Involvement of protein phosphorylation in activation of Ca^{2+} efflux from sarcoplasmic reticulum

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Preincubation of sarcoplasmic reticulum (SR) membranes with a combination of ATP and NaF resulted in inhibition of Ca^{2+} accumulation and stimulation of Ca^{2+} -ATPase and Ca^{2+} efflux. Under the same conditions, the activity of the SR phosphoprotein phosphatase was inhibited and the phosphorylation of two polypeptides with apparent molecular masses of 160 and 150 kDa was obtained. The effect of ATP is specific, since the ATP analogue adenosine 5'-[β - γ -imido]triphosphate did not replace for ATP. In the absence of NaF, ATP was ineffective. The phosphorylation of the 160 kDa and/or 150 kDa proteins and the stimulation of Ca^{2+} efflux are clearly related. The phosphorylation of both proteins and the increase in Ca^{2+} efflux show a similar dependence on the concentration of ATP. The level of protein phosphorylation and the stimulation of Ca^{2+} efflux were also controlled by the NaF concentration which inhibits the phosphoprotein phosphatase. Similar concentrations of NaF were required for the inhibition of phosphoprotein phosphatase and of net Ca^{2+} accumulation, as well as for the stimulation of phosphorylation of both polypeptides. Quantitative analysis revealed a linear correlation between these three activities. Dicyclohexylcarbodi-imide, which inhibited Ca^{2+} efflux, also inhibited the phosphorylation of the two polypeptides. These results suggest the involvement of the phosphorylation/dephosphorylation of 160 kDa and/or 150 kDa polypeptides in the activation of Ca^{2+} release from SR membranes.

INTRODUCTION

The sarcoplasmic reticulum (SR) of skeletal muscle sequesters Ca^{2+} during muscle relaxation and releases it into the cytoplasm upon sarcolemmal depolarization, thereby triggering contraction [1]. Ca^{2+} -release-channel activity has been identified in isolated SR membranes and characterized by flux measurements using $^{45}\text{Ca}^{2+}$ and single-channel measurements using the planar lipid bilayer technique [2–6].

Various studies indicate that more than one Ca^{2+} release pathway co-exist in SR [2–4,6–9]. Ca^{2+} channels of large [5,10] and small [3,4] conductance have been detected in SR membranes. These channels differ both in their distribution across the different structural regions of the SR membranes and in their activation mechanism. One of these channels is the junctional channel present in the SR–T-system junction, which has been identified with the ryanodine receptor/'feet' structures [10–14]. The purified ryanodine receptor is a large 30 S complex comprising a single polypeptide with apparent molecular mass of 350–450 kDa [10,11,14]. There are, however, many indications that other SR proteins, such as the 30 kDa, 60 kDa and 170 kDa proteins, are involved in the operation and/or regulation of Ca^{2+} channels [15–18]. It has been postulated [19,20] that changes in the level of phosphorylation of a specific protein might regulate changes in ionic permeability of the SR membranes. More specifically, the involvement of a calmodulin-dependent phosphorylation of a 60 kDa protein in the control of Ca^{2+} release in isolated SR membranes has been proposed [18,19].

The aim of the present paper is to describe the possible involvement of the Ca^{2+} -dependent endogenous phosphorylation of 160 kDa and 150 kDa proteins (described in the accompanying paper, [21]) in the regulation of Ca^{2+} release from SR membranes.

EXPERIMENTAL

The materials and SR preparations used were the same as those described in the accompanying paper [21]. Protein phos-

phorylation, phosphoprotein phosphatase activity and SDS/PAGE were also carried out as described in the accompanying paper [21].

Assays

Active $^{45}\text{CaCl}_2$ uptake by SR vesicles was determined by Millipore filtration. The basic reaction mixture contained 20 mM-Mops, pH 6.8, 100 mM-KCl, 3 mM-MgCl₂, 3 mM-ATP, 0.5 mM- CaCl_2 (containing ^{45}Ca , about 10^6 c.p.m./ μmol), 0.5 mM-EGTA and 50 mM-potassium phosphate. The uptake was initiated by the addition of 90 μg of SR/ml. After incubation for 1 or 2 min at 30 °C, 0.18 ml samples were filtered through 0.45 μm nitrocellulose filters and washed with 5 ml of 0.15 mM-NaCl. Radioactivity on the filters was measured with a liquid-scintillation counter. ATPase activity was assayed under conditions similar to those used for Ca^{2+} uptake, except that $^{45}\text{CaCl}_2$ was omitted, [γ - ^{32}P]ATP was added to the reaction mixture, and the [^{32}P]P_i released from ATP was determined as described by Avron [22].

For the assay of Ca^{2+} efflux, the vesicles, preincubated under the conditions indicated in the Figure or Table legends, were centrifuged at 100 000 *g* for 30 min, resuspended (2.5 mg/ml) in 20 mM-Mops, pH 6.8, containing 100 mM-KCl and 5 mM- $^{45}\text{CaCl}_2$ (1.5×10^7 c.p.m./ml), and incubated for 2 h at 24 °C. For Ca^{2+} efflux, samples (20 μl) were rapidly filtered and washed for the indicated time with a solution identical with the loading solution, except that CaCl_2 was omitted and 1 mM-EGTA was added. The washing rate was approx. 1 ml/s.

RESULTS

Table 1 shows the effect of preincubation of SR membranes with ATP or adenosine 5'-[β - γ -imido]triphosphate (p[NH]ppA), both with and without NaF, on the ATPase activity and Ca^{2+} accumulation. Preincubation of the SR with either compound, in the absence of NaF, had no significant effect on either Ca^{2+}

Abbreviations used: SR, sarcoplasmic reticulum; p[NH]ppA, adenosine 5'-[β - γ -imido]triphosphate; DCCD, dicyclohexylcarbodi-imide.

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Table 1. Effect of preincubation of SR with ATP or p[NH]ppA with and without NaF, on Ca^{2+} accumulation and ATPase activity

SR membranes (1 mg/ml) were preincubated with the indicated compounds at 30 °C in a solution containing 20 mM-Tricine, pH 8.2, and 100 mM-NaCl. After 4 min, samples were cooled on ice and portions were assayed for Ca^{2+} accumulation and ATPase activity as described in the Experimental section.

Additions to preincubation medium	Ca^{2+} accumulation ($\mu\text{mol}/\text{min}$ per mg of protein)	ATPase activity ($\mu\text{mol}/\text{min}$ per mg of protein)
None	1.19	0.77
NaF (60 mM)	1.21	0.68
ATP (0.2 mM)	1.04	0.76
p[NH]ppA (0.2 mM)	1.05	0.77
NaF + ATP	0.21	2.02
NaF + p[NH]ppA	1.34	0.76

accumulation or ATPase activity. However, preincubation with ATP plus NaF resulted in inhibition of Ca^{2+} accumulation (82%) and about a 2.6-fold stimulation of ATPase activity. Table 1 also shows that this effect was not obtained when ATP was replaced by the non-hydrolysable analogue p[NH]ppA. This apparent uncoupling between Ca^{2+} -accumulation and ATPase activity is dependent on the concentrations of both NaF (Fig. 3) and ATP (Table 2). Table 2 also shows that the loss of Ca^{2+} -accumulation capacity is associated with a greatly accelerated passive efflux of Ca^{2+} from Ca^{2+} -loaded SR vesicles. Thus the inhibition of Ca^{2+} accumulation reflects the stimulation of Ca^{2+} efflux.

Since p[NH]ppA did not replace ATP, and the presence of NaF as a phosphatase inhibitor (Fig. 3) was required for the stimulation of Ca^{2+} efflux by preincubation with ATP, it seems plausible that protein phosphorylation is involved in the enhancement of Ca^{2+} efflux. In the accompanying paper [21], we

Table 2. Effect of preincubation of SR vesicles with NaF and with different concentrations of ATP on their Ca^{2+} accumulation, ATPase activity and Ca^{2+} efflux

SR vesicles (1 mg/ml) were incubated at 30 °C in a solution containing 20 mM-Tricine, pH 8.2, 100 mM-NaCl and 60 mM-NaF and the indicated concentration of ATP. After 4 min the membranes were transferred to ice and then collected by centrifugation (100 000 *g* for 30 min) and resuspended in 100 mM-NaCl/20 mM-Mops, pH 6.8. Samples were assayed for Ca^{2+} accumulation, ATPase activity and Ca^{2+} efflux as described in the Experimental section. Control activities (100%) were 1.7 and 0.94 $\mu\text{mol}/\text{min}$ per mg of protein for Ca^{2+} accumulation and ATPase activity respectively. The Ca^{2+} content of the passively loaded SR vesicles was 24–28 nmol/mg of protein, and the efflux was assayed for 10 s.

[ATP] (μM)	Activity (% of control)		
	Ca^{2+} accumulation	ATPase activity	Ca^{2+} efflux
0	100	100	8
50	97	100	12
80	81	169	32
100	62	184	46
200	14	309	72

have shown an endogenous Ca^{2+} -dependent phosphorylation of two specific proteins with apparent molecular masses of 160 kDa and 150 kDa. We have therefore investigated the involvement of phosphorylation of these two proteins in the regulation of Ca^{2+} release by studying the effects of ATP and NaF on the endogenous phosphorylation and on the stimulation of Ca^{2+} efflux.

Fig. 1(a) shows that the phosphorylation of 160 kDa and 150 kDa proteins is enhanced by ATP in the presence of NaF, and that quercetin increased the level of phosphorylation of both proteins. The quantitative analysis, carried out by densitometric scanning of the autoradiogram and determination of the peak area of the 160 and 150 kDa phosphorylated bands, is shown in

Fig. 1. ATP-dependence of the phosphorylation of the 160 and 150 kDa proteins

SR vesicles (1 mg/ml) were incubated for 4 min at 30 °C in 20 mM-Tricine (pH 8.2)/100 mM-NaCl/60 mM-NaF with various concentrations of [γ - ^{32}P]ATP (2 $\mu\text{Ci}/\text{nmol}$) and then subjected to SDS/PAGE as described [21]. The autoradiogram and the Coomassie-Blue-stained sample (the lane on the right) are shown in (a) and the quantitative analysis of this autoradiogram in (b): ■ and □ indicate the phosphorylation level of the 150 kDa protein obtained in the absence and the presence of 50 μM -quercetin respectively; ○ indicates the phosphorylation level of the 160 kDa protein band. Quantitative analysis of the phosphorylated bands was carried out by densitometric scanning of the autoradiogram with a Cary 219 spectrophotometer, and relative peak areas were estimated from the height $\times$ half-width of each peak. The increased phosphorylation observed with quercetin suggests that ATP hydrolysis takes place during the phosphorylation process and leads to the sigmoidal shape of the curves.

Fig. 2. Relationship between Ca^{2+} efflux and protein phosphorylation

SR vesicles were incubated with various concentrations of ATP in the presence of NaF, collected by centrifugation (100 000 g) and passively loaded with $^{45}\text{CaCl}_2$. Ca^{2+} efflux from the Ca^{2+} -loaded vesicles was assayed as described in the Experimental section. In (a), the Ca^{2+} released per 8 s is plotted as a function of ATP concentration (these data were taken from time courses, some of which are shown in the inset). The time course of Ca^{2+} efflux from SR preincubated with NaF without ATP ($\circ$) or with 100 μM - ($\blacksquare$) or 200 μM - ($\square$) ATP is shown in the inset. In (b), the phosphorylation levels of the 160 kDa ($\circ$) and the 150 kDa ($\bullet$) proteins at various ATP concentrations (results from Fig. 1b) were plotted as a function of the Ca^{2+} efflux obtained at the same ATP concentration. The phosphorylation level of 200 μM -ATP was calculated to be about 70 % of the maximal phosphorylation (according to the intercept of the double-reciprocal plots; result not shown).

Fig. 3. Effect of NaF on protein phosphorylation, phosphoprotein phosphatase and net Ca^{2+} accumulation

SR vesicles were preincubated with various concentrations of NaF, without (for phosphatase activity) or with 100 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (for phosphorylation) or with unlabelled ATP (for Ca^{2+} accumulation) as described in Fig. 1. Samples taken were assayed for these three activities. (a) Protein phosphorylation was carried out as described in Fig. 1: $\square$ and $\blacksquare$ indicate the phosphorylation levels of the 160 and 150 kDa proteins respectively (inset shows the autoradiogram). (b) Phosphoprotein phosphatase activity ($\circ$) was assayed by measuring the release of $[\text{P}^{32}]\text{P}_i$ from ^{32}P -labelled glycogen phosphorylase α as the substrate, as described in the accompanying paper [21]. (c) Ca^{2+} accumulation was assayed as described in the Experimental section: $\triangle$ and $\blacktriangle$ indicate two different experiments.

Fig. 1(b). The level of phosphorylation of each protein is presented as a function of ATP concentration. The stimulation of the phosphorylation by quercetin [an inhibitor of the $(\text{Ca}^{2+} + \text{Mg}^{2+})\text{-ATPase}$] suggests that ATP hydrolysis takes place during the phosphorylation assay.

Fig. 2(a) (inset) shows representative time courses of Ca^{2+} efflux from SR vesicles passively loaded with Ca^{2+} after treatment either with NaF alone or with NaF plus ATP. Preincubation with ATP in the presence of NaF resulted in a rapid Ca^{2+} efflux. In Fig. 2(a), the extent of Ca^{2+} efflux from SR vesicles pretreated with various ATP concentrations is plotted as a function of the ATP concentration, showing that Ca^{2+} efflux was increased by increasing the ATP concentration. The data shown in Figs. 1(b) and 2(a) were replotted in Fig. 2(b) to establish the relationship

between the stimulation of Ca^{2+} efflux and the phosphorylation level of the 150 kDa and 160 kDa proteins: a linear correlation was observed.

The level of protein phosphorylation and stimulation of Ca^{2+} efflux could also be controlled by altering the concentration of NaF in the phosphorylation medium. Fig. 3 shows the effect of preincubation of SR vesicles with ATP, in the presence of different concentrations of NaF, on net Ca^{2+} accumulation (as a reflector of Ca^{2+} efflux; see Table 2), phosphoprotein phosphatase activity and protein phosphorylation. Similar concentrations of NaF were required for the inhibition of protein phosphatase and of net Ca^{2+} accumulation, as well as for stimulation of phosphorylation of the 160 kDa and 150 kDa proteins. Half-maximal inhibition of Ca^{2+} accumulation and phosphatase activity and

Fig. 4. Correlation between phosphorylation, Ca^{2+} accumulation and phosphoprotein phosphatase activity

The data in Fig. 3 have been replotted to show the close correlation between Ca^{2+} accumulation ($\circ$, $\square$), phosphatase activity ($\bullet$, $\blacksquare$) and the phosphorylation of 150 kDa ($\circ$, $\bullet$) and 160 kDa ($\square$, $\blacksquare$) proteins. Ca^{2+} accumulation and phosphatase activities, after preincubation with various NaF concentrations, were plotted as a function of the phosphorylation level of the 150 or 160 kDa proteins obtained at the same NaF concentration.

the stimulation of protein phosphorylation were obtained at 45 mM-, 25 mM- and 40 mM-NaF respectively. In Fig. 4 the extent of inhibition by NaF of the net Ca^{2+} accumulation and of the phosphoprotein phosphatase activity (data from Figs. 3b and 3c) were replotted as a function of the level of phosphorylation of 150 kDa or 160 kDa proteins, at various NaF concentrations (data from Fig. 3a). Fig. 4 shows that, upon varying the concentration of NaF, a linear correlation between the level of phosphorylation of the 150 kDa and 160 kDa proteins and the extent of inhibition of both the net Ca^{2+} accumulation and the phosphoprotein phosphatase activity exists.

We have previously shown that dicyclohexylcarbodi-imide (DCCD) inhibited the Ca^{2+} efflux from SR vesicles activated by different methods [23,24]. Fig. 5 shows that preincubation of SR vesicles with DCCD, before carrying out the phosphorylation assay, resulted in inhibition of phosphorylation of both 160 and 150 kDa polypeptides.

Fig. 6(a) shows that the inhibition of Ca^{2+} accumulation by preincubation of SR vesicles with ATP + NaF is time- and pH-dependent. The Ca^{2+} -accumulation capacity of SR preincubated with NaF alone did not change at pH values between 6.5 and 8.2. However, when ATP was present during the preincubation, an inhibition of Ca^{2+} accumulation, which increased with increasing pH of the preincubation mixture, was observed (Fig. 6a). Preincubation of SR with ATP in the absence of NaF inhibited the Ca^{2+} accumulation only slightly at alkaline pH (results not shown). It should be mentioned that the optimum pH for the phosphorylation is about 7.0 (Fig. 2 in [21]). This discrepancy will be discussed below. The time courses of the phosphorylation of 150 kDa polypeptide and of the inhibition of Ca^{2+} accumulation by preincubation with ATP + NaF at pH 8.2 are compared in Fig. 6(b). The Ca^{2+} -accumulation capacity of SR vesicles, preincubated with NaF at pH 8.2 for 4 min, decreased only slightly (by less than 10%). When ATP was present in the preincubation, however, a strong inhibition of Ca^{2+} accumulation occurred. The phosphorylation level of 150 kDa polypeptide as a function of preincubation time is also shown in Fig. 6(b). The results indicate that similar times are required for the phosphorylation of 150 kDa polypeptide and for the inhibition of Ca^{2+} accumulation to reach their maximal levels.

Fig. 5. Inhibition of protein phosphorylation in DCCD-treated membranes

SR membranes (1 mg/ml) were incubated in 100 mM-NaCl/20 mM-Mes, pH 6.4, with 2% (v/v) ethanol and the indicated concentrations of DCCD for 30 min at 30 °C. Membranes were collected by centrifugation (100000 g), and samples were phosphorylated in the presence of 100 μM -[γ - ^{32}P]ATP and 60 mM-NaF, and then subjected to SDS/PAGE and to autoradiography as described in Fig. 1.

DISCUSSION

In this study, we have found that preincubation of SR membranes with ATP, but not with p[NH]ppA, in the presence of NaF at alkaline pH resulted in the inhibition of Ca^{2+} accumulation, stimulation of ATPase activity and a rapid Ca^{2+} efflux. The requirement for NaF, as an inhibitor of the membrane-associated protein phosphatase (see Fig. 3, and Fig. 7 in [21]), and the observation that the non-hydrolysable ATP analogue p[NH]ppA is ineffective (Table 1), may suggest that the increased membrane permeability for Ca^{2+} produced by preincubation with ATP and NaF is the result of protein phosphorylation.

The experiments reported here suggest that phosphorylation of 160 and/or 150 kDa polypeptides by a Ca^{2+} -dependent endogenous protein kinase (see accompanying paper, [21]) may be involved in the activation of Ca^{2+} efflux from SR vesicles. Our results show that under different preincubation conditions, e.g. different concentrations of ATP or NaF, the enhanced phosphorylation of these two proteins is accompanied by an increase in the membrane permeability to Ca^{2+} . The close correlation between the phosphorylation of 160 and/or 150 kDa proteins and the stimulation of Ca^{2+} efflux is demonstrated by the quantitative analysis of several phosphorylation and Ca^{2+} -experiments. The plots of Ca^{2+} efflux (or the inhibition of net Ca^{2+} accumulation) as a function of the phosphorylation level of the 160 and/or 150 kDa polypeptides showed a linear relationship when the phosphorylation level was controlled by varying either ATP or NaF concentrations (Figs. 2b and 4). Also, the time course of both activities indicates a close correlation between phosphorylation and the stimulation of Ca^{2+} efflux. Thus one of the most important aspects of the present paper is the observation of a direct correlation between the extent of the phosphorylation of the 160 and 150 kDa proteins and the extent of stimulation of Ca^{2+} efflux from SR vesicles.

The findings that DCCD, which inhibits Ca^{2+} release [23,24], also inhibits the phosphorylation of the 160 and 150 kDa proteins (Fig. 5) and that [^{14}C]DCCD was incorporated into the region of 160–170 kDa proteins [24] support the notion that these phos-

Fig. 6. pH- and time-dependence of the inhibition of net Ca^{2+} accumulation by preincubation with ATP + NaF

In (a), SR membranes were preincubated at 30 °C in a solution containing 100 mM-NaCl and 60 mM-NaF at the indicated pH, in the absence (○) or presence of 0.2 mM- (■) or 0.5 mM- (□) ATP. After 4 min, samples were diluted 11-fold and assayed for Ca^{2+} accumulation as described in the Experimental section. Buffers used to control the pH 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. In (b), SR membranes were incubated for the indicated time, with 20 mM-Tricine (pH 8.2)/60 mM-NaF in the absence (▲) or presence of 0.2 mM- (●) or 0.5 mM- (○) ATP, and samples were assayed for Ca^{2+} accumulation. For protein phosphorylation, the membranes were incubated under similar conditions, except that [γ - ^{32}P]ATP (0.2 mM) was used. The level of phosphorylation (150 kDa protein; Δ) was determined by quantitative analysis of the autoradiogram shown in the inset, where lanes 1–7 correspond to phosphorylation times of 10, 15, 20 and 30 s and 1, 2 and 4 min respectively. The phosphorylation of the Ca^{2+} -ATPase in the short incubation time was observed previously [18].

phorylated proteins are associated with the Ca^{2+} -release system(s). The inhibitory effect of DCCD on protein phosphorylation could be attributed to its direct interaction with a Ca^{2+} -release channel, as suggested previously [24]. However, since the phosphorylation of either the 160 or 150 kDa proteins is strongly dependent on Ca^{2+} [21], it is also possible that DCCD modifies a carboxyl residue located on the kinase Ca^{2+} -binding site or on one or on both proteins, thereby preventing the phosphorylation of the 160 and 150 kDa proteins.

In contrast with the close correlation between the level of protein phosphorylation and the Ca^{2+} efflux activity described above, our results show that under similar conditions the optimal pH for protein phosphorylation is about 7.0, whereas for the stimulation of Ca^{2+} efflux it is about 8.0. This observation may suggest that, in addition to phosphorylation, the activation of Ca^{2+} release by phosphorylation proceeds through a multi-state reaction which is pH-dependent.

Since the phosphorylation of the 160 and 150 kDa proteins is accompanied by a significant increase in membrane permeability to Ca^{2+} , it can be assumed that one or both proteins are part of the Ca^{2+} -release channel or part of a system activating Ca^{2+} release. The involvement of 160–170 kDa protein(s) in Ca^{2+} release has been suggested previously [8,15–17]. Zorzato *et al.* [16] showed the binding of doxorubicin to 170 kDa protein, and more recently Rubtsov & Murphy [17], using caffeine, purified a 170 kDa protein, proposing that it is the caffeine receptor of the Ca^{2+} channels. The 170 kDa protein isolated by Rubtsov & Murphy [17] as well as our 160 and 150 kDa proteins were stained blue with the carbocyanine dye Stains All (results not shown).

Since the phosphorylation of these proteins is activated by Ca^{2+} , the phosphorylation/dephosphorylation and the activation/inactivation of Ca^{2+} release could therefore be controlled by the transient increase in the cytoplasmic Ca^{2+} concentration. The approximate steady-state levels of Ca^{2+} in the 'resting' cell are between 10 and 100 nM [25]. Under these conditions and in the presence of Mg^{2+} , the phosphorylation

system would be partially inactivated (the $K_{0.5}$ for Ca^{2+} activation is about 1 nM, with full activation in the micromolar range and Hill coefficient 0.5 (see [21])). Full activation would be obtained by an increase in Ca^{2+} concentration, owing to Ca^{2+} release via the junctional Ca^{2+} -release channels or to Ca^{2+} influx through the voltage-gated Ca^{2+} channels, activated by the depolarization of the T-system. However, protein phosphorylation usually regulates physiological events through an amplification cascade in which many phosphatases and kinases are involved [26]. SR membranes contain not only an endogenous protein kinase(s) but also phosphoprotein phosphatase(s) ([21,27] and Fig. 3). Therefore, the activation of Ca^{2+} release may be dependent on the respective activities of these enzymes. If the SR phosphoprotein phosphatase is more active than the kinase (as was shown by the requirement for a phosphatase inhibitor, such as NaF, in order to detect protein phosphorylation), it is possible that the phosphorylation of these proteins of different drugs which bind to calmodulin [25] was tested. No inhibitory effect was obtained phosphatase. However, the possibility that in our isolated SR membranes the relative activities of the kinase and the phosphoprotein phosphatase are not the same as in the muscle cell must not be overlooked. It is possible that during the isolation of the SR membranes some inactivation and/or activation of the kinase and the phosphatase, respectively, occurred and/or that an activator and/or inhibitor of the kinase and the phosphatase, respectively, were lost.

The mechanism by which the endogenous phosphorylation of 160 and/or 150 kDa proteins stimulates Ca^{2+} efflux is not clear. Based on our previous studies [7,8,28–30] we have suggested that the opening of the non-junctional Ca^{2+} release channel is controlled by a positively charged amino group(s). Because of this group, the Ca^{2+} -release channel could be sensitive to changes in local potential (surface charge) [30,31]. Several mechanisms could cause changes in the surface charge, e.g. the binding of ligand or intramolecular charge movements [31], or, according to our suggestion, by protein phosphorylation, which would add a negative charge(s) to the protein. An alternative possibility is

that phosphorylation is not directly involved, but participates in one of a complex series of molecular interactions which lead to the opening of the Ca^{2+} -release channel. Clearly, further studies are required to clarify the function of this phosphorylation system in the activation of Ca^{2+} release from SR membranes.

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