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Ca^{2+}-induced Ca^{2+} release in myocytes from dyspedic mice lacking the type-1 ryanodine receptor

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While subtypes 1 and 2 of the ryanodine receptor (RyR) function as intracellular Ca^{2+} release channels, little is known about the function of the third subtype (RyR-3), first identified in brain. Myocytes from mice homozygous for a targeted mutation in the RyR-1 gene (dyspedic mice) can now be used for a study on the function of RyR-3, which is predominantly expressed in these cells according to our reverse transcription–polymerase chain reaction analysis. We here demonstrate in these myocytes caffeine-, ryanodine- and adenine nucleotide-sensitive Ca^{2+}-induced Ca^{2+} release with ~10 times lower sensitivity to Ca^{2+} than that of RyR-1. Although RyR-3 does not mediate excitation–contraction coupling of the skeletal muscle type, we propose that RyR-3 may induce intracellular Ca^{2+} release in response to a Ca^{2+} rise with a high threshold.
Key words: caffeine/Ca^{2+}-induced Ca^{2+} release/ cultured myocytes/Fura-2/ryanodine receptor subtypes

Introduction

Intracellular Ca^{2+} stores play a critical role in the regulation of the cytosolic Ca^{2+} concentration in a wide variety of cells (for reviews see Berridge, 1993; Pozzan *et al.*, 1994). Two families of Ca^{2+} release channels have been identified in the stores: the inositol 1,4,5-trisphosphate (IP_3) receptor and the ryanodine receptor (RyR). The function of RyR was first recognized in the skeletal muscle sarcoplasmic reticulum (SR) as that of a Ca^{2+}-induced Ca^{2+} release (CICR) channel, based on the enhancement of channel activity in the presence of micromolar concentrations of cytosolic Ca^{2+} (Endo, 1977). Ca^{2+} release via RyR is also modulated by various compounds, including caffeine, ryanodine and adenine nucleotides (Endo, 1977; Fleischer and Inui, 1989). The purified RyR protein has been shown to form a homo-tetrameric complex with a characteristic 'foot' structure which spans the gap between the SR and transverse (T) tubule in skeletal muscle cells (Block *et al.*, 1988; Lai *et al.*, 1988; Fleischer and Inui, 1989). The Ca^{2+} channel activity and subcellular distribution suggest that skeletal muscle RyR functions as a Ca^{2+} release channel during excitation–contraction (E–C) coupling, in which a T-tubule voltage sensor directly activates the Ca^{2+} release channel in the SR in response to depolarization, without a requirement for Ca^{2+} entry (Schneider and Chandler, 1973). Previous studies have shown that the dihydropyridine (DHP) receptor acts as the T-tubule voltage sensor (Adams and Beam, 1990; Ríos and Pizarro, 1991).

Molecular cloning of cDNAs encoding mammalian RyRs has shown that there are three subtypes of RyR, namely skeletal muscle (RyR-1), cardiac (RyR-2) and brain (RyR-3) subtypes, which are the products of three separate genes (McPherson and Campbell, 1993; Meissner, 1994). The cDNA of rabbit RyR-1 encodes ~5000 amino acid residues, comprising the putative C-terminal channel region and the remaining foot region (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990). RyR-2 and RyR-3 have slightly lower molecular masses, but share characteristic structural features with RyR-1 (Nakai *et al.*, 1990; Otsu *et al.*, 1990; Hakamata *et al.*, 1992). RyR-2 also functions as a CICR channel and mediates cardiac-type E–C coupling, in which Ca^{2+} influx through DHP-sensitive Ca^{2+} channels seems to activate RyR to induce Ca^{2+} release from the SR (Näbauer *et al.*, 1989). RyR-2 is expressed not only in muscle, but also in brain (Furuichi *et al.*, 1994). The full-length cDNA of RyR-3 was first isolated from rabbit brain and it is expressed widely throughout the body (Hakamata *et al.*, 1992). Although the functional properties of RyR-1 and RyR-2 have been studied extensively, those of RyR-3 have not yet been elucidated.

Recently we generated mice with a targeted mutation (*skrr*^{ml}) in the RyR-1 gene (Takeshima *et al.*, 1994). The mutation is thought to be a null mutation of the receptor and mice homozygous for the mutation die perinatally with muscular degeneration. Electron microscopic observation showed triad junctions lacking feet in skeletal muscle cells of these mutant (dyspedic) mice (Takekura *et al.*, 1995). E–C coupling in skeletal muscle is lost in dyspedic mice, indicating that RyR-1 functions as a Ca^{2+} release channel linked to the T-tubule voltage sensor. However, when dyspedic muscle was challenged with a high concentration of caffeine, an activator of CICR, contraction was still observed (Takeshima *et al.*, 1994). This suggests that the skeletal muscle of dyspedic mice expresses CICR channels other than RyR-1. In this report we present evidence that the residual Ca^{2+} release is most likely mediated by RyR-3. Although RyR-3 does not form functional coupling with the DHP receptor to perform skeletal muscle-type E–C coupling, we here indicate that it functions as a CICR channel which is sensitive to both caffeine and ryanodine. We discuss the possible role of RyR-3 in Ca^{2+}-mediated regulation of intracellular Ca^{2+} signalling.

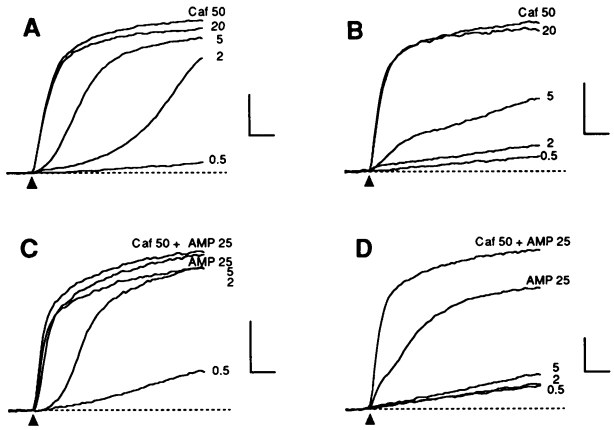

Fig. 1. Caffeine- and AMP-induced Ca^{2+} release in permeabilized control and dyspedic muscle cells. (**A** and **B**) Time course of Ca^{2+} release from the SR in response to 0.5–50 mM caffeine applied at the time point indicated by the arrowhead. (A) Control; (B) dyspedic. (**C** and **D**) Time course of Ca^{2+} release induced by 0–25 mM AMP alone or both 50 mM caffeine and 25 mM AMP applied at the time point indicated by the arrowhead. (C) Control; (D) dyspedic. Active Ca^{2+} loading of the SR was carried out before each challenge with the Ca^{2+} releasing agents. The time course of Ca^{2+} release was measured in the absence of Mg^{2+}, ATP and EGTA using 35 μM Fura-2. Results in each panel were obtained from the same preparation. Representative results of three to four experiments. Horizontal scale, 10 s; vertical scale, 5 μM total Ca^{2+} concentration change within the cuvette.

Results

CICR activity in skeletal muscle cells from dyspedic mice

Thin bundles of muscle fibres were obtained from the skeletal muscle of control and dyspedic mice. They were permeabilized by treatment with saponin and Ca^{2+} release from the SR was examined using Fura-2 as the Ca^{2+} indicator. Figure 1 shows the time course of Ca^{2+} release in response to application of 0.5–50 mM caffeine. Before each caffeine challenge, the SR was actively loaded with Ca^{2+} in the presence of MgATP and then MgATP was removed to inhibit re-uptake of Ca^{2+} during Ca^{2+} release. Both control (Figure 1A) and dyspedic (Figure 1B) muscle cells responded to caffeine, although dyspedic muscle cells were less sensitive to caffeine.

Adenine nucleotides also activate CICR (Endo, 1977; Fleischer and Inui, 1989). Therefore, we examined the Ca^{2+} releasing effect of AMP. As shown in Figure 1, AMP induced Ca^{2+} release from both control (Figure 1C) and dyspedic (Figure 1D) cells, although the sensitivity to AMP was lower in the mutant muscle. While 5 mM AMP induced definite Ca^{2+} release in the control cells, it was barely effective in the dyspedic cells. We also studied the effect of ryanodine. Ryanodine up to 100 μM concentration did not cause immediate Ca^{2+} release, as caffeine and AMP did (data not shown). However, if ryanodine was applied with caffeine, it then irreversibly abolished the subsequent caffeine response in both muscles. Such an effect has been found in rabbit and frog skeletal muscle fibres and has been attributed to locking open of the CICR channels, leading to depletion of the SR (Fleischer *et al.*, 1985; Oyamada *et al.*, 1993). These results clearly indicate that dyspedic muscle has a Ca^{2+} release mechanism with similar pharmacological properties to those of the skeletal muscle and cardiac CICR. Neither control nor dyspedic

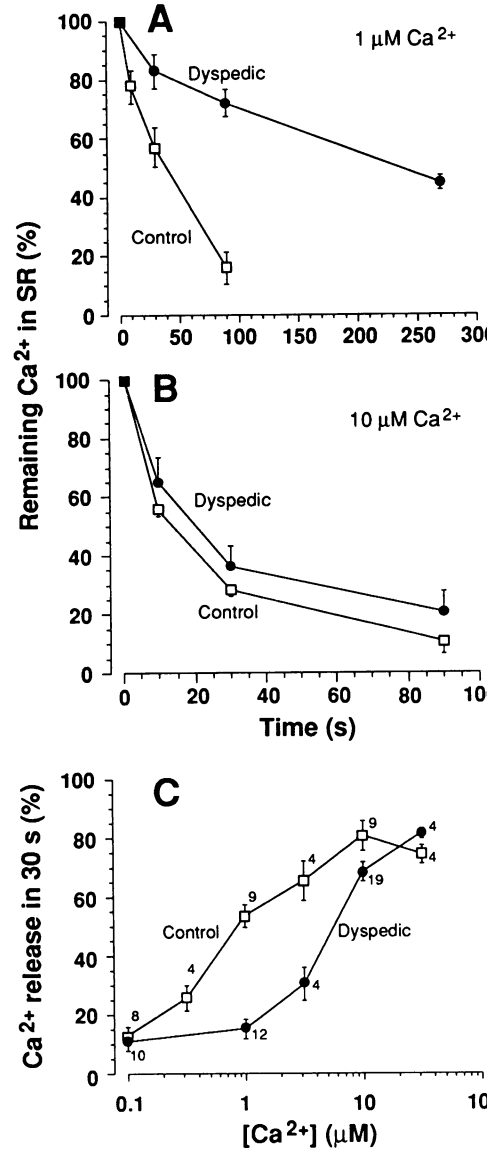

Fig. 2. CICR in control and dyspedic muscle cells. Time course of CICR at 1.0 (**A**) and 10 (**B**) μM Ca^{2+} in permeabilized control (open squares) and dyspedic (closed circles) muscle cells (mean ± SEM, n = 4 for each condition). (**C**) The amount of Ca^{2+} released from the SR within 30 s at 0.1–30 μM Ca^{2+} in control (open squares) and dyspedic cells (closed circles) (mean ± SEM, numbers by the symbols indicate the number of determinations).

muscle cells were sensitive to IP_3, even at a 10 μM concentration (data not shown).

In order to directly demonstrate the presence of the CICR mechanism in dyspedic cells, we studied the time course of Ca^{2+} release at fixed Ca^{2+} concentrations. As shown in Figure 2, Ca^{2+} in the SR declined with Ca^{2+} release time and the rate of Ca^{2+} release increased with increasing concentration of Ca^{2+} from 1 to 10 μM (note the difference in the scales of the abscissae). Unlike the experiments in Figure 1, the ambient Ca^{2+} concentration during CICR was strongly buffered with 10 mM EGTA (see Materials and methods). Although there was no large difference in the rate of Ca^{2+} release at 10 μM Ca^{2+} between the control and dyspedic cells (Figure 2B), Ca^{2+} release rate at 1 μM Ca^{2+} was significantly greater in control cells than that in dyspedic cells (Figure 2A). We

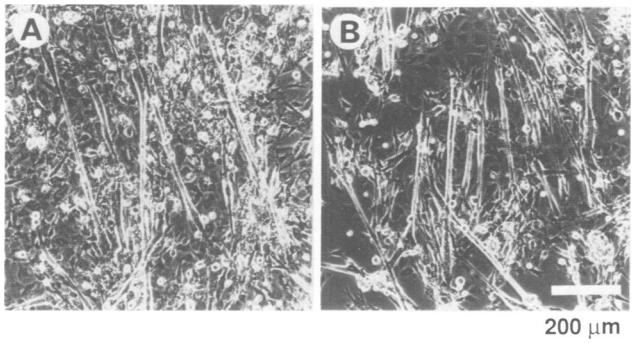

Fig. 3. Primary cultured myocytes. Phase contrast micrographs of myotubes derived from control (**A**) and dyspedic (**B**) mice. The cells were cultured for 7 days as described in Materials and methods. Bar 200 μm.

compared the amount of Ca^{2+} released within 30 s at 0.1–30 μM Ca^{2+} concentrations between control and dyspedic muscle cells (Figure 2C). Although CICR was significantly enhanced at 1 μM Ca^{2+} in control muscle, there was only a slight enhancement in dyspedic muscle at the same Ca^{2+} concentration. However, above 10 μM Ca^{2+} the amount of Ca^{2+} release was nearly the same in control and dyspedic muscle cells. These results show that both control and dyspedic SR have CICR channels and that CICR in the mutant muscle is nearly 10 times less sensitive to Ca^{2+} at the lower levels of activation.

E–C uncoupling and CICR in cultured myocytes from dyspedic mice

To further characterize CICR in the mutant muscle we prepared primary cultured cells from dyspedic neonates. Myocytes with polynuclei developed within 5 days after plating under our culture conditions. Figure 3 shows developed myotubes derived from control (Figure 3A) and dyspedic (Figure 3B) muscle. Myocytes from control mice showed spontaneous contractions and the number of spontaneously contracting cells increased in the course of culture. However, we never observed spontaneous contractions in dyspedic myocytes.

The cultured control myocytes responded with a $[Ca^{2+}]_i$ increase to electrical stimulation or to depolarization by 80 mM $[K^+]$, even in the absence of extracellular Ca^{2+} (Figure 4A and B), which is the hallmark property of skeletal muscle E–C coupling (Armstrong *et al.*, 1972). On the other hand, these stimuli were unable to induce a $[Ca^{2+}]_i$ rise in dyspedic myocytes (Figure 4C and D). Only after an increase in extracellular Ca^{2+} to 10 mM and application of 10 μM Bay K-8644 did electrical stimulation induce a $[Ca^{2+}]_i$ rise of very slow and prolonged kinetics (Figure 4E). Similarly, depolarization by 80 mM $[K^+]$ induced a $[Ca^{2+}]_i$ rise only under increased extracellular Ca^{2+} (10 mM) concentration conditions (Figure 4F). These results are in agreement with those on the contraction of skeletal muscle from dyspedic neonates (Takeshima *et al.*, 1994).

Dyspedic myocytes responded with a $[Ca^{2+}]_i$ rise to application of 25 mM caffeine in physiological salt solution (Figure 5A). Application of up to 100 μM ryanodine alone did not induce any change in $[Ca^{2+}]_i$ for at least 3 min (data not shown). Co-application of caffeine (25 mM) and ryanodine (30 μM) induced a rapid rise in $[Ca^{2+}]_i$, which

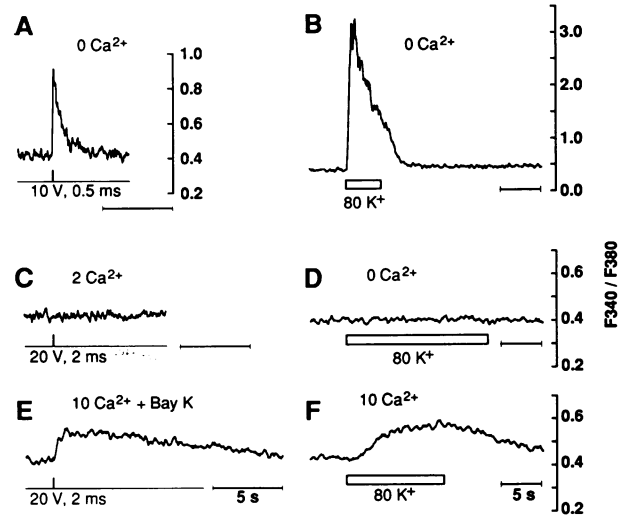

Fig. 4. $[Ca^{2+}]_i$ responses of cultured single myocytes to depolarization. (**A**) $[Ca^{2+}]_i$ change in a control myocyte evoked by field electrical stimulation (10 V, 0.5 ms duration) in Ca^{2+}-free PSS. (**B**) $[Ca^{2+}]_i$ response to depolarization by 80 mM K^+, Ca^{2+}-free PSS in the control myocyte. (**C**) Field electrical stimulation (20 V, 2 ms duration) of a single dyspedic myocyte failed to induce a $[Ca^{2+}]_i$ transient in PSS. (**D**) Depolarization by 80 mM K^+, Ca^{2+}-free PSS failed to induce a $[Ca^{2+}]_i$ rise in the dyspedic cell. (**E**) Electrical stimulation induced a $[Ca^{2+}]_i$ rise with slow kinetics in 10 μM Bay K-8644- and 10 mM Ca^{2+}-containing PSS in the dyspedic myocyte. (**F**) Depolarization of the dyspedic myocyte by 80 mM K^+ induced a $[Ca^{2+}]_i$ rise in the presence of 10 mM Ca^{2+}. Representative results in control myocytes (A and B, n = 7) and in dyspedic myocytes (C–F, n = 7).

was sustained even after withdrawal of both agents (Figure 5B and C). During this sustained rise in $[Ca^{2+}]_i$ further application of caffeine failed to induce any response (Figure 5C). Essentially the same results were obtained in the Ca^{2+}-free condition from dyspedic myocytes (n = 5) and from control myocytes (n = 15), although caffeine response was first observed earlier in control myocytes (day 4 in culture) than in dyspedic cells (day 6 in culture). The results suggest that the SR of both control and dyspedic myocytes was sensitive to caffeine and that Ca^{2+} was translocated from the SR to the cytoplasm, probably due to locking open of the Ca^{2+} release channels after co-application of caffeine and ryanodine. These properties are consistent with those of the permeabilized cells described above and indicate the presence of ryanodine receptors.

Molecular biological identification of ryanodine receptors in cultured dyspedic myocytes

Primary structures of the three members of the RyR family have been determined by the cloning of cDNAs from rabbit tissues (Takeshima *et al.*, 1989; Nakai *et al.*, 1990; Otsu *et al.*, 1990; Zorzato *et al.*, 1990; Hakamata *et al.*, 1992). However, nucleotide sequences of the mouse counterpart cDNAs have not been reported yet. To isolate cDNAs for the mouse RyR subtypes, we screened cDNA libraries derived from mouse skeletal muscle or brain mRNAs by hybridization with a probe derived from the rabbit RyR-1 cDNA. Figure 6 shows nucleotide sequences of 3′-terminal regions of the mouse RyR subtype cDNAs assigned by comparison with those of the rabbit cDNAs. No amino acid substitutions are observed between the

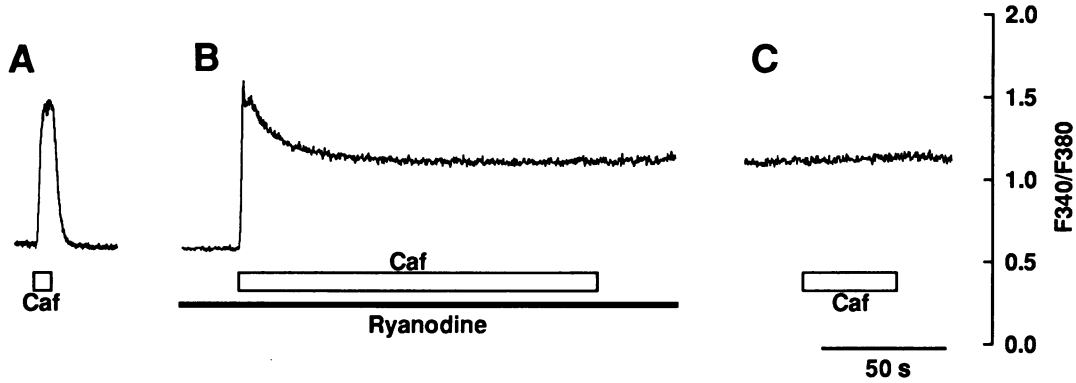

Fig. 5. $[Ca^{2+}]_i$ responses of cultured single dyspedic myocytes to caffeine and ryanodine. (**A**) Caffeine (25 mM) was applied for the period shown by the bar. (**B**) Treatment with 30 μM ryanodine and 25 mM caffeine induced a sustained rise in $[Ca^{2+}]_i$, which continued after withdrawal of both drugs. (**C**) During the sustained $[Ca^{2+}]_i$ rise after caffeine and ryanodine treatment the subsequent caffeine response was abolished. Three parts are shown from a continuous record in the same myocyte. Representative results of 11 cells in PSS.

rabbit and mouse sequences of each subtype pair in the region determined.

To amplify the specific cDNA derived from each RyR subtype mRNA, reverse transcription–polymerase chain reaction (RT-PCR) experiments were performed using three sets of primers from the 3′-non-coding sequences (Figure 7). cDNAs for RyR-1 and RyR-2 were dominantly amplified from RNA preparations from skeletal muscle and heart respectively. In contrast, three amplified cDNA fragments were observed when RNA from brain was used as the template under the same conditions. The results are consistent with the distribution of the RyR subtype mRNAs examined previously by Northern blot and *in situ* hybridization analysis (Hakamata *et al.*, 1992; Takeshima *et al.*, 1993; Furuichi *et al.*, 1994). The $[Ca^{2+}]_i$ rise induced by caffeine results in contractions of cultured muscle cells from both dyspedic and control mice. A significant proportion of the caffeine-sensitive cells detached from the culture dishes due to contraction and we were able to collect only caffeine-responsive myocytes by centrifugation of the medium. When RNA preparations from caffeine-responsive dyspedic myocytes were used as templates in the RT-PCR analysis, cDNAs of both RyR-1 and RyR-3 were amplified (Figure 7). Identification of the amplified cDNAs was further confirmed by sequencing both ends of the DNA fragments. No amplification of cDNA derived from RyR-2 mRNA in myocytes from dyspedic mice was further confirmed using another set of primers. The same results were obtained in an analysis of RNA from cultured control cells (data not shown).

Foot structure in dyspedic muscle cells

RyR-1 exists as a foot protein between the T-tubule and SR membranes at the triad junction (Figure 8A; Block *et al.*, 1988; Lai *et al.*, 1988; Fleischer and Inui, 1989). It is important to determine the location of residual RyR within dyspedic skeletal muscle cells. Formation of the T-tubules and assembly of the triad junctions seem to occur normally in dyspedic muscle (Takekura *et al.*, 1995), although the great majority of triads lack the characteristic foot structure in skeletal muscle from dyspedic mice, probably due to lack of RyR-1 (Figure 8B). However, we were still able to find triads that contained the foot-like proteins as minor examples in the mutant muscle (Figure 8C). The frequency of normal-like triads was <5% of total triads in our observations of hindlimb muscle from dyspedic neonates. The morphological features, such as size and arrangement in triads, of the remaining foot structure are indistinguishable from those of control muscle. The residual staining in the gaps shown in Figure 8B does not have these features. It is interesting to note that the distance between the T-tubule and the SR is reduced where the foot structures are absent, as can be clearly seen in the left hand junction of the triad shown in Figure 8C (Takekura *et al.*, 1995).

Discussion

Our RT-PCR experiments show that cultured muscle cells from dyspedic mice contain RNA species derived from both RyR-1 and RyR-3 genes. The absence of RyR-2 mRNA was confirmed using two sets of PCR primers. As reported previously, mRNA derived from the RyR-1 gene with the *skrr*^{m1} mutation carries translation termination codons and the neomycin resistance gene in its 5′-terminal region, corresponding to exon 2. The mutant mRNA of RyR-1, therefore, does not encode a functional protein (Takeshima *et al.*, 1994). Thus the results of the RT-PCR analysis indicate that RyR-3 exists in the cultured dyspedic myocytes, but neither RyR-1 nor RyR-2 are present. The same may hold in skeletal muscle cells of dyspedic neonates.

RyR-3 does not seem to be expressed in compensation for the failed expression of RyR-1 in dyspedic muscle cells, because RyR-3 cDNA was amplified from not only dyspedic but also control cultured myocytes in the RT-PCR analysis, although the dominant subtype in the control myocytes was RyR-1. On the other hand, almost equal expression of two RyR subtypes has been reported in skeletal muscle from chick and frog (Sutko *et al.*, 1991; Murayama and Ogawa, 1992) and both subtypes from chick were found to function as CICR channels (Percival *et al.*, 1994). In frog the subtypes were shown to be the counterparts of mammalian RyR-1 and RyR-3 by cloning and sequencing the cDNAs (Oyamada *et al.*, 1994). While our results indicate that RyR-1 is the key protein in skeletal muscle E–C coupling, the role of RyR-3 in skeletal muscle cells remains to be clarified. It has been suggested that there is a secondary Ca^{2+} release component that involves CICR in skeletal muscle E–C coupling

A

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 Q  L  S  *
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B

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------(M4)-
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C

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CAATAAAAAGAAGTGCCCACTGCAATAAAGT-(poly A)
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Fig. 6. Nucleotide sequences of 3′-terminal regions of mouse RyR subtype cDNAs and deduced amino acid sequences. The data from cDNAs for mouse RyR-1 (**A**), RyR-2 (**B**) and RyR-3 (**C**) are shown. The one letter amino acid notation is used. Putative polyadenylation signals in nucleotide sequences are underlined and the predicted fourth transmembrane segment (M4) in amino acid sequences are overlined. In the nucleotide sequence of the RyR-2 cDNA N represents an unknown base which could not be determined by sequencing either strand. The GSDB, DDBJ, EMBL and NCBI accession numbers for the sequences reported are D38216–D38218.

(Schneider, 1994). It is, therefore, an interesting possibility that RyR-3 may be responsible for this amplifying process.

Although the properties of RyR-1 and RyR-2 have been studied extensively, little is known about the function of RyR-3. As shown above, the dominant RyRs in skeletal muscle cells from control and dyspedic mice are RyR-1 and RyR-3 respectively. Our results on the functional properties of dyspedic muscle cells, therefore, show that

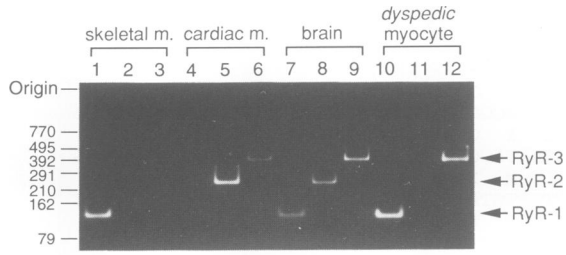

Fig. 7. RT-PCR analysis of RNA from cultured dyspedic myocytes using RyR subtype-specific primers. Templates used were total RNAs from adult mouse skeletal muscle (lanes 1–3), heart (lanes 4–6), brain (lanes 7–9) and cultured dyspedic myocytes (lanes 10–12). Amplifications were carried out with primers from the sequences of cDNAs for RyR-1 (lanes 1, 4, 7 and 10), RyR-2 (lanes 2, 5, 8 and 11) and RyR-3 (lanes 3, 6, 9 and 12). The size markers are indicated in base pairs (bp). Amplified cDNAs of 125, 251 and 378 bp are expected from RyR-1, -2 and -3 mRNAs respectively. Note the differences in amplifying cycles and amounts of templates used between the analysis of RNAs from mouse tissues and cultured cells (see Materials and methods).

RyR-3 functions as a CICR channel with sensitivities to caffeine, adenine nucleotides and ryanodine. Thus these properties are commonly shared by all subtypes of RyRs. There are, however, quantitative differences. The Ca^{2+} sensitivity of CICR mediated by RyR-3 is significantly lower than that of RyR-1 under equivalent conditions (Figure 2). Since the cardiac CICR channel (RyR-2) has similar or even higher Ca^{2+} sensitivity than its skeletal muscle counterpart (Coronado *et al.*, 1994), the Ca^{2+} sensitivity of RyR-3 is different from that of RyR-2. Therefore, our results indicate that RyR-3 has the lowest Ca^{2+} sensitivity among the members of the mammalian RyR family. In the next stage of the study it will be important to compare functional properties using purified RyR proteins. In this respect it is interesting to note that RyR purified from lobster muscle is immunologically distinct from mammalian skeletal and cardiac RyRs and shows channel activity with extremely low Ca^{2+} sensitivity (Seok *et al.*, 1992).

In a previous study cells expressing a RyR-3 homologue were shown to be insensitive to caffeine, although they were sensitive to ryanodine (Giannini *et al.*, 1992). Since the primary effect of caffeine is enhancement of the Ca^{2+} sensitivity of CICR, caffeine-induced Ca^{2+} release is secondary to the positive feedback mechanism of CICR (Endo, 1977). Therefore, cells with low Ca^{2+} content in their stores or expressing low levels of RyR may fail to respond to caffeine, due to an inability to form a sufficiently strong positive feedback loop. The lower sensitivity to Ca^{2+} of RyR-3 is another adverse factor for the formation of a feedback loop, which seems to be the reason why we observed lower sensitivity to caffeine in dyspedic cells (Figure 1). One or a combination of these factors could have been responsible for the failure to observe a caffeine response in the previous study.

Although RyR-3 does not mediate skeletal muscle E–C coupling (Figure 4C and D), Ca^{2+} influx through voltage-dependent Ca^{2+} channels in the plasma membrane may activate RyR-3 through a CICR mechanism. RyR-1 is known to face the DHP-sensitive Ca^{2+} channel, forming a foot structure between the T-tubule and SR membranes (Block *et al.*, 1988). We observed foot structures even in dyspedic skeletal muscle cells, which do not express RyR-1, although their number was greatly reduced (Figure 8). Therefore, not only RyR-1 but also RyR-3 appears to have the potential to form close coupling with the Ca^{2+} channels in the surface membrane. Such localization may be optimal for sensing a local rise in Ca^{2+} concentration resulting from Ca^{2+} influx through Ca^{2+} channels in the

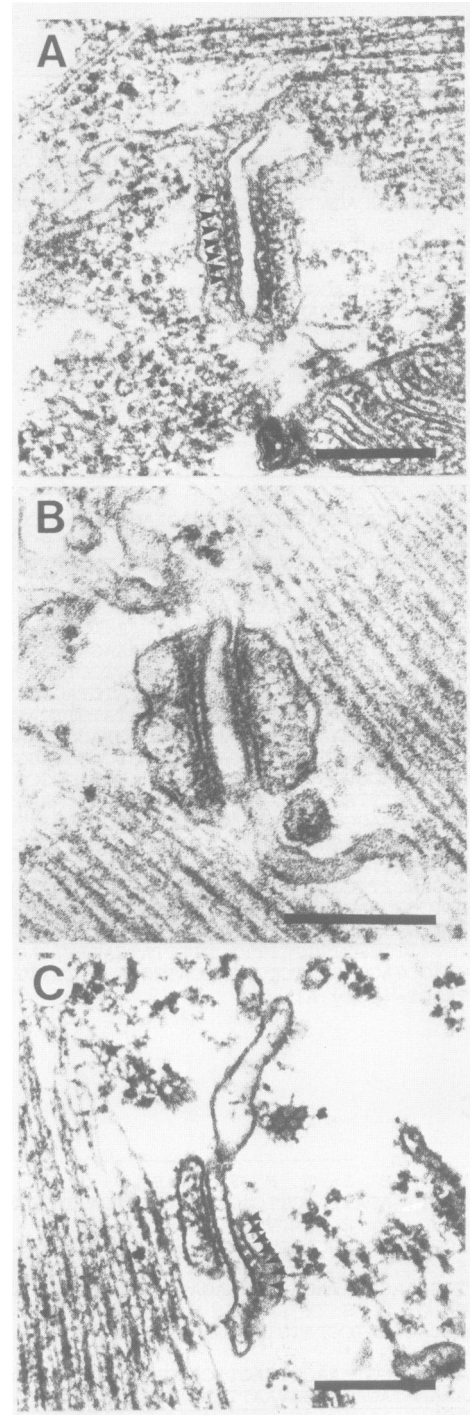

Fig. 8. Triad junctions in skeletal muscle from dyspedic neonates. (**A**) A triad junction in hind limb muscle from a control neonate. Foot structures (arrowheads) are present in junctional gaps between the SR and T tubule. (**B**) A representative triad junction in muscle from a dyspedic neonate. Note the absence of characteristic foot structures in the junctional gap. (**C**) A small fraction of triad junctions bear foot structures (arrowheads) in muscle from dyspedic neonates. Bars 1 μm.

plasma membrane. Since our results indicate that RyR-3 is the least sensitive to Ca^{2+} among the RyR family members, it appears to be tuned to respond specifically to a local Ca^{2+} rise of very high amplitude, ignoring low level inputs. It has been shown that RyR-3 is expressed in various cells, including neuronal cells (Hakamata *et al.*, 1992; Furuichi *et al.*, 1994). Therefore, we propose that RyR-3 may be involved in the voltage- and Ca^{2+}-mediated regulation of intracellular Ca^{2+} signalling with a high threshold in many types of cells.

Materials and methods

Dyspedic mice

Three lines of mice heterozygous for *skrr*^{ml}, which differ in the original embryonic stem cell clone, have been maintained independently (Takeshima *et al.*, 1994). The homozygous mutant neonates were obtained by mating the heterozygotes and their litter mates were used as controls. The genotypes of all the neonates used for the experiments were determined by PCR analysis. The results from heterozygous and wild-type control mice were combined, because we have not found any noticeable difference in the results between the two groups. The experimental data in this report have been confirmed using at least two lines of dyspedic mice.

Ca^{2+} release measurement in permeabilized cells

Muscle bundles were obtained from limbs of newborn mice and were permeabilized by treatment with a relaxing solution [4.76 mM ATP, 5.54 mM Mg methanesulfonate ($MgMs_2$), 108.6 mM KMs, 20 mM NaN_3, 20 mM PIPES, 1 mM EGTA, pH 7.0] containing 50 μg/ml saponin for 60 min. The amount of Ca^{2+} released from permeabilized cells was quantified with Fura-2 (Grynkiewicz *et al.*, 1985) using a method described elsewhere (Iino, 1989). Briefly, thin bundles (150–200 μm in diameter, ~1.5 mm in length) of muscle fibres were dissected from the muscle and were mounted in a capillary cuvette through which solutions can be rapidly perfused using a computer controlled valve and pump system. The cuvette was mounted on the stage of a microscope equipped with a fluorometer for the measurement of the fluorescence intensity of Fura-2 at 510 nm with alternating 340 and 380 nm excitations at room temperature (21–23°C). The SR of the permeabilized cells was actively loaded with Ca^{2+} (0.2 μM Ca^{2+} for 120 s at 4 mM MgATP), then both Ca^{2+} and MgATP were removed from the bathing solution. Finally, a Ca^{2+} releasing stimulus was applied in the presence of 35 μM Fura-2.

To study CICR, Ca^{2+} solution buffered with 10 mM EGTA (to avoid Ca^{2+} concentration changes due to released Ca^{2+}) was applied in the absence of both Mg and ATP (to avoid re-uptake of Ca^{2+}) for the desired length of time after a constant Ca^{2+} loading of the SR. After termination of Ca^{2+} release, Ca^{2+} remaining in the SR was quantified by releasing all Ca^{2+} from the SR with 50 mM caffeine and 25 mM AMP in the presence of Fura-2. Using this method, we were able to study unidirectional Ca^{2+} release without either a feedback effect of released Ca^{2+} or Ca^{2+}-ATPase function (Iino, 1989).

Cell culture

Primary cultures of myoblasts from mouse neonates were prepared as outlined by Beam *et al.* (1986), with minor modifications as below. Minced muscles from limbs were incubated in Ca^{2+}/Mg^{2+}-free physiological salt solution (PSS) containing 2 mg/ml collagenase (Type IA, Sigma) at 37°C for 40 min. The resulting cell suspension was filtered through nylon mesh (70 μm; Falcon), pre-plated for 1 h in a glass dish to remove rapidly adhering cells and then plated onto plastic dishes (Primaria; Falcon) or polylysine-coated coverslips. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum for 3 days and then the medium was replaced with 10% horse serum containing DMEM. The cultured muscle cells were used for $[Ca^{2+}]_i$ measurements and PCR analysis 6–11 days after plating.

$[Ca^{2+}]_i$ measurement in cultured myocytes

Cultured cells were loaded with Fura-2 in PSS (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5.6 mM glucose, pH adjusted to 7.4 with NaOH) by incubation in 10 μM Fura-2AM and 0.005% Pluronic F-127 for 30–35 min at room temperature. The fluorescence intensity of Fura-2 at 510 nm was measured with double wavelength excitation at 340 and 380 nm (alternating at 1000 Hz) using a light source system (CAM-230; JASCO, Japan) attached to an epifluorescence inverted microscope (Nikon) at room temperature. The ratio of the background-corrected fluorescence intensities (F_{340}/F_{380}) was used as the indicator of $[Ca^{2+}]_i$ in cultured myocytes. Cells were viewed using a water immersion objective (WPLAN ×40 UV, NA = 0.7; Olympus). Drugs were applied to the cells using a puffing pipette. Electrical field stimulation was delivered through a pair of platinum wires placed near the cell. $CaCl_2$ (2 mM) in PSS was replaced by equimolar $MgCl_2$ to prepare Ca^{2+}-free PSS. High K^+ (80 mM) PSS was prepared by replacing appropriate amounts of Na^+ and Cl^- in PSS with K^+ and methanesulfonate respectively, so that the $[K^+]$ $[Cl^-]$ product remained constant.

cDNA cloning and RT-PCR analysis

Oligo(dT)-primed mouse skeletal muscle and brain cDNA libraries were constructed in phage λgt10. The libraries were screened with the *Sma*I(13290)–*Sma*I(15181) fragment from clone pRR616 (Takeshima *et al.*, 1989). The cDNA inserts from hybridizing phages were subcloned into pBluescript SK(–) (Stratagene) and analysed by sequencing (Sanger *et al.*, 1977). The cDNA clones were classified into three groups and assigned to the rabbit RyR cDNAs on the basis of the nucleotide sequences obtained. Of the clones obtained, λMSKRR11 and λMSKRR12 were shown to encode mouse RyR-1, λMBRR4 and λMBRR417 to encode mouse RyR-2 and λMBRR24 to encode mouse RyR-3.

Total RNAs were prepared from adult mouse tissues by the GTC/CsCl method (Chirgwin *et al.*, 1979). Cultured muscle cells in a 35 mm dish were treated with 25 mM caffeine and cells detached from the dish (~100 cells) were recovered by centrifugation. RNAs were prepared from the cell precipitates by the AGTPC method (Chomczynski and Sacchi, 1987). After treatment with DNase I (FPLC grade; Pharmacia), RNA samples were used as templates in oligo(dT)-primed reverse transcription by means of a cDNA synthesis system (BRL). PCR reactions (Saiki *et al.*, 1988) were performed using 45 pmol of the primers listed below. The cDNAs transcribed from 0.2 μg RNA derived from mouse tissues were incubated for 27 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The cDNAs from RNAs derived from approximately five myocytes were amplified under the same conditions except that the number of cycles was increased to 35. An equivalent volume of each reaction mixture was analysed on a 5% polyacrylamide gel.

RyR-1 forward: GCTTAGCTGAGGTCTGCAGCTGG
reverse: AGGGGGTGTAGCACAGGATTTAT

RyR-2 forward I: GAATTCATCATGGATACTCTACC
reverse I: GTCATGCACATTATCTTCTGCAT
forward II: TAATTCATTGCATGTTTGTTATGC
reverse II: AAAAGATGGCCTGTCAAGGCGTC

RyR-3 forward: CCTGAGTTCACGACAAGCTACAG
reverse: TAGCTGCTTAAAGCTTTTCAAGC

Electron microscopy

Hind limb muscles of control and dyspedic neonates were fixed *in situ* at resting length for 2 h with 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.4) at 4°C, cut into small pieces and post-fixed for 2 h in 2% OsO_4 (in H_2O) at 4°C. Samples were then *en bloc* stained for 1 h in saturated aqueous uranyl acetate at room temperature, dehydrated in a graded ethanol series (70, 80, 90, 95 and 100%) and embedded in Epon after incubation for 2 h in an Epon:acetone mixture (1:1). Thin sections (<50 nm) were cut using an ultramicrotome (Ultracut E; Reichert-Jung, Austria), stained with lead and uranyl acetate and examined in an electron microscope (JEM-2000EX, JEOL, Japan) at 80 keV.

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Note added in proof

Expression of the RyR-3 gene in mouse skeletal muscle was reported by Giannini *et al.*, 1995 (*J. Cell. Biol.*, **128**, 893–904) after the submission of the manuscript.