Characterization of Ca^{2+} -dependent endogenous phosphorylation of 160 000- and 150 000-Dalton proteins of sarcoplasmic reticulum

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The 160 and 150 kDa proteins of sarcoplasmic reticulum (SR) are phosphorylated endogenously. The phosphorylation of both proteins has a marked requirement for Ca^{2+} . Half-maximal and maximal phosphorylation was obtained at about 1 nM- and 1 μM - Ca^{2+} respectively, and a Hill coefficient of about 0.5 was calculated. The phosphorylation is also dependent on NaF as an inhibitor of the SR phosphoprotein phosphatase. The phosphorylation of these proteins is very rapid, and maximal phosphorylation is achieved in less than 15 s. The phosphorylation of the 160 kDa and 150 kDa polypeptides is completely inhibited by 5 mM- MgCl_2 and by 75 μM - LaCl_3 , by very low concentrations of different detergents, and by preincubation of the SR for 2 min at 60 °C. The inhibition by Mg^{2+} is due to stimulation of ATP hydrolysis, thereby decreasing ATP concentration. Different phosphorylated peptides were obtained by digestion with protease V_8 of the 160 kDa and 150 kDa protein bands, suggesting that the 160 kDa and 150 kDa proteins are distinct. The two phosphorylated proteins are present in different fractions and preparations of SR, with or without [^3H]PN200-110 binding capacity. These and other results suggest that the phosphorylated SR proteins are distinct from the α_1 and α_2 subunits of the voltage-gated Ca^{2+} channel of the T-system membranes. Different inhibitors and activators of protein kinase C and calmodulin-dependent protein kinase have no effect on the endogenous phosphorylation of both polypeptides, suggesting that the phosphorylation is regulated solely by Ca^{2+} . A possible regulatory function for this phosphorylation system is described in the accompanying paper [Gechtman, Orr & Shoshan-Barmatz (1991) *Biochem. J.* 276, 97–102].

INTRODUCTION

Protein phosphorylation stimulated by various protein kinases has been generally recognized as playing a crucial role in the regulatory mechanism of diverse cellular functions such as metabolism and membrane events [1–5]. This type of regulation requires a constant balance between specific protein kinases and specific protein phosphatases, thus endowing the cell with a control mechanism that can be constantly switched on or off, depending on the respective activities of these enzymes.

Protein kinases constitute a very diverse set of enzymes, which are commonly regulated through their interaction with 'second messengers' such as cyclic nucleotides or Ca^{2+} . However, a cyclic-AMP- and Ca^{2+} -independent kinase that specifically phosphorylates the β -adrenergic receptor has also been identified [6]. Two classes of Ca^{2+} -dependent protein kinases have been described in higher animal systems. The first class contains the Ca^{2+} /calmodulin-dependent protein kinases, of which there appears to be a wide variety [7,8]. These kinases are activated by interaction with a Ca^{2+} /calmodulin complex; it is calmodulin which binds Ca^{2+} and senses the changes in cellular Ca^{2+} . The second class is the Ca^{2+} /phospholipid, diacylglycerol-dependent protein kinase, known as protein kinase C. Ca^{2+} and phospholipids are required for activation, but diacylglycerol appears to be the principal regulator of kinase activity [9].

The phosphorylation of skeletal-muscle sarcoplasmic reticulum (SR) has been studied by several investigators [10–14]. Campbell & Shamoo [10] showed that SR membranes contain an endogenous protein-phosphorylation system. The phosphorylation of several proteins, of 64, 42 and 20 kDa, was achieved in the presence of 1 mM-EGTA and 5 mM- MgCl_2 , and was cyclic-AMP-independent [10]. Two high-molecular-mass proteins (> 300 kDa) of SR were dually phosphorylated by an endogenous

Ca^{2+} /calmodulin-dependent protein kinase as well as by an exogenously added catalytic subunit of cyclic-AMP-dependent protein kinase [11]. The Ca^{2+} /calmodulin-dependent phosphorylation of a 60 kDa protein was also demonstrated [12,13]. Consequently, a functional role for the Ca^{2+} /calmodulin-dependent phosphorylation system in the control of Ca^{2+} release in isolated SR membranes has been proposed [12,13]. However, this suggestion was not supported by Plank *et al.* [14].

In the present work, we characterize an endogenous Ca^{2+} -dependent phosphorylation system which catalyses the phosphorylation of two SR proteins of 160 and 150 kDa.

EXPERIMENTAL

Materials

ATP, EGTA, Tris, Mops, NaF, quercetin and *Staphylococcus aureus* (V_8) were obtained from Sigma Chemical Co. Nitrendipine and [^3H]PN200-110 were generously given by Dr. Z. Fogel of the Weizmann Institute, Israel, and phosphorylase *b* and phosphorylase kinase were generously given by Dr. L. M. G. Heilmeyer, Ruhr University, Bochum, Germany. [γ - ^{32}P]ATP was purchased from the Nuclear Research Center, Negev, Israel. ^{32}P -phosphorylase *a* was prepared by phosphorylation of phosphorylase *b* with [γ - ^{32}P]ATP in the presence of phosphorylase kinase, and was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on Sephadex G-50.

Membrane preparations

SR vesicles were prepared from rabbit fast-twitch skeletal muscle as described by MacLennan [15]. Heavy SR (HSR) membranes were isolated as described by Meissner [16]. The membranes were suspended in a final concentration of about 25 mg of protein/ml in a buffer containing 0.2 M-sucrose, 10 mM-

Abbreviations used: SR, sarcoplasmic reticulum; HSR, heavy SR; [^3H]PN200-110, isopropyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethyl-5- $^{[3\text{H}]}$ methoxycarbonylpyridine-3-carboxylate.

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Fig. 1. Phosphorylation of two different membrane preparations and the effect of EGTA and NaF

In (a), SR (1 mg/ml) was phosphorylated for 2 min with 200 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence of 60 mM-NaF and subjected to SDS/PAGE and to autoradiography, as described in the Experimental section. SR was prepared as described by MacLennan [15] and HSR was obtained as described by Meissner [16]. Lanes: 1, stained gel lane for each membrane preparation, illustrating the major SR proteins [ATPase, $(\text{Ca}^{2+}\text{-Mg}^{2+})\text{-ATPase}$; CS, calsequestrin]; 2, autoradiogram illustrating NaF-dependent phosphorylation of two polypeptides of 160 and 150 kDa. In (b), the left lane represents stained gel and the other three lanes are autoradiograms of SR phosphorylated under the indicated conditions: in the absence and the presence of 60 mM-NaF and in the presence of both 60 mM-NaF and 0.5 mM-EGTA.

Tricine, pH 8.0, and 1 mM-histidine, and stored at -70°C . In most of the experiments the membranes were washed with 0.6 M-KCl before use. Protein concentration was determined by the method of Lowry *et al.* [17].

Protein phosphorylation

Phosphorylation of SR membrane (1 mg/ml) was performed at 30°C in 50 μ l of a solution containing 20 mM-Tricine, pH 7.2 or 8.2, 100 mM-NaCl, 100 μ M- or 200 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1 μ Ci/nmol) and other reagents as indicated in the Table or Figure legends. After incubation (0.5–4 min), the reaction was terminated by addition of EGTA (final concn. 5 mM), and samples were assayed for protein phosphorylation by the filtration method or by SDS/PAGE and autoradiography. In the filtration method, protein samples (30 μ g) were filtered through nitrocellulose filters (0.45 μ m pore size) and washed with 3×5 ml of cold buffer containing 0.1 M-NaCl, 20 mM-Tricine, pH 7.2, and 0.2 mM-ATP. The radioactivity retained on the filters was determined by liquid-scintillation counting. When protein phosphorylation was analysed by SDS/PAGE, the reaction was terminated by addition of 20 μ l of a solution containing 8% (w/v) SDS, 200 mM-Tris/HCl, pH 6.8, 4% (w/v) 2-mercaptoethanol, 40% (v/v) glycerol and 0.001% Bromophenol Blue (SDS-quenching solution). Samples were heated for 2 min at 100°C and applied quantitatively to SDS/polyacrylamide slab gels.

For quantification of the incorporation of $[\text{P}^{32}]\text{phosphate}$ into the 160 and 150 kDa proteins, SR membranes were phosphorylated for 1 min in the presence of 60 mM-NaF and 200 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3 μ Ci/nmol). After electrophoresis, the protein bands were cut from the gel and the proteins were extracted by incubation at 50°C for 4 h with 50 mM- Na_2CO_3 (pH 8.5)/0.1% SDS. The radioactivity and protein content of the extracts were determined, and accordingly the phosphorylation stoichiometry was calculated.

Phosphoprotein phosphatase activity of SR membranes

This was measured by the release of $[\text{P}^{32}]\text{P}_i$ from ^{32}P -phosphorylase *a*. The assay conditions were the same as for protein phosphorylation, except that ^{32}P -phosphorylase *a* (1 mg/ml) was added and ATP was omitted from the assay medium.

The reaction was stopped after 10 min of incubation at 30°C by addition of trichloroacetic acid (final concn. 5%). The $[\text{P}^{32}]\text{P}_i$ released was extracted as described previously [18] and measured with a scintillation counter.

Gel electrophoresis and autoradiography

The analysis of phosphoproteins by SDS/polyacrylamide-slab-gel electrophoresis was performed in the discontinuous buffer system of Laemmli [19] in 1.5 mm-thick slab gels with 4–13% acrylamide. Autoradiography of dried gels was carried out with Kodak X-omat film. Molecular-mass standards (Da) were myosin (200000), β -galactosidase (116000), phosphorylase *b* (92500), BSA (66200) and ovalbumin (45000) (Bio-Rad).

Quantitative analysis of the 160 kDa and 150 kDa phosphorylated proteins was performed by densitometric scanning of the autoradiogram, with a Cary 219 spectrophotometer, and relative peak areas were estimated from heights $\times$ half-widths.

RESULTS

The phosphorylation of 160 and 150 kDa proteins is associated with SR

SR membranes were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under the conditions described in the Experimental section (in the absence of exogenous bivalent cations) and in the absence or the presence of 60 mM-NaF and/or 0.5 mM-EGTA, and then submitted to denaturing gel electrophoresis. Fig. 1 shows that, among the several polypeptide chains stained by Coomassie Blue, mainly two specific proteins with apparent molecular masses of 160 kDa and 150 kDa were labelled by ^{32}P . The phosphorylation of these proteins has an absolute requirement for the phosphoprotein phosphatase inhibitor NaF (see Fig. 7). In the presence of 0.5 mM-EGTA no phosphorylation was obtained, suggesting that the phosphorylation obtained with no added Ca^{2+} was due to the presence of an endogenous Ca^{2+} .

Phosphorylation of the 160 and 150 kDa proteins is obtained in different preparations of SR membranes: unfractionated [15] and heavy fraction [16] (Fig. 1a). In most SR preparations the 150 kDa protein is more heavily phosphorylated than the 160 kDa protein. This could be due, at least partially, to their

Table 1. Comparison of protein phosphorylation and [³H]PN200-110 binding of different membrane preparations

Endogenous phosphorylation in the absence or the presence of 60 mM-NaF was carried out as described in the Experimental section. For [³H]PN200-110 binding, the different membrane preparations (1 mg/ml) were incubated with 5 nM-[³H]PN200-110 (sp. radioactivity 55 Ci/mmol) in a solution containing 185 mM-KCl, 1.5 mM-CaCl₂ and 10 mM-Tris/HCl, pH 7.4 (binding buffer), for 30 min at 37 °C. The non-specific binding was determined in the presence of 2 μM-nitrendipine. The unbound [³H]PN200-110 was separated from the protein-bound [³H]PN200-110 by filtration of membrane samples (60 μg) through glass-fibre filters (GF/C), prewashed with 5% polyethyleneimine and washed with 3 × 5 ml of ice-cold binding buffer. The dried filters were counted for radioactivity with a scintillation counter. The results are averages of triplicate samples which differed by 1–5 %.

Membrane	³² P incorporation (nmol/mg of protein)			[³ H]PN200-110 binding (pmol/mg of protein)
	+ NaF	– NaF	Difference	
SR	2.76	0.25	2.51	0.033
HSR	3.39	0.71	2.68	1.386

relative amounts, which did not change when the membranes were prepared in the presence of a variety of protease inhibitors. In most SR preparations the amount of 150 kDa protein is 3–4 times that of the 160 kDa protein (as determined by densitometric scanning of the Coomassie-Blue-stained gel bands). However, the ratio between the two proteins changes as the SR preparation is varied, the amount of the 160 kDa protein being the variable one. In several SDS/PAGE runs, the 160 kDa protein band appeared as two sub-components (data not shown). In the HSR, other protein bands, with apparent molecular masses of about 44 and 33 kDa, were also phosphorylated.

Table 1 compares the incorporation of ³²P from [γ-³²P]ATP and the [³H]PN200-110 binding capacity of the two membrane preparations. NaF stimulated the ³²P incorporation by several-fold in both membrane preparations. In the absence of NaF, the level of incorporated ³²P was nearly 3 times greater in the HSR than in the SR. This is consistent with our observation that other proteins of the HSR (shown in Fig. 1) were also phosphorylated in the absence of NaF (results not shown). Table 1 shows that, although the NaF-dependent ³²P incorporation into the two different membrane preparations was very similar, the [³H]PN200-110 binding capacity of the HSR was about 40 times that of the SR. In subsequent experiments we found that the two membrane preparations possess similar binding affinity for PN200-110 (results not shown). These results suggest that the 160 kDa and 150 kDa proteins, endogenously phosphorylated in the presence of NaF, are different from the α₁ (170 kDa) and α₂ (175 kDa) subunits of the dihydropyridine receptor (DHP)/voltage-sensitive Ca²⁺ channels.

pH-dependence of the phosphorylation

The phosphorylation of both 160 kDa and 150 kDa polypeptides was strongly pH-dependent (Fig. 2a). The quantitative analysis, carried out by densitometric scanning of the autoradiogram and determination of the peak area of both phosphorylated bands, is shown in Fig. 2(b). The optimum pH for the phosphorylation of both proteins was about 7.0, and the level of phosphorylation fell as pH increased. A similar pH profile was obtained for the phosphorylation of 150 kDa both in the absence and in the presence of 50 μM-quercetin, a potent inhibitor of the (Ca²⁺ + Mg²⁺)-ATPase [20]. This suggests that the lower phos-

Fig. 2. pH-dependence of the phosphorylation of 160 and 150 kDa proteins

SR vesicles were phosphorylated as described in Fig. 1, except that the incubation pH was varied as indicated, and where indicated 50 μM-quercetin was added. The buffers used were 20 mM-Mes for pH 5.5 and 6.0, 20 mM-Mops for pH 6.5, 7.0 and 7.2, and 20 mM-Tricine for pH 7.8 and 8.2. The autoradiogram is shown in (a) and the quantitative analysis in (b). Quantitative analysis of the 160 and 150 kDa protein bands was carried out as described in the Experimental section. ■, ○ and □ indicate the phosphorylation level of 150 kDa protein in the absence and the presence of 50 μM-quercetin, and of 160 kDa protein (in the absence of quercetin), respectively. The level of phosphorylation of 150 kDa protein at pH 7.0 in the absence of quercetin was taken as the maximum (1.0 unit). The inset in (b) shows a representative scanning of the two phosphorylated protein bands.

phorylation which occurred at alkaline pH values was not attributable to higher ATPase activity. Because the effect of protein phosphorylation on Ca²⁺ accumulation was pronounced at alkaline pH (see accompanying paper, [21]), several of the experiments were carried out at two different pH values, but some were performed only at pH 7.2.

Time course of 150 kDa-protein phosphorylation

The kinetic experiments were performed under standardized conditions, with 60 mM-NaF, 100 μM-ATP and pH 7.2; the shortest incubation time that could be achieved was 3 ± 0.5 s. Fig. 3 shows that the phosphorylation reached a maximum within about 15 s, followed thereafter by spontaneous dephosphorylation until it reached a constant level. This suggests that in the presence of 60 mM-NaF the phosphoprotein phosphatase was not completely inhibited (see also Fig. 7a). The time course of the phosphorylation of the 150 kDa protein was not affected by quercetin, although the relative intensity was increased.

Fig. 3. Time course of phosphorylation of 150 kDa protein

SR vesicles were phosphorylated at pH 7.2 as described in Fig. 1(a) for the indicated time and subjected to SDS/PAGE, autoradiography and quantitative analysis as described in the Experimental section. Phosphorylation is expressed relative to the phosphorylation of 150 kDa protein after 15 s.

Fig. 4. Ca^{2+} -dependence of phosphorylation of the 160 and 150 kDa polypeptides

Phosphorylation of SR membranes was carried out at pH 7.2 (Δ , $\bullet$) and 8.2 ($\circ$, Δ , $\blacksquare$, $\square$) as in Fig. 1(a), except that 0.1 mM- ($\blacksquare$, $\square$), 0.2 mM- (Δ , $\triangle$, $\circ$) or 0.5 mM- ($\circ$) EGTA and various amounts of Ca^{2+} were also added to the phosphorylation medium. Free Ca^{2+} concentrations were calculated with a computer program using apparent binding constants (K_a) for Ca^{2+} /EGTA of $1.2 \times 10^7 \text{ M}^{-1}$ and $9.96 \times 10^8 \text{ M}^{-1}$ for pH 7.2 and 8.2 respectively. Phosphorylation was quantified as described in the Experimental section. The curve is the best fit to a Hill coefficient of about 0.5 for Ca^{2+} .

Effect of bivalent and trivalent cations

The effect of various concentrations of Ca^{2+} on the phosphorylation of the 150 kDa proteins at two different pH values is shown in Fig. 4. Quantitative analysis of the autoradiogram, presented in Fig. 4, revealed that the phosphorylation of both proteins is sharply activated by Ca^{2+} in the range 0.2 nM – $2 \mu\text{M}$, and that half-maximal stimulation occurred with a free Ca^{2+} concentration of about 1.0 nM and maximal phosphorylation at about $1 \mu\text{M}$ - Ca^{2+} . The calculated Hill coefficient of about 0.5 suggests negative co-operation between two (or more) Ca^{2+} -binding sites located on the same or on different proteins, or indicates heterogeneous Ca^{2+} -binding sites, such as occurs in different kinases with different Ca^{2+} -dependencies.

Fig. 5 demonstrates the effect of Mg^{2+} on the phosphorylation of the 160 and 150 kDa proteins. As shown, Mg^{2+} inhibited the phosphorylation of both proteins. Under the experimental conditions, half-maximal inhibition was obtained at about

Fig. 5. Effect of Mg^{2+} on the phosphorylation of 150 kDa and 160 kDa proteins

Phosphorylation of SR membranes was carried out at pH 7.2 as in Fig. 1(a), except that Mg^{2+} at the indicated concentrations was also added to the phosphorylation medium. Quantitative analysis of the autoradiogram was carried out as described in the Experimental section: $\circ$ and $\bullet$ indicate the relative phosphorylation of 160 and 150 kDa proteins respectively.

Table 2. Effect of incubation time and ATP concentration on the inhibition of phosphorylation by Mg^{2+}

Protein phosphorylation was carried out as in Fig. 1(a), except that in Expt. I the incubation time was 30 or 60 s, and in Expt. II the incubation time was 60 s and ATP concentration was as indicated. Mg^{2+} at the indicated free concentrations was present in the assay medium. Quantitative analysis of the autoradiograms was carried out as in Fig. 2(b). ATP hydrolysis was assayed by transferring $5 \mu\text{l}$ samples into trichloroacetic acid (final concn. 7%) and the $[\text{P}^{32}]\text{P}_i$ released was extracted and measured as described in the Experimental section for assay of phosphatase activity. The amount of ATP hydrolysed during 30 s of incubation (1.0 unit) was 45 nmol/mg of protein.

Expt. I	Reaction time (s)	$[\text{Mg}^{2+}]$ (mM)	150 kDa-protein phosphorylation (relative units)	ATP hydrolysis (relative units)
	30	0.1	1.00	1.0
	30	0.2	0.74	1.5
	30	0.6	0.48	1.8
	60	0.1	1.00	1.5
	60	0.2	0.50	2.2
	60	0.6	0.23	2.8
Expt. II	[ATP] (μM)	150 kDa-protein phosphorylation (% inhibition by 0.2 mM-Mg^{2+})		
	50	80		
	200	35		
	500	0		

0.2 mM and complete inhibition at about 5 mM-Mg^{2+} . This inhibition was decreased when quercetin, an inhibitor of the $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -ATPase [20], was present during the phosphorylation. However, under the same conditions, quercetin prevented the hydrolysis of only about 30% of the ATP (results not shown). These results suggest that part or all of the inhibition by Mg^{2+} is due to stimulation of ATP hydrolysis. This conclusion is strongly supported by the results shown in Table 2. The degree of inhibition of the phosphorylation of 150 kDa protein by Mg^{2+} is increased with increasing reaction time. In parallel, Mg^{2+} stimulated ATP hydrolysis which increased with incubation time

Fig. 6. Effect of La³⁺ on the Ca²⁺-dependent phosphorylation of 160 and 150 kDa proteins

SR vesicles were phosphorylated for 1 min at pH 7.2 as described in Fig. 1(a), except that 5 μM-CaCl₂ and the indicated concentrations of LaCl₃ were also present in the phosphorylation medium.

Fig. 7. SR phosphoprotein phosphatase activity

In (a), phosphoprotein phosphatase activity was assayed in the absence and in the presence of the indicated concentrations of NaF as described in the Experimental section. In (b), protein phosphorylation was carried out as in Fig. 1(a), except that at the indicated time samples were transferred to SDS-quenching solution (see the Experimental section). After 30 s, EGTA was added to a final concentration of 0.5 mM, and then at the indicated times samples were also transferred to SDS-quenching solution. The samples were analysed by SDS/PAGE and autoradiography as described in the Experimental section.

(Expt. I). The degree of inhibition of phosphorylation of 150 kDa protein by 0.2 mM-Mg²⁺ (free concn.) is also a function of ATP concentration (Expt. II). The inhibition is diminished with increasing ATP concentration. The results in Table 2 suggest that the inhibition by Mg²⁺ is a result of a decrease in ATP concentration owing to stimulation of its hydrolysis by Mg²⁺. Accordingly, this inhibition is diminished as the extent of ATP hydrolysis is decreased either by shortening the incubation time or by increasing the ATP concentration.

The effect of various ter- and bi-valent cations on the phosphorylation of the 160 and 150 kDa proteins was examined. Ba²⁺, Cd²⁺ and Ni²⁺ at concentrations of 2–100 μM did not substitute for Ca²⁺, and had no effect on the Ca²⁺-dependent phosphorylation of both proteins (results not shown). On the other hand, La³⁺ inhibited the Ca²⁺-dependent phosphorylation of the two proteins (Fig. 6). In the presence of 5 μM-Ca²⁺, half-

maximal inhibition was obtained at about 5 μM and complete inhibition at about 75 μM-La³⁺.

These results may suggest that La³⁺ competes with Ca²⁺ for the kinase Ca²⁺-binding sites, but, in contrast with Ca²⁺, La³⁺ ions inactivate the kinase. It is possible, however, that the effect of La³⁺ is due to its interaction with the 160 and 150 kDa proteins rather than with the kinase. In any case, La³⁺ is the first inhibitor that we have found, so far, for the phosphorylation system described here.

Phosphoprotein phosphatase activity

Fig. 7(a) shows the effect of NaF on the SR phosphoprotein phosphatase activity (see [22]), with ³²P-phosphorylase *a* as the substrate. This phosphoprotein phosphatase activity is inhibited by NaF, with a half-maximal inhibition at 25 mM and about 90% inhibition at 80 mM-NaF. These results indicate that, in common with other phosphoprotein phosphatases [23,24], this SR phosphoprotein phosphatase is not potently inhibited by NaF.

The action of this protein phosphatase on the phosphorylated 160 and 150 kDa proteins was examined by inhibiting the Ca²⁺-dependent protein kinase, by the addition of EGTA and then monitoring the level of phosphorylation as a function of time. The results shown in Fig. 7(b) indicate that, even in the presence of 60 mM-NaF, the remaining phosphatase activity is able to dephosphorylate both proteins.

Effect of detergents and temperature

Fig. 8(a) shows that the addition of relatively low concentrations of deoxycholate to the phosphorylation medium inhibited the phosphorylation of both proteins. Other detergents such as CHAPS (0.15%) and octyl glucoside (0.3%) were also inhibitors of the phosphorylation at relatively low concentrations (results not shown). Similarly, pre-heating of SR membranes for 2 min at 60 °C resulted in the loss of phosphorylation activity (Fig. 8b), but not the calmodulin-dependent phosphorylation of 60 kDa protein. Fig. 8(b) also shows that pre-heating of the membranes for 1 min stimulated the calmodulin-dependent phosphorylation of the 60 kDa protein. This could be due either to activation of the kinase or to inactivation of the phosphoprotein phosphatase. It is not known whether the kinase or the substrate, or both, were destroyed by detergent or heat. The results indicate, however, that a hydrophobic factor, such as the membrane, is required for the phosphorylation activity.

Effect of calmodulin, cyclic AMP and other activators or inhibitors of protein phosphorylation on the phosphorylation of the 160 and 150 kDa proteins

A list of different compounds that had no effect on the phosphorylation of the 160 and 150 kDa polypeptides is presented in Table 3. Exogenous calmodulin added to the reaction mixture had no effect on the endogenous phosphorylation of the 160 and 150 kDa proteins, but, as was reported previously [12], markedly stimulated the phosphorylation of the 60 kDa protein (see also Fig. 8b). In an attempt to test whether endogenous calmodulin was involved in this phosphorylation, the effect on phosphorylation of these proteins of different drugs which bind to calmodulin [25] was tested. No inhibitory effect was obtained with trifluoperazine or chlorpromazine (up to 400 μM) or with compound 48/80 (40 μg/ml).

Cyclic AMP in the range 0.1–50 μM had no effect on the phosphorylation of either the 160 or 150 kDa proteins and did not stimulate their phosphorylation in the absence of Ca²⁺ with or without added Mg²⁺ (results not shown).

The possible involvement of protein kinase C was also ruled out, because various activators and inhibitors of this kinase had

Fig. 8. Effect of deoxycholate and temperature on the phosphorylation of SR proteins

In (a), SR membranes were phosphorylated at pH 7.2 in the presence of 60 mM-NaF and in the presence of different concentrations of deoxycholate (DOC) and were then subjected to SDS/PAGE and to autoradiography as described in Fig. 1(a) and the Experimental section. In (b), SR membranes were preincubated at 60 °C for the indicated time, after which samples were phosphorylated for 1 min in the absence and in the presence of 0.6 μ M-calmodulin (CM).

Table 3. List of different activators or inhibitors of calmodulin-dependent and cyclic-AMP-dependent protein kinases and of protein kinase C, that have no effect on the phosphorylation of 150 and 160 kDa proteins

Protein phosphorylation was carried out in the presence of the indicated compound as described in Fig. 1(a).

Compound	Concn.
Exogenous calmodulin	10 μ g/ml
Trifluoperazine	Up to 400 μ M
Chlorpromazine	Up to 400 μ M
48/80	Up to 40 μ g/ml
Polymyxin	80 μ M
Heparin	60 μ g/ml
Sphingosine	Up to 100 μ M
Dibucaine	Up to 0.6 mM
Tetracaine	Up to 2 mM
Phosphatidylserine	0.16 mg/ml
Phorbol 12-myristate 13-acetate (PMA)	Up to 1 μ M
Inositol trisphosphate	Up to 3 μ M
AlCl ₃	Up to 35 μ M
Guanosine 5'-[γ -thio]triphosphate	Up to 250 μ M
cAMP	Up to 50 μ M

no effect on the phosphorylation of the 160 and 150 kDa polypeptides. Also, because it has been suggested that F^- (as AlF_4^-) activates the GTP-binding proteins [26], we tested the effect of Al^{3+} on the requirement for NaF. We found that Al^{3+} had no effect on the NaF concentration required for maximal phosphorylation. In addition, guanosine 5'-[γ -thio]triphosphate, under various conditions, had no effect on the phosphorylation of 160 and 150 kDa polypeptides (Table 3).

Relationship between the 160 kDa and 150 kDa proteins

In an attempt to clarify the relationship between these two polypeptides, we compared the pattern of the peptides obtained by proteolytic digestion. The 160 kDa and 150 kDa phosphorylated-protein bands were cut from the gel, subjected to limited proteolysis with *Staphylococcus aureus* protease (V_8) and further electrophoresed. Comparison of the phosphorylated proteolytic fragments of the two proteins revealed different profiles (Fig. 9), indicating that the 150 kDa protein is not a proteolytic product of the 160 kDa protein. However, both proteins are Ca^{2+} -binding proteins as judged by ^{45}Ca -ligand

Fig. 9. Peptide maps of the phosphorylated 160 and 150 kDa protein bands

SR vesicles were phosphorylated at pH 7.2 as described in Fig. 1(a) and were then subjected to SDS/PAGE. The gel was stained with Coomassie Blue and destained briefly. Bands of 150 and 160 kDa were cut out from the gel, rinsed in solution containing 0.125 M-Tris/HCl, pH 6.8, 0.1% SDS and 1 mM-EDTA for 30 min. Proteolysis was carried out as described previously [41]. Briefly, four slices of each protein band were pushed to the bottom of the gel (7.5–20% of acrylamide) well with a spatula and the spaces around the slices filled with the above buffer; the indicated amount of the protease V_8 in the same buffer containing 20% glycerol was then overlaid into each slot. Electrophoresis was performed until the Bromophenol Blue dye approached the bottom of the stacking gel. The current was then turned off for 30 min, and subsequently turned on for several hours. Autoradiography of the dried gel was carried out as described in the Experimental section. The arrows indicate the proteolytic phosphorylated peptides (K = kDa) specific to the 160 and 150 kDa protein bands.

overlay of nitrocellulose blots, and both proteins also stained blue with Stains-All (results not shown).

The phosphoproteins exhibited the characteristics of phosphoesters, because they were not susceptible to degradation by hydroxylamine (results not shown).

The phosphoamino acids in the 160 kDa and 150 kDa proteins were identified by high-voltage paper electrophoresis after partial hydrolysis of the phosphorylated protein bands cut out from the gels. The 160 kDa and 150 kDa proteins contained [³²P]phosphoserine (results not shown). The amount of ³²P incorporated into the 160 and 150 kDa proteins was estimated to be about 8 and 5 mol/mol of protein respectively (see the Experimental section).

DISCUSSION

In the present study we have found an endogenous Ca²⁺-dependent phosphorylation system in skeletal SR membranes which catalyses the specific phosphorylation of 160 kDa and 150 kDa proteins. These two proteins are probably SR proteins, because they were found in different SR preparations (Fig. 1) and remained associated with SR membranes which had been washed with high or low salt concentration, such as 0.6 M-KCl or 10 mM-Tricine, pH 8.0, or 1 mM-EGTA, pH 6.8.

Several proteins with an apparent molecular mass of 160–170 kDa have been observed previously in skeletal-muscle SR [27–33]. An intrinsic glycoprotein (160 kDa) has been identified in SR by Campbell & MacLennan [27], and recently it has been cloned, expressed and designated sarcalumenin [29]. A similar protein has been extracted by Na₂CO₃ treatment [30]. Recently, a SR histidine-rich Ca²⁺-binding protein (HCP) with an apparent molecular mass of 165000 that binds Ca²⁺ and plasma lipoproteins has been purified [31], and its amino acid sequence was deduced from the cDNA [32]. Also, a 170 kDa Ca²⁺ binding protein was purified by affinity chromatography on Reactive Red 120–agarose and was proposed to be the caffeine receptor [33].

The relationship between the 150 and 160 kDa phosphorylated proteins described here and the previously described 160–170 kDa protein remains to be established. It is clear that the two phosphorylated proteins belong to the family of so-called calsequestrin-like proteins [28] because of their staining blue with Stains-All, and their ability to bind Ca²⁺ as shown by the Ca²⁺-overlay technique.

The two proteins are different from the α_1 (170 kDa) and α_2 (175 kDa) subunits of the dihydropyridine receptor (DHP)/voltage-sensitive Ca²⁺ channels (Table 1). This conclusion is further supported by the following observations. (a) The relative mobility of the 160 kDa or 150 kDa proteins in SDS/PAGE did not change after cleavage of disulphide bonds with dithiothreitol or β -mercaptoethanol (results not shown). On the other hand, the 175 kDa subunit was cleaved to 143 kDa and 32 kDa components under reducing conditions [34]. (b) The 150 kDa and 160 kDa proteins bind Ca²⁺, whereas α_1 and α_2 subunits of the DHP receptor have no Ca²⁺-binding activity. (c) In our membrane preparations, the 160 kDa and 150 kDa proteins were phosphorylated by an endogenous kinase, whereas only the 170 kDa (α_1) subunit of the DHP receptor contained the cyclic-AMP-dependent protein kinase phosphorylation site(s) [34].

It has also been shown that the SR 160–170 kDa proteins, which stained blue with Stains-All, were not related to phosphorylase kinase subunits or glycogen-debranching enzyme (145 kDa and 160 kDa respectively) [28].

The phosphorylation systems of the 160 and 150 kDa proteins were quite similar in some respects, such as their Ca²⁺- and pH-dependence, their requirement for NaF, their affinity for ATP [21], their inhibition by Mg²⁺, their inhibition by La³⁺ and their very high sensitivity to detergents and to heating of the SR

membranes, but they differed in their peptide maps. These observations suggest that there is no structural relationship between the two proteins, but that they are phosphorylated by the same protein kinase. The kinase responsible for the phosphorylation of these two proteins has yet to be identified. It is possible that one of these proteins is the kinase that undergoes autophosphorylation. Since the 160 kDa protein has an ATP-binding site [35], the possibility of the 160 kDa protein being the kinase requires investigation.

The phosphorylation of these two polypeptides has not been previously reported, probably because of the high concentrations of NaF (40–60 mM) required. The need for relatively high NaF concentrations is consistent with the inhibition of the phosphoprotein phosphatase of the SR [21]. However, NaF has been shown to modulate protein kinase C [36], to stimulate the production of inositol biphosphate and trisphosphate in WRK₁ cell membranes [37] and to activate several hormonal systems [26]. It has been suggested that the target of F⁻ is the α -subunit of the GTP-binding proteins [38] and that AlF₄⁻ is the active species [26]. In our system, however, Al³⁺ or guanosine 5'-[γ -thio]triphosphate had no effect on NaF-dependent phosphorylation (Table 3).

The Ca²⁺-dependent protein kinase involved in the phosphorylation of the 160 kDa and 150 kDa proteins is different from the previously reported Ca²⁺/calmodulin-dependent protein kinase which phosphorylated the 85, 60, 34 and 20 kDa polypeptides [12,13,39,40]. The two protein kinases differ in that: (a) the optimum pH is below 6.0 for the Ca/calmodulin-dependent kinase ([12]; see also Fig. 2) and is about 7.0 for the Ca²⁺-dependent kinase; (b) exogenous calmodulin and Mg²⁺ are required for the Ca/calmodulin-dependent system, but not for the Ca²⁺-dependent phosphorylation; (c) mild tryptic digestion of SR results in the loss of Ca/calmodulin system [12], but had no inhibitory effect on our phosphorylation system (results not shown); (d) in our experiments (Fig. 8), heating SR membranes at 60 °C for 2 min destroyed one phosphorylating system, but not the other; (e) as expected, the Ca/calmodulin-dependent phosphorylation system was inhibited by trifluoperazine (*K*_i 5 μ M), which had no effect (at least up to 400 μ M) on the phosphorylation described here.

The full activation of 160 kDa and 150 kDa phosphorylation by 1 μ M-Ca²⁺ (Fig. 4) may suggest either that its effect is mediated by calmodulin or that the kinase is of the protein kinase C type. There are several pieces of evidence to suggest that the phosphorylation system described here is neither a Ca²⁺/calmodulin-type nor a protein kinase C. The following observations strongly suggest that calmodulin is not involved: in our experiments, the addition of purified calmodulin failed to increase the phosphorylation above the level obtained with Ca²⁺ alone, although, as reported previously [12,39,40], calmodulin stimulated the phosphorylation of the 60 kDa protein (see Fig. 8). The absence of multiple Ca²⁺-binding sites with a positive co-operativity (Fig. 4) suggests that the effect of Ca²⁺ is not mediated by its binding to calmodulin. Furthermore, a number of drugs which bind to calmodulin with high affinity in a Ca²⁺-dependent fashion [25] have no inhibitory effect on this phosphorylation.

We did not observe any activation by phosphatidylserine, which characteristically activates protein kinase C, nor by tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate ('PMA') [41]. Furthermore, protein kinase C is inhibited by drugs such as trifluoperazine or chlorpromazine, local anaesthetics such as tetracaine and dibucaine, as well as by heparin, sphingosine and polymyxin [41]. These protein kinase C inhibitors were all found to have no inhibitory effect on the phosphorylation of the 160 and 150 kDa proteins, even at relatively high concentrations (see Table 3). Thus no inhibition

of this phosphorylation system was obtained with the various inhibitors of protein phosphorylation. At present, La^{3+} is the only inhibitor that we have found for this Ca^{2+} -dependent phosphorylation system.

It appears that the phosphorylation of the 160 kDa and 150 kDa proteins is regulated solely by Ca^{2+} , and it is therefore a unique Ca^{2+} -dependent protein kinase that is activated directly by Ca^{2+} , independently of calmodulin or phosphatidylserine. A Ca^{2+} -dependent protein kinase from *Paramecium tetraurelia* and from *Chlamydomonas* has been reported previously [42,43].

The possible involvement of the phosphorylation system described here in the control of the membrane permeability for Ca^{2+} is presented in the accompanying paper [21].

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