Chemical Depolarization-Induced SR Calcium Release in Triads Isolated from Rabbit Skeletal Muscle†

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ABSTRACT: Excitation–Ca^{2+} release coupling properties in the heavy microsomal fraction of the rabbit skeletal muscle enriched in triads were investigated by following the same type of approach used for the studies of excitation–contraction coupling in the skinned fiber system. Incubation of the triads with Mg·ATP in a solution containing 150 mM K^+, 15.0–37.2 mM Na^+, 150–180 mM gluconate$^-$, and 150–200 μM Ca^{2+} (priming solution) led to (a) the generation of a T-tubule membrane potential making the cytoplasmic side negative, as assessed by potential-dependent uptake of the potential probe [^{14}C]SCN^- by triads, and (b) active transport of Ca^{2+} into the SR moiety. One volume of the primed (viz., polarized and Ca^{2+}-loaded) triads was mixed with nine volumes of depolarization solution according to Cl^--replacement [Donaldson, S. K. B. (1985) *J. Gen. Physiol 86*, 501–525; Stephenson, E. W. (1985) *J. Gen. Physiol. 86*, 813–832] and Na^+-replacement [Lamb, G. D., & Stephenson, D. G. (1990) *J. Physiol. 423*, 495–517] protocols used for the induction of contraction in skinned fiber system. The ionic replacement procedure by either protocol produced a rapid release of Ca^{2+} from SR as determined by stopped-flow fluorometry using fluo-3 as a Ca^{2+} probe in the presence of BAPTA–calcium buffer. Both the rate constant and the magnitude of Ca^{2+} release increased with the degree of ionic replacement. The ionic replacement-dependent changes in the release kinetics showed a striking similarity to the voltage-dependent changes of the Ca^{2+} transient in the intact fiber system. Blocking of T-tubule polarization by several agents, such as the Na^+–K^+ pump blocker (80 μM digoxin) and the Na^+–K^+ gradient breaker (10 μM monensin together with 10 μM valinomycin), resulted in significant inhibition of ionic replacement-induced SR Ca^{2+} release, showing no effect on SR Ca^{2+} release induced by direct stimulation of the SR channel by polylysine. This indicates that ionic replacement-induced Ca^{2+} release is under the control of T-tubule potential. The above results suggest that essential features of e–c coupling in the skinned (or intact) muscle fiber system are retained in the isolated triad, and the triad preparation can serve as a simplified physiological model useful for the studies of the molecular mechanism of e–c coupling.

The mechanism by which transient changes in the T-tubule[1] membrane potential lead to rapid Ca^{2+} release from the SR is one of the most important unsolved questions in muscle physiology (Endo, 1977; Martonosi, 1984; Caille et al., 1985; Schneider, 1981; Fabiato, 1989; Fleischer & Inui, 1989; Rios & Pizzaro, 1991; Rios & Gonzalez, 1991). Recent studies have resolved two major molecular components involved in the coupling processes. The α_1 subunit of the dihydropyridine (DHP) receptor of the T-tubule plays a critical role, as evidenced by the following facts: this subunit is missing in the dysgenic mouse incapable of e–c coupling (Knudson et al., 1989), while its expression regenerates e–c coupling and charge movement (Tanabe et al., 1988a,b, 1990; Adams et al., 1990), and DHPs block e–c coupling and charge movement (Rios & Brum, 1987). The presence of the putative voltage-sensing region in the DHP receptor (Tanabe et al., 1987; Catterall, 1988) and the fact that the inward Ca^{2+} current through it is not sufficiently rapid to be a prime trigger of Ca^{2+} transient in the skeletal muscle system (Garcia et al., 1989; Schwartz et al., 1985) have suggested that the voltage sensor in the DHP receptor rather than the Ca^{2+} channel serves as a key mechanism in the signal transmission. Another component, the ryanodine receptor, or junctional foot protein (JFP), forms a tetrameric complex whose electron microscopic structure (Imagawa et al., 1987; Lai et al., 1988; Wagenknecht et al., 1989) is essentially identical to that of an individual foot (Ferguson et al., 1984). It behaves like a Ca^{2+} channel when incorporated into lipid bilayers, suggesting that the Ca^{2+} channel resides in the JFP complex (Imagawa et al., 1987; Lai et al., 1988; Hymel et al., 1988; Smith et al., 1985). Thus, the voltage-sensing DHP receptor and the channel-containing JFP are the essential components for e–c coupling.

An elucidation of the molecular mechanism of e–c coupling requires a clearer understanding of protein–protein interactions involved in the coupling process. For this purpose, it will be extremely useful to establish an experimental model that has a much simplified protein composition than the whole-cell system, while retaining essential features of physiological e–c coupling *in vivo*. As described in our previous studies on isolated triads (Ikemoto et al., 1984, 1985) as well as by others (Corbett et al., 1992), rapid SR Ca^{2+} release induced by chemical depolarization mimics some properties of e–c

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DHP, dihydropyridine; e–c coupling, excitation–contraction coupling; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; JFP, junctional foot protein; MES, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SR, sarcoplasmic reticulum; T-tubule, transverse tubular membrane system.

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coupling *in vivo*. In order to firmly establish its eligibility as a physiological model, however, several important questions still remain to be solved. The main purpose of this study is to settle some of these questions.

The voltage dependence of the intracellular Ca^{2+} transient as well as that of charge movement has been thoroughly investigated in the intact muscle fiber system under voltage-clamp conditions [review: Rios and Pizzaro (1991)]. Extensive studies have also been carried out on depolarization-induced tension development in skinned muscle fibers. In the skinned fiber system, where a direct control of the plasma membrane potential by voltage clamping is not applicable, two different types of ionic replacement protocols have been used to generate various degrees of T-tubule depolarization (Donaldson, 1985; Stephenson, 1985; Lamb & Stephenson, 1990a,b). Although direct measurements of T-tubule membrane potential have not yet been done in the skinned fiber system, several inhibitors of T-tubule polarization were shown to inhibit ionic replacement-induced tension development without effect on caffeine-induced tension development (Donaldson, 1985; Lamb & Stephenson, 1990a), indicating that the induced tension development is in fact mediated by T-tubule depolarization. The main purpose of this study is to test the physiological significance of the previously reported Ca^{2+} release in the isolated triads by reproducing the same type of experiments done with the skinned fiber system. In this study, using a highly sensitive Ca^{2+} probe we could follow the release time course at a much higher dilution and ionic replacement than in our previous studies. This permitted us to study the SR Ca^{2+} release by varying the degree of ionic replacement in a broad range. One of the important results described in this paper is the finding that the two different ionic replacement methods used for inducing contraction in the skinned fiber system (Donaldson, 1985; Stephenson, 1985; Lamb & Stephenson, 1990) also induced Ca^{2+} release in the isolated triads. Furthermore, an increased extent of ionic replacement led to an increase in both the rate and the size of induced Ca^{2+} release in an essentially identical fashion as seen in the voltage-clamp experiments with an intact fiber system. We have also investigated the effect of several inhibitors of T-tubule polarization used in the skinned fiber experiments. As shown here, these inhibitors of T-tubule polarization inhibit ionic replacement-induced Ca^{2+} release. The present results suggest that the basic mechanism governing e–c coupling *in vivo* is retained in the triad membranes.

The isolated triad preparation, which is essentially free from the cytoplasmic Ca^{2+} binding protein, has permitted straightforward analysis of the time course of Ca^{2+} release. As shown, it was found that chemical depolarization-induced Ca^{2+} release is virtually independent of the cytoplasmic Ca^{2+} in the range of 0.01–0.5 μM.

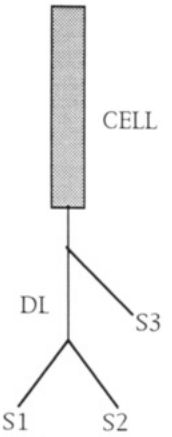
EXPERIMENTAL PROCEDURES

Preparations. The triad-enriched microsomal fraction was prepared from rabbit leg and back muscle by differential centrifugation as described previously (Ikemoto et al., 1984, 1985, 1988). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M potassium gluconate, proteolytic enzyme inhibitors (0.1 mM PMSF, 10 μg/mL aprotinin, 0.8 μg/mL antipain, 2 μg/mL trypsin inhibitor), and 20 mM MES, pH 6.8, at a final protein concentration of 20–30 mg/mL. T-tubules and SR were separated by French press treatment of the triads, followed by sucrose density grandient centrifugation as described previously (Ikemoto et al., 1984); purified T-tubules were on top of the 27% sucrose layer. The fractions were sedimented and homogenized in the same solution as above. The preparations were quickly frozen in liquid nitrogen and stored at −70 °C.

Membrane Potential Assays. The membrane potential of the T-tubule moiety of the triad was monitored using the [^{14}C]SCN^- uptake as described previously (Rottenberg, 1979; Ikemoto et al., 1992). For the assay of T-tubule polarization, the triad vesicles (3.0 mg/mL) were incubated in one of two priming solutions: (solution I) 150 mM potassium gluconate, 30 mM sodium gluconate, 7.2 mM NaCl, 5 mM Mg·ATP, an ATP-regenerating system (2.5 mM PEP and 10 units/mL pyruvate kinase), 20 mM imidazole (pH 6.8), and 13.7 mM [^{14}C]KSCN (56 mCi/mmol, Amersham); or (solution II) 150 mM potassium gluconate, 15 mM NaCl, 5 mM Mg·ATP, an ATP-regenerating system, 20 mM imidazole (pH 6.8), and 13.7 mM [^{14}C]KSCN. Solutions I and II are equivalent to the priming solutions (solution A) used for Cl^- replacement (Table 1) and Na^+ replacement (Table 2) protocols for depolarization-induced Ca^{2+} release, respectively. At various times of incubation at 22 °C, a portion of the solution was diluted with 9 volumes of the same solutions, but without Mg·ATP, ATP-regenerating system, and [^{14}C]KSCN, and immediately filtered through the Whatman GF/F glass microfiber filters using a Hoeffer manifold filtration apparatus. The filters were washed with a 5-mL volume of the same solution, and air-dried, and the radioactivity retained on the filter was counted. Specific [^{14}C]SCN^- uptake was determined by subtracting nonspecific radioligand binding (determined in the absence of added vesicles) from the total count. The specific [^{14}C]SCN^- uptake corresponding to "zero potential" was determined by including both 10 μM monensin and 10 μM valinomycin in the presence of ATP. The specific [^{14}C]SCN^- uptake in the absence of added Mg·ATP was about the same, or slightly higher than, that in the presence of monensin and valinomycin. The ([^{14}C]SCN^- uptake at polarization)/([^{14}C]SCN^- uptake at zero potential in the presence of ionophores) ratio was then calculated. Assuming that this ratio is identical to the C_i/C_o ratio (where C_i and C_o are the intravesicular and extravesicular concentrations of the permeable negatively charged potential probe, respectively; the C_i/C_o ratio can be determined without measuring the lumenal volume of T-tubules), the membrane potential ($\Delta\psi$) is given by $\Delta\psi = -(RT/zF)\ \ln(C_i/C_o)$ (Rottenberg, 1979; Ikemoto et al., 1992).

Assays of the Total Amount of Lumenal Calcium after Ca^{2+} Uptake by SR. For accurate evaluation of the fraction of releasable calcium, it is important to determine the total amount of calcium present in the SR that is accessible to release, since commonly used Ca^{2+} uptake assay methods can determine only the amount of Ca^{2+} taken up. To do this, the triads were incubated in the priming solutions as described. After the steady state of uptake was attained (>5 min), 10 μM neomycin was added to block the Ca^{2+} leak, immediately followed by centrifugation in a Beckmann benchtop ultracentrifuge (TL-100). The sedimented vesicles (1.6 mg/mL in the final concentration) were homogenized in 150 mM KCl, 0.8 mg/mL Triton X-100, and 20 mM MES, pH 6.8. Stepwise addition of EGTA (30–50 μM, per step) to the solution in the presence of 9 μM arsenazo III resulted initially in a sharp linear decrease in the difference absorbance ($A_{650} - A_{700}$), and then in a plateau when the amount of added EGTA became about equal to the amount of the total calcium present. Although the amount of the calcium thus determined includes the externally bound calcium, the portion of the externally bound calcium must be negligible relative to the total amount

Chart 1. Diagrammatic Representation of the Three-Syringe System Used for Stopped-Flow Experiments[a]



[a] DL, delay line; S1, S2, and S3, syringes.

of calcium present in the SR lumen at the steady state of Ca^{2+} uptake.

Production of Various Extents of Ionic Replacement To Induce Ca^{2+} Release from SR. To produce graded depolarization of the T-tubule moiety of the triad, two different methods of ionic replacement used for the skinned muscle fiber system—(a) Cl^--induced (Donaldson, 1985; Stephenson, 1985) and (b) Na^+-induced (Lamb & Stephenson, 1990a,b) Ca^{2+} release—were adopted with some modifications as described below.

(a) Cl^--Induced Ca^{2+} Release. Triads that had been equilibrated in 150 mM potassium gluconate were mixed with 5 mM Mg·ATP in the priming solution (solution A) containing optimum concentrations of Na^+ for T-tubule polarization (Ikemoto et al., 1992) by mediation of the (Na^+–K^+) pump and of Ca^{2+} for active loading of the SR moiety with Ca^{2+}. Solution A was placed in one syringe (S3) of a stopped-flow system (BioLogic SFM-3, see Chart 1) and incubated at 22 °C for 6 min or longer, which was sufficient for the completion of both T-tubule polarization and active loading of SR with Ca^{2+}. Then, 15 μL of solution A (in S3, cf. Chart 1) was mixed with 135 μL of depolarizing solution B (in either S1 or S2) having various ionic compositions to produce various $[Cl^-]_{A+B}/[Cl^-]_A$ ratios ranging from 1 (G1) to 10 (G10) {$[Cl^-]_A$: Cl^- concentration in solution A; $[Cl^-]_{A+B}$: the final concentration of Cl^- after mixing solution A with solution B}. The $[K^+]_{A+B}/[K^+]_A$ ratio changed in a reciprocal fashion, while keeping the $[K^+] \times [Cl^-]$ product constant before and after mixing to avoid osmotic effects (Donaldson, 1985; Stephenson, 1985). It was found that higher concentrations of choline$^+$ present during Ca^{2+} release led to an appreciable inhibition of SR Ca^{2+} release (unpublished data). Therefore, the concentration of choline$^+$ during Ca^{2+} release was kept constant for every mixing protocol (G1 through G10). The ionic strength of the reaction solution was maintained constant before and after mixing with additional amounts of imidazole to either solution A or B. Solution B contained appropriate concentrations of a BAPTA–calcium buffer to define the concentrations of Ca^{2+} (0.01–0.5 μM) at which Ca^{2+} release reaction was initiated (initial $[Ca^{2+}]$, or $[Ca^{2+}]_{t=0}$); BAPTA buffer was chosen because of its much higher Ca^{2+} chelation rate (Tsien, 1980) than EGTA (Smith et al., 1984). The time courses of SR Ca^{2+} release induced at various levels of depolarization were monitored with a stopped-flow fluorometer (BioLogic SFM-3 with MOS-200 optical system). Ionic compositions of priming solution A (A) and after mixing it with depolarizing solution B (A+B) for each level of depolarization are summarized in Table 1.

(b) Na^+-Induced Ca^{2+} Release. Triads that had been equilibrated in 150 mM potassium gluconate reacted with 5 mM Mg·ATP in solution A containing Na^+ and Ca^{2+} for both T-tubule polarization and active loading of SR with Ca^{2+} as described above. Then, 15 μL of solution A (in S3, cf. Chart 1) was mixed with 135 μL of solution B (in either S1 or S2) containing various proportions of Na^+ and K^+ (the sum of $[Na^+] + [K^+]$ was kept constant at 165 mM), appropriate concentrations of a BAPTA-calcium buffer, and Ca^{2+} indicator fluo-3. SR Ca^{2+} release induced by various degrees of depolarization was monitored with a stopped-flow fluorometer as described above. Ionic compositions of solution A (A) and after mixing solution A and solution B (A+B) for each of various magnitudes of Na^+-induced depolarization are summarized in Table 2.

In order to examine possible osmotic effects by this protocol, the same experiments described above were carried out in the absence of added fluo-3. There was no appreciable change in the light scattering of triads, suggesting that this protocol produced little osmotic effect.

Data Collection and Processing. Approximately 30–40 traces of the fluo-3 signal were averaged for each experiment. To calculate the net Ca^{2+} release (nmol/mg) from the fluorescence intensity of fluo-3 in the presence of various concentrations of BAPTA–calcium buffer, each of the 1000 data points of the fluo-3 trace was calculated by using appropriate association constants for all ligands and metals present in the reaction solutions. In this calculation, we assumed that the rates of binding of the released Ca^{2+} to the buffer (Tsien, 1980) and to fluo-3 (Lattanzio & Bartchar, 1991) were significantly higher than the rate of Ca^{2+} release itself. This assumption appeared to be valid for BAPTA–calcium buffer because of its high rate of calcium chelation (Tsien, 1980), but not for EGTA–calcium buffer that produced considerable distortion in the release time course (see Discussion). The coefficient of (fluo-3 signal)/(the change in $[Ca^{2+}]$), required for the above calculation, was determined at each $[Ca^{2+}]$ in the range of 0.01–0.5 μM adjusted with the BAPTA–calcium buffer.

RESULTS

Incubation of Triads in the Priming Solution Produces Polarization of the T-Tubule Moiety and Ca^{2+} Loading of the SR Moiety. Triads were incubated in the priming solutions, and the distribution of the potential-sensitive probe $[^{14}C]SCN^-$ (C_i/C_o) was determined as a function of time. The $[^{14}C]SCN^-$ uptake increased sharply in the initial 2 or 3 min of incubation with Mg·ATP and then reached a plateau at 5 min, which continued until 10 min. The C_i/C_o values (average ± standard deviation) at the steady state of $[^{14}C]SCN^-$ uptake (5–10 min after addition of Mg·ATP) determined in the priming solutions for Cl^--replacement and Na^+-replacement protocols, and the estimated values of T-tubule potential in the primed triads, are summarized in Table 3. The C_i/C_o values obtained with the purified T-tubule preparation under equivalent conditions were in the same range. Since the terminal cisternal vesicles showed no time-dependent $[^{14}C]SCN^-$ uptake after dissociation of T-tubules (Ikemoto et al., 1992), these results indicate that incubation in the priming solutions generated a membrane potential in the the T-tubule moiety (but not in the SR moiety) making the cytoplasmic side of the membrane more negative, which corresponds to the polarization of the plasma membrane and T-tubule membrane in a resting intact muscle fiber.

We also determined the total amount of calcium present in the SR after completion of ATP-dependent Ca^{2+} uptake in

Table 1: Ionic Compositions of Priming Solution (A) and Those after Mixing with Release-Inducing Solution (after A+B) for Producing Various Magnitudes of Depolarization (*G* Values) by Cl^--Replacement Protocol[a]

magnitude of depolarization		mM					
		K^+	Cl^-	Na^+	choline$^+$	gluconate$^-$	bitartrate^{2-}
	A	150.0	7.2	37.2	0	180.0	0
G10	after A+B	15.0	72.0	37.2	71.3	51.5	0
	A	150.0	7.2	37.2	0	180.0	0
G6	after A+B	25.0	43.2	37.2	71.3	61.5	14.4
	A	150.0	7.2	37.2	0	180.0	0
G4	after A+B	37.5	28.8	37.2	71.3	74.0	21.6
	A	150.0	7.2	37.2	0	180.0	0
G2	after A+B	75.0	14.4	37.2	71.3	111.5	28.8
	A	150.0	7.2	37.2	0	180.0	0
G1	after A+B	150.0	7.2	37.2	71.3	186.5	32.4

[a] The *G* values indicate the ratios of $[Cl^-]_{A+B}/[Cl^-]_A$, which are identical to the reciprocal of $[K^+]_{A+B}/[K^+]_A$. General compositions: Priming solution (solution A): 150 mM potassium gluconate, 1.6 mg/mL triad, 37.2 mM Na^+, 5 mM Mg·ATP, an ATP-regenerating system (2.5 mM PEP and 10 units/mL pyruvate kinase), 150–200 μM $CaCl_2$, and 20–142.4 mM imidazole (pH 6.8). Depolarization solution (solution B): various concentrations of potassium gluconate, 7.2–79.2 mM choline chloride, 0–32.4 mM $(choline)_2$ bitartrate (made by mixing choline bitartrate with an equimolar choline base), various concentrations of BAPTA–calcium buffer, 2.5 μM fluo-3, and 20–155.4 mM imidazole (pH 6.8).

Table 2: Ionic Compositions of Priming Solution (A) and Those after Mixing with Release-Inducing Solution (after A+B) for Producing Various Magnitudes of Depolarization (*G* Values) by Na^+-Replacement Protocol[a]

magnitude of depolarization		mM			
		K^+	Na^+	Cl^-	gluconate$^-$
	A	150.0	15.0	15.0	150.0
G10	after A+B	15.0	150.0	15.0	150.0
	A	150.0	15.0	15.0	150.0
G7.75	after A+B	48.75	116.25	15.0	150.0
	A	150.0	15.0	15.0	150.0
G5.5	after A+B	82.5	82.5	15.0	150.0
	A	150.0	15.0	15.0	150.0
G3.25	after A+B	116.25	48.75	15.0	150.0
	A	150.0	15.0	15.0	150.0
G1	after A+B	150.0	15.0	15.0	150.0

[a] General compositions: Priming solution (solution A): 150 mM potassium gluconate, 1.6 mg/mL triad, 15 mM NaCl, 5 mM Mg·ATP, an ATP-regenerating system, 150–200 μM $CaCl_2$, and 20 mM imidazole (pH 6.8). Depolarization solution (solution B): various concentrations of potassium gluconate, sodium gluconate, 15 mM NaCl, various concentrations of BAPTA–calcium buffer, 2.5 μM fluo-3, and 20 mM imidazole (pH 6.8).

Table 3: The Magnitudes of T-Tubule Polarization following Incubation of Triads in Polarizing Solutions of Both Protocols[a]

	C_i/C_o	estimated potential (mV)
Cl^--replacement protocol	14.5 ± 4.3	−68.5 ± 37.4
Na^+-replacement protocol	12.5 ± 3.2	−64.7 ± 29.8

[a] C_i, C_o: the intravesicular and extravesicular concentrations of the $[^{14}C]SCN^-$ probe.

both types of priming solutions. After incubation for 5 min or longer, the total amount of lumenal calcium was 256.3 ± 10.4 nmol/mg of triad protein (n = 8), as determined by the EGTA titration method (Experimental Procedures). As a result of Ca^{2+} accumulation in the SR moiety, the concentration of Ca^{2+} in the priming solution fell as low as 0.2–0.4 μM, as determined by fluorometry with fluo-3.

Increasing Ionic Replacement of the Polarized Triads Produces Graded SR Ca^{2+} Release. On completion of the priming processes (both T-tubule polarization and SR Ca^{2+} uptake), achieved after incubation for more than 5 min, 1 volume of the priming solution (solution A) was mixed with 9 volumes of a depolarizing solution (solution B) using a stopped-flow apparatus. In devising the protocol for chemical depolarization of the T-tubule, we adopted, with some modifications, the two representative methods used for the induction of T-tubule-mediated contraction in the skinned

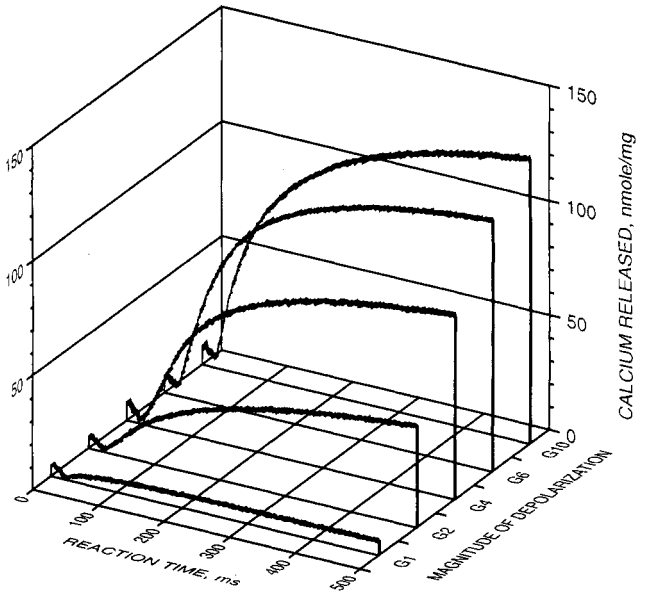


FIGURE 1: Kinetics of SR Ca^{2+} release as a function of ionic replacement according to the Cl^--replacement protocol shown in Table 1. The time courses of SR Ca^{2+} release were monitored with 2.5 μM fluo-3 at 0.2 μM Ca^{2+} buffered with the BAPTA–calcium buffer. Each trace was obtained by signal averaging a total of 200–240 traces originated from 4–5 preparations.

muscle fiber—viz., Cl^--induced Ca^{2+} release (Donaldson, 1985; Stephenson, 1985) and Na^+-induced Ca^{2+} release (Lamb & Stephenson, 1990a,b) as outlined in Tables 1 and 2, respectively. Upon generating T-tubule depolarization by either protocol, the time course of the resultant SR Ca^{2+} release was monitored using fluo-3 as a Ca^{2+} probe at defined initial $[Ca^{2+}]$ (the Ca^{2+} concentration in the reaction solution at the time when SR Ca^{2+} release was triggered: $[Ca^{2+}]_{t=0}$). BAPTA buffer was used to define the $[Ca^{2+}]_{t=0}$ for the following experiments.

We have investigated the kinetics of SR Ca^{2+} release as a function of the extent of ionic replacement (viz., the *G* values, Experimental Procedures). Figure 1 depicts a family of the time courses of Cl^--induced Ca^{2+} release obtained with different *G* values (i.e., $[Cl^-]_{A+B}/[Cl^-]_A = [K^+]_A/[K^+]_{A+B}$ ratios, where $[K^+]_A[Cl^-]_A = [K^+]_{A+B}[Cl^-]_{A+B}$) when Ca^{2+} release was induced at 0.2 μM $[Ca^{2+}]_{t=0}$. In the control (G1), where there was dilution but no ionic replacement, mixing itself produced a decrease of the fluo-3 signal in the initial 25 ms, followed by a small increase and then a plateau. The size of the initial signal was independent of the degree of ionic replacement, suggesting that the initial descending phase

Table 4: The Amount (C), the Rate Constant (k), and the Initial Rate of Release in the Fast Phase of Ca^{2+} Release Induced by Various Degrees of Ionic Replacement According to the Cl^--Replacement Protocol[a]

	G10	G6	G4	G2	G1
C (nmol/mg)	100.8	97.6	77.5	37.6	5.9
k (s^{-1})	19.9	17.5	14.3	12.5	7.5
initial rate [nmol/(mg·s)]	2005.9	1708.0	1108.3	470.0	44.3
C_s (nmol/mg)	83.2	52.3	35.6	22.4	3.2
k_s (s^{-1})	0.25	0.19	0.15	0.11	0.02

[a] The amount (C_s) and the rate constant (k_s) of Ca^{2+} release in the slow phase are also shown. Ionic replacement was created according to the protocol shown in Table 1, and the time course of Ca^{2+} release was monitored as described in the legend to Figure 1. Kinetic parameters shown were calculated by fitting a double-exponential model, $y = C(1 - e^{-kt}) + C_s(1 - e^{-k_s t})$, to the Ca^{2+} release time courses shown in Figure 1, where C and C_s are the magnitudes of Ca^{2+} release in the fast phase and slow phase, respectively; k and k_s are the rate constants of release in the fast phase and slow phase, respectively; $t = t_M - t_L$ (t_M: time after mixing; t_L: lag period). The initial rate of Ca^{2+} release, $(dy/dt)_{t\to 0}$, is $Ck + C_s k_s$. Since Ck was exceedingly larger than $C_s k_s$, $(dy/dt)_{t\to 0}$ was essentially identical with Ck. Therefore, in the table the initial rate was calculated as Ck.

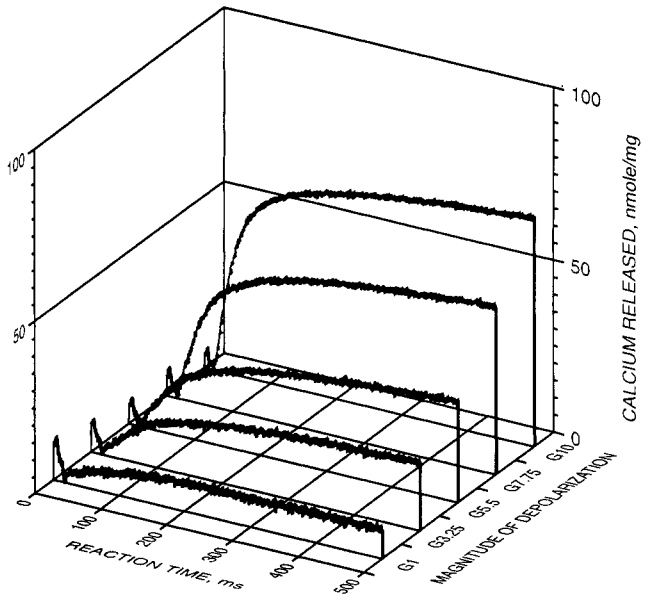


FIGURE 2: Kinetics of SR Ca^{2+} release as a function of ionic replacement according to the Na^+-replacement protocol shown in Table 2. The time courses of SR Ca^{2+} release were monitored with 2.5 μM fluo-3 at 0.2 μM Ca^{2+} buffered with the BAPTA–calcium buffer. Each trace was obtained by signal averaging a total of 200–240 traces originated from 4–5 preparations.

Table 5: The Amount (C), the Rate Constant (k), and the Initial Rate of Release in the Fast Phase of Ca^{2+} Release Induced by Various Degrees of Ionic Replacement According to the Na^+-Replacement Protocol[a]

	G10	G7.75	G5.5	G3.25	G1
C (nmol/kg)	60.5	43.0	24.9	18.3	7.7
k (s^{-1})	21.6	18.2	14.9	12.1	6.3
initial rate [nmol/(mg·s)]	1306.8	782.6	371.0	221.4	48.5
C_s (nmol/mg)	45.2	30.0	21.3	6.4	1.9
k_s (s^{-1})	0.28	0.25	0.18	0.10	0.01

[a] The amount (C_s) and the rate constant (k_s) of Ca^{2+} release in the slow phase are also shown. Various degrees of ionic replacement were created according to the protocol shown in Table 2, and the time course of Ca^{2+} release was monitored as described in the legend to Figure 2. For further details, see the legend to Table 4.

represents a mixing artifact which is unrelated to Ca^{2+} release. The initial mixing artifact was followed by the eminent signal of fast Ca^{2+} release which was dependent upon the degree of ionic replacement. Upon increasing the G value, the rate constant (k), the amplitude (C), and hence the initial rate (Ck, cf. the legend to Table 4) of Ca^{2+} release all increased (Table 4). In the experiment shown in Figure 1, various G values were created by changing $[Cl^-]_{A+B}$ while keeping $[Cl^-]_A$ constant. We also created various G values by varying $[Cl^-]_A$ while keeping the $[Cl^-]_{A+B}$ about constant and obtained essentially the same G value-dependent changes in the Ca^{2+} release kinetics (data not shown). This suggests that the G value, rather than the ion concentration *per se*, is the critical element in characterizing the induced SR Ca^{2+} release.

In agreement with our previous reports that depolarization-induced Ca^{2+} release consists of two phases (Ikemoto et al., 1984, 1985), the rapid Ca^{2+} release was followed by the slow phase of Ca^{2+} release, whose magnitude (C_s) and rate constant (k_s) are shown in Table 4. As seen, both C_s and k_s increased with an increase of the G value, indicating that the slow phase of Ca^{2+} release is also under the control of T-tubule depolarization.

In the experiments shown in Figure 2, SR Ca^{2+} release was induced by another ionic replacement protocol, namely, Na^+-induced Ca^{2+} release. An increase of the G value (in this case, the $[Na^+]_{A+B}/[Na^+]_A$ ratio) again increased all C, k, and Ck as seen in Figure 2 and Table 5. The overall pattern of the G value-dependent changes in the Ca^{2+} release kinetics is essentially identical to that produced by the Cl^--replacement method, although the amount of Ca^{2+} released at an equivalent G value is significantly less in Na^+-induced release than in Cl^--induced release.

Ionic Replacement-Induced SR Ca^{2+} Release Is Mediated by T-Tubule Depolarization Rather Than by Direct Stimulation of SR. The major experimental support to the notion that ionic replacement-induced tension development in the skinned fiber system is induced by mediation of T-tubule depolarization was the observation that several putative blockers of T-tubule polarization, such as ouabain (the inhibitor of the Na^+/K^+ pump: Donaldson, 1985; Lamb & Stephenson, 1990), monensin (Na^+ ionophore: Volpe & Stephenson, 1986), and saponin (Donaldson, 1985; Lamb & Stephenson, 1990), inhibited ionic replacement-induced tension development without affecting caffeine-induced tension development. We have investigated these compounds for their effects on (a) T-tubule polarization, (b) SR Ca^{2+} release induced by Cl^--replacement protocol (Figure 3), (c) Ca^{2+} release induced by Na^+-replacement protocol (Figure 4), and (d) Ca^{2+} release induced by polylysine (Figure 5). The specific $[^{14}C]SCN^-$ uptake after incubation of triads in the priming solution in the presence of 10 μM monensin together with 10 μM valinomycin was about the same as, or even lower than, that after incubation without Mg·ATP (the conditions where there is no activity of the Na^+–K^+ ATPase, viz., no T-tubule polarization). Therefore, under these conditions the T-tubule potential was presumably zero. Incubation of triads in the priming solution in the presence of 80 μM digoxin (relatively membrane-permeable analog of ouabain) produced a significant inhibition of T-tubule polarization. Thus, the level of the polarized potential after priming in the presence of digoxin became 43 ± 12% (n = 5) and 44 ± 14% (n = 6) of the control level, in Cl^-- (Table 1, solution A) and Na^+- (Table 2, solution A) replacement protocols, respectively, as determined by the $[^{14}C]SCN^-$ uptake method (Experimental Procedures). Blocking of T-tubule polarization by the ionophores (monensin plus valinomycin) resulted in almost complete inhibition of SR Ca^{2+} release induced by either Cl^- (G10)-replacement protocol (Figure 3) or Na^+ (G10)-replacement protocol (Figure 4). Partial blocking of T-tubule polarization by digoxin also produced severe inhibiton of SR Ca^{2+} release induced by either

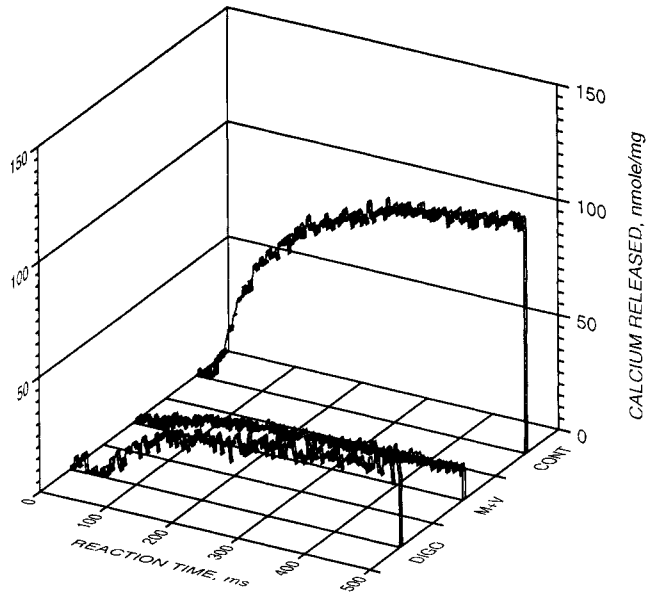


FIGURE 3: Effects of blockers of T-tubule polarization on SR Ca^{2+} release induced by Cl^--replacement protocol. CONT: without addition of blockers (control); M+V: with 10 μM monensin and 10 μM valinomycin; DIGO: with 80 μM digoxin. Triads were incubated in the priming solution (cf. solution A, Table 1), with or without addition of polarization blockers as indicated, and mixed with 9 volumes of depolarization solution (G10 protocol, Table 1). The time course of SR Ca^{2+} release in untreated and blocker-treated triads was monitored with fluo-3 as described in the legend to Figure 1. Each trace was obtained by signal averaging a total of 60–120 traces originated from 2–4 different experiments.

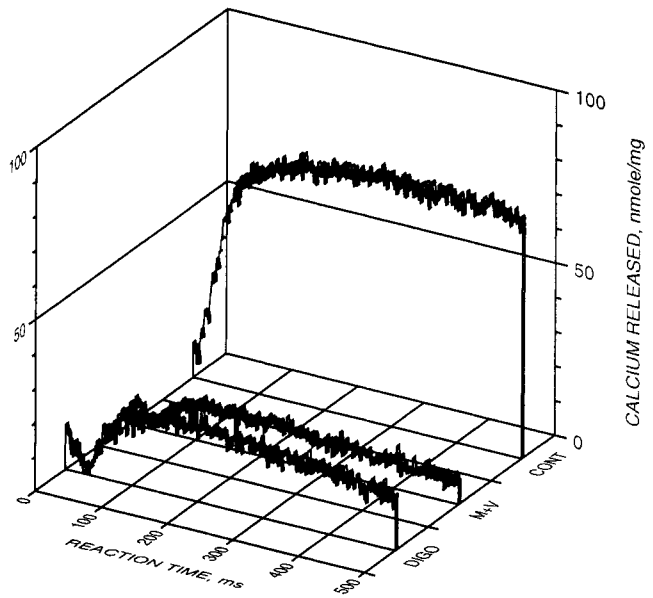


FIGURE 4: Effects of blockers of T-tubule polarization on SR Ca^{2+} release induced by Na^+-replacement protocol. CONT: without addition of blockers (control); M+V: with 10 μM monensin and 10 μM valinomycin; DIGO: with 80 μM digoxin. Triads were incubated in the priming solution (cf. solution A, Table 2), with or without addition of polarization blockers as indicated, and mixed with 9 volumes of depolarization solution (G10 protocol, Table 2). The time course of SR Ca^{2+} release in untreated and blocker-treated triads was monitored with fluo-3 as described in the legend to Figure 2. Each trace was obtained by signal averaging a total of about 90 traces originated from 3 different experiments.

protocol. However, neither ionophore nor digoxin had any appreciable effect on SR Ca^{2+} release, when release was induced by direct stimulation of the ryanodine receptor/Ca^{2+} channel protein by polylysine (Figure 5). These results suggest that ionic replacement-induced SR Ca^{2+} release is in fact mediated by T-tubule depolarization rather than induced by

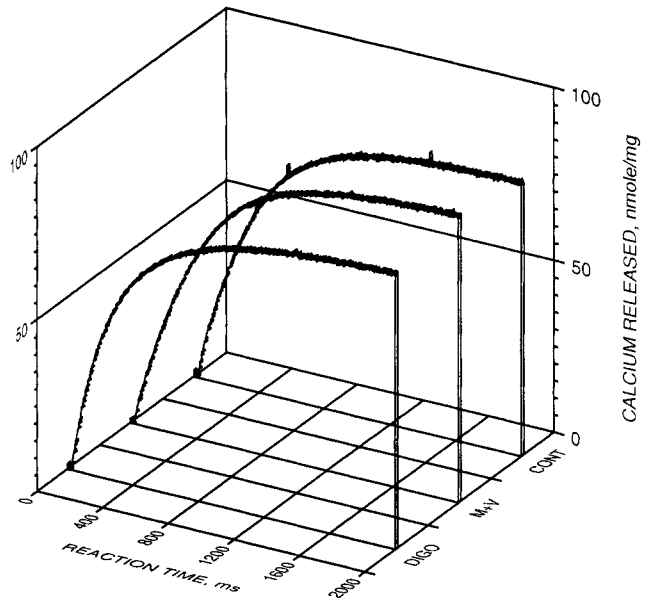


FIGURE 5: Lack of effect of T-tubule polarization blockers on SR Ca^{2+} release if induced by direct stimulation of SR by polylysine. CONT: without addition of blockers (control); M+V: with 10 μM monensin and 10 μM valinomycin; DIGO: with 80 μM digoxin. Triads were incubated in the priming solution (solution A, Table 2) with or without addition of blockers as described in the legend to Figure 4. SR Ca^{2+} release was induced by mixing the primed triads with an equal volume of a solution containing 0.15 M potassium gluconate, 3 μg/mL polylysine (3.3 kDa), 36 μM arsenazo III, and 20 mM imidazole, pH 6.8. Polylysine induced rather poor Ca^{2+} release when release was triggered at $[Ca^{2+}]$ lower than 0.5 μM and monitored with fluo-3. In the experiments shown here, polylysine-induced Ca^{2+} release occurred in the $[Ca^{2+}]$ range from 2 to 60 μM. Each trace was obtained by signal averaging a total of about 90 traces originated from 3 different experiments.

a direct effect of replaced ions on the channel protein. We conclude that saponin, one of the three putative blockers of T-tubule polarization used in the skinned fiber system, is a rather nonspecific blocker of T-tubule-mediated SR Ca^{2+} release at least in the isolated triad system, because (a) it produced only partial inhibition of T-tubule polarization and (b) it produced an appreciable inhibition of polylysine-induced Ca^{2+} release, though to a lesser extent compared with its inhibition of ionic replacement-induced Ca^{2+} release (data not shown).

Depolarization-Induced Ca^{2+} Release Is Virtually Independent of $[Ca^{2+}]_{i=0}$ in the Range of 0.01–0.5 μM. We investigated the $[Ca^{2+}]_{i=0}$ dependence of depolarization-induced Ca^{2+} release in the $[Ca^{2+}]_{i=0}$ range of 0.01–0.5 μM in the presence of appropriate BAPTA–calcium buffers, which was a suitable $[Ca^{2+}]$ range for the measurements with fluo-3. Figures 6 and 7 depict families of time courses of Ca^{2+} release induced by Cl^- (G10)- and Na^+ (G10)-replacement protocols, respectively. The amounts of Ca^{2+} released are essentially identical in this $[Ca^{2+}]_{i=0}$ range.

DISCUSSION

Most of the previous studies on the physiology of e–c coupling have been done with the intact or skinned muscle fiber systems, which are obviously too complex for an analysis of e–c coupling at a molecular level. At the other extreme, purified protein systems have been useful for the studies of intrinsic properties of the individual proteins, as seen in successful conductance measurements of the purified ryanodine receptor protein (Imagawa et al., 1987; Lai et al., 1988). However, they are not suitable for the studies of protein–protein, protein–membrane, and membrane–membrane in-

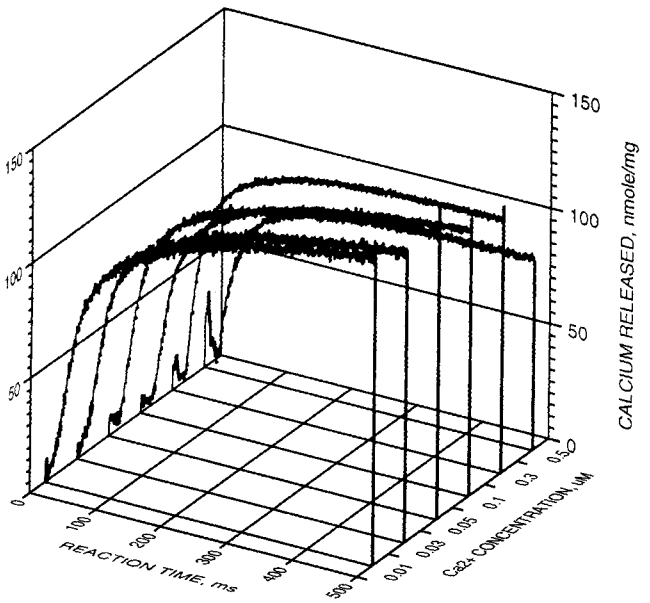


FIGURE 6: The time course of Ca^{2+} release induced by Cl^--replacement protocol at various initial concentrations of Ca^{2+}. The Ca^{2+} concentrations indicated were created with various concentrations of BAPTA-Ca buffers consisting of 0.2 mM BAPTA and various amounts of added calcium. SR Ca^{2+} release was induced by the G10 (Table 1) protocol, and the time course of Ca^{2+} release was determined by stopped-flow flurometry using fluo-3 as a Ca^{2+} probe (cf. Experimental Procedures). Each trace was obtained by signal averaging a total of about 200 traces which originated from 3 preparations.

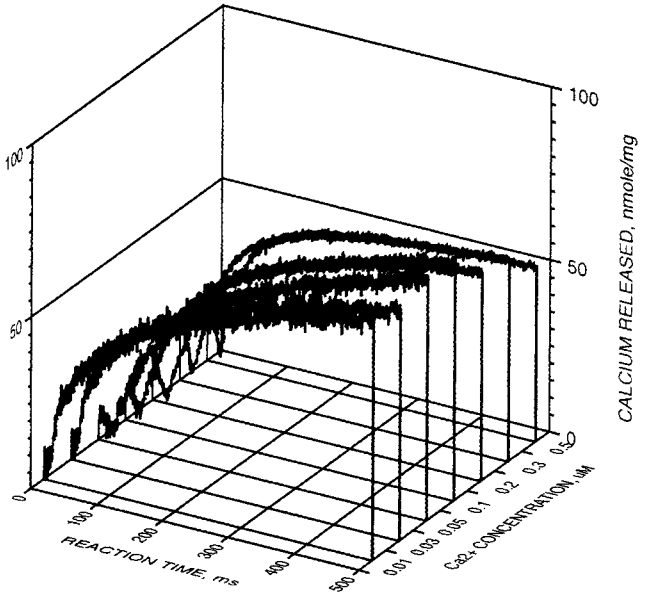


FIGURE 7: The time course of Ca^{2+} release induced by Na^+-replacement protocol at various initial concentrations of Ca^{2+}. The Ca^{2+} concentrations indicated were created with various concentrations of BAPTA–Ca buffers consisting of 0.2 mM BAPTA and various amounts of added calcium. SR Ca^{2+} release was induced by the G10 (Table 2) protocol, and the time course of Ca^{2+} release was determined as described in the legends to Figure 2. Each trace was obtained by signal averaging a total of about 200 traces originated from 3 preparations.

teractions, which are essential elements of e–c coupling. The isolated triad system, which has a much simpler protein composition than the whole-cell system would promise new possibilities for characterizing proteins and their modes of involvement in the e–c coupling mechanism. In order to establish its eligibility as a physiolgically relevant model, however, one must prove that the isolated triad retains essential features of e–c coupling observed in the intact skinned muscle fiber systems. In this study we attempted to achieve the same type of experiments done with skinned fibers in the isolated triad system.

The major conclusions derived from the skinned fiber experiments are as follows. (a) Appropriate changes in the ionic composition of the reaction solution after priming the fiber induced contraction. (b) The ionic replacement-induced contraction was inhibited by several specific blockers of T-tubule polarization, suggesting that the ionic replacement produced T-tubule depolarization. (c) The magnitude of tension development increased with the increased extent of ionic replacement. The main point of this paper is that all of these aspects could be reproduced in the isolated triad preparation.

Most of our previous stopped-flow studies of depolarization-induced Ca^{2+} release were carried out by mixing the primed triad with an equal volume of depolarization solution, which limited the degree of ionic replacement. In this study, using a highly sensitive Ca^{2+} probe, it became possible to follow the release time course at a much larger extent of ionic replacement, hence a much higher chemical depolarization, than before. Another important aspect in this study is that we investigated both representative ionic replacement protocols described in the skinned fiber literature. We found that a systematic increase of the degree of ionic replacement, expressed by the G value, from 1 to 10, according to either the Cl^--replacement protocol (Table 1) or Na^+-replacement protocol (Table 2), results in a progressive increase both in the rate constant (k) and the amplitude of induced Ca^{2+} release (C), and consequently in the increase of the initial rate (Ck). As shown here, the G value-dependent changes in the Ca^{2+} release kinetics show an essentially identical pattern with the graded tension development reported in the skinned fiber system. Furthermore, the graded increases in both the magnitude and the rate constant of Ca^{2+} release shown here are essentially identical to the effects of increasing plasma membrane depolarization on the Ca^{2+} transient in an intact muscle fiber under voltage-clamp conditions [review: Rios and Pizzaro (1991)].

We have investigated all of the putative blockers of T-tubule polarization used in the control experiments with skinned fibers, with some modifications. Dissipation of the T-tubule membrane potential by monensin (Volpe & Stephenson, 1986), added together with valinomycin, blocked both T-tubule polarization and SR Ca^{2+} release induced by either ionic replacement protocol. This was not due to the effect of ionophores on the SR moiety, because direct stimulaltion of SR by polylysine induced a normal Ca^{2+} release. Prevention of T-tubule polarization by blocking the Na^+–K^+ pump with digoxin, the membrane-permeable analog of ouabain used to block T-tubule polarization in the skinned fiber experiments (Donaldson, 1985; Lamb & Stephenson, 1990a,b), inhibited ionic replacement-induced SR Ca^{2+} release, again without affecting polylysine-induced Ca^{2+} release. Since ionic replacement procedures will not produce a depolarization signal in the already depolarized T-tubule, these results support the notion that SR Ca^{2+} release induced by either ionic replacement protocol is mediated by T-tubule depolarization.

In order to establish a direct correlation between the degree of ionic replacement and the magnitude of T-tubule depolarization, appropriate methods to follow rapid changes of T-tubule membrane potential have to be developed, even though such measurements have not yet been done even in the skinned fiber system. In our preliminary study (Ikemoto & Antoniu, 1994), we stained the purified T-tubule vesicles with the membrane-impermeable fluorescent potential-sensitive dye, WW781, from the extravesicular (cytoplasmic) side. After

polarization of these vesicles by incubating in the priming solutions with Mg·ATP, the fluorescence intensity of the membrane-bound WW781 increased. Upon generating various degrees of ionic replacement according to either Cl^--replacement or Na^+-replacement protocol, the fluorescence intensity decreased, whose magnitude was approximately proportional to the degree of ionic replacement, indicating that the ionic replacement procedures described here in fact produce graded T-tubule depolarization. Our current effort is to establish the quantitative assays of both membrane depolarization and the resultant SR Ca^{2+} release in the same stained triad preparation with a high time resolution.

The isolated triad system, which is free from cytoplasmic proteins, permits straightforward analysis of the Ca^{2+} release time course without performing the rather complicated analytical procedures required for the whole-cell system that contains number of Ca^{2+} binding proteins. One of the new aspects of this study is that we induced Ca^{2+} release at well-defined Ca^{2+} concentrations using appropriate calcium buffers. In the initial phase of this study, release was triggered at 1–3 μM $[Ca^{2+}]_{t=0}$ using an EGTA–calcium buffer. However, two problems were encountered under these conditions. First, in the presence of the EGTA–calcium buffer the shape of the release curve was considerably changed as the buffer concentration increased. Second, since the $[Ca^{2+}]$ of the priming solution (solution A) became as low as 0.2–0.5 μM as a result of active Ca^{2+} uptake (see Results), mixing of the primed triads with the depolarizing solution (solution B) with 1–3 μM Ca^{2+} produced a significant Ca^{2+} jump (from submicromolar to several micromolar), in turn producing a significant depolarization-independent (actually Ca^{2+} jump-dependent) component of Ca^{2+} release. Both of these problems could be circumvented by using BAPTA–calcium buffer and by inducing Ca^{2+} release at much lower Ca^{2+} concentrations ($\leq$0.5 μM). Thus, the Ca^{2+} release curves obtained under the present conditions are virtually devoid of the depolarization-independent/Ca^{2+} jump-dependent component. As shown here, the amplitude of Ca^{2+} release is virtually identical in the $[Ca^{2+}]_{t=0}$ range of 0.01–0.5 μM. This observation is in a good agreement with our previous finding that the amounts of Ca^{2+} released upon chemical depolarization at different $[Ca^{2+}]_{t=0}$ in the zero to submicromolar range were essentially identical (Ikemoto et al., 1985). As previously described (Ikemoto et al., 1985), further increase of $[Ca^{2+}]_{t=0}$ in a submicromolar to several micromolar range produced a sharp increase in the amount of Ca^{2+} released. This part of the increase seems to reflect, at least partly, an increase of the Ca^{2+} jump-dependent component described above.

In conclusion, the present study provides evidence that the isolated triads retain essential features of e–c coupling *in vivo*. (1) Both of the two representative ionic replacement methods used for the induction of T-tubule-mediated tension development in the skinned fiber system produced Ca^{2+} release from triads at a physiological rate. (2) There is a close correlation between the extent of ionic replacement (or chemical depolarization of the T-tubule) and the kinetics of induced SR Ca^{2+} release as expected for a physiological e–c coupling. (3) The ionic replacement-dependent changes in the release kinetics showed a striking similarity to the voltage-dependent changes of the Ca^{2+} transient in the intact fiber system. Thus, the isolated triad preparation may serve as a simplified physiological model useful for the studies of e–c coupling at a molecular level.

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