

Absence of Ca^{2+} current facilitation in skeletal muscle of transgenic mice lacking the type 1 ryanodine receptor

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1. Whole-cell patch-clamp recordings were used to study voltage-dependent facilitation of Ca^{2+} currents and excessive Ca^{2+} tail currents in skeletal myoballs cultured from wild-type and transgenic mice expressing a null mutation of the ryanodine receptor (RyR) type 1 (dyspedic myoballs).
2. Ca^{2+} current density in dyspedic myoballs was reduced by about 60% compared with wild-type cells, with dihydropyridine-binding capacity largely retained.
3. Strong and long-lasting depolarizations (+80 mV and 600 ms), which normally produce excessive tail currents upon repolarization in control cells, failed to do so in dyspedic myoballs.
4. Dyspedic myoballs also failed to produce both Ca^{2+} current facilitation and the left shift of the current–voltage (I – V) curve induced by paired-pulse stimulation.
5. We propose that excessive tail currents and facilitation arise from silent Ca^{2+} channels acting as the voltage sensors in excitation–contraction coupling.

The two major proteins thought to be involved in the initial step of skeletal muscle excitation–contraction (E–C) coupling (Schneider & Chandler, 1973) are the dihydropyridine receptor (DHPR) in the plasma membrane (Rios & Brum, 1987) and the ryanodine receptor (RyR) in the sarcoplasmic reticulum (SR) (Melzer, Herrmann-Frank & Lüttgau, 1995). Both proteins are thought to be physically linked to each other and failure to express either one abolishes E–C coupling (Beam, Knudson & Powell, 1986; Takeshima *et al.* 1994). Recent evidence indicates that mice lacking the type 1 RyR show reduced Ca^{2+} currents, suggesting that functional RyRs affect Ca^{2+} channel properties (Nakai, Dirksen, Nguyen, Pessah, Beam & Allen, 1996). On the other hand, there is now compelling evidence that a large fraction of DHPRs in skeletal muscle are silent Ca^{2+} channels (Lamb, 1992), which normally do not contribute to macroscopic Ca^{2+} current, but which may conduct Ca^{2+} after strong and long-lasting depolarizations, producing excessively large tail currents followed by a short period in which a subsequent depolarization yields facilitated Ca^{2+} currents (Fleig & Penner, 1995, 1996). The recruitment of excessive tail currents and facilitation occur in parallel and seem to be linked to the RyR, since ryanodine and caffeine selectively inhibit both tail currents and facilitation but not the normal Ca^{2+} current (Fleig & Penner, 1996). This raises the question of whether functional or silent DHPRs are coupled to RyRs. We report here that myoballs from transgenic mice expressing a null mutation of the type 1 RyR gene have reduced Ca^{2+} currents (in agreement with Nakai *et al.* 1996),

but, more importantly, completely fail to produce excessive tail currents and facilitation. This suggests that the strongest impact of RyR deletion is on the functioning of excessive tail currents and facilitation and that the RyR is crucial for controlling the behaviour of the underlying Ca^{2+} channels.

METHODS

Control skeletal myoballs were prepared from newborn wild-type C57BL mice (1–3 days old) as described before (Fleig & Penner, 1995). For each wild-type preparation, one donor animal was killed by decapitation. Dyspedic myoballs were prepared from homozygous mutant neonates obtained by mating mice heterozygous for *skrr^{m1}* (Takeshima *et al.* 1994). Of thirty-five neonates obtained from five crosses between mice heterozygous for *skrr^{m1}*, eight died perinatally and were used for myoball cultures. All cells studied from these five preparations consistently showed the same electrophysiological characteristics we describe here. Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 23–27 °C in an external solution containing (mM): NaCl, 140; KCl, 2.8; CaCl_2 , 10; MgCl_2 , 2; glucose, 11; tetraethylammonium chloride (TEA-Cl), 10; Hepes, 10; tetrodotoxin (TTX), 0.03; pH 7.2 (equilibrated with HCl). Sylgard-coated patch pipettes had resistances of 1.5–3 M Ω after filling with the standard intracellular solution which contained (mM): *N*-methyl-D-glucamine, 140; NaCl, 8; MgCl_2 , 1; Cs-EGTA, 20; Mg-ATP, 4; GTP, 0.3; Hepes, 10; pH 7.2 (equilibrated with HCl). In some experiments Cs-EGTA was 10 mM.

Membrane currents were acquired by a computer-based patch-clamp amplifier system (EPC-9; HEKA, Lambrecht, Germany). Capacitive currents were determined and compensated before each voltage pulse using the automatic capacitance neutralization of the

EPC-9. Holding potential was -70 mV. Currents evoked during the depolarizing test pulses are referred to as 'pulse currents'. Currents evoked upon repolarization to more negative voltages than the test pulse are referred to as 'tail currents'. Pulse and tail current amplitudes are given as peak amplitudes. Series resistance compensation was not performed. Maximal voltage errors due to membrane capacitance (C_m) and series resistance (R_s) were estimated for control cells to be about 10 mV at -40 mV with time-to-clamp values of around 180 μ s (based on mean R_s values of 3.5 ± 0.4 M Ω , mean C_m values of 51 ± 5 pF and mean peak tail currents of 3 ± 0.4 nA ($n = 14$)). Currents were low-pass filtered at 8 kHz and digitized at 100 μ s intervals. For analysis and presentation, currents were filtered digitally to 1–3 kHz.

The current–voltage relationships of Ca^{2+} currents were fitted with a Boltzmann function, a linear term, and a term for positive block according to:

$$I_{\text{Ca}} = g_{\text{max}}(V_m - E_{\text{rev}}) \times \frac{1}{1 + \exp(-[V_m - V_{1/2}]/\text{slope})} \times \frac{1}{1 + \exp(-[V_m - V_{1/2,\text{block}}]/\text{slope}_{\text{block}})}$$

where g_{max} is the maximal conductance, V_m the test potential, E_{rev} the reversal potential, and $V_{1/2}$ the voltage at half-maximal activation.

Throughout, average data are given as means \pm s.e.m. with n determinations. For the dihydropyridine (DHP)-binding assay microsomal samples were prepared from neonate hindlimbs according to the method of Saito, Seiler, Chu & Fleischer (1994). The DHP-binding assay was performed using [^3H]PN200-110 (New England Nuclear, Boston, MA, USA) as described previously (Nishimura, Takeshima, Hofmann, Flockerzi & Imoto, 1993). Non-specific binding was determined in the presence of 20 μM unlabelled nifedipine.

RESULTS

Given the intimate link between DHPR and RyR in E–C coupling, we examined the characteristics of Ca^{2+} currents in skeletal myoballs of wild-type and homozygous offspring of transgenic parents with a targeted mutation (*skrr^{ml}*) of the gene for the skeletal muscle type 1 RyR (referred to as 'dyspedic', as they lack the so-called 'feet' corresponding to the cytoplasmic domain of RyRs; Takeshima *et al.* 1994; Takekura, Nishi, Noda, Takeshima & Franzini-Armstrong, 1995). Figure 1A illustrates whole-cell patch-clamp measurements of Ca^{2+} currents recorded in wild-type and dyspedic myoballs evoked by depolarizing voltage pulses to various

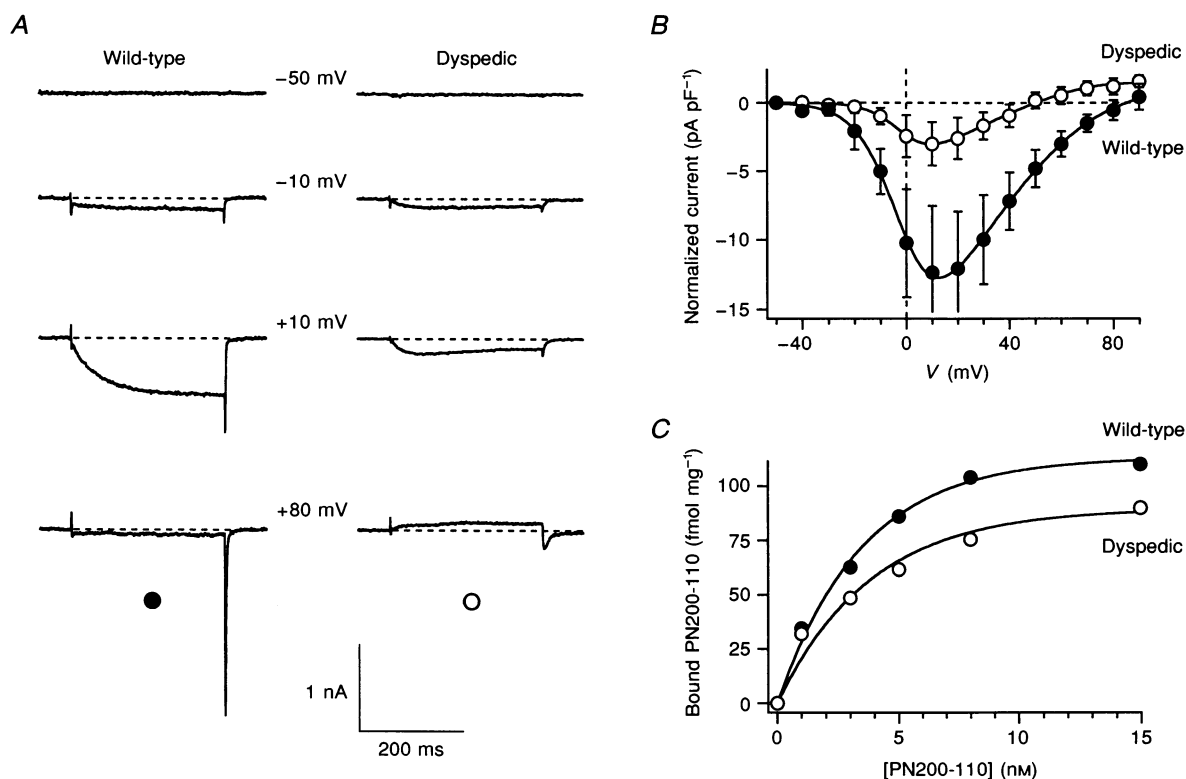


Figure 1. Dyspedic myoballs have reduced Ca^{2+} currents

A, average Ca^{2+} currents recorded in mouse wild-type (left panel; $n = 3$) and dyspedic myoballs (right panel; $n = 4$) evoked by depolarizations to -50 , -10 , $+10$ and $+80$ mV. B, average current–voltage relationships of Ca^{2+} currents recorded in wild-type (\bullet ; $n = 5$) and dyspedic (\circ ; $n = 4$) myoballs. Data points of the I – V curve represent mean current amplitudes (I_{Ca}) measured as peak amplitudes and plotted as a function of test potential. Test-pulse length was 300 ms. The fit to the mean I – V curve for wild-type cells yielded the following values: $V_{1/2} = -1$ mV, slope = 7.8 mV. The values for the mean I – V curve of dyspedic myoballs were: $V_{1/2} = -1$ mV, slope = 6.4 mV. C, saturation curve of [^3H]PN200-110 binding to microsomal samples prepared from neonate hindlimbs of wild-type (\bullet) and dyspedic (\circ) mice. Values are means from duplicate reactions.

Table 1. Comparison between wild-type and dyspedic skeletal myoballs

| Parameter | Wild-type | n | Dyspedic | n |
|---|------------|----|-----------|----|
| V _{1/2} (control) (mV) | -1 | 5 | -1 | 4 |
| Slope factor (control) (mV) | 7.8 | 5 | 6.4 | 4 |
| V _{1/2} (conditioned) (mV) | -14 | 5 | -5 | 4 |
| Slope factor (conditioned) (mV) | 12 | 5 | 9.8 | 4 |
| Current density (pA pF ⁻¹)* | 6.5 ± 0.6 | 14 | 2.3 ± 0.4 | 32 |
| Control pulse (pA)* | 320 ± 39 | 14 | 113 ± 19 | 32 |
| Facilitation pulse (pA)* | 508 ± 65 | 14 | 111 ± 17 | 32 |
| Tail current (pA)† | 3081 ± 395 | 14 | 281 ± 38 | 32 |
| Cells without current (%) | 0 | 26 | 30.3 | 66 |
| Cells without facilitation (%) | 3.8 | 26 | 100 | 66 |

'Conditioned' test pulses were preceded by a 600 ms prepulse to +80 mV and a brief return to -40 mV for 20 ms. 'Control' test pulses were evoked without the prepulse. * Measured at a test potential of 0 mV; † measured after 600 ms and at -40 mV.

potentials. Calcium currents of wild-type myoballs showed the classical voltage-dependent characteristics of L-type Ca²⁺ channels, activating at about -30 mV with peak currents at about +10 mV. Table 1 compares the properties of wild-type and dyspedic myoballs. While all wild-type myoballs examined expressed measurable pulse currents, 30% of dyspedic myoballs had no measurable Ca²⁺ current (see Table 1). One striking observation in the remaining dyspedic myoballs was that, on average, their pulse currents were significantly smaller than in wild-type myoballs. The current-voltage relationships of wild-type and dyspedic myoballs are shown in Fig. 1B and they illustrate that, aside from their magnitude, the voltage dependence of Ca²⁺ channels in both types of myoballs appears similar. The difference in the apparent reversal potential may not be significant, since small contaminating outward currents through potassium channels might affect the measured reversal potential more strongly in dyspedic myoballs than in wild-type cells because of their smaller Ca²⁺ currents. These results are in good agreement with the findings of a recent study which also found that pulse currents were reduced in dyspedic mice (Nakai *et al.* 1996). Possible reasons for the reduced Ca²⁺ currents might be that the absence of the RyR in dyspedic mice either affects the expression level of functional DHPRs or alters their functional properties. To distinguish between these possibilities, we carried out DHP-binding assays. The results show that mutant muscle largely retains DHP-binding capacity (Fig. 1C), suggesting that the number of DHPRs in skeletal muscle of mutant mice is similar to that of wild-type animals. Although DHP binding does not address the question of DHPR location, it seems likely that the DHP receptors are indeed incorporated somewhere in the plasma membrane, since gating charge movements remain unaffected in muscle cells of dyspedic mice (Nakai *et al.* 1996).

We have previously suggested the presence of at least two subsets of DHP-sensitive Ca²⁺ channels in rat skeletal myoballs. One subset has classical L-type channel character-

istics and another has anomalous gating behaviour that is 'activated' or 'primed' by strong and long-lasting depolarizations without conducting significant Ca²⁺ current (silent Ca²⁺ channels; Fleig & Penner, 1995); however, upon repolarization, this second subset of channels generates large excessive tail currents. The bottom traces of Fig. 1A with depolarizations to +80 mV reveal that the tail-current amplitude in dyspedic myoballs is greatly reduced. In fact, the tail currents in these cells are even more strongly suppressed than the pulse currents. They amount to only 10% of the size in the wild-type, whereas pulse currents are reduced to one-third (see Table 1). This suggests that the RyR in some way controls the manifestation of excessive tail currents.

To examine further the differences between tail currents in the two types of cells, we took advantage of the fact that short depolarizations primarily recruit tail currents associated with the normal pulse currents, whereas longer depolarizations recruit the additional excessive tails corresponding to the postulated silent Ca²⁺ channels (Fleig & Penner, 1995). This phenomenon is depicted in Fig. 2A for wild-type myoballs, where tail currents recorded at various repolarization potentials following short (50 ms) and long (600 ms) depolarizations to +80 mV are compared. It is seen that long depolarizations produce significantly larger tail currents for each of the three example repolarizations. The repolarization potential affects both the peak amplitude of the tail current (which is determined by the driving force) and the decay kinetics (which is determined by the deactivation rate of the Ca²⁺ channels; Fleig & Penner, 1995). Figure 2B quantifies the tail-current amplitude as a function of the repolarization voltage for both short and long depolarizations. The difference between the two data sets largely reflects the size of the excessive tail currents produced by the silent Ca²⁺ channels, which suggests about 2-3 times as many silent as normal Ca²⁺ channels and is similar to our previous determination in rat skeletal myoballs (Fleig & Penner, 1995). Under identical experimental

protocols, the recordings from dyspedic myoballs fail to show the large tail currents following long (600 ms) depolarizations to +80 mV (Fig. 2C and D). Thus, functional RyRs are needed to generate excessive tail currents following long depolarizations.

Since we have previously established that excessive tail currents and facilitation of Ca^{2+} currents develop in parallel (Fleig & Penner, 1996), we would predict that the absence of excessive tails also abolishes facilitation. As illustrated in Fig. 3, this is indeed the case. Figure 3A provides typical examples of Ca^{2+} currents evoked by a stimulus protocol designed to reveal paired-pulse facilitation. Two identical voltage pulses of 300 ms to 0 mV were separated by a conditioning pulse of 600 ms to +80 mV and a brief return to -40 mV for 20 ms. The conditioning pulse induced a large tail current and a marked augmentation of the subsequent pulse current in wild-type but not in dyspedic myoballs (Fig. 3A). From the average current-voltage relationships of control and facilitated Ca^{2+} currents ($n = 5$) it is seen that the voltage dependence of facilitated Ca^{2+} currents in wild-type

cells is shifted by about 10 mV to the left, as compared with the control currents. Thus, facilitation is prominent at potentials more negative than +10 mV, in close agreement with the facilitation characteristics observed in rat skeletal myoballs (Sculptoreanu, Scheuer & Catterall, 1993; Fleig & Penner, 1996). However, dyspedic myoballs lack both excessive tails and facilitation (Fig. 3A and B, right-hand panels), which suggests that neither phenomenon is an intrinsic property of the normal Ca^{2+} channels. Instead, they might be due to the recruitment of additional Ca^{2+} channels that exhibit a slight left shift in their voltage dependence and remain silent during the depolarization (Fleig & Penner, 1996). As witnessed by the absence of tail currents and facilitation in dyspedic myoballs, these additional Ca^{2+} channels require the expression of RyRs. These results also suggest that the type 3 RyR, which is expressed at lower levels in wild-type muscle and remains present in dyspedic myoballs (Takeshima *et al.* 1994), cannot substitute for type 1 RyR to induce excessive tails and facilitation.

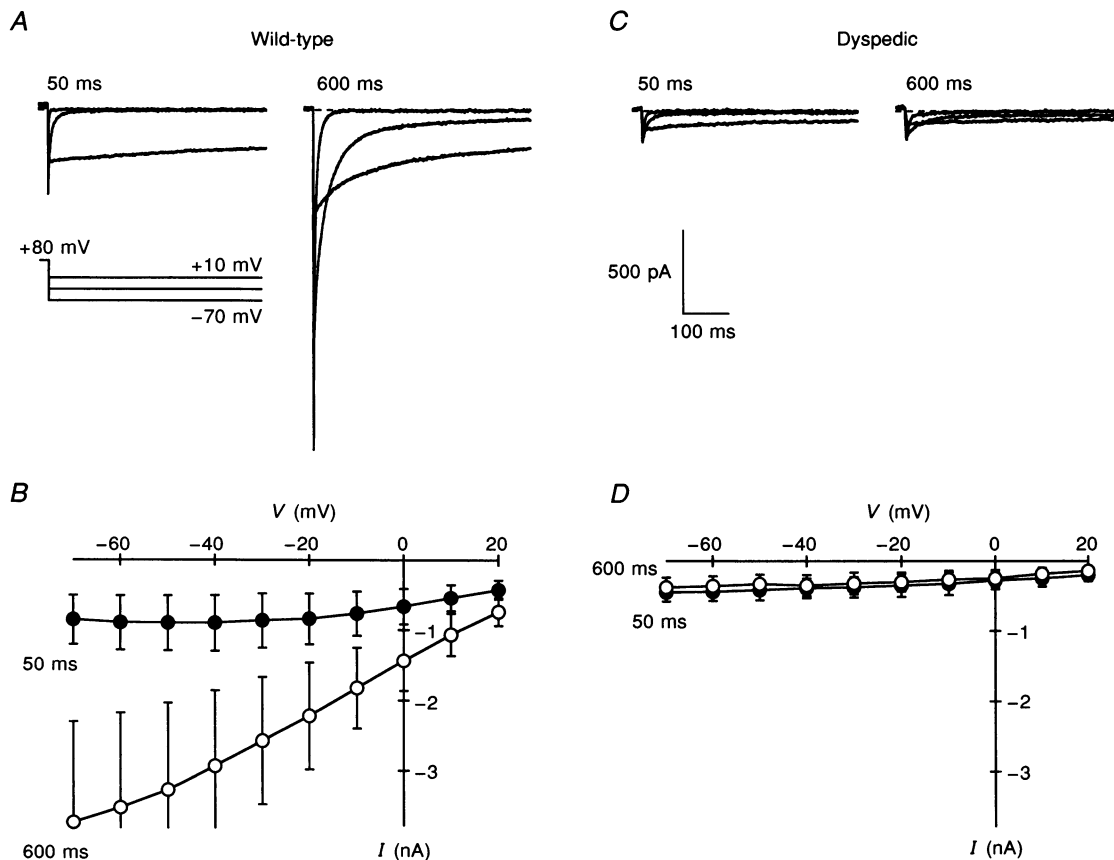


Figure 2. Dyspedic mice lack excessive tail currents

A, superimposed tail currents averaged from 5 different wild-type myoballs evoked by depolarizations of 50 ms (left) or 600 ms duration (right) to +80 mV and repolarization to -70, -30 and +10 mV. B, average tail-current amplitudes of wild-type cells plotted as a function of repolarization potential following depolarizations to +80 mV for 50 ms (●) or 600 ms (○). Data are from the same 5 cells. C, mean tail currents of dyspedic cells ($n = 4$) evoked by the same stimulus protocol as described in A. D, average tail-current amplitudes of dyspedic myoballs shown as a function of repolarization potential following depolarizations to +80 mV for 50 ms (●) or 600 ms (○). Data are from the same 4 cells.

DISCUSSION

In summary, our results indicate that myoballs derived from mice lacking functional skeletal muscle RyRs of type 1 express reduced Ca^{2+} currents and are essentially devoid of excessive tail currents and Ca^{2+} current facilitation. It is therefore clear that the RyR can modify the behaviour of DHPRs in the plasma membrane and this effect is not simply due to a change in the expression level of DHPRs, since the DHP binding assays (Fig. 1C) and gating charge measurements (Nakai *et al.* 1996) do not indicate a vastly reduced expression of DHPRs in dyspedic mice. The interpretation of our results is not straightforward, since there is no general consensus on the crucial question of whether all DHPRs can serve a dual role as voltage sensors in E–C coupling and functional Ca^{2+} channels (Lamb, 1992; Melzer *et al.* 1995) or whether there exist different functional types of DHPRs exclusively serving Ca^{2+} entry and voltage sensing (Fleig & Penner, 1995, 1996; Melzer *et al.* 1995).

Let us first assume there is only one type of DHPR functioning both as normal Ca^{2+} channel and voltage sensor. The reduced pulse-current amplitude could be interpreted such that the disruption of the physical link between RyRs and DHPRs in dyspedic myoballs decreases the Ca^{2+} current by reducing the open probability and/or the unitary conductance of the channels (Nakai *et al.* 1996). However, the most striking effect observed in dyspedic myoballs, namely the complete block of excessive tail currents and Ca^{2+} current facilitation, is not easily reconcilable with the dual function hypothesis. In particular, we find it very difficult to explain why the remaining Ca^{2+} channels, which are otherwise normal, do not show the characteristic excessive tail currents and facilitation if these were a property of a single class of DHPRs.

The alternative scenario postulates two functional types of DHPRs, which operate either as Ca^{2+} channels or as voltage sensors. We have previously presented evidence that, in

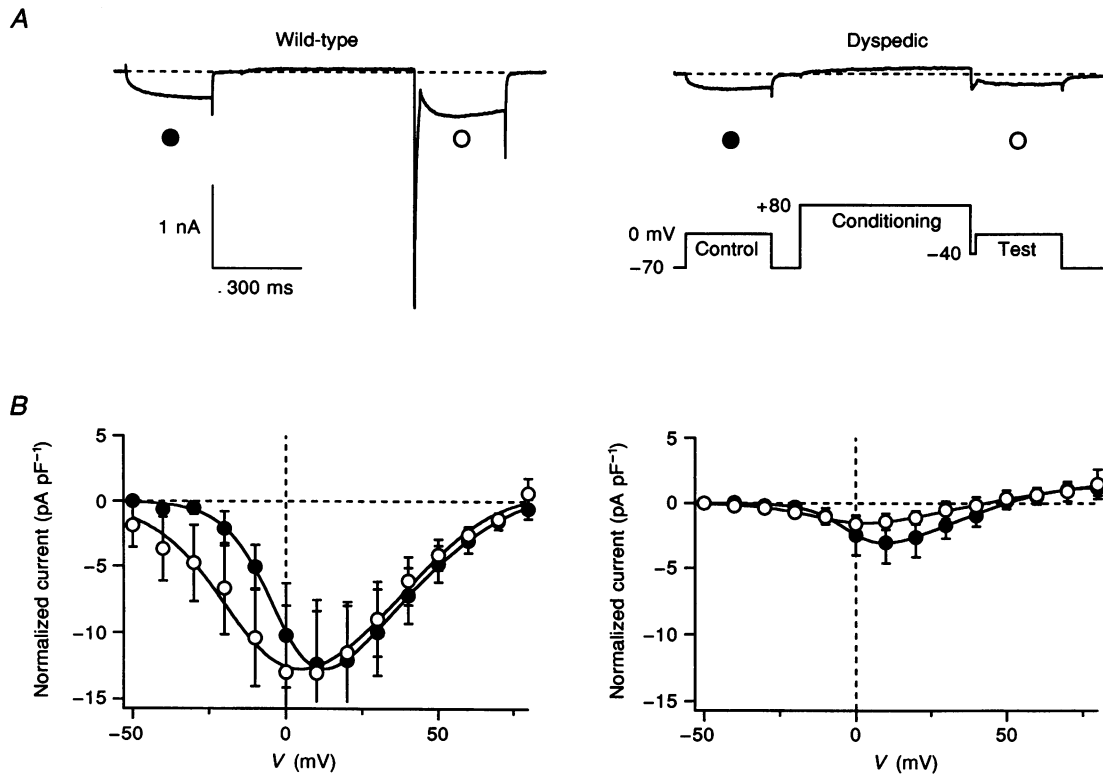


Figure 3. Dyspedic mice lack facilitation of Ca^{2+} currents

A, average Ca^{2+} currents recorded in wild-type (left panel; $n = 6$) and dyspedic myoballs (right panel; $n = 6$) evoked by a double-pulse protocol with a control pulse (●) and a test pulse (○) to 0 mV. The test pulse was preceded by a conditioning pulse of 600 ms to +80 mV and a short (20 ms) repolarization to -40 mV, and the conditioning pulse was applied 100 ms after the control pulse. *B*, average current–voltage relationships of Ca^{2+} currents for control (●) and conditioned test pulses (○) recorded in wild-type (left panel; $n = 5$) and dyspedic myoballs (right panel; $n = 4$). Same stimulus protocol as in *A* with variable test pulses between -50 and +80 mV. Data points of the *I*–*V* curve represent mean current amplitudes measured as peak amplitudes and plotted as a function of test potential. The fit to the conditioned *I*–*V* curve for wild-type cells yielded the following values: $V_{1/2} = -14$ mV, slope = 12 mV. The values for the conditioned *I*–*V* curve of dyspedic myoballs were: $V_{1/2} = -5$ mV, slope = 9.8 mV. See legend to Fig. 1 and Table 1 for the values of the control *I*–*V* curves.

addition to the classical Ca^{2+} channels responsible for the pulse currents, a large fraction of DHPRs in skeletal muscle are silent Ca^{2+} channels (Schwartz, McCleskey & Almers, 1985; Fleig & Penner, 1995). We hypothesized that the silent Ca^{2+} channels are the voltage sensors in E-C coupling, which have similar characteristics to normal Ca^{2+} channels, but do not conduct Ca^{2+} , because they are normally blocked by the RyR itself or some linking protein. However, strong and long depolarizations can cause a transient unblock of these channels, which then gives rise to excessively large tail currents, followed by a short period in which a subsequent depolarization yields facilitated Ca^{2+} currents (Fleig & Penner, 1996). If one accepts this hypothesis, the absence of excessive tails in dyspedic muscle would suggest that the silent Ca^{2+} channels when not coupled to RyRs either experience a complete loss of function or that they still activate but remain permanently blocked.

The first possibility might result if the RyRs provide some structural prerequisite for the functional integrity of the channels, maybe as a 'subunit' of a multimolecular complex. Indeed, electron microscopic analysis suggests that in wild-type muscle some of the DHPRs are arranged into tetrads and arrays of tetrads colocalized with RyRs. This arrangement is disturbed in dyspedic muscle, where the particles are not organized into these structures (Takekura *et al.* 1995). If the second possibility is true, and the channels are intact but permanently blocked, then the blocking particle must be some unidentified protein rather than the RyR itself. Nevertheless, the RyR might play a role in targeting this blocking protein to the relevant DHPRs.

But why are the normally conducting Ca^{2+} channels, which are presumably not 'coupled' to RyRs, also inhibited in dyspedic mice? One possibility is that the absence of the RyR prevents proper targeting of the blocking particle to the 'coupled' DHPRs, such that the blocking particle is now directed indiscriminately towards all DHPRs, even previously 'non-coupled' ones. Another possibility is that the product of the mutated gene construct, which yields a short peptide of twenty-four amino acids (Takeshima *et al.* 1994), might act as a blocking particle of Ca^{2+} channels, although synthetic peptides with the corresponding amino acid sequence (MGDGGEGEDEVQFLRTDDEVVLQ) at concentrations of 1 mg ml^{-1} ($385 \mu\text{M}$) failed to affect either pulse currents or facilitation ($n = 3$; data not shown). Finally, one might consider an indirect effect on pulse currents by the absence of depolarization-induced Ca^{2+} release in dyspedic muscle, as it has been reported that Ca^{2+} release itself can potentiate pulse currents (Feldmeyer, Melzer, Pohl & Zöllner, 1993). If this latter possibility applies, then the primary effect of RyR deletion might be on the silent Ca^{2+} channels (which are totally abolished) and a secondary effect would reduce the pulse currents due to the absence of Ca^{2+} release.

Clearly, the dramatic block of facilitation observed in myoballs lacking the RyR highlights the intimate relationship between Ca^{2+} channels and RyRs. It also suggests that the communication between the two proteins is not unidirectional in that the voltage sensors simply cause the opening of the RyRs, but that the RyR itself controls an important function of the DHPRs, namely facilitation, which could serve a crucial purpose in refilling Ca^{2+} stores and maintaining Ca^{2+} homeostasis during high-frequency firing of skeletal muscle.

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