Identification of a Ryanodine Receptor in Rat Heart Mitochondria

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Recent studies have shown that, in a wide variety of cells, mitochondria respond dynamically to physiological changes in cytosolic Ca2+ concentrations ([Ca2+]c). Mitochondrial Ca2+ uptake occurs via a ruthenium red-sensitive calcium uniporter and a rapid mode of Ca2+ uptake. Surprisingly, the molecular identity of these Ca2+ transport proteins is still unknown. Using electron microscopy and Western blotting, we identified a ryanodine receptor in the inner mitochondrial membrane with a molecular mass of approximately 600 kDa in mitochondria isolated from the rat heart. [3H]Ryanodine binds to this mitochondrial ryanodine receptor with high affinity. This binding is modulated by Ca2+ but not caffeine and is inhibited by Mg2+ and ruthenium red in the assay medium. In the presence of ryanodine, Ca2+ uptake into isolated heart mitochondria is suppressed. In addition, ryanodine inhibited mitochondrial swelling induced by Ca2+ overload. This swelling effect was not observed when Ca2+ was applied to the cytosolic fraction containing sarcoplasmic reticulum. These results are the first to identify a mitochondrial Ca2+ transport protein that has characteristics similar to the ryanodine receptor. This mitochondrial ryanodine receptor is likely to play an essential role in the dynamic uptake of Ca2+ into mitochondria during Ca2+ oscillations.

Mitochondria play a central role in numerous fundamental cellular processes ranging from the generation of ATP to the regulation of cytosolic Ca2+ homeostasis and apoptosis (1–3). Impairment of intracellular Ca2+ homeostasis and mitochondrial function has been implicated in the development of neurodegenerative diseases, diabetes, and cardiomyopathy (4–6). Measuring changes in mitochondrial Ca2+ concentrations ([Ca2+]m)1/2 in living cells with high sensitive fluorophores and targeted luminescence probes has drawn new attention on the regulation of mitochondrial Ca2+ homeostasis and its biological implications (7–9). Several studies show that, in many cell types, mitochondria respond dynamically to physiological oscillations of free [Ca2+]m (10–14).

In cardiac muscle cells, mitochondria also respond to physiological changes in [Ca2+]c (15). However, controversy remains whether the mitochondrial Ca2+ uptake mechanisms can sequester Ca2+ rapidly on a beat-to-beat basis (15). Established Ca2+ uptake mechanisms are the CaUP and rapid mode of Ca2+ uptake (1, 16). The mitochondrial CaUP is activated by Ca2+ concentrations greater than 10 μM, which are usually only achieved within cytosolic microdomains (9). In isolated liver mitochondria, rapid mode of Ca2+ uptake responds with mitochondrial Ca2+ uptake in response to Ca2+ pulses of less than 300 ms (16). Although these Ca2+ uptake mechanisms are kinetically and pharmacologically well characterized, their molecular identity has yet to be determined.

An intriguing observation is the considerable similarity in biochemical and pharmacological properties between the mitochondrial CaUP and the SR-RyR (1, 17). Both, the CaUP and the SR-RyR are activated by changes in [Ca2+]c and inhibited by adenine nucleotides, Mg2+, and RR. The strikingly similar properties of these proteins led to our hypothesis that mitochondria contain a RyR. Here we show with immunological, biochemical, pharmacological, and physiological techniques that heart mitochondria contain a functional RyR within the IMM. This mRyR underlies fast mitochondrial Ca2+ uptake and, therefore, is uniquely positioned to regulate dynamically Ca2+-mediated cellular processes such as ATP production during heartbeat (18).

EXPERIMENTAL PROCEDURES

Materials—Ryanodine was purchased from Calbiochem and [3H]ryanodine from Amersham Pharmacia Biotech. Antibodies against RyR (clone 54C), developed by J. Airey and J. Sutko, were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences of the University of Iowa (Iowa City, IA) and from RBI/Sigma. The antibodies against the voltage-dependent anion channel (VDAC) and the sarco- and endoplasmic reticulum Ca2+-ATPase (SERCA) were obtained from Calbiochem and Santa Cruz, respectively. All other chemicals were purchased from Sigma unless noted.

Isolation of Rat Heart Mitochondria—Heart mitochondria were isolated in isotonic ice-cold mannitol/sucrose buffer (M/S buffer; in mM: 225 mannitol, 10 sucrose, 0.5 EGTA, 1 glutathione; 10 HEPES, pH 7.4) by differential centrifugation and subsequent purification on a Percoll gradient (19). The final two washes were done in EGTA-free buffer. The isolated mitochondria were stored on ice and used for experiments for up to 4 h after finishing the isolation procedure. Experiments were done with mitochondria having intact RR- and carbonyl cyanide 3-chlorophenylhydrazone-sensitive Ca2+ uptake mechanisms, as measured with a Ca2+-sensitive microelectrode.

Preparation of Mitochondrial Subfractions—Mitochondrial subfractions were prepared as described previously (20). Briefly, isolated mi-
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FIG. 1. Immunogold labeling and Western blot analysis of a RyR in the IMM. A, immunogold labeling of RyR (arrows) in the IMM of isolated adult rat heart mitochondria (original magnification, ×50,000). Mitochondria were isolated as described under “Experimental Procedures.” The final sediment containing the purified mitochondria was fixed in paraform aldehyde and embedded in low temperature resin prior to immunogold labeling. B, as a negative control the gold-labeled secondary antibody was applied in absence of the RyR antibody, yielding no significance detection of RyR protein. C, Western blot analysis against RyR of mitochondrial subfractions from isolated and osmotically shocked heart mitochondria. Characterized subfractions were centrifuged for 90 min at 300,000 × g to sediment the RyR. C, SR containing cytosol; M, purified intact mitochondria; CS, contact sites; anti-RyR, labeling against RyR; anti-SERCA and anti-VDAC, Western blots against SERCA and VDAC as negative controls for contamination of the IMM with cytosolic membrane fragments and OMM. Arrows indicate the position and the molecular weight of the marker molecules. D, cytosol and mitochondria of four independent performed preparations were probed against SERCA. Lanes 1C–4C represent the cytosolic fractions obtained after the first centrifugation of the homogenate at 12,000 × g. Lanes 1M–4M represent the corresponding mitochondria after Percoll purification. C, cytosol; M, mitochondria.

FIG. 2. [3H]Ryanodine binding to isolated mitochondria in the presence or absence of modulators of the RyR. All assays were done in triplicate, and all figures represent a mean of at least three independent experiments. A, ryanodine binding to isolated heart mitochondria. Data were fit for a single class of binding sites by Scatchard plot (inset). Bmax was 398 ± 12 fmol/mg of protein, and the Kd was 9.52 nM (r = 0.97 for linear regression). B, bound [3H]ryanodine; F, free [3H]ryanodine. B, Ca2+ dependence of [3H]ryanodine binding. 100 μg of mitochondrial protein was incubated with various concentration of Ca2+ in presence of 9 nM [3H]ryanodine. C, inhibition of [3H]ryanodine binding with different concentrations of MgCl2. D, inhibition of [3H]ryanodine binding by RR.

Isolated heart mitochondria were embedded in Lowicryl mixture at −20 °C. Gold labeling of ultrathin sections of embedded mitochondria against RyR was performed as described previously (21, 22).

Immunogold Labeling Methods—For immunogold labeling of RyR, isolated heart mitochondria were embedded in Lowicryl mixture at −20 °C. Gold labeling of ultrathin sections of embedded mitochondria against RyR was performed as described previously (21, 22).

Denaturing SDS-Gel Electrophoresis—For the immunologcal detection of the RyR and SERCA, 100 μg of protein was loaded on a 5% SDS-polyacrylamide gel. The separated proteins were transferred onto a nitrocellulose membrane for 90 min at 100 V. For VDAC, 50 μg of protein was loaded onto a 12% SDS-polyacrylamide gel and transferred for 45 min at 100 V onto a nitrocellulose membrane. Western blots were performed using the Aurora chemiluminescence assay (ICN) with an alkaline phosphatase-linked secondary antibody.

[3H]Ryanodine Binding—For binding assays, 100 μg of mitochondrial protein were incubated with different concentrations of [3H]ryanodine in 0.5 ml of binding buffer (in mM: 170 KCl, 0.02 CaCl2, 10 MOPS, pH 7.0) for 16 h at 25 °C. For nonspecific binding, Ca2+ was replaced by 6 mM EGTA. At the end of the incubation time, the reaction mixture was filtered under reduced pressure through glass fiber filters (Whatman) and washed with ice-cold buffer (170 mM KCl, 10 mM MOPS).
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RESULTS

Immunological Detection of RyR in Isolated Heart Mitochondria—Electron microscopic analysis revealed that ~70% of isolated adult rat heart mitochondria, treated with gold-labeled antibodies against SR-RyR, were labeled with 1–4 gold particles (Fig. 1A). The majority of the gold particles were found in the cristae membrane of IMM (73.3% of total), although some labeling was detected in the peripheral IMM. In contrast, no significant labeling was found in the OMM and extramitochondrial membranes (3.5% of total counted gold particles). In absence of the RyR antibody, only 5% of the mitochondria showed labeling with a maximum of 1 gold particle (Fig. 1B).

Using Western blot analysis, we confirmed the specific detection of RyR-like protein in the IMM from osmotically shocked rat heart mitochondria (Fig. 1C). Western blots performed on the cytosolic and mitochondrial subfractions demonstrated immunoreactivity against RyR in all fractions except the OMM (Fig. 1C, anti-RyR). A positive signal against RyR protein in the IMM was obtained in all preparations (n = 6). In all tested fractions, the RyR antibody labeled a protein of ~680 kDa. The purity of the IMM fraction was verified by the presence of marker pump proteins (Fig. 1D), indicating that the intact IMM fraction is devoid of any significant contamination from SR and OMM fragments. In addition, SERCA pump proteins were not detected in four independently isolated mitochondrial preparations (Fig. 1D), indicating that even the intact IMM fraction had minimal SR contamination.

An extensive effort to exclude the possibility that the mRyR was described previously (23). Briefly, isolated heart mitochondria (1 mg of protein) were diluted in 1 ml of modified M/S buffer (in mM: 120 KCl, 65 mannitol, 30 sucrose, 10 succinate, 5 Na2HPO4/NaH2PO4, 10 HEPES, pH 7.2). The absorbance was recorded with a spectrophotometer (Spectronic) at 540 nm for 2–3 min to obtain a stable baseline, followed by the addition of Ca2+ to induce mitochondrial swelling.
was not caused by contamination with SR-RyR was deemed essential, because mitochondria are located in close proximity to the SR in cardiac muscle cells (14, 24). The chosen marker proteins, such as SERCA, VDAC, and SDH, are specific for their intracellular location. Labeling of the RyR in the purified IMM with a specific antibody is consistent with the result obtained by electron microscopy, showing that the mRyR is localized within the IMM (Fig. 1A). The molecular weight of the detected RyR proteins in the IMM and the cross-reactivity with the used SR-RyR specific antibody suggests that the mRyR is structurally homologous to the SR-RyR.

\[ \text{[3H]} \text{Ryanodine Binding to Isolated Heart Mitochondria—} \]

Various physiological and pharmacological effectors including Ca\(^{2+}\), caffeine, Mg\(^{2+}\), and RR modulate ryanodine binding to the SR-RyR (25, 26). To characterize the pharmacological properties of mRyR, we studied \( \text{[3H]} \text{ryanodine binding to isolated heart mitochondria in presence of these modulators.} \]

\[ \text{[3H]} \text{Ryanodine bound to isolated heart mitochondria in presence of 20 \( \mu \text{M Ca}^{2+} \) with an apparent affinity (} K_a \text{)} \text{ of 9.8} \pm 2.1 \text{ nM} (n = 3, \text{ in triplicate for all binding experiments; Fig. 2A). Depending on the experimental conditions employed, it has been reported that SR-RyR exhibited an apparent } K_a \text{ between 2 and 200 nM for ryanodine (27, 28). The maximal density of mRyR binding sites (} B_{\text{max}} \text{) was 398.4} \pm 12 \text{ fmol/mg of protein} (n = 3, \text{ Scatchard plot in Fig. 2A), which is } \sim 10 \text{ times less than that described for } \text{[3H]} \text{ryanodine binding to purified SR membranes under similar experimental conditions (28, 29). To confirm that}\]

\[ \text{[3H]} \text{ryanodine binding was not due to contamination by SR, binding was performed with equal amounts of proteins from intact isolated mitochondria and mitochondrial subfractions. The results showed that } 90 \pm 5\% (n = 3) \text{ of the total } \text{[3H]} \text{ryanodine bound to mitochondria was due to binding to the IMM and not to other fractions.} \]

\[ \text{The amount of } \text{[3H]} \text{ryanodine binding to mitochondria as a function of free Ca}^{2+} \text{ concentration in the assay media was biphasic. Binding increased at pCa between 5 and 7 and decreased at pCa between 3 and 4 with maximal binding at pCa 5.3 (Fig. 2B). Surprisingly, unlike the cardiac SR-RyR, caffeine did not enhance mitochondrial ryanodine binding (n = 3). Caffeine-insensitive RyR, however, have been described in canine salivary glands (30) and in Jurkat cells (31). Mg}^{2+} \text{ is known to decrease the } B_{\text{max}} \text{ for } \text{[3H]} \text{ryanodine binding and to inhibit SR Ca}^{2+} \text{ efflux in skeletal and cardiac muscle (26, 32, 33). This is consistent with single-channel studies,} \]
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where millimolar concentrations of Mg\(^{2+}\) reduced the open probability of the RyR and maintained the channel in a closed state (25, 26, 32). Accordingly, we studied the effects of Mg\(^{2+}\) on \(^{3}H\)ryanodine binding in isolated heart mitochondria and observed a 50% inhibition in presence of 0.33 mM Mg\(^{2+}\) (n = 3, Fig. 2C). Under comparable experimental conditions, it has been shown that up to 1 mM Mg\(^{2+}\) did not have any inhibitory effects on ryanodine binding in cardiac SR-RyR (34–36). This suggests that, in cardiac muscle cells, the mRyR is more sensitive to Mg\(^{2+}\) inhibition of \(^{3}H\)ryanodine binding than the SR-RyR.

RR inhibits the release of Ca\(^{2+}\) from the SR in skeletal and cardiac muscles by decreasing the open probability of the RyR (26, 37). After RR treatment, \(^{3}H\)ryanodine binding to isolated rat heart mitochondria was strongly inhibited with IC\(_{50}\) = 105 nM (n = 3, Fig. 2D). This suppression of mitochondrial \(^{3}H\)ryanodine binding by RR is much more potent than that observed in cardiac SR-RyR, which had IC\(_{50}\) values between 290 and 1,000 nM (34, 35). Consistent with the binding data, we have shown that RR (1–5 μM) blocks mitochondrial Ca\(^{2+}\) uptake without much effect on SR Ca\(^{2+}\) release in chemically skinned cardiac myocytes (14). These results indicate that significant differences exist in the potency of RR inhibiting mRyR and SR-RyR.

The binding data provide pharmacological evidence of the existence of mRyR in the IMM. They also show that there are distinct differences between mRyR and SR-RyR with respect to their abundance and their sensitivities to caffeine, Mg\(^{2+}\), and RR. Therefore, the mRyR and SR-RyR would operate at different capacities under similar conditions and could be regulated and modulated differentially.

**Ryanodine Inhibition of Mitochondrial Ca\(^{2+}\) Uptake**—We next investigated functional aspects of mRyR in the sequestration of Ca\(^{2+}\) using two different methods. Adding mitochondria to a buffer containing 60 μM free Ca\(^{2+}\) caused a significant mitochondrial Ca\(^{2+}\) uptake, as measured by a decrease in the extramitochondrial Ca\(^{2+}\) concentration with a Ca\(^{2+}\)-selective microelectrode (Fig. 3A, control). In the presence of 100 or 10 μM ryanodine, mitochondrial Ca\(^{2+}\) uptake was suppressed by 60 ± 2.7% or 41.2 ± 1.9%, respectively (Fig. 3A, control). Removal of the OMM with digitonin (25 μg/mg of mitochondrial protein for 30 s) to minimize possible SR-contamination had no significant effect on mitochondrial Ca\(^{2+}\) uptake in the presence or absence of ryanodine (Fig. 3D).

The same inhibitory effect in mitochondrial Ca\(^{2+}\) uptake was observed with dantrolene, a compound that has been shown to inhibit the skeletal muscle SR-RyR and therefore Ca\(^{2+}\) release (17). The effect of dantrolene on the cardiac SR-RyR is still controversial (38–40). However, incubation of isolated mitochondria with 10 μM dantrolene decreased mitochondrial Ca\(^{2+}\) uptake by 55.9 ± 7.8% (Fig. 3, B and D; n = 5). Finally, the presence of 30 μM cyclopiazonic acid (CPA), an inhibitor of SERCA, did not alter mitochondrial Ca\(^{2+}\) uptake compared with untreated mitochondria (96 ± 2.4%, n = 3), indicating little contamination of the SR-RyR (Fig. 3, C and D).

The relatively slow response time of Ca\(^{2+}\) microelectrodes made them unable to follow the rapid mitochondrial Ca\(^{2+}\) uptake during Ca\(^{2+}\) pulses. To investigate whether the mRyR contributes to rapid Ca\(^{2+}\) uptake, we measured the Ca\(^{2+}\) responses of single or small clusters of isolated, rhod-2-loaded mitochondria using high speed digital imaging in combination with pulsatilie flash photolysis of caged Ca\(^{2+}\). As shown in Fig. 4 (A and C), Ca\(^{2+}\) uptake was stimulated in rhod-2-loaded isolated heart mitochondria after flash photolysis of caged Ca\(^{2+}\) in the external solution. The slow decay in the fluorescence may result from absence of Na\(^{+}\) in the solution that inhibited Na\(^{+}\)-dependent Ca\(^{2+}\) efflux. In Fig. 4B, a low affinity Ca\(^{2+}\) indicator Oregon Green Bapta-5N was used in separate experiments to confirm that pulsatilie flash photolysis resulted

![Fig. 5](http://www.jbc.org/)

**Fig. 5. Ryanodine inhibits Ca\(^{2+}\)-induced mitochondrial swelling.** In all experiments, 1 mg of mitochondrial protein were dissolved in modified M/S buffer and swelling was induced by the addition of 100 μM Ca\(^{2+}\). A, this figure shows a representative concentration-response curve of ryanodine-inhibited mitochondrial swelling. Heart mitochondria were preincubated for 15 min at room temperature with the indicated concentration of ryanodine. Ca\(^{2+}\) was added at time 0 (arrow), and the change in absorbance at 540 nm was followed. Data are expressed as the ratio of the absorbance at any given time (A) divided by the baseline absorbance at time 0 (Ao). B, averaged changes of the inhibitory effect of ryanodine (Rya) on mitochondrial swelling. Each bar represents at least six independently performed experiments. Data are expressed as mean values ± S.E., and p values <0.01 by paired t test were considered statistically significant (asterisks).

![Fig. 6](http://www.jbc.org/)

**Fig. 6. Mitochondrial swelling in the presence or absence of modulators of the RyR or SERCA.** Each bar represents the averaged decrease in absorbance of at least three independently performed experiments. Experiments were performed as in Fig. 5. Ca\(^{2+}\); 100 μM Ca\(^{2+}\); CPA, incubation with 50 μM CPA for 10 min at room temperature prior the addition of Ca\(^{2+}\); Dan, preincubation with 10 μM dantrolene for 10 min; Rya, 20 μM ryanodine. C, SR containing cytosolic fraction. Data are expressed as mean values ± S.E.
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**FIG. 7. A model of Ca**

\(^{2+}\) **dynamics in cardiac myocytes.** Mitochondria sense microdomains with high Ca\(^{2+}\) following activation of voltage-gated Ca\(^{2+}\) channels (VGCC) and Ca\(^{2+}\) release from intracellular stores. These raises could trigger fast mitochondrial Ca\(^{2+}\) uptake via the mRyR and/or CaUP, which may ultimately affect physiological and pathological processes.

in elevations in extramitochondrial Ca\(^{2+}\) that reached to their peak value within sampling time of one frame (250–500 ms). After pre-incubating mitochondria with 100 \(\mu\)M ryanodine, a significant component of the evoked change in mitochondrial fluorescence was suppressed (Fig. 4C). The cumulative change in fluorescence (\(\Delta F\)) evoked by each flash was 0.14 ± 0.02, 0.34 ± 0.05, 0.58 ± 0.08, 0.85 ± 0.04, and 1 for control; and 0.05 ± 0.02, 0.12 ± 0.04, 0.25 ± 0.07, 0.40 ± 0.09, and 0.60 ± 0.13 for ryanodine-treated mitochondria (\(n = 6\), Fig. 4D). The magnitude of \([\text{Ca}^{2+}]_m\) at higher number of flashes in the control solution could be an underestimation, due to the saturation of rhod-2 (\(K_d = 0.6\)–0.8 \(\mu\)M). This could account for the decrease in the slope of control curve between flash number 4 and 5 in Fig. 4D. Finally, this mitochondrial Ca\(^{2+}\) uptake was not due to release of Ca\(^{2+}\) from SR contamination, because repetitive UV flashes applied to rhod-2-loaded mitochondria in a droplet containing 100 \(\mu\)M t-myo-inositol 1,4,5-triphosphate, P\(^{4,5,6}\)-1-(2-nitrophenyl)/ethyl ester, a concentration of caged inositol 1,4,5-triphosphate shown to evoke robust Ca\(^{2+}\) release from reticular stores (41), induced no measurable \(\Delta F\).

The ability of mitochondria to sequester significant amount of Ca\(^{2+}\) through a ryanodine inhibitory pathway suggests that the mRyR could play an important role in buffering high concentrations of [Ca\(^{2+}\)]\(_m\). Consistent with our experiments, a decrease in [Ca\(^{2+}\)]\(_m\) in the presence of ryanodine has been observed in A10 cells when perfused with more than 1 \(\mu\)M Ca\(^{2+}\) (42). Moreover, the ability of mitochondria to respond instantaneously to fast Ca\(^{2+}\) pulses through a ryanodine inhibitable pathway suggests that the mRyR may be responsible for Ca\(^{2+}\) sequestration during heartbeats.

**Inhibition of Mitochondrial Swelling by Ryanodine—Excessive accumulation of Ca**

\(^{2+}\) in the mitochondrial matrix depolarizes the mitochondrial membrane potential and disrupts fundamental mitochondrial functions like oxidative phosphorylation and ATP production, which results in opening of the mitochondrial permeability transition pore in isolated heart and liver mitochondria (23, 43). This is accompanied by mitochondrial swelling and can be measured by the decrease of absorbance at 540 nm. Inducing mitochondrial swelling in isolated intact heart mitochondria with 100 \(\mu\)M Ca\(^{2+}\) led to a decrease in absorbance of 19.5 ± 3.4\% (\(n = 8\)). Upon preincubation with 2–20 \(\mu\)M ryanodine, we observed a concentration-dependent inhibition of Ca\(^{2+}\)-induced mitochondrial swelling (Fig. 5A). Interestingly, less then 2 \(\mu\)M ryanodine significantly enhanced mitochondrial swelling in all experiments (Fig. 5, A and B; \(n = 6\)). CPA had no effect on the inhibition of mitochondrial swelling after treatment with 20 \(\mu\)M ryanodine (Fig. 6). In addition, the concentration dependence in ryanodine-mediated inhibition of mitochondrial swelling by ryanodine was also not altered by CPA treatment (data not shown). An accelerated mitochondrial swelling in the presence of less than 2 \(\mu\)M ryanodine or the inhibition of swelling by higher ryanodine concentrations is in agreement with the literature, where low ryanodine concentrations switch the SR-RyR into an open state and higher concentrations keep the Ca\(^{2+}\) channel in the RyR closed (17, 44).

Dantrolene (10 \(\mu\)M) was as effective as 20 \(\mu\)M ryanodine in blocking mitochondrial swelling (Fig. 6). Finally, the SR-containing cytosolic fraction itself revealed no significant changes in absorbance after the addition of Ca\(^{2+}\) (Fig. 6). In these experiments the same amount of cytosolic protein and experimental protocol was used as that described for mitochondrial swelling. Therefore, the possibility that the effect of mitochondrial swelling could be mimicked by cytosolic components like the SR was excluded.

Mitochondrial swelling due to the opening of the mitochondrial permeability transition pore has been implicated in triggering apoptosis and necrosis of several cell types (45). The ability of ryanodine to prevent such mitochondrial swelling by blocking influx of Ca\(^{2+}\) may provide some insights into the development of novel therapeutic agents for mitochondria-mediated cell injury and death.

**DISCUSSION**

The present study demonstrates that mitochondria contain a RyR within the IMM, which shares several, similar biochemical, pharmacological, and physiological properties with both the SR-RyR and the CaUP. Based on the results presented here, a contamination of the purified mitochondria by SR-RyR can be excluded for several reasons. 1) Preparations of isolated mitochondria were free of detectable amounts of SERCA protein. 2) The separated OMM fraction tested positive for VDAC but negative for SDH activity. 3) The separated IMM tested positive for SDH but negative for the OMM protein VDAC and the SR protein SERCA. 4) CPA had no effect on mitochondrial Ca\(^{2+}\) uptake and mitochondrial swelling. 5) \(^{[3H]}\)Ryanodine binding was more sensitive to Mg\(^{2+}\) and RR inhibition. 6) Caffeine had no effect on ryanodine binding.

The localization of mRyR in the IMM may raise the question of whether the RyR, like other intracellular membrane proteins, could have multiple intracellular locations. It has been shown that the inositol 1,4,5-triphosphate receptor is localized in the nucleus (46, 47) and the plasma membrane (48) in addition to the ER and SR. Likewise, proteins of the cell death-regulating Bcl-2 family have been shown to be localized in the OMM, the nucleus membrane, and the ER membranes (49). A
Na⁺/Ca²⁺ exchanger is localized in the IMM but also in the plasma membrane (1).

An increase in [Ca²⁺]i activates the RyR in the SR or ER to release Ca²⁺ from intracellular stores due to an outwardly directed Ca²⁺ electrochemical gradient. Conversely, the inwardly directed Ca²⁺ electrochemical gradient in mitochondria could result in Ca²⁺-induced Ca²⁺ uptake following the activation of mRyR. Finally, because of the pharmacological similarities to the CaUP, it is tempting to speculate that the mRyR is the CaUP.

The proximity between the mitochondria and other Ca²⁺ transport proteins, such as SR- or ER-RyR and L-type Ca²⁺ channels, would allow mitochondria to sense microdomains with Ca²⁺ concentrations sufficient to open the mRyR (Fig. 7). Ca²⁺ released by SR-RyR has been shown to activate mitochondrial Ca²⁺ uptake and finally, because of the pharmacological similarities to the CaUP, it is tempting to speculate that the mRyR is the CaUP.

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**REFERENCES**

1. Gunter, T. E., Gunter, K. K., Sheu, S.-S., and Gavin, C. E. (1994) Am. J. Physiol. 267, C313–C339
2. Duchen, M. R. (2000) J. Physiol. (London) 529, 57–68
3. Bernardi, P., Petronilli, V., Di Lisa, F., and Forte, M. (2001) Trends Biochem. Sci. 26, 112–117
4. Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Mateumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I., and Bronsme, M. (2000) Nature 404, 787–790
5. Beal, M. F. (1996) Curr. Opin. Neurobiol. 6, 661–666
6. Wallace, D. C. (1999) Science 283, 1482–1488
7. Babcock, D. F., Herrington, J., Goodwin, P. C., Park, Y. B., and Hille, B. (1997) J. Cell Biol. 136, 833–844
8. Sheu, S.-S., and Jou, M. J. (1994) Bioenerg. Biomembr. 26, 487–493
9. Rizzuto, R., Brini, M., Murgia, M., and Pozzan, T. (1993) Science 262, 744–747
10. Simpson, P. B., and Russell, J. T. (1990) J. Biol. Chem. 265, 33483–33501
11. Hajnoczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) Cell 82, 415–424
12. Chacon, E., Ohata, H., Harper, I. S., Trellinger, D. R., Herman, B., and Lemasters, J. J. (1996) FEBS Lett. 382, 31–36
13. Duchen, M. R., Lysens, A., and Crompton, M. (1998) J. Cell Biol. 142, 57–68
14. Sharma, V., Ramesh, V., Franzini-Armstrong, C., and Sheu, S.-S. (2000) J. Bioenerg. Biomembr. 32, 97–104
15. Hueser, J., Blatter, L. A., and Sheu, S.-S. (2000) J. Bioenerg. Biomembr. 32, 27–33
16. Sparagna, G. C., Gunter, K. K., Sheu, S.-S., and Gunter, T. E. (1995) J. Biol. Chem. 270, 27510–27515
17. Franzini-Armstrong, C., and Prostasj, F. (1997) Physiol. Rev. 77, 699–729
18. McCormack, J. G., and Denton, R. M. (1993) Biochem. Soc. Trans. 21, 793–799
19. Rehneron, S., Mela, L., and Siesjo, B. K. (1979) Stroke 10, 437–446
20. Ohlendieck, K., Riesing, I., Adams, V., Krause, J., and Bredica, D. (1986) Biochim. Biophys. Acta 860, 672–689
21. Herrera, G. A. (1989) Ultrastruct. Pathol. 13, 485–499
22. de Mesy Jensen, K. L., and di Sant’Agnese, P. A. (1992) Ultrastruct. Pathol. 16, 51–59
23. Petronilli, V., Cola, C., Massari, S., Colonna, R., and Bernardi, P. (1993) J. Biol. Chem. 268, 21839–21845
24. Ogata, T., and Yamazaki, Y. (1997) Anat. Rec. 248, 214–223
25. Pessah, I. N., Stambuk, R. A., and Casida, J. E. (1987) Mol. Pharmacol. 31, 232–238
26. Meissner, G., Darling, E., and Eveleth, J. (1998) Biochemistry 25, 236–244
27. Inui, M., Saito, A., and Fleischer, S. (1987) J. Biol. Chem. 262, 15637–15642
28. Lindsay, A. R., and Williams, A. J. (1991) Biochim. Biophys. Acta 1064, 89–102
29. Kijima, Y., Saito, A., Jetton, T. L., Magirus, M. A., and Fleischer, S. (1993) J. Biol. Chem. 268, 3489–3506
30. Yamaki, H., Morita, K., Kitayama, S., Imai, Y., Itadani, K., Akagaya, Y., and Dohi, T. (1998) J. Dent. Res. 77, 1807–1816
31. Hakamata, Y., Nishimura, S., Nakai, J., Nakashima, Y., Kita, T., and Imoto, K. (1994) FEBS Lett. 352, 206–210
32. Meissner, G., and Henderson, J. S. (1987) J. Biol. Chem. 262, 3065–3073
33. Liu, W., Pasek, D. A., and Meissner, G. (1998) Am. J. Physiol. 274, C129–C128
34. Pessah, I. N., Waterhouse, A. L., and Casida, J. E. (1985) Biochim. Biophys. Res. Commun. 128, 449–456
35. Holmberg, S. B., and Williams, A. J. (1990) Biochim. Biophys. Acta 1022, 187–193
36. Zimanyi, I., and Pessah, I. N. (1991) J. Pharmacol. Exp. Ther. 256, 938–946
37. Xu, L., Tripathy, A., Pasek, D. A., and Meissner, G. (1999) J. Biol. Chem. 274, 32680–32691
38. Frue, B. R., Mickelson, J. R., and Louis, C. F. (1997) J. Biol. Chem. 272, 26965–26971
39. Meissner, G., Min, Y. J., Haake, N., Hirt, S., and Simon, R. (1999) Eur. J. Heart Fail. 1, 177–186
40. Fratea, S., Langeron, O., Lecarpentier, Y., Coriat, P., and Rios, B. (1997) Anesthesiology 86, 205–215
