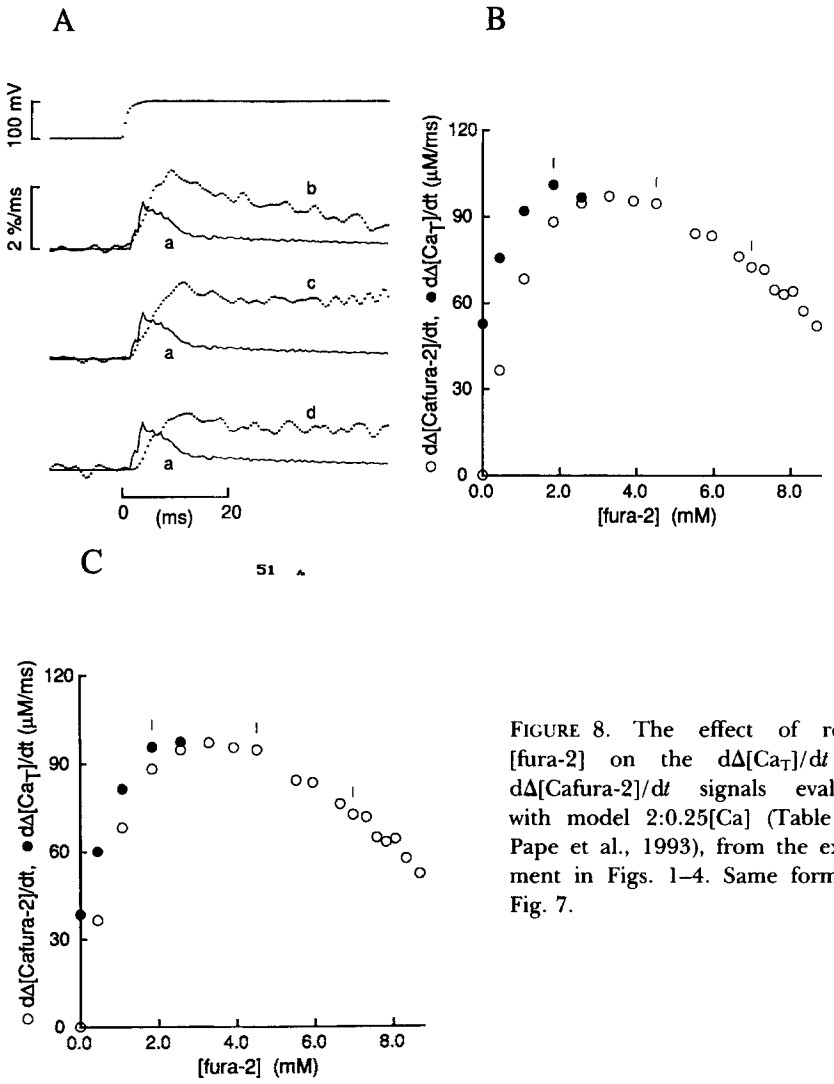

FIGURE 8. The effect of resting [fura-2] on the $d\Delta[Ca_T]/dt$ and $d\Delta[Cafura\text{-}2]/dt$ signals evaluated with model 2:0.25[Ca] (Table I in Pape et al., 1993), from the experiment in Figs. 1–4. Same format as Fig. 7.

increase in $d\Delta[Ca_T]/dt$ observed with an increase in resting [fura-2] from 0 to 1 mM (compare Fig. 8 *C* with Fig. 8 *B*). Although we think that the unscaled Δ[Ca] signal obtained with PDAA represents the correct calibration, the reduction of the amplitude of the Ca signal with model 2:0.25Δ[Ca] was made to facilitate the comparison between our results and those of Jacquemond et al. (1991), who used antipyrylazo III

to measure Ca; the amplitude of a Δ[Ca] signal measured with antipyrylazo III is expected to be only 0.2–0.25 times that measured with PDAA (Maylie et al., 1987*a;* Maylie, Irving, Sizto, and Chandler, 1987*b;* Hirota, Chandler, Southwick, and Waggoner, 1989; Konishi and Baylor, 1991; Konishi, Hollingworth, Harkins, and Baylor, 1991). The results in Figs. 8 *A* and 8, *B* and *C*, are similar to those in Figs. 2 *B* and 3 *A*, respectively, and thus are very different from those of Jacquemond et al. (1991).

Estimates of SR Ca release were also made with model 1 modified to include the rate of Ca removal by the SR Ca pump according to the reaction cycle of Fernandez-Belda et al. (1984). For the five runs with resolvable Ca transients that were used in Fig. 3 *A* (filled circles), the peak values of $d\Delta[Ca_T]/dt$ calculated from model 1 with and without the pump contribution are, respectively, 73 and 64 μM/ms ([fura-2] = 0 mM), 92 and 86 μM/ms (0.438 mM), 105 and 101 μM/ms (1.065 mM), 109 and 107 μM/ms (1.825 mM), and 104 and 103 μM/ms (2.561 mM). Thus, if the rate of Ca removal by the SR Ca pump is calculated with the model of Fernandez-Belda et al. (1984) and then added to $d\Delta[Ca_T]/dt$ calculated with model 1, the peak value of $d\Delta[Ca_T]/dt$ monotonically increases when the concentration of fura-2 increases from 0 to 1.825 mM, contrary to the rather complex relation obtained with model 2:Δ[Ca] (Fig. 7 *B*).

In summary, the rate of SR Ca release has been calculated in several different ways for the experiment in Figs. 1–4 and 7 and 8. The most reliable estimates are probably those based on model 1, with or without Ca removal by the SR Ca pump as estimated from the reaction cycle of Fernandez-Belda et al. (1984). The estimate based on model 2:Δ[Ca] with Ca removal determined from the cycle of Fernandez-Belda et al. (1984) may also be reliable, although the value of K_D for the Ca-regulatory sites on troponin, 7.7 μM, is larger than values measured under in vitro conditions with rabbit troponin reconstituted on thin filaments (K_D = 2 μM at 20°C, Rosenfeld and Taylor, 1985; K_D = 1.3 μM with 1 mM Mg at 25°C, Zot and Potter, 1987). The value of 7.7 μM for the K_D is also larger than the value of free [Ca] required to give a half-maximal contraction in skinned fibers from *Rana pipiens* (1.3 μM at 15°C and pH 7.00, Godt and Lindley, 1982) or from *Rana temporaria* (2.1 μM at 16°C and pH 7.24, Brotto, M. A. P., and R. E. Godt, personal communication). All these estimates show that the peak rate of SR Ca release increases as the value of resting [fura-2] is increased from 0 to 1.825 mM. Consequently, we are inclined to accept this monotonic increase as reliable. Model 2:Δ[Ca], with Ca removal by the SR Ca pump determined from the reaction of Jacquemond et al. (1991), probably fails to give a reliable estimate of the rate of SR Ca release (Fig. 7, *A* and *B*).

Effect of Fura-2 on the Peak Rate of SR Ca Release during a Voltage Pulse to 10 mV in Fibers from Rana temporaria and Rana pipiens

Table I (*Rana temporaria*) and Table II (*Rana pipiens*) provide a summary of the effect of resting fura-2 concentration on the peak rate of SR Ca release estimated with models 1, 2:Δ[Ca], and 2:0.25Δ[Ca]; the calculations with model 2 used the reaction of Jacquemond et al. (1991) to estimate Ca removal by the SR Ca pump rather than the reaction cycle proposed by Fernandez-Belda et al. (1984). The format of the tables is similar to Table IV in Pape et al. (1993) except that the last column gives

values of the fractional rate of SR Ca release, obtained from column *3* after correction for SR Ca depletion. With the exception of fiber O20921 in Table II, the condition of which is somewhat questionable (see the text discussion of Fig. 6), the calculations with model 1 and model 2:0.25Δ[Ca] show that 0.5–2 mM fura-2 (Tables I and II) and 2–3 mM fura-2 (Table I only) increased the peak value of $d\Delta[Ca_T]/dt$ with respect to that estimated at 0 mM fura-2. The calculations with model 2:Δ[Ca] show

TABLE I

Effect of 0–3 mM Fura-2 on the Peak Rate of SR Ca Release during a Voltage Step to 10 mV in Fibers from Rana temporaria

(1) Fiber	(2) Method	(3) 0 mM	(4) 0.5–2.0 mM	(5) 2.0–3.0 mM	(6) 0 mM
		$d\Delta[Ca_T]/dt$			
		μM/ms	*μM/ms*	*μM/ms*	*%/ms*
325921	Model 1	45	n.m.	n.m.	0.96
(2/8)	Model 2:Δ[Ca]	60	n.m.	n.m.	1.26
	Model 2:0.25Δ[Ca]	37	n.m.	n.m.	0.79
616922	Model 1	44	59 (1.34)	97 (2.20)	1.20
(1/8)	Model 2:Δ[Ca]	70	67 (0.96)	99 (1.41)	1.87
	Model 2:0.25Δ[Ca]	36	58 (1.61)	95 (2.64)	0.97
617921	Model 1	27	47 (1.74)	51 (1.89)*	0.44
(1/8)	Model 2:Δ[Ca]	57	50 (0.88)	51 (0.89)*	0.91
	Model 2:0.25Δ[Ca]	22	46 (2.09)	51 (2.32)*	0.35
618921	Model 1	64	103 (1.61)	102 (1.59)	1.40
(0/8)	Model 2:Δ[Ca]	115	106 (0.92)	98 (0.85)	2.49
	Model 2:0.25Δ[Ca]	53	96 (1.81)	97 (1.83)	1.15
Mean	Model 1	47	70 (1.56)	83 (1.89)	1.00
	Model 2:Δ[Ca]	77	74 (0.92)	83 (1.05)	1.63
	Model 2:0.25Δ[Ca]	39	67 (1.84)	81 (2.26)	0.82

n.m., no measurements were made at these concentrations of fura-2. Column *1* gives the fiber reference and, in parentheses, the values of [Cafura-2] and [fura-2_T] in millimolar in the end pool solutions separated by a slash mark. Column *2* gives the method used to estimate SR Ca release from myoplasmic free [Ca] (Table I in Pape et al., 1993 with SR Ca removal in model 2 described by the reaction of Jacquemond et al., 1991). Columns *3–5* give peak values of $d\Delta[Ca_T]/dt$ obtained with 2.2–2.9 mM PDAA and 0, 0.5–2.0, or 2.0–3.0 mM fura-2 at the optical site, as indicated. In each row, the numbers in parentheses in columns *4* and *5* represent the values in these columns divided by the value in column *3*. Column *6* gives the values of $d\Delta[Ca_T]/dt$ from the traces used for column *3*, corrected for SR Ca depletion and expressed in units of percent per millisecond.

*The Δ[Ca] signal was unreliable so that the value of $d\Delta[Ca_T]/dt$ was determined from $d\Delta$[Cafura-2]/dt alone.

either no change or a decrease with [fura-2] = 0.5–2 mM, but, as mentioned above, these calculations are considered to be unreliable.

At concentrations of fura-2 >2–3 mM, the peak rate of SR Ca release was only slightly greater than the quasi-steady rate. The effect of fura-2 on the quasi-steady level of Ca release is described in the next section.

Effect of Fura-2 on the Quasi-steady Rate of SR Ca Release during a Voltage Pulse to 10 mV in Fibers from Rana temporaria and Rana pipiens

Table III summarizes our results on the effect of 2–8 mM fura-2 on the quasi-steady rate of SR Ca release, expressed as the fractional rate of release, during a step depolarization to 10 mV. Parts *A* and *B* give results obtained with fibers from *Rana temporaria* and *Rana pipiens*, respectively. Column *1* gives the fiber reference with the values of [Cafura-2] and [fura-2_T] in the end pool solutions given in parentheses. Column *2* gives the peak values of $d\Delta[Ca_T]/dt$ calculated with model 1 from the PDAA [Ca] transient obtained with [fura-2] = 0 mM; these are the same as the values

TABLE II

Effect of 0–2 mM Fura-2 on the Peak Rate of SR Ca Release during a Voltage Step to 10 mV in Fibers from Rana pipiens

(1) Fiber	(2) Method	(3) 0 mM	(4) 0.5–2.0 mM	(5) 0 mM
		$d\Delta[Ca_T]/dt$		
		μM/ms	*μM/ms*	*%/ms*
O19921	Model 1	60	75 (1.25)	2.60
(0/8)	Model 2:Δ[Ca]	122	90 (0.74)	5.18
	Model 2:0.25Δ[Ca]	51	69 (1.35)	2.28
O20921	Model 1	42	30 (0.71)	5.30
(0/8)	Model 2:Δ[Ca]	67	37 (0.55)	8.47
	Model 2:0.25Δ[Ca]	34	25 (0.74)	4.15
O20922	Model 1	53	83 (1.57)	2.19
(0/8)	Model 2:Δ[Ca]	89	90 (1.01)	3.46
	Model 2:0.25Δ[Ca]	43	82 (1.90)	1.81
Mean	Model 1	52	63 (1.21)	3.36
	Model 2:Δ[Ca]	93	72 (0.77)	5.70
	Model 2:0.25Δ[Ca]	43	59 (1.37)	2.75

This table is similar to Table I except that fibers from *Rana pipiens* were used. The information corresponding to column *5* in Table I has been omitted because the PDAA [Ca] transient with 2.0–3.0 mM fura-2 was essentially zero (similar to the signal in Fig. 1 *C*) and did not have a positive phase to use for the calculation with model 1 or 2. Consequently, column *5* in this table is similar to column *6* in Table I. PDAA concentration at the optical site, 2.4–3.0 mM.

in the last column of Tables I and II, model 1. Similar calculations were carried out with models 2:Δ[Ca] and 2:0.25Δ[Ca] and the peak values of $d\Delta[Ca_T]/dt$ are also given in the last column of Tables I and II.

Column *3* of Table III gives the quasi-steady values of $d\Delta[Ca_T]/dt$ calculated with model 1 ([fura-2] = 0 mM). Similar calculations were carried out with models 2:Δ[Ca] and 2:0.25Δ[Ca] and with model 1 modified to include Ca removal by the SR as estimated with the 11-step reaction cycle of Fernandez-Belda et al. (1984). The mean values, given in the legend of Table III, are in the range 0.32–0.34 %/ms for fibers from *Rana temporaria* and 0.98–1.22 %/ms for fibers from *Rana pipiens*. These values

TABLE III

Effect of 2–8 mM Fura-2 on the Quasi-steady Rate of SR Ca Release during a Voltage Step to 10 mV

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Fiber	0 mM $d\Delta[Ca_T]/dt$		2.0–3.0 mM	5.0–6.0 mM	7.0–8.0 mM	SR Ca content
			$d\Delta$[Cafura-2]/dt			
	Peak	Steady	Steady	Steady	Steady	
	%/ms	%/ms	%/ms	%/ms	%/ms	μM
A Rana temporaria						
325921 (2/8)	0.96	0.25	n.m.	1.07 (4.3)	n.m.	4,873
616922 (1/8)	1.20	0.28	1.89 (6.8)	2.83 (10.1)	1.35 (4.8)	3,818–4,312
617921 (1/8)	0.44	0.11	0.80 (7.3)	0.55 (5.0)	0.14 (1.3)	6,371
618921 (0/8)	1.40	0.26	1.51 (5.8)	1.67 (6.4)	1.20 (4.6)	4,689–4,446
Mean	1.00	0.23	1.40 (6.6)	1.53 (6.5)	0.90 (3.6)	
SEM	0.21	0.04	0.32 (0.4)	0.49 (1.3)	0.38 (1.1)	
B Rana pipiens						
O19921 (0/8)	2.60	0.43	1.72 (4.0)	1.27 (3.0)	0.94 (2.2)	2,476–2,372
O20921 (0/8)	5.30	1.06	1.54 (1.5)	1.37 (1.3)	1.15 (1.1)	702–1,078
O20922 (0/8)	2.19	0.39	2.29 (5.9)	1.71 (4.4)	1.01 (2.6)	2,649–2,492
Mean	3.36	0.63	1.85 (3.8)	1.45 (2.9)	1.03 (2.0)	
SEM	0.98	0.22	0.23 (1.3)	0.13 (0.9)	0.06 (0.5)	

n.m., no measurements were made at these concentrations of fura-2. Column *1* gives the fiber reference and, in parentheses, the values of [Cafura-2] and [fura-2_T] in millimolar in the end pool solutions separated by a slash mark. Column *2* gives the peak values of $d\Delta[Ca_T]/dt$ calculated from model 1 with [fura-2] = 0 mM. Similar calculations were carried out with model 2 with Jacquemond et al.'s (1991) model of SR Ca removal; the values are given in column *6* in Table I and column *5* in Table II. Calculations were also carried out with model 1 and the model of Fernandez-Belda et al. (1984) for SR Ca removal; values for the fiber used for Figs. 1–4, 7, and 8 are given in the text just after the discussion of Fig. 8. Column *3* gives the quasi-steady values of $d\Delta[Ca_T]/dt$ calculated from model 1 with [fura-2] = 0 mM. Calculations carried out with model 2 and Jacquemond et al.'s (1991) model of SR Ca removal gave the following mean values (±SEM) of the quasi-steady rate of release: for *Rana temporaria*, 0.32 (±0.04) %/ms with model 2:Δ[Ca] and 0.32 (±0.05) %/ms with model 2:0.25Δ[Ca]; for *Rana pipiens*, 1.22 (±0.60) %/ms with model 2:Δ[Ca] and 1.04 (±0.42) %/ms with model 2:0.25Δ[Ca]. Calculations with model 1 and the model of Fernandez-Belda et al. (1984) for SR Ca removal gave 0.34 (±0.05) %/ms for *Rana temporaria* and 0.98 (±0.57) %/ms for *Rana pipiens*. Columns *4–6* give the quasi-steady values of $d\Delta$[Cafura-2]-dt obtained in the presence of 2.0–3.0, 5.0–6.0, or 7.0–8.0 mM fura-2 at the optical site, as indicated; each number in parentheses represents the ratio of the number in the column to that in column *3*. The values in columns *2–6* have been corrected for SR Ca depletion and expressed as fractional rates of SR Ca release, as described in the text. The quasi-steady values in column *3* were averaged from 15–20 to 30 ms after the depolarization; the values in columns *4–6* represent similar averages, usually over the interval 30–100 ms after the depolarization. Column 7 gives the range of the values of SR Ca content that were used for the depletion corrections in columns *2–6*; these were determined from plateau values of Δ[Cafura-2] and are expressed in units of myoplasmic concentration. The experiments in *A* and *B* were carried out on fibers from *Rana temporaria* and *Rana pipiens*, respectively. The concentration of PDAA at the optical site was 2.2–3.0 mM in *A* and 2.4–3.0 mM in *B*. Additional information is given in Tables I and II.

are somewhat larger than those calculated with model 1 without Ca removal by the SR pump, 0.23 and 0.63 %/ms, respectively (column *3*).

Column 7 of Table III gives the range of values of SR Ca content that were used for the SR Ca depletion corrections. Interestingly, the SR Ca content in the voltage-clamp experiments on fibers from *Rana temporaria* (*A*) was two to three times that in the action potential experiments, which were also carried out on fibers from *Rana temporaria* (column *8* of Table III in Pape et al., 1993). This difference is considered to be genuine since the experiments were done on the same batches of frogs and even on the same days (compare fiber references in column *1* of Table III in this article with those in column *1* of Table III in Pape et al., 1993). This suggests that the Ca content of the SR is influenced by the ionic composition of the internal or external solution. A possible explanation, for which we have no evidence, is that resting myoplasmic free [Ca] was maintained at a lower value in the action potential experiments because of the action of the Na-Ca exchanger. Since external Na was not present in the voltage-clamp experiments, myoplasmic [Ca] may have been higher, thus allowing the SR Ca pump to accumulate more Ca inside the SR.

Columns *4–6* of Table III give the quasi-steady values of $d\Delta[\text{Cafura-2}]/dt$ obtained with 2–3, 5–6, and 7–8 mM fura-2 at the optical site, as indicated. The values in parentheses, which have been normalized by the corresponding values in column *3*, are all greater than unity. Thus, fura-2 in the range of concentrations used in columns *4–6* (2–8 mM) always increased the quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ above that estimated with 0 mM fura-2.

Table III *A* shows that the action of fura-2 was similar in the four fibers studied from *Rana temporaria*. The quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ with either 2–3 or 5–6 mM fura-2 (columns *4* and *5*) exceeded both the peak and quasi-steady values of $d\Delta[Ca_T]/dt$ estimated with [fura-2] = 0 mM (columns *2* and *3*). With 7–8 mM fura-2 (column *6*), the quasi-steady value of $d\Delta[\text{Cafura-2}]/dt$ exceeded that of $d\Delta[Ca_T]/dt$ with 0 mM fura-2 in all three fibers tested, but exceeded the peak value in only one fiber (616922).

On average, the quasi-steady values of $d\Delta[\text{Cafura-2}]/dt$ for 2–3, 5–6, and 7–8 mM fura-2 were 6.6, 6.5, and 3.6 times the quasi-steady values of $d\Delta[Ca_T]/dt$ estimated with model 1 for 0 mM fura-2 (values in parentheses, columns *4–6* in Table III *A*). Similar comparisons were made using model 2 to estimate the quasi-steady values of $d\Delta[Ca_T]/dt$ with [fura-2] = 0 mM (values for individual fibers not given). The corresponding factors were 4.4, 4.5, and 2.5 with both model 2:Δ[Ca] and model 2:0.25Δ[Ca]. Thus, even at concentrations as large as 7–8 mM, fura-2 increased considerably the quasi-steady rate of SR Ca release in fibers from *Rana temporaria*.

Fura-2 had a similar, but less pronounced, effect on the quasi-steady rate of SR Ca release in fibers from *Rana pipiens* (Table III *B*). All the values in columns *4–6* are larger than the corresponding values for 0 mM fura-2 (column *3*). Only one of them, however (2.29 %/ms for fiber O20922 in column *4*), is larger than the corresponding peak value (column *2*). The ratios of the values in columns *4–6* to those in column *3* (numbers in parentheses in columns *4–6*) are roughly half those in part *A;* the mean values are 3.8, 2.9, and 2.0 for 2–3, 5–6, and 7–8 mM fura-2, respectively. When model 2 was used to estimate the quasi-steady values of $d\Delta[Ca_T]/dt$ with 0 mM fura-2,

the corresponding values were 2.4, 1.8, and 1.2 with model 2:Δ[Ca] and 2.4, 1.9, and 1.2 with model 2:0.25Δ[Ca].

Other differences between the results from *Rana temporaria* (Table III *A*) and *Rana pipiens* (Table III *B*) are that (*a*) both the peak and quasi-steady levels of the fractional rate of SR Ca release with 0 mM fura-2 are greater in Table III *B* than in Table III *A* (columns *2* and *3*) and (*b*) the SR Ca content is two to three times greater in Table III *A* than in Table III *B* (column *7*). Fiber O20921, the questionable fiber used for Fig. 6, had the largest fractional rates of SR Ca release (columns *2* and *3*) and the smallest SR Ca content (column *7*). These differences between fibers from *Rana temporaria* and *Rana pipiens* may be due to a species difference, to the difference in the time of year when the experiments were carried out (March and June for *Rana temporaria* and October for *Rana pipiens*), or to some unknown factor.

A Possible Effect of SR Ca Content on the Peak Rate of SR Ca Release

The data in Table III suggest that a correlation may exist between the fractional rate of SR Ca release and SR Ca content. Fig. 9 *A* shows the peak value of $d\Delta[Ca_T]/dt$ with [fura-2] = 0 mM (expressed in terms of the fractional rate of release, from column *2* in Table III), plotted as a function of SR Ca content. The circles and squares are from fibers from *Rana temporaria* and *Rana pipiens*, respectively. The left-most point, with 900 μM SR Ca content, is from fiber O20921, which, as discussed above in connection with Fig. 6, may be unreliable. The points in Fig. 9 *A* appear to fall on the same curve. This suggests that, if the fractional rate of SR Ca release with [fura-2] = 0 mM is affected by SR Ca content, the effect is the same for fibers from *Rana temporaria* and *Rana pipiens*. It is difficult to be certain about this, however, because the SR Ca contents of the fibers from *Rana pipiens* were all less than those from *Rana temporaria* and the two species of frogs were studied at different times of the year. In spite of these uncertainties, the data in Fig. 9 *A* will now be discussed as though they represent a reliable relation between the peak value of $d\Delta[Ca_T]/dt$ and SR Ca content.

The peak values of $d\Delta[Ca_T]/dt$ in Fig. 9 *A* span a 12-fold range (including fiber O20921) or a sixfold range (excluding fiber O20921) and vary inversely with SR Ca content. This inverse relation arises because, with the possible exception of fiber 617921, the peak value of $d\Delta[Ca_T]/dt$ in micromolar per millisecond (model 1 in column *3* of Tables I and II) is relatively independent of SR Ca content. (Note that [$d\Delta[Ca_T]/dt$ in μM/ms] ≅ [$d\Delta[Ca_T]/dt$ in %/ms] × [SR Ca content] ÷ 100). Fig. 9 *B* shows $\Delta[Ca_T]$ at the time of the peak rate of SR Ca release plotted against SR Ca content. Except for the right-most point (fiber 617921), the values are relatively constant and show that, at the time of peak rate of release, the amount of Ca that had been released from the SR was relatively independent of SR Ca content.

A possible explanation for the inverse relation in Fig. 9 *A* and the constant relation in Fig. 9 *B* is that Ca inactivation of Ca release, which depresses the peak value of $d\Delta[Ca_T]/dt$, becomes more effective as the SR Ca content increases. This is plausible since, at any moment of time after an activating depolarization, the rate of SR Ca release and thus the value of free [Ca] are expected to increase with SR Ca content.

Suppose for the moment that, in the absence of Ca inactivation of Ca release, the peak rate of fractional SR Ca release were constant and independent of SR Ca

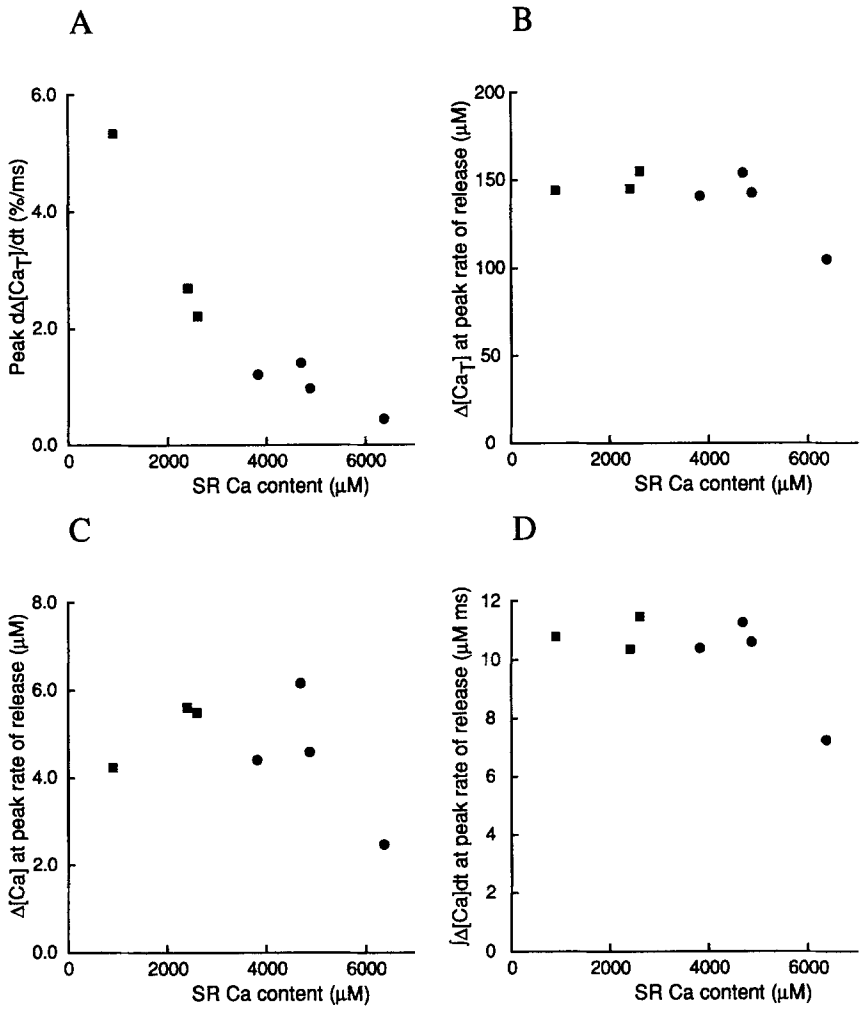

FIGURE 9. Effect of SR Ca content on peak rate of SR Ca release in fibers from *Rana temporaria* (*filled circles*) and *Rana pipiens* (*filled squares*). Each point represents the mean value of measurements obtained from a single fiber with 0 mM fura-2. In all panels, the abscissa represents SR Ca content. *A* shows the peak value of $d\Delta[Ca_T]/dt$ estimated with model 1, expressed in terms of the fractional rate of release. *B* shows the value of $\Delta[Ca_T]$ at the time of peak $d\Delta[Ca_T]/dt$. *C* shows the value of Δ[Ca] at the time of peak $d\Delta[Ca_T]/dt$. *D* is similar to *C* except that the ordinate is the integral of Δ[Ca] from the time of depolarization to the time when $d\Delta[Ca_T]/dt$ reached its peak value. From the experiments listed in Table III.

content. In this case, the value of the noninactivated rate of release would be expected to be at least as large as the maximal rate plotted in Fig. 9 *A*, which is given by the value of the point at 900 μM SR Ca content. To explain the peak value of $d\Delta[Ca_T]/dt$ associated with the largest SR Ca content, 6,371 μM, the SR Ca release pathway would have to have been >90% inactivated at the time of the peak rate of release.

It therefore seemed of interest to determine the amplitude of the Ca signal at the time of the peak rate of SR Ca release, to see whether larger Ca signals, and presumably larger extents of Ca inactivation of Ca release, were associated with larger SR Ca contents. Fig. 9 *C* shows the values of myoplasmic free Δ[Ca] measured at the time of the peak of the $d\Delta[Ca_T]/dt$ signal, plotted as a function of SR Ca content. Fig. 9 *D* shows a similar plot of the integral of Δ[Ca] from the beginning of the depolarization to the time of the peak rate of release. Except possibly for the right-most point in each panel (from fiber 617921), the data in Fig. 9, *C* and *D*, show no consistent dependence on SR Ca content. Therefore, they provide no direct support for the idea that a large SR Ca content produces a large Δ[Ca] signal which then produces a large amount of Ca inactivation of Ca release.

It seems possible, however, that Ca inactivation of Ca release is modulated by the concentration of Ca near the release sites rather than in the bulk myoplasmic solution. Near the release sites, the value of free Δ[Ca] is expected to be given by the sum of two terms: Δ[Ca] in the bulk myoplasmic solution (approximated by the data in Fig. 9 *C*) and a "convergence" term (or terms) that is directly proportional to the Ca flux through a site or sites nearby. The $d\Delta[Ca_T]/dt$ signal (in units of micromolar per millisecond), suitably scaled, might be used as a first approximation of the Ca flux through the nearby site (or sites). As mentioned above, however, the peak value of $d\Delta[Ca_T]/dt$ (in units of micromolar per millisecond) shows no consistent variation with SR Ca content so that the convergence term would be expected to be relatively independent of SR Ca content. Thus, the inverse relation in Fig. 9 *A* and the constant relation in Fig. 9 *B* cannot be explained by Ca inactivation of Ca release that is regulated primarily by either free [Ca] in the bulk myoplasmic solution or free [Ca] near release sites that pass a flux of Ca that is proportional to $d\Delta[Ca_T]/dt$.

A possible explanation for the inverse relation in Fig. 9 *A* and the constant relation in Fig. 9 *B* is given in the Discussion. The essential idea is that a single SR Ca release channel is regulated by locally induced Ca inactivation and that this regulation allows it, on activation, to pass a fixed number of Ca ions, independent of the magnitude of the single channel flux, before inactivation stops the flow of ions through the channel. Regardless, however, of whether this or some other explanation is correct, experiments such as that in Figs. 1–4 clearly show that 0.5–2 mM fura-2 is able to reduce Ca inactivation of Ca release and that this reduction is similar to the reduction of free Δ[Ca] in bulk myoplasm estimated with PDAA.

A Possible Effect of SR Ca Content on the Quasi-steady Rate of SR Ca Release

Fig. 10 shows the quasi-steady values of $d\Delta[Ca_T]/dt$ plotted against SR Ca content, from the experiments illustrated in Fig. 9. The filled symbols show the values with [fura-2] = 0 mM (from column *3* of Table III). The data show an inverse relation with respect to the SR Ca content, similar to that observed in Fig. 9 *A* and possibly due to Ca inactivation of Ca release. The open symbols in Fig. 10 show the quasi-steady values of $d\Delta[Cafura\text{-}2]/dt$ that were obtained after the resting concentration of fura-2 at the optical site had reached 5–6 mM (from column *5* of Table III).

The scatter in the data with 5–6 mM fura-2 makes it difficult to tell whether the quasi-steady rate of SR Ca release was constant or varied with SR Ca content. All the points, however, lie above the corresponding points with 0 mM fura-2, indicating that

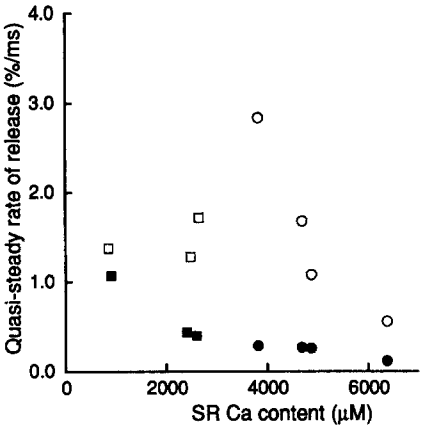

FIGURE 10. Effect of SR Ca content on quasi-steady rate of SR Ca release in fibers from *Rana temporaria* (*circles*) and *Rana pipiens* (*squares*). The filled and open symbols show, respectively, the values obtained with 0 and 5–6 mM fura-2 at the optical site. From the experiments used in Fig. 9 and listed in Table III.

5–6 mM fura-2 is able to reduce Ca inactivation of Ca release. This large concentration of fura-2 may also have other effects (see Discussion), such as a reduction of Ca-induced Ca release or a pharmacological action unrelated to Ca buffering.

DISCUSSION

The primary purpose of this section is to discuss the differences between our results and those of Jacquemond et al. (1991). In both sets of experiments, the rate of SR Ca release was estimated in voltage-clamped fibers during a depolarization to 10–20 mV. In our experiments, 0.5–3 mM fura-2 increased the quasi-steady rate of release in all fibers studied and increased the peak rate of release in all but one of these fibers. In contrast, Jacquemond et al. (1991) found no increase of the rate of SR Ca release with 1.5–2 mM fura-2; the quasi-steady rate of release was unchanged and the peak rate was reduced to the quasi-steady level. If these actions of fura-2 are due to its ability to complex Ca and thereby reduce free [Ca], our results would be consistent with a reduction of Ca inactivation of Ca release, whereas their results would be consistent with a reduction of Ca-induced Ca release. These two types of regulation of SR Ca release are discussed below.

Ca Inactivation of Ca Release

The determination of whether fura-2 increases or decreases the rate of SR Ca release depends on a comparison of the rates of release in the absence and presence of fura-2. Different methods were used by Jacquemond et al. (1991) and by us to estimate the rate of SR Ca release with 0 mM fura-2 and the associated resting Ca content of the SR; the value of the SR Ca content is required to correct the rate of SR Ca release, in units of micromolar per millisecond, for SR Ca depletion to give the fractional rate of release, in units of percent per millisecond. The discussion below shows that these different methods are expected to give similar values for the rate of SR Ca release. Consequently, it is unlikely that they account for the marked differences observed by Jacquemond et al. (1991) and us on the effect of 0.5–3 mM fura-2 on SR Ca release.

COMPARISON OF THE METHODS USED TO ESTIMATE SR Ca RELEASE WITH O mM FURA-2

SR Ca content. In the experiments reported here and by Jacquemond et al. (1991), the rate of SR Ca release was frequently normalized by SR Ca content to give the fractional rate of release in units of percent per millisecond. To make this conversion, the amount of Ca inside the SR before stimulation must be known.

In our experiments, SR Ca content was determined from the value of Δ[Cafura-2] measured after a long depolarization with an excess concentration of fura-2 in the myoplasm (Fig. 1 *D*). One advantage of this method is that it is direct. A disadvantage is that it can only be used when the concentration of fura-2 in the myoplasm exceeds that of Ca in the SR (expressed in terms of myoplasmic concentration). In most experiments, once resting [fura-2] exceeded the plateau value of Δ[Cafura-2] during a long-lasting depleting depolarization, there was a range of fura-2 concentrations where the estimated SR Ca content was relatively constant (Figs. 3 *B*, 5 *D*, and 6 *D*). Consequently, the value of SR Ca content at smaller fura-2 concentrations was usually taken to be the same as that estimated when resting [fura-2] was ~ 1 mM larger than the plateau value of Δ[Cafura-2]. The implicit assumption is that the Ca content of the SR does not change during the 30–50-min period that separates the measurements made with resting [fura-2] = 0 mM and with resting [fura-2] > the plateau value of Δ[Cafura-2].

Although it seems likely that the SR Ca content was reasonably constant during this period (before the first reliable estimate of content could be obtained) the possibility of a change in content should be kept in mind. During the first few depolarizations (given every 5 min), some of the Ca that is initially inside the SR might leave and fail to be reaccumulated by the SR Ca pump, especially if Ca-free fura-2 were used in the end pool solutions as was done in one experiment with *Rana temporaria* and in all three experiments with *Rana pipiens*. Thus, the SR Ca content might progressively decrease during the first few measurements so that the initial value with [fura-2] = 0 mM would be underestimated. If this happened, our estimated fractional rates of SR Ca release with [fura-2] = 0 mM (in units of percent per millisecond) would be too large and, consequently, the reported increases with fura-2 would be too small.

Jacquemond et al. (1991) estimated SR Ca content with an indirect method: the value was adjusted computationally until the rate of SR Ca release, corrected for SR Ca depletion, was constant by the end of a 200-ms pulse, as would be expected if the Ca permeability of the SR membrane were constant and the driving force were directly proportional to SR content. Their Figs. 1–5 show results from four injection experiments, one with fura-2 alone and three with a mixture of BAPTA plus fura-2. Surprisingly, in every experiment the content was increased after the injection, although the value of resting myoplasmic free [Ca], which was given for two experiments, decreased. The range of their values of SR Ca content, 1,200–2,550 μM before injection and 1,600–3,200 μM after injection, is similar to our range with the same species of frog (*Rana pipiens*), 760–2,649 μM (Table III, column 7). Because the two methods give similar estimates of SR Ca content, it seems reasonable to tentatively conclude that the *Rana pipiens* fibers used by Jacquemond et al. (1991) and by us had similar SR Ca contents.

Peak rate of SR Ca release without fura-2. Since the experiments carried out by Jacquemond et al. (1991) and by us show different effects of fura-2 on the peak rate of SR Ca release, it is important to compare the methods used to estimate SR Ca release in the absence of fura-2, since this gives the control or baseline value. Jacquemond et al. (1991) used model 2 (Table I in Pape et al., 1993) to calculate SR Ca release from the antipyrylazo III [Ca] transient. Our method was similar except that we used the [Ca] transient measured with PDAA and relied primarily on calculations with model 1 (Table I in Pape et al., 1993).

Fig. 6 in Jacquemond et al. (1991) gives a mean value of 1.73 %/ms for the peak rate of SR Ca release, corrected for SR Ca depletion, in 16 experiments with [fura-2] = 0 mM. Our mean value with *Rana pipiens,* 3.36 %/ms, is almost twice as large (model 1, column *5,* Table II).

We also calculated SR Ca release with model 2 so that our estimates of the rate of SR Ca release could be compared directly with those of Jacquemond et al. (1991). The first calculations were carried out with the unscaled PDAA [Ca] transient, model 2:Δ[Ca]. As mentioned above in connection with Fig. 7 *A,* the estimates of the rate of SR Ca release calculated with model 2:Δ[Ca] are probably unreliable. Calculations were then carried out with the PDAA [Ca] transient scaled by 0.25, model 2:0.25Δ[Ca]. Although the unscaled PDAA Δ[Ca] signal is expected to represent a reliable estimate of myoplasmic free Δ[Ca], the factor 0.25 was introduced to compensate for the expectation that the amplitude of the antipyrylazo III Δ[Ca] signal is only 0.2–0.25 times that of the PDAA Δ[Ca] signal (Maylie et al., 1987*a, b;* Hirota et al., 1989; Konishi and Baylor, 1991; Konishi et al., 1991). Consequently, our estimates of SR Ca release with model 2:0.25Δ[Ca] and PDAA [Ca] transients can be compared directly with those of Jacquemond et al. (1991), who used antipyrylazo III [Ca] transients. With fibers from *Rana pipiens,* the mean peak value of the rate of SR Ca release was 2.75 %/ms (model 2:0.25Δ[Ca], column *5,* Table II), which is 1.6 times that observed by Jacquemond et al. (1991).

Since the values of the peak rate of SR Ca release in the absence of fura-2 are greater when calculated with model 1 than with model 2:0.25Δ[Ca], the difference between the two models cannot account for the fact that, in five out of six fibers, we found an increase in the peak rate of release with 0.5–2 mM fura-2, whereas Jacquemond et al. (1991) always found a decrease. This difference in the effect of 0.5–2 mM fura-2 suggests that the condition of our fibers was different from that of the fibers studied by Jacquemond et al. (1991).

Quasi-steady rate of SR Ca release without fura-2. Fig. 6 in Jacquemond et al. (1991) gives a mean value of 0.42 %/ms for the quasi-steady rate of SR Ca release in 16 fibers with [fura-2] = 0 mM. Our mean values with fibers from *Rana pipiens* are larger, 0.63 %/ms with model 1 (column *3* in Table III), 1.04 %/ms with model 2:0.25Δ[Ca] (Table III, legend), and 0.99 %/ms with model 1 modified to include the rate of Ca removal by the SR Ca pump according to the reaction cycle of Fernandez-Belda et al. (1984) (Table III, legend). The increase in the quasi-steady rate of SR Ca release that we find with 2–3 or 5–6 mM fura-2 (columns *3–5,* Table III *B*) is independent of whether model 1 or model 2:0.25Δ[Ca] is used to estimate $d\Delta[Ca]_T/dt$ with 0 mM fura-2.

COMPARISON OF THE EFFECTS OF FURA-2 ON THE QUASI-STEADY RATE OF SR Ca RELEASE

Jacquemond et al. (1991) found that 1.5–2 mM fura-2 or 3.8 mM BAPTA eliminated the early transient component of SR Ca release without affecting the quasi-steady component. Fig. 4 in Jacquemond et al. (1991) gives a value of ~0.36 %/ms for the quasi-steady rate of SR Ca release in a fiber with resting [fura-2_T] = 2.2 mM (estimated resting [fura-2] = 1.5 mM); their mean value with 3.8 mM BAPTA was similar, 0.44 %/ms (16 fibers). In our experiments with *Rana pipiens*, the mean value of the quasi-steady rate of release was 1.85 %/ms with 2–3 mM fura-2.

Both sets of values of the quasi-steady rate of SR Ca release were obtained directly from fura-2 signals without calculations with model 1 or 2. As discussed above, it seems likely that the values of SR Ca content were similar in the two sets of experiments. Thus, although Jacquemond et al. (1991) do not give the value of the rate of SR Ca release in units of micromolar per millisecond, the fivefold difference between our value of the fractional rate of release, 1.85 %/ms, and their value, 0.36 %/ms, probably reflects a comparably large difference in the corresponding rates of SR Ca release expressed in units of micromolar per millisecond. This large difference in the quasi-steady rates of SR Ca release observed with 2–3 mM fura-2 suggests, again, that the condition of our fibers was different from that of the fibers studied by Jacquemond et al. (1991).

The general conclusions from this section are that (*a*) in the presence of 0.5–3 mM fura-2, our quasi-steady rates of SR Ca release estimated from $d\Delta[\text{Cafura-2}]/dt$ are about five times those of Jacquemond et al. (1991), and (*b*) the qualitatively different effect of fura-2 on the peak and steady rates of SR Ca release observed by Jacquemond et al. (1991) and by us is not due to the different models used to calculate the rate of release with 0 mM fura-2. Consequently, the condition of the fibers studied by us appears to have been different from that of the fibers studied by Jacquemond et al. (1991).

Ca-induced Ca Release

Block, Imagawa, Campbell, and Franzini-Armstrong (1988) studied freeze-fracture sections of tubular membranes from rabbit skeletal muscle with the electron microscope and visualized tetramers of molecules suggested to represent dihydropyrydine (DHP) receptors, the likely voltage sensors in excitation–contraction coupling (Rios and Brum, 1987; Tanabe, Beam, Powell, and Numa, 1988). Since the density of the adjacent "foot" structures is about twice that of the DHP tetramers, Block et al. (1988) suggested that half the foot structures are associated with DHP tetramers and the other half are not. The foot structure appears to be the ryanodine receptor protein (Inui, Saito, and Fleischer, 1987; Block et al., 1988; Lai, Erickson, Rousseau, Liu, and Meissner, 1988), which has been identified as a large-conductance SR Ca release channel (Lai et al., 1988).

Based on this and other evidence, Rios and Pizarro (1988) proposed that there are two functionally distinct types of SR Ca release channels in frog muscle. One type is gated by the voltage across the tubular membranes and the other type is a Ca-induced Ca release channel. According to their ideas, the Ca that is used to gate

each Ca-induced Ca release channel comes solely from neighboring voltage-gated channel(s). Ca from Ca-induced Ca release channels is assumed to be ineffective so that SR Ca release is always under the control of the voltage across the tubular membranes. The Ca fluxes through the two types of channels are assumed to have similar amplitudes.

These ideas and those of Jacquemond et al. (1991) have provided the following hypothesis about the dual regulation of SR Ca release under physiological conditions. After depolarization of the tubular membranes, some of the voltage-gated channels open and release Ca from the SR into the myoplasm. Since voltage-gated channels are assumed to be insensitive to Ca inactivation, they are able to remain open to provide a maintained rate of Ca release. Some of the Ca that moves through the voltage-gated channels produces Ca-induced Ca release in adjacent channels. These channels are assumed to be sensitive to Ca inactivation so that the Ca flux through them is transient. According to Jacquemond et al. (1991), 1.5–2 mM fura-2 reduces the myoplasmic free [Ca] transient and thereby blocks the Ca-induced component of SR Ca release; it has no effect on the voltage-gated component.

In our experiments with fura-2, the concentration required to depress SR Ca release was larger than that used by Jacquemond et al. (1991). In the experiment in Fig. 3 (fiber 618921 from *Rana temporaria*), the SR Ca content was 4,689–4,446 μM (Table III, column 7). In this fiber, the ability of fura-2 to reduce the peak and quasi-steady rates of SR Ca release was not clearly observed until its concentration exceeded 4 mM. On the other hand, in the experiment in Fig. 6 (fiber O20921 from *Rana pipiens*), the SR Ca content was 702–1,078 μM and 1.7 mM fura-2 was able to produce nearly maximal reduction of the peak and quasi-steady rates of release. (As mentioned in the text discussion of Fig. 6, caution should be taken in the interpretation of results from this fiber since both its Δ[Ca] signal and its SR Ca content were unusually small; consequently, the fiber may have been damaged or have been otherwise abnormal.) In the fiber used for Fig. 5 (fiber O20922 from *Rana pipiens*), the SR Ca content was 2,649–2,492 μM, and the relations between the peak and quasi-steady rates of release and resting [fura-2] were intermediate between those in Figs. 3 and 6.

If it is valid to directly compare these results obtained from fibers from *Rana temporaria* and *Rana pipiens,* it appears that SR Ca release is more sensitive to depression by fura-2 when the SR Ca content is small than when it is large. Although this is qualitatively consistent with a reduction of Ca-induced Ca release by fura-2, it might also be due to some effect of a large concentration of fura-2 unrelated to its ability to complex Ca. Furthermore, it is difficult to tell whether the mechanism of the depression of the peak (Fig. 3 *A*) and quasi-steady (Fig. 3 *C*) rates of SR Ca release by [fura-2] $\geq$ 3–4 mM is the same as that of the depression of only the peak rate of release by 1.5–2 mM fura-2 that was observed by Jacquemond et al. (1991). If all these effects are due to a decrease in Ca-induced Ca release, it follows that this process is more sensitive to fura-2 in their fibers than in ours. In any event, a difference between the condition of their fibers and ours is again suggested.

Another relevant finding is that an increase in the resting concentration of fura-2 $>$ 3 mM delays the onset of SR Ca release. For example, in the experiment shown in Figs. 1–4 (see text discussion of Fig. 3 *C*), an increase in resting fura-2 concentration

from 3.286 to 8.668 mM increased the time to half-peak of the rate of SR Ca release from ~4 ms to 6–7 ms. If voltage-gated channels provide Ca for activation of the Ca-induced Ca release channels, as suggested by Rios and Pizarro (1988) and Jacquemond et al. (1991) as described above, the earliest component of SR Ca release must utilize voltage-activated channels, which are assumed to be insensitive to Ca inactivation of Ca release. Since the Ca flux through the Ca-induced Ca release channels would be expected to occur somewhat later, a reduction of this component would not be expected to delay the onset of SR Ca release. Consequently, the delay produced by fura-2 at concentration >3 mM seems inconsistent with the dual regulation hypothesis and a simple Ca buffering action of fura-2. Perhaps the possibility should be considered that such a large concentration of fura-2 might have an action not related to Ca complexation.

A Hypothetical Channel Designed to Pass a Fixed Number of Ions

The results in Fig. 9 *B* raise the possibility that an SR Ca channel, once activated, will pass a relatively fixed number of ions regardless of SR Ca content. This type of regulation might be advantageous for a cell like a striated muscle fiber in which activation requires that sufficient Ca be released from the SR to complex a fixed concentration of Ca-regulatory sites on troponin. We will now show that one way to design such a channel is to have its inactivation regulated by a Ca receptor that senses [Ca] very close to its mouth.

If the mouth of the channel is approximated by a hemispherical source in a planar membrane, the steady-state concentration of free [Ca] at the inactivation receptor, $[Ca]_R$, is given by

$$[Ca]_R = \frac{\phi_{Ca}}{2\pi D_{Ca} r} + [Ca]_0 \tag{1}$$

ϕ_{Ca} represents the flux of Ca ions through the channel, D_{Ca} represents the diffusion constant of Ca in the myoplasm surrounding the mouth, and r represents the distance of the receptor from the origin of the mouth. $[Ca]_0$ represents the concentration of free Ca at a distance from the mouth that is sufficiently large that the first term on the right-hand side of the Eq. 1 can be neglected.

If the receptor is sufficiently close to the mouth of the channel, the first term on the right-hand side of Eq. 1 is much larger than the second term and

$$[Ca]_R \cong \frac{\phi_{Ca}}{2\pi D_{Ca} r} \tag{2}$$

The essential assumption of Eq. 2 is that the concentration of Ca that is sensed by the inactivation receptor is determined only by the flux of Ca ions through that channel.

For simplicity, assume that a channel has only one inactivation receptor that is either Ca free, denoted by R, or Ca complexed, denoted by CaR. A channel in the CaR state is assumed to be completely inactivated. Transitions between R and CaR are assumed to obey the reaction

$$Ca + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CaR \tag{3}$$

Schneider and Simon (1988) determined the time constant for recovery from inactivation in voltage-clamped cut fibers and obtained an average value of 90 ms at 6–10°C. If the above scheme is used to represent the reaction between Ca and R, and if only one site is associated with each channel, $1/k_{-1} = 90$ ms.

In the experiments used for Fig. 9, *A* and *B*, the time to the peak value of $d\Delta[Ca]_T/dt$ after a depolarization to 10 mV varied between 6 and 8 ms and showed no consistent dependence on SR Ca content (not shown). Since this period of time is much less than 90 ms, the dissociation of CaR can be ignored and the reaction between Ca and R can be represented as

$$Ca + R \xrightarrow{k_1} CaR \tag{4}$$

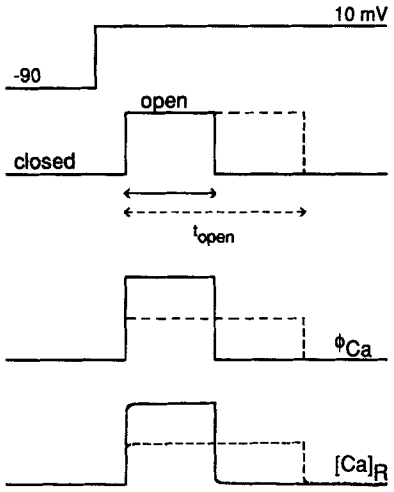

FIGURE 11. Time course of events associated with locally induced Ca inactivation of a hypothetical SR Ca release channel. The top trace shows a voltage step from −90 to 10 mV. The first pair of traces shows the period when the channel was open; t_{open} denotes the channel open time. The middle pair of traces shows ϕ_{Ca}, the flux of Ca ions through the channel. The bottom pair of traces shows $[Ca]_R$, the concentration of Ca at the site of the inactivation receptor that is located near the mouth of the channel. Within each pair, the continuous curve shows the response associated with the larger value of SR Ca content. See text for additional information.

After a fiber is depolarized and an SR Ca channel is gated open, the concentration of free Ca at the receptor site increases to a new value $[Ca]_R$. It is easy to show that, if the receptor is located within a few nanometers of the mouth of the channel, the change in free [Ca] occurs within a few microseconds (see Eq. 2 in section 10.4 of Carslaw and Jaeger, 1959). If the channel remains gated open, as would be expected for a depolarization to 10 mV, Ca is expected to bind to the receptor with a macroscopic rate constant $k_1[Ca]_R$ and, on binding, to completely inactivate the channel.

Fig. 11 illustrates this kind of Ca inactivation of a Ca channel. The top trace shows the voltage. The next three pairs of traces show results from two hypothetical trials in which the SR is assumed to contain different amounts of Ca; the continuous curves illustrate the trial with the larger amount. The first pair of traces shows the opening of the channel that occurs soon after the depolarization. Although the delay is

expected to vary from trial to trial, it is taken to be the same in the two trials illustrated in the figure. The channel open time is denoted by t_{open}.

The middle pair of traces shows ϕ_{Ca}, with a time course that is identical to that of the channel opening; the implicit assumption is that the driving force for Ca is constant, which, in the absence of SR Ca depletion, is expected to hold once the value of [Ca] at the mouth of the channel reaches a steady value (Eq. 1 or 2). The bottom pair of traces shows $[Ca]_R$, which tracks ϕ_{Ca} with a slight diffusional delay, as mentioned above.

According to reaction 4, t_{open} has a probability density distribution that is proportional to $\exp(-k_1[Ca]_R t_{open})$ and a mean open time, $\langle t_{open} \rangle$, given by

$$\langle t_{open} \rangle = 1/(k_1[Ca]_R) \tag{5}$$

$\langle t_{open} \rangle \phi_{Ca}$ gives the amount of Ca, on average, that passes through a channel before it inactivates. According to Eqs. 2 and 5, it is given by

$$\langle t_{open} \rangle \phi_{Ca} \cong \frac{2\pi D_{Ca} r}{k_1} \tag{6}$$

which is independent of ϕ_{Ca}.

Although this model of Ca channel inactivation has been developed for a channel that is inactivated by Ca binding to a single receptor site located near its mouth, it can be extended to the case in which inactivation requires that Ca bind to several such receptor sites. In many such cases, such as identical receptor sites that function independently, the mean open time is expected to be inversely proportional to the single channel Ca flux, as expected for the case of a single receptor site. An SR Ca channel that is regulated in this manner would be expected, on activation, to release a constant mean quantity of Ca, independent of ϕ_{Ca} or SR Ca content, before it becomes inactivated.

The requirement that a channel pass a constant amount of Ca before inactivation is not exactly the same as the requirement that it pass a constant amount of Ca at the time of peak $d\Delta[Ca_T]/dt$, as illustrated in Fig. 9 *B*, although both requirements are clearly similar. To further evaluate this idea and compare its predictions with data such as those in Fig. 9, information is required about how SR Ca release is activated by intramembranous charge movement and how the release flux itself is able to influence charge movement, as found by Csernoch, Pizarro, Uribe, Rodríguez, and Ríos (1991), García, Pizarro, Ríos, and Stefani (1991), Szűcs, Csernoch, Magyar, and Kovács (1991), Pizarro, Csernoch, Uribe, Rodríguez, and Ríos (1991), Jong, Pape, and Chandler (1992), and Pape, Jong, and Chandler (1992). Information is also required about whether SR Ca content influences other processes that may regulate Ca flux through SR Ca channels.

Final Conclusions

The main conclusion of this and the preceding (Pape et al., 1993) article is that 0.5–2 mM fura-2 increases the rate of SR Ca release that accompanies either action potential stimulation or a voltage-clamp depolarization to 10 mV. This result is in general agreement with the proposal of Baylor and Hollingworth (1988) and Hollingworth et al. (1992) that fura-2, in this range of concentrations, reduces Ca

inactivation of Ca release because of the Ca buffering capacity of fura-2. In our voltage-clamp experiments, this reduction of Ca inactivation is manifest as an increase in both the peak and quasi-steady rates of SR Ca release.

This augmentation of the rate of Ca release by fura-2 stands in contrast to the findings of Jacquemond et al. (1991). These authors found that 1.5–2 mM fura-2 (2.2–2.8 mM fura-2_T) eliminated the transient component of SR Ca release but left the quasi-steady level unchanged. They suggested that the transient component was caused by Ca-induced Ca release and that this component was blocked by the Ca buffering action of fura-2. We are particularly surprised that Jacquemond et al. (1991) did not observe any augmentation of the quasi-steady level of the rate of SR Ca release with fura-2, since this effect was very prominent in our experiments.

This difference between their results and ours is probably due to a difference in the physiological state of the fibers. For example, different temperatures were used in the different experiments. Jacquemond et al. (1991) worked at 8–10°C, whereas Baylor and Hollingworth (1988), Hollingworth et al. (1992), Pape et al. (1993), and we worked at 14–16°C. Hollingworth et al. (1992) also studied two fibers at 8.5 and 10.5°C but obtained the same result that they found at 16°C, namely, that 2–3 mM fura-2 increased the peak rate of SR Ca release. Thus, the difference in temperature probably does not account for the difference in the physiological state of the fibers. Another difference in the experimental conditions used with cut fibers is that Jacquemond et al. (1991) used notches whereas we used a brief saponin treatment to allow the end pool solutions to exchange with the myoplasmic fluid. Since action potential–elicited Ca transients are similar with the two methods (Figs. 1 *A* and 6 in Maylie et al., 1987*b*), it seems likely that the physiological state of a fiber does not depend on whether notches or saponin treatment is used.

Although other differences may exist between the cut fiber method used by Jacquemond et al. (1991) and that used by us, a possibly relevant difference is the length of time that a fiber was kept depolarized before the start of an experiment. In experiments by both groups, a muscle was removed from a frog, placed in Ringer's solution, and, after preliminary dissection, was depolarized by relaxing solution. After contraction and subsequent relaxation, a segment of single fiber was isolated and mounted in a double Vaseline-gap chamber. In our experiments, the chamber was then mounted on the optical apparatus, the fiber was polarized to −90 mV, and indicator was introduced into the end pools. In the experiments of Jacquemond et al. (1991), the fiber was kept depolarized during the period when indicator (antipyrylazo III) diffused into the fiber and was polarized just before the experimental measurements began. Although Jacquemond et al. (1991) do not give the total duration of the period of depolarization, it was probably two to three times that used in our experiments. We have no information, however, on whether the different periods of depolarization used by Jacquemond et al. (1991) and us might account for any difference in fiber condition. Clearly, a useful goal of future experiments would be to understand the reason for this difference.

When the concentration of fura-2 was increased to 4–8 mM, we found that the peak and quasi-steady rates of SR Ca release were decreased and that the rising phase of the rate of release signal was delayed. In contrast, Jacquemond et al. (1991) found that fura-2 (1.5–2 mM) decreased only the peak rate of release and left the

quasi-steady rate unchanged. Because of this difference and because of the different fura-2 concentrations required to decrease the rate of SR Ca release, it is difficult to know whether the inhibitory effects of fura-2 in our experiments and theirs have a common underlying mechanism. In our experiments, the inhibitory effect of 4–8 mM fura-2 on SR Ca release might be caused by a reduction of Ca-induced Ca release due to the Ca buffering capacity of fura-2 or it might be due to a pharmacological action of fura-2 unrelated to its ability to complex Ca.

We thank the staff of the Biomedical Instrumentation Laboratory of the Yale Department of Cellular and Molecular Physiology for help with the design and construction of equipment. We also thank Dr. Steve Hollingworth for helpful discussion during the experiments and for reading the manuscript and Dr. Martin Schneider for helpful discussion and for furnishing the parameters used to calculate SR Ca release with model 2 (Table I *B* in Pape et al., 1993).

This work was supported by U.S. Public Health Service grant AM-37643.

Original version received 5 March 1993 and accepted version received 27 May 1993.

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