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The Voltage Dependence of Depolarization-induced Calcium Release in Isolated Skeletal Muscle Triads

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Abstract. We demonstrate for the first time in this study that triadic vesicles derived from skeletal muscle display a voltage dependence of depolarization-induced calcium release similar to that found in intact muscle. We confirm previous studies by Dunn (1989) which demonstrated that changes in extravesicular potassium induced membrane potential changes in isolated transverse tubules with the voltage sensitive dye DiSC(3)-5. Depolarization-induced calcium release was studied in isolated triadic vesicles through similar changes in extravesicular [K] while clamping extravesicular Ca^{++} to submicromolar concentrations. The amplitude of fast phase of calcium release, identified as depolarization-induced calcium release, varied with the percentage of transverse tubules in the preparation (determined through ^{3}H-PN200-110 specific activity) and different levels of depolarization. Threshold activation of calcium release was obtained with a 40.5 mV potential change; maximal calcium release was obtained with a 75 to 81 mV potential change. Boltzmann fits to the normalized depolarization induced calcium release plotted against the membrane potential change yielded a voltage dependence (k = 4.5 mV per e-fold change) very similar to that found in intact muscle (k = 3–4 mV per e-fold change; Baylor, Chandler & Marshall 1978, 1983; Miledi et al., 1981). Substitution of methanesulfonate for propionate as the impermeant ion or addition of valinomycin in the depolarizing solutions had little effect on the voltage dependence of calcium release.

Key words: Voltage-dependence — Calcium release — Skeletal muscle — Triads — Fluorescence — Terminal cisternae

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Introduction

Excitation-contraction coupling has focused on how excitation of the muscle membrane translates into the resultant release of calcium from its internal stores. As one begins to probe the molecular mechanism by which this signal transduction occurs, it would be to the experimenter's advantage to use the simplest system which retains all of the physiological characteristics of the intact muscle. Depolarization-induced calcium release has been studied using intact (Miledi, Parker & Schaow, 1977, Miledi et al., 1981; Baylor et al., 1978, 1983), and skinned fibers from frog (Donaldson, 1985; Stephenson, 1985), cut fibers from frog and rat (Szücs, Simon & Schneider, 1984; Melzer et al., 1986; Klein, Simon & Schneider, 1992; Delbono & Stefani, 1993), and isolated triadic vesicles from rabbit (Ikemoto, Antoniu & Kim, 1984; Corbett et al., 1992). Work with intact muscle fibers has defined the normal physiological characteristics of depolarization-induced calcium release in skeletal muscle. The membrane potential has been controlled in studies using either intact or cut muscle fibers with Vaseline gap voltage clamp techniques and the calcium transients obtained upon depolarization have been measured with calcium-sensitive optical dyes such as arsenazo III and Fura-2. Miledi et al., (1977, 1981) found that the calcium transients from intact muscle fibers displayed voltage-dependent properties with a threshold voltage for calcium release usually around –40 mV, a steeply voltage-dependent range of release from –40 to 0 mV, with maximal release occurring at about 0 to 10 mV. This voltage-dependent activation of calcium release was well fit with a Boltzmann equation, which defines the probability that the calcium release channels will be in an open or a closed state at a given depolarization, yielding slope factors (k values) of 3–4 mV per e-fold change (Baylor et al., 1978, 1983; Miledi et al., 1981). In stud-

ies that used cut skeletal fibers from frog, Boltzmann fits to the peak rate of calcium released revealed k values between 10–12 mV per e-fold change (Szücs et al., 1984; Melzer et al., 1986; Klein et al., 1992), showing slightly less voltage dependence than that obtained in intact fibers. Delbono and Stefani (1993) used cut fibers from rat and also found that depolarization-induced calcium release was voltage dependent, obtaining a k value of 11 mV for the Boltzmann fit; the kinetics of calcium release at 17°C had a time constant of 13.8 msec for the rising phase of calcium release when Fura-2 was used as the calcium indicator.

Donaldson (1985) examined the effects of depolarization of the transverse tubule, accomplished by ionic replacement of potassium propionate with choline chloride while maintaining a constant [K][Cl] product, and measured the isometric tension transients of skinned fibers that resulted from Ca^{2+} release from the terminal cisternae. These studies indicated that different levels of depolarization elicited different magnitudes of tension transients, consistent with a voltage-dependent process, although the rate of release was slow (limited by diffusion of the depolarizing ions). Donaldson (1985) and Volpe and Stephenson (1986) further demonstrated that repolarized transverse tubules were vital in depolarization-induced calcium release by showing that incubating the fiber in a ouabain solution (prior to skinning) or digoxin (after skinning), which would effectively depolarize the transverse tubule membrane, eliminated the tension transient upon subsequent depolarization. In similar studies, Lamb and Stephenson (1990), found that previously depolarizing a skinned fiber with a Na^+ substitution for K^+ for a substantial period of time inactivated the voltage sensor in the transverse tubule such that it could no longer respond to depolarization initiated by a choline chloride wash.

Triadic vesicles isolated terminal cisternae have been used by a number of investigators to study different mechanisms of calcium release (Ikemoto et al., 1984; Meissner, 1984; Palade, 1987; Corbett et al., 1992). Some studies, using isolated terminal cisternae, have examined calcium-induced calcium release, which is believed to be a direct stimulation of the sarcoplasmic reticulum: this form of calcium release requires micromolar free Ca^{2+} as a trigger, is enhanced by ATP and is inhibited by physiological levels of free Mg^{2+} (Meissner, 1984, 1986; Ikemoto, Bozena & Meszaros, 1985). In preparations with low triadic content, where depolarization-induced calcium release may be expected to be small, the use of 10 μM Fura effectively clamps free calcium to submicromolar levels dramatically reducing any calcium-induced calcium release from uncoupled ryanodine receptors (Corbett et al., 1992).

Depolarization-induced calcium release in skeletal muscle vesicles was initially studied using muscle microsomes: the vesicles were subjected to chemical depolarizations by rapid replacement of K gluconate with choline chloride and Ca^{2+} efflux was measured by absorbance changes of arsenazo III using a stopped flow apparatus (Ikemoto et al., 1984). In these early studies, a small depolarization of the vesicles (1:1 dilution) was produced, resulting in relatively slow release (time constant of approximately 20–30 msec) of dihydropyridine-sensitive depolarization-induced calcium release (Ohkusa et al., 1991). The voltage dependence of release was not addressed in this early work. In recent work by Ikemoto and Antoniu (1993), it was found that increasing the amount of T-tubular depolarization increased both the amount and the rate of calcium release measured by the fluorescent dyes fluo-3 and calcium green-5N, although a quantitative analysis of the voltage dependence of depolarization-induced calcium release was not given.

Triads represent the smallest component of skeletal muscle that contain all of the elements essential for depolarization-induced calcium release. To prove that depolarization-induced calcium release from isolated triad vesicles was physiological, we have performed experiments which: (i) demonstrate that changes in extravesicular K^+ concentrations depolarize the vesicles through the use of a voltage-sensitive dye; (ii) show that depolarization of the transverse tubule membrane was the mediator for calcium release; and (iii) show that the voltage dependence of depolarization-induced calcium release was similar to values obtained in cut and intact fibers.

Materials and Methods

Triad and T-Tubule Vesicle Preparation

The triad and transverse tubule vesicles were prepared from the leg fast twitch muscle of 5–7 lb New Zealand white rabbits using a modification of the procedure of Corbett et al. (1992). The animals were sacrificed by cervical dislocation and subsequent exsanguination. The leg fast twitch muscles were removed and minced into 500 ml of ice-cold Sucrose-EDTA buffer (250 mM sucrose, 2 mM EDTA, pH 7.0) containing protease inhibitors (100 μg/ml 1,10-phenanthroline, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 2 μg/ml pepstatin, 34 μg/ml PMSF).

The rest of the preparation was similar to that used by Corbett et al. (1992). The minced muscle was homogenized in a Waring Blender for a total of 1.5 min using three 30-sec rests to prevent heating of the sample. The homogenate was subjected to a 14,000 × *g* spin in a Beckman JA-10 rotor for 20 min at 5°C. The supernatant was poured through three layers of cheesecloth to remove floating material, the pH adjusted to 7.0 with NaOH, and centrifuged at 123,000 × *g* in a Beckman Ti45 rotor for 45 min at 5°C. The supernatant was discarded; the pellets were rehomogenized in a sucrose-histidine buffer (250 mM sucrose, 20 mM histidine, pH 7.0) and again centrifuged at 123,000 × *g* for 45 min at 5°C. The supernatant was discarded and the pellet resuspended in a small volume (25–30 ml) of sucrose-histidine buffer.

Microsomal fractions were separated on continuous (15 to 65% w/w) sucrose gradients which had been chilled to 5°C. A portion (5–6 ml) of the rehomogenized microsomes were loaded onto the top of each gradient and spun at 27,000 rpm in a Beckman SW-28 rotor for 12–17

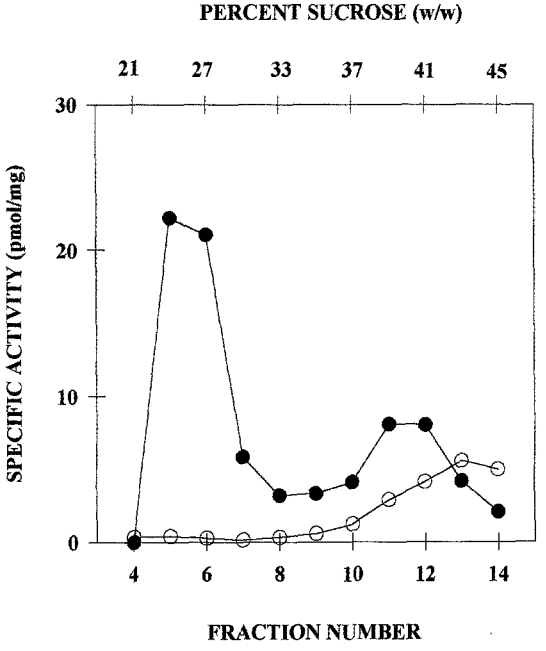

Fig. 1. Radioligand characterization of skeletal muscle vesicles separated on sucrose gradients. Skeletal muscle vesicles were prepared as described in Materials and Methods. Crude microsomes from the 3/31/93 preparation were separated on a continuous 15–45% sucrose gradient, which was fractionated into 2 ml aliquots (bottom x-axis). Small aliquots from each 2 ml fraction were assayed for protein content, ^{3}H-PN200-110 (closed circles) and ^{3}H-ryanodine (open circles) binding. Binding of the radioligand was normalized to the amount of protein and expressed as a specific activity (pmol bound radioligand/mg protein): each point represents the mean of two replicates at two different dilutions. The percentage of sucrose (w/w) for each fraction is shown on the top x-axis.

hr. The gradients were fractionated into 2 ml aliquots and small aliquots of each fraction (40–50 µl) were assayed for ^{3}H-PN200-110 or ^{3}H-nitrendipine (transverse tubule marker) binding, ^{3}H-ryanodine (calcium release channel marker) binding, protein content, and the percentage sucrose (w/w) (Fig. 1). Based on the elevated ^{3}H-PN200-110 and ^{3}H-ryanodine binding in Fig. 1, the fractions at 35–45% sucrose contained a mixture of triads and terminal cisternae. Fractions at 19–23% sucrose were determined to contain free transverse tubule vesicles because of very high specific binding for ^{3}H-PN200-110 but little binding for ^{3}H-ryanodine. Fractions of interest were pooled, diluted slowly into sucrose-histidine buffer, and concentrated through centrifugation (123,000 × g_{av} for 45 min at 5°C) and rehomogenization in a small volume of sucrose-histidine buffer. The concentrated samples were divided into small aliquots (250 µl/vial) and quick-frozen by immersing the cryovials in a mixture of acetone and dry ice; samples were stored at −70°C.

Radioligand Binding Assays

Assays for ^{3}H-PN200-110 or ^{3}H-nitrendipine (T-tubule marker) and ^{3}H-ryanodine binding (terminal cisternae marker) were performed for every triad preparation. Samples were incubated in a 50 mM Tris HCl solution (pH 7.0) that contained 3 nM ^{3}H-PN200-110 or ^{3}H-nitrendipine at room temperature in subdued light for 30 min. Nonspecific binding was determined through the addition of 20 µM cold nitrendipine. The samples were filtered through glass fiber filters and washed three times with 4.5 mls of 140 mM choline chloride. The filters were counted by liquid scintillation. B_{max} values for specific binding were obtained with the following equation:

$$B_{max} = \text{specific binding} \cdot ((K_D/[\text{free ligand}]) + 1) \quad (1)$$

where the K_D for PN200-110 was 0.4 nM and the K_D for nitrendipine was 13.67 nM.

^{3}H-ryanodine binding was performed according to the method of Imagawa et al., (1987) with minor modifications. Samples were diluted and incubated in (mM) 150 KCl, 3 Na-ATP, 10 HEPES, 260 µM $CaCl_2$, and 5 nM ^{3}H-ryanodine at room temperature for 1 hr. Nonspecific binding was determined through the addition of 10 µM cold ryanodine. Samples were filtered through glass fiber filters and washed three times with 4.5 ml of a wash solution (150 mM KCl, 10 mM HEPES, pH 7.4). Filters were counted by liquid scintillation. B_{max} values were obtained using the above formula, using a K_D for ryanodine of 14 nM (obtained by Scatchard analysis).

Table 1. Ionic composition of load buffer solutions

Load buffer A	Load buffer C
100 K Propionate*	4 K Propionate
2 $MgCl_2$	96 TEACl
	2 $MgCl_2$
Load buffer B	Load buffer C
98 K Propionate*	4 K Propionate
2 KCl	98 TEACl
1 $MgCl_2$	1 $MgCl_2$
Nondepolarizing	Nonhyperpolarizing
96 K Propionate*	4K Propionate
4 KCl	100 TEACl

* In some experiments K methanesulfonate replaced K propionate. All solutions used in isolated *triad vesicle* experiments included an ATP regenerating system (2 mM NaATP, 15 mM Na creatine phosphate, and 15 units/ml creatine phosphoinase), 1 mM Na azide, 36 mM imidazole, and 10 µM Fura-2, pH = 7. Some solutions used in isolated *transverse tubule vesicle* experiments did not contain an ATP-regenerating system while all containined DiSC(3)-5(2.5 µM after dilution) instead of Fura-2.

Protein Assay

Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard. The protein determined for a given sample represents the mean of three different dilutions of the sample, each containing two replicates.

Solutions

All solutions were designed to maintain a constant [K][Cl] product after dilution to prevent osmotic shock to the vesicles (Stephenson, 1985). All load buffers used with triadic vesicles (for calcium loading) contained 36 mM imidazole, 1 mM Na azide, 10 µM Fura-2 (impermeant), 100 µl/ml of an ATP-regenerating system (2 mM Na ATP, 15 mM Na creatine phosphate, and 15 units/ml creatine phosphokinase), pH 7.0. The major ionic content for the incubating load buffers which varied is shown in Table 1. Load solution (A) contained (in mM) either 100 K propionate or 100 KMES, 2 $MgCl_2$. (Note: For the 1:5 dilution, Load Solution (B) contained (in mM): 98 K Propionate or 98 KMES, 2

Table 2. Depolarizing and hyperpolarizing solutions

Dilution	Depolarizing solutions (mM)	Hyperpolarizing solutions (mM)	EST. mV · Δ
1:5	25 TEACl	24 K Propionate	±40.5
1:10	44 TEACl	44 K Propionate	±58.0
1:12	52 TEACl		+63.0
1:14	60 TEACl		+66.5
1:15	64 TEACl	64 K Propionate	±68.0
1:20	84 TEACl	84 K Propionate	±75.5
1:25	104 TEACl		+81.0

In all solutions NMG propionate was added to match the osmotic strength of the Load buffer. In experiments where K methanesulfonate replaced propionate in the Load buffer, NMG methanesulfonate replaced replaced NMG propionate in the release solutions. All solutions used in isolated *triad vesicle* experiments included an ATP-regenerating system (2 mM NaATP, 15 mM Na creatine phosphate, and 15 units/ml creatine phosphoinase), 1 mM Na azide, 36 mM imidazole, and 10 μM Fura-2, pH 7. Some solutions used in isolated *transverse tubule vesicles* experiments did not contain an ATP regenerating system and DiSC(3)-5 (2.5 μM after dilution) was used instead of Fura-2.

KCl, and 1 $MgCl_2$). The ionic content of the Load solutions used in the membrane potential studies with transverse tubule vesicles was the same except some experiments did not contain an ATP-regenerating system and the membrane potential sensitive dye DISC(3)-5 (5 μM final) was used instead of Fura-2.

All depolarizing solutions used with triadic vesicles contained 36 mM imidazole, 1 mM Na Azide, 10 μM Fura-2 (impermeant) and an ATP-regenerating system (2 mM NaATP, 15 mM Na creatine phosphate, and 15 units/ml creatine phosphokinase). The depolarizing solutions contained varying amounts of TEACl with NMG propionate or NMG MES (*see* Table 2). All dilutions greater than 1:10 received additional amounts of $MgCl_2$ to ensure that the final total Mg^{2+} concentration following dilution of loaded vesicles was 0.2 mM.

In experiments designed to hyperpolarize transverse tubules, the vesicles were incubated with Load solution (C) (Table 1) containing DiSC(3)-5,(mM)4K-Propionate, 2 $MgCl_2$, 96 TEACl, 1 Na Azide, 36 imidazole, pH 7.0 (*Note:* The load solution (D) for a 1:5 dilution contained 1 mM $MgCl_2$, and 98 mM TEACl).

The basic hyperpolarizing solutions contained varying amounts of K-propionate and NMG propionate (*see* Table 2). All dilutions greater than 1:10 received additional amounts of $MgCl_2$ to ensure a final concentration of 0.2 mM following dilution.

Calcium Loading

The protocol for calcium loading of the triads was the same as Corbett et al., (1992). Briefly, isolated triads were diluted into Load solution (A or B). The "Load" mixture (0.9 ml) was placed in a 1 ml quartz cuvette and aliquots (3.6 μl) of a 10 mM $CaCl_2$ stock were added. Vesicles typically received 5–6 additions of calcium.

Calcium uptake and release were measured by changes in extravesicular Fura-2 fluorescence using an SLM Model 8000 spectrofluorimeter. The ratio of two excitation wavelengths (340 and 380 nm) was monitored continuously with a 100 Hz chopper at a constant emission wavelength of 505 nm. The fluorescence ratio varied directly with changes in extravesicular calcium (i.e., an increase in the fluorescence ratio indicated an increase in extravesicular calcium.)

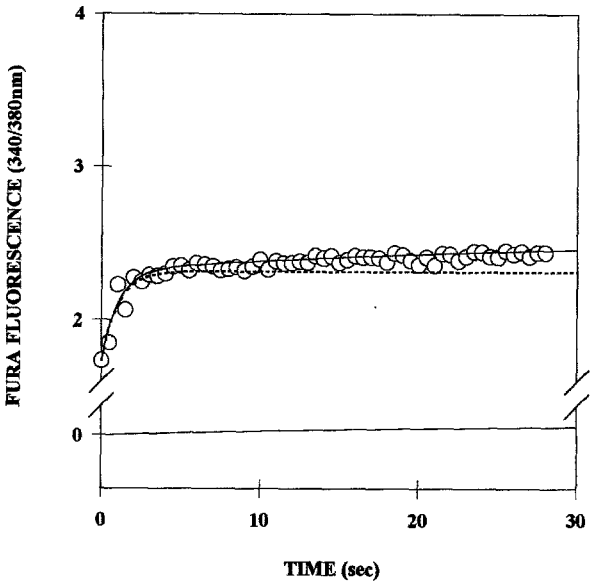

Fig. 2. Double exponential fit of depolarization-induced calcium release. Triad vesicles were incubated in Load buffer A with an ATP-regenerating system (Materials and Methods) and 10 μM of the calcium-sensitive dye Fura-2. The vesicles were manually diluted (1:10) into a depolarizing solution (58 mV potential change: *see* Table 1) and Fura-2 fluorescence changes were monitored at 340 and 380 nm on an SLM spectrofluorimeter using a 100 Hz chopper. The fura-2 fluorescence ratio (340/380 nm) was sampled every 500 msec and plotted against time (open circles). The changes in fluorescence displayed a biphasic form with a fast phase (depolarization-induced calcium release) and a slow phase (calcium leakage from the vesicles). The data were fit (continuous line) with a double exponential equation (Eq. 2) using a least squares fitting program (Nfit: Island products). The broken line is the fast exponential with a constant added to bring the first point up to the origin. The continuous line beginning at 0 is the form of the slow exponential. The difference between the origin of the fast exponential and its plateau was used to calculate the percentage of depolarization-induced calcium released.

Calcium Release

The calcium loaded triadic vesicles were manually diluted into different TEACl solutions which were designed to depolarize the vesicles as well as maintain a constant $[K^+][Cl^-]$ product (*see* Tables 1 and 2). Loaded triadic vesicles released calcium upon depolarization, resulting in an increase of the 340/380 fluorescence ratio of Fura-2. This release displayed both a fast and a slow phase of release. The fast phase of release lasted approximately 4–10 seconds when vesicles were manually mixed and had characteristics consistent with depolarization-induced calcium release (Corbett et al., 1992). The slow phase was observed with all calcium loaded vesicles (i.e., isolated terminal cisternae and longitudinal reticulum) and probably represents a slow calcium leak. The biphasic rise in the fluorescence ratio (*shown* in Fig. 2) was fit with a double exponential equation (*shown below*) using a nonlinear least squares fitting program (Nfit: Island Products):

$$y = a + (b \cdot \exp(-x/T1)) + (c \cdot \exp(-x/T2)) \quad (2)$$

where $T1$ is the time constant for the Slow Exponential, $T2$ is the time constant for the Fast exponential and a, b, and c are constants. The fast

exponential phase, $y = (C \cdot \exp(-x/T2)$, was calculated and a constant added to bring the y-value up to the origin of release (*shown* by dashed line in Fig. 2). The change in Fura fluorescence between the origin of release (*shown* here at 0 sec) and the plateau value was used to determine the change in extravesicular calcium (Fig. 2).

Calibration of the release solution was performed according to Corbett et al. (1992). Briefly, unloaded vesicles were diluted into the corresponding release solutions and known amounts of calcium (1 and 5 μM) were added, producing stepwise changes in Fura fluorescence until the dye was saturated. The percentage of Fura-2 saturation was calculated for each calcium addition by using the equation:

$$\%\text{Fura-2 Saturation} = k \cdot (R - R_{min})/[R_{max} - (k \cdot R_{min}) - (R \cdot (1 - k)] \quad (3)$$

where k is the ratio of the fluorescence of free Fura-2 to the fluorescence of the complexed Fura-2 at 380 nm. R_{max} and R_{min} are the values of R for calcium saturated and calcium free Fura-2 (Grynkiewicz, Poenie & Tsien, 1985). The percent saturation was plotted against the amount of calcium added and fit with the equation:

$$y = ((x - A) \cdot B)/((x - A) + M) \quad (4)$$

where y is the percent Fura-2 saturation, x is the known amount of added calcium (total), A is the contaminating calcium prior to calcium additions, B is the maximum saturation, and M is proportional to the K_D of the dye. Values of A, B, and M were obtained using a nonlinear least squares curve fitting program (Nfit: Island Products). This curve fit was used to calculate the change in extravesicular calcium from the fluorescence changes.

The percent saturation was calculated for the fast phase fluorescence change of each release. The amount of calcium released was calculated using the calibration curve described above and expressed as a percentage of the total calcium loaded into the vesicles. The percentage of calcium released was plotted against the estimated potential change for each dilution (calculated from the Nernst equation). The data was then fit with a Boltzmann function:

$$y = \max/(1 + \exp((V_{0.5} - x)/k)) \quad (5)$$

where max is the maximum percent of calcium released, $V_{0.5}$ is the potential at which the percentage of calcium released is half maximal, and k is the slope factor.

Transverse Tubule Depolarization

Transverse tubules were incubated in a Load A or B buffer minus the ATP regenerating system for 45 min and centrifuged to concentrate the vesicles (Table 1). A voltage-sensitive fluorescent dye, 3,3′-dipropylthiadicarbocyanine iodide (DiSC3(5)), was allowed to equilibrate with the vesicles for 15 min. The vesicles were diluted into a nondepolarizing control and different depolarizing solutions (*see* Table 2). The final protein concentration after dilution was 50 μg/ml and final dye concentration was 5 μM for Fig. 3 (Excitation Scan) and 2.5 μM for all other experiments. The fluorescence was monitored at a 647 nm excitation wavelength and a 670 nm emission wavelength. Valinomycin (63 μM Final) was added to give the final fluorescence change. Diluting the T-Tubules into a depolarizing solution produced a smaller fluorescence signal compared to dilution into a nondepolarizing solution. This was expressed as a percent decrease in fluorescence. Control dilutions into nondepolarizing solution were performed for each experiment to determine the relative fluorescence change. Additional experiments were also performed in the presence of an ATP regenerating system (2 mM Na ATP, 15 mM Na creatine phosphate, and 15 units/ml creatine phosphokinase).

Transverse Tubule Hyperpolarization

All experiments were performed as explained above except the T-Tubules were incubated in a low K load (C or D) solution for 45 min, centrifuged to concentrate the vesicles, and diluted into a high K-release solution (Tables 1 and 2). Diluting the T-Tubules into a hyperpolarizing solution produced a larger fluorescence signal than that obtained when vesicles were diluted into a nondepolarizing solution. This was expressed as a percentage increase in fluorescence. Control dilutions into nondepolarizing solution were included in each experiment to determine the relative fluorescence change under various conditions.

Results

T-Tubule Depolarization and Hyperpolarization

Preliminary experiments, examining the binding of ^{3}H-ouabain and ^{3}H-digoxin, have been performed to determine the orientation and the integrity of the transverse tubule membrane vesicles (Hidalgo et al. 1986). Ouabain or digoxin only binds to the Na,K-ATPase in the presence of ATP. The binding site for ATP on the Na,K-ATPase is located on the cytoplasmic face of the transverse tubule in intact muscle fibers while the ouabain or digoxin binding site is located on the extracellular side. Using our isolated transverse tubule vesicles, ^{3}H-ouabain binding increased in the presence of ATP when saponin was added (*data not shown*). This revealed that 76% of transverse tubule vesicles were sealed and that either the ATP or ^{3}H-ouabain did not have access to its binding site inside the vesicle until saponin was added. In other experiments, the binding activity of ^{3}H-digoxin, a membrane permeable analog of ouabain, remain unchanged upon the addition of saponin in the presence of ATP. This demonstrated that the vesicles were oriented 100% inside-out with the digoxin binding site inside the vesicle and the ATP binding site on the extravesicular side. Our preliminary results compare well with Hidalgo et al. (1986). They found that 82.5% of their isolated transverse tubule vesicles were sealed and 100% were oriented inside-out.

The following experiments were designed to test whether isolated transverse tubule membrane vesicles could be chemically depolarized by incubating the vesicles in a high K (100 mM) load buffer A or B (Table 1) and diluting them into a release solution to effectively lower the extravesicular concentration of K^+ (Table 2). To hyperpolarize the vesicles, they were incubated in

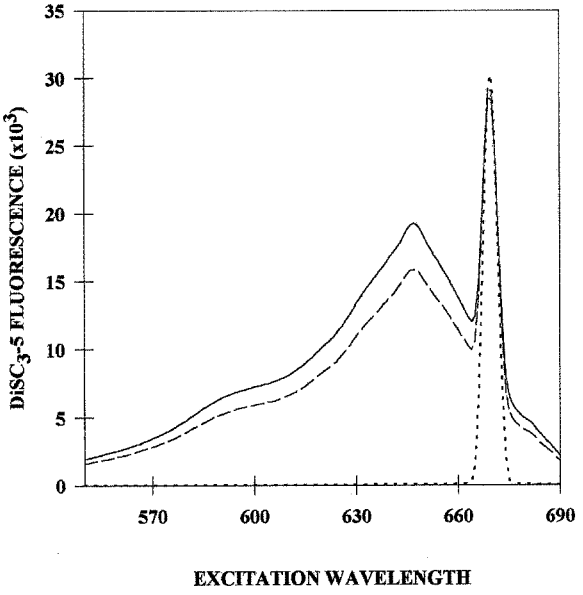

Fig. 3. Excitation scans of isolated transverse tubules with the voltage-sensitive dye DiSC(3)-5 at different polarized states. Isolated transverse tubule vesicles (1 mg/ml from the 8/3/92 preparation) were incubated in load buffer A with DiSC(3)-5 (50 μM) for 15 min. The vesicles were diluted (1:20) into a nondepolarizing solution (Table 1) and the excitation scan (continuous line) was performed from 550 to 700 nm at an emission wavelength of 670 nm on an SLM spectrofluorimeter (continuous line). In the second experiment, the transverse tubules were diluted (1:20) into a depolarizing solution (75.5 mV potential change: Table 1), indicated by the broken line. Valinomycin (63 μM) was present in both the nondepolarizing and depolarizing solutions. An excitation scan (550–700 nm) was performed using isolated transverse tubules, without incubation with DiSC(3)-5, which were diluted (1:20) into a nondepolarizing solution (dotted line). Light scattering from the vesicles was evident from 665–680 nm, but did not contribute more than 0.4% to the fluorescence measurement at 647 nm excitation wavelength.

low K^+ Load C or D buffer (Table 1) and diluted into high K release solutions (Table 2). To detect the amount of depolarization caused by the various dilutions, the voltage-sensitive dye DISC(3)-5 was used. Cabrini and Verhman (1986), using biological membrane and PC vesicles, determined that an induced membrane potential primarily resulted in DiSC(3)-5 being translocated between sites at the inner and outer membrane leaflets. Secondary effects of an induced membrane potential resulted in dimerization and membrane partitioning of the voltage-sensitive dye. Sims, Wang & Hoffman, (1974) demonstrated that the partitioning of a cyanine dye between the extravesicular solution and the vesicle was influenced by membrane potential.

In Fig. 3, transverse tubules were isolated as described in Materials and Methods, incubated with 8 mls of Load A buffer for a total of 45 min while being concentrated through centrifugation. This long incubation was necessary to ensure that K had fully equilibrated across the membrane, producing a lumenal K concentration near 100 mM. After the spin, the vesicles were rehomogenized and suspended in Load A buffer. The T-tubule vesicles (526.8 μg/ml) were then incubated with DiSC(3)-5 (50 μM) for 15 min. After incubation, the T-Tubules were diluted into a nondepolarizing solution that contained the K ionophore valinomycin (63 μM final) and an excitation scan was performed from 550–700 nm with an emission wavelength of 670 nm on an SLM 8000 Spectrofluorimeter (continuous line). The scan showed a small shoulder at 600 nm and peaks at 647 nm and 670 nm. The second peak, where excitation wavelength equals emission wavelength, reflects light scattering from the vesicles. In another experiment, vesicles were diluted into a 1:20 depolarizing solution and an excitation scan was performed (broken line). This line showed the same characteristics of the nondepolarizing scan except the fluorescence level was reduced. The dilution of the vesicles into the 1:20 solution depolarized the vesicles due to dilution of extravesicular K^+ from 100 to 5 mM. Translocation of DiSC(3)-5 from the external to the internal membrane leaflet resulted in a quenched fluorescence of DiSC(3)-5. (*Note:* In vesicles that are oriented outside-out this would be referred to as a hyperpolarization. Our vesicles are oriented inside-out so the convention is reversed: this is referred to as a depolarization.) 647 nm was determined to be the optimal excitation wavelength because maximal differences were observed between the nondepolarizing and depolarizing fluorescence levels at this wavelength. An additional experiment was performed where no dye was present to determine the contribution of light scatter from the vesicles to our signal at 647 nm. A peak appeared between 660 and 675 nm (Fig.3 : dotted line). This experiment confirmed that virtually none of the light scatter contributed to our signal at 647 nm.

Figure 4 shows the results from experiments designed to measure membrane depolarization of the isolated transverse tubule vesicles. Transverse tubule vesicles were diluted with 8 mls of Load A buffer and spun down at 125,000 × *g* in a Ti45 rotor for 45 min to ensure that the concentration of K inside the vesicles was 100 mM. After the spin, the vesicles were rehomogenized and suspended in Load A buffer. The T-tubules were then incubated for 15 min with DiSC(3)-5 before being diluted into different release solutions (1:5–1:25, Table 2). The extravesicular K concentration was effectively diluted down to 0.020 M in the 1:5 dilution and 0.004 M in a 1:25 dilution. The log of these extravesicular K concentrations (M) were plotted on the bottom x-axis. Valinomycin (63 μM), a K ionophore, was added to each experiment to increase the conductance of K^+. The Nernst equation was used to calculate the membrane potential change for each dilution, assuming that the largest conductance in the presence of valinomycin was K. In the first set of experiments (continuous line: circles), the lumenal [K] was assumed to be in equilibrium with ex-

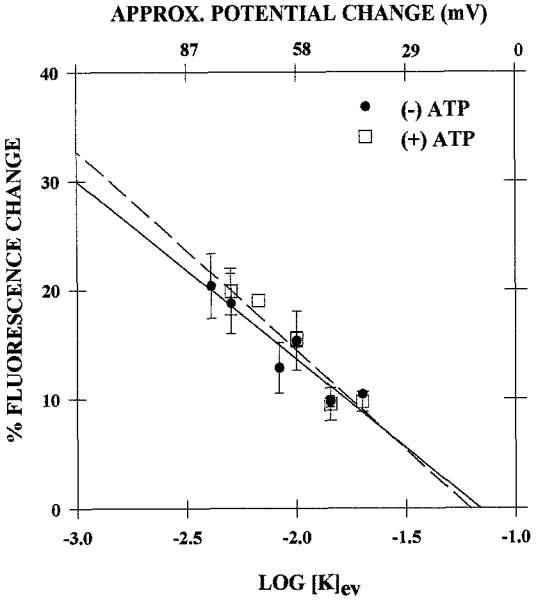

Fig. 4. Correlation of DiSC(3)-5 fluorescence changes (quench) with changes in extravesicular K concentrations upon depolarization. Isolated transverse tubule vesicles were incubated in Load buffer A (for dilutions greater than 1:5) or buffer B (for a 1:5 dilution) for 45 min and with the voltage-sensitive dye DiSC(3)-5 for an additional 15 min (2.5 μM after dilution) with (circles) or without (squares) an ATP regenerating system. The vesicles were then diluted into different depolarizing solutions (Table 1): the changes in DiSC(3)-5 fluorescence were monitored at an excitation wavelength of 647 nm and an emission wavelength of 670 nm. All release solutions contained 63 μM valinomycin. For all experiments a control dilution into a nondepolarizing solution was performed. The fluorescence levels between the nondepolarizing and depolarizing dilutions were compared: the percent fluorescence change was calculated and plotted against the log of the extravesicular concentration of K. The upper x-axis displays the calculated Nernst potential changes for changes in extravesicular [K], assuming a lumenal [K] of 100 mM. Each data point represents the mean of 2–6 values ± SD (standard deviation). For solutions that did not contain an ATP-regenerating system (continuous line, closed circles), linear regression (r^2 = 0.7) yielded a slope of –0.281 mV^{-1} and an x-intercept of approximately –1.15 (the log of estimated lumenal [K], or approximately 71 mM K). For solutions which did contain an ATP-regenerating system (broken line, open squares), the regression line (r^2 = 0.9) a slope of –0.315 mV^{-1} was obtained.

travesicular [K] in the load solution, or 0.1 M: ATP was absent, so there should be no Na-K ATPase activity. We therefore assume that the membrane potential in the load solution and in the nondepolarizing solution is 0 mV because both lumenal and extravesicular [K] are equal (100 mM). In each experiment DiSC(3)-5 fluorescence in the presence of valinomycin under depolarizing conditions was compared to a nondepolarizing fluorescent level and the percentage of fluorescent change was calculated: this was plotted on the y-axis. As can be seen in Fig. 4, greater dilutions of extravesicular K^+, which produce greater calculated potential changes (top x-axis), generated increasingly greater percentage fluorescent changes in the voltage-sensitive dye. A regression line was fit through the points; the x-axis intercept at zero fluorescence change should be equal to the log of lumenal [K], or –1.0 for an anticipated lumenal [K] of 0.1 M. The x-intercept was –1.15 (or about 71 mM lumenal K) and the slope of the fit line was –0.281 mV^{-1}. A second line (broken: squares) is shown for vesicles that were incubated in load A solution plus an ATP regenerating system, and then diluted into various depolarizing solutions in the presence of the voltage-sensitive dye plus valinomycin. Through other preliminary studies with this voltage-sensitive dye, we estimate that the resting membrane potential after addition of the ATP regenerating system is approximately –68 mV. In this figure, however, so that the degree of depolarization for both the system with and without ATP may be compared, we have plotted the estimated potential change from nondepolarizing rather than the actual calculated membrane potentials. It is clear that the slopes obtained for the two systems using the same depolarizing solutions is very similar (+ATP: –0.315 mV^{-1}; –ATP: –0.281 mV^{-1}), indicating that the same degree of depolarization (or potential change) is obtained with these solutions and is not dependent on the initial resting membrane potential.

Figure 5 displays experiments that were designed to measure the hyperpolarization of the transverse tubule vesicles. The preparation of the transverse tubules was the same as described above except they were diluted with low [K] Load C or D solutions, centrifuged, and resuspended to ensure a lumenal concentration of 4 mM K. The vesicles were incubated with DISC(3)-5 (2.5 μM final after dilution) for 15 min. The T-Tubules were then diluted into different hyperpolarizing solutions (1:5–1:20), the fluorescence allowed to stabilize, and then valinomycin (63 μM final) added. When vesicles were incubated in the 4 mM K Load buffer and then diluted into a 1:5 hyperpolarizing solution the extravesicular K concentration was effectively changed to 0.020 M, while for a 1:20 dilution the extravesicular K concentration became 0.080 M. The log values for these concentrations were plotted on the bottom x-axis. The Nernst potential for K, assuming a lumenal [K] of 4 mM, was calculated and plotted on the top x-axis. The hyperpolarizing dilution's percentage fluorescent change relative to the control (nonhyperpolarizing) dilution was calculated and plotted on the y-axis. The points were fit with a regression line which was expected to intercept the x-axis at a log [K] value of –2.39, the log value for a lumenal [K] of 0.004 M. The regression line intercepted the x-axis at –2.18 (or about 6.6 mM lumenal K) with a slope of 0.151 mV^{-1}.

By looking at the x-intercepts of the regressions under both depolarizing and hyperpolarizing conditions, we see that the lumenal concentration of K is extremely close to either 100 mM under depolarizing conditions or 4 mM K under hyperpolarizing conditions. This leads us

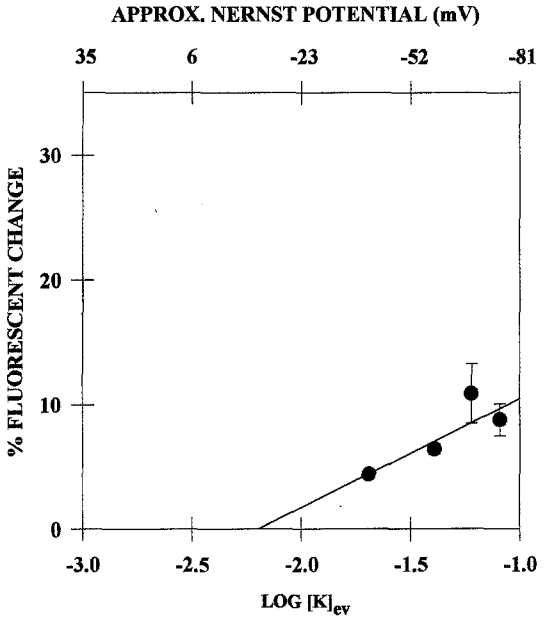

Fig. 5. Correlation of DiSC(3)-5 fluorescence changes (increase) with changes in extravesicular K concentrations upon hyperpolarization. Isolated transverse tubule vesicles were incubated in Load buffer C (for dilutions greater than 1:5) or buffer D (for a 1:5 dilution) with DiSC(3)-5 for 15 min. The vesicles were diluted into different hyperpolarizing solutions (*see* Table 1) that contained 63 μM valinomycin. The fluorescence of DiSC(3)-5 was monitored at an excitation wavelength of 647 nm and an emission wavelength of 670 nm. For each experiment, vesicles were diluted into a control nonhyperpolarizing solution as well as a hyperpolarizing solution. The different response in these two solutions was used to calculate the percent fluorescence change (relative to the control) and was plotted against the log of the extravesicular concentration of K. The Nernst potential was calculated for the change of extravesicular K (upper x-axis), assuming a lumenal [K] of 4 mM. Each data point represents the mean of 2–3 values ± SD. The data were fit with a linear regression line ($r^2 = 0.6$) that had a slope of -0.151 mV^{-1} and an x-intercept of approximately -2.18 (log of estimated lumenal [K], or approximately 6.6 mM K).

to believe that the incubation time of 1 hr in the Load solutions is sufficient for equilibration of K across the membrane of the transverse tubule vesicle. If equilibration was not occurring in this time, the x-intercepts would not be so close to the predicted values (−1.0, −2.39).

Role of Transverse Tubules in Depolarization-induced Calcium Release

The next set of experiments was designed to determine whether calcium release from triad vesicles was due to the depolarizing solutions acting on the calcium release channel or the transverse tubule component that contains the DHP receptor. To accomplish this, two separate populations of vesicles were isolated. The first was composed of triadic and terminal cisternae vesicles that banded at 39% on a continuous sucrose gradient: ^{3}H-nitrendipine-specific activity was 4.85 pmol/mg and ^{3}H-ryanodine specific activity was 6.93 pmol/mg. A second population of vesicles, primarily terminal cisternae, was obtained from a 41% band on the sucrose gradient. Its ^{3}H-nitrendipine specific activity was lower, 1.5 pmol/mg, indicating a lower percentage (approximately one-third) of transverse tubules, or triadic vesicles. ^{3}H-ryanodine-specific activity was approximately the same, 5.91 pmol/mg, indicating a similar population of terminal cisternae.

The triad vesicles were incubated in a Load A buffer (*see* Table 1) that contained 10 μM Fera-2 and an ATP-regenerating system. 40 μM additions of calcium were added to 900 μl of the vesicle mixture. Uptake was monitored on an SLM 8000 spectrofluorimeter (*not shown*). After making 4–6 calcium additions, a baseline Fura-2 fluorescence ratio (340/380 nm) was monitored (Fig. 6*A*, "load"). The load cuvette was removed and replaced by a cuvette that contained 760 μl of 1:20 release solution (*see* Table 2). A baseline fluorescence ratio was recorded and 40 μl of the loaded vesicles were mixed into the release solution. Calcium release from the triad vesicles was measured by an increase in the Fura-2 fluorescence ratio which, with manual mixing, displayed an initial fast phase that lasted approximately 10 sec followed by a slow phase of release (Fig. 6*A;* Release 1,3). Unloaded vesicles were added to a separate Release cuvette and the Fura-2 fluorescence showed a small slow increase that bore little resemblance to the biphasic release seen in Rel 1 and 3 (Fig. 6*A;* Release 2).

In Fig. 6*B*, terminal cisternae vesicles with a low amount of triads were prepared as described above and diluted into a similar 1:20 release solution (Fig. 6*B;* Release 1,3). In this case, the calcium release displayed the biphasic nature as seen before, but the amplitude of the initial fast phase of release was greatly reduced. As a result of these experiments, we concluded that the depolarizing release solutions act on the transverse tubule element and not directly on the calcium release channel. If the solutions did act directly on the isolated terminal cisternae, one would have expected to see similar calcium release from both vesicle populations.

The Voltage-dependence of Depolarization-induced Calcium Release

The voltage dependence of depolarization-induced calcium release from skeletal muscle triadic vesicles was examined using a previously described fluorescence assay (Corbett et al., 1992). Depolarization was accomplished by diluting the vesicles into different release solutions (*see* Table 2). If calcium release from the triads was voltage dependent, one would expect to observe: (i) some threshold depolarization at which calcium begins

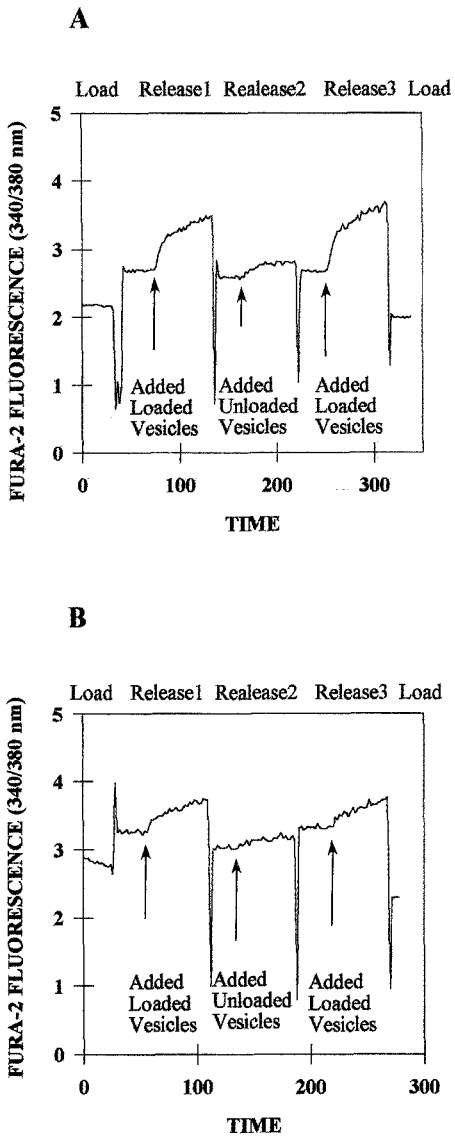

Fig. 6. Depolarization-induced calcium release in triads *vs.* terminal cisternae. (*A*) triad/terminal cisternae vesicles,with a ^{3}H-nitrendipine specific activity of 4.85 pmol/mg (transverse tubule marker) and a ^{3}H-ryanodine specific activity of 6.93 pmol/mg (terminal cisternae marker), were incubated in Load buffer A with Fura-2 (10 μM) and an ATP-regenerating system and loaded with five 36 μM additions of $CaCl_2$. The baseline fluorescence of the loaded triads were monitored at excitation wavelengths 340 and 380 nm (ratio of 340/380 nm plotted) before and after releases 1, 2, 3 on an SLM spectrofluorimeter using a 100 Hz chopper. Release 1 and 3 show the changes in the fluorescence ratio (340/380 nm) when calcium-loaded triad vesicles were diluted (1:20) into a depolarizing solution (approximately 75.5 mV potential change). Release 2 shows the fluorescence response when unloaded triad vesicles were diluted (1:20) into the depolarizing solution. (*B*) The same experiments were repeated but terminal cisternae were used (^{3}H-nitrendipine specific activity of 1.5 pmol/mg and a ^{3}H-ryanodine specific activity of 5.91 pmol/mg). The experimental protocol for Release 1 through 3 was the same as that used in *A*. The same amount of protein and calcium loading was used in each experiment (*A vs. B*): a reduced amplitude of fast phase of calcium release, or depolarization-induced calcium release, was apparent in the experiments which used terminal cisternae.

to be released, (ii) some depolarization at which maximal calcium release is achieved, and (iii) a graded response in between the threshold and maximal response potentials.

In Fig. 7*A*, triad vesicles were prepared from rabbit skeletal muscle as described in Materials and Methods and incubated (0.5 mg/ml) in Load buffer A. The terminal cisternae of the vesicles were loaded with calcium as described previously. After the fluorescence ratio of the loading vesicles acquired a steady baseline (approximately 2 or 3 for 340/380 nm Fura fluorescence), the vesicles were diluted into various release solutions (Table 2). The depolarized vesicles released calcium causing the Fura-2 fluorescence ratio to increase. The fluorescence response was calibrated, and the percentage of calcium release calculated (*see* Materials and Methods). The maximum percentage calcium release varied from preparation to preparation, so we normalized the percentage release values by setting the maximum release to equal "one" and the nondepolarizing release to equal "zero." All of the other percentage releases were expressed as values between zero and one. The mean percentage calcium release for each dilution was plotted against the calculated potential change obtained from the Nernst equation. All calcium release experiments were performed in the presence of an ATP regenerating solution, which activates the Na/K ATPase in the transverse tubule membrane, making it impossible to accurately estimate the concentration of K^+ inside the transverse tubule. Therefore, we could not calculate the true membrane potential of the T-tubule membrane potential after dilution using the Nernst equation. However we could use the Nernst equation to calculate the change in membrane potential after dilution into the release solution: this was determined from the ratio of extravesicular $[K]^+$ before and after dilution. These values are plotted on the x-axis. As the dilution of the vesicles was increased from 1:5 to 1:25 the percentage of calcium released from the triads also increased obtaining a maximum at about a 1:20 dilution (75 mV potential change). The range between the minimum and maximum percentage release was approximately 35–40 mV. The data points were fit very well with a Boltzmann function, yielding a slope factor of 4.5 ± 1.5 mV per e-fold change and a $V_{0.5}$ value of 51.3 ± 1.8 mV potential change.

A similar set of experiments was performed using release solutions which contained 63 μM valinomycin to see if the voltage-dependent activation curve would be altered. In Fig. 7*B*, the percentage of calcium releases were calculated and normalized as described above. The data points were fit with a Boltzmann function and a slope factor of 5.3 ± 1.4 mV per e-fold change and a $V_{0.5}$ value of 55.6 ± 1.7 mV potential change were obtained.

In Fig. 8, skeletal muscle triad vesicles were incubated in Load buffer A that contained KMES instead of K propionate. The triads were diluted into TEACl depolarizing solutions that contained NMG-Mes (Table 2).

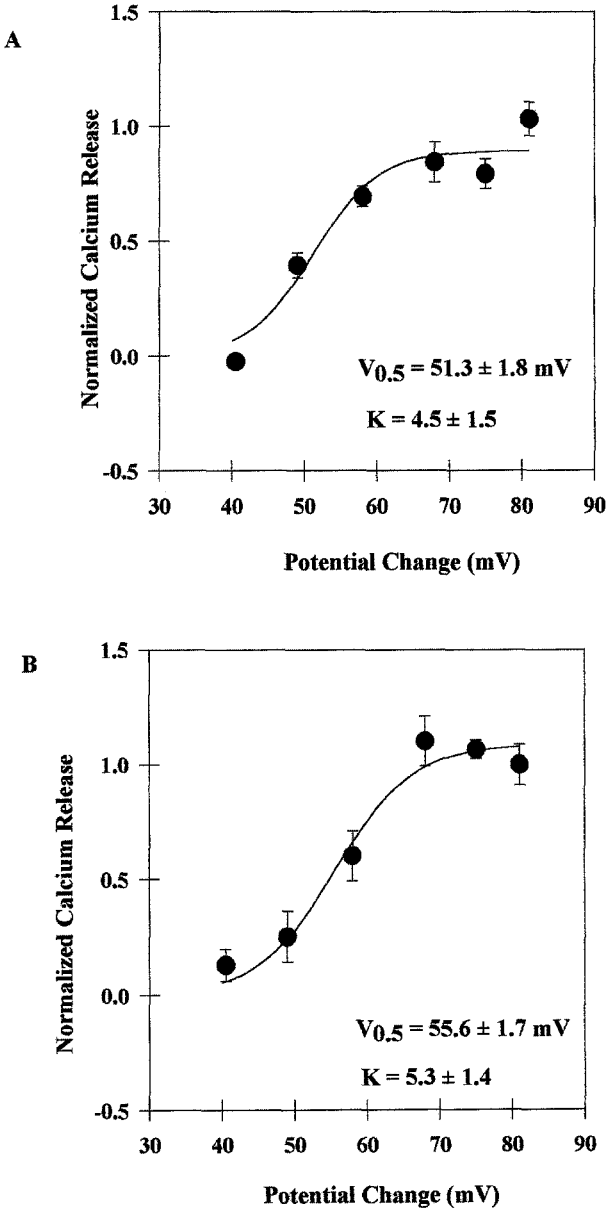

Fig. 7. The voltage dependence of depolarization-induced calcium release in isolated triads using propionate as the impermeant anion. (*A*) Triad vesicles were incubated in Load A (for dilutions greater than 1:5) or (*B*) (for a 1:5 dilution) with an ATP-regenerating system (Materials and Methods) and 10 μM Fura-2. The vesicles were loaded with calcium (five 36 μM additions) and diluted (1:5–1:25) into different depolarizing solutions. The changes in the Fura-2 fluorescence ratio (340/380 nm) were monitored on an SLM spectrofluorimeter with a 100 Hz chopper (EM = 510 nm). The percentage of total calcium loaded that was released upon depolarization was calculated (8.1% release; 19 nmoles Ca^{++}/mg protein) and normalized by setting the maximal release equal to 1 and the nondepolarizing release equal to 0. The normalized releases were plotted against the Nernst potential change calculated for the change in extravesicular K upon dilution and all data points were fit with a Boltzmann function (Eq. 5). Each data point represents the mean of 3–7 values ± SEM. The parameters of the fit were $V_{0.5}$ = 51.3 ± 1.8 mV potential change and k = 4.5 ± 1.5 mV. (*B*) The experiments described in (*A*) were repeated with 63 μM valinomycin present in the depolarizing release solutions. Each data point represents the mean of 2–6 values ± SEM. The maximal calcium release obtained with depolarization was 7.8% or 17.6 nmoles Ca^{++}/mg. The normalized releases were fit with a Boltzmann function yielding similar parameters ($V_{0.5}$ = 55.6 ± 1.7 mV potential change and k = 5.3 ± 1.4 mV).

The percentage of calcium released was calculated and normalized for each dilution. The percentage of calcium released was plotted against the calculated potential change (using Nernst equation: *see above*) and fit with a Boltzmann function. The *k* factor was calculated to be 5.7 ± 1.2 mV per e-fold change and the $V_{0.5}$ was calculated to be 57.9 ± 1.0 mV potential change.

Discussion

The Voltage-dependence of Depolarization-induced Calcium Release

To prove that we were indeed depolarizing the vesicles in question with our solution changes, we examined changes in membrane potential elicited by identical solution changes using isolated transverse tubules and the voltage-sensitive fluorescent dye DiSC(3)-5. Studies previously performed by Dunn (1989) used this voltage-sensitive dye to demonstrate that isolated T-tubule vesicles could generate and maintain membrane potentials in response to K^+ gradients established across the membrane in the presence of valinomycin. In these studies, vesicles incubated in 150 mM K gluconate were diluted into a solution containing 145 mM choline chloride and 5 mM K gluconate to induce depolarization, a fixed amount of the DiSC(3)-5 dye was added and fluorescence recorded at excitation wavelength of 622 and emission wavelength of 670. Following a brief incubation, 0.1 μM valinomycin was added, and fluorescence changes were again recorded. The fluorescence changes induced by the depolarizing solution were compared with control nondepolarizing response and were presented as a change in fluorescence (% control) *vs.* the log of extravesicular K concentration. Fluorescence changes varied linearly with extravesicular K concentrations for both depolarizing and hyperpolarizing conditions in this study, although absolute slopes were not given.

In our studies, we used K propionate solutions to incubate the transverse tubules and depolarized them by diluting them into a solution containing TEACl: all of our solutions were balanced so that [K][Cl] products were equal in both the incubation and depolarizing or hyperpolarizing solution. Although the solutions used in Dunn (1989) were iso-osmotic, the KCl product was not maintained upon dilution, possibly resulting in osmotic changes across the transverse tubule membrane. We also found that under our ionic conditions, the maximal fluorescence difference upon depolarization occurred at 647

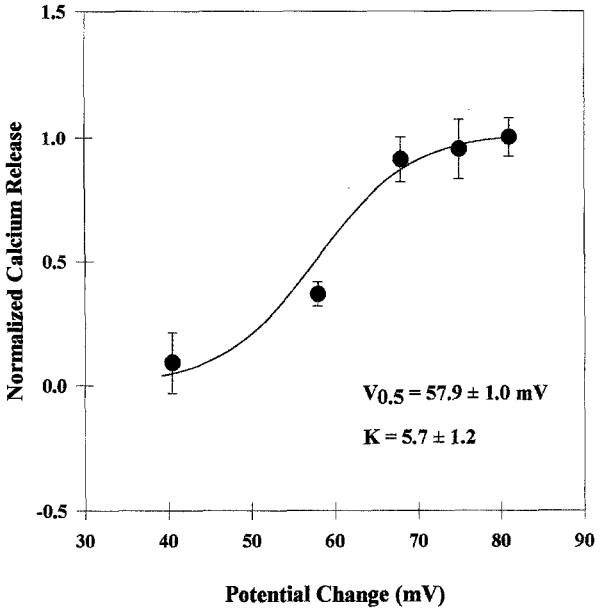

Fig. 8. The voltage dependence of depolarization-induced calcium release in isolated triads using methanesulfonate as the impermeant anion. Isolated triad vesicles were incubated in Load buffer A (for dilutions greater than 1:5) or B (for a 1:5 dilution) with K methanesulfonate used in place of K propionate. The load solutions contained 10 μM Fura-2 and an ATP-regenerating system (*see* Materials and Methods). The vesicles were loaded with five 36 μM additions of $CaCl_2$ and then were diluted (1:5–1:25) into different depolarizing solutions (Table 1) that contained NMG methanesulfonate instead of NMG propionate. The changes in the Fura-2 fluorescence ratio (340/380 nm) were monitored on an SLM spectrofluorimeter using a 100 Hz chopper (EM = 510 nm). The percentage of total calcium loaded which was released upon depolarization was calculated (5.7%) and normalized by setting the maximum release equal to 1 and the nondepolarizing release equal to 0. Each data point represents the mean of 4–10 values ± SEM. The points were fit with a Boltzmann function (Eq. 4) yielding the following parameters: $V_{0.5}$ = 57.9 ± 1.0 mV potential change and k = 5.7 ± 1.2 mV.

nm excitation wavelength while holding emission at 670 nm (*see* Fig. 3). Since our excitation wavelength was very close to our emission wavelength, we performed a control with transverse tubules and no dye to assure that light scattering signal from the vesicles did not significantly contribute to our measurement at 647 nm. We found that a constant protein:dye ratio was essential at each depolarization or hyperpolarization change for reproducible results (all depolarizations and hyperpolarizations had a constant final concentration of protein and dye). We also found that an incubation of the vesicles with DiSC(3)-5 for 10–15 min prior to dilution into control (nondepolarizing) or depolarizing solutions was essential for reproducibility: addition of the dye after dilution produced extreme variations in the response. We used a higher concentration of valinomycin (63 μM) in our experiments to ensure optimal effects: lowering the concentration to 30 μM had no effect on our responses. Like Dunn (1989), our fluorescence change varied linearly with the amount of extravesicular K in both the depolarizing (slope of -0.281 mV^{-1}) and hyperpolarizing experiments (slope of 0.151 mV^{-1}). It was surprising that the magnitude of the slopes obtained under depolarizing and hyperpolarizing conditions were not equal: this may have been due to different ionic solution used to achieve these effects or the fact that a small percentage of our dye appeared to be associated with the membrane (approximately 30–40% based on maximal changes in fluorescence). Dunn (1989) found that fluorescence changes in the hyperpolarized direction dissipated more rapidly than those established in the depolarizing direction, with a half time of approximately 10 min rather than 1–2 hr, suggesting fundamental differences in the fluorescence response.

When we depolarized triadic vesicles, we incubated the vesicles in 100 mM K propionate, 2 mM $MgCl_2$ and then added an ATP-regenerating system to initiate transverse tubule repolarization through activation of the Na-K ATPase. We used identical solutions in our experiments with isolated transverse tubules and DiSC(3)-5, except the ATP-regenerating system was absent. In order to calibrate our response with isolated transverse tubules, it was necessary to eliminate the ATP-regenerating system so that we could assume that the lumenal K concentration was equivalent to the incubation solution: this allowed us to determine the corresponding membrane potential upon depolarization using the lumenal and extravesicular K concentrations in the Nernst equation. It was therefore essential that we repeat the above experiments with isolated transverse tubules in the presence of the same ATP-regenerating system used in our triad experiments. In these experiments, the potential change was calculated by using the ratio of the extravesicular K concentration in the two solutions (incubation or loading versus depolarizing) in the Nernst equation. In experiments, we determined that depolarization in the presence of the ATP-regenerating system (linear relationship with a slope of -0.315 mV^{-1}) was very similar to that in its absence (slope of -0.281 mV^{-1}).

Ideally, one would like to measure potential changes in the transverse tubules of isolated triads. However, voltage-sensitive dyes such as DiSC(3)-5 are able to translocate across all membranes, so addition of vesicles other than transverse tubules lowers the signal-to-noise ratio. This was confirmed for us in some experiments in which some triadic vesicles were used with the voltage-sensitive dye: little or no response was seen upon depolarization. This suggests indirectly that the contaminating terminal cisternae or longitudinal reticulum are not able to be depolarized by these solution changes. This was confirmed in a study by Ikemoto et al. (1992) which looked at uptake of ^{14}C-SCN into T-tubules, triads, and SR vesicles in different depolarized states. The signal difference between polarized and depolarized vesicles was smaller (approximately half) in triads *vs.* isolated

Table 3. Comparison of slope factors (voltage dependence) of depolarization-induced calcium release in different skeletal muscle preparations

Reference	Temp. °C	k (mV)
A. Frog intact fibers		
Miledi et al. (1981)	5.5–7	3.6
Baylor et al. (1983)	15–17	2.9
Baylor et al. (1983)	15–17	3.3
Baylor et al. (1979)		3–4
B. Frog cut fibers		
Klein et al. (1992)	6–10	10
Szücx at al. (1984)	5–8	12
Melzer et al. (1986)	5–10	11.4
C. Rat cut fibers		
Delbono and Stefani (1993)	17	11
D. Rabbit vesicles		
Kramer and Corbett		
Propionate[b]	r.t.[a]	4.5
Prop. + Val.[b]	r.t.[a]	5.3
KMES[b]	r.t.[a]	5.7

[a] r.t. = 22–25°C

[b] Values for rabbit vesicles refer to Figs. 7 and 8 in this paper.

transverse tubules and no signal change was observed in SR vesicles.

In previous studies using intact (Miledi et al., 1981; Baylor et al., 1978, 1983) and cut (Szücs et al., 1984; Melzer et al., 1986; Klein et al., 1992) skeletal muscle fibers from frog and cut skeletal muscle fibers from rat (Delbono & Stefani, 1993) both calcium release and charge movement showed a steep voltage dependence, with k values of 2.9–12 mV for each e-fold change (*see* Table 3). In order for triadic vesicles to be considered physiological, they must display a similar voltage dependence upon T-tubule depolarization, they must display a threshold voltage at which calcium release begins, a region of steep voltage-dependent calcium release, and a level of depolarization at which maximal calcium release is obtained. In studies using intact or cut muscle fibers, the rate of calcium release is usually plotted against voltage to obtain the voltage-dependent curve. This basically provides an estimate of the probability that the calcium release channel is open at given voltages: as a larger percentage of total channels open, the rate of release increases until a maximal response is obtained when all functioning channels are open. In our studies with isolated triadic vesicles, we had to use a number of different dilutions to obtain the varying levels of depolarization (*see* Table 1). In all of our studies, manual mixing was used to perform the dilutions: current-stopped flow devices are not capable of obtaining all of the ratios we needed to test. Therefore, the rate of release in our experiments with triadic vesicles simply reflected the speed of mixing (time constant of approximately 2 sec) rather than the physiological rate of calcium release from our vesicles (J.W. Kramer and A.M. Corbett, *in press*). In our studies with triadic vesicles, the percentage of total calcium loaded which was released at a given depolarization was taken to be equivalent to the probability of the calcium release channel opening (P_o) in response to given voltage changes. As might be expected, different triad preparations displayed different maximal levels of calcium release. It was essential that we normalize these values in order to compare the voltage dependence of calcium release in different preparations. This was done by assuming that release into a nondepolarizing solution represented minimal release (or $P_o = 0$) and setting maximal percentage of calcium release equal to 1 (reflecting all functional calcium release channels in triads open).

In four different triad preparations, we were able to obtain full voltage-dependent calcium release curves in the absence of valinomycin: all of these curves showed a threshold response at the 1:5 dilution (40.5 mV potential change), k values which ranged from 4.5 to 8.1 mV for each e-fold change, and $V_{0.5}$ values which ranged from 51.2 to 56.1 mV potential change. The maximal percentage of release ranged from 3.2 to 8.1% with a mean of 5.2%: we found that the maximal percentage of release varied directly with ^{3}H-nitrendipine or ^{3}H-PN200-110 specific activity (pmoles binding/mg protein) of the triad preparation. Once the maximal percentage of release was normalized and plotted against calculated potential changes, however, the values obtained from the Boltzmann fit of the data were remarkably consistent from preparation to preparation. In our studies with voltage-sensitive dyes and isolated transverse tubules, full depolarization (as determined by dye quench) was achieved only after addition of valinomycin to the vesicles. Valinomycin enhances the K conductance across the T-tubule membrane, thus rendering the opposing Cl conductance insignificant when calculating potential changes with the Nernst equation. Although valinomycin had a significant effect on the voltage-sensitive dye quench, there was little effect on the voltage-dependence of depolarization-induced calcium release in isolated triads ($k = 5.3$ and $V_{0.5} = 55.6$). This would suggest that Cl conductance contributes little to the generation of membrane potentials across the transverse tubules in triadic vesicles under our conditions. There has also been evidence (Stephenson, 1985) that propionate is semipermeable to the frog skeletal muscle membranes, having approximately 2–3 times the permeability of methanesulfonate and approximately one-eighth the permeability of Cl. The ratios of propionate, Cl and methanesulfonate permeabilities in isolated transverse tubules from rabbit skeletal muscle, however, are uncertain. To ensure that we were indeed obtaining optimal depolarizations based on our extravesicular K concentrations, we changed our impermeant anion to methanesulfonate in a series of experiments. We found very little difference in the voltage

dependence of calcium release upon depolarization when methanesulfonate replaced propionate as the impermeant anion ($k = 5.7$ and $V_{0.5} = 57.9$).

Role of the Transverse Tubule in Depolarization-induced Calcium Release

In voltage-clamped experiments with intact or cut fibers, depolarization of the external membrane, specifically the transverse tubules (Schneider & Chandler, 1973; Ríos & Brum, 1987) is critical for the initiation of depolarization-induced calcium release. In skinned fibers experiments, it was shown that depolarizing the transverse tubule with either ouabain or digoxin prior to the test depolarization (induced by solution changes similar to those used in the experiments shown here) abolished calcium release (Donaldson, 1985; Volpe & Stephenson, 1986). These experiments targeted the Na-K ATPase in the transverse tubules and clearly showed that a repolarized transverse tubule is essential for the initiation of depolarization-induced calcium release in skinned fibers. In isolated skeletal muscle vesicles, it was demonstrated that dihydropyridine receptor antagonists (Ohkusa et al., 1991) inhibited the slow phase but not the fast phase of depolarization-induced calcium release. In these initial experiments, a 1:1 dilution, which would be expected to produce a small depolarization was used. Similar experiments, using a monoclonal antibody to the alpha$_1$-subunit of the dihydropyridine receptor, also reduced the slow phase of depolarization-induced calcium release in isolated triads (Ohkusa, Smilowitz & Ikemoto, 1990). In our experiments, we have evaluated the role the transverse tubule in depolarization-induced calcium release by comparing triad preparations (high ^{3}H-nitrendipine or ^{3}H-PN200-110 specific activity, 5–10 pmol/mg ^{3}H-ryanodine specific activity) with terminal cisternae (little ^{3}H-nitrendipine or ^{3}H-PN200-110 specific activity, 5–10 pmol/mg ^{3}H-ryanodine specific activity). In these experiments, it was critical that the relative number of ^{3}H-ryanodine binding sites (determined by the ryanodine specific activity and amount of protein) be as close as possible in the two preparations (triads versus terminal cisternae), with only the number of dihydropyridine binding sites changing. The amount of protein used in each experiment was identical and vesicle types were loaded to the same degree. When there were very few triads in the preparation (indicated by low ^{3}H-PN200-110 specific activity), the amplitude of the fast phase of release was reduced. This experiment clearly demonstrates that transverse tubules are essential for depolarization-induced calcium release in isolated triads. As our triads preparations have improved (greater ^{3}H-PN200-110 specific activity) over the years, we have found that the maximal percentage of calcium release achieved upon depolarization has increased dramatically from an original 3% to our present release of 16%.

In conclusion, we have demonstrated for the first time that depolarization-induced calcium release in isolated triadic vesicles, derived from rabbit skeletal muscle, displays voltage-dependent properties similar to that seen in intact muscle. This work firmly establishes triadic vesicles as a physiological model for skeletal muscle which may be used to examine the molecular mechanism of signal transduction in excitation-contraction coupling.

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