Evidence for the transport of glutathione through ryanodine receptor channel type 1

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In the present study, we have investigated the role of RyR1 (ryanodine receptor calcium channel type 1) in glutathione (GSH) transport through the sarcoplasmic reticulum (SR) membrane of skeletal muscles. Lanthanum chloride, a prototypic blocker of cation channels, inhibited the influx and efflux of GSH in SR vesicles. Using a rapid-filtration-based assay and lanthanum chloride as a transport blocker, an uptake of radiolabelled GSH into SR vesicles was observed. Pretreatment of SR vesicles with the RyR1 antagonists Ruthenium Red and ryanodine as well as with lanthanum chloride blocked the GSH uptake. An SR-like GSH uptake appeared in microsomes obtained from an HEK-293 (human embryonic kidney 293) cell line after transfection of RyR1. These observations strongly suggest that RyR1 mediates GSH transport through the SR membranes of skeletal muscles.

Key words: cation channel, glutathione, lanthanum chloride, ryanodine receptor calcium channel type 1, sarcoplasmic reticulum.

INTRODUCTION

Glutathione (GSH and GSSG) constitutes the most important redox buffer of animal cells both in the cytosol and in organelles. Since each intracellular compartment is characterized by a particular redox potential [1], the transport of glutathione through endomembranes should play a crucial role in both the maintenance and regulation of redox conditions. Although direct experimental evidence is missing, by generalizing the observations related to the endoplasmic reticulum (ER) lumen [2], it is supposed that the lumen of the sarcoplasmic reticulum (SR) has a higher redox potential and, consequently, a lower [GSH]/[GSSG] ratio when compared with the cytosol.

Redox potentials on the cytosolic and luminal surfaces of the SR membrane have a fundamental role in the regulation of calcium fluxes. A major mechanism for increasing cytosolic Ca^{2+} is the release of Ca^{2+} from internal stores via the members of a superfamily of intracellular calcium-release channels including RyRs (ryanodine receptors) [3–5].

Hypersensitive thiols of RyRs are the subject of oxidoreduction, which causes the activation or inhibition of Ca^{2+} release [6–9]. Generally speaking, thiol oxidation by reactive oxygen species, glutathione disulphide and other thiol-specific reagents activates a channel, whereas reducing agents, such as GSH, dithiothreitol and 2-mercaptoethanol, inhibit a channel. Recent observations indicate a more sophisticated mechanism: RyR1 (RyR calcium channel type 1) from skeletal muscles can function as a transmembrane redox sensor [10]. A large transmembrane redox potential gradient inhibits a channel, whereas dissipation of this gradient activates a channel. Therefore it is logical to consider that GSH/GSSG transport through the SR membrane is involved in the control of the local redox potential gradient necessary for the redox regulation of RyR1.

Relatively few experimental results are available on the transport of glutathione through the ER/SR membrane. It has been reported that GSH is transported through the membrane of hepatic ER at a relatively low rate, whereas the membrane is practically impermeable to GSSG [11]. Recent results showed that both compounds could permeate the membrane of SR vesicles from skeletal muscles, although with a different velocity [10]. Subsequently, we provided evidence for the possible involvement of RyR1 in GSH/GSSG transport through the SR membrane of skeletal muscles, and suggested that RyR1 may act as a glutathione transporter on its own or, alternatively, may interact with a putative GSH/GSSG transporter [12].

The molecular nature of the ER/SR GSH/GSSG transporter(s) is currently unknown. A major aim of the present study was to validate experimentally the hypothesis that the RyR1 channel itself is a GSH transporter. To this end, first, we re-investigated the transport of GSH in skeletal-muscle SR vesicles using a suitable rapid-filtration assay and, secondly, we employed microsomes from cells stably transfected with the RyR isoform type 1 and compared them with the microsomes of wild-type cells, which do not constitutively express this receptor [13]. The results obtained strongly suggest that the RyR1 channel can directly allow the permeation of GSH through the SR membrane.

EXPERIMENTAL

Cell culture and transfection

HEK-293 (human embryonic kidney 293) cells were maintained and transfected as described in [13]. Briefly, cells were cultured in α-minimal essential medium, supplemented with 2 mM glutamine (Bio-Whittaker, Walkersville, MD, U.S.A.), 100 mg/ml streptomycin, 100 units/ml penicillin (Bio-Whittaker), 1 mM sodium pyruvate (Bio-Whittaker) and 10 % (v/v) heat-inactivated foetal calf serum (Bio-Whittaker) at 37 °C under 5 % CO_2. Transfections of the pcDNA3-RyR1 clones were performed by the GenePORTER™ method (Gene Therapy Systems, San Diego, CA, U.S.A.) according to the manufacturer's instructions. For stable transfections, Geneticin sulphate (G418; Life Technologies, Groningen, The Netherlands) was added 48 h after transfection

Abbreviations used: ER, endoplasmic reticulum; RyR, ryanodine receptor; RyR1, RyR calcium channel type 1; SR, sarcoplasmic reticulum.

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at a final concentration of 800 mg/ml. Single colonies were transferred to a 96-well multiplate, expanded and tested for RyR expression.

Cells were harvested by a rubber scraper, pelleted in 50 ml tubes and homogenized in ice-cold buffer A (320 mM sucrose/5 mM Na-Hepes, pH 7.4/0.1 mM PMSF) using a Teflon potter. 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 fractions was quantified using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The presence of RyR1 in microsomes of transfected cells was verified by Western-blot analysis, [^{3}H]ryanodine binding assay and caffeine-induced Ca^{2+} release [13].

Preparation of SR vesicles

SR vesicles were prepared from the dominantly white hind limb skeletal muscles of New Zealand White rabbits by the method of Saito et al. [14]. Intactness of the vesicles was assessed by the light-scattering method (see below) using non-permeant compounds (i.e. sucrose, maltose and UDP-glucuronate). The integrity of SR vesicles was also assessed on the basis of their ATP-dependent Ca^{2+} accumulation as described by Fulceri et al. [15]. Microsomal preparations were frozen and maintained in liquid N_2 until use.

Transport measurements by the rapid-filtration method

Rapid-filtration experiments were essentially performed as described in [16,17]. Briefly, microsomal vesicles (0.5–1 mg of protein/ml) were incubated at room temperature (22 °C) in a buffer containing 100 mM KCl, 20 mM NaCl, 20 mM Mops and either 1 mM GSH and its radiolabelled analogue [^{3}H]GSH (10 μCi/ml) or 20 μM $CaCl_2$ and trace amounts of $^{45}Ca^{2+}$ (10 μCi/ml). At the indicated times, aliquots (0.1 ml) were withdrawn, filtered through cellulose acetate/nitrate filter membranes (pore size, 0.22 μm) and washed quickly on the filter with the same ice-cold buffer. The radioactivity retained on the filter was measured by liquid scintillation. Alamethicin (50 μg/mg of protein) was included in parallel incubates to distinguish the intravesicular and the bound radioactivities. Alamethicin, a pore-forming antibiotic, makes the microsomal vesicles permeable to various hydrophilic compounds [16–18], including GSH and GSSG [11]. The alamethicin-treated vesicles were recovered on filters and washed as above. More than 95 % of the microsomal proteins were retained by the filters, indicating that alamethicin treatment did not affect the vesicular structure of microsomes as reported in [18]. The alamethicin-releasable portion of radioactivity (assumed as intravesicular) was calculated by subtraction.

Transport measurements by light-scattering techniques

Permeability of the microsomal membranes to GSH was also measured by continuous monitoring of osmotically induced changes in the size and shape of microsomal vesicles [19]. SR/ER vesicles (50 μg of protein/ml) were equilibrated for 2 h in a hypo-osmotic medium (5 mM K-Pipes, pH 7.0). Then, light scattering of the microsomes was monitored at 400 nm, using a fluorimeter (Hitachi F-4500) equipped with a temperature-controlled cuvette holder (37 °C) and a magnetic stirrer. The addition of a small volume (< 5 % of the total incubation volume) of a concentrated and neutralized solution of GSH resulted in a rapid increase in light scattering (owing to the shrinkage of the vesicles). This peak is followed by a gradual decrease in light scattering (caused by vesicle swelling as GSH concentration equilibrates between the extra- and intravesicular spaces). The relative heights of the peak and the slope of the curve depend on the GSH permeability of the vesicular membrane.

Fluorescence measurement of Ca^{2+} fluxes

The incubation medium consisted of 10 mM KCl, 20 mM NaCl, 3.5 mM $MgCl_2$, 20 mM Mops (pH 7.2), 3 mM ATP, 20 mM phosphocreatine and 10 mM P_i (as potassium phosphate buffer, pH 7.2). Creatine kinase (10 units/ml) was also added. Incubation (at 37 °C) was started by adding SR vesicles (1 mg of protein) to make a final volume of 2 ml in a fluorimeter cuvette, and uptake and release of Ca^{2+} were measured by using the fluorescent Ca^{2+} indicator, Fluo 3 free acid (1 μM), as described previously [15]. The excitation and emission wavelengths were 506 and 526 nm respectively.

Materials

GSH, Ruthenium Red, ryanodine and alamethicin were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H]GSH and $^{45}CaCl_2$ were obtained from NEN® Life Science Products (Boston, MA, U.S.A.). Dithiothreitol was removed from the [^{3}H]GSH solution by extraction as described by Butler et al. [20]. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

In the present study, we have developed an experimental approach suitable to allow the confident use of a filtration-based transport assay for GSH. Although the light-scattering technique [12] suggested the presence of glutathione transport in SR vesicles, the rapid-filtration assay was unable to demonstrate intraluminal glutathione accumulation in vesicles incubated with GSH. To discriminate the net intravesicular GSH content from the binding of GSH to vesicles, radioactivity associated with SR vesicles was measured using intact vesicles (intravesicular and bound GSH) as well as vesicles permeabilized with the pore-forming compound alamethicin (bound GSH). The amount of vesicle-associated GSH was virtually the same in both cases (Figure 1, upper panel, closed and open circles), indicating that very little or no free GSH was recovered in the SR vesicles. This was presumably caused by glutathione efflux during the filtration process. Therefore we tried to trap the intravesicular glutathione by adding potential efflux blockers to the SR vesicles just before the filtration. In particular, the RyR antagonists Ruthenium Red (5 μM) and ryanodine (300 μM) and the cation channel blocker lanthanum chloride (5 μM) were added to each sample 20 s before the filtration; the same concentration of each inhibitor was also included in the washing buffer.

As shown in Figure 1 (upper panel, closed and open squares), termination of GSH uptake using lanthanum chloride resulted in a large increase in the radioactivity associated with SR vesicles at any incubation time when compared with the radioactivity associated with alamethicin-treated SR vesicles. Therefore a significant net intravesicular accumulation of free GSH was observed. On the other hand, termination of GSH uptake using the RyR antagonists, Ruthenium Red (5 μM) or ryanodine (300 μM), did not result in significant increases in (vesicular) GSH compared with the level of GSH non-specifically associated with vesicular membranes (Figure 1, lower panel). On the basis of these results,

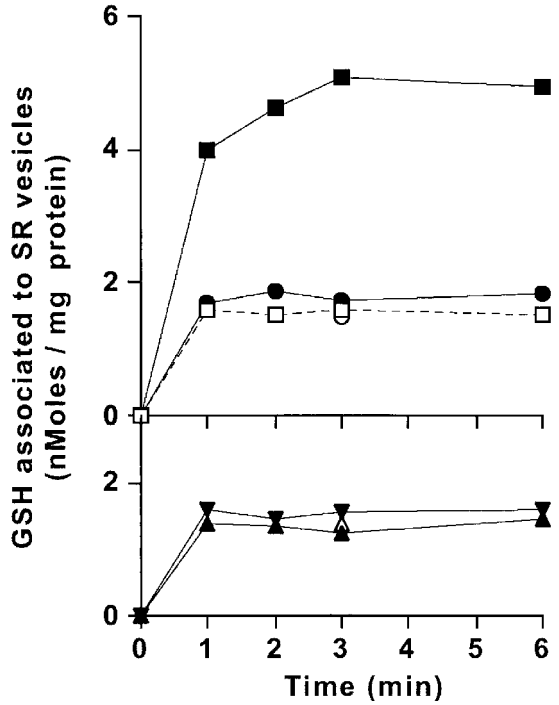

Figure 1 Blocking of GSH efflux from SR vesicles by lanthanum chloride

SR vesicles (1 mg of protein/ml) were incubated in the presence of 1 mM GSH and trace amounts of [^{3}H]glutathione (10 μCi/ml) at room temperature. At the indicated time points, aliquots (0.1 ml) were withdrawn to measure the ^{3}H associated with the vesicles. Lanthanum chloride (5 μM; ■), Ruthenium Red (5 μM; ▲) or ryanodine (300 μM; ▼) were added to vesicle suspensions 20 s before performing the rapid-filtration assay; the same concentration of each inhibitor was also included in the washing buffer to avoid GSH leakage during the filtration assay. ●, Control incubates (i.e. without terminating inhibitors). The corresponding open symbols represent parallel incubates, including alamethicin (50 μg/ml), which were run to evaluate the level of GSH non-specifically associated with vesicles. Results are means from 3–7 independent experiments; S.E.M. <10 % of means are omitted for clarity. $P<0.01$ compares lanthanum-terminated incubates (■) with controls (●).

we logically concluded that (i) intravesicularly accumulated GSH is rapidly released during the filtration-based procedure and (ii) lanthanum chloride, but not Ruthenium Red or ryanodine, can block the efflux of GSH from GSH-loaded SR vesicles.

On the basis of these results, lanthanum chloride was used to terminate the incubations in subsequent rapid-filtration measurements. Using this method, very little or no intravesicular free GSH was measured in vesicles that had been pretreated with Ruthenium Red or ryanodine before GSH uptake starts (Figure 2). This indicates that both antagonists of RyR can block the influx of GSH into SR vesicles, and it is consistent with the previous results obtained by using the light-scattering technique [12]. Pretreatment of the SR vesicles with lanthanum chloride had a similar effect (Figure 2), indicating that the tervalent cation can block both the influx and efflux of GSH in SR vesicles.

Inhibition of the vesicular GSH influx by lanthanum chloride was also proved using the light-scattering method. The addition of GSH (25 mM) caused a transient shrinking followed by a swelling phase, indicating the permeation of the molecule (Figure 3, trace *b*). When GSH was added to SR vesicles pretreated with lanthanum chloride, a more prolonged and sustained shrinking phase was observed, which reflects an inhibition of GSH influx (Figure 3, trace *a*).

Whereas lanthanum chloride inhibited both the influx and efflux of GSH into/from SR vesicles, Ruthenium Red and ryanodine appeared to inhibit only the influx of GSH. A possible logical

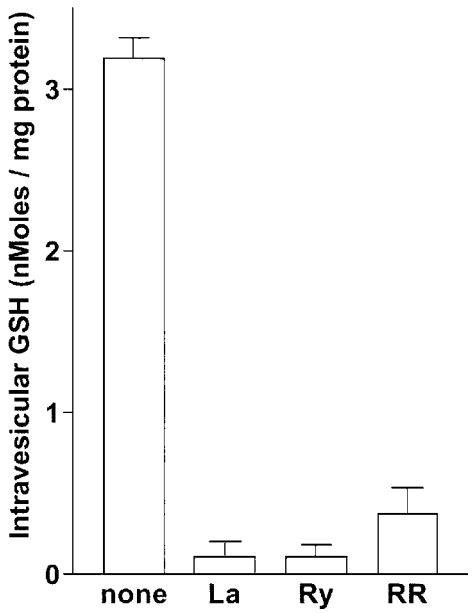

Figure 2 Prevention of the accumulation of GSH in SR vesicles by RyR inhibitors

SR vesicles (1 mg protein/ml) were incubated in the presence of 1 mM GSH and trace amounts of [^{3}H]glutathione (10 μCi/ml) for 3 min, and aliquots (0.1 ml) of the incubates were withdrawn to measure the ^{3}H associated with the vesicles by rapid filtration as detailed in the Experimental section. Intravesicular GSH values were calculated from the difference of vesicle-associated radioactivity in the absence and in the presence of alamethicin in the incubation system. GSH uptake was uniformly terminated by adding lanthanum chloride, SR vesicles were preincubated with lanthanum chloride (La; 5 μM), Ruthenium Red (RR; 5 μM) or ryanodine (Ry; 300 μM) for 1 min, before the addition of GSH. Results are means $\pm$ S.E.M. from 3–7 independent experiments.

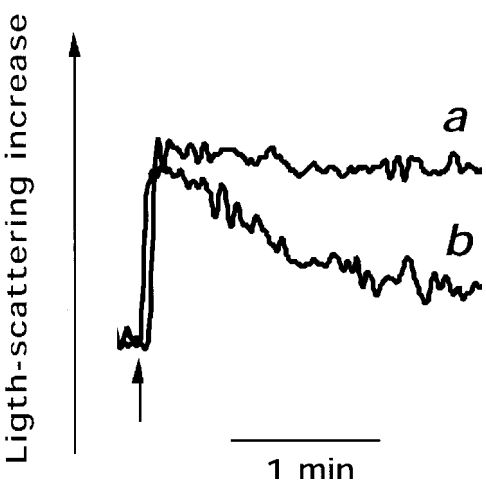

Figure 3 Inhibition of GSH uptake by lanthanum chloride in SR vesicles

GSH uptake was detected by light scattering as detailed in the Experimental section. Osmotically induced changes in light-scattering intensity after the addition of GSH (25 mM; marked with an arrow) were measured in control (trace *b*) and in La^{3+}-pretreated (5 μM, for 3 min; trace *a*) SR vesicles. Representative traces of 4–6 similar experiments are shown.

explanation is that intraluminal GSH renders RyR1 insensitive to these inhibitors. Alternatively, it can be assumed that different transporters facilitate the influx and efflux of GSH. To test these hypotheses, we determined whether intravesicular GSH influences the effect of RyR inhibitors on (i) the calcium efflux from SR vesicles or (ii) the GSH uptake into SR vesicles.

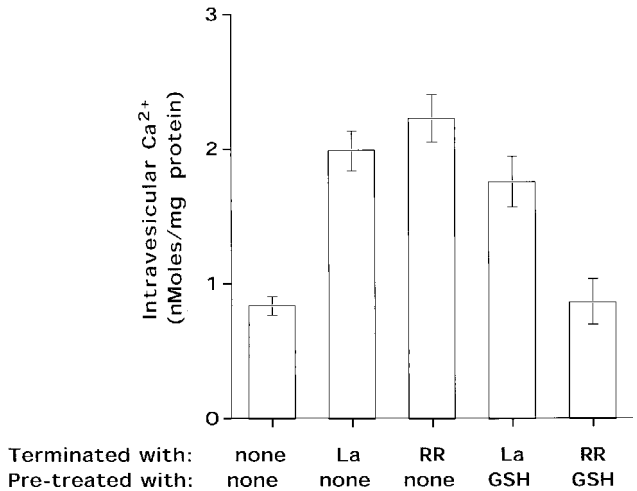

Figure 4 Insensitivity of calcium efflux to Ruthenium Red, but not to lanthanum chloride in SR vesicles after GSH uptake

SR vesicles (1 mg of protein/ml) were incubated in the presence of 20 μM $CaCl_2$ and trace amounts of $^{45}Ca^{2+}$ (10 μCi/ml) for 3 min, and aliquots (0.1 ml) of the incubates were withdrawn to measure the radioactivity associated with the vesicles by rapid filtration as detailed in the Experimental section. Levels of intravesicular Ca^{2+} were calculated from the difference in vesicle-associated radioactivity in the absence and in the presence of alamethicin in the incubation system. Ca^{2+} uptake was terminated by adding lanthanum chloride (La; 5 μM) or Ruthenium Red (RR; 5 μM) to incubates 20 s before performing the rapid-filtration assay. SR vesicles were preincubated with or without GSH (1 mM, for 10 min) before the addition of $^{45}Ca^{2+}$. Results are means $\pm$ S.E.M. from three independent experiments.

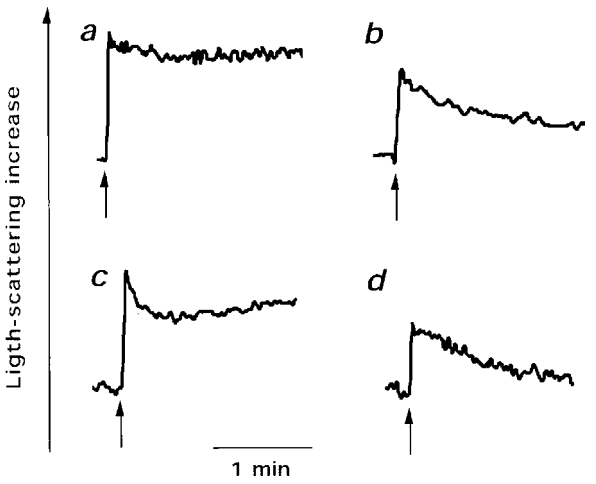

Figure 5 Non-inhibition of GSH transport in GSH-pretreated SR vesicles owing to RyR antagonists

GSH uptake was detected by light scattering as detailed in the Experimental section. Osmotically induced changes in light-scattering intensity were caused by the addition of GSH (25 mM, marked with arrows). SR vesicles were preincubated in the absence (traces *a* and *c*) or presence (traces *b* and *d*) of 1 mM GSH for 10 min at room temperature and, subsequently, with 300 μM ryanodine (traces *a* and *b*) or 5 μM Ruthenium Red (traces *c* and *d*) for 2 min, before the addition of 25 mM GSH. Representative traces of 4–6 similar experiments are shown.

First, we measured the effect of Ruthenium Red on the calcium efflux from SR vesicles with or without GSH pretreatment. The vesicles were passively loaded with radiolabelled Ca^{2+}. Steady-state levels of Ca^{2+} uptake were attained at 2–3 min of incubation (results not shown) similar to the case of GSH uptake (see Figure 1). As shown in Figure 4, termination of the incubation using either lanthanum chloride or Ruthenium Red largely increased the steady-state level of Ca^{2+} transport when compared with control incubates (i.e. washed without inhibitors), which indicates that both inhibitors can prevent the efflux of Ca^{2+} from SR vesicles. In vesicles preincubated with calcium in the presence of GSH (Figure 4), Ruthenium Red no longer inhibited the Ca^{2+} efflux, i.e. termination of the inhibition using lanthanum chloride resulted in a significant increase in the intravesicular Ca^{2+} levels. As the Ca^{2+} efflux from SR vesicles is attributed to RyR1, these results confirm that intraluminal GSH makes RyR1 resistant to Ruthenium Red.

Secondly, using the light-scattering method, we compared the effects of Ruthenium Red and ryanodine on GSH uptake with or without glutathione preloading. As shown in Figure 5, the pre-equilibration of extra- and intravesicular GSH (1 mM) resulted in a large decrease in the inhibitory effects of both Ruthenium Red and ryanodine on the influx of a subsequently added higher dose of GSH. This result suggests that intraluminal GSH affects the characteristics of glutathione influx and efflux as well as calcium efflux in a similar manner and, therefore, further supports our first hypothesis.

Glutathione specificity of the phenomenon was tested by measuring the effect of Ruthenium Red (5 μM) or ryanodine (300 μM) on the calcium-activated calcium efflux in SR vesicles. As detailed in the Experimental section, SR vesicles were loaded with Ca^{2+} in the presence of an ATP-generating system and the calcium fluxes were monitored using the fluorescent Ca^{2+} indicator Fluo 3. Both Ruthenium Red and ryanodine effectively delayed the calcium-activated calcium release (results not shown), indicating that RyR1 was sensitive to them in the presence of conventional agonists such as calcium and ATP.

It has been shown previously that a rapid equilibration of the intra- and extravesicular GSH concentrations favours the open probability of the RyR channel [10]. Our findings indicate that the RyR channel becomes insensitive to Ruthenium Red and ryanodine under these conditions.

Collectively, the above results show that lanthanum chloride is a useful agent for performing sensitive filtration assays of SR GSH transport. Moreover, they further support the possible direct involvement of the RyR1 channel in the transport of GSH. In fact, it is reasonable to assume that the tervalent cation may act preferentially by occluding the RyR channel, since it is well known that the tervalent cation blocks the permeability of a wide variety of unrelated cation channels.

Using lanthanum chloride as a channel blocker for the filtration-based transport assay, GSH transport was then investigated in microsomes from HEK-293 cells stably transfected for the RyR1. Wild-type HEK-293 cells do not constitutively express any RyR channel, whereas the stably transfected cell line expresses a functioning RyR1 channel (see the Experimental section). If RyR1 acts as a 'channel' for GSH, a skeletal-muscle SR-like GSH transport may also be detected in ER vesicles from cells expressing this channel isoform.

As shown in Figure 6, a substantial GSH transport was present in ER vesicles from both wild-type and RyR1-expressing cells. In microsomes from wild-type cells, a liver-like [11] GSH transport was observed, which was not at all affected by RyR1 antagonists. However, in microsomes from RyR1-expressing cells, the GSH transport appeared to have the same properties as that observed in skeletal-muscle SR vesicles. In particular, (i) the intravesicular level of transported GSH was severalfold higher in samples terminated by adding lanthanum chloride when compared with control samples (Figure 6A); (ii) GSH uptake could be inhibited by Ruthenium Red and ryanodine; and (iii) GSH uptake was also inhibited by high concentrations of Mg^{2+} ions (Figure 6B), which inhibit both RyR activity [21–23] and GSH transport in SR

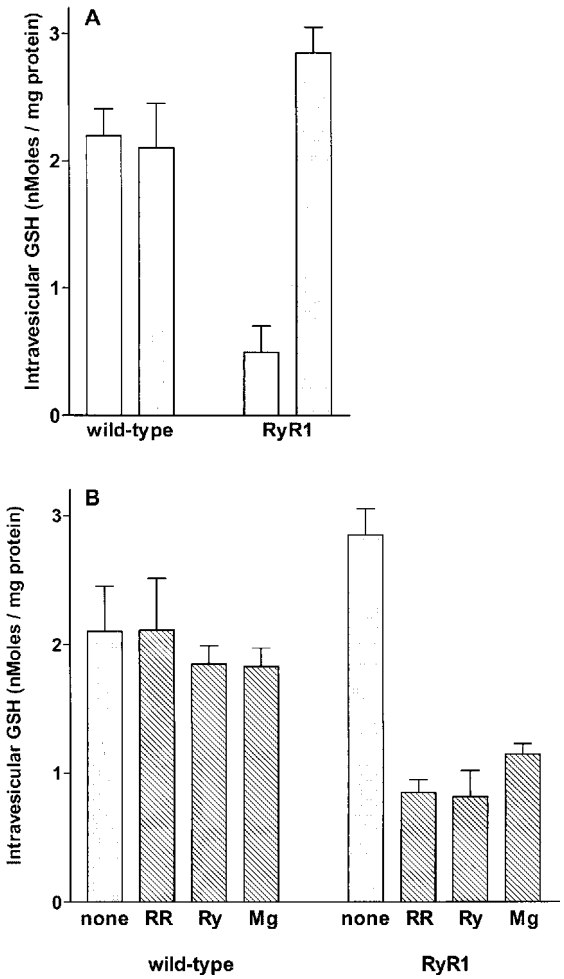

Figure 6 Uptake of GSH in microsomal vesicles prepared from RyR1-expressing HEK-293 cells

Microsomes were prepared from wild-type and RyR1-expressing HEK-293 cells as detailed in the Experimental section. Microsomal vesicles (0.5 mg of protein/ml) were incubated in the presence of 1 mM GSH and trace amounts of [^{3}H]glutathione (10 μCi/ml) for 3 min, and aliquots (0.1 ml) of the incubates were withdrawn to measure the ^{3}H associated with the vesicles by rapid filtration. Intravesicular GSH values were calculated from the difference of vesicle-associated radioactivity in the absence and in the presence of alamethicin in the incubation system. (**A**) GSH uptake was terminated by adding either lanthanum chloride (5 μM; shaded bars) or the solvent alone (5 μl of incubation buffer; open bars) to the incubates 20 s before performing the rapid-filtration assay. (**B**) Microsomal vesicles were preincubated with Ruthenium Red (RR; 5 μM), ryanodine (Ry; 300 μM) or $MgCl_2$ (Mg; 10 mM) for 1 min, before the addition of GSH. GSH uptake was uniformly terminated by adding lanthanum chloride. Results are means $\pm$ S.E.M. from 3–5 independent experiments.

vesicles [12]. The last two observations suggest that the expression of RyR1 somehow suppressed the basal GSH transport activity present in wild-type cells. Since the GSH transporter of the ER has not been explored at the molecular level, the exact mechanism underlying this phenomenon is still obscure.

A glutathione uptake has been recently observed in skeletal-muscle SR vesicles [10]. The uptake is dependent on the abundance of RyR1 and can be influenced by the physiological and experimental agonists/antagonists of RyR1 [12]. In the present study, we show that the channel blocker lanthanum chloride blocks both the influx and efflux of GSH in SR vesicles and the expression of RyR1 in cells constitutively lacking this channel results in the appearance of a skeletal-muscle phenotype of microsomal GSH transport. These results strongly suggest that the RyR1 channel mediates the permeation of GSH through the SR membrane. Our observation is novel but not unprecedented, as it has been proven earlier that RyR is not strictly specific to Ca^{2+} ions. The permeation of cations [choline and tris(hydroxymethyl) aminomethane] and neutral compounds (glucose, xylose and glycine) of low molecular mass through the RyR channel has been reported previously [24–26]. Recent observations show that a bulky cation such as neomycin is also capable of permeating the channel [27]. Moreover, glutathione fluxes through other ion channels (e.g. cystic fibrosis transmembrane conductance regulator [28]) have also been demonstrated.

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