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Article:
Depletion of Ca\textsuperscript{2+} from the sarcoplasmic reticulum of cardiac muscle prompts phosphorylation of phospholamban to stimulate store refilling

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Edited by Clara Franzini-Armstrong, The University of Pennsylvania School of Medicine, Philadelphia, PA, and approved December 12, 1997

(received for review August 13, 1997)

**ABSTRACT** Nonmuscle cells have almost ubiquitously evolved a mechanism to detect and prevent Ca\textsuperscript{2+} store depletion—store operated calcium entry. No such mechanism has, as yet, been reported in cardiac myocytes. However, it is conceivable that such a mechanism may play an important role in cardiac Ca\textsuperscript{2+} homeostasis to ensure the availability of sufficient stored Ca\textsuperscript{2+} to maintain normal excitation contraction coupling. We present data that confirms the presence of a mechanism that is able to monitor the Ca\textsuperscript{2+} load of the SR and initiate a signaling process to accelerate Ca\textsuperscript{2+} uptake by the SR when store depletion is detected. Depletion of SR Ca\textsuperscript{2+} activates a protein kinase, the principal SR substrate of which is phospholamban. Phosphorylation of this SR protein promotes Ca\textsuperscript{2+} pump activity and therefore store refilling. Furthermore, a protein kinase activity associated with the SR that is inhibited by Ca\textsuperscript{2+} ions has been identified. We have measured luminal [Ca\textsuperscript{2+}] by using a fluorescent Ca\textsuperscript{2+} indicator and found that by initiating Ca\textsuperscript{2+} uptake and increasing Ca\textsuperscript{2+} load, we can inhibit the protein kinase activity associated with the SR. This confirms that a protein kinase, that is regulated by luminal [Ca\textsuperscript{2+}], has been identified and represents part of a previously unidentified signalling cascade. This local feedback mechanism would allow the myocyte to detect and prevent SR Ca\textsuperscript{2+} load depletion.

The calcium required for cardiac muscle contraction is derived from two sources, the intracellular store [the sarcoplasmic reticulum (SR)] that represents a finite reserve, and the extracellular milieu, which is effectively infinite. The quantitative importance of each of these sources in excitation contraction coupling is the inverse of their size. The SR contributes \(\approx 75\%\) of the Ca\textsuperscript{2+} for each contraction (variable with animal species) with the remainder entering from the extracellular environment (1). The maintenance of the intracellular Ca\textsuperscript{2+} store, suitably filled with Ca\textsuperscript{2+}, is a prerequisite for excitation contraction coupling, and is achieved by the appropriate competition between Ca\textsuperscript{2+} transport systems of the SR and plasma membrane (1). In many other cell types the intracellular Ca\textsuperscript{2+} store also performs an important function. Ca\textsuperscript{2+} release enables the cell to effectively translate extracellular signals into functional responses. To guard against Ca\textsuperscript{2+} store depletion and loss of this capability, cells have evolved a mechanism that allows constant replenishment of the Ca\textsuperscript{2+} store. Store operated Ca\textsuperscript{2+} entry initiated by retrograde signaling pathways have been described in a wide variety of cell types, including smooth muscle cells and nonmuscle cells (2–4). The exact nature of the retrograde signal remains a matter of some debate. One example, however, is a protein phosphatase in human platelets that is responsive to the Ca\textsuperscript{2+} content of platelet stores and modifies the phosphorylation status of targets in response to store depletion (5). A second example may involve the mammalian homologue of the serine/threonine kinase IRE1 (6). Inhibition of Ca\textsuperscript{2+} uptake by intracellular Ca\textsuperscript{2+} stores of Chinese hamster ovary cells results in the increased expression of the molecular chaperone grp78 (6), an effect that is exaggerated by expression of the yeast kinase IRE1. By analogy, the Ca\textsuperscript{2+} concentration within the lumen of the SR of cardiac muscle may control kinase/phosphatase enzymes and thereby influence the rate of Ca\textsuperscript{2+} accumulation by the store. This could serve to safeguard the SR from progressive Ca\textsuperscript{2+}-loss if the Ca\textsuperscript{2+}-transport systems of the plasma membrane and SR were not balanced appropriately. Phospholamban represents a plausible target in this hypothesis; normally an inhibitor of SR Ca\textsuperscript{2+} pump activity, phosphorylation of phospholamban in response to Ca\textsuperscript{2+} store depletion would be expected to accelerate Ca\textsuperscript{2+}-uptake by the SR (7) and facilitate store refilling. In the present study, experimental evidence is provided in support of this suggestion. First, freshly isolated cardiac myocytes (from the rat) responded to SR Ca\textsuperscript{2+} depletion by phosphorylating phospholamban on Ser-16. Second, a protein kinase that copurifies with the SR is active at low [Ca\textsuperscript{2+}] (\(\approx 3\ \mu M\)), but inhibited by high [Ca\textsuperscript{2+}] (\(\approx 30\ \mu M\)). This activity represents a potential candidate mediating the myocyte response to Ca\textsuperscript{2+} store depletion. Finally, we have demonstrated that the site of this regulation by Ca\textsuperscript{2+} is from the luminal aspect of the SR membrane ([Ca\textsuperscript{2+}]\textsubscript{l}). Manipulating luminal Ca\textsuperscript{2+} alters phospholamban phosphorylation in a manner consistent with our hypothesis, i.e., at steady-state loading, which represents [Ca\textsuperscript{2+}]\textsubscript{l}, of 35 \(\mu M\) phospholamban phosphorylation is inhibited. Collapse of the Ca\textsuperscript{2+} gradient and a return to low [Ca\textsuperscript{2+}]\textsubscript{l}, (3 \(\mu M\)) results in a concomitant increase in phospholamban phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials.** Cardiac SR vesicles were prepared as described in Li et al. (8), phospholamban antibodies as described in Drago and Colyer (9). The catalytic subunit of protein kinase A was purified from bovine heart according to Peters et al. (10). The synthetic peptide PLI919Y (RSAIIRASTIEY) was purchased from Neosystem (Strasbourg, France) and used as received (\(\approx 80\%\) pure).

**Ca\textsuperscript{2+} Store Depletion in Cardiac Myocytes.** Ventricular myocytes were isolated from adult Wistar rats (\(\approx 250\) g as described in ref. 11), loaded with Fura-2AM (Molecular...
Phosphorylation of Phospholamban.

In Vitro Phosphorylation of Phospholamban. Phosphorylation was performed at 30°C in the absence and presence of 200 nM c-protein kinase A in 50 mM histidine (pH 7.0), 5 mM MgSO_4, 0.25 mM NaF, 1 mM vanadate, 1 mM EGTA, 2 μM A23187, 0.5 mg/ml cardiac SR, 0.1 mM [γ-32P]-ATP (100 cpm/pmol), and CaCl_2 to achieve free Ca^{2+} concentrations of 3, 30, 300, and 3,000 μM. The phosphorylation reaction was terminated by the addition of Laemmli sample buffer (13) and the sample proteins in whole-cell extracts. Rat cardiac myocytes were isolated as described in ref. 11 and resuspended at a density of ~10^6 cells/ml in 5 mM Hepes (pH 7.3), 113 mM NaCl, 1 mM Na_2HPO_4, 1 mM MgSO_4, 5 mM KCl, 10 mM glucose, 20 mM Na acetate, 5 units/ml insulin, 0.75 mM CaCl_2. A single sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitor [thapsigargin (Tg, 2.5 μM), 2,5-di-(t-butyl)-1,4-hydroquinone (t-BHQ, 30 μM), and cyclopiazonic acid (CPA, 50 μM)] for 15 min. The SR Ca^{2+} content was assessed by challenge with 10 mM caffeine after 15 min exposure to drug.

Peptide Phosphorylation and Dephosphorylation. Phosphorylation of the synthetic peptide RSA IRR ASTIEY amide was performed by centrifugation (as above) and washed twice in buffer for 120 min at 4°C. Loaded vesicles were exposed to sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase inhibitors [thapsigargin (Tg, 2.5 μM), 2,5-di-(t-butyl)-1,4-hydroquinone (t-BHQ, 30 μM), and cyclopiazonic acid (CPA, 50 μM)] for 15 min. The SR Ca^{2+} content was assessed by challenge with 10 mM caffeine after 15 min exposure to drug.

RESULTS AND DISCUSSION

Interested in the signaling events triggered by Ca^{2+} store depletion in many cells, we sought to investigate the possibility of an analogous process occurring in cardiac muscle. Our proposal was that manipulation of the phosphorylation status of phospholamban would be an appropriate response to the depletion of Ca^{2+} from the SR. We would anticipate an increase in the phosphorylation of phospholamban, because this would promote store refilling through increased Ca^{2+}-ATPase activity. To evaluate this hypothesis we first investigated the response of a population of freshly isolated rat cardiac myocytes to Ca^{2+} store depletion. The SR was depleted of Ca^{2+} by treating cells with three structurally dissimilar Ca^{2+}-ATPase inhibitors, Tg, t-BHQ, and CPA. Fig. 1 illustrates the effectiveness of this strategy: Ca^{2+} transients from individual rat ventricular myocytes (evoked by electrical stimulation) were of uniform amplitude and short duration (Fig. 1a, c, and e). The Ca^{2+} content of the SR was determined by exposure to 10 mM caffeine (12, 16). This maneuver opens the Ca^{2+} release channels in the SR (17) and mobilizes stored Ca^{2+}. The Ca^{2+} transient evoked by caffeine was larger than that arising from electrical stimulation, indicative of a large amount of Ca^{2+} mobilized from the SR. The effects of caffeine were fully reversible. Removal of caffeine solutions and restoration of electrical stimulation was seen to refill the SR back to steady state Ca^{2+} transient levels. Exposure of the myocytes to each of the three SR Ca^{2+} pump inhibitors Tg (18) (2.5 μM, Fig. 1b), t-BHQ (19) (30 μM, Fig. 1d), or CPA (20) (50 μM, Fig. 1f) decreased the amplitude and increased the duration of the Ca^{2+} transient, consistent with inhibition of uptake by the SR.

Under these conditions caffeine mobilizes little or no calcium after 15-min exposure to these drugs (Fig. 1, b, d, and f), confirming depletion of SR Ca^{2+} stores. According to our hypothesis a coincident increase in phospholamban phosphorylation would be anticipated.

The effect of store depletion on the phosphorylation of Ser-16 of phospholamban was measured by using polyclonal antibodies specific for this phosphorylation site (9). These antibodies are wholly specific for phospholamban phosphorylated at this site and facilitate immunodetection of the phosphoprotein in whole-cell extracts. Rat cardiac myocytes exhibit low basal phosphorylation of phospholamban on Ser-16; this was increased dramatically on exposure of the cells to the β-adrenergic agonist, isoprorenaline (Fig. 2, C and I). A similar dramatic increase in the phosphorylation of phospholamban was provoked by depletion of the SR Ca^{2+} stores by 5 min exposure to each of the three Ca^{2+} pump inhibitors [Fig. 2, Tg (2.5 μM), BHQ (30 μM), CPA (50 μM)]. The identical effect of the three Ca^{2+} pump inhibitors suggest that the phosphorylation of phospholamban was provoked by their common action, i.e., Ca^{2+} pump inhibition and the subsequent depletion of Ca^{2+} stores. These data were reproduced in five other experiments with rat ventricular myocytes and a sixth with ferret ventricular myocytes (although not all drugs were
facilitated refilling of the SR and recovery of the peak amplitude of the Ca^2+ pump (Fig. 1) was not responsible for the increasing Ser^16 phosphorylation (in experiments of Fig. 2), because a similar elevation of cytoplasmic Ca^2+, achieved by raising extracellular [Ca^2+] (21), was without effect on phospholamban phosphorylation (data not shown). Thus, depletion of the Ca^2+ store provides the signal to increase the phosphorylation of phospholamban. This in turn would promote refilling of the Ca^2+ store through stimulation of Ca^2+ pump activity (7).

The time course of Ca^2+ loss from the SR (illustrated by a progressive reduction in the amplitude of the Ca^2+ transient) upon exposure to sarcoplasmic/endoplasmic reticulum Ca^2+-ATPase 2 inhibitors was very similar to that of the increase in Ser^16 phosphorylation. Negretti et al. (12) demonstrated that Tg (2.5 μM) reduced the caffeine-induced Ca^2+ mobilization of Ca^2+ from the SR of rat ventricular myocytes (which is believed to equate to SR load) by =50% after 2 min. In our experiments Tg (2.5 μM) induced phosphorylation of phospholamban was first evident at 3 min (data not shown). Thus the signaling process that conveys the luminal Ca^2+ status to phospholamban is activated upon partial loss of Ca^2+ from the SR, and may be an important control point to balance the Ca^2+-sequestration activity of the SR with those of the sarclemma.

**Identification of a State of Filling Kinase (SOF Kinase).**

The next step in this investigation was to identify enzymes involved in mediating the phosphorylation of phospholamban in response to Ca^2+ store depletion. In the first series of experiments we sought to determine whether the candidate enzymes were associated with cardiac SR vesicles. Phosphorylation of phospholamban, catalyzed by enzymes associated with SR vesicles was investigated as a function of [Ca^2+]. SR vesicles are generally of right side out orientation (22), therefore a Ca^2+ ionophore (A23187) was included in these experiments to facilitate manipulation of luminal as well as cytosolic [Ca^2+]. The phosphorylation of phospholamban was observed at low [Ca^2+] (3 μM) upon addition of ATP, but was inhibited progressively as the [Ca^2+] rose (30–3000 μM) (Fig. 3D). Under these conditions phosphorylation of phospholamban was on Ser-16 exclusively (data not shown) as had been the case in vivo (Fig. 2). Other SR proteins phosphorylated in these experiments were unaffected by titration of Ca^2+ (data not shown). Thus the phosphorylation of phospholamban by enzyme(s) associated with the SR was inhibited by high [Ca^2+].

The concentration dependence of this phenomenon was appropriate for cytoplasmic Ca^2+, but within the range of measured [Ca^2+], it found in the literature, which vary from 12 μM to 2 mM (25–26).

Two separate mechanisms could account for the reduced phosphorylation of phospholamban at high [Ca^2+]. Either the inhibition of a protein kinase or the activation of a phosphatase could underlie these observations. The phosphorylation of a synthetic peptide derived from the primary sequence of phospholamban (residues 9–19 plus a carboxy terminal tyrosine amide, refs. 9 and 27) was used to identify which of these enzymes in cardiac SR was Ca^2+ sensitive. A Ca^2+ ionophore (A23187) was included in the assay to ensure manipulation of both luminal and extravesicular [Ca^2+]. The concentration dependence of this phenomenon was appropriate for cytoplasmic Ca^2+, but within the range of measured [Ca^2+], it found in the literature, which vary from 12 μM to 2 mM (25–26).

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response to Ca-dependent cardiac SR and represents a potential candidate kinase that is distinct from PKA and PKC is associated with I and microsystin, both of which inhibit SOF kinase. Thus a Furthermore, protein kinase C is unaffected by protein kinase this enzyme’s preferred site of phosphor ylation is Ser-10. Ser-16 phosphor ylation of phospholamban by protein kinase C, because this enzyme phosphor ylates phospho-

however, Ser-16 phosphorylation of phospholamban by protein kinase C was Ca-sensitive insensitively (Fig. 3B). The kinase cannot be Cam kinase II, because this enzyme phosphorylates phospholamban on Thr-17 exclusively. Neither is it protein kinase C; this enzyme’s preferred site of phosphorylation is Ser-10. Furthermore, protein kinase C is unaffected by protein kinase I and microsystin, both of which inhibit SOF kinase. Thus a kinase that is distinct from PKA and PKC is associated with cardiac SR and represents a potential candidate mediating the response to Ca store depletion in cardiac myocytes. The Ca profile of the kinase would tend to preclude regulation by cytosolic Ca, because even during a Ca transient, the [Ca] would barely reach concentrations required to cause its inhibition. However, because both lumenal and cytosolic Ca were manipulated in the experiment of Fig. 3, it is possible that the inhibitory effects of high Ca were achieved from the lumenal aspect of the SR. To investigate the site of regulation further, we investigated the correlation between luminal [Ca] and phospholamban phosphorylation. Intravesicular Ca was measured by using the Ca indicator Magfura-5. Incubation of the membrane permeable form (acetoxymethyl) with cardiac SR vesicles resulted in significant deesterification of the dye. Thus loading of vesicles with the Ca responsive form was clearly shown by a strong fluorescence signal in the excitation spectrum 250–450 nm (Fig. 4A). Addition of Mn (20 μM) caused quenching of the extracellular signal (Fig. 4B). This was repeated in all experiments. Permeabilizing the vesicular membrane by introducing ionomycin in the presence of Mn resulted in almost complete quench (Fig. 4C), indicating that the majority (~80%) of the fluorescent signal originated from within the membrane vesicles, i.e., from dye trapped in the lumen.

Calibration of Magfura-5. The response of Magfura-5 to [Ca] in our system was markedly nonlinear. The method of Loomis-Husselbee and Dawson (29) was used to calibrate the dye. A line of best fit to the response of the dye to a stepwise increase in free [Ca] was used to derive an empirical relationship between fluorescence and [Ca] (Fig. 5). This equation was then applied to raw fluorescence data to convert to [Ca]. Addition of Ca (to a final concentration of 1 mM) to a standard uptake assay, indicated a lumenal [Ca] of 100 μM. This represents the extent of passive loading. However, introduction of ionomycin results in a measured [Ca] of 1 mM, correctly reflecting the free [Ca] of the assay. Therefore, application of the experimentally derived equation is an accurate method of determining [Ca].

[Ca]L Following Active Ca Transport. Calcium uptake into Magfura-5 loaded vesicles was initiated by the addition of

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**Fig. 3.** Inhibition of phospholamban phosphorylation at high Ca. The phosphorylation of phospholamban in SR vesicles prepared from canine heart (8) was catalyzed by an endogenous kinase (A) or 200-nM Ca-dependent protein kinase (B) at various free Ca concentrations in the presence of A23187 (2 μM) for the times indicated. Phosphoproteins (10 μg) were separated by SDS/15% PAGE (13) and an autoradiograph of the region of the gel containing phospholamban displayed. These data were reproduced in six separate experiments by using different SR preparations. The phosphorylation (C) and dephosphorylation (D) of an exogenous synthetic peptide substrate derived from the sequence of phospholamban (RSAIRRSTIE, single letter codes) was performed at either 3 μM (●) or 3 mM (●) CaCl_2 in the presence of A23187 (2 μM) by enzymes associated with the SR vesicles (see Materials and Methods). Data shown are typical of four independent experiments.

**Fig. 4.** Excitation wavelength scans indicate substantial luminal de-esterification of Magfura-5 AM in canine cardiac SR vesicles. Vesicles were incubated with Magfura-5 AM (20 μM) for 120 min at 4°C. Each assay consists of 100 μg of protein in 1.5 ml of assay buffer (150 mM sucrose/50 mM KCl/5% PEG/10 mM Hepes, pH 7.0). Assays were performed at 30°C in a stirred cuvette. Traces describe excitation scans from 260–450 nm measuring emission at 510 nm; (a) Control; (b) addition of 20 μM Mn (to quench external Magfura-5); (c) Addition of ionomycin (5 μM) plus Mn (50 μM). The ion selectivity of ionomycin necessitates an increase in [Mn] to observe rapid quenching of luminal Magfura-5.
Mg-ATP (1.5 mM). After a rapid increase in lumenal \([Ca^{2+}]_l\), a steady-state loading of \(\approx 35 \mu M\) was achieved and maintained after 10 min (Fig. 6B). Tg released \(\approx 70\%\) of sequestered \(Ca^{2+}\) (Fig. 6B, trace i) and addition of Tg before ATP resulted in no significant uptake (Fig. 6B, trace ii), confirming the majority of \(Ca^{2+}\) uptake was into SR vesicles. Extravesicular \([Ca^{2+}]_e\) in this experiment would be \(\approx 2.5 \mu M\), a condition that supports full SR kinase activity in vitro. However, if the effect of \(Ca^{2+}\) is manifest from the SR lumen, then we would expect some inhibition of SOF kinase activity at this \([Ca^{2+}]_l\), \(\approx 35 \mu M\) (Fig. 3A). Cardiac SR vesicles not loaded with Magfura-5 were treated in the same way and used in the standard uptake assay. Calcium uptake was again initiated by the addition of Mg-ATP. Aliquots of assay medium (7.5 \(\mu g\) protein) were removed at several points before and after the addition of Mg-ATP and quenched by using Laemmli sample buffer (13). Fig. 6A describes the phosphorylation of phospholamban during the course of uptake. The level of phospholamban phosphorylation is low before the addition of Mg-ATP. On addition of Mg-ATP an increase in phosphorylation is observed, which is consistent with our hypothesis, because the vesicles at this point would contain a low \([Ca^{2+}]_l\). As steady state was achieved \((\approx 10\ min\ of\ uptake,\ Fig.\ 6B,\ trace\ i)\) and maintained, a gradual decrease in phospholamban phosphorylation is observed (Fig. 6A, 20', 25'). When \([Ca^{2+}]_l\) is reduced to 2.5 \(\mu M\) following introduction of ionomycin an increase in phospholamban phosphorylation was observed (Fig. 6A, 30', 35') consistent with activation of the SR kinase (SOF kinase) in response to \(Ca^{2+}\) store depletion. Keeping \([Ca^{2+}]_l\) low by adding ionomycin and Mg-ATP simultaneously results in SOF kinase activity and a high level of phospholamban phosphorylation throughout the time course of \(Ca^{2+}\) uptake (not shown). Thus, \([Ca^{2+}]_l\) has been directly measured in intact vesicles and manipulations of [\(Ca^{2+}]_l\) have resulted in the predicted changes in the phosphorylation of phospholamban. Therefore, a direct causal link between \([Ca^{2+}]_l\) and phospholamban phosphorylation has been established, indicating that SOF kinase is indeed regulated at the lumenal face of the SR membrane.

In conclusion, cardiac myocytes increase phospholamban phosphorylation in response to store depletion. A phospholamban kinase associated with cardiac SR vesicles and sensitive to high \([Ca^{2+}]_l\) has been identified. Furthermore, loading Magfura-5 into the lumen of these SR vesicles has enabled measurement of active \(Ca^{2+}\) transport, a \([Ca^{2+}]_l\) of \(\approx 35 \mu M\) has been observed. This figure is unlikely to reflect SR \([Ca^{2+}]_l\) in vivo (see ref. 30), however, has allowed us to determine the site of SOF kinase regulation. Measurement of the time course of phospholamban phosphorylation during active \(Ca^{2+}\) transport indicated inhibition of SOF kinase activity when \([Ca^{2+}]_l\) rose to 35 \(\mu M\). This inhibition is consistent with both the \(Ca^{2+}\) profile of SOF kinase activity (Fig. 3) and the time taken to reach steady state \(Ca^{2+}\) uptake (\(\approx 10\ min\); Fig. 6B, trace i). This evidence strongly supports the presence of a local feedback mechanism in cardiac SR. Depletion of \(Ca^{2+}\) load activates SOF kinase, which in turn phosphorylates phospholamban. In its phosphorylated state phospholamban can no longer exert its inhibitory influence on sarcoplasmic/endoplasmic reticulum \(Ca^{2+}\)-ATPase 2, therefore an increase in \(Ca^{2+}\) pump activity promotes the refilling of the \(Ca^{2+}\) store.
We are grateful for the help and advice of Professor Clive H. Orchard throughout these studies and for the provision of myocytes. The financial support of the British Heart Foundation (PG/96125) and the Medical Research Council (G9428756MA) is gratefully acknowledged. J.C. is a British Heart Foundation lecturer (BS/6).