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Kinetics of Depolarization-Induced Calcium Release from Skeletal Muscle Triads *In Vitro*[1]

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Calcium release from the sarcoplasmic reticulum (SR) depending on depolarization of the transverse tubular membrane (TTM) caused by rapid ionic replacement was measured in skeletal muscle triadic vesicles using a stopped-flow apparatus and Fura-2, a membrane-impermeable Ca^{2+} indicator. Calcium release was triggered by an increase in the magnitude of depolarization. This Ca^{2+} release was inhibited by ruthenium red, digoxin and dantrolene, and enhanced by caffeine. Thus, Ca^{2+} release was found to occur through the SR Ca^{2+} release channel *via* TTM depolarization and to be able to cause skeletal muscle contraction. Calcium release curves could be divided into two phases. In contrast to other previous studies, in the fast phase the amount of released Ca^{2+} increased with an increase in the magnitude of depolarization but the Ca^{2+} release rate did not; on the other hand, in the slow phase the Ca^{2+} release rate increased but the amount of Ca^{2+} did not. Furthermore, the Ca^{2+} release rate was controlled by the luminal Ca^{2+} concentration of the SR only in the fast phase. These independent dual kinetics of Ca^{2+} release were explained by the calsequestrin regulation model.

Key words: calcium release channel, calsequestrin, excitation-contraction coupling, sarcoplasmic reticulum, transverse tubule.

In skeletal muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) caused by depolarization of the transverse tubular membrane (TTM) leads to muscle contraction. However, signal transduction between the TTM and the SR, and the gating properties of the Ca^{2+} release channel of SR are unclear. These are difficult problems regarding excitation-contraction coupling (E-C coupling) in skeletal muscle.

In an early study (*1*), Endo *et al.* found that Ca^{2+} release from the SR was controlled by the cytoplasmic Ca^{2+} concentration. In cardiac muscle, this mechanism is thought to operate physiologically, that is, Ca^{2+} enters from the extracellular side through the voltage-dependent Ca^{2+} channel [dihydropyridine (DHP) receptor] in the TTM according to depolarization, this Ca^{2+} triggers the Ca^{2+}-induced Ca^{2+} release channel (ryanodine receptor) in the SR, and Ca^{2+} is released from the SR. However, in skeletal muscle, this is not thought to occur for following reasons; the activation kinetics of the DHP receptor/Ca^{2+} channel are very slow compared to muscle contraction (*2*), functional Ca^{2+} channels comprise only 2 to 3% of the DHP receptors in skeletal muscle (*3*), and Ca^{2+} could be released from the SR through depolarization even when extracellular Ca^{2+} has been removed (*4*). On the contrary, Schneider and Chandler found that the charge movement in the TTM observed on TTM depolarization exhibited the same period of activation as skeletal muscle contraction, and thus proposed that the voltage sensor in the TTM could detect membrane potential changes as conformation changes with this charge movement and then transduce the depolarization signal to the Ca^{2+} release channel in the SR allosterically (*5*). This mechanism is now called mechanical coupling or depolarization-induced Ca^{2+} release (DICR), and the sensor for membrane potential changes proved to be the DHP receptor (*6*). Furthermore, in a recent study, triadin, which can interact with both the DHP receptor and the ryanodine receptor, was found and was suggested to contribute to the mechanical coupling (*7*). For these reasons, understanding of E-C coupling as signal transduction between intrinsic factors and functional analysis of each factor in DICR are important.

Until now, many investigators have studied DICR through measurement of Ca^{2+} release or tension development using skinned muscle fibers and/or intact muscle fibers depolarized by ionic replacement (*8, 9*) or under a voltage clamp (*10*). However, such experiments are not suitable for analysis of the gating mechanism of the Ca^{2+} release channel in the SR or signal transduction between intrinsic factors, because muscle tension does not directly correspond to Ca^{2+} channel gating and many cytoplasmic proteins (especially Ca^{2+} binding proteins) which are not directly concerned with E-C coupling exist; thus the amount of Ca^{2+} released from the SR cannot be measured

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Abbreviations: CICR, Ca^{2+}-induced Ca^{2+} release; DHP receptor, dihydropyridine receptor; DICR, depolarization-induced Ca^{2+} release; E-C coupling, excitation-contraction coupling; SR, sarcoplasmic reticulum; TC, terminal cisternae; TEA, tetraethylammonium; TTM, transverse tubular membrane.

accurately. On the contrary, triads, which are membrane complexes that maintain the binding between the SR and the TTM, are the simplest and most useful preparations for studying DICR as to the gating properties of the Ca^{2+} channel and signal transduction between intrinsic factors which regulate E-C coupling. Recently, Corbett *et al.* (*11*) measured DICR using Fura-2 as a Ca^{2+} indicator and diluting triads loaded with Ca^{2+} in several volumes to provide various magnitudes of depolarization of the TTM, though the time resolution was not good compared to that in the case of stopped-flow measurement. Furthermore, Ikemoto *et al.* (*12*) measured DICR at various magnitudes of depolarization using a stopped-flow measuring apparatus, in which a sample solution including triads could be mixed with several volumes of other solutions.

In the present study, we established an assay system for DICR, and measured DICR from skeletal muscle triads using a stopped-flow apparatus and Fura-2 according to the method of Corbett *et al.* (*11*) and/or Ikemoto *et al.* (*12*) in order to analyze the kinetics of DICR, *i.e.*, the gating properties of the SR Ca^{2+} release channel depending on depolarization of the TTM. As a result, DICR was found to be divisible into two (fast and slow) phases and the kinetics of these phases were substantially different from each other. These kinetic results are different from the results reported by Ikemoto *et al.* (*12*), and furthermore it was found that only the fast phase was controlled by the luminal Ca^{2+} concentration of the SR.

MATERIALS AND METHODS

Membrane Preparation—A triad and terminal cisternae mixture (TC/triads) was prepared from rabbit fast twitch muscle under 4°C according to the method of Ikemoto *et al.* (*13*) with slight modifications. Dorsal and hind leg muscles were homogenized with 4 volumes of 0.1 M NaCl, 10 mM Tris-maleate (pH 7.0), 1 mM EGTA, and protease inhibitors (0.5 μg/ml aprotinin, 1 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM PMSF), using a mixer (Toshiba MX A30G, Tokyo), for a total of 2 min with five 30 s intervals. The homogenate was centrifuged at $10,000 \times g$ (av.) for 3 min and the resultant supernatant was centrifuged at $10,000 \times g$ (av.) for 40 min. The resultant precipitate was suspended in 100 ml of 10 mM Tris-maleate (pH 7.0) and protease inhibitors (0.25 μg/ml aprotinin, 0.5 μg/ml antipain, 0.5 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 0.1 mM PMSF), and then homogenized in a Teflon-glass homogenizer at 2,500 r.p.m. with 5 strokes. The homogenate obtained on further dilution to 200 ml with 10 mM Tris-maleate (pH 7.0) was centrifuged at $16,000 \times g$ for 35 min. The final precipitate was suspended in 10 mM Tris-maleate (pH 7.0), homogenized as described above and then stored at 0°C. For most experiments, the isolated TC/triads were used within 10 days. In some experiments, freshly obtained TC/triads were mixed with 10% (w/v) sucrose (final concentration), frozen in liquid nitrogen and then stored at −80°C.

The membrane fraction of TC/triads treated with a high concentration salt buffer was prepared as follows. TC/triads prepared as described above were diluted to 1.4 mg protein/ml with 0.6 M KCl and 10 mM Tris-maleate (pH 7.0), mixed in a vortex mixer and then incubated for 5 min at 0°C. This solution was centrifuged at $120,000 \times g$ for 1 h, and the resultant precipitate was suspended in 10 mM Tris-maleate (pH 7.0) and homogenized. The homogenate was centrifuged at $120,000 \times g$ for 1 h again. The final precipitate was suspended in 10 mM Tris-maleate (pH 7.0), homogenized and then stored at 0°C.

The protein concentration was determined by means of the biuret reaction, with calibration by nitrogen determination.

Loading of Ca^{2+} into the Sarcoplasmic Reticulum—We loaded Ca^{2+} into the fraction of the SR in TC/triads in order to measure DICR. The TC/triads were first diluted to 1.5 mg protein/ml with a loading solution [100 mM K-propionate, 2 mM $MgCl_2$, and 20 mM Tris-maleate (pH 7.0)] and then incubated for 20 min at room temperature. After that, we added an ATP regeneration system (15 mM Na_2-phosphocreatine, 15 units/ml creatine phosphokinase, and 2 mM Na_2-ATP) and 50 or 30 μM $CaCl_2$ in order to load Ca^{2+} into the SR through Ca^{2+}-Mg^{2+}-ATPase in the SR, and to polarize the TTM by means of Na^+-K^+-ATPase at the same time. Ten minutes after the addition, the measurement of DICR was performed. The concentrations of all reagents given are the final concentrations.

In some experiments, we added 10 μM Fura-2 (membrane impermeable) to the loading solution and monitored the Ca^{2+} uptake as changes in Fura-2 fluorescence intensity at an excitation wavelength of 340 nm using a fluorescence spectrophotometer (Union Giken FS-501, Osaka). As a result, most of the added Ca^{2+} was taken into the SR and the extravesicular Ca^{2+} concentration was submicromolar.

Measurement of Depolarization-Induced Ca^{2+} Release from TC/Triads—We induced depolarization stimuli in the TTM in TC/triads by ionic replacement according to the method of Corbett *et al.* (*11*), and monitored DICR from the SR using membrane-impermeable Fura-2 and a stopped-flow spectrofluorimeter (Applied Photophysics SX. 17MW, UK). With this stopped-flow apparatus, the dead time of mixing is less than 1.5 ms; thus we can measure DICR on a short time scale. Ionic replacement means that the extravesicular concentrations of K^+ and Cl^- are changed without changing the osmotic pressure or the $[K^+] \cdot [Cl^-]$ product; thus we used solutions which depolarized the TTM (Depo Solutions), as described in the legend to Table I. We used Depo Solutions A, B, C, and D for diluting the extravesicular (cytoplasmic) K^+ concentration 1, 2, 6, and 11 times, respectively. The TC/triads loaded with Ca^{2+} as described above were mixed with 10 volumes of a Depo Solution in the stopped-flow apparatus, and the Ca^{2+} released from the SR depending on TTM depolarization was monitored as the change in extravesicular Fura-2 fluorescence intensity. Changes in Fura-2 fluorescence intensity were measured at an excitation wavelength of 340 nm through a 475 nm cutoff filter. When TC/triads were mixed with a Depo Solution, the extravesicular K^+ concentration decreased and the Cl^- concentration increased; on the other hand, the Na^+ concentration did not change, as shown in Table I. According to the Nernst equation, the magnitudes of depolarization from the resting potential of the TTM, which was induced by Na^+-K^+-ATPase activity, were 0, 17.5, 45.1, and 60.4 mV for 1, 2, 6, and 11 times dilution of the extravesicular (cytoplasmic) K^+ concentration, respectively. All experiments were performed at room temperature.

Data Analysis—The stopped-flow apparatus has two

TABLE I. **Extravesicular (cytoplasmic) ionic compositions before and after the mixing of TC/triads with a Depo Solution.** Depo Solution A contained 100 mM K-propionate and 4 mM TEA-Cl. Depo Solution B contained 45 mM K-propionate, 8.4 mM TEA-Cl, and 50.6 mM TEA-propionate. Depo Solution C contained 8.3 mM K-propionate, 26 mM TEA-Cl, and 69.7 mM TEA-propionate, and Depo Solution D contained 48 mM TEA-Cl and 56 mM TEA-propionate. All solutions contained 20 mM Tris-maleate (pH 7.0), an ATP regeneration system (15 mM Na_2-phosphocreatine, 15 units/ml creatine phosphokinase, and 2 mM Na_2-ATP), and 11 μM Fura-2 (membrane impermeable) in common. PC means phosphocreatine. All values are given in millimolar.

	K^+	Cl^-	Na^+	Mg^{2+}	TEA^+	Propionate$^-$	PC^{2-}	ATP^{2-}
Before	100	4	34	2	0	100	15	2
0 mV (+Solution A)	100	4	34	2/11	40/11	100	15	2
17.5 mV (+Solution B)	100/2	8	34	2/11	590/11	96	15	2
45.1 mV (+Solution C)	100/6	24	34	2/11	957/11	80	15	2
60.4 mV (+Solution D)	100/11	44	34	2/11	1040/11	60	15	2

TABLE II. **Several parameters of DICR.** The total amounts of Ca^{2+} released from triads at +17.5, +45.1, and +60.4 mV depolarization were calculated as $C_f + C_s$ in Eq. 1. At +0 mV and +60.4 (KCl) mV depolarization, the total amounts were estimated by subtracting the average for the initial 20 ms from the average for the final 20 ms in the 500 ms DICR curve. The values of C_f, C_s, k_f, and k_s in Eq. 1 were used as data for the Ca^{2+} release rate (Rate) and the amounts of released Ca^{2+} (Calcium) in the fast and slow phases of DICR at +17.5, +45.1, and +60.4 mV depolarization. One datum was calculated from the averaged trace in a single sample experiment as described in "*Data Analysis*" under "MATERIALS AND METHODS." All values represent the average for several different samples ± SD.

	Total	Fast phase		Slow phase	
	Calcium (nmol/mg)	Rate (k_f) (1/s)	Calcium (C_f) (nmol/mg)	Rate (k_s) (1/s)	Calcium (C_s) (nmol/mg)
0 mV (n=7)	0.73±0.26				
17.5 mV (n=10)	3.84±0.58	28.70±3.05	1.81±0.39	4.15±0.58	2.03±0.30
45.1 mV (n=10)	4.74±0.56	28.47±2.22	2.75±0.59	7.50±0.95	2.00±0.27
60.4 mV (n=10)	5.58±0.83	27.57±2.27	3.38±0.72	7.54±1.37	2.20±0.35
60.4 mV (KCl) (n=6)	1.35±0.20				

cylinders; one for the TC/triads loaded with Ca^{2+} and the other for the Depo Solution, and parts of the solutions are mixed transiently; thus several data can be obtained sequentially in a single sample experiment. In our experiments using the stopped-flow apparatus, we collected 4–10 traces of Fura-2 fluorescence intensity changes reflecting DICR sequentially per sample; thus we regarded the average of these traces as a single sample datum. In Fig. 4B and Table II, we summarize several data for different samples originating from a few rabbits. One trace consists of 400 points of Fura-2 fluorescence intensity.

The amounts of Ca^{2+} released from the TC/triads (nmol Ca^{2+}/mg protein) were calculated from the fluorescence intensity using a Ca^{2+}-Fura-2 calibration curve obtained as follows. TC/triads not loaded with Ca^{2+} were mixed with 10 volumes of a Depo Solution containing various concentrations of Ca^{2+} (0.5–50 μM), and the mixtures were each placed in the mixing cell of the stopped-flow apparatus. The Fura-2 fluorescence intensity of each mixture was measured and plotted against the Ca^{2+} concentration. This curve was fitted by a single-site titration curve, and the dissociation constant and the amplitude of fluorescence intensity were used to calculate the amount of Ca^{2+} released from the TC/triads.

The values calculated from statistics show average ± standard deviation.

RESULTS

Depolarization-Induced Ca^{2+} Release from TC/Triads—First we measured DICR from TC/triads with different magnitudes of TTM depolarization. As shown in Fig. 1, the amount of Ca^{2+} released from the TC/triads increased depending on the increase in the magnitude of TTM depolarization, and at +0 mV depolarization, the amount of released Ca^{2+} was very small. In the study of Ikemoto *et al.* (*12*), the DICR curves were shown to consist of two phases (fast and slow Ca^{2+} release phases) and could be fitted by a double-exponential equation. In our experiments, the DICR curves at +17.5, +45.1, and +60.4 mV depolarization could be fitted by the same equation, as follows:

$$C_t = C_f(1-\exp(-k_f t)) + C_s(1-\exp(-k_s t)) + A \qquad (1)$$

where C_t (nmol/mg protein) is the total amount of extravesicular Ca^{2+} at t, C_f and C_s (nmol/mg protein) are the amounts of released Ca^{2+} in the fast and slow phases, respectively, k_f and k_s (1/s) are the Ca^{2+} release rates in the fast and slow phases, respectively, and A is a constant that is much smaller than C_f and C_s. The value of $C_f + C_s$ in Eq. 1 means the total amount of Ca^{2+} released from the TC/triads, and this value was 3.84±0.58 (n=10), 4.74±0.56 (n=10), and 5.58±0.83 nmol/mg protein (n=10) for +17.5, +45.1, and +60.4 mV depolarization, as shown in Table II. In order to confirm that this Ca^{2+} release was from the triads, which maintained the binding between the TTM and the SR functionally, we measured DICR using TC/triads treated with a high concentration salt buffer ("MATERIALS AND METHODS"). As shown in Table II, little Ca^{2+} was released from the TC/triads even at +60.4 mV depolarization. When the TC/triads were treated with buffer including 0.6 M KCl, it was thought that the TTM dissociated from the SR and that the functional triads might be broken; thus, this means that the DICR in our study is not from the TC but from the triads. Because the DICR curves for +0 and +60.4 mV depolarization of the TC/triads treated with 0.6 M KCl [60.4 mV (KCl) in Table II] could not be fitted by Eq. 1, the total amounts of Ca^{2+} were estimated by subtracting the average for the initial 20 ms from the average for the final 20 ms in the 500 ms DICR curve, and the values being 0.73±0.26 (n=7) and 1.34±0.20 nmol/mg protein (n=6) for +0 and +60.4 mV (KCl-treated), respectively.

Next we investigated the effects of several drugs on DICR. Caffeine is known as an activator of the SR Ca^{2+} release channel in skeletal muscle (*14*). As shown in Fig. 2, when the Depo Solution A included 5.5 mM caffeine (final 5 mM), DICR at +0 mV depolarization was enhanced and much Ca^{2+} was released compared with when the Depo Solution A did not include caffeine (control; shown in Fig. 1 and Table II). The total amount of released Ca^{2+} ($C_f + C_s$ in Eq. 1) on activation by caffeine was 5.49 ± 0.69 nmol/mg protein ($n=5$), which was about 7.5 times larger than the control value. In Fig. 3 the effects of three types of blocker are shown. Ruthenium red is one of the specific inhibitors of the SR Ca^{2+} release channel (*15*), and in the present study we investigated its effect on DICR at +60.4 mV depolarization using a Depo Solution D including 1.1 μM ruthenium red (final 1 μM). Digoxin, a membrane permeable analog of ouabain, is a blocker of Na^+-K^+-ATPase of the TTM and can permeate the plasma membrane from the cytoplasmic side of the TTM and bind to the K^+ binding site of Na^+-K^+-ATPase; therefore, the TTM cannot polarize (*8, 12*). In order to determine whether or not polarization of the TTM before depolarization is required for DICR, in the present study we added 300 μM digoxin to TC/triads not loaded with Ca^{2+} and incubated the mixture at room temperature for 20 min, the TTM not being polarized when an ATP regeneration system and Ca^{2+} were added after that. Dantrolene is a medical drug for malignant hyperthermia that is closely related to the abnormality in the SR Ca^{2+} channel of skeletal muscle, and in skeletal muscle fibers it is known that dantrolene causes muscle relaxation (*16*) and inhibits Ca^{2+} release from the SR (*17*); thus dantrolene is thought to cause skeletal muscle relaxation through SR Ca^{2+} channel inhibition, though the dantrolene receptor is not known. If dantrolene inhibits DICR, the possibility that the DICR in the present study triggers skeletal muscle contraction will increase. We investigated the effect of dantrolene using incubated TC/triads including 10 μM dantrolene at 37°C for 20 min before Ca^{2+} was loaded. As judged from the results in Fig. 3, all of the drugs blocked DICR, and the values of $C_f + C_s$ in Eq. 1 (which for ruthenium red and dantrolene were the total amounts of Ca^{2+} estimated by subtracting the average for the initial 20 ms from the average for the final 20 ms in the 500 ms DICR curves because the curves could not be fitted by Eq. 1 as well as +0 mV depolarization) were 0.82 ± 0.39 ($n=6$), 3.59 ± 0.56 ($n=5$), and 0.98 ± 0.27 nmol/mg protein ($n=5$) for ruthenium red, digoxin, and dantrolene, respectively. Clearly these amounts of released Ca^{2+} are smaller than that in the case of +60.4 mV depolarization shown in Fig. 1 (control in Fig. 3) and Table II. These results for the effects of an activator and blockers show that the DICR observed in the present study is from the SR Ca^{2+} release

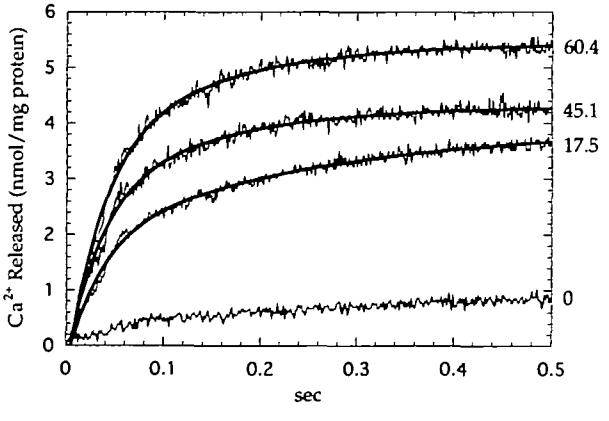

Fig. 1. **Ca^{2+} release from triads at several magnitudes of depolarization of the TTM.** Each trace is the average of 4-10 traces collected sequentially in a single sample experiment, and the fitting curve with Eq. 1 is also shown with each trace. The variance of each trace around the double-exponential function (Eq. 1) is the least among various exponential functions. The values of C_f, C_s, k_f, k_s, and A in Eq. 1 are 3.87, 2.17, 28.23, 7.65, and −0.61 for the +60.4 mV trace, respectively. For the +45.1 mV trace, the values are 2.85, 1.82, 29.32, 7.60, and −0.37, respectively, and for the +17.5 mV trace, 2.20, 2.06, 28.18, 4.01, and −0.32, respectively. The dimension of C_f, C_s, and A is nmol/mg protein, and that of k_f and k_s is 1/s. The values indicated at the right of the panel represent the magnitudes of depolarization in mV.

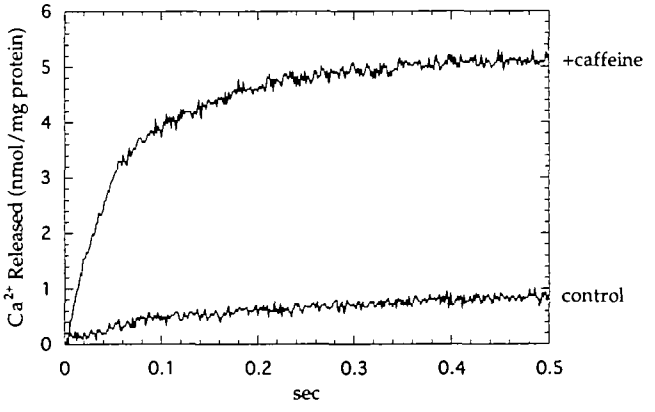

Fig. 2. **The effect of caffeine on DICR from triads at +0 mV depolarization.** Both traces are the average of 4-10 traces collected sequentially in a single sample experiment. The words, control and +caffeine, at the right of the panel represent in the absence and presence of 5 mM caffeine, respectively. The data for the control are the same as those for +0 mV in Fig. 1.

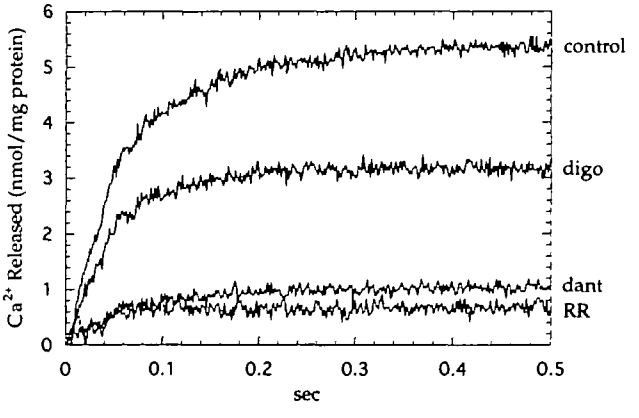

Fig. 3. **The effects of several blockers on DICR from triads at +60.4 mV depolarization.** Each trace is the average of 4-10 traces collected sequentially in a single sample experiment. The words, control, RR, digo, and dant, at the right of the panel represent the absence of a blocker, the presence of 1 μM ruthenium red, using TC/triads treated with 300 μM digoxin, and ones treated with 10 μM dantrolene, respectively. The data for the control are the same as those for +60.4 mV in Fig. 1.

channel *via* depolarization of the TTM, and the possibility that this Ca^{2+} release causes contraction of skeletal muscle *in vivo* was suggested.

Kinetics Analysis of DICR from TC/Triads—In the present study, we determined the amounts of released Ca^{2+}, and the rate constants of Ca^{2+} release in the fast and slow phases because we could measure DICR on a time scale of 500 ms. Table II shows the amounts of released Ca^{2+}, and the rate constants of Ca^{2+} release in the fast and slow phases of DICR curves at +17.5, +45.1, and +60.4 mV depolarization. In the fast phase (C_f in Eq. 1), the amount increased depending on the magnitude of depolarization; on the other hand, in the slow phase (C_s in Eq. 1), the amount did not change regardless of the magnitude of depolarization. As far as the rate constants of Ca^{2+} release are concerned, in the fast phase (k_f in Eq. 1), the rate constant did not change regardless of the magnitude of depolarization; on the other hand, in the slow phase (k_s in Eq. 1), it increased depending on the magnitude of depolarization. Summarizing these results in Table II briefly, in the fast phase the amount of Ca^{2+} depends on the magnitude of depolarization but the Ca^{2+} release rate does not; on the other hand, in the slow phase the Ca^{2+} release rate depends on the magnitude of depolarization but the amount of Ca^{2+} does not. These results are different from the results of Ikemoto *et al.* (*12*). They showed that all values in Eq. 1 increased with the magnitude of depolarization (see "DISCUSSION").

In previous studies (*18-20*), the gating properties of the SR Ca^{2+} release channel on Ca^{2+}-induced Ca^{2+} release (CICR) were shown to be regulated by luminal Ca^{2+} of the SR through calsequestrin; the major Ca^{2+} binding protein on the luminal side of the SR (*21*). In the present study, we investigated the effect of luminal Ca^{2+} on DICR by changing the amount of Ca^{2+} loaded into the SR. In the experiments shown in Figs. 1-3 and Table II, we added 50 μM $CaCl_2$

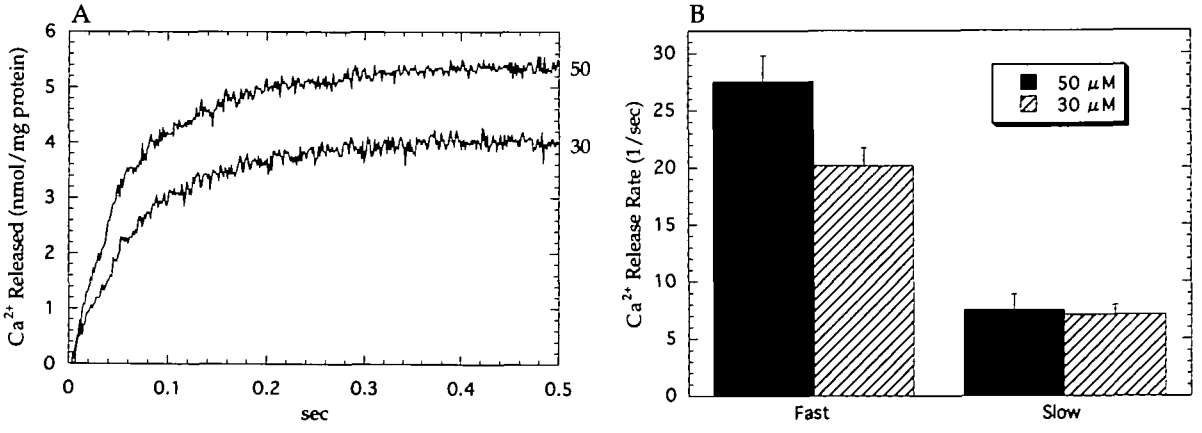

Fig. 4. **The effect of luminal Ca^{2+} of the SR on DICR at +60.4 mV depolarization.** (A) Ca^{2+} release from triads at +60.4 mV depolarization of the TTM on changing of the luminal Ca^{2+} concentration. Both traces are the average of 4-10 traces collected sequentially in a single sample experiment. The values at the right of the panel represent the added Ca^{2+} concentration (μM) when Ca^{2+} was loaded. (B) Rate constants of Ca^{2+} release in the fast and slow phases at different luminal Ca^{2+} concentrations. The words, fast and slow, under the panel represent the fast phase and slow phases, respectively. The filled and striped columns represent 50 and 30 μM added Ca^{2+}, respectively. The values of k_f and k_s in Eq. 1 were used as data. One datum was calculated from the averaged trace in a single sample experiment as described in "*Data Analysis*" under "MATERIALS AND METHODS." All columns with error bars represent the average for several different samples + SD, and the data at 50 μM are the same as in Fig. 1 and Table II.

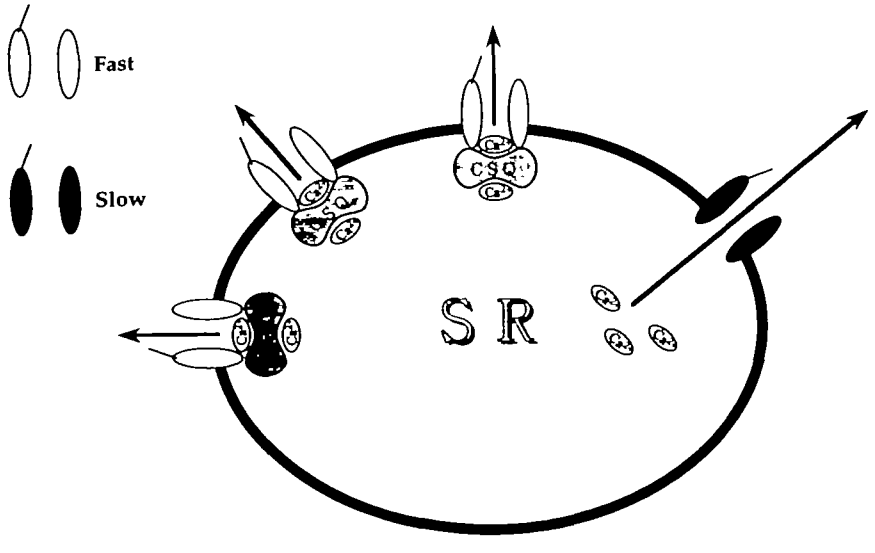

Fig. 5. **The model of Ca^{2+} release in the fast and slow phases of DICR.** CSQ represents calsequestrin, and the open and filled figures represent SR Ca^{2+} channels contributing to the fast and slow phases of DICR, respectively. A third protein, for example, triadin (not shown in the figure), exists strictly between the SR Ca^{2+} channel and calsequestrin according to the previous paper (*28*).

when Ca^{2+} was loaded into the SR ("MATERIALS AND METHODS"); however, in this experiment we added 30 μM $CaCl_2$ and measured DICR at +60.4 mV depolarization. Of course the amount of released Ca^{2+} at 30 μM was smaller than that at 50 μM (Fig. 4A); however, as shown in Fig. 4B, the rate constant of Ca^{2+} release at 30 μM was smaller than that at 50 μM only in the fast phase, and the rate in the slow phase did not change regardless of the luminal Ca^{2+} concentration. The values of k_f in Eq. 1 are 27.57±2.27 (n=10) and 20.18±1.58 (1/s) (n=6), and those of k_s are 7.54±1.37 (n=10) and 7.10±0.84 (1/s) (n=6) for 50 and 30 μM, respectively. This shows that only the fast phase of the DICR observed in the present study is regulated by luminal Ca^{2+} and that this regulation probably occurs through calsequestrin according to the previous papers (*18-20*). Furthermore, the results in Table II can be explained by the calsequestrin regulation or non-regulation model (see Fig. 5, shown below).

DISCUSSION

One of the purposes of the present study was to establish an assay system for DICR *in vitro*. Earlier, Ikemoto *et al.* (*13*) measured DICR from skeletal muscle triads induced by ionic replacement with a stopped-flow spectrophotometer. However, with their method some problems remained, as follows: (1) Ca^{2+}-induced Ca^{2+} release was not eliminated perfectly; and (2) triads loaded with Ca^{2+} were diluted in only 2 volumes, thus the magnitude of depolarization was limited and great depolarization could not be caused by ionic replacement. On the contrary, Corbett *et al.* (*11*) solved these problems by using Fura-2 as a Ca^{2+} indicator as well as a Ca^{2+} chelator for elimination of CICR, and by diluting the triads in several volumes to induce various magnitudes of depolarization in the TTM, though the time resolution was not adequate compared to that with a stopped-flow system. We measured DICR using a stopped-flow system and Fura-2, and it was certain that CICR was eliminated for the following reasons: we also used Fura-2 as a Ca^{2+} chelator and, moreover, the amount of loaded Ca^{2+} was too small to cause CICR even if all of it was released.

As shown in Fig. 1, Ca^{2+} release was triggered depending on the magnitude of depolarization, which is consistent with the results of previous studies involving intact muscle fibers under a voltage clamp (*10*). They showed that the Ca^{2+} transient in skeletal muscle increased depending on the increase in the membrane potential. Though in the present study we used membrane vesicles instead of intact muscle fibers in order to simplify the assay system, our results in Fig. 1 are very similar to theirs. Therefore the assay system used in the present study is thought to be a more conventional and simpler system for analyzing E-C coupling. At +0 mV depolarization, a little Ca^{2+} was released, which was thought to be caused by artifacts of the stopped-flow system, in which the TC/triads solution and the Depo Solution were mixed under 2 kg/cm^2 pressure with nitrogen gas.

In the present study, we did not use purified triads but a terminal cisternae and triad mixture (TC/triads) as the membrane fraction, because in order to obtain purified triads from TC/triads, more centrifugations must be performed, including a long centrifugation for the sucrose density gradient which easily results in breaking of the binding between the TTM and the SR. However, as shown in Table II, DICR in the present study was certainly from only the triads. This also means that DICR cannot occur only at the SR. In some experiments, we confirmed that most of the added Ca^{2+} (50 μM, that is 33.3 nmol/mg protein) was loaded into TC/triads, as described in "*Loading of Ca^{2+} into the Sarcoplasmic Reticulum*" under "MATERIALS AND METHODS." Since the released Ca^{2+} is the greatest at +60.4 mV depolarization (5.58±0.83 nmol/mg protein), if the added Ca^{2+} is loaded into TC and triads equally, the percentage of triads in TC/triads is more than 17% (5.58/33.3) on average. The small amount of Ca^{2+} released from TC/triads treated with a high concentration salt buffer is thought to be from triads protecting the membrane complex against the treatment. When we prepare the TTM in usual experiments, two times treatment of the membrane complex with a high concentration salt buffer is performed, in each step the supernatant of the centrifuged sample is collected, and the TTM is finally purified from the supernatant by the sucrose density gradient method. The first supernatant contains most of the TTM, however, the second supernatant also contains a little TTM. In the present study we treated it one time; thus a small amount of triads protecting the membrane complex is thought to have remained, which might have resulted in the low DICR observed in Table II.

Based on the effects of several drugs shown in Figs. 2 and 3, the DICR in the present study was proved to occur through the SR Ca^{2+} release channel *via* depolarization of the TTM. DICR curves modulated by caffeine and digoxin could be fitted by Eq. 1, and both the fast and slow phases were modulated by caffeine (data not shown). As described below, the two phase kinetics of the DICR in the present study reflect two kinds of gating mechanism of SR Ca^{2+} channels, and caffeine is thought to modulate each type of channel directly. On the contrary, those obtained with ruthenium red and dantrolene were not fitted, and fitting with digoxin was thought not to be strictly correct. The reasons are as follows: (1) for the curves with ruthenium red and dantrolene, the signal-to-noise ratio was so small that fitting was impossible, and (2) the effect of digoxin is dual, *i.e.*, it blocks Na^+-K^+-ATPase of the TTM and activates the SR Ca^{2+} release channel as previously reported for cardiac muscle (*22*); thus the phases of DICR became more complex. Because the amount of released Ca^{2+} was sufficiently decreased by digoxin, it is certain that the DICR in the present study occurred *via* depolarization of the TTM. In spite of blocking of TTM polarization by digoxin, Ca^{2+} was released, as shown in Fig. 3, though the amount was smaller than that in the control experiment. This release was thought to be caused by the direct activation of SR Ca^{2+} channels by digoxin described above. In fact, digoxin-induced Ca^{2+} release was observed at +0 mV depolarization in our stopped-flow measurements (data not shown). Dantrolene was shown to inhibit skeletal muscle contraction (*16*) and Ca^{2+} release from the SR (*17*). In the present study dantrolene also inhibited DICR, as shown in Fig. 3, and the possibility that DICR in the present study cause muscle contraction was suggested. The binding site of dantrolene was shown to be not on the TTM but on the SR (*23*). However, in a recent study dantrolene was shown not to bind to the SR Ca^{2+} release channel (*24*), thus the dantrolene binding protein is probably the third protein

regulating DICR, *i.e.*, E-C coupling. Therefore, analysis of the dantrolene effect on DICR in our assay system as to the function of the E-C coupling regulator is effective, because in our assay system the manipulation of an intrinsic factor (for example, application of antibodies) can be performed easily.

The main finding in the present study concerns the kinetics of the fast and slow phases of DICR. With the method of Corbett *et al.* (*11*) shown above, the time resolution of DICR is not adequate, thus kinetic analysis of Ca^{2+} release could not be performed. On the contrary, in a recent study by Ikemoto *et al.* (*12*), the time resolution was improved with the use of a stopped-flow apparatus, and of course CICR was thought to be eliminated. In the case of their kinetic results regarding DICR, the amount of released Ca^{2+}, the Ca^{2+} release rates of both the fast and slow phases, and the initial rate of Ca^{2+} release ($C_f \cdot k_f + C_s \cdot k_s$ in Eq. 1) all increased depending on the magnitude of depolarization. In our experiments, the initial rate of Ca^{2+} release increased in a manner similar to in the case of Ikemoto's results (*12*). However, the kinetics of the fast and slow phases in our case are definitely different from those of Ikemoto *et al.* (*12*). In the present study, the amount of released Ca^{2+} in the fast phase increased with an increase in the magnitude of depolarization but the Ca^{2+} release rate did not; on the other hand, the Ca^{2+} release rate in the slow phase increased but the amount did not, as shown in Table II. The large differences between the results of Ikemoto *et al.* and ours are in the data analysis and data accuracy. First, they regarded the average of many DICR traces for several different preparations as one datum. Since variance among different samples can exist, their data analysis is thought not to be as adequate as ours. On the contrary, we got one datum from a single sample experiment. Second, though they measured DICR on a 500 ms time scale, the time constant of the slow phase in their study was more than 3.5 s. Therefore, at least for the slow phase the fitting is thought not to be correct compared to our fitting. Third, since they used the average of 60-240 traces, the signal-to-noise ratio of one trace is thought to be small. In fact the data for several drug effects in their paper are noisy in spite of being the averages of 60-120 traces. On the contrary, though our data were the averages of 4-10 traces, they could be fitted by Eq. 1 adequately; thus the accuracy of our data is thought to be higher than that of those of Ikemoto *et al.* Because of these three reasons, our kinetics results are thought to be different from those of Ikemoto *et al.*

Our kinetic results indicate that there are two types of gating mechanism in the SR Ca^{2+} channel. In the fast phase, Ca^{2+} release seems to be the quantal release observed as IP_3-induced Ca^{2+} release (*25*), and recently in DICR using a confocal microscope system (*26*). Furthermore, luminal Ca^{2+} of the SR affected the Ca^{2+} release rate only in the fast phase, as shown in Fig. 4B. In previous studies (*18-20*), regulation of the SR Ca^{2+} release channel by luminal Ca^{2+} was shown to be mediated by calsequestrin. Thus the results in Fig. 4B suggest that the Ca^{2+} release channels contributing to the fast phase are regulated by calsequestrin and those contributing to the slow phase are not. We also examined the effect of a much lower level of luminal Ca^{2+} on the fast phase of DICR by changing the amount of Ca^{2+} when Ca^{2+} was loaded into the SR. However, in the experiments involving 15 μM Ca^{2+}, the amounts of released Ca^{2+} in the slow phase were very small, so fitting of the DICR curves by Eq. 1 (double exponential equation) might be incorrect. Therefore we fitted the curves by a single exponential equation, and as a result the rate of Ca^{2+} release was found to be 15.20 ± 1.22 (1/s) ($n=3$). This value is smaller than the rate of the fast phase at 30 μM, and the calsequestrin regulation model described above can probably be applied at 15 μM Ca^{2+}. Based on these facts, the results shown in Table II can be explained by the model shown in Fig. 5. In the fast phase, the number of the Ca^{2+} channels opening increases depending on the magnitude of depolarization, and the amount of Ca^{2+} releasable from one channel is restricted by calsequestrin functioning as a Ca^{2+} pool. On the contrary, in the slow phase the gating of each channel is activated depending on the magnitude of depolarization, and Ca^{2+} releasable through the channels is luminal free Ca^{2+}, which is all the Ca^{2+} not bound to calsequestrin; thus the total amount of released Ca^{2+} in the slow phase does not change. In the slow phase, the difference in the release rate between +45.1 and +60.4 mV depolarization was not significant, as shown in Table II; therefore the SR Ca^{2+} channels contributing to the slow phase were thought to be fully activated at +45.1 mV depolarization. This result indicated that the maximal rate of the slow phase under fully activated conditions is about one-third that of the fast phase. The reason for this is thought to be that the channels for the slow phase do not interact with calsequestrin also acting as a channel regulator, as shown in previous studies (*18-20*). Though several Ca^{2+} binding proteins other than calsequestrin, such as sarcalumenin (*27*), exist on the luminal side of the SR, calsequestrin is certain to act as a Ca^{2+} pool in the fast phase of DICR judging from the quantity of molecules and localization in the TC where the Ca^{2+} channel is localized (*21*). Furthermore, it is probable that calsequestrin detects the depolarization signal from the TTM voltage sensor through a third protein, for example, triadin, which binds to the TTM voltage sensor, the SR Ca^{2+} channel and calsequestrin (*7, 28*), and regulates the number of Ca^{2+} channels opening contributing to the fast phase.

In conclusion, we analyzed the kinetics of DICR by dividing it into two phases, and these kinetics are substantially different from the results previously reported. Furthermore, these kinetics are explained by an intrinsic factor regulation model; thus from now on experiments on DICR as to signal transduction between intrinsic factors should be performed.

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