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Involvement of the 60 kDa phosphoprotein in the regulation of Ca^{2+} release from sarcoplasmic reticulum of normal and malignant hyperthermia susceptible pig muscles

Do Han Kim [a,d], Frank A. Sreter [a,b,c], Noriaki Ikemoto [a,c]

[a] *Department of Muscle Research, Boston Biomedical Research Institute,* [b] *Department of Anesthesia, Massachusetts General Hospital,* [c] *Department of Neurology, Harvard Medical School, Boston, MA and* [d] *Department of Medicine, Cardiology Division, University of Connecticut Health Center, Farmington, CT (U.S.A.)*

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Junctional sarcoplasmic reticulum (SR) vesicles isolated from back muscles of normal and malignant hyperthermia susceptible (MHS) pigs were phosphorylated by addition of MgATP in the presence of 5 mM Ca^{2+} and 1 μM calmodulin (CaM). The major site of phosphorylation was a 60 kDa protein both in normal and MHS SR. The maximal amount of phosphorylation in MHS SR (5 pmol P/mg SR) was significantly lower than that in the normal SR (12 pmol P/mg SR). The phosphorylated 60 kDa protein was spontaneously dephosphorylated both in normal and MHS SR. Ca^{2+} release from the passively loaded SR was induced by a Ca^{2+}-jump, and monitored by stopped-flow fluorometry using chlorotetracycline. In the absence of preincubation with MgATP, no significant difference was found in any of the kinetic parameters of Ca^{2+} release between normal and MHS SR. Upon addition of 20 μM MgATP to the passively loaded SR to phosphorylate the 60 kDa protein, the initial rate of Ca^{2+} release in normal SR significantly decreased from 659 ± 102 to 361 ± 105 nmol Ca^{2+}/mg SR per s, whereas in MHS SR the rate decreased from 749 ± 124 to 652 ± 179 nmol Ca^{2+}/mg SR per s. Addition of 20 μM adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA) did not significantly alter the initial rate of Ca^{2+} release both in normal and MHS SR. These results suggest that the previously reported higher Ca^{2+} release rate in MHS SR (Kim et al. (1984) Biochim. Biophys. Acta 775, 320–327) is at least partly due to the reduced extent of the Ca^{2+}/CaM-dependent phosphorylation of the 60 kDa protein. Two-dimensional gel electrophoresis study showed that amount of a protein with $M_r = 55000$ was significantly lower in MHS SR than in normal SR suggesting that the abnormally lower amount of 55 kDa protein would cause the lower amount of phosphorylation of the 60 kDa protein in MHS SR.

Introduction

Malignant hyperthermia is a pharmaco-genetic disorder involving abnormal intracellular Ca^{2+} movements in muscle cells during exposure to volatile anaesthetics such as halothane [1–6]. On the basis of various studies using fragmented SR vesicles [7–12], skinned fiber preparation [13] or muscle biopsies [14–17], it has been proposed that sites of the primary defects are in the sarcoplasmic reticulum (SR). Recent evidence has shown that a mechanism involved in the communication be-

Correspondence: Do Han Kim, Department of Medicine, Cardiology Division, University of Connecticut Health Center, Farmington, CT 06032, U.S.A.

tween transverse tubule and SR may also be abnormal in MHS muscle [18].

Our previous studies [11] have suggested that in MHS pig muscle the putative Ca^{2+} release channels located in the SR appear to be altered in such a way that all types of Ca^{2+} release investigated occur at higher rates than normal. Recently, we have shown that Ca^{2+}/calmodulin (CaM)-dependent phosphorylation of a 60 kDa protein inhibits Ca^{2+} release in the skeletal muscle SR [19,20], suggesting that the 60 kDa protein is involved in the regulation of Ca^{2+} release. The aim of this study is to test the hypothesis that the altered Ca^{2+} release in MHS SR [11] is due to an abnormal regulatory mechanism including phosphorylation of the 60 kDa protein. The results show that in MHS SR the level of phosphorylation of the 60 kDa protein is lower and the extent of reduction of the initial Ca^{2+} release rate is significantly lower in MH SR than in normal SR. This alteration appears to be at least in part a cause for the higher Ca^{2+} release rates in the MHS SR reported previously [11].

Materials and Methods

Care and screening of pigs for MHS trait were carried out as described previously [11].

Preparation of sarcoplasmic reticulum. A junctional SR fraction from pig back muscle (primarily fast twitch muscle) was prepared by differential centrifugation as described previously [21]. The final pellets were suspended in a solution containing 0.15 M KCl, 20 mM Mes (pH 6.8), 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/l aprotinin, 0.8 mg/l antipain, 2 mg/l trypsin inhibitor, and 0.3 M sucrose. The final protein concentration was adjusted to 20–30 mg/ml and the samples immediately frozen in liquid nitrogen and stored at −80 °C were used within 3–4 weeks.

Phosphorylation of the SR proteins. In order to phosphorylate the SR membrane proteins, 0.5 mM [γ-^{32}P]ATP (300 cpm/pmol) and equimolar $MgCl_2$ were added to a solution containing 15 mg/ml SR, 0.15 M KCl, 20 mM Mes (pH 6.8), 5 mM $CaCl_2$ and 1 μM calmodulin in the presence of the various proteinase inhibitors described above. The phosphorylation reaction was quenched by 10-fold dilution of the reaction mixture with a solution containing 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 10% glycerol, and 3.3 μg/ml Bromophenol blue. The proteins in the quenched solution were separated by 7 or 10% SDS-polyacrylamide gel electrophoresis according to Laemmli [22]. The gels were stained by Coomassie brilliant blue and after destaining, the gels were dried with cellophane paper backing. The dried gels were autoradiographed using Kodak X-Omat film. For determining the amount of ^{32}P incorporated into the proteins, the gels were sliced into 1 mm pieces and before the scintillation counting the gel slices were incubated in 0.2 ml of 7.5% H_2O_2 solution overnight at 50 °C.

Ca^{2+} release assay. For stopped-flow fluorometry using chlorotetracycline, SR incubated in a solution containing 0.15 M KCl, 20 mM Mes (pH 6.8), and 5 mM $CaCl_2$ for 4–5 h at 2–4 °C was further incubated for 20 min in a solution containing 0.15 KCl, 20 mM Mes (pH 6.8), 5 mM $CaCl_2$, and 20 μM chlorotetracycline. In order to phosphorylate the SR proteins, calmodulin (CaM) (67 pmol/mg SR) and MgATP (33 nmol/mg SR) were added to the SR loaded with 5 mM $CaCl_2$ and 20 μM chlorotetracyline. The solution was then loaded in syringe A of a stopped-flow apparatus (Dionex model D-100). Ca^{2+} release was triggered by mixing the content of syringe A with an equal volume of a solution in syringe B containing 0.15 M KCl, 20 mM Mes (pH 9.3), 6.35 mM EGTA, and 20 μM chlorotetracyline. The free Ca^{2+} concentration was 2 μM after mixing and the pH of the solution was equilibrated to 6.8 within 2 ms after mixing. Changes in the fluorescence intensity of chlorotetracycline [23–26] during Ca^{2+} release assay were measured using the computerized stopped-flow fluorometer (excited at 395 nm, and the emitted light was collected through an interference filter at 510 nm). Various kinetic parameters of the Ca^{2+} release were calculated by an iterative fitting algorithm [27].

Two-dimensional gel electrophoresis. The proteins in normal and MHS SR were separated by two-dimensional gel electrophoresis system. The SR vesicles solubilized in 9.5 M urea, 2% Nonidet P-40, 2% ampholine and 5% β-mercaptoethanol were separated by isoelectric focusing (pH 4.5 and 7.5) and 10% SDS gel electrophoresis according to the method of O'Farrell [28]. The gels were stained

by Coomassie brilliant blue or Stains-all [29]. The relative protein amount was determined by densitometry scanning and calculation of area of stained protein spots. Identification of glycoproteins by treatment with Endo H was carried out using the method of Campbell et al. [30,31].

Miscellaneous. Chlorotetracyline, and calmodulin were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents used were of analytical grade. Protein concentrations were determined by the Lowry method using bovine serum albumin as a standard [32].

Results

The SR vesicles from normal and MHS pig back muscles were incubated with 0.5 mM [γ-^{32}P]ATP and equimolar $MgCl_2$ in the presence of 5 mM $CaCl_2$ and 1 μM CaM in order to phosphorylate the membrane proteins. Fig. 1 depicts the autoradiograms of 10% SDS-polyacrylamide gel representing the sites of ^{32}P incorporation into

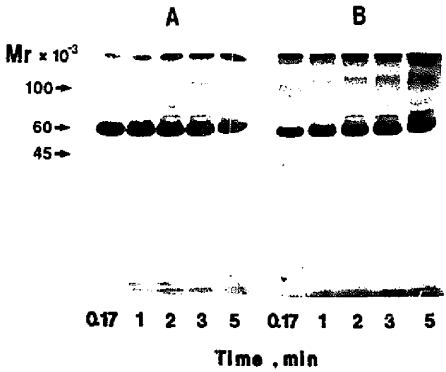

Fig. 1. Autoradiogram of phosphorylated proteins of sarcoplasmic reticulum vesicles of normal (A) and malignant hyperthermia susceptible pig muscle (B). To phosphorylate the SR components, 0.5 mM Mg[γ-^{32}P]ATP (300 cpm/pmol) was added to a solution containing 15 mg/ml SR, 0.15 M KCl, 20 mM Mes (pH 6.8), and 5 mM $CaCl_2$, and various proteinase inhibitors (see Materials and Methods) at 21°C in the presence of 1 μM calmodulin. After incubation for various times as indicated, 20 μl of the reaction solution was diluted 10 times with the SDS quenching solution (see Materials and Methods). The samples were analyzed by 10% SDS-gel electrophoresis according to Laemmli's method [22], and the phosphoproteins were analyzed by autoradiography as described under Materials and Methods.

normal (A) and MHS (B) SR proteins at the indicated times after the addition of 0.5 mM [γ-^{32}P]ATP. The major ^{32}P-incorporation site is the 60 kDa protein in both normal and MHS SR (cf. Refs. 19, 20). In 7% SDS-polyacrylamide gel, no 200–450 kDa phosphoproteins were observed. The bands near the top of the 10% gel (Fig. 1) appear to be aggregation products. The extent of phosphorylation of the 60 kDa protein in MHS SR is significantly lower than in the normal SR (Fig. 2). In the normal SR, the maximal phosphorylation (12 pmol P/mg SR) was reached within 1 min after addition of MgATP. The maximal phosphorylation (5 pmol P/mg SR) in the MHS SR was less than 50% of the normal value. Further incubation of the SR vesicles after the maximal phosphorylation had been reached, led to spontaneous dephosphorylation in both types of SR. This suggests that the isolated SR of the normal and malignant hyperthermic pig muscle contain endogenous protein phosphatases [19,20].

Fig. 3 illustrates the time course of passive Ca^{2+} release from normal (A) or MHS SR (B). The time courses of Ca^{2+} release from SR loaded passively were monitored by stopped-flow fluorometry using chlorotetracycline as a fluorescence probe of the intravesicular [Ca^2] [23–26]. The time course of Ca^{2+} release consists of two phases both in normal and MHS SR. Statistical analyses of the kinetic parameters of Ca^{2+} release summarized in Table I show that there is no significant difference in any of the kinetic parameters of Ca^{2+} release (amount, rate constant and initial rate) between normal and MHS SR under the conditions in which no phosphorylation of the 60 kDa protein occurs.

In an effort to examine the role of the 60 kDa phosphoprotein in Ca^{2+} release from normal and MHS SR, the initial rates of Ca^{2+} release were determined in the absence (Fig. 4, column A) or in the presence (Fig. 4, column C) of MgATP using the stopped-flow fluorometry. Since the phosphorylated 60 kDa protein is spontaneously dephosphorylated (Figs. 1 and 2), the incubation time of SR with MgATP was fixed at 2–3 min. The results show that the initial rate of Ca^{2+} release in the normal SR significantly decreased by the phosphorylation (659 ± 102 vs. 361 ± 105 nmol Ca^{2+}/mg SR per s, $\bar{x} \pm$ S.D.), whereas much

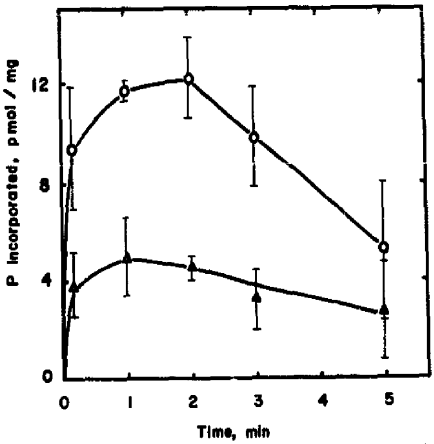

Fig. 2. Time course of phosphorylation of the 60 kDa protein in normal (○) and malignant hyperthermia susceptible sarcoplasmic reticulum (▲). Phosphorylation of the SR proteins was carried out as described in the legend to Fig. 1. and the amount of ^{32}P incorporation into the 60 kDa protein was determined by scintillation counting (see Materials and Methods). Data in the figure represent means ± S.D. for three normal and three MHS pig SR preparations. The values of normal SR are significantly larger than the ones of MHS SR between 10 s and 3 min ($P < 0.05$).

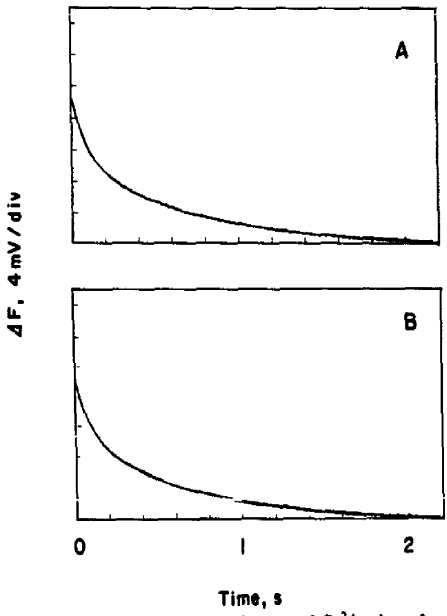

Fig. 3. Stopped-flow fluorometric trace of Ca^{2+} release from normal (A) and malignant hyperthermic sarcoplasmic reticulum (B). 1.8 mg of SR vesicles in 3 ml of a solution containing 0.15 M KCl, 20 mM Mes (pH 6.8), 5 mM $CaCl_2$, and 20 μM CTC was loaded in syringe A of a stopped-flow apparatus (Dionex model D-100). Ca^{2+} release was triggered by mixing syringe A solution with syring B solution containing 0.15 M KCl, 20 mM Mes (pH 9.3), 6.35 mM EGTA, and 20 μM chlorotetracycline. Changes in the fluorescence intensity were measured as described under Materials and Methods. Each figure was obtained by signal averaging a total 30–40 traces originated from four normal and four MHS SR preparations. 1 mV corresponds to 6.25 nmol Ca^{2+}/mg SR.

less inhibition was observed in MHS SR (749 ± 124 vs. 652 ± 179 nmol Ca^{2+}/mg SR per s, $\bar{x} \pm$ S.D.). In the light of the fact that adenine nucleotide activates Ca^{2+} release in skeletal muscle SR [33], it might be assumed that the difference in Ca^{2+} release rates between normal and MHS SR would be due to different nucleotide sensitivity. This possibility was investigated by addition of 20 μM p[NH]ppA, the same concentration as that of MgATP used for phosphorylation (Fig. 4, column B) to the loaded SR, since p[NH]ppA cannot be used to phosphorylate the 60 kDa protein. The addition of 20 μM p[NH]ppA to the loaded SR did not significantly affect the Ca^{2+} release rate both in normal and MHS SR. Similarly, no further Ca^{2+} fluxes were induced by 20 μM MgATP when MgATP was included in Syringe B of the stopped-flow system, under which conditions there

TABLE I

PASSIVE Ca^{2+} EFFLUX FROM SARCOPLASMIC RETICULUM OF NORMAL AND MALIGNANT HYPERTHERMIA SUSCEPTIBLE (MHS) PIG MUSCLES

The experiment was carried out as described in the legend to Fig. 1. The passive Ca^{2+} efflux experiments and calculation of the kinetic parameters of fast and slow phases of Ca^{2+} release (amount; rate constant; rate) were done as described in Materials and Methods. Data represent means ± S.D. for four determinations.

	Phase	Amount (nmol Ca^{2+} per mg)	Rate constant (s^{-1})	Rate (nmol Ca^{2+}/ per mg s)
Normal	Fast	61.9 ± 11.3	11.0 ± 1.4	681 ± 131
	Slow	65.5 ± 13.2	1.4 ± 0.1	87 ± 15
MHS	Fast	68.1 ± 14.4	10.7 ± 0.3	729 ± 138
	Slow	60.5 ± 6.9	1.4 ± 0.1	86 ± 16

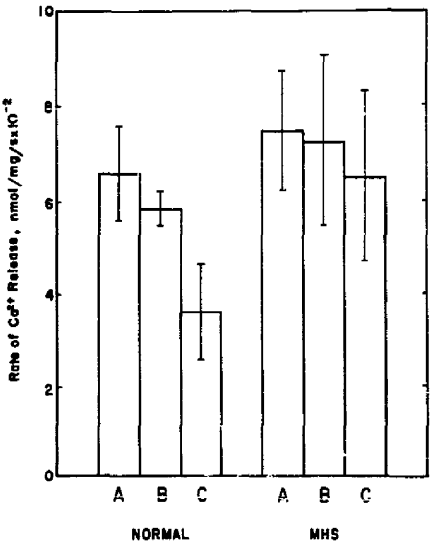

Fig. 4. Initial rate of Ca^{2+} release from normal and malignant hyperthermia susceptible sarcoplasmic reticulum in the absence (A), or in the presence of p[NH]ppA (B) and MgATP (C). Ca-induced Ca^{2+} release were triggered and monitored as described in the legend to Fig. 3. Adenine nucleotide sensitivity of normal and MHS SR was examined by addition of 20 μM p[NH]ppA (33 nmol/mg SR) to the loaded SR before triggering the Ca^{2+} release. For the control and p[NH]ppA experiments, 7–10 traces of Ca^{2+} release were signal-averaged for each SR preparation. For the phosphorylation experiment, when the phosphorylation of the 60 kDa protein reached a maximal level at 2–3 min after addition of MgATP (33 nmol/mg of SR) and calmodulin (67 pmol/mg SR), Ca^{2+} release was triggered by mixing the phosphorylated SR with the syringe B solution. Total eight traces of Ca^{2+} release from phosphorylated normal or MHS SR were used for the statistical analysis. The initial rate of Ca^{2+} release (fast phase) was determined by computer fitting [27]. Three normal and three MHS pigs were used for these studies. Each column shows the average (top margin of the column) ± S.D. The phosphorylated normal SR (C) has significantly lower Ca^{2+} release rates than control or p[NH]ppA added SR ($P < 0.05$).

was no phosphorylation of the 60 kDa protein. These results indicate that the concentration of p[NH]ppA and ATP used for these experiments (20 μM) is far below the range of activating concentrations (e.g. 1 mM, Ref. 33). Calmodulin (67 pmol/mg SR) alone did not affect the Ca^{2+} release rates both in normal and MHS SR (data, not shown). These results suggest that higher Ca^{2+} release rates in MHS SR previously found [11] may in fact be due to reduced inhibition owing to reduced phosphorylation of the 60 kDa protein.

Proteins responsible for the alteration of the extent of phosphorylation of the 60 kDa protein in MHS SR (Figs. 1 and 2) were investigated using two-dimensional gel electrophoresis system (Fig. 5). In the isoelectric point range between 4.5 and 7.5, two proteins with $M_r = 64000$ and 55000 (arrow) were detected both in normal and MHS SR. The 64 kDa protein appears to be calsequestrin, since the protein is acidic (pI = 5.0) and stained with Stains-all [29,30]. The 60 kDa phosphoprotein was not detected in this gel system due to an extremely low content of the pro-

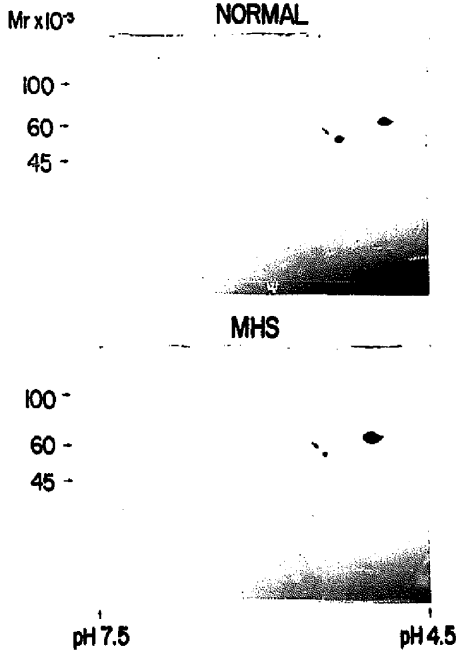

Fig. 5. Separation of proteins in normal and MHS skeletal sarcoplasmic reticulum by two-dimensional gel electrophoresis. 200 μg of SR proteins solubilized in the lysis buffer containing 9.5 M urea, 2% Nonidet P-40, 2% ampholines and 5% β-mercaptoethanol was subjected to isoelectric focusing gel electrophoresis according to the method of O'Farrell [28]. The proteins in the isoelectric focusing gel were separated further by 10% SDS-polyacrylamide gel electrophoresis [28]. The gel was stained by Coomassie brilliant blue. The pH of the isoelectric focusing gel in 5-mm sections was measured in vials containing 2 ml of degased H_2O on a pH meter.

tein in the SR [19]. The amount of a protein with $M_r = 55{,}000$ was significantly lower in MHS SR than normal SR (58 ± 24 vs. $100 \pm 24\%$, $\bar{x} \pm$ S.D., $N = 5$), whereas no significant difference was found in that of calsequestrin. The possibility that the 55 kDa protein might be the intrinsic glycoprotein [30] was investigated by treating normal and MHS SR by Endo H [30,31]. The result showed that the mobility of the 55 kDa protein was not altered by Endo H treatment, indicating that the 55 kDa protein is not the intrinsic glycoprotein. It should be noted that in this particular gel system other major SR proteins such as the Ca^{2+}-ATPase, did not enter the gel [34].

Discussion

Our previous studies on the kinetics of Ca^{2+} release from SR isolated from pig muscles [11] have shown that the initial rates of all types of Ca^{2+} release investigated are significantly higher in MHS SR than in normal. Since these types of Ca^{2+} release (e.g. halothane-induced, and depolarization-induced release) seem to be induced by different triggering mechanisms but occur through a common channel [35], the above finding led us to propose that the putative Ca^{2+} channels rather than triggering mechanisms are altered in MHS SR [11]. One of the most important findings in the present study is that MHS pig SR shows a significantly lower Ca^{2+}/CaM-dependent phosphorylation of a 60 kDa protein than normal. In view of our recent finding that the 60 kDa protein phosphorylation inhibits Ca^{2+} release [19], the above finding suggests that the faster Ca^{2+} release in MHS SR is produced not by actual activation of Ca^{2+} release, but in fact by de-suppression of Ca^{2+} release owing to the lower, inhibitory, phosphorylation. This view is further supported by our observation that the Ca^{2+} efflux rates are indistinguishable between normal and MHS SR if no preincubation is made with MgATP before Ca^{2+} release (cf. Fig. 3). The above view also provides a good explanation for the recent observation of Michelson et al. that the addition of $MgCl_2$, ATP and calmodulin to the passively loaded SR significantly inhibits (about 50%) the Ca^{2+} release from normal SR, whereas no significant inhibition was observed in the passively loaded MHS SR [12].

The actual mechanism by which the extent of the Ca^{2+}/CaM-dependent phosphorylation is suppressed in MHS SR remains to be further investigated. This might be due to some structural alterations or lower amount of the 60 kDa phosphoprotein polypeptide. However, an accurate quantitation of the 60 kDa protein using densitometry scanning of the stained gel is extremely difficult, since the 60 kDa protein is a minor protein in skeletal SR (estimated protein amount in the SR is approximately in the range between 1 and 4 μg/mg SR). Alternatively, the lower extents of phosphorylation of the 60 kDa protein may be caused by altered functions or amounts of the enzymes involved in the phosphorylation and dephosphorylation processes, e.g. a Ca^{2+}/CaM-dependent protein kinase and protein phosphatase. From the estimated rate constants of spontaneous dephosphorylation of the 60 kDa protein in the normal and MHS SR (Fig. 2), it appears that difference in the protein phosphatase activity is not the cause for the lower extents of phosphorylation in MHS SR. Fig. 5 shows that the amount of a 55 kDa protein in the MHS SR is less than that in the normal SR. Study using Endo H suggests that the 55 kDa protein is not the intrinsic glycoprotein in the SR [32]. Although further study is necessary to characterize the functional role of the 55 kDa protein, we tentatively propose that the 55 kDa protein may be one of the subunits of the Ca^{2+}/CaM-dependent protein kinase which catalize phosphorylation of the 60 kDa protein [36,37], and hence the reduced amount of kinase in MHS SR may lead to the reduced phosphorylation of the 60 kDa protein, in turn leading to the apparent activation of Ca^{2+} release.

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