

# L-Type $\text{Ca}^{2+}$ Channel Charge Movement and Intracellular $\text{Ca}^{2+}$ in Skeletal Muscle Fibers from Aging Mice

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**ABSTRACT** In this work we tested the hypothesis that skeletal muscle fibers from aging mice exhibit a significant decline in myoplasmic  $\text{Ca}^{2+}$  concentration resulting from a reduction in L-type  $\text{Ca}^{2+}$  channel (dihydropyridine receptor, DHPR) charge movement. Skeletal muscle fibers from the *flexor digitorum brevis* (FDB) muscle were obtained from 5–7-, 14–18-, or 21–24-month-old FVB mice and voltage-clamped in the whole-cell configuration of the patch-clamp technique according to described procedures (Wang, Z.-M., M. L. Messi, and O. Delbono. 1999. *Biophys. J.* 77:2709–2716). Total charge movement or the DHPR charge movement was measured simultaneously with intracellular  $\text{Ca}^{2+}$  concentration. The maximum charge movement ( $Q_{\text{max}}$ ) recorded (mean  $\pm$  SEM, in nC  $\mu\text{F}^{-1}$ ) was  $53 \pm 3.2$  ( $n = 47$ ),  $51 \pm 3.2$  ( $n = 35$ ) (non-significant, ns), and  $33 \pm 1.9$  ( $n = 32$ ) ( $p < 0.01$ ), for the three age groups, respectively.  $Q_{\text{max}}$  corresponding to the DHPR was  $43 \pm 3.3$ ,  $38 \pm 4.1$  (ns), and  $25 \pm 3.4$  ( $p < 0.01$ ) for the three age groups, respectively. The peak intracellular  $[\text{Ca}^{2+}]$  recorded at 40 mV (in  $\mu\text{M}$ ) was  $15.7 \pm 0.12$ ,  $16.7 \pm 0.18$  (ns), and  $8.2 \pm 0.07$  ( $p < 0.01$ ) for the three age groups, respectively. No significant changes in the voltage distribution or steepness of the Q-V or  $[\text{Ca}^{2+}]$ -V relationship were found. These data support the concept that the reduction in the peak intracellular  $[\text{Ca}^{2+}]$  results from a larger number of ryanodine receptors uncoupled to DHPRs in skeletal muscle fibers from aging mammals.

## INTRODUCTION

The mechanisms underlying decline in muscle power and fatigue in aging mammals, including humans, are not known. Studies on muscle contractility in rodents and humans in vivo and in vitro have demonstrated that skeletal muscle strength declines with aging (Booth et al., 1994; Brooks and Faulkner, 1988; Brooks and Faulkner, 1994b; Eddinger et al., 1985; Edstrom and Larsson, 1987; Renganathan et al., 1998). Several mechanisms have been postulated to explain the age-related skeletal muscle weakness (for a review see Loeser and Delbono, 1999). It is evident that the loss of muscle mass (atrophy) does not explain entirely the decrease in contractile properties with aging (Brooks and Faulkner, 1994a,b; Moore, 1975). This means that the conservation of the muscle mass over ages does not ensure a complete preservation of the muscle tension. Studies on in vitro contractility showed that when the maximum isometric force for aged mice and rats is normalized to the smaller total muscle fiber cross-sectional area, a significant deficit in specific isometric force remains unexplained by atrophy (Brooks and Faulkner, 1988; Brooks and Faulkner, 1994b; Renganathan et al., 1998). These data suggest that other factors in addition to reductions in contractile proteins are contributing to muscle weakness in muscles from aging mammals. Studies on skinned muscle fibers demonstrated

that the force generated per cross-sectional area does not differ in adult and old mice during isometric and shortening contraction (Brooks and Faulkner, 1994a). Therefore, it is possible that earlier steps in excitation-contraction coupling are altered. An incomplete  $\text{Ca}^{2+}$  activation might account for differences in normalized tension in muscles from adult and aged mammals. In the present work we investigated whether excitation-intracellular  $\text{Ca}^{2+}$  coupling is a potential defective step in the contraction of fast-twitch muscles from aging mammals. Specifically, this study has been designed to test the hypothesis that in addition to already identified determinants of muscle impairment with aging a reduction in L-type  $\text{Ca}^{2+}$  channel (dihydropyridine receptor, DHPR) charge movement results in a significant reduction in intracellular  $\text{Ca}^{2+}$  mobilization. To test this hypothesis, single fibers from the *flexor digitorum brevis* (FDB) muscle from young, middle-aged, and old mice have been voltage-clamped with the whole-cell configuration of the patch-clamp technique, and sarcolemmal currents and intracellular  $\text{Ca}^{2+}$  have been recorded simultaneously as described previously (Wang, 1999b). The application of this technique for the first time to skeletal muscle from aging mouse allowed us to assess changes in charge movement and intracellular  $\text{Ca}^{2+}$  under very stable recording conditions as described previously for adult muscle fibers (Wang, 1999b). Furthermore, the simultaneous recording of L-type  $\text{Ca}^{2+}$  channel charge movement and intracellular  $\text{Ca}^{2+}$  in the muscle fiber permits a direct analysis of these associated cellular events instead of relying on the statistical correlation of both variables studied in separate sets of experiments.

Intramembrane charge movement associated with the L-type  $\text{Ca}^{2+}$  channel and sarcoplasmic reticulum  $\text{Ca}^{2+}$  influx are part of a signaling cascade that, among other factors,

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determine the magnitude of the muscle contraction tension (Ashley et al., 1991; Melzer et al., 1995). Therefore, the reduction in L-type  $\text{Ca}^{2+}$  channel charge movement will have an impact on the levels of cytosolic  $\text{Ca}^{2+}$  available for skeletal muscle force development in aging mammals. The present data and previous studies on the number of DHPR and ryanodine receptors (Renganathan et al., 1997, 1998) allow us to conclude that the reduction in the peak intracellular  $[\text{Ca}^{2+}]$  results from a larger number of ryanodine receptors uncoupled to DHPRs (excitation-contraction uncoupling) in skeletal muscle fibers from aging mammals.

## METHODS

### Mouse skeletal muscle single fibers

Single skeletal muscle fibers from the FDB muscle were obtained from 5–7- (young group), 14–18- (middle-aged), or 21–24- (old) month-old FVB mice raised in a pathogen-free area at the Animal Research Program of Wake Forest University School of Medicine (WFUSM). The maximum life expectancy of FVB mice is 27 months. Animal handling and procedures followed an approved protocol by the Animal Care and Use Committee of WFUSM. FDB muscles were dissected in a solution containing 155 mM cesium aspartate, 5 mM magnesium aspartate<sub>2</sub>, and 10 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) (pH 7.4 with CsOH) (Beam and Franzini-Armstrong, 1997). Muscles were treated with 2 mg/ml collagenase (Sigma, St. Louis, MO) in a shaking bath at 37°C. After 3 h of enzymatic treatment, FDB muscles were dissociated into single fibers with Pasteur pipettes of different tip sizes.

### Charge movement and calcium current recordings

Muscle fibers were voltage-clamped using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA) in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) according to procedures previously described (Wang et al., 1999b). Muscle fibers were transferred to a small flow-through Lucite chamber positioned on a microscope stage. Fibers were continuously perfused with the external solution (see below) using a push-pull syringe pump (WPI, Saratoga, FL). Only fibers exhibiting a clean surface and lack of evidence of contracture were used for electrophysiological recordings. Patch pipettes were pulled from borosilicate glass (Boralex) using a Flaming Brown micropipette puller (P97; Sutter Instrument Co., Novato, CA) and then fire-polished to obtain electrode resistance ranging from 450 to 650 k $\Omega$ . For these measurements the pipettes were filled with “pipette” solution. The pipette was filled with the following “pipette” solution (mM): 140 cesium aspartate; 2 magnesium aspartate<sub>2</sub>; 0.2 Cs<sub>2</sub>EGTA (ethylene glycol-bis( $\alpha$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), and 10 HEPES, with pH adjusted to 7.4 with CsOH (Adams et al., 1990; Wang et al., 1999a). The external solution used for  $\text{Ca}^{2+}$  current recording contained (in mM): 150 TEA (tetraethylammonium hydroxide)-CH<sub>3</sub>SO<sub>3</sub>, 2 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, 10 Na-HEPES and 0.001 tetrodotoxin (Delbono, 1992; Delbono et al., 1997b). Solution pH was adjusted to 7.4 with CsOH. Both the pipette and the bath solution were selected based on the ease of membrane seal formation and cell stability over time. For charge movement recording,  $\text{Ca}^{2+}$  current was blocked with the external solution containing 0.5 mM Cd<sup>2+</sup> and 0.3 mM La<sup>3+</sup> (Adams et al., 1990; Wang et al., 1999b).

Whole-cell currents were acquired and filtered at 5 kHz with pCLAMP 6.04 software (Axon Instruments). A Digidata 1200 interface (Axon Instruments) was used for A-D conversion. Membrane current during a voltage pulse, *P*, was initially corrected by analog subtraction of linear

components. The remaining linear components were digitally subtracted on-line using hyperpolarizing control pulses of one-quarter test pulse amplitude ( $-P/4$  procedure) (Bezanilla, 1985; Delbono, 1992; Delbono and Stefani, 1993a) as described for rat and mouse muscle fibers (Delbono, 1992; Delbono et al., 1997). Four control pulses were applied before the test pulse. Charge movements were evoked by 25-ms depolarizing pulses from the holding potential ( $-80$  mV) to command potentials ranging from  $-70$  to  $70$  mV with 10-mV increments. Intramembrane charge movement was calculated as the integral of the current in response to depolarizing pulses (charge on,  $Q_{\text{on}}$ ) and is expressed per membrane capacitance (coulombs per farad). The complete blockade of the inward  $\text{Ca}^{2+}$  current was verified by the  $Q_{\text{on}}-Q_{\text{off}}$  linear relationship (Wang et al., 1999b).

### Intracellular $\text{Ca}^{2+}$ transient recording

Intracellular  $\text{Ca}^{2+}$  transients were recorded simultaneously with sarcolemmal currents in single voltage-clamped FDB muscle fibers. A group of experiments done in 0.05 and 0.1 mM EGTA did not show significant changes in the intracellular  $\text{Ca}^{2+}$  transient kinetics (data not shown). The fluorescent indicator calcium green-5N (200  $\mu\text{M}$ ) (Molecular Probes, Eugene, OR) with low affinity for  $\text{Ca}^{2+}$  was used as the  $\text{Ca}^{2+}$  probe. The fibers were loaded with the  $\text{Ca}^{2+}$  dye via the patch pipette. After the whole-cell voltage-clamp was attained the dye was allowed to diffuse for 20–30 min before the fiber was pulsed. As the fiber sarcomere space was  $\sim 1.5$   $\mu\text{m}$ , only slight movements were detected in some fibers on one or both ends that did not interfere with the fluorescent recording in the center of the fiber (see below). For fluorescent recordings, the fiber was illuminated with a 75 W xenon lamp through a 20 $\times$  Fluar objective (Zeiss, Oberkochen, Germany). The light beam passed through an excitation filter centered at 485 nm wavelength with 10 nm bandwidth (Omega Optical Inc., Brattleboro, VT) mounted in a computer-controlled filter wheel (Ludl Electronics, Hawthorne, NY). The light was reflected by a dichroic mirror centered at 505 nm (DRLPO2, Omega Optical) and at a 45° angle. The emitted light was collected by a frame-transferred CCD camera (PXL-EEV-37, Photometrics, Tucson, AZ) after passing through an emission filter centered at 535 (DF35) (Omega Optical). Hardware control, image acquisition, and processing were done with ISee software (Inovision, Durham, NC) run in a SUN (Mountain View, CA) or a Silicon Graphics O<sub>2</sub> (Mountain View, CA) workstation. Although the fluorescence was recorded from the whole cell, only a rectangular region of interest (ROI) of  $\sim 2000$  to 3000 pixels near the patch pipette was analyzed. The patch pipette was not included in the ROI. Mean values of fluorescence changes normalized to basal fluorescence were plotted over time. Sequences of images for up to 2 s were acquired at 50 frames/s (20-ms interval). All of the records were corrected for background fluorescence (optical pathway) and photobleaching. Data are expressed as a percentage of change in fluorescence relative to basal fluorescence ( $100 \times \Delta F/F$ ) (Finch and Augustine, 1998).

### Calculation of intracellular $\text{Ca}^{2+}$ transients from calcium green-5N signals

The in vivo calibration of the dye and calculations of the kinetic constants of the calcium green-5N/ $\text{Ca}^{2+}$  reaction followed described procedures (Shirokova et al., 1996; Tsugorka et al., 1995) with some modifications. Changes in fluorescence intensity, normalized to basal fluorescence ( $\Delta F/F$ ) recorded in individual muscle fibers, were scaled to the concentration of the dye bound to  $\text{Ca}^{2+}$  ( $[\text{dye}/\text{Ca}^{2+}]$ ) with the following equation

$$[\text{dye}:\text{Ca}^{2+}] = [\text{dye}_T](F - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}}) \quad (1)$$

where  $[\text{dye}_T]$  is the total dye concentration and  $F_{\text{min}}$  and  $F_{\text{max}}$  are the minima (absence of  $\text{Ca}^{2+}$ ) and maxima (saturating  $\text{Ca}^{2+}$ ) of fluorescence.

The time course of  $[Ca^{2+}](t)$  was calculated from the dye signal recorded in the muscle fiber as

$$[Ca^{2+}]_{on}^{-1} dF/dt + K_d(F - F_{min})/(F_{max} - F) \quad (2)$$

where  $k_{on}$  is the dye/ $Ca^{2+}$  association rate constant and  $K_d$  the dissociation constant.  $K_d$  was measured in non-contracting fibers from young mice equilibrated with different  $Ca^{2+}$  concentrations (0–100 mM) (Beuckelmann and Wier, 1988). Free  $Ca^{2+}$  and  $Mg^{2+}$  concentrations in solutions were calculated according to Fabiato (1988). The  $K_d$  values recorded in muscle fibers from young mice ( $33 \pm 1.8 \mu M$ ;  $n = 20$ ) did not differ from those recorded in fibers from middle-age and old mice ( $31 \pm 2.1$  and  $29 \pm 3.1$ , respectively,  $n = 18$ ;  $p > 0.05$ ). The  $k_{on}$  value was calculated as  $k_{off}/K_d$ , and  $k_{off}$  was obtained as the reciprocal of  $\tau_{off}$  for each experiment. Fig. 1 shows representative records of the dye/ $Ca^{2+}$  complex and transient changes in intracellular  $[Ca^{2+}]$ .

All of the experiments were carried out at room temperature (22°C). Data values are given as means  $\pm$  SEM with the number of observations ( $n$ ). Experimental groups have been statistically analyzed using Student's unpaired  $t$ -test, and  $p < 0.05$  was considered significant.

## RESULTS

### Charge movement in skeletal muscle fibers from young, middle age and old mice

Intramembrane charge movement was recorded after blocking the inward  $Ca^{2+}$  current (see Methods). Fig. 2 *A* shows a group of charge movement traces recorded in a muscle

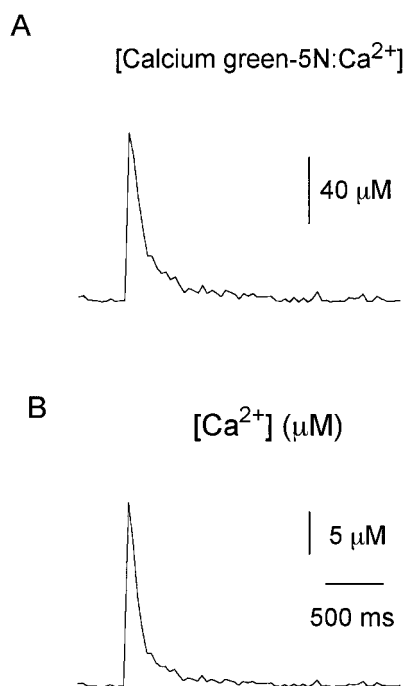


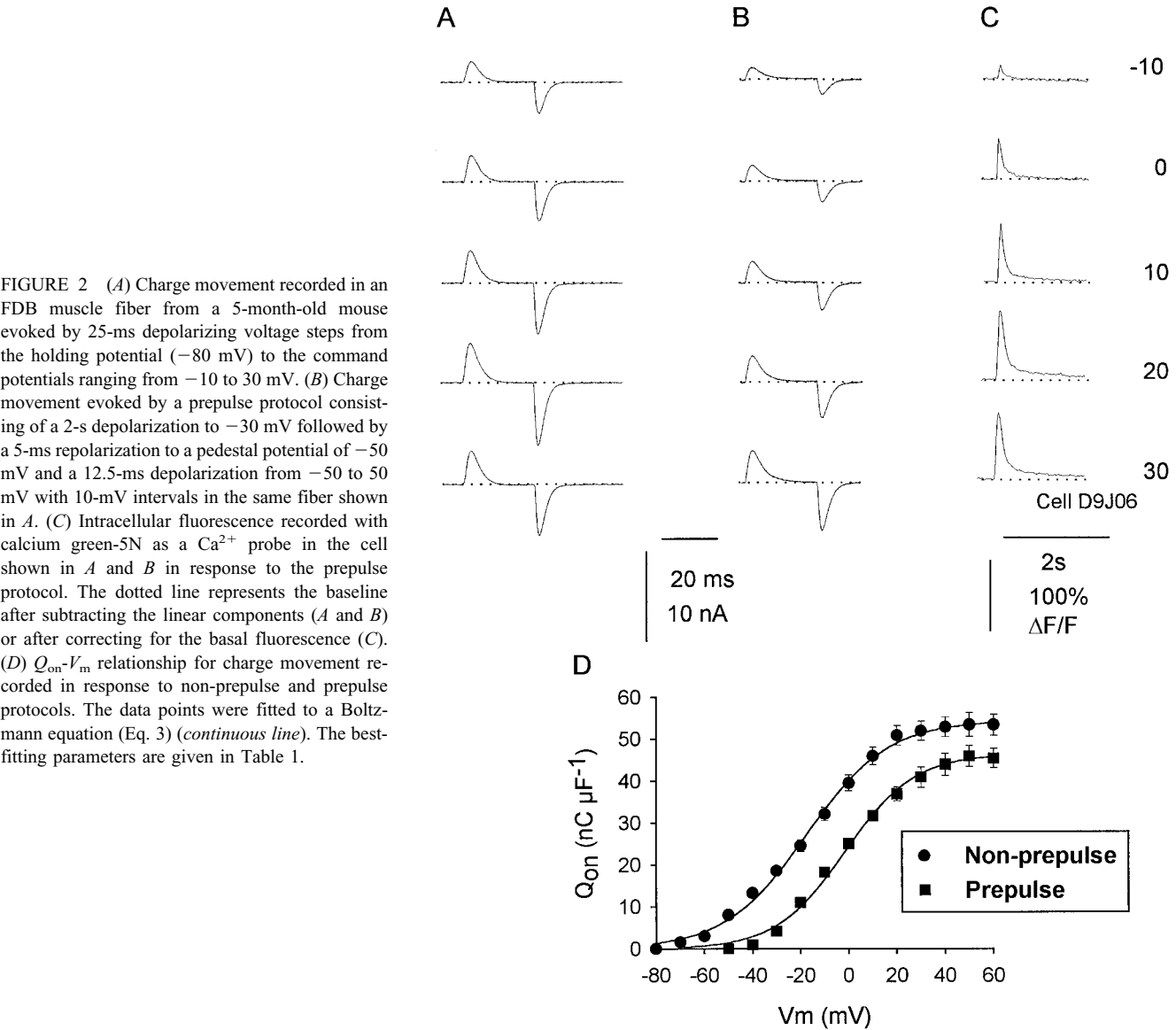
FIGURE 1 Calculation of intracellular  $Ca^{2+}$  concentration. (A) FDB muscle fiber voltage-clamped in the whole-cell configuration of the patch-clamp technique. The calcium green-5N/ $Ca^{2+}$  signal was elicited by a 25-ms depolarizing pulse from the holding potential of  $-80$  mV to  $20$  mV (command potential). (B) Transient increase in intracellular free  $Ca^{2+}$  concentration calculated as explained in Methods.  $K_d = 33 \mu M$ ,  $\tau_{off} = 3.7$  ms,  $k_{off} = 0.27 \text{ ms}^{-1}$ , and  $k_{on} = 0.0082 \mu M^{-1} \text{ ms}^{-1}$ .

fiber from a young (5-month-old) mouse. Charge movements have been evoked by applying 25-ms depolarizing voltage steps from the holding potential ( $-80$  mV) to the command potentials ranging from  $-10$  to  $30$  mV. The current recorded after blocking the  $Ca^{2+}$  current is the intramembrane charge movement because it shows saturation at both extremes of the voltage range, and the amount of charge moved during depolarization ( $Q_{on}$ ) is equal to the charge that returns during the repolarization ( $Q_{off}$ ). This has been demonstrated previously for adult skeletal muscle fibers voltage-clamped in the whole-cell configuration of the patch-clamp technique (Wang et al., 1999b). We have also recorded the charge movement corresponding to gating of the L-type  $Ca^{2+}$  channel/DHPR (Adams et al., 1990). To this end, we used a pulse protocol consisting of a 2-s prepulse to  $-30$  mV and a subsequent 5-ms repolarization to a pedestal potential of  $-50$  mV, followed by a 12.5-ms depolarization from  $-50$  to  $50$  mV with 10-mV intervals (Adams et al., 1990). The optimal duration of the prepulse has been defined as the value at which no further immobilization of charge movement is attained. The optimal duration of the prepulse has been determined to be 2 s after testing a range of prepulses from 1 to 6 s. Prepulses longer than 2 s did not significantly modify the integral of the charge movement elicited by the test pulse in fibers from young, middle-aged, and old mice (data not shown). Therefore, a 2-s prepulse protocol was applied systematically to all of the muscle fibers included in the present study. Fig. 2 *B* shows charge movement traces in response to the application of the prepulse protocol from  $-10$ -mV to  $30$ -mV command pulses to the same fiber recorded in Fig. 2 *A*. Total charge movement and the immobilization-resistant charge have been plotted in Fig. 2 *D*. For the analysis of the voltage-dependence of the charge, data points were fitted to a Boltzmann equation of the form:

$$Q_{on} = Q_{max}/\{1 + \exp[z_Q F(V_{1/2Q} - V_m)/RT]\}, \quad (3)$$

where  $Q_{max}$  is the maximum charge,  $V_m$  is the membrane potential,  $V_{1/2Q}$  is the charge movement half-activation potential,  $z_Q$  is the effective valence, and  $F$ ,  $R$ , and  $T$  have their usual thermodynamic meanings. The best-fitting parameters for  $Q_{max}$ ,  $V_{1/2Q}$ , and  $z_Q$  recorded in response to either the prepulse or the non-prepulse protocol in muscle fibers from young mice are included in Table 1. From these measurements we conclude that  $19 \pm 2.1\%$  ( $n = 47, 35$ , and  $32$  for young, middle-aged, and old fibers, respectively) of the total charge is immobilized by the prepulse depolarization, and the voltage distribution of the charge is shifted toward more negative potentials.

Fig. 3 shows representative traces of charge movement recorded in response to either the non-prepulse (*A*) or the prepulse (*B*) protocol in a muscle fiber from a middle-aged (15-month-old) mouse. The maximum charge movement and its voltage distribution in both recording conditions are



represented in Fig. 3. The best-fitting parameters for  $Q_{max}$ ,  $V_{1/2Q}$ , and  $z$  are given in Table 1. The statistical analysis of these parameters did not show significant differences with those recorded in muscle fibers from young mice.

Fig. 4 shows a set of charge movement traces recorded in a single muscle fiber from an old mouse (22 months old) in response to the non-prepulse (A) and prepulse (B) protocol. The  $Q_{on}$ -voltage relationships have been plotted in Fig. 3 D. Figs. 2 D, 3 D, and 4 D have been plotted in the same scale to compare the magnitude of the age-related decline in the maximum charge movement.  $Q_{max}$  recorded in fibers from the older group was significantly smaller than in fibers from young and middle-aged mice, whereas no statistically significant differences in  $V_{1/2Q}$  and  $z$  have been recorded (Table 1). The fraction of immobilized charge recorded in muscle fibers from either middle-aged or old mice ( $25 \pm 2.9$

and  $24 \pm 2.7\%$ , respectively) was similar to that recorded in fibers from young mice.

### Intracellular $Ca^{2+}$ recording in FDB muscle fibers from young, middle-aged, and old mice

Intracellular  $Ca^{2+}$  has been recorded with the relatively low affinity  $Ca^{2+}$  fluorescent dye calcium-green-5N. This  $Ca^{2+}$  indicator has been selected based on  $K_d$  measurements in this and previous works (Wang et al., 1999b) and on the peak of intracellular  $Ca^{2+}$  recorded in rat skeletal muscle fibers (Delbono and Stefani, 1993b; Garcia and Schneider, 1993). Therefore, saturation of the calcium green-5N with  $Ca^{2+}$  is not expected. This dye also exhibits an acceptable quantum yield that allows for recordings of changes in the



**TABLE 1** Best-fitting parameters describing the voltage-dependence of charge movement and intracellular  $\text{Ca}^{2+}$  simultaneously recorded in FDB muscle fibers

Best-fitting parameters		5–7-month-old mice ( <i>n</i> = 47)	14–18-month-old mice ( <i>n</i> = 35)	21–24-month-old mice ( <i>n</i> = 32)
Charge movement	Non-prepulse			
	Prepulse			
Intracellular $[\text{Ca}^{2+}]$				

*n*, Number of muscle fibers; ns, non-significant statistically.

dye/ $\text{Ca}^{2+}$  complex with the photo-detector device used in the present work. Figs. 2 *C*, 3 *C*, and 4 *C* show representative traces of intracellular  $\text{Ca}^{2+}$  fluorescence recorded simultaneously with DHPR charge movement (prepulse protocol). It is apparent that the peak fluorescent signal recorded in the fiber from the old mouse (Fig. 4 *C*) is significantly lower than in fibers from young mice. Fig. 5 shows the voltage-dependence of the calculated peak  $\text{Ca}^{2+}$  transient recorded in muscle fibers from young, middle-aged, and old mice. The data points were fitted to Eq. 3 and the best-fitting parameters are included in Table 1. No significant differences between muscle fibers from young and middle-aged mice were found. However, the peak  $\text{Ca}^{2+}$  concentration in fibers from old mice was significantly reduced compared with fibers from middle-aged and old mice without. No significant changes in the  $V_{1/2Q}$  and *z* parameters were recorded among fibers from the same animal groups (Table 1).

## DISCUSSION

In this work we demonstrate that the peak intracellular  $\text{Ca}^{2+}$  evoked by sarcolemmal depolarization under voltage-clamp conditions in muscle fibers from old mice is significantly smaller than that recorded in fibers from middle-aged or young mice. Intracellular  $\text{Ca}^{2+}$  measurements performed in muscle fibers from mice of different ages demonstrates that it is senescence and not maturation which determines a substantial reduction in intracellular  $\text{Ca}^{2+}$  concentration upon fiber activation. The sarcoplasmic reticulum  $\text{Ca}^{2+}$  release is a crucial step in sarcolemmal excitation-contrac-

tion coupling because the levels of free cytosolic  $\text{Ca}^{2+}$  regulate muscle tension (Ashley et al., 1991). In the present study we demonstrate that muscle fibers from old mice exhibit a reduction of ~48% of the peak  $\text{Ca}^{2+}$  concentration recorded in fibers from young and middle-aged mice. This significant reduction in free  $\text{Ca}^{2+}$  available to bind to contractile proteins might account for the reported decline in specific muscle tension (tension normalized to cross-sectional area) not explained by atrophy in skeletal muscle fibers from aging mammals (Brooks and Faulkner, 1994b).

Absolute reductions in the number and/or function of the DHPR and/or RyR1 are potential explanations for the age-related impairment in intracellular  $\text{Ca}^{2+}$  mobilization in skeletal muscle from aging mammals (Delbono et al., 1997a). The percent decline in myoplasmic  $\text{Ca}^{2+}$  concentration mentioned above is very similar to the magnitude of the decrease in total charge movement and in the charge movement corresponding to the DHPR. The application of a prolonged depolarizing pulse to the muscle fiber induces inactivation of voltage-gated ion channels that potentially contribute to the charge movement recorded (Adams et al., 1990). We tested different prepulse durations and the amount of charge immobilization was similar (see above). The charge immobilized by the prepulse protocol in muscle fibers from young, middle-aged, and old mice is approximately half of that reported in myotubes (40%) (Adams et al., 1990). This means that the contribution of voltage-gated ion channels other than the DHPR to the charge movement recorded in adult fibers is less prominent than in myotubes, and it does not change significantly at older ages.

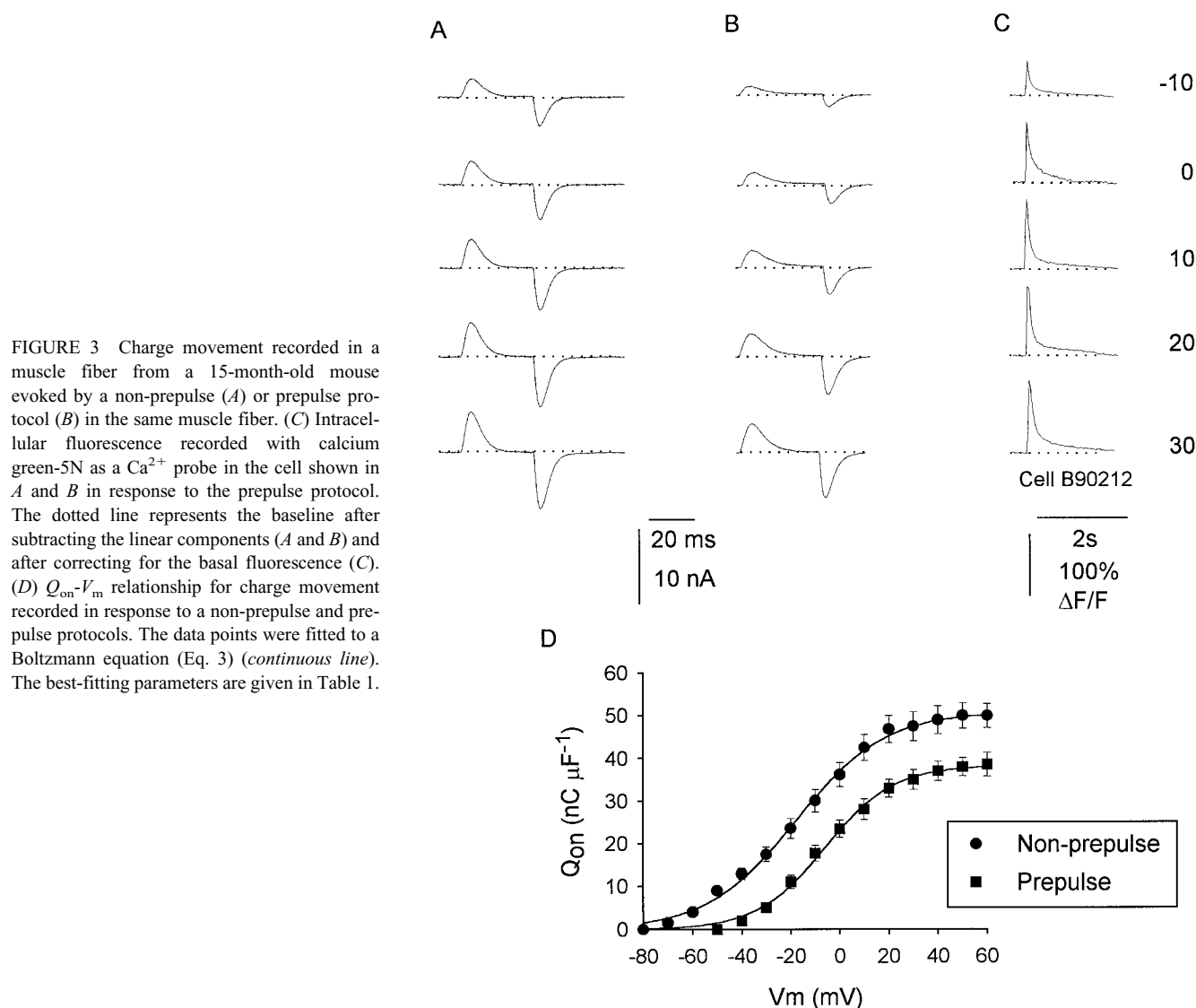
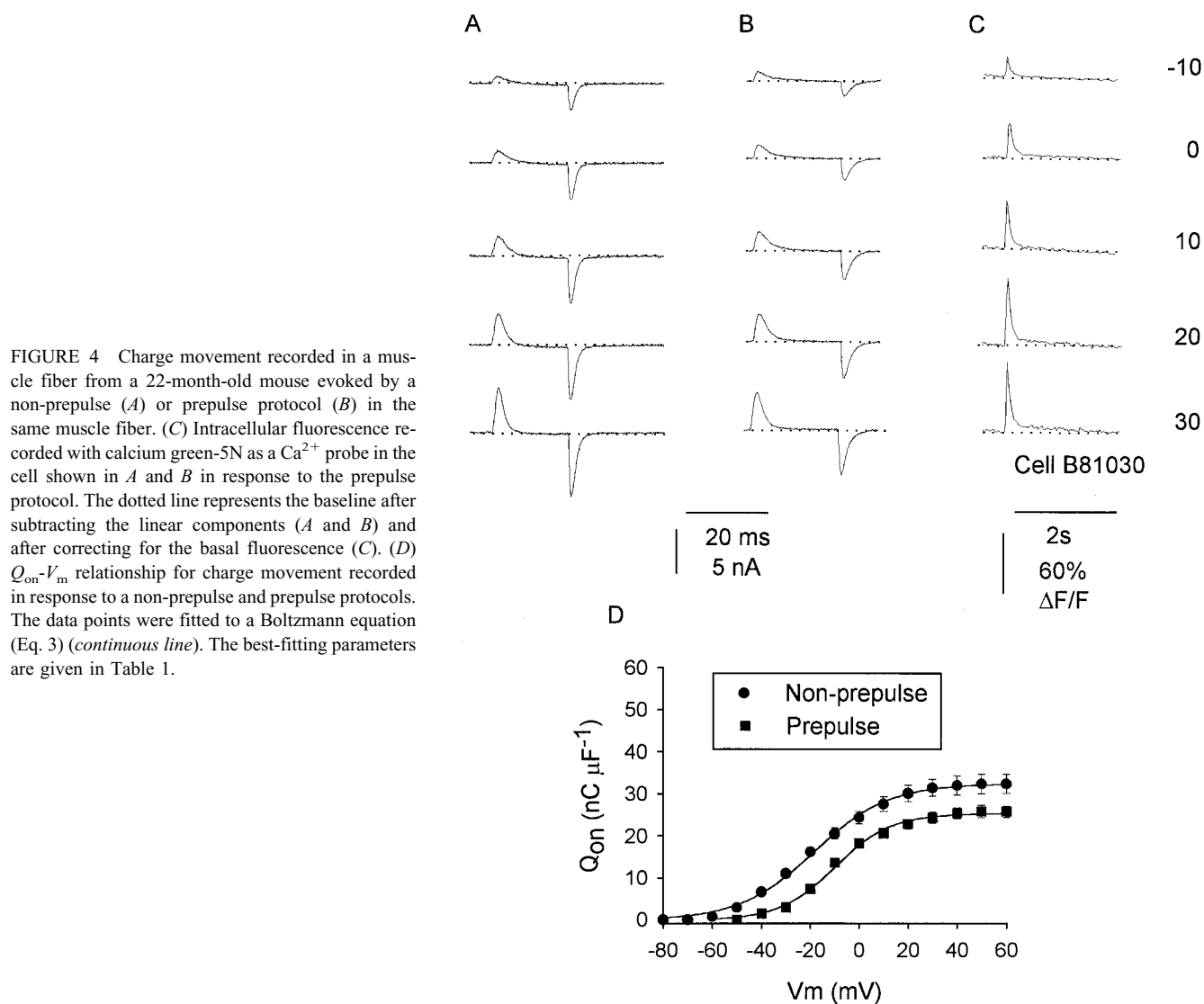


FIGURE 3 Charge movement recorded in a muscle fiber from a 15-month-old mouse evoked by a non-prepulse (A) or prepulse protocol (B) in the same muscle fiber. (C) Intracellular fluorescence recorded with calcium green-5N as a  $\text{Ca}^{2+}$  probe in the cell shown in A and B in response to the prepulse protocol. The dotted line represents the baseline after subtracting the linear components (A and B) and after correcting for the basal fluorescence (C). (D)  $Q_{\text{on}}-V_m$  relationship for charge movement recorded in response to a non-prepulse and prepulse protocols. The data points were fitted to a Boltzmann equation (Eq. 3) (continuous line). The best-fitting parameters are given in Table 1.

The percent decrease in total and DHPR charge movement recorded in fibers from young or middle-aged to old mice is similar (see above). Although the decrease in charge movement and myoplasmic  $\text{Ca}^{2+}$  concentration at older ages is similar, it may be possible that the smaller peak  $\text{Ca}^{2+}$  in fibers from older animals does not result only from the deficit in charge movement. Therefore, evidences that support this concept and some alternative explanations will be discussed. Previous studies from our laboratory demonstrated that the number of DHPR expressed in mouse EDL muscles decreases with aging, whereas no significant changes in the number of RyR1 were detected in fast-twitch muscles from the same group of mice (Renganathan et al., 1997, 1998). In these publications no significant changes in the pharmacological properties of the two receptors for their high-affinity ligands were detected. The reduction in the number of DHPR measured by radioligand binding assay is consistent with the decrease in charge movement corre-

sponding to the DHPR reported here. The ratio between the number of DHPRs and RyR1s in adult EDL muscle showed a mean value of 0.92. This ratio suggests that every fourth RyR1 is linked to a group of four DHPR (Delbono and Meissner, 1996). The reduction in DHPR charge movement in fibers from aging mice is consistent with measurements of DHPR maximum binding capacity (Renganathan et al., 1997). The magnitude of this reduction indicates that every sixth to eighth RyR1 is linked to a group of four DHPR in muscles from aging mice.

The lack of changes in the receptor affinity for the ligand does not rule out completely functional alterations in the DHPR. To completely ascertain this point, single DHPR recordings would be needed in muscle fibers. However, the DHPR is not accessible to patch pipettes due to its location at the t-tubule membrane. Similar considerations can be applied to the RyR1. Although the maximum binding capacity and affinity of the RyR1 for its ligand did not change



in fast-twitch muscle fibers from aging mice, single RyR1 recordings *in vivo* would be desirable. Single RyR1 recordings in living muscle fibers have not been reported yet due to technical difficulties in accessing an intracellular organelle (sarcoplasmic reticulum). It has been demonstrated that confocal microscopy is a very powerful technique to assess the function of a single RyR1 or a group of RyR1 in living frog muscle cells (Klein et al., 1996; Tsugorka et al., 1995). However, this technique failed to show  $Ca^{2+}$  sparks in mammalian skeletal muscle (Shirokova et al., 1998; Niggli, 1999). These studies suggest that technical development is required to address whether the age-related decrease in the voltage-activated  $Ca^{2+}$  release in skeletal muscle is associated with alterations in DHPR and/or RyR1 function.

Another potential explanation for the lower peak myoplasmic  $Ca^{2+}$  concentration in muscle fibers from old mammals is a sarcoplasmic reticulum  $Ca^{2+}$  depletion faster than

in fibers from young mice. Shorter depolarizations could deplete SR luminal  $Ca^{2+}$  in fibers from older mammals. This alternative has not been fully explored. There are some indications that there is a residual free luminal  $Ca^{2+}$  in the fibers from older humans even after prolonged depolarizations. This is based on the observation that caffeine can elicit further increases in myoplasmic  $Ca^{2+}$  concentration after a maximal activation (Delbono et al., 1995). To explore this issue in more depth, direct recordings of sarcoplasmic reticulum luminal  $Ca^{2+}$  in muscle fibers from animals of different ages are needed.

In summary, the lower peak myoplasmic  $Ca^{2+}$  concentration recorded in fibers from the FDB muscle is associated with a reduction in DHPR charge movement and in the number of DHPR  $\alpha 1$  subunits (Renganathan et al., 1997). The impact of these events on single muscle fiber contractility remains to be addressed and it is the subject of ongoing studies in our laboratory (González and Delbono, 2000).

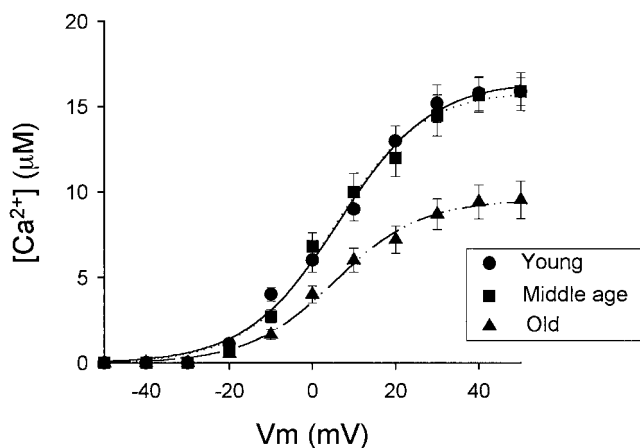


FIGURE 5 Peak intracellular  $\text{Ca}^{2+}$  concentration-membrane voltage relationship recorded in FDB muscle fibers from young, middle-aged, and old mice in response to the prepulse protocol. The data points were fitted to a Boltzmann equation (Eq. 3) and represented as a continuous, dotted, or dotted-dashed line for experiments in muscle fibers from young, middle-aged, or old mice.

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