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Sarcoplasmic Reticulum Ca^{2+} Release in Rat Slow- and Fast-Twitch Muscles

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Abstract. The same isoform of ryanodine receptor (RYR1) is expressed in both fast and slow mammalian skeletal muscles. However, differences in contractile activation and calcium release kinetics in intact and skinned fibers have been reported. In this work, intracellular Ca^{2+} transients were measured in *soleus* and *extensor digitorum longus* (EDL) single muscle fibers using mag-fura-2 (K_D for Ca^{2+} = 49 μM) as Ca^{2+} fluorescent indicator. Fibers were voltage-clamped at V_h = −90 mV and sarcoplasmic reticulum calcium release was measured at the peak (*a*) and at the end (*b*) of 200 msec pulses at +10 mV. Values of *a-b* and *b* were assumed to correspond to Ca^{2+}-gated and voltage-gated Ca^{2+} release, respectively. Ratios (*b/a-b*) in *soleus* and EDL fibers were 0.41 ± 0.05 and 1.01 ± 0.13 (n = 12), respectively. This result suggested that the proportion of dihydropyridine receptor (DHPR)-linked and unlinked RYRs is different in *soleus* and EDL muscle. The number of DHPR and RYR were determined by measuring high-affinity [^{3}H]PN200-110 and [^{3}H]ryanodine binding in *soleus* and EDL rat muscle homogenates. The B_{max} values corresponded to a PN200-110/ryanodine binding ratio of 0.34 ± 0.05 and 0.92 ± 0.11 for *soleus* and EDL muscles (n = 4–8), respectively. These data suggest that *soleus* muscle has a larger calcium-gated calcium release component and a larger proportion of DHPR-unlinked RYRs.

Key words: Skeletal muscle — Sarcoplasmic reticulum — Calcium release — Soleus — Slow-twitch muscle — Dihydropyridine receptor — Ryanodine receptor

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Introduction

Sarcolemmal depolarization in skeletal muscle results in sarcoplasmic reticulum (SR) Ca^{2+} release into the myoplasm. The transduction of the sarcolemmal voltage signal into an intracellular calcium response is mediated by the voltage-sensor/dihydropyridine receptor (DHPR) which activates the SR Ca^{2+} release channel/ryanodine receptor (RYR) by a mechanism not well understood. Ultrastructural and ligand binding studies support a direct linkage of four DHPRs to some but not all RYRs. The existence of uncoupled receptors has suggested that the skeletal muscle RYR is activated basically by two mechanisms, (i) a voltage-dependent mechanism in which DHPR transduces sarcolemmal voltage into SR Ca^{2+} release through a hypothetical mechanical interaction or (ii) by a Ca^{2+}-dependent process (Franzini-Armstrong, 1994; Meissner, 1994; Schneider, 1994; Melzer, Herrmann-Frank & Lüttgau, 1995). The reuptake of Ca^{2+} by SR is mediated by a Ca^{2+}-transporting ATPase, which causes muscle to relax by restoring the intracellular Ca^{2+} concentration from ~10^{-6}–10^{-5} M to ~10^{-7} M (Inesi et al., 1990).

SR Ca^{2+} release and myoplasmic Ca^{2+} removal are considerably slower in *soleus* than EDL fibers. Differences in muscle relaxation and calcium binding protein: Ca^{2+} and SR calcium pump: Ca^{2+} kinetics between fast- and slow-twitch muscles have been reported (Robertson, Johnson & Potter, 1981; Gillis et al., 1982; Heizmann, Berchtold & Rowlerson, 1982; Leberer & Pette, 1986; Klein et al., 1991).

SR Ca^{2+} release in intact or cut muscle fibers has been investigated mainly in amphibian (Melzer et al., 1995) and much less in mammals. More recently, the regulation of SR calcium release in mammals was explored in fast-twitch rat muscle fibers (*extensor digi-*

torum longus, EDL) (Delbono & Stefani, 1993; García & Schneider, 1993; Delbono, 1995; Shirokova, Garcia, Pizarro & Rios, 1996). By comparison, information on the kinetics of SR Ca^{2+} release in slow-twitch (type I) fibers is sparse. Differences in intracellular Ca^{2+} transients kinetics and sensitivity of SR Ca^{2+} release to caffeine in *soleus* and EDL muscles have been reported (Eusebi, Miledi & Takashi, 1985; Fryer & Neering, 1989). Differences in SR Ca^{2+} kinetics in both skinned fibers (Salviati & Volpe, 1988) and isolated membrane fractions (Lee et al., 1991) were also observed, even though it is known that the same isoform of the RYR (RYR1) is expressed in both fast- and slow-twitch mammalian skeletal muscles.

In this study, we analyzed intracellular Ca^{2+} transients and measured the number of DHPR and RYR binding sites in muscle homogenates of *soleus* and EDL muscles, to investigate the mechanism(s) involved in triggering intracellular Ca^{2+} release in mammalian muscles. These results lead us to propose that rat *soleus* muscle has a larger proportion of DHPR-unlinked RYRs and a larger SR calcium-gated Ca^{2+} release component than fast-twitch muscles.

Materials and Methods

Fiber Preparation

Adult Wistar rats of an average weight of 200 g were used. *Soleus* and EDL muscles were dissected after sacrificing the rat in a carbon dioxide chamber. For biochemical studies, muscles were immediately frozen and stored at −135°C. For intracellular Ca^{2+} measurements, muscles were placed in a modified Krebs solution (*see below*). Fibers were isolated within 3 hr of muscle dissection. In most of the experiments, fibers were stretched to 3.6–3.8 μm sarcomere length to avoid contraction artifacts during fluorescent measurements.

Solutions

Modified Krebs solution (mM): 145 NaCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgSO_4$, 10 HEPES-Na, 10 glucose. The external solution contained (mM): 150 TEA (tetraethylammonium)-$MeSO_3$ (methanesulphonic acid), 2 $CaCl_2$, 2 $MgCl_2$, 2 TEA-HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) and 0.001 TTX (tetrodotoxin), 1 9 anthracenecarboxylic acid, and 1 3-4 diaminopyridine. The internal solution contained (mM): 98 K-glutamate; 0.1 K_2-EGTA; 0.0082 $CaCl_2$; 5 Na_2ATP; 5.5 $MgCl_2$; 5 glucose; 5 K-HEPES; 5 Na_2-phosphocreatine. The osmolarity of the solutions was 300 mOsm and the pH was 7.2. Mag-fura-2 (Molecular Probes, Eugene, OR) was added from 5 mM stock solutions (in deionized water) to final concentrations of 400 μM. Free Ca^{2+} concentration in solutions were calculated according to Fabiato (1988) using the following equation: $[\text{Free } Ca^{2+}] = K_D\text{EGTA} \cdot [Ca^{2+}\text{EGTA}]/[\text{EGTA}]$. The K_D for mag-fura-2 was 49 μM (Delbono et al., 1993).

Setup

Fibers were voltage-clamped using the double Vaseline gap technique (Kovacs, Ríos & Schneider, 1983; Delbono et al., 1991) at a holding potential (V_h) of −90 mV. Temperature was monitored with a thermistor probe positioned close to the fiber in the middle pool. For fluorescent recordings, the fiber was epi-illuminated with a 75-W xenon lamp. The UV beam passed through 340, 350 or 380 nm excitation wavelength filters, with 10-nm bandwidth (Omega Optical, Brattleboro, VT) mounted in a filter-wheel (Ludl, Hawthorne, NY). Switching from one to another excitation filter was performed by computer control of the filter-wheel. The UV light was reflected downward by a long pass dichroic mirror centered at 400 nm (DC 400 LP, Omega Optical, Brattleboro, VT) and at 45° angle. The light beam was focused onto the fiber with a 100× (N.A. 1.3) fluar objective (Zeiss). After illuminating the fiber, the upward emitted light beam was collected by the objective and passed through a dichroic mirror and an emission filter centered at 510 nm with 40 nm bandwidth. Light intensity was determined with a photodiode (UDT-455UV, UDT Sensors, Hawthorne, CA). The photodiode current was recorded with a current-to-voltage converter with 1 gigaohm feedback resistance to improve the signal-to-noise ratio. The fiber was periodically illuminated during stimulation using a computer-controlled shutter mounted in a filter wheel. All the records were corrected for photobleaching using control records without fiber stimulation.

Stimulation, Recording and Data Analysis

An IBM compatible personal computer was used. D-A and A-D conversions, were done by a Labmaster Computer Interface (Axon Instruments, Foster City, CA). Stimulation protocols are detailed in Results for each group of experiments. Fluorescent signals were filtered at 0.3 of the sampling frequency (−3 Db point) with a 4-pole Butterworth low-pass filter (Frequency Devices, Haverhill, MA). Fluorescence signals were normalized to basal dye fluorescence in resting conditions and transformed into free Ca^{2+} concentration. All the experiments were carried out at 20–22°C. Values are means ± SEM and number of observations (*n*).

In Vivo Calibration

Intracellular Ca^{2+} concentrations were calculated with the equation: $[Ca^{2+}] = K_D\,(R - R_{min})/(R_{max} - R)$ (Grynkiewicz, Poenie & Tsien, 1985) where K_D is the dissociation constant of the Ca^{2+}-dye reaction, $R = F_{380}/F_{350}$, R_{min} is the R value at 0% saturation of the dye by Ca^{2+} and R_{max} is the R value at 100% saturation. R_{max} was determined in the fiber with the following procedure. Fibers were isolated in the dissecting solution and placed in the recording chamber that contained the mounting solution with 10 mM glutaraldehyde grade II (Sigma Chemical, St. Louis, MO) (Delbono & Stefani, 1993). After the Vaseline seals were built, the solution in the central pool was replaced by the external solution plus the same glutaraldehyde concentration, and the solution in the lateral pools by the internal solution plus 400 μM mag-fura-2. Changes in basal fluorescence (350 and 380 nm) were determined every 5 min. After 40 min, when the dye reached about 80% of the concentration in the lateral pools, the external solution was substituted with modified Krebs solution plus 1 μM ionomycin (Calbiochem, La Jolla, CA) and 10 mM glutaraldehyde. With this procedure, Ca^{2+} entered into the cell in sufficient quantity to saturate mag-fura-2 without causing mechanical movement and/or disruption of the cellular architecture. This resulted in a decrease of the basal fluorescence at 380 nm wavelength. The fluorescence measured at the isosbestic point was not modified. R_{max} was calculated from the minimum fluorescence values after loading the cell with Ca^{2+}. R_{max} was also determined after metabolic poisoning of the muscle cell following procedures described (Delbono et al., 1993). Five μM carbonyl cyanide m-

chlorophenylhydrazone (CCCP) (Sigma Chemical) and 2 μM rotenone (Sigma Chemical) were used to avoid fiber movement. Both procedures gave consistent results for R_{max} determinations (0.35 ± 0.08, $n = 8$).

R_{min} was determined in the presence of 10 mM EGTA and 400 μM mag-fura-2. In these conditions, neither the replacement of the external solution with a higher Ca^{2+} concentration, nor depolarizing pulses under voltage clamp conditions further reduced the basal fluorescence. This indicates the adequacy of intracellular Ca^{2+} chelation. The R_{min} value from 8 determinations was 2.66 ± 0.17.

Calculation of SR Ca^{2+} Release

SR Ca^{2+} release throughout fiber stimulation was calculated according to Melzer, Ríos & Schneider (1987). The adequacy of the application of this algorithm to rat skeletal muscle fibers has been discussed recently (Delbono, O'Rourke & Ettinger, 1995; Shirokova et al., 1996). This method obtains the rate of Ca^{2+} release from $[d\mathrm{Ca}^{2+}/dt]$ = input-output flux. The input flux corresponds to the SR Ca^{2+} release, while the output flux corresponds to the activity of the Ca^{2+} removal system. As SR Ca^{2+} release terminates shortly after the end of the pulse (Delbono et al., 1993), the Ca^{2+} removal function can be obtained by fitting the decaying phase of the Ca^{2+} transient to the following differential equation: $d[\mathrm{Ca}^{2+}]/dt = -(d[\mathrm{CaTN}]/dt) - (d[\mathrm{CaPARV}]/dt) - (MF^2) - (d[\mathrm{Ca}P]/dt) - (d[\mathrm{Ca}D]/dt) - (d[\mathrm{CaEGTA}]/dt) + L)$, where TN is troponin; PARV parvalbumin; M maximum SR Ca^{2+} pump rate; F fractional occupancy of pump sites (P); D dye concentration and L the Ca^{2+} leak from the SR. PARV equilibrates with [CaPARV] and [MgPARV] and MF^2 corresponds to $M(\mathrm{Ca}P]/[Pt])^2$ where Pt is the total concentration of pump sites. The parameters were: [TN], K_{on}[CaTN], K_{off}[CaTN], [PARV], K_{on}[CaPARV], [Mg^{2+}], K_{on}[MgPARV], K_{off}[MgPARV], M, Pt, [DYE], K_{on}[CaDYE], K_{off}[CaDYE], [EGTA], K_{on}[CaEGTA], and K_{off}[Ca-EGTA]. The rate constants were fitted with boundaries close to the corresponding biochemical determinations. Biochemical measurements of parvalbumin concentration in *soleus* (0.7 μM) and EDL (1 mM) muscles were used (Heizmann et al., 1982). A least-square-fit to the Ca^{2+} transient decaying phase 14 msec after fiber repolarization was used.

Corrections for SR calcium depletion were done by scaling the SR Ca^{2+} release function at time t, by $C_o/(C_o - \int R_{rel}\, dt)$, where C_o is the SR Ca^{2+} content before the pulse and $\int R_{rel}\, dt$ is the amount of Ca^{2+} released from the start of the depolarization until time t. For these calculations it is assumed that the rate of Ca^{2+} release reaches a steady state by the end of fiber depolarization (Schneider, Simon & Szücs, 1987; Jacquemond, Kao & Schneider, 1991).

Preparation of Muscle Homogenates

Muscle homogenates were prepared as described (Anderson, Cohn & Meissner, 1994; Anderson & Meissner, 1995). Briefly, whole *soleus* or EDL muscles were homogenized at 4°C in a Waring blender in 8 vol of 0.1 M NaCl, 5 mM tris((hydroxy-methyl)aminomethane (Tris) maleate, pH 6.8, 2 mM EDTA, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetracetic acid (EGTA), and various protease inhibitors (0.2 mM PMSF, 100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM benzamidine) and stored at −135°C. Protein concentrations were determined by the Lowry method with bovine serum albumin as the protein calibration standard.

Radioligand Binding Studies to DHPR and RYR

DHPR and RYR concentrations were determined using the radioligands [^{3}H]PN200-110 and [^{3}H]ryanodine, respectively. Homogenates were incubated either with 4 nM (+)-[Methyl-^{3}H]PN200-110 (Du Pont-New England Nuclear) for 1 hr at 23°C in 50 mM Tris.HCl, pH 7.5, 10 μM Ca^{2+}, 1 mM diisopropyl fluorophosphate (DIFP) and 5 μM leupeptin, or 50 nM [^{3}H]ryanodine (Du Pont-New England Nuclear) for 24–48 hr at 12°C in 20 mM Na piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.0, 1.0 NaCl, 100 μM Ca^{2+}, 5 mM AMP, 0.2 mM Pefabloc, and 20 μM leupeptin. The radioligand concentrations used resulted in occupancy of >95% of the high-affinity binding sites (Anderson et al., 1994). Membrane bound [^{3}H]PN200-110 and [^{3}H]ryanodine were determined by filtration through Whatman GF/B filters. Nonspecific [^{3}H]PN200-110 and [^{3}H]ryanodine were assessed in the presence of 1 μM unlabeled nifedipine (Sigma Chemical, St. Louis, MO) or PN200-110 (Sandoz Pharmaceutical, East Hanover, NJ) and 10 μM unlabeled ryanodine (Calbiochem, San Diego, CA), respectively (Anderson et al., 1994).

Results

Voltage- and Ca^{2+}-gated SR Ca^{2+} release mechanisms are thought to supply Ca^{2+} required for contractile protein activation. To assess the contribution of these two processes to SR Ca^{2+} release in *soleus* and EDL muscles, we measured the inactivable- and noninactivable SR Ca^{2+} release components in voltage-clamped single muscle fibers using a well established fluorescent technique (Delbono, 1995). The extent of DHPR-RYR interactions were assessed by determining their maximum high affinity binding capacity (B_{max}) in muscle homogenates using two specific radioligands.

Intracellular Ca^{2+} transients were monitored in EDL and *soleus* fibers with the low affinity Ca^{2+} indicator mag-fura-2 (K_D = 49 μM) to prevent dye saturation at micromolar [Ca^{2+}]. Figure 1 illustrates the voltage dependence of SR Ca^{2+} release in a *soleus* fiber. Calcium transients were elicited by 50 msec pulses from V_h −90 mV to depolarizing potentials ranging from −60 to +30 mV (Fig. 1*A* and *C*). A small Ca^{2+} signal was detected when the membrane potential was lowered to −60 mV. The magnitude of this signal increased as increasing depolarizing pulses up to +30 mV. Ca^{2+} signals reached a maximum value at the end of the 50 msec pulse and slowly returned to prestimulation values with fiber repolarization. Mag-fura-2 reacts with Ca^{2+} with 1:1 stoichiometry, and it has been shown that the magnitude of the myoplasmic Ca^{2+} signals measured by the dye are directly related to the amount of Ca^{2+} mobilized during fiber depolarization (Konishi et al., 1991; Delbono et al., 1993). Figure 1*B* shows the calculated rate of SR Ca^{2+} release. An initial fast rising and decaying phase is followed by a slower decaying phase. A fast decline in the rate of Ca^{2+} release followed by a slower decaying phase has been observed in studies in frog (Melzer et al., 1987), rat (Delbono et al., 1993; García & Schneider, 1993; Delbono, 1995) and human fast- and slow-muscle fibers (Delbono et al., 1995). It has been suggested that SR Ca^{2+} release is rapidly inhibited by a Ca^{2+}-dependent mechanism in amphibian and mammalian muscle fibers (Baylor, Chandler & Marshall, 1983; Delbono et al.,

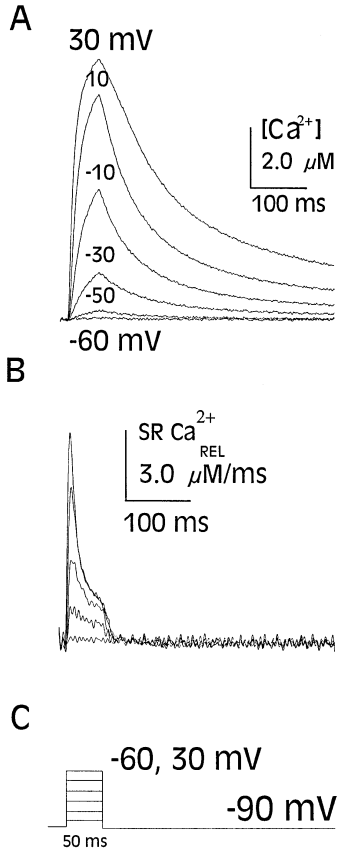

Fig. 1. Voltage dependence of SR Ca^{2+} release in rat *soleus* muscle fiber. Ca^{2+} transients were elicited by 50-msec pulses from V_h −90 mV to the potentials indicated close to the traces (*A*). Experimental records are expressed in Ca^{2+} concentration ([Ca^{2+}]). Ca^{2+} transients were interrupted by repolarization to −90 mV (*C*). SR Ca^{2+} release (Ca^{2+}_{REL}) calculations for pulses from −50 to +30 mV are shown in *B*. Fibers were stained with 400 μM mag-fura-2 for 50 min and the records were obtained at room temperature (20–22°C).

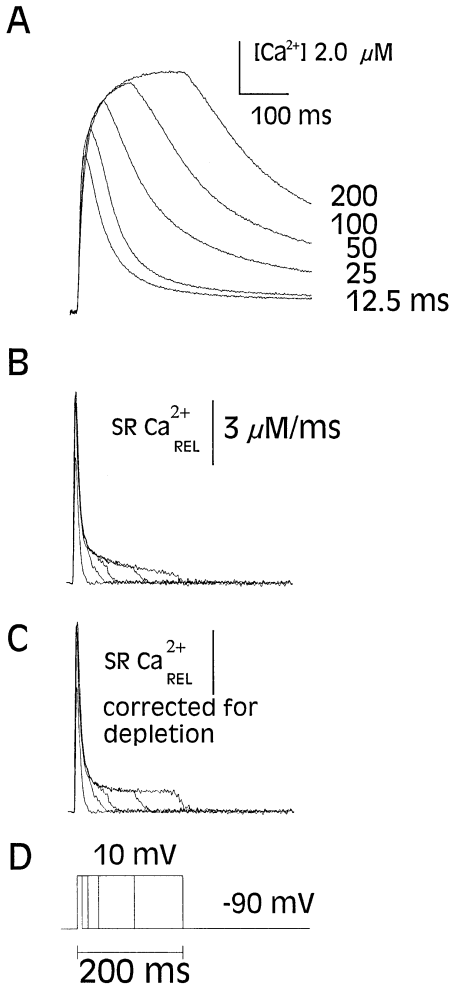

Fig. 2. Time dependence of SR Ca^{2+} release in rat *soleus* muscle fiber. Ca^{2+} transients (*A*) were evoked with pulses of different duration (values near traces) from V_h −90 mV to +10 mV (*D*). SR Ca^{2+} release is shown in (*B*). SR Ca^{2+} release traces corrected for SR Ca^{2+} depletion are shown in (*C*).

1995; *see also* Schneider, 1994). In addition, there is a voltage-dependent and Ca^{2+}-independent component that slowly declines as a result of SR Ca^{2+} depletion (Melzer et al., 1987; Delbono et al., 1993, 1995).

Figure 2 shows Ca^{2+} transients (*A*) evoked by depolarizing pulses of different duration. This protocol was applied to better resolve the fast and slow phases of the SR Ca^{2+} release waveform in *soleus* muscle (Fig. 2*B* and *C*). Depolarizing pulses to +10 mV of different duration (12.5 to 200 msec) are represented at the bottom of the figure (*D*). Figure 2*B* shows a rapid rise and decay in the rates of SR Ca^{2+} release followed by a slower second phase that rapidly declined when fibers were repolarized. A slow decrease in the rate of release with time over the second phase suggests SR Ca^{2+} depletion. It is known that prolonged depolarizations induce a more accentuated decline in the second phase of Ca^{2+} release than shorter pulses, as shown in frog and rat muscle fibers (Melzer et al., 1987; Delbono et al., 1993). Figure 2*C* shows the effect of correcting the calculated Ca^{2+} release waveform (*B*) to SR Ca^{2+} depletion following methods described above. As a result of this procedure a rapid and transient Ca^{2+} release phase was followed by a steady-state voltage-dependent Ca^{2+} release phase. We used corrected traces to calculate the peak and steady-state rates of SR Ca^{2+} release.

Table 1 lists the peaks (*a*) and steady-state (*b*) rates of SR Ca^{2+} release in *soleus* muscle. Calculations of *b*, in *soleus* and EDL fibers, were done at 200 msec after the beginning of fiber depolarization with 200 msec pulses to +10 mV. As the values at (*a*-*b*) and (*b*) correspond to Ca^{2+}- and voltage-gated SR Ca^{2+} release, respectively (Jacquemond et al., 1991), the ratio (*b*/*a*-*b*) represents the proportion of Ca^{2+}-independent SR Ca^{2+} release during the peak of release. All three values (*a*-*b*,

Table 1. Calcium release (CR) in *soleus* and EDL muscle fibers

Fiber	*Soleus*		
	CR peak amplitude (*a*)	CR amplitude at the end of the pulse (*b*)	Ratio (*b/a − b*)
t420405	9.0	2.9	0.42
t420417	10.4	3.4	0.49
t420703	8.6	2.5	0.41
t420714	11.2	3.0	0.37
t421803	13.1	3.4	0.35
t432306	7.7	2.2	0.40
t432315	9.5	2.8	0.42
t432326	12.1	3.3	0.38
t441804	12.6	3.1	0.33
t442013	10.3	3.5	0.52
t452503	9.9	2.9	0.41
t452716	10.6	3.3	0.45
Average (n = 12)	10.4 ±1.6	3.0 ±0.39	0.41 ±0.05
	EDL		
t440505	14.4	7.6	1.12
t440527	15.6	7.9	1.03
t441807	15.8	8.2	1.08
t441817	16.6	7.3	0.79
t442709	15.2	7.5	0.97
t451106	13.3	6.6	0.98
t451115	14.4	7.8	1.18
t452612	15.7	8.3	1.12
t452633	16.8	8.5	1.02
t461604	14.9	7.7	1.07
t461612	18.0	7.9	0.78
t463008	16.2	7.4	0.84
Average (n = 12)	15.6 ±1.3	7.7 ±0.5	1.01 ±0.13

Calcium release (CR) is expressed in μM/msec. Values are means ± SEM.

b and *b/a-b*) were significantly different from those in EDL fibers (*see below*).

Ca^{2+} transients in *soleus* fibers were compared with those in EDL fibers (Fig. 3). Intracellular Ca^{2+} responses in EDL fibers were evoked with a pulse protocol identical to that used for *soleus* fibers (Fig. 3*C*). The maximally determined free [Ca^{2+}] during a 200-msec pulse was about 5 to 6 μM higher in EDL than in *soleus* muscle (≈9 μM and ≈16 μM in Figs. 2*A* and 3*A*, respectively). Despite an increased Ca^{2+} level, we observed that, in agreement with previous studies, EDL muscles removed faster myoplasmic Ca^{2+} than *soleus* muscles. Figure 3*B* shows the rates of Ca^{2+} release, corresponding to the traces in *A*, corrected for SR Ca^{2+} depletion. Values for Ca^{2+} release at the peak (*a*), steady-state phase of Ca^{2+} release (*b*) and (*b/a-b*) ratio for EDL fibers are shown in Table 1. Differences for the three parameters with respect to *soleus* fibers were statistically significant ($P < 0.01$). This comparison raised the possibility that a smaller proportion of RYRs in *soleus* than in EDL muscles are under the direct control of DHPR.

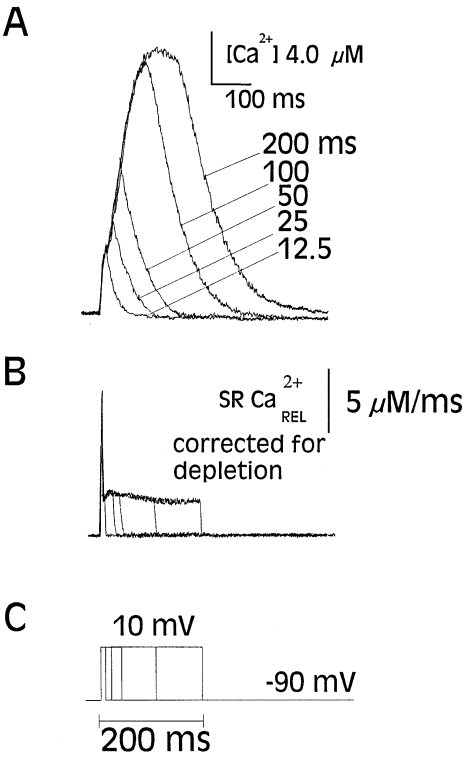

Fig. 3. Voltage dependence of SR Ca^{2+} release in EDL muscle fiber. Ca^{2+} transients were elicited by 50-msec pulses from V_h −90 mV to the potentials as indicated (*A*). Experimental records are expressed in calcium concentration ([Ca^{2+}]). SR Ca^{2+} release (Ca^{2+}_{REL}) traces corrected for SR Ca^{2+} depletion are shown in *B.* Fibers were stained with 400 μM mag-fura-2 for 50 min. The experiments were carried out at room temperature (20–22°C).

DHPR and RYR concentrations in rat *soleus* and EDL muscles were determined in whole muscle homogenates using two specific high-affinity radioligands. Three to four *soleus* and EDL muscles were pooled to prepare muscle homogenates. [^{3}H]PN200-110 binding to transverse tubule DHPRs and [^{3}H]ryanodine binding to SR RYR/Ca^{2+} release channels were measured under conditions that resulted in >95% occupation of the respective high-affinity binding sites (Anderson et al., 1994). The ratio of the B_{max} values of [^{3}H]PN200-110 binding and [^{3}H]ryanodine binding in EDL muscles (Table 2) did not differ from that determined for rabbit white leg muscles and psoas. However, the binding ratio for *soleus* muscles was significantly lower than for EDL. A smaller [^{3}H]PN200-110/[^{3}H]ryanodine binding ratio supported the concept of fewer DHPR-linked RYR in *soleus* muscle, thereby rendering the SR Ca^{2+} release process more dependent on released Ca^{2+} than in EDL fibers.

Table 2. [^{3}H]PN200-110/[^{3}H]Ryanodine binding ratio

	Soleus	EDL
[^{3}H]PN200-110 (pmol/mg protein)	12.4 ± 1.7	62 ± 6.0
[^{3}H]Ryanodine (pmol/mg protein)	40.9 ± 3.8	63 ± 9.0
[^{3}H]PN200-110/ [^{3}H]Ryanodine ratio	0.34 ± 0.05	0.92 ± 0.11

Values are mean ± SEM of 4–8 experiments.

Discussion

This study shows that there exists significant differences in the mechanism of SR Ca^{2+} release in *soleus* and EDL muscle fibers. Measurements of intracellular Ca^{2+} transients and determination of the number of DHPRs and RYRs supported the idea that a smaller fraction of RYRs are under the direct control of the DHPR in slow-twitch than fast-twitch muscles.

Previous studies in intact, skinned fibers and skeletal muscle microsomes support differences in SR Ca^{2+} release in fast- and slow-twitch muscles. Intracellular Ca^{2+} in rat *soleus* muscle was measured with aequorin. Ca^{2+} transients in *soleus* muscle showed a smaller amplitude, slower half-rise, mean decay times, and a higher sensitivity to caffeine compared with EDL (Eusebi et al., 1985; Dulhunty & Gage, 1985; Fryer & Neering, 1989). Studies with aequorin have several disadvantages such as: (i) low signal/noise ratio, (ii) light emission that appears to vary as $[Ca^{2+}]^{2.5}$ and (iii) the use of an indicator which does not react rapidly with myoplasmic Ca^{2+} (time constant of about 10 msec at 20°C) (Baylor et al., 1982). In this work, we used the low-affinity fluorescent calcium indicator mag-fura-2 (furaptra). It has been demonstrated that this indicator adequately traces changes in intracellular Ca^{2+} in frog and rat skeletal muscle fibers, in the micromolar range (Konishi et al., 1991; Delbono et al., 1993; Delbono, 1995).

Previous studies with ^{45}Ca-loaded SR were interpreted to show an initial rate of Ca^{2+} release three times slower in slow- than in fast-twitch muscles. The sensitivity to caffeine, doxorubicin, Ca^{2+}, Mg^{2+}, ruthenium red and tetracaine differed only slightly between both muscle types (Lee et al., 1991). In fibers, the kinetics of SR Ca^{2+} release is mainly determined by the number of RYRs, DHPRs and the modulatory channel environment (Ca^{2+}, Mg^{2+}, ATP, among others). Our data support the idea of a larger number of RYR uncoupled to DHPR in *soleus* than in EDL muscle as the underlying mechanism for the larger Ca^{2+}-sensitive Ca^{2+} release component in *soleus*. The slower Ca^{2+} release rate in *soleus* muscle was consistent with a lower number of RYRs compared to EDL (Table 2). Other mechanisms that may slow and reduce SR Ca^{2+} release are Ca^{2+}-dependent inactivation of the RYR (Meissner, 1994), SR Ca^{2+} depletion (Baylor et al., 1983, 1988) and RYR adaptation (Györke et al., 1993) in skeletal muscle. A Ca^{2+}-dependent inactivation of RYR has been described in EDL fibers (Delbono et al., 1993; García et al., 1993; Delbono, 1995) but not in *soleus* fibers. Double pulse experiments will clarify the contribution of this mechanism to the kinetics of SR Ca^{2+} release in *soleus* muscle. SR Ca^{2+} depletion was prominent in our records in *soleus* muscle. Therefore, all records were corrected for SR Ca^{2+} depletion. Adaptation of slow-twitch RYR has not been studied yet.

Although there is consensus about a Ca^{2+}-dependent SR calcium release channel inactivation in frog (Baylor et al., 1983; Schneider & Simon, 1988; Simon & Schneider, 1991) and rat (Delbono et al., 1993, 1994; García et al., 1993, 1994; Delbono, 1995) skeletal muscle fibers, the existence of a calcium-induced SR calcium release process is more controversial (Pape, Jong & Chandler, 1995; Jong et al., 1995). Some evidence has been obtained in support of a calcium-gated mechanism in amphibian and mammalian muscle fibers. The concept of an inactivable SR calcium release channel population corresponding to uncoupled RYR-DHPR in skeletal muscle has been proposed by Jacquemond et al. (1991) and confirmed subsequently by the same (Csernoch, Jacquemond & Schneider, 1993) and other (reviewed in Pizarro & Ríos, 1991; Delbono et al., 1994) groups. Despite some publications that contradict these results (Pape et al., 1995; Jong et al., 1995), an increasing number of studies support the presence of a Ca^{2+} regenerative process in skeletal muscle. These include: (i) RYR is directly activated by Ca^{2+} ions in bilayers (for a review *see* Meissner, 1994) with kinetic rate constants conceivable for a physiological process (Györke, 1993; Valdivia et al., 1995); (ii) The finding of junctional and extrajunctional uncoupled RYR1 to DHPRs determined by other methodologies to those used in this study (Dulhunty et al. 1992; Franzini-Armstrong, Ferguson & Champ, 1988; Block et al., 1988); and (iii). Control of T-tubule depolarization-induced SR Ca^{2+} release by DHPR- and Ca^{2+}-dependent mechanisms in cell homogenates from rabbit skeletal muscle (Anderson & Meissner, 1995).

A large variation in the DHPR/RYR ratio has been reported in muscles from different animal species (Lamb, 1992; Margreth, Damiani & Tobaldin, 1993). The variation in receptor coupling deduced from morphological and electrophysiological measurements is probably due to methodological differences and species-specific triadic arrangements. Tetrads, groups of four T-tubule particles, presumed to represent four DHPRs, have been located almost exactly opposite the four subunits of the RYR (Block et al., 1988; Franzini-Armstrong, Pincon-Raymond & Rieger, 1991). If there is one high-affinity

ryanodine binding site and four DHPR per foot/RYR tetramer, the DHPR/RYR ratio is 4. This assertion is valid as long as all DHPRs and RYRs are located in the T-tubule-SR junction, tetrads interacts with only one RYR and all DHPRs and RYRs bind their specific ligands (Anderson et al., 1994). A binding ratio of 0.92 (Table 2) suggests that in EDL muscles every fourth RYR is linked to a group of four DHPRs. In *soleus* muscles a lower ratio of 0.34 suggests that only every twelfth RYR is coupled to a group of four DHPRs. Such a low coupling ratio is consistent with our SR Ca^{2+} release measurements which showed a significantly larger Ca^{2+}-dependent SR Ca^{2+} release component in *soleus* than in fast-twitch muscles.

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