The EMBO Journal Vol.17 No.10 pp.2790–2798, 1998

Functional properties of the ryanodine receptor type 3 (RyR3) Ca^{2+} release channel

Alois Sonnleitner, Antonio Conti[1], Federica Bertocchini[1], Hansgeorg Schindler and Vincenzo Sorrentino[1,2,3]

Institute for Biophysics, University of Linz, Austria, [1]DIBIT, San Raffaele Scientific Institute, Milan and [2]Department of Biomedical Sciences, School of Medicine, University of Siena, Siena, Italy

[3]Corresponding author
e-mail: sorrenv@dibit.hsr.it

Single-channel analysis of sarcoplasmic reticulum vesicles prepared from diaphragm muscle, which contains both RyR1 and RyR3 isoforms, revealed the presence of two functionally distinct ryanodine receptor calcium release channels. In addition to channels with properties typical of RyR1 channels, a second population of ryanodine-sensitive channels with properties distinct from those of RyR1 channels was observed. The novel channels displayed close-to-zero open-probability at nanomolar Ca^{2+} concentrations in the presence of 1 mM ATP, but were shifted to the open conformation by increasing Ca^{2+} to micromolar levels and were not inhibited at higher Ca^{2+} concentrations. These novel channels were sensitive to the stimulatory effects of cyclic adenosine 5′-diphosphoribose (cADPR). Detection of this second population of RyR channels in lipid bilayers was always associated with the presence of the RyR3 isoform in muscle preparations used for single-channel measurements and was abrogated by the knockout of the RyR3 gene in mice. Based on the above, we associated the novel population of channels with the RyR3 isoform of Ca^{2+} release channels. The functional properties of the RyR3 channels are in agreement with a potential qualitative contribution of this channel to Ca^{2+} release in skeletal muscle and in other tissues.

Keywords: Ca^{2+}-release channels/Ca^{2+} signalling/cyclic adenosine 5′-diphosphoribose/ryanodine receptor type 3

Introduction

Two families of intracellular Ca^{2+} release channels are known (Berridge, 1993): one is sensitive to the second messenger inositol trisphosphate ($InsP_3$) and is referred to as the $InsP_3$ receptor family (Furuichi *et al*., 1994; Kasai and Petersen, 1994); the other is characterized by channels named ryanodine receptors (RyRs) since they are able to bind the plant alkaloid ryanodine (Inui *et al*., 1987; Meissner, 1994; Sorrentino, 1995). Ca^{2+} release through $InsP_3$ receptors is a ubiquitous mechanism for regulation of intracellular Ca^{2+} levels (Berridge, 1993). At least three different $InsP_3$ receptors are known and all are activated to release Ca^{2+} upon $InsP_3$ binding (Furuichi *et al.*, 1994). Regulation of RyR channels is less understood and apparently more complex. Three genes coding for RyRs have been identified, including the so-called skeletal and cardiac isoforms, RyR1 and RyR2, and the more recently identified RyR3 (Meissner, 1994; Sorrentino, 1995; Sutko and Airey, 1996). All available data indicate that RyRs are homotetrameric channels formed by four identical protomers (Sutko and Airey, 1996).

Studies in vertebrate striated muscles have provided the most advanced information on how RyRs are activated. Depolarization of the plasma membrane triggers Ca^{2+} release from the sarcoplasmic reticulum through the RyRs, and the resulting increase in Ca^{2+} concentration activates contraction of the myofibrillar apparatus (Rios *et al*., 1992; Schneider, 1994).

The presence of RyRs has also been documented in non-contractile cells (Berridge, 1993; Sorrentino, 1995). Obviously, knowledge of the regulatory properties of the three RyR isoforms is essential in order to understand the mechanisms through which the single RyR isoforms are activated both in excitable and in non-excitable cells. RyR channel activity is activated by several compounds including Ca^{2+} at micromolar concentrations, ATP, adenine nucleotides, caffeine, halothane, ryanodine at nanomolar concentrations, sulfhydryl reagents and cyclic adenosine diphosphoribose (cADPR). RyR channel activity is blocked by Mg^{2+}, Ca^{2+} at millimolar concentrations, ryanodine at micromolar concentrations and by ruthenium red (Fleischer and Inui, 1989; Coronado *et al*., 1994; Meissner, 1994). The FK506-binding protein (FKBP12) has also been shown to regulate RyR channel properties (Brillantes *et al*., 1994). An interesting potential candidate for an intracellular regulator/modulator agent that can activate Ca^{2+} release through RyRs is cADPR, a ubiquitous metabolite of nicotinamide adenine dinucleotide (NAD^+) (Galione *et al*., 1991; Lee, 1993; Galione and Summerhill, 1995). The ability of cADPR to activate Ca^{2+} release through RyRs is well established, especially in sea urchin (Galione *et al*., 1991; Galione, 1992; Lee, 1993). However, its ability to activate mammalian RyRs and its mechanisms of action are highly debated (Lee, 1997). cADPR has been shown to stimulate Ca^{2+} release through RyR2 channels, but not RyR1 channels (Mészáros *et al*., 1993). RyR1 activation by cADPR has also been reported by Williams and co-workers, who have also proposed that cADPR may work through the ATP-binding site of RyRs (Sitsapesan *et al*., 1994, 1995; Sitsapesan and Williams, 1995b).

Of the three RyRs known, the third isoform, RyR3, is the least well characterized (Sorrentino, 1995). The RyR3 isoform, initially identified on the basis of its inducibility by transforming growth factor-β in an epithelial cell line (Giannini *et al*., 1992), has been shown to be widely expressed in mammalian and chicken tissues (Giannini *et al*., 1995; Ottini *et al*., 1996), but it has not

been associated with a specific physiological function (Takeshima *et al*., 1996). Recent reports have, however, started to shed some light on the regulatory properties of this isoform (Chen *et al*., 1997; Murayama and Ogawa, 1997). It has been shown recently that RyR3 is widely expressed in murine skeletal muscles during the post-natal phase of muscle development, while in adult animals its expression is restricted mainly to the diaphragm and the soleus muscles (Conti *et al*., 1996; Bertocchini *et al*., 1997; Tarroni *et al*., 1997). Knockout of the RyR3 gene has been shown to impair skeletal muscle contractility in neonatal mice, supporting a possible physiological role of this isoform in optimizing the contractile response in neonatal skeletal muscles (Bertocchini *et al*., 1997). These results suggest that RyR3 may contribute to the amplification component proposed in skeletal muscle excitation–contraction coupling.

Based on the evidence that, among mammalian skeletal muscles, diaphragm expresses the highest levels of RyR3, we performed single-channel experiments with bovine diaphragm muscle preparations as a source of vesicles containing both RyR1 and RyR3. Abdominal and other skeletal muscles were used as a source of vesicles containing only RyR1. Vesicles prepared from the diaphragm muscle of normal and RyR3 knockout mice were used as a further control. The results obtained indicate that: (i) at variance with RyR1 channels, RyR3 channels were not activated by Ca^{2+} at nanomolar concentration in the presence of 1 mM ATP; (ii) a shift to high open-probability was observed when Ca^{2+} concentrations were increased to the micromolar range; (iii) RyR3 channels were not inactivated at Ca^{2+} concentrations up to 1 mM; and (iv) under the condition used, RyR3 channels were activated by cADPR while RyR1 channels were not.

Results

Two distinct populations of RyR channels in sarcoplasmic reticulum from bovine diaphragm muscle

The recent identification of the RyR3 isoform in diaphragm muscles of mammals prompted us to measure the single-channel conductance and the properties of channels incorporated into planar lipid bilayers using sarcoplasmic reticulum vesicles prepared from bovine diaphragm. For comparison, microsomal vesicles prepared from muscles of the neck and abdomen of adult bovines were fused to planar lipid bilayers. Following the oriented incorporation of the channel, its cytosolic (*cis*) side was exposed to millimolar concentrations of ATP while Ca^{2+} was titrated from nanomolar to millimolar concentrations.

ATP at millimolar concentrations can activate Ca^{2+} release through RyR1 channels prepared from skeletal muscles at nanomolar Ca^{2+} concentrations (Sitsapesan

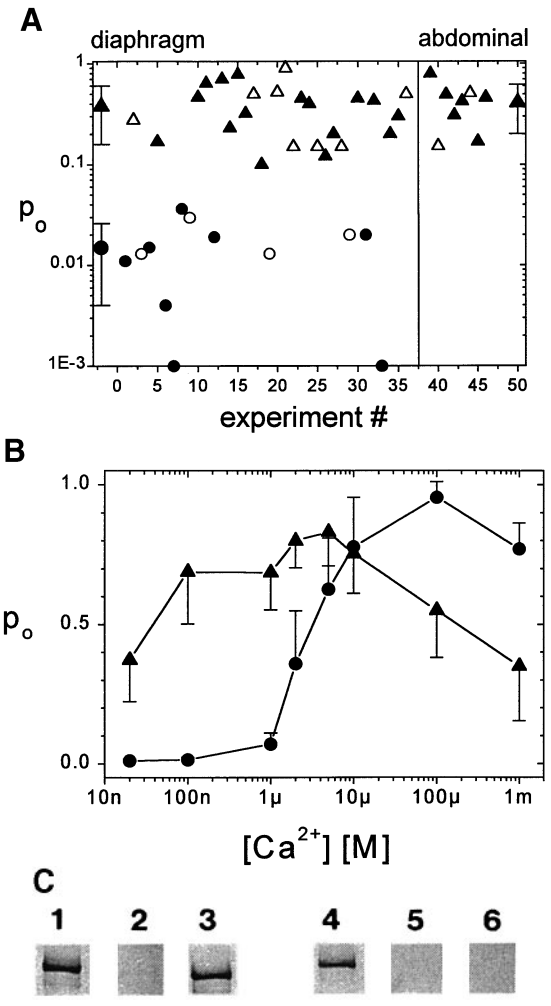

Fig. 1. (**A**) Single-channel open probability at millimolar ATP and 20 nM free Ca^{2+}. The open probability for 36 and eight experiments with channels from microsomes of bovine diaphragm and abdomen, respectively, fused to planar lipid bilayers are plotted. *Cis* buffer was 0.5–2 mM ATP, 1 mM dibromo-BAPTA, <10 μM residual Ca^{2+} (giving a free Ca^{2+} concentration <20 nM), 250 mM HEPES/110 mM Tris, pH 7.4; *trans* buffer was 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4; holding potential = 0 mV. The experiments for different muscle preparations are separated by a vertical line. Filled symbols represent experiments where an additional Ca^{2+} titration was carried out. For the experiments represented by the open symbols, the membrane broke before additional conditions could be tested. Triangles, experiments showing a bell-shaped response; circles, experiments with a sigmoidal Ca^{2+} response to increasing Ca^{2+} (see also legend to Figure 2A). The open probability for each condition was determined from at least 2 min of continuous recordings in 1024 ms bins, based on a 50% criterion. The symbols with error bars show the mean ± SD of the open probability of the RyR1 and RyR3 type channels for the diaphragm muscle on the left and the RyR1 channel from the abdominal muscle on the right. (**B**) Ca^{2+} dependence. Single-channel open probability as a function of free Ca^{2+} concentration. Holding potential, 0 mV; buffer conditions: *cis* buffer, 0.5–2 mM ATP, 1 mM dibromo-BAPTA, 10, 39, 290, 451, 676, 813, 1076 and 2076 μM $CaCl_2$ to adjust the free Ca^{2+} to 20 nM, 100 nM, 1, 2, 5, 10 and 100 μM, and 1 mM respectively, 250 mM HEPES/110 mM Tris, pH 7.4; *trans* buffer, 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4. Titrations are for independent channels from low to high Ca^{2+} concentrations. ●: mean single channel open probability from three independent experiments with bovine diaphragm muscle microsomes, representing typical behaviour for the close-to-zero open-probability population from (A). ▲: average open probability of three single-channel experiments with bovine diaphragm microsomes and three with bovine abdominal muscle microsomes, typical of the non-zero open probability population in (A). Open probabilities for each condition were determined from at least 2 min of consecutive channel trace. (**C**) RyR3 expression in diaphragm muscle but not in abdominal muscles. Microsomal proteins were prepared from bovine diaphragm (lanes 1–3) and abdominal muscles (lanes 4–6). Aliquots of 3, 50 and 30 μg of protein were loaded for RyR1 (lanes 1 and 4), RyR2 (lanes 2 and 5) and RyR3 (lanes 3 and 6) detection, respectively. Gel electrophoresis and Western blot analysis were performed as described in Materials and methods.

and Williams, 1995a; Sonnleitner *et al*., 1997). Under these conditions, in diaphragm microsomes (Figure 1A, left), two populations of Ca^{2+} channels were detected. One group of channels (24 out of 36, indicated by triangles), which are active under these conditions, match p_o values found in most skeletal muscles (e.g. Figure 1A, right panel). In contrast, the novel group of channels observed in diaphragm microsomes (12 out of 36 channels analysed) displayed p_o values (circles in Figure 1A) which are significantly lower, scattering between 0.001 and 0.036 (i.e. channels that are only a little sensitive, if not completely insensitive, to activation at nanomolar Ca^{2+} concentrations in the presence of micromolar ATP concentrations).

The difference between the two groups of channels observed in diaphragm was confirmed further by experiments in the presence of increasing Ca^{2+} concentration (Figure 1B). Starting from p_o values found at the lowest Ca^{2+} concentration (see conditions for Figure 1A), the ATP gated channels (Figure 1B, triangles) showed a bell-shaped Ca^{2+} response, as expected for RyR1 channels (see Ma and Zhao, 1994 for 6 μM Ca^{2+} *trans* ± 1 mM ATP; Copello *et al*., 1997 for 50 mM Ca^{2+} *trans*). The second class of channels, not gated by ATP (Figure 1B, circles) showed gating by Ca^{2+} with a steep characteristic Hill coefficient (2.7 ± 0.7) and an EC_{50} = 3.2 ± 0.4 μM. Activation at higher Ca^{2+} concentrations distinguishes the novel class of channels observed in diaphragm microsomes from the so-called 'low activity' RyR1 channels. Low activity RyR1 channels are not activated by ATP and are also poorly responsive to increasing concentrations of Ca^{2+} (Copello *et al*., 1997). Another clear characteristic of the novel channels found in diaphragm was their lower sensitivity to inactivation at high Ca^{2+} concentrations (i.e. the decrease in p_o values is shifted at least one order of magnitude to higher Ca^{2+} concentrations compared with RyR1 channels).

Western blot analysis of microsomes prepared from bovine diaphragm and abdominal muscles yields a clear correlation between the occurrence of two RyR channel populations and the presence of RyR isoforms. In fact, while the presence of the RyR1 isoform was observed in both abdominal and diaphragm microsomes (Figure 1C, lanes 1 and 4), the RyR3 isoform was detected only in diaphragm muscle (Figure 1C, lane 3) but not in abdominal muscle (Figure 1C, lane 6), while the cardiac isoform, RyR2, was found not to be expressed in either muscle preparation (Figure 1C, lanes 2 and 5). This evidence further suggests that the second type of channels observed in diaphragm muscle microsomes are contributed by the RyR3 isoform. Characteristic for the RyR3 channels are: (i) the lack of activation by ATP; (ii) the steep activation by Ca^{2+}; and (iii) the low inactivation at high Ca^{2+}. Results recently reported (Chen *et al*., 1997) using transfected cells expressing recombinant RyR3 channels report Ca^{2+} sensitivity for the recombinant RyR3 channels essentially similar to those of Figure 1B.

Comparison of the properties of RyR1 and RyR3 channels in bovine diaphragm muscle

Figure 2A compares typical channel traces for RyR3 and RyR1. The left panel gives channel traces for a single-channel experiment, where the channel initially was not activated by 1 mM ATP at <20 nM free Ca^{2+}, a response we assigned to RyR3. When Ca^{2+} was increased up to 2 μM, the channel was almost completely open and did not close at free Ca^{2+} concentrations as high as 1 mM. The right panel gives the typical response of a RyR1 channel, which is characterized initially by its ability to be activated by ATP at low free Ca^{2+} concentrations <20 nM. This initial activity increases as Ca^{2+} rises to 10 μM and then decreases with increasing free Ca^{2+}. Characteristic of RyRs is their response to ryanodine, a plant alkaloid, and to ruthenium red (Coronado *et al*., 1994; Meissner, 1994). As shown in Figure 2B, both RyR1 and RyR3 channels from diaphragm muscle were blocked by 10 μM ryanodine (top traces) at 41 ± 5 and 51 ± 5 pS conductance, respectively. The RyR1 and RyR3 channels were completely blocked by ruthenium red (bottom traces). RyRs can also be identified by their conductivity, as shown in Figure 2C: RyR3 showed a single-channel conductance of 105 ± 8 pS, comparable with the observed RyR1 single-channel conductance of 96 ± 11 pS.

RyR3 channels respond to cADPR

Reports describing responsiveness to cADPR for RyR1 and RyR2 have been published over the last years (Mészáros *et al*., 1993; Sitsapesan *et al*., 1994, 1995; Sitsapesan and Williams, 1995a,b). We tested whether cADPR would affect RyR3 channel open probability. Figure 3 shows a typical response of a RyR3 channel to 1 μM cADPR. RyR3 channels were identified by their low activity in the presence of ATP at nanomolar concentrations of Ca^{2+}. These channels, however, were activated at much lower levels of Ca^{2+} in the presence of cADPR. cADPR sensitivity was observed in six out of 10 experiments with RyR3 channels in the presence of 0.5–1 μM cADPR. No difference was observed between these two concentrations of cADPR. In the presence of cADPR, the average EC_{50} was 0.38 ± 0.2 μM, which is a shift to lower levels compared with the absence of cADPR (EC_{50} = 3.2 ± 0.2 μM; dashed line: Hillfit to circles in Figure 1B). The average Hill coefficient (n_H = 2.3 ± 0.5) was not altered significantly compared with the Hill coefficient for experiments under identical conditions but in the absence of cADPR (n_H = 2.7 ± 0.7; experiments with sigmoidal Ca^{2+} response from Figure 1B). For the six observed cADPR-sensitive RyR3 channels, the average p_o increased from 0.02 ± 0.02 to 0.41 ± 0.15 upon addition of cADPR.

The subsequent addition of the antagonist 8-Br-cADPR reduced the open probability to control levels in both RyR3 channels that had been activated by cADPR (not shown). RyR1 channels were not found to respond to cADPR in five out of five cases in single-channel experiments and in seven out of seven cases in multichannel experiments. No effect of a subsequent addition of 8-Br-cADPR was observed in all three single-channel experiments with RyR1 channels.

RyR3 channel characteristics are absent in RyR3 knockout mice diaphragm

The previous data indicated that the presence of the novel population of RyR channels, with functional properties distinct from RyR1 channels, appeared to reflect the

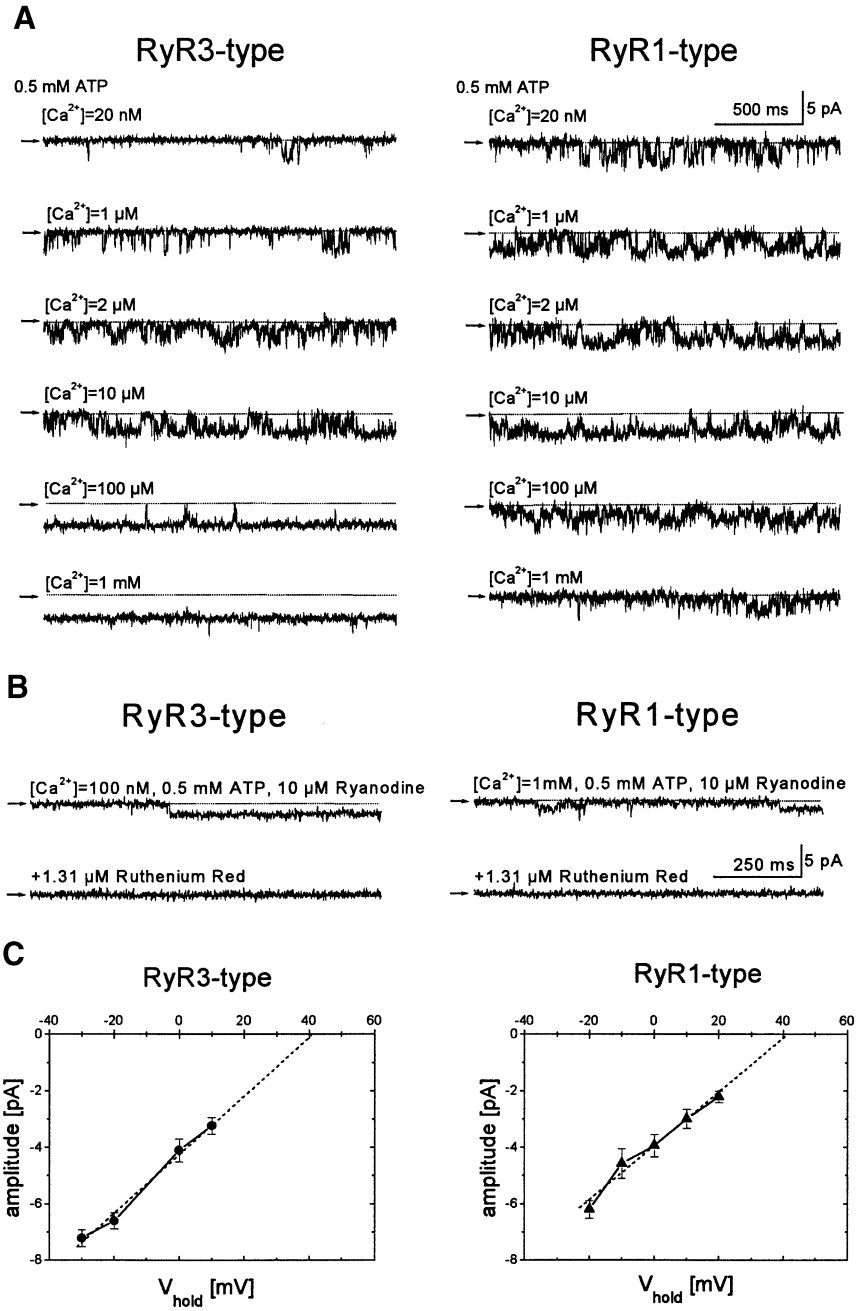

Fig. 2. (**A**) The left panel (RyR3 type) shows typical channel traces from a single-channel experiment with bovine diaphragm microsomes for channels with a sigmoidal Ca^{2+} response in Figure 1B. The right panel (RyR1 type) shows typical single-channel traces for channels with a bell-shaped Ca^{2+} response. Channel openings are shown as downward deflections for the indicated concentrations of free Ca^{2+}. Holding potential = 0 mV. The baseline is indicated by the dashed line and the arrow. The channel traces were filtered at 300 Hz. For buffer conditions see legend to Figure 1B. (**B**) Ryanodine and ruthenium red response of RyR1 and RyR3 channels. The upper traces in the left and right panel give the response of a RyR3 and RyR1 type channel to 10 µM ryanodine at subactivating conditions. *Cis* buffer: 250 mM HEPES, 110 mM Tris pH 7.4, 0.5 mM ATP, 1 mM dibromo-BAPTA, 39 or 2079 µM $Ca(OH)_2$/HEPES for RyR3 and RyR1, respectively. *Trans* buffer: 250 mM HEPES/50 mM $Ca(OH)_2$ pH 7.4. Holding potential = 0 mV. Openings are shown as downward deflections from the closed channel level indicated by the arrow and dashed line. Subconductance levels were 51 ± 5% and 41 ± 5% of the full conductance under comparable conditions for RyR3 and RyR1, respectively. Bottom traces: channel activity after addition of 1.31 µM ruthenium red. (**C**) Conductivity. The left and right panel give the mean single channel current amplitude as a function of the holding potential for RyR3 and RyR1, respectively. Dotted line: linear fit giving 105 ± 8 pS (RyR3) and 96 ± 9 pS (RyR1). Data points are the means of three and four independent experiments for RyR3 and RyR1, respectively; error bars show the standard deviation. Buffer conditions: *cis* buffer, 250 mM HEPES, 110 mM Tris, pH 7.4, 0.5 mM ATP, 1 mM dibromo-BAPTA, 2076 and 0 µM $Ca(OH)_2$/HEPES (free Ca^{2+}: 1 mM and <20 nM) for RyR3 and RyR1, respectively; *trans* buffer, 250 mM HEPES/50 mM $Ca(OH)_2$, pH 7.4.

presence of the RyR3 isoform in the different muscle preparations that were analysed. To establish further a link between this population of channels and the RyR3 isoform of the Ca^{2+} release channel, we took advantage of the development in our laboratory of a strain of knockout mice for the RyR3 gene (Bertocchini *et al.*, 1997). Therefore, microsomes were prepared from the diaphragm of normal and RyR3 knockout mice. Under conditions similar to those of Figure 1A, in microsomes prepared from diaphragm muscle of wild-type mice, two

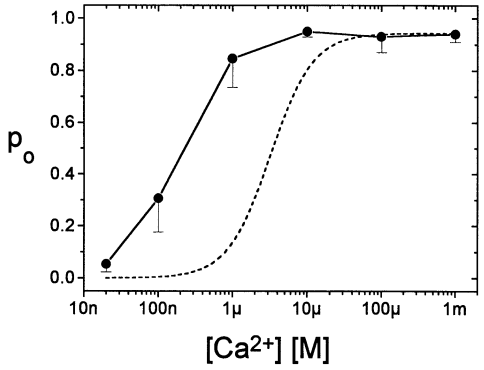

Fig. 3. cADPR effects on a RyR3 channel. Buffer conditions: *cis* buffer, 1 µM cADPR, 1 mM ATP, 1 mM dibromo-BAPTA, 39, 290, 451, 676, 813, 1076 and 2076 µM $CaCl_2$ to adjust the free Ca^{2+} to 100 nM, 1, 2, 5, 10 and 100 µM, and 1 mM, respectively, 250 mM HEPES/110 mM Tris pH 7.4; *trans* buffer, 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4. Holding potential = 0 mV. The channel from bovine diaphragm muscle, displaying close to zero p_o in the presence of 1 mM ATP but absence of Ca^{2+} (<20 nM), therefore identified as RyR3, was activated by Ca^{2+} in the presence of 1 µM cADPR. The error bars give the standard deviation of the open probability in 2048 ms bins of a 2 min recording for each condition. Dashed line: Hillfit to the data with sigmoidal Ca^{2+} response from Figure 1B.

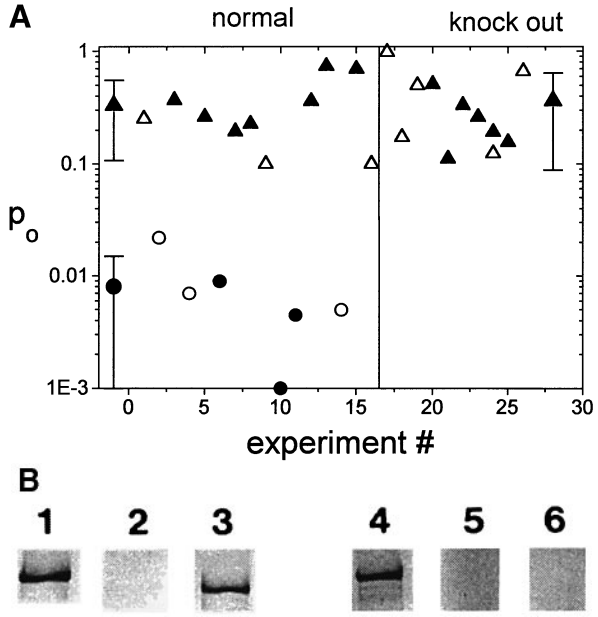

Fig. 4. (**A**) Single-channel open probability at millimolar ATP and 20 nM free Ca^{2+}. The open probability for 16 and 11 experiments with channels from microsomes of normal and RyR3 knockout mice, respectively, fused to planar bilayers is plotted. *Cis* buffer, 0.5–1 mM ATP, 1 mM dibromo-BAPTA, <10 µM residual Ca^{2+} (<20 nM free Ca^{2+}), 250 mM HEPES/110 mM Tris, pH 7.4; *trans* buffer, 50 mM $Ca(OH)_2$/250 mM HEPES, pH 7.4. Holding potential = 0 mV. The open probability was determined from at least 2 min of continuous recordings in 1024 ms bins, based on a 50% criterion. The symbols with error bars show the mean ± SD of the open probability of the RyR1 and RyR3 type channels for the diaphragm muscle of normal mice on the left and of the RyR1 channel for the knockout mouse on the right. (**B**) RyRs in diaphragm muscles from normal and RyR3 knockout mice. Microsomal proteins were prepared from diaphragm muscle of adult normal (lanes 1–3) and of RyR3 knockout mice (lanes 4–6). Aliquots of 3, 50 and 30 µg of protein were loaded for RyR1 (lanes 1 and 4), RyR2 (lanes 2 and 5) and RyR3 (lanes 3 and 6) detection, respectively. Gel electrophoresis and Western blot analysis were performed, as described in Materials and methods.

populations of channels occurred (Figure 4A, left). The two populations of channels observed in murine diaphragm showed two different Ca^{2+} responses, comparable with those observed in bovine diaphragm (Figure 1B). Circles in Figure 4A (left) denote six out of a total of 16 channels analysed which were not activated by ATP at low Ca^{2+} concentrations, but presented a sigmoidal Ca^{2+} response when exposed to increasing Ca^{2+} concentrations. In Figure 4A (left), triangles denote channels which were activated by millimolar ATP concentration and that displayed a bell-shaped Ca^{2+} response, i.e. were inactivated at higher Ca^{2+} concentrations.

In contrast, in microsomes prepared from the diaphragm of RyR3 knockout mice, only one population of channels, with typical RyR1 properties, was observed (Figure 4A, right). All 11 channels from diaphragm of RyR3 knockout mice were gated by ATP at nanomolar Ca^{2+} concentrations with p_o values between 0.11 and 0.99 resulting in a mean ± SD of 0.44 ± 0.32 (Figure 4A, right), which is comparable with the p_o of 0.52 ± 0.25 observed for bovine abdominal muscle in Figure 1A.

Biochemical analysis of the RyR isoforms present in the microsomal fraction of diaphragm from normal and RyR3 knockout mice is shown in Figure 4B. The RyR1 isoform is present in both mouse strains (Figure 4B, lanes 1 and 4) while the RyR3 isoform was detected only in the preparation from normal mice (Figure 4B, lane 3). As expected, the RyR2 isoform was not detected in either of the two preparations (Figure 4B, lanes 2 and 5). Thus we conclude that the channels which depends on micromolar Ca^{2+} concentrations for their activation are embodied by the RyR3 isoform.

Discussion

Taking advantage of the differential distribution of RyR3 in mammalian skeletal muscles (Conti *et al*., 1996; Tarroni *et al*., 1997) and of the development of a RyR3 knockout strain of mice (Bertocchini *et al*., 1997), we have analysed the single-channel properties of the native mammalian RyR3 channels using sarcoplasmic reticulum vesicles prepared from bovine and murine diaphragm muscle. In addition to the expected RyR1 channels usually observed in skeletal muscles, a second population of RyR channels was detected in a sizeable fraction of total fusion events with sarcoplasmic reticulum fractions from diaphragm. At variance with the RyR1 channels which are characterized by the ability to be activated by ATP at nanomolar Ca^{2+} concentrations in the presence of millimolar *trans* Ca^{2+} concentrations (Sitsapesan and Williams, 1995a; Sonnleitner *et al*., 1997), the novel population of channels observed in diaphragm vesicles could not be activated under these conditions. However, when Ca^{2+} in the *cis* chamber was increased to micromolar concentrations, the single-channel open probability increased to almost unity. On this evidence, the novel class of channels observed in diaphragm microsomes differs from the so-called 'low activity' RyR1s that do not respond to increasing concentrations of Ca^{2+} (Copello *et al*., 1997). Given that the second class of ryanodine receptors observed in diaphragm muscle was associated with the expression of the RyR3 protein and that it was not observed in diaphragm muscles of RyR3 knockout mice, we conclude that the second

channel population is contributed by the RyR3 isoform of Ca^{2+} release channel.

The relatively high frequency with which the RyR3 channels have been detected in single-channel analysis in our studies appears to contrast with the relatively low abundance of the RyR3 isoform with respect to the RyR1 isoform in skeletal muscles (Conti *et al*., 1996; Murayama and Ogawa, 1997). Although we have no explanation for this phenomenon, it is noteworthy that it strikingly resembles what was reported for the chicken β/RyR3 channels that were found to be incorporated into lipid bilayers much more readily than α/RyR1 channels (Percival *et al*., 1994).

Properties of RyR3 channels

The conductance properties observed for the two channel populations present in diaphragm muscle were similar, since the RyR3 channel exhibited a conductance of 105 ± 8 pS (with 50 mM Ca^{2+} as the charge carrier) comparable with that observed with RyR1 channels (96 ± 9 pS). The first differences between RyR1 and RyR3 channels derive from their response to activating concentrations of Ca^{2+} in the presence of ATP. At constant concentrations of ATP (millimolar range), RyR1 channels can be activated by a resting concentration of Ca^{2+} (i.e. in the nanomolar range), while it appears that RyR3 channels require cytosolic $[Ca^{2+}]_i$ in the micromolar range in order to be activated. The second difference appears when Ca^{2+} is increased to millimolar levels, which inactivates RyR1 but leaves RyR3 active. A third difference between the two channels appeared when sensitivity to Ca^{2+} activation was tested in the presence of cADPR. cADPR initially has been identified on the basis of its ability to cause release of Ca^{2+} from sea urchin eggs through ryanodine-sensitive channels (Galione *et al*., 1991). Work with mammalian cells has shown that cADPR can cause Ca^{2+} release from cardiac and brain microsomes (which contain mainly the RyR2 isoform) and that it can increase the open probability of RyR2 but not that of RyR1 channels incorporated in planar lipid bilayers (Mészáros *et al*., 1993). Furthermore, Williams and co-workers have found that the RyR1 channels also are sensitive to cADPR (Sitsapesan *et al*., 1995). This group has also provided evidence that cADPR may work through the ATP-binding site of RyRs (Sitsapesan *et al*., 1994, 1995).

In our experiments, 1 μM cADPR, at 100 nM free extravesicular Ca^{2+} and 1 mM ATP, significantly enhanced the Ca^{2+} activity of the novel channel type specifically detected in diaphragm vesicles, resulting in an ~1 log lower threshold for Ca^{2+} activation of the RyR3 channels in the presence of cADPR. No effect was observed on RyR1 channels under identical buffer conditions, although it should be noted that our conditions differ from those of Sitsapesan *et al*. (1994) and Sitsapesan and Williams (1995b). Whether the different experimental conditions used can account for the observed lack of cADPR effect on RyR1 channels in our experiments is being investigated. cADPR levels have been shown to vary between 20 and 600 nM among cells and tissues, including skeletal muscles (Lee, 1993; Galione and Sommerhill, 1995). Therefore, since both cADPR and ATP are present in skeletal muscles, this combination could shift the sensitivity of the RyR3 channels to Ca^{2+} activation. According to Williams and co-workers, ATP would compete with cADPR in activating RyR2 channels (Sitsapesan *et al*., 1994). In our case, however, only cADPR (and not ATP) activated Ca^{2+} release through RyR3 channels. It should be noted that cytosolic proteins have been proposed to be necessary for cADPR to activate RyR channel activity. Among these factors, calmodulin has been shown to be essential for cADPR-induced Ca^{2+} release in sea urchin egg microsomes (Lee *et al*., 1994). cADPR has been reported to bind to the FK506-binding proteins (FKBP12.6) and it has been proposed that this interaction is important in order to activate Ca^{2+} release in pancreatic islet microsomes (Noguchi *et al*., 1997). Therefore, when comparing data from different laboratories, one should consider the possibility that either vesicles or purified RyR preparations may differ in their relative content of accessory proteins. In this respect, it is noteworthy that Chen *et al*. (1997), working with recombinant RyR3 channels expressed in HEK293 cells, did not find a stimulating effect of 10 μM cADPR in the presence of 1 μM calmodulin at 88 nM and 50 μM free Ca^{2+}. While all other characteristics found by Chen *et al*. (1997). agree well with our data, we currently have no explanation for the observed difference with respect to cADPR sensitivity of RyR3 channels in the two laboratories. The actual mechanisms of action of cADPR might be more complex, and obviously a more complete characterization of the effects of cADPR and of other agonists and antagonists of RyRs on RyR3 channels is required to allow a more detailed comparative analysis of the channels embodied by the three isoforms.

Comparison with non-mammalian RyRs

The observed properties of RyR3 channels found in mammalian diaphragm muscle can be compared with data reported for the α and β isoforms of Ca^{2+} release channels of non-mammalian skeletal muscles, which in frogs and chickens correspond to the RyR1 and RyR3 isoforms of mammals (Oyamada *et al*., 1994; Marziali *et al*., 1996; Ottini *et al*., 1996). Single-channel data on β/RyR3 from chicken indicated a higher sensitivity of β/RyR3 than α/RyR1 to activation by Ca^{2+} (Percival *et al*., 1994). This contrasts with the data we obtained with both bovine and murine RyR3 channels, which are not active at nanomolar Ca^{2+} and require Ca^{2+} concentrations in the micromolar range in order to be activated. The high threshold for Ca^{2+} activation of RyR3 observed in this study is in good agreement with studies performed with skeletal muscles of knockout mice for either the RyR1 or the RyR3 gene. Activation of a calcium-induced calcium release mechanism in skeletal muscles of RyR1 knockout mice (containing only RyR3) seems to require higher Ca^{2+} concentrations compared with those required for activation of contraction in wild-type muscles (containing both RyR1 and RyR3) and RyR3 knockout mice (containing only the RyR1 isoform) (Takeshima *et al*., 1994, 1995). Therefore, in both *in vitro* and *in vivo* conditions there is agreement in observing that mammalian RyR3 channels have a lower sensitivity to Ca^{2+} than do RyR1 channels. Mammalian RyR1 and RyR3 channels display similar conductances (in the range of 100 pS with 50 mM Ca^{2+} as charge carrier) (Coronado *et al.*, 1994; Meissner, 1994; Sitsapesan *et al*., 1995). These values are similar to those reported

for α/RyR1 and β/RyR3 in chicken (Percival *et al*., 1994; Sutko and Airey, 1996), while in fish different conductance values for the two isoforms have been observed (O'Brien *et al*., 1995). Both chicken and fish β/RyR3 and mammalian RyR3 display a lower sensitivity to Ca^{2+} inactivation, up to the millimolar range, in the presence of ATP (Percival *et al*., 1994; O'Brien *et al*., 1995; Sutko and Airey, 1996). Sensitivity to cADPR has not been reported for RyRs of non-mammalian vertebrates.

RyR3 properties and the role of the two Ca^{2+} release channels in vertebrate skeletal muscle excitation-coupling

The finding that mammalian RyR3 channels possess functional properties distinctive from those of RyR1 and, to a certain extent, also from those of RyR2 channels, demonstrates that the three isoforms of the RyR family embody channels which differ in their sensitivity to agonists such as Ca^{2+}, ATP and cADPR. This evidence confirms the expectations that differences in the regulatory properties of the individual isoforms are the basis for the existence of three RyR genes and eventually of splicing variants of the different gene products. The contribution of different channels in a single cell may be important to allow a more precise regulation of cellular function (Berridge, 1993; Clapham, 1995). Although use of more Ca^{2+} release channel isoforms is common to several cell types, vertebrate skeletal muscles have long provided a useful model for studying the regulation of a cellular function, e.g. muscle contraction, by intracellular Ca^{2+} concentration. In non-mammalian vertebrates, such as frogs and birds, the α/RyR1 isoform is present only in extra-fast muscles, in contrast to the use of both α/RyR1 and β/RyR3 in most body muscles (O'Brien *et al*., 1993; Sutko and Airey, 1996). In mammals, RyR1 is the predominant isoform in skeletal muscles (Meissner, 1994). However, RyR3 is widely expressed during the neonatal phase of development in rodent skeletal muscles (Bertocchini *et al*., 1997), while in adult muscles it displays a more restricted pattern of expression and is found mainly in diaphragm and soleus muscles (Giannini *et al*., 1992, 1995; Conti *et al*., 1996; Tarroni *et al*., 1997). Knockout of the RyR3 gene has shown that lack of RyR3 channels results in a marked reduction in neonatal muscle contractility, which contrasts with the apparent low level of abundance of this isoform (Bertocchini *et al*., 1997). How the different properties, observed at the single-channel level, between RyR1 and RyR3 channels integrate in the regulation of skeletal muscle contraction is not known. Strong evidence has been obtained in favour of a central role for the RyR1 isoform in excitation–contraction coupling (Takeshima *et al*., 1994; Fleig *et al*., 1996; Nakai *et al*., 1996). It is possible that the RyR3 channels may be activated by the rising Ca^{2+} concentration provided by the initial opening of the RyR1. The lower sensitivity of RyR3 channels to inactivation at high Ca^{2+} concentration may be relevant to maintain a sustained Ca^{2+} release after deactivation of RyR1 (Rios *et al*., 1992; Schneider, 1994). These properties may comply with RyR3 contributing an amplification component for Ca^{2+} release from the skeletal sarcoplasmic reticulum (Rios *et al*., 1992; Schneider, 1994; Bertocchini *et al*., 1997). The evidence that RyR3 channels are also sensitive to the activating effects of cADPR calls for additional work in order to understand the regulatory/modulatory role of this compound in the regulation of Ca^{2+} release from RyRs. A role for cADPR in the regulation of cardiac contraction under physiological conditions has been demonstrated (Rakovic *et al*., 1996). An effect of cADPR in isolated membrane fractions from skeletal muscle has also been reported (Yamaguchi and Kasai, 1997). Sensitivity of the RyR3 isoform to cADPR suggests that this compound may activate and/or modulate Ca^{2+} release through RyR channels in skeletal muscles as well as in the many other cell types that express these channels. Several other factors, including the effect of the FKBP12 protein in optimal gating and regulation of the RyR3 isoform as well as the effect of alternative splicing and of phosphorylative events, remain to be analysed in order to attain a better understanding of the molecular mechanisms underlying the contribution of the RyR3 channels to Ca^{2+} release.

Materials and methods

Microsomal vesicle preparation

Skeletal muscles isolated from either bovine or mouse tissues were used to prepare the microsomal fractions. Bovine skeletal muscles were obtained from a local slaugtherhouse. Knockout mice were obtained as described elsewhere (Bertocchini *et al*., 1997). Briefly, chimeric males carrying a mutated RyR3 allele were bred to C57BL/6 females. Heterozygous mice were bred to obtain homozygous mice. Mice were genotyped by Southern blotting and the absence of the RyR3 protein was verified by Western blot. The tissues were cleaned carefully from any contaminating connective tissue and quickly frozen in liquid nitrogen. Microsomes were prepared as previously described (Conti *et al*., 1996). Tissue samples were homogenized in ice-cold buffer A [320 mM sucrose, 5 mM Na–HEPES pH 7.4 and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] using a Dounce homogenizer. Homogenates were centrifuged at 7000 *g* for 5 min at 4°C. The supernatant obtained was centrifuged at 100 000 *g* for 1 h at 4°C. The microsomes were resuspended in buffer A and stored at –80°C. Protein concentration of the microsomal fraction was quantified using the Bradford protein assay kit (Bio-Rad).

Bilayer measurements

The bilayer apparatus was described previously (Schindler, 1989). Planar bilayers were formed from a mixture of PE:PS:PC (phosphatidylethanolamine:L-α-phosphatidylserine:1,2 diphytanoyl-*sn*-glycero-3-phosphocholine, all from Avanti Polar Lipids, AL) in the ratio 5:4:1 across a 180 μM hole in a 6 μm thick Teflon sheet, which separated two chambers containing 250 mM HEPES/53 mM $Ca(OH)_2$ pH 7.4, *trans*, and 250 mM HEPES/110 mM Tris pH 7.4, *cis*. Bilayers had specific membrane capacitances of 0.5–0.6 μF/cm^2. For fusion, 750 mM CsCl and 2 mM $Ca(OH)_2$/250 mM HEPES were added to the *cis* buffer. Fusion events occurred within 5 min after addition of 1–5 μl (1–5 μg protein) of microsomes and were monitored by chloride channels, also contained in the membrane. After up to five fusion events, which typically led to incorporation of 1–2 activatable RyR channels, the *cis* chamber was perfused with 250 mM HEPES/110 mM Tris pH 7.4 at 4 ml/min for 3 min. Ca^{2+} currents were amplified and low pass filtered at 300 or 1000 Hz with 12 dB/octade with a pre-amplifier (SR 560, Stanford Research, CA) at low noise setting. Data was stored on a computer (Pentium, 133 MHz) using an analog digital interface (TL-1-40, Axon Instruments, CA). Open probabilities were determined from at least 1 min of consecutive traces in 1024 ms bins based on a 50% criterion using PCLAMP 6.0.3 (Axon Instruments, CA).

In all experiments, Ca^{2+} concentrations were adjusted to free Ca^{2+} concentrations of 20 and 100 nM, 1, 2, 5, 10 and 100 μM, and 1 mM using 1 mM dibromo-BAPTA (Fluka, Switzerland) and addition of 0, 39, 290, 450, 676, 813, 1076 and 2076 μM $CaCl_2$ to the *cis* buffer as calculated from $K_d = 3\times10^5$/M (Harrison and Bers, 1987, assuming an ionic strength of 350 mM), and measured with a Ca^{2+} electrode (Orion SA 720, Orion, Boston, MA). cADPR and 8-Br-cADPR, kindly provided by A.Galione (Oxford University, UK), were added at the indicated concentrations to the *cis* side. If channel activity was not observed in the presence of 10 μM Ca^{2+} and >0.5 mM ATP after fusion, the

membrane was considered not to contain a RyR channel and the experiment was cancelled.

Western blot analysis

Microsomal proteins were separated by SDS–PAGE, as described (Conti *et al*., 1996). Proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell) by blotting gels for 5 h at 350 mA at 4°C in a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS and 10% methanol. Filters were incubated for 3 h in a blocking buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.2% Tween-20, 5% no-fat milk. Primary antibodies (diluted 1:3000) were incubated with membranes overnight at room temperature. Polyclonal rabbit antisera able to distinguish the three RyRs were developed against purified GST fusion proteins corresponding to the region of low homology situated between the transmembrane domains 4 and 5 (divergent region 1, or D1) of the RyR1, RyR2 and RyR3 proteins, as previously described (Giannini *et al*., 1995). These antibodies have been shown not to cross-react with each other (Tarroni *et al*., 1997). Antigen detection was performed using the alkaline phosphatase detection method.

Acknowledgements

This work was supported by EC project PL960592 and by Telethon grant No. 653.

References

Berridge,M.J. (1993) Inositol trisphosphate and calcium signalling. *Nature*, **361**, 315–325.

Bertocchini,F., Ovitt,C., Conti,A., Barone,V., Scholer,H.R., Bottinelli,R., Reggiani,C. and Sorrentino,V. (1997) Requirement of the ryanodine receptor type 3 for efficient contraction in neonatal skeletal muscles. *EMBO J.*, **16**, 6956–6963.

Brillantes,A.-M.B. *et al*. (1994) Stabilization of calcium release channel (ryanodine receptor) function by FK506-binding protein., *Cell*, **77**, 513–523.

Chen,W.S.R., Li,X., Ebisawa,K. and Zhang,L. (1997) Functional characterization of the recombinant type 3 Ca^{2+} release channel (ryanodine receptor) expressed in HEK293 cells. *J. Biol. Chem.*, **272**, 24234–24246.

Clapham,D.E. (1995) Calcium signaling. *Cell*, **80**, 259–268.

Conti, A., Gorza,L. and Sorrentino,V. (1996) Differential distribution of ryanodine receptor type 3 (RyR3) gene product in mammalian skeletal muscles. *Biochem. J.*, **316**, 19–23.

Copello,J.A., Barg,S., Onoue,H. and Fleischer,S. (1997) Heterogeneity of Ca^{2+} gating of skeletal muscle and cardiac ryanodine receptors. *Biophys. J.*, **73**, 141–156.

Coronado,R., Morrissette,J., Sukhareva,M. and Vaughan,D.M. (1994) Structure and function of ryanodine receptors. *Am. J. Physiol*., **266**, C1485–C1504.

Fleig,A., Takeshima,H. and Penner,R. (1996) Absence of Ca^{2+} current facilitation in skeletal muscle of transgenic mice lacking the type 1 ryanodine receptor. *J. Physiol.*, **496**, 339–345.

Fleischer,S. and Inui,M. (1989) Biochemistry and biophysics of excitation–contraction coupling. *Annu. Rev. Biophys. Biophys. Chem*., **18**, 333–364.

Furuichi,T., Kohda,K., Miyawaki,A. and Mikoshiba,K. (1994) Intracellular channels. *Curr. Opin. Neurobiol*., **4**, 294–303.

Galione,A. (1992) Calcium-induced calcium release and its modulation by cyclic ADP-ribose. *Trends Pharmacol*., **13**, 304–306.

Galione,A. and Summerhill,R. (1995) Regulation of ryanodine receptors by cyclic ADP-ribose. In Sorrentino,V. (ed.), *Ryanodine Receptors*. CRC Press, Boca Raton, FL, pp. 51–70.

Galione,A., Lee,H.C. and Busa,W.B. (1991) Calcium-induced calcium release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science*, **253**, 1143–1146.

Giannini,G., Clementi,E., Ceci,R., Marziali,G. and Sorrentino,V. (1992) Expression of a ryanodine receptor-Ca^{2+} channel that is regulated by TGF-beta. *Science*., **257**, 91–94.

Giannini,G., Conti,A., Mammarella,S., Scrobogna,M. and Sorrentino,V. (1995) The ryanodine receptor/calcium channel genes are widely and differentially expressed in murine brain and peripheral tissues. *J. Cell Biol.*, **128**, 893–904.

Harrison,S.M. and Bers,D.M. (1987) The effect of temperature and ionic strength on the apparent Ca-affinity of EGTA and the analogous Ca-chelators BAPTA and dibromo-BAPTA. *Biochim. Biophys. Acta*, **925**, 133–143.

Inui,M., Saito,A. and Fleischer,S. (1987) Purification of the ryanodine receptor and identity with feet structures of junctional terminal cisternae of SR from fast skeletal muscle. *J. Biol. Chem.*, **262**, 1740–1747.

Kasai,H. and Petersen,O.H. (1994) Spatial dynamics of second messengers: IP_3 and cAMP as long-range and associative messengers. *Trends Neurosci*., **17**, 95–101.

Lee,H.C. (1993) Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. *J. Biol. Chem.*, **268**, 293–299.

Lee,H.C. (1997) Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol. Rev.*, **77**, 1133–1164.

Lee,H.C., Aarhus,R., Graeff,R., Gurnach,M.E. and Walseth,T.F. (1994) Cyclic ADP-ribose activation of the ryanodine receptor is mediated by calmodulin. *Nature*, **370**, 307–309.

Ma,J. and Zhao,J. (1994) Highly cooperative and hysteretic response of the skeletal muscle ryanodine receptor to changes in proton concentrations. *Biophys. J.*, **67**, 626–633.

Marziali,G., Rossi,D., Giannini,G., Charlesworth,A. and Sorrentino,V. (1996) cDNA reveals a tissue specific expression of alternatively spliced transcripts of the ryanodine receptor type 3 (RyR3) calcium release channel. *FEBS Lett*., **394**, 76–82.

Meissner,G. (1994) Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu. Rev. Physiol.*, **56**, 485–508.

Mészáros,L.G., Bak,J. and Chu,A. (1993) Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca^{2+} channel. *Nature*, **364**, 76–79.

Murayama,T. and Ogawa,Y. (1997) Characterization of type 3 ryanodine receptor (RyR3) in mammalian diaphragm muscle. *J. Biol. Chem.*, **272**, 24030–24037.

Nakai,J., Dirksen,R.T., Nguyen,H.T., Pessah,I.N., Beam,K.G. and Allen,P.D. (1996) Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature*, **380**, 72–75.

Noguchi,N., Takasawa,S., Nata,K., Tohgo,A., Kato,I., Ikehata,F., Yonekura,H. and Okamoto,H. (1997) Cyclic ADP-ribose binds FK506-binding protein 12.6 to release calcium from islet microsomes. *J. Biol. Chem.*, **272**, 3133–3136.

O'Brien,J., Meissner,G. and Block,B. (1993) The fastest contracting muscles of nonmammalian vertebrates express only one isoform of the ryanodine receptor. *Biophys. J.*, **65**, 2418–2427.

O'Brien,J., Valdivia,H.H. and Block,B.A. (1995) Physiological differences between the α and β ryanodine receptors of fish skeletal muscle. *Biophys. J.*, **68**, 471–482.

Ottini,L., Marziali,G., Conti,A., Charlesworth,A. and Sorrentino,V. (1996) Alpha and beta isoforms of ryanodine receptor from chicken skeletal muscle are the homologues of mammalian RyR1 and RyR3. *Biochem. J.*, **315**, 207–216.

Oyamada,H., Murayama,Y., Takagi,T., Iino,M., Iwabe,N., Miyata,T., Ogawa,Y. and Endo,M. (1994) Primary structure and distribution of ryanodine-binding protein isoforms of the bullfrog skeletal muscle. *J. Biol. Chem.*, **269**, 17206–17214.

Percival,A., Williams,A., Kenyon,J., Grinsell,M., Airey,J.A. and Sutko,J.L. (1994) Chicken skeletal muscle ryanodine receptor isoforms: ion channel properties. *Biophys. J.*, **67**, 1834–1850.

Rakovic,S., Galione,A., Ashamu,G.A., Potter,B.V.L. and Terrar,D.A. (1996) A specific cyclic ADP-ribose antagonist inhibits cardiac excitation–contraction coupling. *Curr. Biol.*, **6**, 989–996.

Rios,E., Pizarro,G. and Stefani,E. (1992) Charge movement and the nature of signal transduction in skeletal muscle excitation–contraction coupling. *Annu. Rev. Physiol.*, **54**, 109–133.

Schindler,H. (1989) Planar lipid–protein membranes: strategies of formation and of detecting dependencies of ion transport functions on membrane conditions. *Methods Enzymol*., **171**, 225–253.

Schneider,M.F. (1994) Control of calcium release in functioning skeletal muscle fibers. *Annu. Rev. Physiol.*, **56**, 463–484.

Sitsapesan,R. and Williams,A.J. (1995a) The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+}-release channel is regulated by luminal Ca^{2+}. *J. Membr. Biol*., **146**, 133–144.

Sitsapesan,R. and Williams,A.J. (1995b) cADPR and related compounds activate sheep skeletal sarcoplasmic reticulum Ca^{2+}-release channel. *Am. J. Physiol*., **268**, C1235–C1240.

Sitsapesan,R., McGarry,S.J. and Williams,A.J. (1994) Cyclic ADP-ribose competes with ATP for the adenine nucleotide binding site on the cardiac ryanodine receptor Ca^{2+}-release channel. *Circ. Res*., **75**, 596–600.

Sitsapesan,R., McGarry,S.J. and Williams,A.J. (1995) Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. *Trends Pharmacol.*, **16**, 386–391.

Sonnleitner,A., Fleischer,S. and Schindler,H. (1997) Gating of the skeletal calcium release channel by ATP is inhibited by protein phosphatase 1 but not by Mg^{2+}. *Cell Calcium*, **21**, 283–290.

Sorrentino,V. (1995) *Ryanodine Receptors*. CRC Press, Boca Raton, FL.

Sutko,J.L. and Airey,J.A. (1996) Ryanodine receptor Ca^{2+} release channels: does diversity in form equal diversity in function? *Physiol. Rev.*, **76**, 1027–1071.

Takeshima,H., Iino,M., Takekura,H., Nishi,M., Kuno,J., Minowa,O., Takano,H. and Noda,T. (1994) Excitation–contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. *Nature*, **369**, 556–559.

Takeshima,H.,Yamazawa,T., Ikemoto,T., Takekura,H., Nishi,M., Noda,T. and Iino,M. (1995) Calcium-induced calcium release in myocytes from dyspedic mice lacking the type-1 ryanodine receptor. *EMBO J.*, **14**, 2999–3006.

Takeshima,H. *et al*. (1996) Generation and characterization of mutant mice lacking ryanodine receptor type 3. *J. Biol. Chem.*, **271**, 19649–19652.

Tarroni,P., Rossi,D., Conti,A. and Sorrentino,V. (1997) Expression of the ryanodine receptor type 3 (RyR3) calcium release channel during development and differentiation of mammalian skeletal muscle cells. *J. Biol. Chem.*, **272**, 19808–19813.

Yamaguchi,N. and Kasai,M. (1997) Potentiation of depolarization-induced calcium release from skeletal muscle triads by cADPR and inositol 1,4,5-trisphosphate. *Biochem. Biophys. Res. Commun*., **240**, 772–777.

Received August 13, 1997; revised March 20, 1998; accepted March 24, 1998