BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 544–548 (1997)
ARTICLE NO. RC977305

Phosphorylation of Cardiac Na^+-K^+ ATPase by Ca^{2+}/Calmodulin Dependent Protein Kinase

Thomas Netticadan, Kiminori Kato, Paramjit Tappia, Vijayan Elimban, and Naranjan S. Dhalla[1]
Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre and Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

Received July 30, 1997

Na^+-K^+ ATPase is known to be involved in the transport of sodium and potassium across the cell membrane. We describe here a novel mechanism for the regulation of cardiac Na^+-K^+ ATPase through phosphorylation by a Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) present in the sarcolemmal membrane. Incubation of cardiac sarcolemma in the presence of Ca^{2+} and calmodulin resulted in phosphorylation of a 110 kDa protein, identified as the α-subunit of Na^+-K^+ ATPase. The compound W-7, a potent inhibitor of calmodulin, caused significant inhibition of the CaM kinase-mediated phosphorylation while ouabain, a potent inhibitor of Na^+-K^+ ATPase, had no effect. Furthermore, phosphorylation of the sarcolemmal membrane with Ca^{2+}/calmodulin caused significant reduction in the activity of Na^+-K^+ ATPase. These results suggest that phosphorylation of the α-subunit of Na^+-K^+ ATPase by an endogenous CaM kinase may lead to an inhibition of its catalytic activity.

By virtue of its ability to control fluxes of Na^+ and K^+ across the sarcolemmal membrane, Na^+-K^+ ATPase is known to maintain the resting membrane potential and is thus considered to play an important role in the process of cardiac excitation-contraction coupling (1). Earlier studies have revealed that Na^+-K^+ ATPase is made up of two subunits, namely α and β subunits. The α subunit, which is responsible for the catalytic activity of the enzyme, has a molecular mass of 110 kDa. On the other hand, the β subunit is a glycoprotein with a molecular mass of 32 kDa and is considered to be involved in α-β assembly, cell to cell communication and modulation of the affinity for K^+ (2). It has also been shown that the α-subunit of Na^+-K^+ ATPase may be regulated by cAMP dependent protein kinase and protein kinase C mediated phosphorylations (3-10). Although the presence of CaM kinase has been reported in the cardiac sarcolemmal membrane (11), its role in the regulation of Na^+-K^+ ATPase has not been examined thus far. In this report we present evidence for the phosphorylation of the α-subunit of cardiac Na^+-K^+ ATPase by an endogenous CaM kinase. We also demonstrate a marked decrease in the sarcolemmal Na^+-K^+ ATPase activity by endogenous CaM kinase phosphorylation.

MATERIALS AND METHODS

Preparation of heart sarcolemmal membranes. Rat heart sarcolemma was isolated by the method of Pitts (12) as modified by Kaneko et al (13). The membranes were suspended in 0.25 M sucrose, 10 mM histidine, pH 7.0; protein concentration was measured by the method of Lowry et al (14). The sarcolemmal preparations were quickly frozen in liquid nitrogen and stored at −90°C for up to 3 weeks. The purity of the membrane preparations was examined by comparing the activities of marker enzymes such as Na^+-K^+ ATPase, cytochrome C oxidase, and rotenone-insensitive NADPH-cytochrome C reductase in both the homogenate and sarcolemmal membrane according to the procedures used in our laboratory (13, 15). Marker enzyme activities showed that the isolated sarcolemmal fraction was enriched with Na^+-K^+ ATPase (18- and 20-fold purification) and had negligible cross-contamination by the sarcoplasmic reticulum fragments or mitochondria. Each assay in this study was carried out by employing 4 to 6 different sarcolemmal preparations and the results were analyzed statistically by using the Students' "t"-test.

Determination of Ca^{2+}/calmodulin-dependent protein phosphorylation. Protein phosphorylation due to endogenous CaM kinase was determined by the procedure described by Netticadan et al (16). The incubation medium (total volume 50 μl) for phosphorylation contained 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM EGTA, 100 μM $CaCl_2$, 2 μM calmodulin, 0.8 mM [γ-^{32}P]ATP (specific activity 200-300 cpm/pmol), and sarcolemma (30-40 μg protein). Free Ca^{2+} concentration in the medium, determined according to the computer program of Fabiato (17), was 3.7 μM. Assay was performed at 37°C.

[1] Address correspondence to: Dr. Naranjan S. Dhalla, Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, 351 Tache Avenue, Winnipeg, Manitoba, R2H 2A6, Canada. Fax: (204) 233-6723. E-mail: cvso@sbrc.umanitoba.ca.

Abbreviations used: Na^+-K^+ ATPase, Sodium-potassium stimulated adenosine triphosphatase; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; PEP, phosphoenol pyruvate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-291X/97 $25.00

The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP following preincubation of the assay components for 45 sec. Ca^{2+}/calmodulin dependence of phosphorylation was shown in the absence of calmodulin and Ca^{2+} in the assay medium containing 1 mM EGTA. Reaction was terminated after 45 sec unless otherwise indicated by the addition of 15 μl of SDS sample buffer, and the samples were subjected to SDS-PAGE in 4-18% gradient slab gels (18). The gels were stained with Coomassie brilliant blue, dried, and autoradiographed (19).

Measurement of Na^+-K^+ ATPase activity. Estimation of Na^+-K^+ ATPase activity was carried out by a method described previously (20) with some modification. Briefly, phosphorylated and unphosphorylated membranes were assayed for total ATPase activity in a medium containing 50 mM Histidine-HCl, pH 7.4, 5 mM NaN_3, 6 mM $MgCl_2$, 100 mM NaCl and 10 mM KCl, 2.5 mM phosphoenolpyruvate (PEP), and 10 IU/ml pyruvate kinase. PEP and pyruvate kinase were used as an ATP-regenerating system to maintain the concentration of ATP in the incubation medium. The medium was preincubated at 37°C for 5 min. The reaction was started immediately after the transfer of the phosphorylated and unphosphorylated membranes by the addition of 0.025 ml of 80 mM ATP, pH 7.4, and terminated 5 min after with 0.5 ml of ice-cold 12% trichloroacetic acid. The liberated phosphate was measured by the method of Taussky and Shorr (21). Mg^{2+}-ATPase activity of the phosphorylated and unphosphorylated membranes was also determined in a similar manner except that both NaCl and KCl were omitted from the reaction medium. Na^+-K^+ ATPase activity was calculated as the difference between the total ATPase and Mg^{2+}-ATPase activities. In order to further verify the effect of CaM kinase mediated phosphorylation on the enzyme activity, digitoxigenin-sensitive Na^+-K^+ ATPase was determined by measuring the total ATPase activity in the absence or presence of 200 μM digitoxigenin, a well known inhibitor of Na^+-K^+ ATPase. All measurements were carried out in duplicate.

Immunoblotting analysis. The Western immunoblotting procedure was used to identify the alpha subunit of the Na^+-K^+ ATPase. For this, the phosphorylated and unphosphorylated sarcolemma samples were subjected to SDS-PAGE (10% minigel) and electroblotted to Immobilon-P transfer membrane (Millipore Corporation, Bedford, MA). These preparations were incubated with monoclonal α-1 Na^+-K^+ ATPase-specific antibody (Upstate Biotechnology, Lake Placid, NY). Antibody binding was detected by enhanced chemiluminescence using a peroxidase-conjugated sheep anti-mouse IgG secondary antibody and other reagents in an Amersham kit following instructions of the manufacturer (Amersham Corporation, Arlington Heights, IL).

Immunoprecipitation of the α-subunit of Na^+-K^+ ATPase. Sarcolemmal membrane proteins were extracted using buffer containing 1% w/v SDS, 50 mM HEPES (pH 7.2), 200 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml soya bean trypsin inhibitor and rotated for 2 hr at 4°C. The sample was centrifuged (280,000 g $\times$ 25 min) and the supernatant recovered as the solubilized membrane fraction. The membrane extract was incubated overnight at 4°C with monoclonal anti-rabbit Na^+-K^+ ATPase α_1 (1:70 antibodies to membrane extract). The immunocomplex was captured with 100 μl (50 μl packed beads) of washed Protein G sepharose at 4°C by rotation for 2 hr. The agarose beads were collected by pulsing (5 sec) at 10,000 g and used for the immunoblotting analysis.

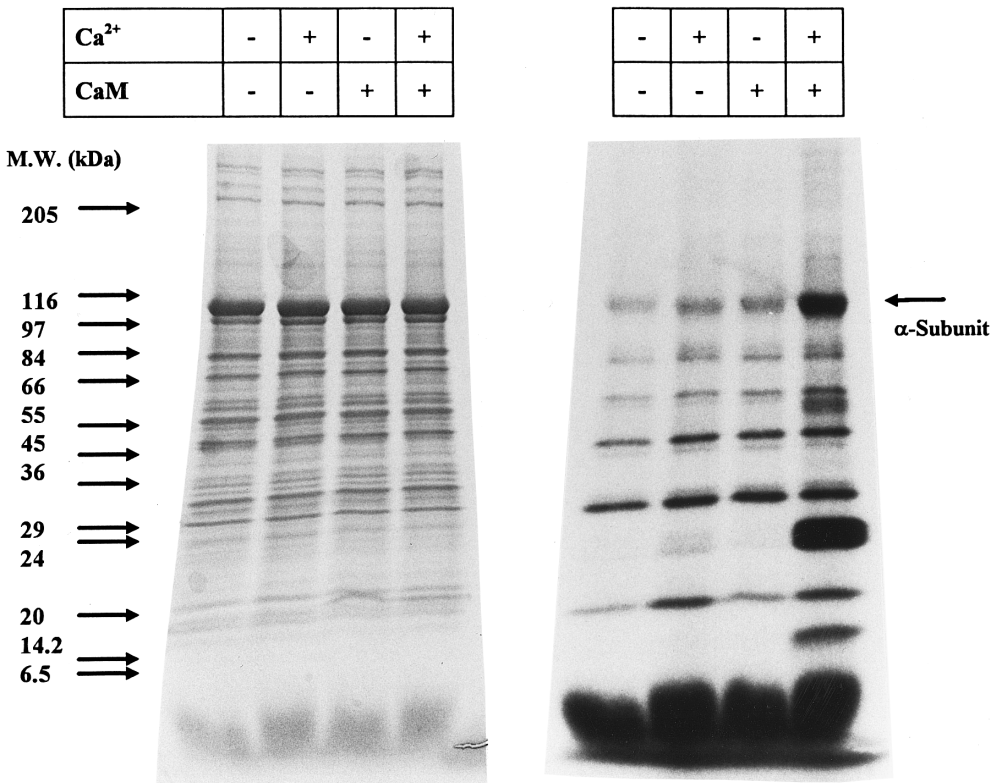

FIG. 1. Endogenous CaM kinase mediated phosphorylation of cardiac sarcolemmal proteins. Panel on the left shows the Coomassie blue-stained 4-18% SDS-polyacrylamide gel depicting the protein profile whereas panel on the right side is the corresponding autoradiogram showing the incorporation of radioactive label. Experimental details are as described under "Materials and Methods".

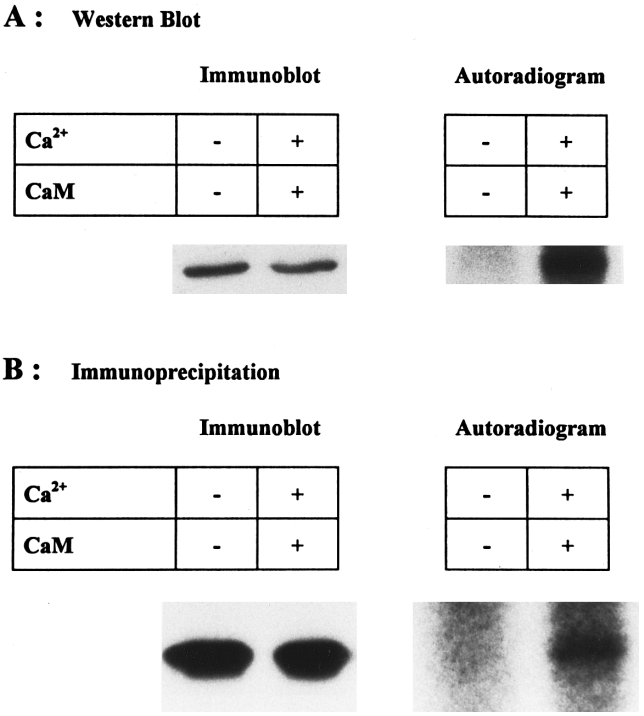

FIG. 2. (A) Western immunoblot identifying the 110 kDa protein band (left side) as the α-subunit of Na^+-K^+ ATPase and the corresponding autoradiogram (right side) showing Ca^{2+} and calmodulin dependent protein kinase phosphorylation. (B) Immunoprecipitation of the α-subunit of Na^+-K^+ ATPase. The immunoblot identifying the 110 kDa protein band (left side) as the α-subunit of Na^+-K^+ ATPase and the corresponding autoradiogram (right side) showing Ca^{2+} and calmodulin dependent protein kinase phosphorylation. The phosphorylation reaction was carried out for 45 sec in the presence and absence of Ca^{2+} and calmodulin as indicated. Experimental details are as described under "Materials and Methods".

RESULTS AND DISCUSSION

When the sarcolemmal preparation was incubated in the phosphorylation assay medium in the presence of Ca^{2+} and calmodulin, a protein band of approximate molecular mass of 110 kDa was phosphorylated (Fig. 1). Although two other bands having approximate molecular mass of 26 kDa and 6 kDa were also phosphorylated, no effort was made to identify these proteins. On the other hand, the identity of the 110 kDa band, as the α-subunit of Na^+-K^+ ATPase, was confirmed by Western immunoblotting analysis (Fig. 2A) and immunoprecipitation technique (Fig. 2B) utilizing a monoclonal antibody against the α-subunit of Na^+-K^+ ATPase. The phosphorylation of Na^+-K^+ ATPase demonstrated here may not be due to the formation of the acylphosphate (aspartyl phosphate) intermediate of Na^+-K^+ ATPase because: a) phosphorylation was observed only in the presence both of Ca^{2+} and calmodulin but not Ca^{2+} or calmodulin alone; and b) acylphosphate does not survive the alkaline conditions of SDS-PAGE (22). A time-course study revealed that the phosphorylation by endogenous CaM kinase in cardiac membranes occurred rapidly, reaching a maximum within 45 sec (Fig. 3). An additional band having an approximate molecular mass of 115 kDa appeared after longer incubation times (4-5 min). In another set of experiments, the effect of a calmodulin inhibitor, compound W-7, was tested on the phosphorylation of the α-subunit of Na^+-K^+ ATPase by endogenous CaM kinase. Compound W-7 has been reported to be a potent inhibitor of calmodulin activated reactions in the heart at concentrations ranging from 10-300 μM (23-25). Fig. 4 shows a marked inhibition of phosphorylation with compound W-7; high concentrations of compound W-7

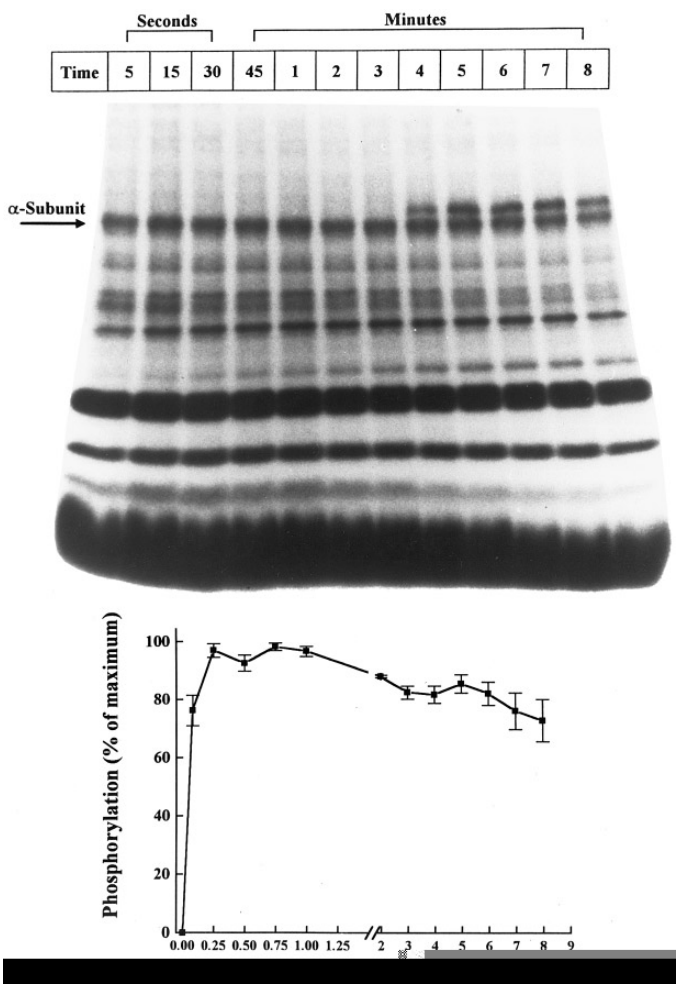

FIG. 3. Time course of endogenous CaM kinase mediated phosphorylation of the α-subunit of the Na^+-K^+ ATPase. The top is an autoradiogram showing time of the phosphorylation reaction whereas below is the densitometric scan analysis of the data. The SL membranes were incubated in the standard assay medium in the presence of Ca^{2+} and calmodulin (see "Materials and Methods"), and the time course of phosphorylation was monitored by removing aliquots of the reaction mixture at various time intervals indicated.

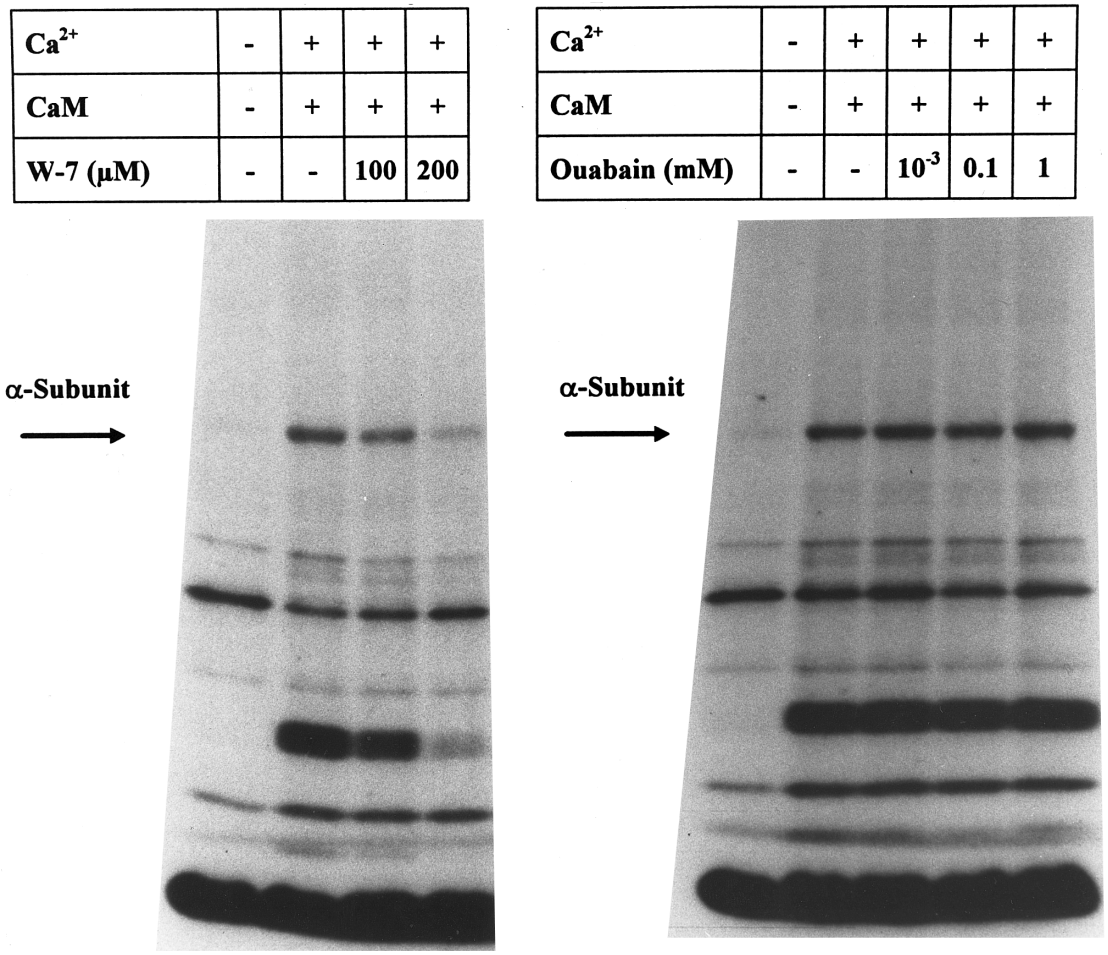

FIG. 4. Effects of compound W-7, a calmodulin inhibitor, and ouabain, an inhibitor of Na^+-K^+ ATPase, on the endogenous CaM kinase mediated phosphorylation of the α-subunit of Na^+-K^+ ATPase. The autoradiogram on the left side shows the effect of 100 and 200 μM concentrations of compound W-7 whereas that on the right side shows the effect of 1 μM, 0.1 mM and 1 mM concentrations of ouabain. The phosphorylation reaction was carried out in the presence and absence of varying concentrations of these inhibitors. Experimental details are as described under "Materials and Methods".

(200 μM) were required to completely inhibit CaM kinase phosphorylation. This may be due to the presence of a significant amount of endogenous calmodulin in the rat heart sarcolemma in addition to the added exogenous calmodulin (2 μM). In fact endogenous calmodulin in rabbit myocardial sarcolemma has been reported to be 1.4 micrograms/mg membrane protein (24). Nonetheless, our results with compound W-7 provide further confirmation of the calmodulin dependence of the endogenous CaM kinase. On the other hand, different concentrations of ouabain, an inhibitor of Na^+-K^+ ATPase (26), did not exert any effect on the endogenous CaM kinase phosphorylation (Fig. 4). Likewise, digitoxigenin (200 μM), another inhibitor of Na^+-K^+ ATPase, was also found to show no action on the CaM kinase mediated phosphorylation.

In order to examine whether the catalytic function of Na^+-K^+ ATPase is regulated by CaM kinase, the effect of CaM kinase phosphorylation on cardiac sarcolemmal Na^+-K^+ ATPase activity was studied. Membranes were phosphorylated in the presence and absence of Ca^{2+} and calmodulin for 45 sec and the Na^+-K^+ ATPase activity was estimated. Fig. 5 shows a marked inhibition of the Na^+-K^+-stimulated ATPase activity ($\sim$ 50%) and digitoxigenin-sensitive Na^+-K^+ ATPase activity ($\sim$40%) by phosphorylation due to CaM kinase. Digitoxigenin was utilized in preference to ouabain because digitoxigenin, unlike ouabain, inhibits the Na^+-K^+ ATPase activity markedly in the membrane vesicular preparation due to its freely permeable nature (27). The marked inhibition of the enzyme activity due to CaM kinase phosphorylation observed in this study is consistent with a report indicating 50% inhibition of the ouabain-sensitive Na^+-K^+ ATPase activity in hearts from spontaneously hypertensive rats in the presence of calmodulin (28). Calmodulin was also

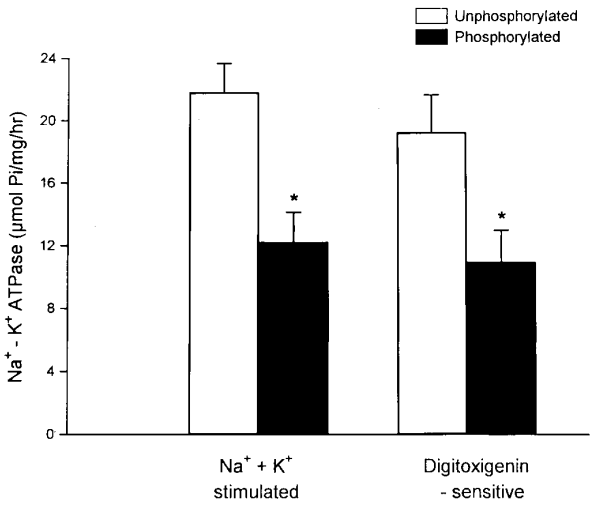

FIG. 5. Effect of endogenous CaM kinase mediated phosphorylation on Na^+-K^+ ATPase activity. Phosphorylated and unphosphorylated membranes were assayed for Na^+-K^+ ATPase activity. Experimental details are described under "Materials and Methods". *-Significantly different ($P < 0.05$) from the value for unphosphorylated membrane. Mg^{2+}-ATPase activities were 162.0 ± 8.2 and 155.4 ± 12.3 μmol Pi/mg/hr in the unphosphorylated and phosphorylated conditions, respectively; the difference between these values was not statistically significant ($P < 0.05$).

shown to inhibit Na^+-K^+ ATPase of the red cell hemolysates (29). A role for calmodulin in the modulation of Na^+-K^+ ATPase was suggested when this enzyme and calmodulin were co-localized by immunohistochemical staining in rat heart sarcolemma (30). Although other investigators (31-33) have also suspected the role of Ca^{2+} and calmodulin in the regulation of Na^+-K^+ ATPase, no information regarding the mechanism of such a regulatory effect was available in the literature. Thus the observations reported in the present study provide evidence that CaM kinase-mediated phosphorylation at the α-subunit of the enzyme may be involved in the regulation of Na^+-K^+ ATPase function.

ACKNOWLEDGMENTS

The research work reported here was supported by a grant from the Medical Research Council of Canada (MRC Group in Experimental Cardiology). Dr. K. Kato was a postdoctoral fellow of the Heart and Stroke Foundation of Canada.

REFERENCES

1. Vasilets, L. A., and Schwarz, W. (1993) *Biochim. Biophys. Acta* **1154,** 201–222.
2. Arystarkhova, E., Gibbons, D. L., and Sweadner, K. J. (1995) *J. Biol. Chem.* **270,** 8785–8796.
3. Chibalin, A. V., Lopina, O. D., Petukhov, S. P., and Vasilets, L. A. (1993) *J. Bioenerg. Biomemb.* **25,** 61–66.
4. Feschenko, M. S., and Sweadner, K. J. (1994) *J. Biol. Chem.* **269,** 30436–30444.
5. Cornelius, F., and Logvinenko, N. (1996) *FEBS. Letts.* **380,** 277–280.
6. Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C., and Greengard, P. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11359–11362.
7. Feschenko, M. S., and Sweadner, K. J. (1995) *J. Biol. Chem.* **270,** 14072–14077.
8. Fisone, G., Cheng, S. X. J., Nairn, A. C., Czernik, A. J., Hemmings, H. C., Jr., Hoog, J.-O., Bertorello, A. M., Kaiser, R., Bergman, T., Jornvall, H., Aperia, A., and Greenard, P. (1994) *J. Biol. Chem.* **269,** 9368–9373.
9. Beguin, P., Beggah, A. T., Chibalin, A. V., Burgener-Kairuz, P., Jaisser, F., Mathews, P. M., Rossier, B. C., Cotecchia, S., and Geering, K. (1994) *J. Biol. Chem.* **269,** 24437–24445.
10. Turi, A., and Somogyi, J. (1988) *Biochim. Biophys. Acta* **940,** 77–84.
11. Tuana, B. S., Murphy, B. J., and Schwarzkopf, C. (1987) *Mol. Cell. Biochem.* **78,** 47–54.
12. Pitts, B. J. R. (1979) *J. Biol. Chem.* **245,** 6232–6235.
13. Kaneko, M., Beamish, R. E., and Dhalla, N. S. (1989) *Am. J. Physiol.* **256,** H368–H374.
14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
15. Ganguly, P. K., Pierce, G. N., Dhalla, K. S., and Dhalla, N. S. (1983) *Am. J. Physiol.* **244,** E528–E535.
16. Netticadan, T. J., Xu, A., and Narayanan, N. (1996) *Arch. Biochem. Biophys.* **333,** 368–376.
17. Fabiato, A. (1988) *Meth. Enzymol.* **157,** 378–417.
18. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
19. Jiang, M. T., and Narayanan, N. (1990) *Mech. Ageing Dev.* **54,** 87–101.
20. Pierce, G. N., and Dhalla, N. S. (1983) *Am. J. Physiol.* **245,** C241–C247.
21. Taussky, H., and Shorr, E. (1953) *J. Biol. Chem.* **202,** 678–685.
22. Buss, J. E., and Stull, J. T. (1983) *Meth. Enzymol.* **99,** 7–14.
23. Zimmer, M., Gobel, C., and Hofman, F. (1984) *Eur. J. Biochem.* **139,** 295–301.
24. Rudinger, A., Mylotte, K. M., Davis, P. J., Davis, F. B., and Blas, C. D. (1984) *Arch. Biochem. Biophys.* **229,** 379–385.
25. Toyama, J., Sugiura, H., Kamiya, K., Kodama, I., Terasawa, M., and Hidaka, H. (1994) *J. Mol. Cell. Cardiol.* **26,** 1007–1015.
26. Vemuri, R., Longoni, S., and Philipson, K. D. (1989) *Am. J. Physiol.* **256,** C1273–C1276.
27. Pitts, B. J. R., and Okhuysen, C. H. (1980) *Ann. N.Y. Acad. Sci.* **358,** 357–358.
28. David-Dufilho, M., Pernollet, M., Sang, H. L., Benlian, P., Mendonca, M. D., and Grichois, M. (1986) *J. Cardiovasc. Pharmacol.* **8,** S130–S135.
29. Kassir, S., and Meltzer, H. L. (1991) *Biol. Psych.* **30,** 631–634.
30. Nagakawa, R., Qiao, Y., and Asano, G. (1990) *Nippon. Ika. Daigaku. Zasshi.* **57,** 541–546.
31. Yingst, D. R., Ye-Hu, J., Chen, H., and Barett, V. (1992) *Arch. Biochem. Biophys.* **295,** 49–54.
32. Yingst, D. R. (1988) *Annu. Rev. Physiol.* **50,** 291–303.
33. Powis, D. A. (1985) *Experentia* **41,** 1048–1051.