*Advances in Cyclic Nucleotide and Protein Phosphorylation Research, Vol. 17,*
edited by P. Greengard et al.
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# A Calmodulin-Dependent Protein Kinase System from Skeletal Muscle Sarcoplasmic Reticulum

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The sarcotubular system in muscle cells is a membrane network that surrounds each myofibril and is responsible for the regulation, through uptake and release, of sarcoplasmic free $Ca^{2+}$ concentrations (11). The network is extensive and has the capacity to raise and lower $Ca^{2+}$ concentrations within milliseconds, thus accounting for the rapidity of muscle movement: $Ca^{2+}$ is taken up from the sarcoplasm by the pumping action of a $Ca^{2+}$-dependent ATPase, which has a threshold of activation of about 0.2 to 0.3 $\mu$M free $Ca^{2+}$, and is stored preferentially in the lumen of the terminal cisternae (26), the site of localization of the $Ca^{2+}$ binding protein, calsequestrin (13,20).

There is no evidence, as yet, of any controlling mechanism other than the concentration of free $Ca^{2+}$ for the $Ca^{2+}$-ATPase of skeletal muscle sarcoplasmic reticulum. In cardiac sarcoplasmic reticulum, however, there is a second subunit, pliospholamban (27), which is phosphorylated by either the cyclic-AMP-dependent protein kinase or by a calmodulin-dependent kinase (16), thereby regulating $Ca^{2+}$-ATPase and $Ca^{2+}$ uptake activity.

Release of $Ca^{2+}$ is controlled through electrical currents that pass into the muscle cell by way of the transverse tubular system. There is no evidence that depolarizing currents pass into the sarcoplasmic reticulum to initiate $Ca^{2+}$ release, and studies showing that electrical depolarization of the sarcoplasmic reticulum membrane can initiate $Ca^{2+}$ release arc not very convincing (cf. 21). Tins suggests that a second messenger, generated in the triad region in response to the depolarizing current, may be responsible for the initiation of $Ca^{2+}$ release.

We have been interested in the question of whether phosphorylalion might be a regulator of sarcoplasmic reticulum function. Phosphorylation of skeletal muscle sarcoplasmic reticulum proteins of $M_r$ 46,000, 42,000, and 20,000 was observed by Campbell and Shamoo (5), who measured phosphorylation in the presence of 1 mM EGTA and absence of cyclic AMP or exogenous protein

kinase. We (1,3) observed phosphorylation of three proteins of 85,000, 60,000, and 20,000 daltons in the presence of $Ca^{2+}$ and calmodulin. The 85,000-dalton protein did not appear to be of sarcoplasmic reticulum origin because it could be removed on further purification of the sarcoplasmic reticulum vesicles. The content of 60,000- and 20,000-dalton proteins, however, was increased with each step of purification, suggesting that they were, indeed, of sarcoplasmic reticulum origin. Chiesi and Carafoli (6) also observed calmodulin-dependent phosphorylation of 57,000-, 35,000-, and 20,000-dalton sarcoplasmic reticulum proteins.

We found that the calmodulin dependence of the phosphorylation reaction could be satisfied with an EGTA extract of the sarcoplasmic reticulum, demonstrating that calmodulin bound to the sarcoplasmic reticulum membrane was extracted into EGTA solution (3). Calmodulin-dependent incorporation of $^{32}P$ into the 60,000-dalton protein was increased about threefold following EGTA extraction, suggesting that the basal content of phosphoryl groups was lowered when endogenous calmodulin was removed. Chiesi and Carafoli (6) also reported that calmodulin is bound to the sarcoplasmic reticulum. They estimated its concentration at 0.5 to 1 $\mu$g/mg protein.

Phosphorylation of the 60,000- and 20,000-dalton proteins occurred at different rates. With low ATP concentrations, we observed maximal phosphorylation of the 60,000-dalton protein at 50 to 60 pmole/mg protein after 90 sec and maximal phosphorylation of the 20,000-dalton proteins of about 30 pmole/mg only after about 5 min. Chiesi and Carafoli (7), using more nearly optimal conditions, reported much faster rates of phosphorylation but confirmed that the 60,000-dalton protein was more rapidly phosphorylated than the 20,000-dalton protein. We found that maximal phosphorylation occurred in the presence of NaF to inhibit phosphatase activity and in the presence of 0.6 $\mu$M calmodulin and 0.3 $\mu$M free $Ca^{2+}$. The pH optimum for phosphorylation was very low. The maximal phosphorylation fell off dramatically above pH 6.0 and by pH 8.0 was only 10 to 15% of that at pH 6.0.

Analysis of the proteins that were phosphorylated proved them to be previously unidentified sarcoplasmic reticulum proteins (3,7). The 60,000-dalton component was not calsequestrin ($M_r$ 63,000), the intrinsic glycoprotein ($M_r$ 53,000), or the regulatory subunit of cyclic-AMP-dependent protein kinase ($M_r$ 53,000), which adheres to sarcoplasmic reticulum preparations (2). It did not appear to be a glycoprotein, since its molecular weight was insensitive to digestion with endoglycosidase H or D. The residues phosphorylated were found to be an approximately equal mixture of phosphoserine and phosphothreonine (3), ruling out the possibility that the protein was pp60, the 60,000-dallon product of the *src* gene, which has been shown to be an autophosphorylated tyrosine kinase (8).

The question of whether the phosphorylated proteins of 60,000 and 20,000 daltons are kinases that are autophosphorylated or whether they are substrates

for an independent kinase can best be answered by purification and reconstitution. Two potential candidates as membrane-bound kinases are the high-mannose intrinsic glycoproteins of $M_r$ 53,000 and 160,000 found in the sarcoplasmic reticulum (2,22). The 53,000-dalton glycoprotein bears a resemblance to the 53,000-dalton subunit of the $Na^+$, $K^+$-ATPase (15). The glycoprotein cannot be removed from the $Na^+$, $K^+$-ATPase without loss of activity, but the 53,000-dalton glycoprotein is readily separated from the 105,000-dalton $Ca^{2+}$-ATPase without effect on either $Ca^{2+}$-ATPase or $Ca^{2+}$ uptake.

The function of the glycoproteins is unknown. Campbell and MacLennan (4) used 8-azido[$\alpha$-$^{32}$P]ATP (8-$N_3$-ATP) as a photoaffinity label for ATP binding sites in the sarcoplasmic reticulum membrane. At concentrations between 0.14 and 0.6 $\mu$M, 8-$N_3$-ATP bound specifically to proteins of 53,000, 105,000, and 160,000 daltons and nonspecifically to proteins of 35,000 and 95,000 daltons. The 105,000-dalton protein was the $Ca^{2+}$, $Mg^{2+}$-dependent ATPase; the 53,000- and 160,000-dalton proteins were shown to be the intrinsic glycoproteins since they bound to concanavalin A columns, their molecular weights were reduced on digestion with endoglycosaminidase H, and they were immunoprecipitated with an antibody against the 53,000-dalton glycoprotein that cross reacted with both glycoproteins. The binding of ATP with high affinity suggested that these proteins might be membrane-bound protein kinases and that 8-$N_3$-ATP might be bound to the catalytic portion of these enzymes. This possibility has made it important to attempt to purify the two glycoproteins and the two phosphorylated proteins under conditions in which they would retain function.

The 53,000-dalton protein has been purified using SDS as a solubilizing agent (2). This permitted chemistry to be carried out on the protein but did not permit functional assays. The two intrinsic glycoproteins were partially purified in undenatured form on concanavalin A columns, but complete purification was not achieved under nondenaturing conditions. Chiesi and Carafoli (6) were able to bind the 53,000-dalton glycoprotein to CAPP-sepharose 4B columns in the presence of $Ca^{2+}$ and elute it in nearly pure form with EGTA. Although they did not report any activity associated with the purified protein, they observed that a $Ca^{2+}$-dependent inhibition of the ATPase by trifluoperazine (TFP) was largely lost after removal of the glycoprotein. This suggested that the glycoprotein was the site of TFP binding and that it might regulate the ATPase through protein-protein interaction.

Only partial purification of the 60,000- and 20,000-dalton proteins has been achieved under nondenaturing conditions. A fraction containing a small amount of phosphorylatable 60,000-dalton protein is released by EGTA extraction of the membranes. The remainder of the protein can only be dissolved with detergent (*unpublished observations*). The use of calmodulin columns for affinity chromatography has not been successful. Indeed, Chiesi and Carafoli (7) have reported that none of the phosphorylated proteins interact with calmodulin. They have, however, purified the 20,000-dalton protein as a proteolipid

in acidified chloroform-methanol. Obviously, considerably more work is necessary to achieve purification of all of the proteins of interest so that the question of what is a substrate and what is a kinase can be resolved.

Another approach to the question of function of the phosphorylation system has been to probe the effect of inhibition of phosphorylation on the membrane system. Trifluoperazine, a potent inhibitor of calmodulin-dependent reactions, was shown to inhibit the calmodulin-dependent phosphorylation in sarcoplasmic reticulum (3). It was also an inhibitor of $Ca^{2+}$ uptake (3,6), but TFP inhibition of $Ca^{2+}$ uptake was overcome with excess $Ca^{2+}$ (6). Even at a concentration of 100 $\mu$M, TFP did not enhance $Ca^{2+}$ release from preloaded vesicles, suggesting that it did not, of itself, cause membrane damage leading to $Ca^{2+}$ release or directly stimulate $Ca^{2+}$ release. Trifluoperazine also inhibited $Ca^{2+}$-ATPase activity and so was far from an ideal reagent for analyzing specific effects of phosphorylation on the function of $Ca^{2+}$ uptake. However, when comparisons were made between the concentration of TFP required to inhibit either $Ca^{2+}$-ATPase or $Ca^{2+}$ uptake, $Ca^{2+}$ uptake was inhibited preferentially over $Ca^{2+}$-ATPase, suggesting that TFP might act to uncouple $Ca^{2+}$ uptake from $Ca^{2+}$-ATPase activity (3).

Recently, Gietzen et al. (12) reported that the compound 48/80 is a potent and specific inhibitor of calmodulin-dependent reactions. In contrast to TFP, it does not inhibit the $Ca^{2+}$-ATPase. We have examined the effect of this drug on $Ca^{2+}$ uptake and found that it does inhibit $Ca^{2+}$ uptake rather dramatically in the range of 10 to 50 $\mu$g/ml, where it is a powerful inhibitor of calmodulin-dependent reactions (Fig. 1). When we examined the effect of this compound on calmodulin-dependent phosphorylation of the 60,000- and 20,000-dalton proteins, we found that it also inhibited these reactions. Therefore, we have achieved an inhibition of calmodulin-dependent phosphorylation and of $Ca^{2+}$ uptake under conditions in which the $Ca^{2+}$-ATPase is fully functional. In other words, $Ca^{2+}$ uptake and $Ca^{2+}$-ATPase activity have been uncoupled.

We have observed this type of uncoupling previously. Extraction of the sarcoplasmic reticulum with EGTA led to loss of $Ca^{2+}$-dependent $Ca^{2+}$ accumulation while causing an enhancement of $Ca^{2+}$-ATPase activity (9,17). Diamond et al. (9) have shown that $Ca^{2+}$ permeability of the membranes is enhanced greatly after EGTA extraction. We have shown (3) that EGTA extraction leads to removal of calmodulin from the membranes and to a lower level of membrane phosphorylation. In a previous study (17), we showed that $Ca^{2+}$ uptake by EGTA-extracted membranes could be largely reconstituted by preincubation with $Mg^{2+}$, $Ca^{2+}$, ATP, and a supernatant factor that was probably calmodulin. These studies now suggest that an inhibition of the calmodulin-dependent phosphorylation system by calmodulin removal could have led to an uncoupling of $Ca^{2+}$ transport from $Ca^{2+}$-ATPase activity.

It has long been known that the pH optimum for $Ca^{2+}$ uptake is below 7.0 and that the efficiency of $Ca^{2+}$ uptake (the $Ca^{2+}$/ATP ratio) falls off dramatically

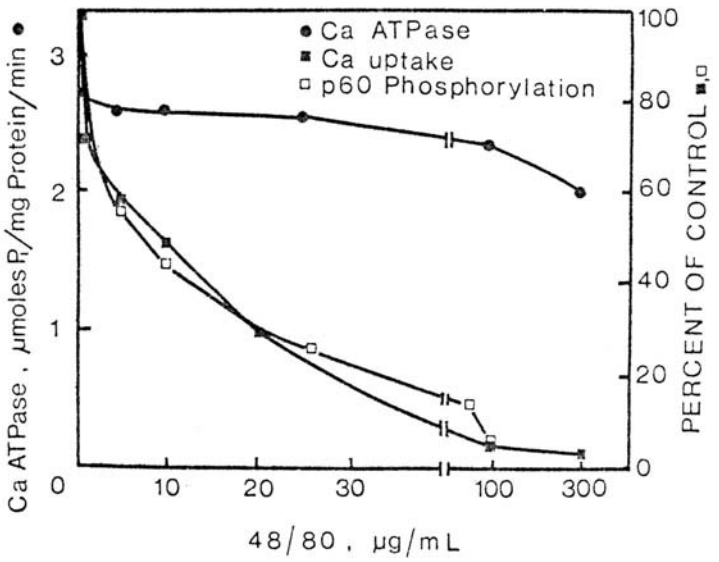


**FIG. 1.** Effect of 48/80 on $Ca^{2+}$ ATPase, $Ca^{2+}$ uptake, and calmodulin-dependent phosphorylation of the 60,000-dalton protein in sarcoplasmic reticulum.

above that pH. This is in spite of the fact that the optimal pH for ATPase activity is 7.5 (18). We have observed that the pH optimum for calmodulin-dependent phosphorylation is also below 7.0 and that the amount of phosphorylation falls off below pH 6.0 on a curve similar to that of the diminution of the $Ca^{2+}$/ATP ratio with increased pH (Fig. 2). Thus, elevated pH, like 48/80 and EGTA extraction, leads to uncoupling of $Ca^{2+}$ uptake from $Ca^{2+}$-ATPase activity.

What is the mechanism of uncoupling? In another publication (25), we showed that tetraphenylboron ($TPB^-$) is an uncoupler with similar apparent effects to 48/80. We showed that $TPB^-$ does not affect ATPase activity but has the ability to release $Ca^{2+}$ from loaded sarcoplasmic reticulum either in vesicular form or in skinned fibers. In this case, the $Ca^{2+}$ release was undoubtedly initiated by alterations in charge on one or the other surface of the membrane as $TPB^-$ oriented itself according to the membrane potential. This demonstration raises the probability that uncoupling is no more than an enhancement of $Ca^{2+}$ release over $Ca^{2+}$ uptake. These two processes have been demonstrated to occur concomitantly in functional sarcoplasmic reticulum vesicles (14).

An effect of phosphorylation on $Ca^{2+}$ release could he rationalized if we postulate that there is a $Ca^{2+}$ release channel that is phosphorylatable in a calmodulin-dependent reaction. When dephosphorylated, it would be open; when phosphorylated, it would be closed. The consequences would fit a very

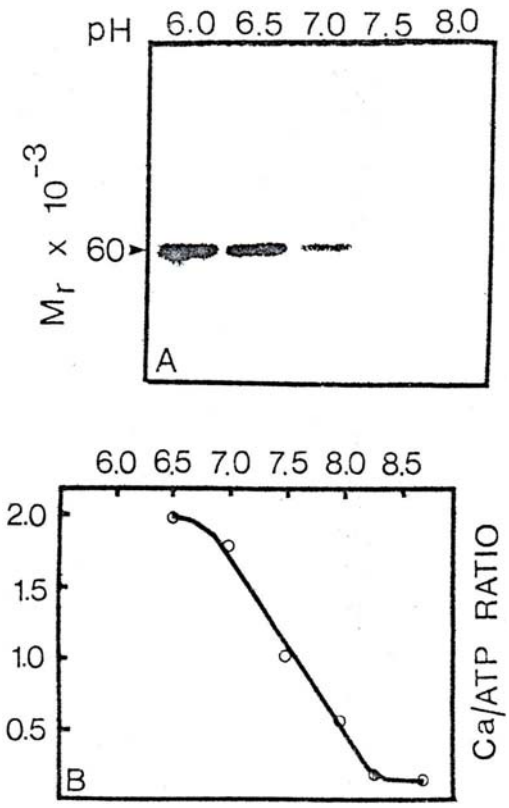


**FIG. 2.** Effect of pH on (**A**) calmodulin-dependent phosphorylation of the 60,000-dalton protein of sarcoplasmic reticulum and (**B**) the efficiency (Ca/ATP ratio) of $Ca^{2+}$ uptake by sarcoplasmic reticulum vesicles.

simple uptake and sequestration model. The addition of external (cytoplasmic) $Ca^{2+}$ and ATP to membranes would phosphorylate the channel and close it, allowing $Ca^{2+}$ to be pumped inside and retained. When cytoplasmic $Ca^{2+}$ was lowered below the point of activation of calmodulin, the pump would be dephosphorylated, and $Ca^{2+}$ would start to leak out. This would result in an oscillation of $Ca2^{2+}$ release and sequestration (cf. 14) but would maintain cytoplasmic $Ca^{2+}$ below concentrations that would initiate contraction. This simple model would explain why perturbation of the ability of the 60,000- and 20,000-dalton proteins to be phosphorylated would lead to open channels and to uncoupling.

Such a model would not explain how a burst of $Ca^{2+}$ release of sufficient magnitude to initiate muscle contraction would be achieved. The fact that a rapid transient $Ca^{2+}$ release docs occur under physiological conditions in response to electrical stimulation would suggest that there is a signal acting more directly

on the release process, perhaps in concert with the putative phosphorylation control. We could, therefore, assume that there are two control sites or gates (Fig. 3). One would respond to $Ca^{2+}$ by phosphorylation, the other to alternate signals. Cytoplasmic $Ca^{2+}$ would stimulate phosphorylation of one gate to close it, permitting coupled $Ca^{2+}$ uptake. The process of $Ca^{2+}$ uptake would create a signal leading to closing of the second gate by an unknown gate-closing mechanism. If $Ca^{2+}$ could not accumulate inside the vesicle, for example, when the first gate could not be phosphorylated to close it, then the second gate would also remain open. This would account for our observation that procedures that inhibit phosphorylation lead to uncoupling of $Ca^{2+}$ accumulation from $Ca^{2+}$-ATPase activity. When cytoplasmic $Ca^{2+}$ was lowered, permitting dephosphorylation, the dephosphorylated gate would open, but the second gate would remain closed until signaled to open by an unknown $Ca^{2+}$ release signal. Release of $Ca^{2+}$ would then close the phosphorylatable gate to start the cycle again.

We have previously developed this idea using a proton gradient as the second

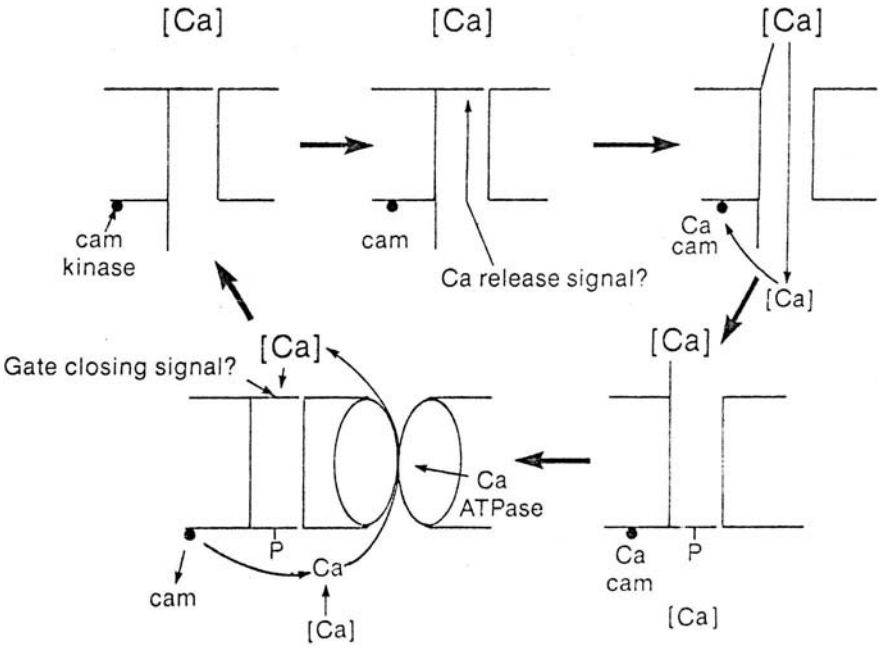


**FIG. 3.** A model for the control of $Ca^{2+}$ release through phosphorylation. In the resting state, the $Ca^{2+}$ release channel is closed at the upper gate. and cytoplasmic $Ca^{2+}$ is very low. In response to a $Ca^{2+}$ release signal, the upper gate opens, permitting $Ca^{2+}$ release. The increase in cytoplasmic $Ca^{2+}$ leads to $Ca^{2+}$- and calmodulin-dopenrient phosphorylation of the lower gate and to its closure; $Ca^{2+}$ can now be pumped inward and accumulate, loading to closure of the upper gate. When cytoplasmic $Ca^{2+}$ falls, the lower gale begins to dephosphorylate, but the upper gate remains closed.

signal (3). A proton gradient can control $Ca^{2+}$ release in skinned muscle fibers and, when disrupted by $H^+$ ionophores or elevated pH, causes $Ca^{2+}$ release (24) Although we originally thought that the regulatory pH gradient was in the sarcoplasmic reticulum (24), we later suggested that the gradient might lie elsewhere, perhaps in the transverse tubular system (19). This change in view was based on our realization that pH-induced $Ca^{2+}$ release from isolated vesicles probably occurs through sites in the ATPase molecule that are sensitive to quercetin (23) and on the finding by Donaldson (10) that the transverse tubular system might become polarized in skinned muscle fibers by the action of the $Na^+$,$K^+$-ATPase.

There are several major problems in studying $Ca^{2+}$ release from sarcopiasmic reticulum. We do not know whether $Ca^{2+}$ release occurs throughout the membrane system or only in junctional regions. We do not have any idea what the $Ca^{2+}$ release channels are composed of, and we do not know what the signals are that initiate $Ca^{2+}$ release. Our rationalization of the finding of a calmodulin. dependent kinase system in skeletal muscle sarcoplasmic reticulum suggests that there may be regulatory mechanisms for $Ca^{2+}$ release. Further investigation of these mechanisms might lead to an understanding of the entire $Ca^{2+}$ release system in the sarcoplasmic reticulum.

## ACKNOWLEDGMENTS

This research was supported by grant MT 3399 to D.H.M. and a postdoctoral fellowship to K.P.C. from the Medical Research Council of Canada and by a grant to D.H.M. and postdoctoral fellowships to H.T. and B.S.T. from the Muscular Dystrophy Association of Canada.

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