Glucocorticoid modulation of protein phosphorylation and sarcoplasmic reticulum function in rat myocardium

M. K. RAO, A. XU, AND N. NARAYANAN
Department of Physiology, The University of Western Ontario, London, Ontario, Canada N6A 5C1

Received 10 August 2000; accepted in final form 5 March 2001

Rao, M. K., A. Xu, and N. Narayanan. Glucocorticoid modulation of protein phosphorylation and sarcoplasmic reticulum function in rat myocardium. Am J Physiol Heart Circ Physiol 281: H325–H333, 2001.—To decipher the mechanisms underlying glucocorticoid action on cardiac contractile function, this study investigated the effects of adrenalectomy and dexamethasone treatment on the contents of sarcoplasmic reticulum (SR) Ca$^{2+}$-cycling proteins, their phosphorylation by endogenous Ca$^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II), and SR Ca$^{2+}$-sequestration in the rat myocardium. Cardiac SR vesicles from adrenalectomized rats displayed significantly diminished rates of ATP-energized Ca$^{2+}$ uptake in vitro compared with cardiac SR vesicles from control rats; in vivo administration of dexamethasone to adrenalectomized rats prevented the decline in SR function. Western immunoblotting analysis showed that the relative protein amounts of ryanodine receptor, Ca$^{2+}$-release channel, Ca$^{2+}$-ATPase, and calsequestrin, and phospholamban were neither diminished significantly by adrenalectomy nor elevated by dexamethasone treatment. However, the relative amount of SR-associated CaM kinase II protein was increased 2.5- to 4-fold in dexamethasone-treated rats compared with control and adrenalectomized rats. Endogenous CaM kinase II activity, as judged from phosphorylation of ryanodine receptor, Ca$^{2+}$-ATPase, and phospholamban protein, was also significantly higher (50–80% increase) in the dexamethasone-treated rats. The stimulatory effect of CaM kinase II activation on Ca$^{2+}$-uptake activity of SR was significantly depressed after adrenalectomy and greatly enhanced after dexamethasone treatment. These findings identify the SR as a major target for glucocorticoid actions in the heart and implicate modification of the SR CaM kinase II system as a component of the mechanisms by which dexamethasone influences SR Ca$^{2+}$-cycling and myocardial contractile function. 

A number of studies have suggested an apparent involvement of corticosteroids in the maintenance of myocardial function. Thus it is well known that adrenalectomized animals, unless supported by maintenance doses of corticosteroids, gradually develop a form of circulatory decompensation (12). Lefer (19) observed a marked time-dependent decrease in contractile force development by papillary muscles isolated from adrenalectomized rats. Treatment of adrenalectomized rats in vivo or cardiac muscle in vitro with the synthetic glucocorticoid dexamethasone prevented the deterioration in contractile performance of cardiac muscle, and it was suggested that dexamethasone exerted a direct effect on the myocardium, possibly via effects on glycogen metabolism and on electrolyte balance (19). It has also been reported that dexamethasone treatment significantly enhanced the development of contractile tension and increased the velocity of contraction and relaxation in cardiac muscle from dogs, cats, and rabbits (31). While these observations suggest a likely role for glucocorticoids in the maintenance of normal contractile function of the heart, the cellular processes affected by glucocorticoids and the biochemical mechanisms underlying their action(s) have not been clarified.

By virtue of its ability to control cytosolic Ca$^{2+}$ concentration, the sarcoplasmic reticulum (SR) plays a central role in contractile force development and the speed of contraction and relaxation in heart muscle (3). Conceivably, the ability of glucocorticoids to augment cardiac contractile function may arise from their ability to influence the Ca$^{2+}$-sequestration and Ca$^{2+}$-release functions of the SR. Consistent with this possibility, we observed previously (23) that cardiac SR vesicles isolated from adrenalectomized rats exhibit diminished rates of ATP-energized Ca$^{2+}$ uptake compared with SR vesicles from control rats and that dexamethasone treatment of adrenalectomized rats results in improved Ca$^{2+}$ uptake activity of SR. The major Ca$^{2+}$-cycling proteins in the SR include the Ca$^{2+}$-sequestering ATPase (Ca$^{2+}$-ATPase), the Ca$^{2+}$-storage protein calsequestrin (22), the ryanodine receptor/Ca$^{2+}$-release channel (RyR-CRC) (4), and the Ca$^{2+}$-ATPase-regulatory protein phospholamban (17, 34, 38). In its unphosphorylated state, phospholamban is thought to diminish the Ca$^{2+}$ sensitivity of Ca$^{2+}$-ATPase; phosphorylation of phospholamban by cAMP-dependent protein kinase (PKA) or Ca$^{2+}$/calmodulin-dependent protein kinase II (CaM kinase II) restores the Ca$^{2+}$ sensitivity (17, 34, 38). Besides phospholamban, calmodulin and CaM kinase are tightly associated with cardiac SR and have been implicated in the mod-

Address for reprint requests and other correspondence: N. Narayanan, Dept. of Physiology, Medical Sciences Bldg., Univ. of Western Ontario, London, Ontario, Canada N6A 5C1 (E-mail: njanoor.narayanan@med.uwo.ca).

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ulation of the Ca\(^{2+}\) uptake and release functions of the SR through direct phosphorylation of Ca\(^{2+}\)-ATPase (11, 26–28, 30, 40, 44–47) and RyR-CRC (10, 39, 43). As part of an attempt to decipher the mechanisms underlying glucocorticoid modulation of cardiac contractile function, the present study investigated the effects of adrenalectomy and dexamethasone treatment on the contents of major SR Ca\(^{2+}\)-cycling proteins, their phosphorylation by SR-associated CaM kinase II, and SR Ca\(^{2+}\) sequestration function in the rat myocardium.

**METHODS**

**Chemicals.** Reagents for electrophoresis were obtained from Bio-Rad laboratories (Mississauga, ON, Canada), \([\gamma^{32}P]ATP\) was purchased from Amersham (Oakville, ON, Canada), and \(45\text{CaCl}_2\) was obtained from NEN (Mississauga, ON, Canada). Dexamethasone was obtained from Organon Teknika (Toronto, ON, Canada). Monoclonal antibodies against the protein(s) constituting the RyR, SR Ca\(^{2+}\)-ATPase, and calsequestrin were purchased from Affinity BioReagents (Golden, CO). Antiphospholamban monoclonal antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-5-CaM kinase II polyclonal antibody was a generous gift from H. A. Singer (Albany Medical College, Albany, NY). All other chemicals were obtained from Sigma (St. Louis, MO).

**Animals.** Male Wistar rats weighing 250–300 g were purchased from Charles River (St. Constant, PQ, Canada). On arrival, the rats were housed individually in plastic cages in the Health Sciences Center animal care facility of this institution at 23°C on a 12:12-h light-dark cycle. The investigations were conducted under guidelines approved by the local Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care. The rats were anesthetized with metofane, and bilateral adrenalectomy was performed as described previously (23). Control animals were sham operated. The adrenalectomized animals were divided into two groups: one group received dexamethasone via a subcutaneously implanted ALZET osmotic mini pump (model 2001, flow rate 1 \(\mu\)l/h) that delivered dexamethasone at a rate of 4 \(\mu\)g/h for 7 days, and the second group received no dexamethasone. The adrenalectomized animals were maintained on normal saline to prevent volume depletion; the control animals were given tap water. All animals had free access to food (Purina Chow containing 20% protein). The animals were killed 7 days after surgery, and the ventricular myocardium was used for experiments.

**Isolation of SR vesicles.** SR membrane vesicles were isolated from the ventricular myocardium of control, adrenalectomized, and dexamethasone-treated rats according to the procedure described previously (15). After isolation, the SR vesicles were suspended in 10 mM Tris-maleate (pH 6.8) containing 100 mM KCl, quick-frozen in liquid N\(_2\), and then stored at −80°C. Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as standard. The yield of SR membranes from the hearts of the three groups of rats was similar (−1.5 mg protein/g wet tissue). The relative purity of the cardiac SR vesicles from the three groups of rats did not differ as judged from essentially similar protein profiles revealed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, see RESULTS).

**Preparation of muscle homogenates.** In addition to the SR, homogenates from control, adrenalectomized, and dexamethasone-treated rat hearts were also used in some experiments. The homogenates were prepared by homogenizing the ventricular tissue in 10 volumes (based on tissue weight) of 10 mM Tris-maleate-100 mM KCl buffer (pH 6.8) using a polytron PT-10 homogenizer (three 15-s bursts with 30-s intervals between bursts, setting 6, Brinkman; Westbury, NY). The homogenates were filtered through four layers of cheese cloth and used for experiments.

**Ca\(^{2+}\) transport and Ca\(^{2+}\)-ATPase assays.** ATP-dependent, oxalate-facilitated Ca\(^{2+}\) uptake by cardiac SR vesicles was determined using the Millipore filtration technique as described previously (25). The standard incubation medium for Ca\(^{2+}\) uptake (total volume 250 \(\mu\)l) contained (in mM) 50 Tris-maleate (pH 6.8), 5 MgCl\(_2\), 5 Na\(_2\)ATP, 120 KCl, 0.1 EGTA, 5 potassium oxalate, 5 ATP and 0.1 \(45\text{CaCl}_2\) (−8,000 cpm/nmol, 5.2 \(\mu\)M free Ca\(^{2+}\)) and cardiac SR vesicles (7.5 \(\mu\)g of protein). In experiments where Ca\(^{2+}\) concentration dependence was studied, the EGTA concentration in the assay medium was held constant at 0.1 mM and the amount of total \(45\text{CaCl}_2\) added was varied to yield the desired free Ca\(^{2+}\). The initial free Ca\(^{2+}\) concentration was determined using the computer program of Fabiato (9). To evaluate the effects of endogenous CaM kinase II-mediated phosphorylation on Ca\(^{2+}\) uptake, the assays were performed in the absence of calmodulin and in the presence of 1 \(\mu\)M calmodulin in the incubation medium. Other modifications to the standard assay medium are specified in the figure legends. The Ca\(^{2+}\) uptake reaction was initiated by the addition of SR to the rest of the assay components, preincubated for 3 min at 37°C, and allowed to proceed for 2 min, during which the Ca\(^{2+}\) uptake rates were found to be linear. The data on Ca\(^{2+}\) concentration dependance on Ca\(^{2+}\) uptake were analyzed by nonlinear regression curve fitting using the SigmaPlot scientific graph program (Jandel Scientific) run on an IBM personal computer. The data were fit to the equation

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v = V_{\text{max}}[\text{Ca}^{2+}]^n/(K_{\text{m}}[\text{Ca}^{2+}]+[\text{Ca}^{2+}]^n)
\]

where \(v\) is the measured Ca\(^{2+}\) uptake rate at a given Ca\(^{2+}\) concentration, \(V_{\text{max}}\) is the maximum rate reached, \(K_{\text{m}}\) is the Ca\(^{2+}\) concentration giving half of \(V_{\text{max}}\), and \(n\) is the equivalent to the Hill coefficient.

**Ca\(^{2+}\)-ATPase activity of the SR membrane vesicles was determined as described previously (25) using the assay conditions specified below. The incubation medium used for the Ca\(^{2+}\)-ATPase assay was identical to that described for Ca\(^{2+}\) uptake except that \([\gamma^{32}P]ATP\) was used instead of nonradioactive ATP and nonradioactive \(\text{CaCl}_2\) was used instead of \(45\text{CaCl}_2\). The assays were performed in the absence and presence of thapsigargin (TG). When present, the concentration of TG in the assay medium was 0.1 \(\mu\)M, the concentration found to produce complete inhibition of Ca\(^{2+}\) sequestration by the SR (35). In these experiments, the TG-inhibitable ATP hydrolysis was defined as the Ca\(^{2+}\)-ATPase activity (designated “TG-sensitive Ca\(^{2+}\)-ATPase activity” in RESULTS). The ATPase reaction was initiated by the addition of SR after preincubation of the rest of the assay components for 3 min at 37°C and was allowed to proceed for 3 min. The longer reaction time used for the measurement of Ca\(^{2+}\)-ATPase activity (i.e., 3 min as opposed to 2 min used for the measurement of Ca\(^{2+}\) uptake) permitted better quantitative resolution of Ca\(^{2+}\)-ATPase activity from the high level of basal Mg\(^{2+}\)-ATPase activity associated with rat cardiac SR vesicles (23).

**Immunoblotting of SR Ca\(^{2+}\)-cycling proteins.** Western immunoblotting techniques were used for the detection and estimation of the relative amounts of SR Ca\(^{2+}\)-cycling proteins in the rat heart. For immunossay of RyR-CRC, Ca\(^{2+}\)-ATPase, phospholamban, calsequestrin, and CaM kinase II, rat heart homogenate (25 \(\mu\)g protein/lane) and cardiac SR
vesicles (25 μg protein/lane) were first subjected to SDS-PAGE in 6% (for RyR-CRC), 10% (for Ca²⁺-ATPase, calseen, and CaM kinase), or 15% (for phospholamban) gels. The protein samples separated by gel electrophoresis were then transblotted to nitrocellulose membranes. The membranes were probed with antibodies specific for cardiac RyR-CRC [monoclonal (1), dilution 1:2,500], cardiac SR Ca²⁺-ATPase [monoclonal (16), dilution 1:2,500], phospholamban [monoclonal (37), 0.5 μg/ml], calseen [monoclonal (18), dilution 1:1,000], or CaM kinase II (polyclonal, dilution 1:1,000) or anti-rabbit (for CaM kinase II) IgG at a dilution of 1:5,000 was used as the secondary antibody. Protein bands reactive with antibodies were visualized using the enhanced chemiluminescence detection system from Amersham. The images of the protein bands were optimized, captured, and analyzed by ImageMaster VDS gel documentation system (Pharmacia Biotech; San Francisco, CA). The Western blotting detection system was determined to be linear with respect to the amount of SR/homogenate protein in the range of 10–40 μg using this camera-based densitometry system.

Phosphorylation assay. Phosphorylation of SR proteins by endogenous CaM kinase II was determined as described previously (44). The assay medium (total volume 50 μl) for phosphorylation by endogenous CaM kinase II contained 50 mM HEPES (pH 7.4), 10 mM MgCl2, 0.2 mM CaCl2, 0.2 mM EGTA, 1 μM calmodulin, 0.8 mM [γ-32P]ATP (specific activity 200–300 cpm/pmol), and SR (25 μg protein). The initial free Ca²⁺ concentration, determined using the computer program of Fabiato (9), was 5.4 μM. The phosphorylation reaction was initiated by the addition of [γ-32P]ATP after preincubation of the rest of the assay components for 3 min at 37°C. Reactions were terminated after 2 min by adding 15 μl of SDS-sample buffer, and the samples were subjected to SDS-PAGE in 4–18% gradient gels, stained with Coomassie brilliant blue, dried, and autoradiographed (14). Quantification of phosphorylation was carried out by liquid scintillation counting after careful excision of the radioactive bands from the gels (14).

Data analysis. Results are presented as means ± SE. Statistical significance was evaluated with a single-factor analysis of variance with the Tukey multiple comparison test. P < 0.05 was taken as a level of significance.

RESULTS

Effects of adrenalectomy and dexamethasone treatment on the Ca²⁺-sequestration function of cardiac SR. The ATP-dependent, oxalate-facilitated Ca²⁺ uptake by SR vesicles is a useful parameter commonly used to measure the Ca²⁺-pump (Ca²⁺-ATPase) function of SR in vitro. The results presented in Fig. 1 compare the rates of ATP-driven Ca²⁺ uptake into cardiac SR vesicles from control, adrenalectomized, and adrenalectomized/dexamethasone-treated rats, measured in the absence and presence of Ca²⁺-release channel blockers. These assays were performed at a fixed Ca²⁺ concentration (8.2 μM) adequate for maximal activation of Ca²⁺ uptake (cf Fig. 2). At concentrations known to block Ca²⁺ release (5, 48), ruthenium red (25 μM) and ryanodine (625 μM) both stimulated the rates of Ca²⁺ uptake by SR significantly in control and adrenalectomized/dexamethasone-treated rats. A similar tendency was also observed in the adrenalectomized group, but the difference was not statistically significant. The membranes from adrenalectomized rats showed significantly reduced (~40% decrease) rates of Ca²⁺ uptake compared with the membranes from control animals in both the absence and presence of Ca²⁺ release channel blockers. The membranes from adrenalectomized/dexamethasone-treated animals showed restoration of the higher rates of Ca²⁺ uptake compared with those from adrenalectomized animals in both the absence and presence of Ca²⁺-release channel blockers.

In additional experiments, Ca²⁺ uptake by SR was measured at varying Ca²⁺ concentrations in the presence of ruthenium red (25 μM) in the assay medium, and the results are summarized in Fig. 2. At the wide range of Ca²⁺ concentrations tested, the rate of Ca²⁺ uptake by cardiac SR vesicles from adrenalectomized animals was significantly lower than that of the membranes from control animals. A significantly higher rate of Ca²⁺ uptake by cardiac SR vesicles from adrenalectomized/dexamethasone-treated compared with...
untreated adrenalectomized animals could be observed at all Ca\(^{2+}\) concentrations. The kinetic parameters derived from the data shown in Fig. 2 are summarized in Table 1. Adrenalectomy and dexamethasone treatment did not appear to significantly influence the concentrations of Ca\(^{2+}\) required for half-maximal velocity \((K_{0.5})\) or the Hill coefficient \((n_H)\), whereas the \(V_{\text{max}}\) values were diminished significantly.

**Effects of adrenalectomy and dexamethasone treatment on the energy transduction function of Ca\(^{2+}\)-ATPase in cardiac SR.** The effect of adrenalectomy and dexamethasone treatment on the energy transduction function of the Ca\(^{2+}\)-ATPase was assessed by measuring TG-sensitive ATP hydrolysis in cardiac SR vesicles. As shown in Fig. 3, the rate of ATP hydrolysis measured was not affected significantly by adrenalectomy. Treatment of adrenalectomized animals with dexamethasone, however, led to a significant increase (~70\%) in the rate of ATP hydrolysis. The stoichiometry of Ca\(^{2+}\) uptake/ATP hydrolysis by cardiac SR vesicles was not improved by treatment of adrenalectomized animals with dexamethasone. The estimated ratios of Ca\(^{2+}\) uptake to TG-sensitive ATP hydrolysis were as follows: control, 0.59; adrenalectomized, 0.37; and adrenalectomized/dexamethasone-treated, 0.33.

As discussed elsewhere (23), such low stoichiometry between Ca\(^{2+}\) uptake and ATP hydrolysis has been reported in several published studies using rat cardiac SR vesicles; the reasons for the apparently low efficiency of coupling ATP hydrolysis to Ca\(^{2+}\) transport in rat cardiac SR vesicles in vitro remain unclear.

**Effects of adrenalectomy and dexamethasone treatment on cardiac SR Ca\(^{2+}\)-cycling proteins and their phosphorylation by endogenous CaM kinase II.** Major Ca\(^{2+}\)-cycling proteins in the SR include the RyR-CRC responsible for Ca\(^{2+}\) release into the cytosol on myocyte excitation to induce muscle contraction (4); Ca\(^{2+}\)-ATPase, which actively sequesters Ca\(^{2+}\) back into the SR lumen to promote muscle relaxation (22); calsequestrin, which serves to bind and store Ca\(^{2+}\) within the SR lumen (22); and phospholamban, which serves to regulate Ca\(^{2+}\)-ATPase function (17, 34, 38). In the present study, we used antibodies specific for each of these SR Ca\(^{2+}\)-cycling proteins to perform Western blotting analysis of their relative amounts in cardiac SR isolated from control, adrenalectomized, and adrenalectomized/dexamethasone-treated rats. The results of these experiments are summarized in Fig. 4. No significant change was evident in the relative amounts of RyR-CRC, Ca\(^{2+}\)-ATPase, calsequestrin, and phospholamban in cardiac SR after adrenalectomy or dexamethasone treatment. Similar findings were also obtained in experiments in which Western blot analysis of these proteins was performed using unfractionated cardiac muscle homogenates from all three groups (re-
sults not shown). In additional experiments, Western blot analysis of cardiac SR membranes from a group of rats that was not adrenalectomized but received dexamethasone treatment did not show any significant change in the level of the SR Ca\(^{2+}\)-cycling proteins when compared with a corresponding control group that received no dexamethasone treatment (results not shown).

CaM kinase II associated with the SR (and present in the cytosol) is implicated in the regulation of the Ca\(^{2+}\)-uptake and -release functions of the cardiac SR through phosphorylation of phospholamban (17, 34, 38), RyR-CRC (10, 19, 43), and Ca\(^{2+}\)-ATPase (11, 26–28, 30, 40, 44–47). We determined endogenous CaM kinase-catalyzed protein phosphorylation in cardiac SR vesicles isolated from control, adrenalectomized, and adrenalectomized/dexamethasone-treated rats. Figure 5 shows the protein profiles of cardiac SR vesicles isolated from each experimental group and the corresponding autoradiogram depicting protein phosphorylation. In the presence of Ca\(^{2+}\) and calmodulin, SR-associated CaM kinase II catalyzed the phosphorylation of RyR-CRC, Ca\(^{2+}\)-ATPase, and phospholamban. No significant change in the phosphorylation of these Ca\(^{2+}\)-cycling proteins was evident after adrenalectomy. On the other hand, dexamethasone treatment of adrenalectomized animals led to a significantly higher level of phosphorylation of all three CaM kinase II substrates. In several experiments, phosphorylation was quantified by determining \(^{32}\)P incorporation into the protein bands excised from gels (see METHODS). The results confirmed the significantly higher level (50–80% increase) of substrate phosphorylation in the dexamethasone-treated group (Fig. 6).

Endogenous CaM kinase II levels in control, adrenalectomized, and dexamethasone-treated rats. We utilized a polyclonal antibody, specific for the \(\delta\)-isoform of CaM kinase II predominantly expressed in the heart (2, 7, 33), to perform Western blotting analysis of this enzyme in cardiac SR from control, adrenalectomized, and adrenalectomized/dexamethasone-treated rats. As shown in Fig. 7, the relative amount of \(\delta\)-CaM kinase II protein was found to be 2.5–4-fold higher in the adrenalectomized/dexamethasone-treated group compared with control or adrenalectomized groups. No statistically significant difference was observed in the level of SR-associated CaM kinase II protein in the adrenalectomized group compared with the control group.

Effect of activation of endogenous CaM kinase II on Ca\(^{2+}\) uptake by SR. To compare the effect of endogenous CaM kinase II activation on Ca\(^{2+}\) uptake by cardiac SR from the three groups of rats, ATP-dependent Ca\(^{2+}\) uptake by SR was determined in the absence and presence of calmodulin in the assay medium. Under the experimental conditions employed, the addition of calmodulin to the Ca\(^{2+}\) uptake assay medium promotes phosphorylation of CaM kinase II substrates. These experiments were performed in the absence of ruthenium red in the assay medium because this drug...
has an inhibitory effect on Ca\(^{2+}\)-ATPase phosphorylation by CaM kinase II (28). As shown in Fig. 8, the presence of calmodulin (1 μM) in the assay medium resulted in stimulation of Ca\(^{2+}\) uptake by cardiac SR vesicles from control, adrenalectomized, and adrenalectomized/dexamethasone-treated rats. The stimulatory effect of calmodulin was most pronounced in the dexamethasone-treated group (74% increase) and was minimal in the adrenalectomized group (25% increase).

**DISCUSSION**

In this study, we made the following key observations: 1) The ATP-dependent Ca\(^{2+}\) uptake rate (Ca\(^{2+}\)-pump function) of cardiac SR membrane vesicles in...
of cardiac SR from adrenalectomized animals reported here correlates well with the depression of myocardial contractility observed in adrenal deficiency in vivo (41) and in vitro (19). The Ca\(^{2+}\)-ATPase content (Fig. 4) and Ca\(^{2+}\)-ATPase activity (Fig. 3) of cardiac SR were not altered significantly by adrenalectomy. Therefore, the observed decline in SR Ca\(^{2+}\)-pump function is likely due to impaired Ca\(^{2+}\) translocation rather than energy transduction. This apparent uncoupling of ATP hydrolysis and Ca\(^{2+}\) transport does not appear to be due to enhanced Ca\(^{2+}\) leak from the SR because SR Ca\(^{2+}\)-release channel blockers did not abolish or attenuate the depression in Ca\(^{2+}\)-uptake activity of SR from adrenalectomized animals (Fig. 1). The impaired SR Ca\(^{2+}\)-pump function after adrenalectomy and the improvement after dexamethasone treatment may involve alterations in the membrane-associated glyco-
genolytic pathway. Previous studies have demonstrated a marked and selective depletion and restoration of both active and total phosphorylase activities in the rat heart microsomes after adrenalectomy and dexamethasone treatment, respectively (24). A strong association of substantial amounts of phosphorylase, glycogen, and other enzymes linked to glyco-
genolysis with the SR membrane in cardiac muscle has been documented in earlier studies (8), suggesting that the glyco-
genolytic pathway present in the membrane might serve as a link between excitation-contraction coupling and intermediary metabolism. It is possible that the loss of phosphorylase from the SR membrane in adrenal insufficiency might result in derangement of the link between excitation-contraction coupling and intermediary metabolism.

To our knowledge, the present study is the first to provide evidence of phosphorylation-dependent glucocorticoid modulation of SR function. Our results revealed a significant increase in the Ca\(^{2+}\) kinase II-mediated phosphorylation of RyR-CRC, Ca\(^{2+}\)-ATPase, and phospholamban after dexamethasone treatment, although no significant change was observed due to adrenalectomy per se (Figs. 5 and 6). Because dexamethasone treatment did not significantly alter the levels of Ca\(^{2+}\) kinase II substrates, this increase in phosphorylation may be attributed to the observed increase in the amount of Ca\(^{2+}\) kinase II, which is the predominant Ca\(^{2+}\) kinase II isoform present in cardiac cytosol and SR (2, 7, 33). The functional consequence of cardiac RyR-CRC phosphorylation has not been clearly established. Recently, Ca\(^{2+}\) kinase II inhibitors as well as protein phosphatases have been found to reduce SR Ca\(^{2+}\)-release channel activity in intact cardiomyocytes (6, 20). These findings are consistent with an increased SR Ca\(^{2+}\)-release channel activity on RyR-CRC phosphorylation by Ca\(^{2+}\) kinase II. In any case, the observed increase in the RyR-CRC phosphorylation after dexamethasone treatment can be expected to impact on the modulation of SR Ca\(^{2+}\) release and, therefore, myofilament activation. Phosphorylation of phospho-
lamban by PKA (at Ser\(^{16}\)) and Ca\(^{2+}\) kinase II (at Thr\(^{17}\)) is well known to stimulate Ca\(^{2+}\) uptake by SR, apparently by relieving an inhibitory effect exerted by de-

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**Fig. 8.** Effect of activation of endogenous Ca\(^{2+}\) kinase II by CaM on Ca\(^{2+}\) uptake activity of cardiac SR isolated from control, ADX, and ADX + DEX rats. Ca\(^{2+}\) uptake reaction was carried out under standard assay conditions in the absence of CaM (-CaM) and in the presence of 1 \(\mu\)M CaM (+CaM) as described in METHODS. Ca\(^{2+}\) uptake data from experiments using 4 separate cardiac SR preparations from each group of rats are presented as means ± SE. \(*P < 0.05\) (absence vs. presence of CaM); \(\times P < 0.05\) (control vs. ADX); \(\#P < 0.05\) (ADX vs. ADX + DEX).

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vitro is markedly reduced following adrenalectomy; treatment of adrenalectomized animals with dexamethasone prevents this decline in SR Ca\(^{2+}\) transport function. 2) The levels of the major Ca\(^{2+}\)-cycling pro-
teins (Ca\(^{2+}\)-ATPase, RyR-CRC, calsequestrin, and phospholamban) in cardiac SR are not altered signif-
cantly after adrenalectomy or dexamethasone treat-
ment. 3) Treatment of adrenalectomized animals with dexamethasone leads to a striking increase in the amount of \(\delta\)-CaM kinase II associated with cardiac SR, as well as significantly enhanced substrate (Ca\(^{2+}\)-ATPase, RyR-CRC, and phospholamban) phosphoryla-
tion by the endogenous CaM kinase II. These findings clearly identify the SR membrane as a major subcellu-
lar target for glucocorticoid actions in the heart, and, as discussed below, they provide insights into the mechanisms underlying glucocorticoid modulation of cardiac contractile function.

Although systematic studies on the rates of contrac-
tion and relaxation of cardiac muscle from adrenal-
 deficient animals seem to be lacking, cardiac muscle from dexamethasone-treated intact animals has been shown to display markedly increased contractile ten-
sion as well as rates of contraction and relaxation (31). The present findings suggest strongly that the dexamethasone-mediated increase in the velocity of muscle relaxation might arise, at least in part, from the ability of this glucocorticoid to augment the SR Ca\(^{2+}\)-pump activity. Because the SR Ca\(^{2+}\)-pump activity is a major determinant of SR Ca\(^{2+}\) load (and hence, the amount of Ca\(^{2+}\) available for release) (13, 36, 42), the increased SR Ca\(^{2+}\)-pump activity may also contribute to the enhanced velocity of contraction (31) and contractile tension (19) observed in dexamethasone-treated ani-
mals. Conversely, the diminished Ca\(^{2+}\)-pump activity
phosphorylated phospholamban on the Ca\textsuperscript{2+}-ATPase (17, 34, 38). Recently, Ser\textsuperscript{38} phosphorylation of the cardiac SR Ca\textsuperscript{2+}-ATPase by CaM kinase II also was shown to result in stimulation of ATP hydrolysis (44) and Ca\textsuperscript{2+} transport (11, 27, 30, 40, 45). Although some studies (29, 32) have questioned the physiological role of Ca\textsuperscript{2+}-ATPase phosphorylation, evidence from more recent studies (45, 46) strongly supports the view that Ca\textsuperscript{2+}-ATPase phosphorylation is a physiological event (47) that results in stimulation of the $V_{\text{max}}$ of Ca\textsuperscript{2+} pumping in native cardiac SR. The positive $V_{\text{max}}$ effect of Ca\textsuperscript{2+}-ATPase phosphorylation (11, 40, 44, 45) and the enhancement in Ca\textsuperscript{2+} affinity of the ATPase due to phosphorylamin phosphorylation (17, 34, 38) may provide a powerful, mutually complementary mechanism for the stimulation of Ca\textsuperscript{2+} pumping in native cardiac SR. The present results showing increments in SR-associated CaM kinase II and CaM kinase II-mediated phosphorylation of SR Ca\textsuperscript{2+}-cycling proteins in cardiac muscle after dexamethasone treatment of adrenalectomized animals suggest an important modulatory role for glucocorticoids in the maintenance of normal SR function and, therefore, cellular Ca\textsuperscript{2+} homeostasis in the myocardium. In this regard, it is also noteworthy that the stimulatory effect of protein phosphorylation by endogenous CaM kinase II on the Ca\textsuperscript{2+}-uptake function of SR was clearly more pronounced in the dexamethasone-treated animals (Fig. 8). Thus modification of SR-associated CaM kinase II system appears to be a key component of the mechanisms by which dexamethasone influences SR Ca\textsuperscript{2+}-cycling and myocardial contraction. It is intriguing that adrenalectomy per se did not result in a significant decline in the level of CaM kinase II protein in the cardiac SR membrane. Therefore, it is not clear whether endogenously occurring glucocorticoids influence the SR-associated CaM kinase II system in a manner similar to that observed in this study after exogenous administration of the synthetic glucocorticoid dexamethasone.

We thank Bruce Arppe for preparing photographs of illustrations and Lily Jiang for secretarial assistance.

This work was supported by Grant T-3682 from the Heart and Stroke Foundation of Ontario.

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