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Modulation of the skeletal muscle ryanodine receptor by endogenous phosphorylation of 160/150-kDa proteins of the sarcoplasmic reticulum

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Abstract

This paper demonstrates and characterizes the inhibition of ryanodine binding caused by the phosphorylation of the 160/150-kDa proteins in skeletal muscle sarcoplasmic reticulum (SR). Inhibition of ryanodine binding was obtained by preincubation of SR membranes with ATP + NaF. The inhibition was characterized by the following findings: (a) If ATP was replaced by AdoPP[NH]P, inhibition of ryanodine binding activity was not observed. (b) The inhibitory effect of preincubation with ATP + NaF, like the phosphorylation of 150/160-kDa proteins, was Ca^{2+} dependent. (c) Inhibition of ryanodine binding, as the protein phosphorylation, was not observed if NaF (> 30 mM) was replaced with okadaic acid. (d) The optimal pH for the inhibition and the phosphorylation was about 7.0. (e) Both the phosphorylation of the 160/150-kDa proteins and inhibition of ryanodine binding were prevented by dichlorobenzimidazole riboside and hemin, inhibitors of casein kinase II. (f) Dephosphorylation of the 160/150-kDa proteins prevented the inhibition of ryanodine binding. (g) The presence of NP-40 during the phosphorylation prevented both the 160/150-kDa phosphorylation and the inhibition of ryanodine binding. Furthermore, a linear relationship was obtained between the degree of ryanodine binding inhibition and the level of phosphorylation of the 160/150-kDa proteins, as controlled by ATP or NaF concentrations. The binding affinity for Ca^{2+} of the ryanodine receptor (RyR) was modified by phosphorylation of the 160/150-kDa proteins, decreasing by up to 100-fold. The phosphorylation of the SR membranes resulted in an elimination of ryanodine binding sites with slight effect on the ryanodine binding affinity. These results suggest the modulation of the properties of the RyR by phosphorylation/dephosphorylation of the 160/150-kDa proteins. The identification of the phosphorylated 160/150-kDa proteins, their kinase, and the structural interactions between them and the RyR are presented in the accompanying paper.

Keywords: Ryanodine receptor; Sarcoplasmic reticulum; Protein phosphorylation

1. Introduction

Recent interest in the regulation of ionic conductances in excitable membranes has focused on biochemical mechanisms such as protein phosphorylation [1–3]. The ryanodine-binding proteins of skeletal and cardiac muscles have been identified as the Ca^{2+}-release channel of the junctional sarcoplasmic reticulum (SR) [4,5]. Several types of regulatory mechanism for Ca^{2+} release have been postulated, including the involvement of Ca^{2+} [4,5]; calmodulin [6,7]; reactive amino group(s) [8] or phosphorylation–dephosphorylation [9–15].

In skeletal muscle, the ryanodine receptor (RyR)/Ca^{2+}-release channel has been shown to be a substrate both for an endogenous Ca^{2+}/ calmodulin-dependent protein kinase and for an exogenously added catalytic subunit of cAMP-dependent protein kinase [9]. Cardiac RyR has also been shown to be phosphorylated by exogenously added calmodulin-, cAMP-, and cGMP-dependent protein kinases [9–12]. Recently it has been shown that purified skeletal muscle RyR is also phosphorylated by cAMP-, cGMP-, and Ca^{2+}/calmodulin-dependent protein kinases, exclusively in serine-2843 [13]. It was suggested, however, that this phosphorylation plays a physiological role in the regulation of cardiac RyR but not skeletal RyR activity [14].

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Mops, 3-(*N*-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SR, sarcoplasmic reticulum; HSR, heavy SR; RyR, ryanodine receptor; C_{50}, the concentration required for 50% of maximal effect; AdoPP[NH]P, adenosine 5'-[β,γ-imido]triphosphate.

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Evidence for a relationship between RyR phosphorylation and Ca^{2+} conductance comes from studies in which protein kinases and protein phosphatases were introduced. In cardiac muscle, phosphorylation of serine-2809 of membrane-bound or purified RyR reconstituted into a planar lipid bilayer led to activation of the Ca^{2+}-release channel [15]. In skeletal muscle, phosphorylated RyR was purified from SR endogenously phosporylated and then incorporated into planar lipid bilayers, showed an enhanced open channel probability due to an increase in the channel sensitivity towards Ca^{2+} and ATP [16]. The *enhancement* in the channel activity by phosphorylation could be reversed by exogenously added protein phosphatase 2A [16]. However, *inactivation* of the skeletal muscle RyR/Ca^{2+}-release channel in excised patches from SR under conditions favorable for protein phosphorylation has been reported [17]. These controversial observations regarding the effect of phosphorylation on channel activity [16,17] may result from phosphorylation not of the RyR but of closely associated proteins.

In our previous studies [18,19] we have characterized an endogenous, Ca^{2+}-dependent phosphorylation system which phosphorylates exclusively two SR polypeptides migrating on SDS-PAGE with apparent molecular masses of 150- and 160-kDa. In parallel to the phosphorylation of these polypeptides, Ca^{2+} release is activated. In the present study we investigated whether this phosphorylation system modulates the properties of the RyR.

2. Experimental procedures

2.1. Materials

ATP, EGTA, Tris, Mops, GTP, hemin, heparin, NaF, Nonidet P-40, AdoPP[NH]P and dichlorobenzimidazole riboside were obtained from Sigma. [^{3}H]Ryanodine (60 Ci/mmol) was purchased from New England Nuclear, and unlabeled ryanodine was obtained from Calbiochem. [γ-^{32}P]ATP was obtained from Amersham. [^{32}P]Phosphorylase kinase was prepared by phosphorylation with [γ-^{32}P]ATP in the presence of the catalytic subunit of cAMP-dependent protein kinase, and was separated from excess reagents by chromatography on Sephadex G-50. Okadaic acid was obtained from Research Biochemical International.

2.2. Membrane preparations

Junctional SR membranes were prepared from rabbit fast twitch skeletal muscle as described by Saito et al. [20] or were isolated according to Lai et al. [21]. In all, these SR preparations, the proteinase inhibitors PMSF (0.2 mM), benzamidine (0.8 mM), and leupeptin (0.5 μg/ml) were included in all solutions. The membranes were suspended at a final concentration of about 25 mg protein/ml in a buffer containing 0.2 M sucrose, 10 mM Tricine, pH 8.0, and 1 mM histidine and stored at -70°C. Protein concentration was determined by the method of Lowry et al. [22].

2.3. Protein phosphorylation

Phosphorylation of SR membranes (1 mg/ml) was performed at 30°C in 50 μl of a solution containing 20 mM Tricine, pH 7.2, 100 mM NaCl, 200 or 400 μM ATP or [γ-^{32}P]ATP (0.5 to 1.0 μCi/nmol), 40–60 mM NaF and other reagents as indicated in the table or figure legends. After incubation some of the samples were diluted (1:1) with the reaction mixture for ryanodine binding as indicated. After 20 min the samples were assayed for ryanodine binding or diluted with 1/3 volume of buffer containing 260 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, and 4% (v/v) β-mercaptoethanol, and incubated for 3 min at 100°C.

2.4. Gel electrophoresis and autoradiography

Analysis of the protein profile and of the phosphoproteins by SDS-PAGE was performed with the discontinuous buffer system of Laemmli [23] in 1.5 mm thick slab gels with 6% or 3–13% acrylamide, using a 3% stacking gel. Gels were stained with Coomassie brilliant blue. Molecular weight standards were: myosin, 200 000; β-galactosidase, 116 000; phosphorylase *b*, 97 400; bovine serum albumin, 66 200; and ovalbumin, 42 700 (Bio-Rad). Kodak X-omat film was used for autoradiography of the dried gels. Quantitative analysis of the protein bands and of the phosphorylated protein bands was performed by densitometric scanning of the gels or autoradiograms, with a computing densitometer (Molecular Dynamics) using Image Quant software provided by the manufacturer. The maximal amount of ^{32}P incorporated into the 160- and 150-kDa proteins was estimated (in different SR preparations) to be about 8 and 5 mol/mol of protein, respectively. These values were also estimated by cutting the labeled bands from the gels and counted in a liquid scintillation counter.

2.5. [^{3}H]Ryanodine binding

Unless otherwise indicated, phosphorylated or non-phosphorylated SR membranes (final concentration of 0.5 mg/ml) were incubated with 20 nM [^{3}H]ryanodine in a standard binding solution containing Ca^{2+} at the concentrations indicated in the table or figure legends, 1 M NaCl, and 20 mM Mops, pH 7.4, for 20 min at 37°C. The unbound ryanodine was separated from the protein-bound ryanodine by filtration of 50-μg aliquots of protein through Whatman GF/C filters, followed by washing three times with 5 ml of ice-cold buffer containing 0.2 M NaCl, 5 mM Mops, pH 7.4, and 50 μM $CaCl_2$. The filters were dried, and the radioactivity was determined by a liquid scintillation counting technique. Non-specific binding was deter-

mined in the presence of a 1250-fold excess of unlabeled ryanodine.

3. Results

Junctional SR membranes were incubated with [γ-^{32}P]ATP in the absence or presence of 60 mM NaF and/or 0.5 mM EGTA, and then subjected to denaturing gel electrophoresis. Fig. 1 shows the Coomassie stained gel and corresponding autoradiogram. Among the several polypeptide chains stained by Coomassie blue, the ^{32}P label was mainly incorporated into two specific proteins with apparent molecular masses of 160-kDa and 150-kDa. As shown previously for the non-junctional SR [18], the phosphorylation of these proteins has an absolute requirement for the phosphoprotein phosphatase inhibitor NaF (see Table 2) as well as for Ca^{2+}. Fig. 1 also shows that the peptide inhibitor specific for the cAMP-dependent protein kinase, PKA-I, has no effect on the phosphorylation of 150- 160-kDa proteins. In some experiments a few other proteins were also phosphorylated. However, their phosphorylation was not always observed and was either NaF or Ca^{2+} - independent and was not sensitive to some inhibitors. Also, their phosphorylation did not correlate with the inhibition of ryanodine binding. Using specific antibodies, the 95 kDa was identified as phosphorylase b and the 110 kDa protein as the Ca^{2+}-ATPase which was phosphorylated under some conditions such as at low NaF concentrations.

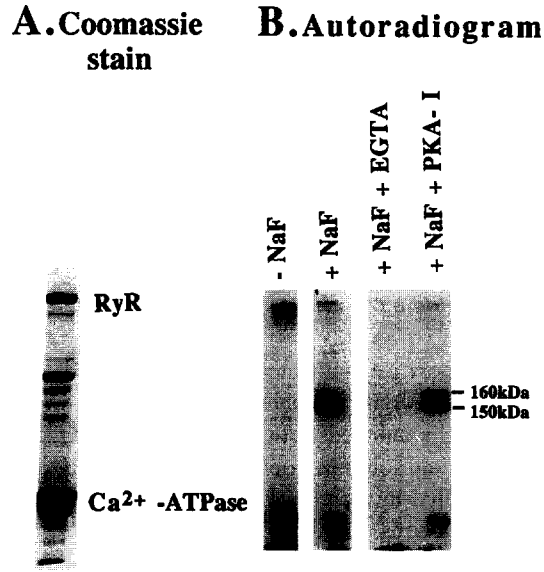

Fig. 1. Ca^{2+}- and NaF-dependent phosphorylation of the 160/150-kDa polypeptides. HSR (1 mg/ml) was phosphorylated for 2 min at 30°C with 200 μM [γ-^{32}P]ATP, at pH 7.2, in the absence or presence of 60 mM NaF or 0.5 mM EGTA, and subjected to SDS-PAGE and autoradiography, as described under Section 2. PKA-I indicates the presence of 5 μM of protein kinase A inhibitor peptide (PKI (6–22) amide). The figure presents a representative experiment from 20 similar experiments.

Table 1
Effect of preincubation of HSR with ATP or AdoPP[NH]P, with and without NaF, on the ryanodine-binding capacity

Additions to preincubation medium	Bound ryanodine (% of control)
None (control)	100
NaF (60 mM)	78
AdoPP[NH]P (0.4 mM)	125
ATP (0.4 mM)	130
NaF + AdoPP[NH]P	112
NaF + ATP	23
NaF (20 mM) + AdoPP[NH]P	100
NaF (20 mM) + ATP	64
Okadaic acid (10 μM) + AdoPP[NH]P	100
Okadaic acid + ATP	102
NaF (20 mM) + okadaic acid + AdoPP[NH]P	99
NaF (20 mM) + okadaic acid + ATP	58

HSR membranes (1 mg/ml) were preincubated with the indicated compounds at 30°C in a solution containing 20 mM Tricine, pH 7.2, and 100 mM NaCl. After 2 min, samples were diluted (1:1) with the reaction mixture for ryanodine binding which contained 40 nM ryanodine and 5 μM $CaCl_2$, and assayed for [^{3}H]ryanodine bound after 20 min of incubation at 37°C. Control activity (100%) was 4.4 pmol/mg protein. The table presents a representative experiment of six similar experiments.

Table 1 shows the effect of preincubation of junctional SR membranes with ATP or AdoPP[NH]P, both with and without NaF, on their ryanodine binding capacity. Preincubation of the membranes with either compound in the absence of NaF had a stimulatory effect on ryanodine binding. However, preincubation with ATP plus NaF resulted in inhibition of ryanodine binding. This inhibitory effect on ryanodine binding was not obtained when ATP was replaced by the non-hydrolyzable analog AdoPP[NH]P. Since AdoPP[NH]P could not substitute for ATP, and the presence of NaF as phosphatase inhibitor was required, it seems plausible that protein phosphorylation is involved in the inhibition of ryanodine binding. Another phosphoprotein phosphatase inhibitor, okadaic acid [25], could not substitute for NaF in either the inhibition of ryanodine binding or the phosphorylation of 160/150-kDa proteins when present during the preincubation of the SR with ATP (Table 1).

Table 2 shows the protein phosphatase activities associated with heavy SR (HSR) membranes. Protein phosphatases type 1 and 2A are inhibited by okadaic acid, whereas type 2C which is not affected by okadiac acid [25] is activated by Mg^{2+} [24]. The data presented indicate the presence of each of these phosphatases in the HSR membranes used. Since 160/150-kDa proteins were not phosphorylated when NaF was replaced by okadaic acid (data not shown), it is possible that phosphoprotein phosphatase type 2C is involved in the dephosphorylation of the 150/160-kDa proteins.

The time course of ryanodine binding by membranes preincubated with AdoPP[NH]P and NaF (control) or with ATP and NaF (phosphorylated) is shown in Table 3. Ryanodine binding to control membranes reached a plateau

Table 2
Protein phosphatase activities of junctional SR membranes

Assay conditions	Protein phosphatase activity (% of control)
Control	100
+ NaF, 50 mM	1
+ Mg^{2+}, 8 mM	106
+ okadaic acid, 1 μM	11
+ okadaic acid, 5 μM	4
+ okadaic acid, 5 μM + Mg^{2+}	50
+ okadaic acid, 5 μM + Mg^{2+} + NaF	6

Protein phosphatase activities were determined by measuring the release of ^{32}P from [^{32}P]phosphorylase kinase as the substrate. HSR membranes (5 μg) were incubated for 30 min at 30°C with [^{32}P]phosphorylase kinase (containing $6 \cdot 10^6$ cpm/ml) in a solution (30 μl) containing 100 mM NaCl, 20 mM Tricine, pH 7.2, and the indicated reagents. The reaction was stopped by the addition of 5 μg of bovine serum albumin and trichloroacetic acid to a final concentration of 16%. After 2 min the samples were centrifuged for 5 min at 4°C, and then samples of the supernatant were counted in a scintillation counter. This table presents a representative experiment of four similar experiments.

after about 30 min. Under the conditions used (1:1 dilution with ryanodine-binding medium), the ryanodine binding capacity of the phosphorylated membranes remained low (between 11% to 22% of that of control membranes) during the assay period. Table 3 also shows a parallel experiment in which the membranes were phosphorylated with [γ-^{32}P]ATP and then diluted with reaction mixture for ryanodine binding and after the indicated incubation time were subjected to SDS-PAGE. The degree of phosphorylation of the 160/150-kDa proteins only slightly decreased during the 40 min following dilution. Thus, in subsequent experiments ryanodine binding was assayed after 20 or 30 min, depending on the particular SR preparation, probably due to the relative kinase and phosphatase activities.

Table 3
Time course of ryanodine binding by control and phosphorylated SR membranes

Ryanodine binding assay time (min)	Bound ryanodine (pmol/mg protein)		Phosphorylated 160/150-kDa proteins (% of control)
	control	phosphorylated	
10	4.4	1.0	100
20	6.5	1.0	100
30	7.3	0.9	86
40	6.4	0.8	83

HSR membranes (1 mg/ml) were incubated for 2 min at 37°C with 60 mM NaF and 0.4 mM AdoPP[NH]P (control) or 0.4 mM ATP or [γ-^{32}P]ATP (phosphorylated). The membranes were diluted 1:1 with reaction mixture for ryanodine (20 nM) binding containing 10 μM $CaCl_2$, and the time course of ryanodine binding was determined, as described in Section 2. The phosphorylation level with [γ-^{32}P]ATP of the 160- and 150-kDa proteins was determined in parallel at the indicated times after the dilution (with the reaction mixture for ryanodine binding) as described in Section 2. Control (100%) for the phosphorylated 160/150-kDa proteins was taken as the phosphorylation level of the membranes before the dilution. This is a representative experiment from five similar experiments.

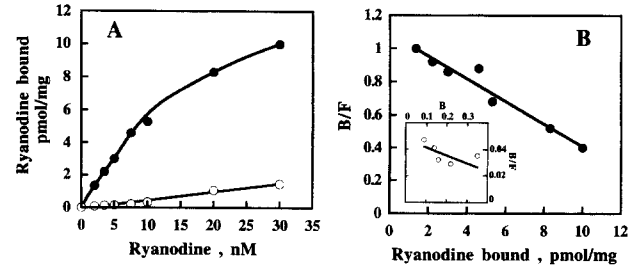

Fig. 2. Effect of phosphorylation of the 150/160-kDa proteins on the affinity of the ryanodine binding site. HSR membranes were incubated for 2 min at 30°C, at pH 7.2, with 0.4 mM ATP or AdoPP[NH]P and 60 mM NaF. (A) [^{3}H]Ryanodine binding to unphosphorylated (incubated with AdoPP[NH]P and NaF) (●) and phosphorylated membranes (○) (incubated with ATP and NaF) as a function of ryanodine concentration was assayed for 60 min in the presence of 10 μM $CaCl_2$ as described in Table 1. (B) Scatchard plot analysis; inset shows the Scatchard plot analysis of the phosphorylated membranes.

The binding of [^{3}H]ryanodine to membranes incubated with NaF + AdoPP[NH]P (control) or with NaF + ATP (phosphorylated) as a function of ryanodine concentration is shown in Fig. 2. Treatment of SR membranes with ATP + NaF, which leads to phosphorylation of the 160/150-kDa proteins, decreased ryanodine binding. Scatchard plot analysis showed that phosphorylation conditions decreased the apparent binding affinity by 1.8-fold (K_D) (from 11.3 ± 2 nM, $n = 2$ to 16.4 ± 2 nM, $n = 3$) and the B_{max} value by 92% (from 12.1 ± 2.8 pmol/mg, $n = 3$ to 0.99 ± 0.4 pmol/mg, $n = 3$). These results suggest that the conditions which led to the phosphorylation of the 160/150-kDa proteins modified the receptor affinity for ryanodine and eliminated ryanodine binding sites.

The effect of phosphorylation of the 150/160-kDa proteins on the Ca^{2+} dependence of ryanodine binding is shown in Fig. 3. Under the conditions used (1.0 M NaCl and pH 7.4), the Ca^{2+} dependence of the phosphorylated

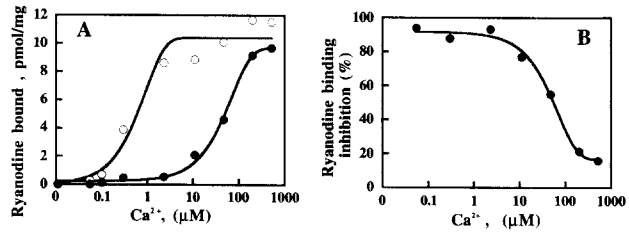

Fig. 3. Ca^{2+} dependence of ryanodine binding in control and 150/160-kDa phosphorylated membranes. HSR membranes were phosphorylated with 0.4 mM ATP and 60 mM NaF (●) and then diluted 1:1 with the reaction mixture for ryanodine binding which contained 0.4 mM EGTA and the indicated concentrations of free Ca^{2+}. Free Ca^{2+} concentrations were calculated with a computer program as previously described [38]. Control membranes (○) were incubated under the same conditions but with AdoPP[NH]P instead of ATP. (A) [^{3}H]Ryanodine binding (20 nM) was assayed for 20 min in the presence of the indicated free Ca^{2+} concentration as described in Section 2. (B) Inhibition of ryanodine binding by the phosphorylation is plotted as a function of the free Ca^{2+} concentration in the ryanodine-binding medium.

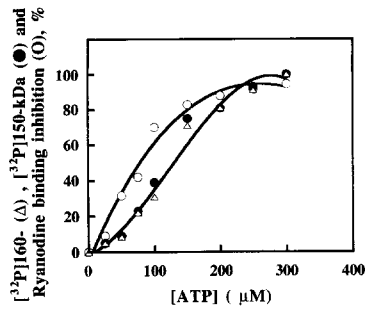

Fig. 4. Phosphorylation of 160/150-kDa polypeptides and inhibition of ryanodine binding as a function of ATP concentration in the preincubation medium. HSR vesicles (1 mg/ml) were incubated for 2 min at 30°C in 20 mM Tricine pH 7.2, 100 mM NaCl, and 60 mM NaF with different concentrations of [γ-^{32}P]ATP (1 μCi/nmol) and then diluted with the reaction mixture for ryanodine binding. After 20 min they were assayed for ryanodine binding or subjected to SDS-PAGE, Coomassie staining, autoradiography and quantitative analysis as described in Section 2. For [^{3}H]ryanodine binding unlabeled ATP was used, and control membranes were incubated with NaF and AdoPP[NH]P instead of ATP. The figure presents a representative experiment of four similar experiments.

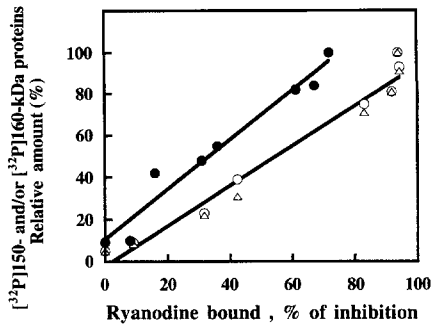

Fig. 5. Correlation between phosphorylation of 160/150-kDa polypeptides and inhibition of ryanodine binding. The data from Fig. 4 were replotted to show the linear relationship between the inhibition of ryanodine binding and the stimulation of 150-kDa (O) and 160-kDa (Δ) phosphorylation. The degree of inhibition of ryanodine binding was plotted as a function of the level of phosphorylation obtained at the same ATP concentration. (●) represent the results from similar experiments carried out with SR vesicles isolated according to Lai et al. [21], in which the phosphorylation of 160/150-kDa proteins was plotted as a function of the degree of inhibition ryanodine binding.

membranes (incubated with ATP + NaF) differed from that of the unphosphorylated membranes (incubated with AdoPP[NH]P + NaF). Half-maximal stimulation (C_{50}) of ryanodine binding was obtained with about 0.35 μM Ca^{2+} in the control and with about a 100-fold higher concentration of Ca^{2+} (30 μM) in the phosphorylated membranes (Fig. 3A). The change in the Ca^{2+}-binding affinity of the ryanodine receptor due to the phosphorylation of the

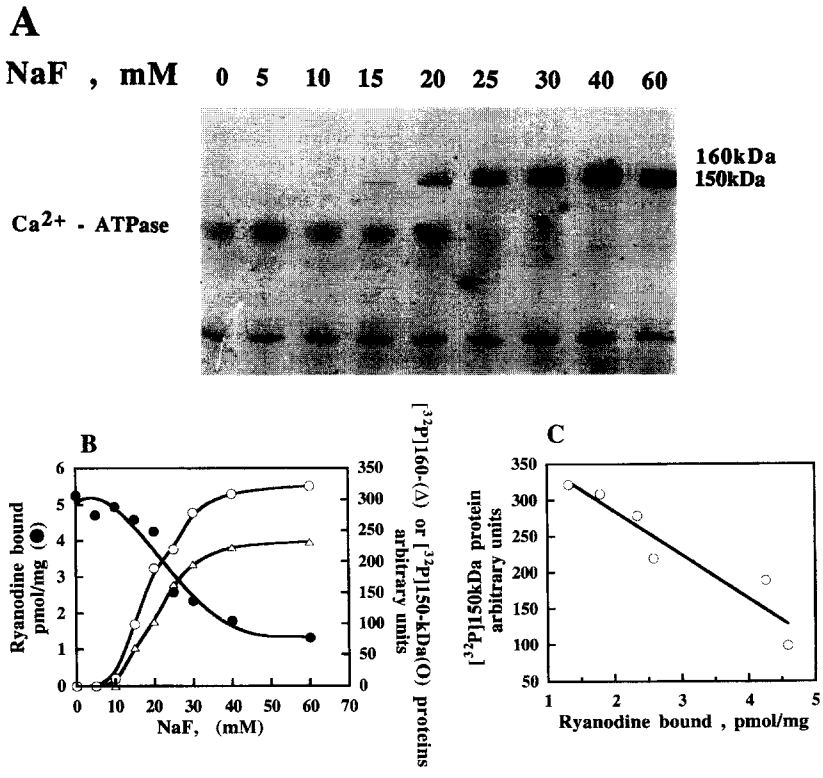

Fig. 6. NaF stimulation of the phosphorylation of the 160/150-kDa proteins and the inhibition of ryanodine binding. HSR vesicles were preincubated with different concentrations of NaF and 0.4 mM [γ-^{32}P]ATP as described in Fig. 4. The autoradiogram is shown in A. For the inhibition of ryanodine, HSR vesicles were preincubated with different concentrations of NaF and with 0.4 mM ATP and then ryanodine binding was assayed as in Fig. 4. In B the phosphorylated 160- (Δ) and 150- (O) kDa proteins and the ryanodine binding capacity of the phosphorylated membranes (●) is presented as a function of NaF concentration. In C, the amount of ^{32}P-labeled 150-kDa protein formed as a function of the ryanodine binding capacity (at different NaF concentrations) is presented.

160/150-kDa proteins was dependent on the level of phosphorylation and the particular SR preparation used. Thus, in the different experiments, the C_{50} for Ca^{2+} activation of ryanodine binding was higher than for control membranes by 30- to 100-fold and was not observed when the membranes were incubated with either ATP or NaF alone. Fig. 3B shows that the degree of inhibition of ryanodine binding by phosphorylation of the 160/150-kDa proteins decreased with increasing Ca^{2+} concentration – from about 90% inhibition at < 11 μM Ca^{2+} to only 16% inhibition in the presence of 500 μM Ca^{2+}. Phosphorylation had no effect on the Ca^{2+} affinity of the low-affinity inhibitory Ca^{2+}-binding site(s) (data not shown). The results suggest that preincubation of the SR under conditions that led to phosphorylation of the 160/150-kDa proteins altered the binding of Ca^{2+} to the high-affinity site of the RyR.

The following experiments demonstrate a correlation between the phosphorylation of the 160- and/or 150-kDa proteins and inhibition of ryanodine binding.

Fig. 4 shows the phosphorylation of 160-kDa and of 150-kDa proteins and the inhibition of ryanodine binding as a function of ATP concentration in the preincubation medium. In the presence of NaF, both the level of phosphorylation and the inhibition of ryanodine binding were increased by increasing the ATP concentration, with the half-maximal effect (C_{50}) being obtained at 120 and 80 μM of ATP, respectively. However, the C_{50} was dependent on the type of SR preparation and the concentration of the membranes in the preincubation medium. This variability may result from the different relative activities of the kinase and phosphatase in the different SR preparations. To establish the relationship between the phosphorylation level of the 160- and 150-kDa proteins and the inhibition of ryanodine binding the data shown in Fig. 4 were replotted (Fig. 5). A linear correlation between the two activities was obtained. A similar linear relationship was also found for SR vesicles isolated as described by Lai et al. [21] (Fig. 5).

Both the level of phosphorylation of the 160/150-kDa proteins and inhibition of ryanodine binding in the presence of 0.4 mM ATP were found to be controlled by altering NaF concentration (Fig. 6). Quantitative analysis of the phosphorylated bands shows that similar concentrations of NaF were required for the inhibition of ryanodine binding and for the stimulation of 160/150-kDa phosphorylation (Fig. 6B). Half-maximal effects were obtained at about 20 mM NaF. Replotting the data from Fig. 6B in the form shown in Fig. 6C demonstrated a linear correlation between the level of phosphorylation of the 160/150-kDa proteins and the inhibition of ryanodine binding. These linear relationships between the level of phosphorylation of the 160/150-kDa proteins and the degree of inhibition of ryanodine binding, produced by preincubation of the SR under phosphorylation conditions, suggest that the phosphorylation of 160/150-kDa proteins is responsible for the inhibition of ryanodine binding. This suggestion is further supported by the results presented in Tables 4 and 5.

As we have previously shown [18], the phosphorylation of the 160/150-kDa proteins is Ca^{2+}-dependent (see also Fig. 1). Table 4 shows that the inhibition of ryanodine binding by preincubation with NaF and ATP also required the presence of Ca^{2+}, as did the phosphorylation of the 160/150-kDa proteins.

Table 5 shows that the inhibitors of casein kinase II hemin and the membrane permeable dichlorobenzimidazole riboside (DRB) decreased both the phosphorylation of the 160/150-kDa proteins and the inhibition of ryanodine binding produced by preincubation of SR membranes with ATP and NaF.

We have shown in previous work [18], that the presence of detergents during the phosphorylation reaction prevented the endogenous phosphorylation of 160/150-kDa proteins. Similar results are presented in Table 6, indicating that NP-40 inhibits the phosphorylation as well as decreases the inhibition of ryanodine binding produced by preincubation under the phosphorylation conditions. The stimulation of ryanodine binding by detergents such as CHAPS has been observed previously [21].

The lack of the inhibition of ryanodine binding when the phosphorylated 160/150-kDa proteins were dephosphorylated is shown in Table 7. Preincubation of SR membranes with ATP + NaF resulted in about 70% inhibition of their ryanodine binding activity compared with membranes incubated with ATP alone or with AdoPP[NH]P + NaF. The removal of Ca^{2+} required for the phosphorylation of the 160/150-kDa proteins (Fig. 1, Table 4 and Ref. [18]), by addition of EGTA, resulted in the dephosphorylation of both proteins and in the lack of ryanodine binding inhibition. The level of phosphorylation

Table 4
Ca^{2+} requirement for the phosphorylation of the 160/150-kDa proteins and inhibition of ryanodine binding

Preincubation conditions	Bound ryanodine (% of control)	P-labeled 150-kDa proteins (relative amount)
1. AdoPP[NH]P	100	–
2. AdoPP[NH]P + EGTA (0.2 mM)	91	–
3. AdoPP[NH]P 10 μM free Ca^{2+}	100	–
4. ATP	32	100
5. ATP + EGTA (0.2 mM)	73	26
6. ATP + 10 μM free Ca^{2+}	34	125

HSR were incubated for 2 min with 60 mM NaF and with the compounds shown, as described in Table 1. The incubation mixtures were then diluted 1:1 with the reaction mixture for ryanodine binding (20 nM). After 20 min at 37°C, the samples were assayed for ryanodine binding or subjected to SDS-PAGE as described in Section 2. For samples 3 and 6, Ca^{2+} was added to a final concentration of 10 μM free Ca^{2+}. Unlabeled ATP (0.2 mM) was used for the ryanodine binding experiment, and [γ-^{32}P]ATP for the phosphorylation experiment. Bound ryanodine (100%) = 12 pmol/mg protein. The table presents one representative experiment of three similar experiments.

Table 5
Effect of hemin and dichlorobenzimidazole riboside (DRB) on the phosphorylation of the 160/150-kDa proteins and on the concomitant inhibition of ryanodine binding

Preincubation conditions	Bound ryanodine		P-labeled 160/150-kDa proteins (relative amounts)
	pmol/mg	% of inhibition	
Expt. I			
AdoPP[NH]P (0.3 mM)	4.1		
AdoPP[NH]P + hemin (50 μM)	3.8		
AdoPP[NH]P + hemin (100 μM)	3.4		
AdoPP[NH]P + hemin (200 μM)	3.2		
ATP (0.3 mM)	0.5	88	1.0
ATP + hemin (50 μM)	2.0	49	0.7
ATP + hemin (100 μM)	2.1	39	0.47
ATP + hemin (200 μM)	2.3	26	0.25
Expt. II			
AdoPP[NH]P (0.2 mM)	3.8		
AdoPP[NH]P + DRB (0.5 mM)	4.2		
AdoPP[NH]P + DRB (2.0 mM)	5.8		
AdoPP[NH]P + DRB (3.0 mM)	5.8		
ATP (0.2 mM)	1.0	74	1.0
ATP + DRB (0.5 mM)	1.6	62	0.77
ATP + DRB (2.0 mM)	2.8	52	0.50
ATP + DRB (3.0 mM)	3.3	43	0.41

HSR membranes were incubated for 2 min with [γ-^{32}P]ATP or with unlabeled ATP (for ryanodine binding) in the presence of 60 mM (Expt. I) or 30 mM (Expt. II) NaF, and the indicated concentration of hemin or DRB as described in Table 1. Control membranes were incubated with AdoPP[NH]P instead of ATP. Hemin and DRB were dissolved in dimethylsulfoxide (DMSO), and the final DMSO concentration in the control and hemin- or DRB-containing samples did not exceed 2%. Hemin inhibition of ryanodine binding was prevented by DTT to a final concentration of 1 mM prior to the assay for ryanodine binding. The results are given for one representative experiment of three similar experiments.

of the 160/150-kDa proteins before and after exposure to dephosphorylation conditions (Table 7) provides an indication of the correlation between the phosphorylation state of the proteins and the inhibition of ryanodine binding. The results in Tables 4–7 also indicate that alteration in the free Ca^{2+} concentration could not be the cause of ryanodine binding inhibition by preincubation with ATP + NaF (in comparison to AdoPP[NH]P).

In view of the above results, it may be expected that alteration of the Ca^{2+}- and ryanodine-binding affinities by phosphorylation of the 160/150-kDa proteins would also result in modification of the ryanodine-sensitive Ca^{2+} release channel activity. We found that the phosphorylation of 160/150-kDa proteins at pH 7.2, which inhibits ryanodine binding, has no significant effect on the SR membrane Ca^{2+} permeability. This could result from the fact that the concentrations of ryanodine (μM) required for Ca^{2+}-release activation are several-fold higher than those for ryanodine binding activity (nM). However, using HSR we found, as in our previous study with non-junctional SR [19], that only when the phosphorylation was carried out at pH 8.2, but not at pH 7.2, activation of Ca^{2+} efflux from SR vesicles passively loaded with Ca^{2+} was observed (data not shown). The Ca^{2+} efflux activated by phosphorylation at pH 8.2 was only slightly inhibited by Ruthenium red or ryanodine and was activated in non-junctional SR (Ref. [19] and data not shown). These results may suggest that phosphorylation conditions at pH 8.2 activate Ca^{2+} efflux via another unidentified channel, which is probably distinct from the RyR/Ca^{2+}-release channel [27,28]. This suggestion has been discussed in our previous work [19] and is the subject of another study in which reconstitution

Table 6
Nonidet P-40 (NP-40) inhibits the phosphorylation of 160/50-kDa proteins and prevents the concomitant inhibition of ryanodine binding

Preincubation conditions	NP-40	Ryanodine bound		P-labeled 160/150-kDa proteins (relative amount)
	(%)	pmol/mg	%	
AdoPP[NH]P	0	2.0	100	–
ATP	0	0.5	25	1.0
AdoPP[NH]P + NP-40	0.02	4.1	100	–
ATP + NP-40	0.02	1.6	39	0.61
AdoPP[NH]P + NP-40	0.04	5.2	100	–
ATP + NP-40	0.04	3.2	62	0.32
AdoPP[NH]P + NP-40	0.05	5.6	100	–
ATP + NP-40	0.05	5.2	91	0.27

SR membranes were incubated for 90 s with 0.2 mM of ATP, [γ-^{32}P]ATP or AdoPP[NH]P in the presence of 30 mM NaF, and with and without NP-40. The samples were then diluted 2-fold with reaction mixture for ryanodine binding (10 nM) and after 30 min at 37°C assayed for protein phosphorylation or ryanodine binding as described in Fig. 4. Quantitation of phosphorylated 160/150-kDa proteins was carried out as in Table 3. The results are the averages of two different experiments.

Table 7
Dephosphorylation of the 160/150-kDa proteins prevents the ryanodine binding inhibition

Preincubation conditions		Ryanodine binding activity (pmol/mg protein)		Phosphorylation (relative units)	
Stage I	Stage II	after Stage I	after Stage II	after Stage I	after Stage II
A. AdoPP[NH]P + NaF	–	8.2	7.9	–	–
B. AdoPP[NH]P + NaF	EGTA	8.2	6.4	–	–
C. ATP + NaF	–	0.8	1.2	1.0	1.0
D. ATP + NaF	EGTA	0.8	6.0	1.0 (0.3) [a]	0.36

HSR membranes (1 mg/ml) were preincubated with 0.4 mM ATP or AdoPP[NH]P and 60 mM NaF at 30°C in a solution containing 20 mM Tricine, pH 7.2, and 100 mM NaCl. After 2 min, aliquots were either assayed for ryanodine binding or protein phosphorylation (Stage I) or EGTA was added to aliquots to a final concentration of 0.5 mM, and 2 min later aliquots were removed for assay of phosphorylation. The samples were then centrifuged for 30 min at 30000 × g. The pellets were resuspended in the phosphorylation medium containing either AdoPP[NH]P and NaF (samples A and B) or ATP and NaF (samples C and D) and assayed for ryanodine binding (Stage II). The pellets of the samples phosphorylated with [γ-^{32}P]ATP were resuspended in a sample buffer for SDS-PAGE. Ryanodine binding, SDS-PAGE, autoradiography and quantitative analysis were carried out as described in Section 2.
[a] The figure in parentheses indicates the phosphorylation level 2 min after the addition of EGTA and before centrifugation.

of SR channel(s) into planar membranes will be carried out.

4. Discussion

This study indicates the possible role of phosphorylation/dephosphorylation of the 150/160-kDa proteins in the modulation of the properties of the RyR. Our observations have indicated that, under the specific conditions that lead to phosphorylation of the 150/160-kDa proteins, the ryanodine binding capacity of the phosphorylated membranes is diminished. The decrease in ryanodine binding is due to a decrease in the Ca^{2+}- and ryanodine-binding affinities of the ryanodine receptor by up to the 2-fold and 100-fold, respectively (Figs. 2 and 3).

The relationship between the phosphorylation of the 160/150-kDa proteins and the modulation of RyR, as reflected in the inhibition of the ryanodine binding activity, was demonstrated in several different experiments. The phosphorylation and the inhibition of ryanodine binding involved a number of common features: (a) an absolute requirement for NaF, which could not be replaced by the phosphoprotein phosphatase inhibitor – okadaic acid (Fig. 1 and Table 1); (b) a requirement for Ca^{2+} (Fig. 1 and Table 4); (c) similar time courses, optimal pH values, and NaF and ATP concentration dependences (Figs. 4 and 6); (d) prevention by hemin, DRB and NP-40 of the phosphorylation of 160/150-kDa proteins and the concomitant inhibition of ryanodine binding (Tables 5 and 6); and (e) dephosphorylation of the 160/150-kDa phosphoproteins prevents the inhibition of ryanodine binding by the preincubation with ATP + NaF (Table 7). Furthermore, analysis of our data showed a linear relationship between the phosphorylation level of the 160/150-kDa proteins and the degree of inhibition of ryanodine binding (Figs. 4 and 5). Thus, the data presented here suggest that there is a relationship between the state of phosphorylation, specifically of the 160/150-kDa proteins, and the modulation of the properties of the RyR as reflected in the ryanodine- and Ca^{2+}-binding affinities. The requirement for NaF for the phosphorylation of the 160/150-kDa proteins (Figs. 1 and 6), the presence of endogenous phosphatases in the SR (Table 1), and the prevention of the inhibition of ryanodine binding (produced by preincubation at the phosphorylation conditions) by dephosphorylation conditions (Table 7) suggest that the modulation of RyR properties is controlled by a reversible phosphorylation–dephosphorylation cycle. The question of identity of the 160/150-kDa proteins, their kinase, and their association with the RyR are addressed in the companion paper [29].

The mechanism(s) underlying the requirement for Ca^{2+} for the phosphorylation of the 160/150-kDa proteins and for the subsequent ryanodine binding inhibition are not known. The kinase, which catalyses their phosphorylation – casein kinase II [29] – has no requirement for Ca^{2+} [26]. Furthermore, phosphorylation of purified 160/150-kDa proteins by exogenously added casein kinase II is also independent of Ca^{2+} [29]. It should be noted, however, that both the 160- and 150-kDa proteins bind Ca^{2+} [30,31] and can be extracted from the SR by EGTA [29–31]. The control mechanisms of the kinase and phosphatase activities are not known. The phosphorylation of luminally located 160/150-kDa proteins [29,30] requires the transport of ATP into the lumen of the SR, the association of the kinase with these proteins, and the inactivation of the phosphatase. Thus, Ca^{2+} could be involved in any step in this multistep process. Further studies are therefore needed to determine the role of Ca^{2+} in the phosphorylation of the membrane-associated 160/150-kDa proteins.

While the results presented here are consistent with the suggestion that there is a linear relationship between the phosphorylation of the 150/160-kDa proteins and the inhibition of ryanodine binding, the mechanism by which this phosphorylation affects ryanodine binding is not clear. The observation that the phosphorylation of 160/150-kDa proteins modifies the Ca^{2+} dependence of ryanodine binding (Fig. 3) suggests that the phosphorylation of one protein or both proteins modifies the Ca^{2+}-binding affinity of the RyR, by unknown mechanism. There is no indica-

tion for a direct interaction between the RyR and either or both 160/150-kDa proteins [29]. Thus, this effect may be propagated to the RyR via other proteins that interact directly with the RyR [29]. It is possible that the phosphorylation of the 160/150-kDa proteins participates in one of a complex series of molecular interactions which lead to modification of the properties of the RyR. The phosphorylation state of the 160/150-kDa proteins might affect their interaction with other proteins [29]. Recent results point to the modulation of RyR activities by protein–protein interactions [32–35]. The lumenal Ca^{2+}-binding proteins calsequestrin [32,33] and annexin VI [34] have been shown to modify the activity of the RyR/Ca^{2+} release channel: the channel reconstituted into planar lipid bilayers became insensitive to Ca^{2+} after calsequestrin removal [33], and annexin VI increased in a Ca^{2+}-dependent manner the activity of the channel from the lumenal side [34]. The activation of the skeletal muscle RyR/Ca^{2+} release channel by a cytoplasmic loop of the dihydropyridine receptor has also been demonstrated [35].

Our results suggest the modulation of the properties of the RyR by a regulatory pathway involving the phosphorylation of the 150/160-kDa polypeptides. The phosphorylation of HCP by 60-kDa calmodulin-dependent protein kinase and its possible involvement in the modulation of the functional state of the RyR has been presented recently [36]. Also recently, the modulation of single channel activity of junctional SR reconstituted into planar bilayer by phosphorylation/dephosphorylation of non defined protein(s) has been demonstrated [37].

For determination of the exact role of the phosphorylation of the 150-kDa and/or 160-kDa proteins in the modulation of the properties of the RyR, reconstitution of a functional system including RyR, phosphorylated and unphosphorylated 150-kDa and/or 160-kDa proteins, and other proteins such as calsequestrin, triadin and the 53-kDa glycoprotein is required.

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