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EFFECTS OF THE PHOSPHATASE INHIBITOR CALYCULIN A ON THE PHOSPHORYLATION OF C-PROTEIN IN MAMMALIAN VENTRICULAR CARDIOMYOCYTES

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Abstract—The effects of inhibitors of protein phosphatase activity on C-protein phosphorylation were studied in preparations from mammalian ventricles. Calyculin A (CyA), an inhibitor of type 1 and 2A protein phosphatases, was studied. CyA concentration- and time-dependency increased the phosphorylation state of C-protein in isolated ^{32}P-labelled guinea pig ventricular cardiomyocytes. C-protein was identified by its reaction with a polyclonal antibody and immunoprecipitation. It is concluded that C-protein in intact cardiomyocytes could be a substrate for type 1 and 2A protein phosphatases.

Key words: protein phosphorylation; phosphatase inhibitor; calyculin A; C-protein

C-protein is located in the thick filaments of the cardiac contractile apparatus and has an apparent molecular weight of 130,000–160,000 [1]. The skeletal muscle isoforms of C-protein are immunologically distinct from the cardiac form of C-protein [2]. C-protein can modulate the ATPase activity of cardiac myosin [3–5]. Removal of C-protein from skinned cardiac cells resulted in an increase in isometric tension at submaximal levels of Ca^{2+}, suggesting that the protein plays an important role in force regulation [6]. After β-adrenergic stimulation the phosphorylation state of a protein, tentatively identified as C-protein, was enhanced in bovine atria, amphibian cardiac preparations, isolated rat and rabbit hearts [7–11]. In addition, it has been suggested that the relaxant properties of β-adrenergic stimulation (at least in amphibian preparations) may be linked to C-protein phosphorylation [10]. Furthermore, the phosphorylation state of a protein similar in size to C-protein was enhanced by β-adrenergic stimulation in isolated rat cardiomyocytes [12, 13]. However, C-protein was not immunologically identified in these isolated cells.

All these findings have led to the assumption that some of the contractile effects of β-adrenergic catecholamines might be mediated via C-protein phosphorylation. Hence, the mechanism of phosphorylation of C-protein has been studied more extensively *in vitro*. It was shown that isolated C-protein from chicken heart could be phosphorylated *in vitro* by cAMP-dependent protein kinase as well as by Ca^{2+} calmodulin-dependent protein kinase [14]. C-protein in myofibrillar fractions from rat cardiomyocytes was phosphorylated at least *in vitro* by both cAMP-dependent protein kinase and protein kinase C [13]. Whereas C-protein phosphorylation has been extensively studied, dephosphorylation has only recently received attention. It was reported that *in vitro* purified C-protein could be dephosphorylated by purified type 1 and 2A protein phosphatases [15]. Depending on their substrate specificities protein phosphatases may be classified as type 1, 2A, 2B and 2C [16]. Recently, cell membrane permeable inhibitors of protein phosphatase activity (phosphatase inhibitors for short) have become available as tools for the study of dephosphorylation. For instance, CyA** inhibits the activity of isolated type 1 and 2A phosphatases in nanomolar concentrations [17].

Hence, we hypothesized that phosphatase inhibitors should increase the phosphorylation state of C-protein in intact mammalian cardiac cells. Therefore, C-protein was identified in isolated ^{32}P-labelled guinea pig ventricular cardiomyocytes by means of a polyclonal antiserum. Thereafter, the effects of CyA on the phosphorylation state of C-protein were studied.

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** Abbreviations: CyA, calyculin A; Iso, (-)isoproterenol bitartrate; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TnI, troponin I; MLC, myosin light chain-2.

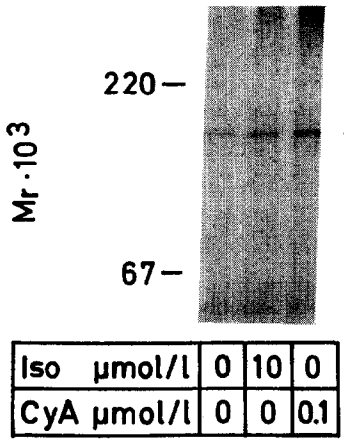


Fig. 1. The autoradiogram of a 5% polyacrylamide gel. Immunoprecipitation of C-protein in guinea pig ventricular cardiomyocytes. Incubation of the cells with ^{32}P, gel electrophoresis and immunoprecipitation of C-protein were performed as described in the Materials and Methods section. Cells were incubated with Iso or CyA as indicated. Molecular weight standards are indicated on the left.

MATERIALS AND METHODS

Isolation of cardiomyocytes. Cardiomyocytes were isolated as described [18] with minor modifications [19]. In brief, guinea pig hearts were mounted on a modified Langendorff perfusion system and retrogradely perfused with a buffer and collagenase. Later, atria were cut off, ventricles minced and cardiomyocytes isolated.

Labelling of cardiomyocytes. Isolated cells were incubated with ^{32}P-labelled orthophosphate as described previously [19].

Protein phosphorylation. Adenosine deaminase (10 units/mL) was added to prevent interference from endogenous adenosine during the preparation of cardiomyocytes and upon treatment. The drug solution (150 µL) was preincubated for 2 min at 37° prior to mixing with 150 µL of the diluted cardiomyocytes, kept at 37°. At the indicated periods of time, the reaction was stopped by adding 150 µL SDS stop solution [19] and the mixture then frozen.

SDS-PAGE and autoradiography. Samples were thawed and then heat-treated for 10 min at 95°. An aliquot of 100 µL was applied to each lane. Gels were run according to [19] in order to measure the phosphorylation of the inhibitory subunit of TnI and MLC. We have identified these latter proteins immunologically [20]. Furthermore, only 5% polyacrylamide was used in the separating gels to separate C-protein. Staining, drying and autoradiography were performed as described previously [19]. Bands corresponding to the proteins of interest were cut from dried gels and counted in a liquid scintillation counter as published [19, 20].

Raising of antibody. A crude preparation of bovine cardiac C-protein was prepared from washed, glycerinated myofibrils using DEAE sepharose CL-6B to separate C-protein from myosin (a modified procedure of [1]). The C-protein was subjected to SDS-PAGE on a 6% gel. The proteins were visualized with 0.2 M KCl and C-protein was electroeluted and concentrated using a Centricon filter. Polyclonal antibody was raised in a rabbit using Freund's adjuvant and the IgG fraction of the serum was collected by ammonium sulphate (45%).

Immunological identification of C-protein. Isolated guinea pig ventricular cardiomyocytes were labelled with ^{32}P and incubated with isoproterenol (1 µmol/L) for 1 min at 37° as described above. Samples were subjected to gel electrophoresis. Separated proteins were electrophoretically transferred to nitrocellulose membranes as described [20] in 50 mmol/L sodium phosphate buffer (pH was adjusted to 7.4) for 180 min at 3 A at a constant temperature of 4°. A nitrocellulose strip was incubated with a polyclonal antibody from a rabbit raised against C-protein (see above). Proteins binding the antibody were visualized colorimetrically using alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G.

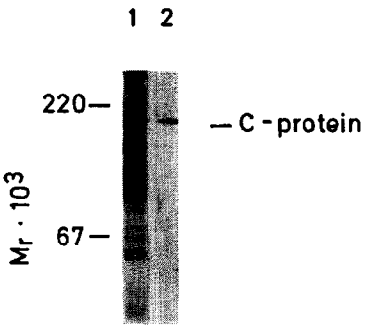
Immunoprecipitation of C-protein. Cells were labelled with ^{32}P and incubated as above. Reactions were stopped by adding SDS solution. Samples were diluted twice with a buffer in order to obtain the following final concentrations (in mmol/L): Tris (pH = 7.5) 25, NaCl 200, EDTA 5, NaF 100, sodium pyrophosphate 20, 1% Nonidet P-40. These samples were incubated with the antibody (1 : 100) overnight and were incubated thereafter for 3 hr with a protein A complex. Pellets were collected by centrifugation, washed and dissolved in Laemmli-sample buffer [19]. Subsequently, electrophoresis and autoradiography were performed as above. Due to insufficient amounts of antibody, samples were not routinely immunoprecipitated except for experiments in Fig. 1. However, all radioactive material migrating at approx. 150 kDa could be immunoprecipitated.

Statistics. Values are means ± standard error of the mean. Statistical significance was estimated using Student's *t*-test for unpaired observations; $P < 0.05$ was considered significant.

Chemicals. Compounds used were adenosine deaminase, collagenase (Boehringer Mannheim, Germany), alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA, U.S.A.), BSA (A 9647), Iso (Sigma Chemical Co., Munich, Germany), nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, *p*-toluidine salt (Biomol Feinchemikalien, GmbH, Hamburg, Germany), CyA (Biotrend GmbH, Köln, Germany), and ^{32}P-labelled orthophosphate (Du Pont de Nemours, Dreieich, Germany). All materials for SDS-PAGE were purchased from Bio-Rad (Munich, Germany). All other chemicals were of analytical or best commercial grade available. Deionized and twice distilled water was used throughout.

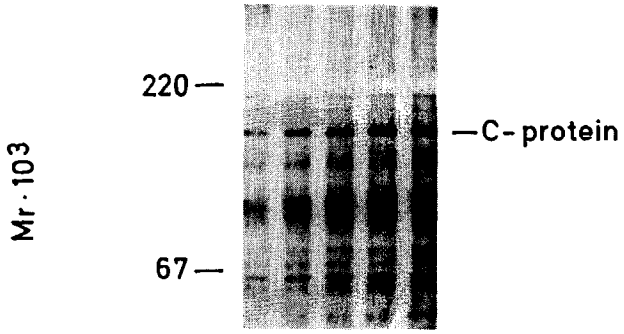
RESULTS

In our own earlier studies C-protein phosphorylation was not easily detectable because gels containing 10% acrylamide were used [19, 20]. However, by using separating gels with a lower acrylamide content C-protein could be resolved in



1 Ctr, autoradiogram
2 C-protein, alkaline phosphatase, photograph

Fig. 2. Autoradiogram and photograph of a 5% polyacrylamide gel transferred to nitrocellulose membranes. Immunological identification of C-protein in guinea pig ventricular cardiomyocytes. Incubation of the cells with ^{32}P, gel electrophoresis and immunological identification of C-protein were performed as described in the Materials and Methods section. Lane 1 depicts an autoradiogram of a nitrocellulose strip obtained from unstimulated cardiomyocytes. A nitrocellulose strip was treated with an antibody against C-protein and alkaline phosphatase-conjugated antibody against rabbit immunoglobulin G and a colour reagent (lane 2). A photograph was obtained. Molecular weight standards are indicated on the left.



CyA 0.1µmol/l	+	+	+	+	+
Time (min)	0	1	5	15	30

Fig. 3. The autoradiogram of a 5% polyacrylamide gel. Time-dependent effects of CyA on phosphorylation of C-protein in guinea pig ventricular cardiomyocytes. Isolated cardiomyocytes were labelled with ^{32}P and incubated with CyA (0.1 µmol/L) at 37° for the indicated times. The reaction was terminated by addition of SDS stop solution and samples were heat-treated prior to electrophoresis and autoradiography. Molecular weight standards are indicated on the left.

cardiomyocytes. The identification of C-protein is dependent on several criteria. Iso increased the phosphorylation state of a protein of approx. 150 kDa apparent molecular weight (Fig. 2). Furthermore, the direct activator of cAMP-dependent protein kinase N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate (1 mmol/L, 30 min) and the inhibitor of phosphodiesterase activity 3-isobutyl-1-methylxanthine (100 µmol/L, 30 min) increased the phosphorylation of the 150 kDa protein (data not shown). When contractile proteins from perfused guinea pig ventricles enriched in C-protein were prepared according to Rapundalo [21], a prominent protein comigrated on Coomassie stains of gels with the protein of 150 kDa in isolated cardiomyocytes (data not shown). Furthermore, Western blots of ^{32}P-labelled cardiomyocytes were performed. Autoradiograms of transferred proteins were obtained and the same nitrocellulose strips incubated with an antiserum raised against C-protein. As shown in Fig. 2, C-protein could be immunologically identified at the expected molecular weight. When ^{32}P-labelled cardiomyocytes were immunoprecipitated against C-protein with an antiserum a distinct protein was detected in autoradiograms, the phosphorylation state of this protein being enhanced after pretreatment with Iso (Fig. 1). Thus, we regard the 150 kDa substrate as C-protein.

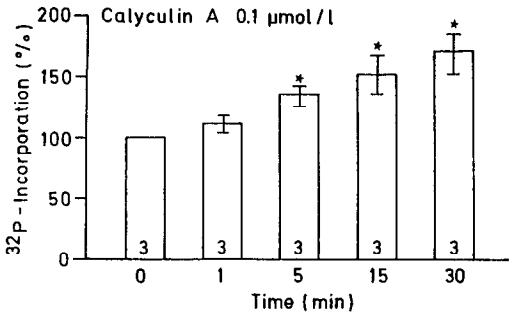


Fig. 4. Time-dependent effects of CyA (0.1 µmol/L) on phosphorylation of C-protein in guinea pig ventricular cardiomyocytes. Isolated cardiomyocytes were labelled with ^{32}P and incubated with CyA at 37° for the indicated times. The reaction was terminated by addition of SDS stop solution and samples were heat-treated prior to electrophoresis and autoradiography. C-protein phosphorylation was quantified as indicated in the Materials and Methods section. * Indicates significant differences versus time = 0 min.

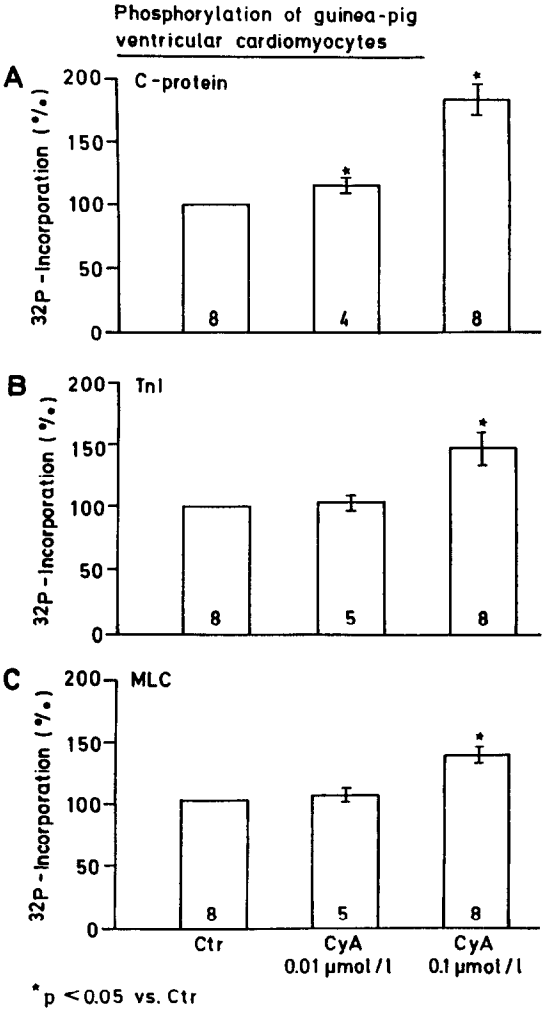


Fig. 5. Concentration-dependent effects of CyA on the phosphorylation of C-protein (A), the inhibitory subunit of TnI (B) and MLC (C) in guinea pig ventricular cardiomyocytes. Isolated cardiomyocytes were labelled with ^{32}P and incubated with CyA for 30 min at 37°. The reaction was terminated by addition of SDS stop solution and samples were heat-treated prior to electrophoresis and autoradiography. C-protein, TnI and MLC phosphorylation were quantified as indicated in the Materials and Methods section. * Indicates significant differences versus Ctr (= solvent control).

Next, the effects of CyA, the inhibitor of type 1 and type 2A phosphatase activities, were tested. CyA (0.1 µmol/L) time-dependently enhanced the phosphorylation state of C-protein as shown in a typical autoradiogram (Fig. 3). The phosphorylation of additional proteins of unknown nature was also enhanced, suggesting that the phosphorylation state of various proteins is affected by type 1 and/or 2A phosphatases. The time-dependent effect of CyA on C-protein phosphorylation was quantified in several independent experiments and is summarized in Fig. 4. Subsequent experiments (Fig. 5) were performed at 30 min of incubation. In addition, the effect of CyA on the phosphorylation of the inhibitory subunit of TnI and MLC were measured (Fig. 5(B) and (C)). The effects of CyA on phosphorylation of C-protein were concentration-dependent starting at 0.01 µmol/L CyA. Figure 1 depicts the effect of CyA (0.1 µmol/L) on phosphorylation of C-protein after immunoprecipitation. Furthermore, the effect of 0.01 µmol/L CyA could be amplified by additional application of isoprenaline (0.1 µmol/L, data not shown).

DISCUSSION

C-protein phosphorylation has hitherto been described in a preliminary way. For instance, in ^{32}P-labelled rat cardiomyocytes, isoproterenol enhanced the phosphorylation state of a protein with an apparent molecular weight of approximately 150,000 [12, 13]. However, the authors did not identify C-protein with an antiserum and did not report an enhanced phosphorylation in the presence of a phosphatase inhibitor. Antisera have been employed to detect C-protein in avian cardiac preparations [22] but protein phosphorylation was not examined. Others have measured C-protein phosphorylation in bovine atria and used an antibody in order to identify C-protein purified from rabbit hearts [9]. This is the first work combining the immunological identification of C-protein and the assessment of its phosphorylation state in the same cardiac preparation.

The present study identified C-protein based on several pharmacological and immunological criteria in ^{32}P-labelled mammalian ventricular cardiomyocytes. β-Adrenoceptor-mediated activation of cAMP-dependent protein kinase in the presence of isoproterenol increased the phosphorylation state of a 150 kDa protein in guinea pig ventricular cardiomyocytes in agreement with earlier reports in perfused hearts and rat cardiomyocytes [7–10, 12, 13]. This phosphorylated protein comigrated on Coomassie stains with C-protein enriched with a myofibrillar preparation of perfused guinea pig

hearts. The very same ^{32}P-labelled 150 kDa protein reacted with an antiserum raised against C-protein on Western blots and could be immunoprecipitated and detected by autoradiography. The immunoprecipitation with the antibody *per se* does not prove that the labelled protein is indeed C-protein. However, all biochemical and pharmacological observations combined provide strong circumstantial evidence that this is actually the case.

Inhibition of phosphatase activity led to an increased phosphorylation state of C-protein. The effects of CyA were time- and concentration-dependent and could be enhanced by isoproterenol. At nanomolar concentrations, CyA is known to inhibit both type 1 and 2A phosphatase activities (with similar IC_{50} values) in *skeletal* muscle [17] but not type 2B or 2C phosphatase activities. Moreover, CyA concentration-dependently inhibited phosphorylase phosphatase activity of guinea pig *ventricular* phosphatases (IC_{50}: 22 nmol/L [23]).

These findings agree with previous *in vitro* work which demonstrated that phosphorylated purified C-protein is a substrate for purified type 1 as well as type 2A phosphatases [15]. The present data suggest that both phosphorylation by different kinases as well as impairment of dephosphorylation can enhance the phosphorylation state in intact cells. The inhibition of phosphatase activity does not specifically enhance the phosphorylation of C-protein alone. The phosphorylation of, for instance, the inhibitory subunit of troponin is likewise enhanced by CyA. On the other hand, the present data do not rule out the involvement of CyA-insensitive phosphatases in the regulation of C-protein phosphorylation.

It may be asked what functional consequence follows CyA-induced C-protein phosphorylation. We have shown that CyA (starting at 1 μmol/L) can increase the force of contraction in isolated guinea pig papillary muscle [23]. Hence, it is conceivable that C-protein phosphorylation may be causally related to the positive inotropic effects of the phosphatase inhibitor CyA. However, the inotropic effect of CyA was not accompanied by a relaxant effect [23]. From earlier work [10] one might have predicted that phosphorylation of C-protein could lead to relaxation. However, as CyA affects the phosphorylation of several proteins, the resultant effects might neutralize each other. Further work on the functional role of C-protein is clearly warranted.

The main contribution of this work is the immunological identification of C-protein in ^{32}P-labelled mammalian cardiomyocytes and the demonstration that the phosphorylation state of cardiac C-protein can be enhanced by the phosphatase inhibitor CyA.

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REFERENCES

1. Offer G, Moos C and Starr R, A new protein of the thick filaments of vertebrate skeletal myofibrils. *J Mol Biol* **74**: 653–676, 1973.
2. England PJ, Pask HT and Miller D, Cyclic AMP-dependent phosphorylation of cardiac contractile proteins. *Adv Cyclic Nucleotide Protein Phosphorylation Res* **17**: 383–390, 1984.
3. Yamamoto K and Moos C, The C-proteins of rabbit red, white, and cardiac muscles. *J Biol Chem* **258**: 8395–8401, 1983.
4. Hartzell HC, Effects of phosphorylated and unphosphorylated C-protein on cardiac actomyosin ATPase. *J Mol Biol* **186**: 185–195, 1985.
5. Lim MS and Walsh MP, Phosphorylation of skeletal and cardiac muscle C-proteins by the catalytic subunit of cAMP-dependent protein kinase. *Biochem Cell Biol* **64**: 622–630, 1986.
6. Hofmann PA, Hartzell HC and Moos RL, Alterations in Ca^{2+} sensitive tension due to partial extraction of C-protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J Gen Physiol* **97**: 1141–1163, 1991.
7. Ray KP and England PJ, Phosphorylation of the inhibitory subunit of troponin and its effect on the calcium dependence of myofibril adenosine triphosphatases. *FEBS Lett* **70**: 11–16, 1976.
8. Jeacocke SA and England PJ, Phosphorylation of a myofibrillar protein of Mr 150 000 in perfused rat heart, and the tentative identification of this as C-protein. *FEBS Lett* **122**: 129–132, 1980.
9. Hartzell HC and Titus L, Effects of cholinergic and adrenergic agonists on phosphorylation of a 165,000-dalton myofibrillar protein in intact cardiac muscle. *J Biol Chem* **257**: 2110–2120, 1982.
10. Hartzell HC, Phosphorylation of C-protein in intact amphibian cardiac muscle. *J Gen Physiol* **83**: 563–588, 1984.
11. Solaro RJ, Protein phosphorylation and the cardiac myofilaments. In: *Protein Phosphorylation in Heart Muscle* (Ed. Solaro RJ), pp. 129–156. CRC Press Inc, Boca Raton, FL, U.S.A., 1987.
12. George EF, Romano FD and Dobson JR Jr, Adenosine and acetylcholine reduce isoproterenol-induced protein phosphorylation of rat myocytes. *J Mol Cell Cardiol* **23**: 749–764, 1991.
13. Venema RC and Kuo JF, Protein kinase C-mediated phosphorylation of troponin I and C-protein in isolated myocardial cells is associated with inhibition of myofibrillar actomyosin MgATPase. *J Biol Chem* **268**: 2705–2711, 1993.
14. Hartzell HC and Glass DB, Phosphorylation of purified cardiac muscle C-protein by purified cAMP-dependent and endogenous Ca^{2+}-calmodulin-dependent protein kinases. *J Biol Chem* **259**: 15587–15596, 1984.
15. Schlender KK, Hegazy MG and Thysseril TJ, Dephosphorylation of cardiac myofibril C-protein by protein phosphatase 1 and protein phosphatase 2A. *Biochim Biophys Acta* **928**: 312–319, 1987.
16. Shenolikar S and Nairn AC, Protein phosphatases: Recent progress. *Adv Second Messenger Phosphoprotein Res* **23**: 1–121, 1991.
17. Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Fusetani N, Watabe S, Hashimoto K, Uemura D and Hartshorne DJ, Calyculin A and okadaic acid: inhibitors of protein phosphatase activity. *Biochem Biophys Res Commun* **159**: 871–877, 1989.
18. Neumann J, Schmitz W, Scholz H and Stein B, Effects of adenosine analogues on contractile response and cAMP content in guinea-pig isolated ventricular myocytes. *Naunyn-Schmiedebergs Arch Pharmacol* **340**: 689–695, 1989.
19. Neumann J, Boknik P, Herzig S, Gupta RC, Schmitz W, Scholz H and Watanabe AM, Evidence for physiological functions of protein phosphatases in the

heart. Evaluation with okadaic acid. *Am J Physiol* **265**: H257–H266, 1993.

20. Neumann J, Bokník P, Bodor GS, Jones LR, Schmitz W and Scholz H, Effects of adenosine receptor and muscarinic cholinergic receptor agonists on cardiac protein phosphorylation. Influence of pertussis toxin. *J Pharmacol Exp Ther* **269**: 1310–1318, 1994.
21. Rapundalo ST, Solaro RJ and Kranias EG, Inotropic responses to isoproterenol and phosphodiesterase inhibitors in intact guinea pig hearts: Comparison of cyclic AMP levels and phosphorylation of sarcoplasmic reticulum and myofibrillar proteins. *Circ Res* **64**: 104–111, 1989.
22. Takano-Ohmuro H, Goldfine SM, Kojima T, Obinata T and Fischman DA, Size and charge heterogeneity of C-protein isoforms in avian skeletal muscle. Expression of six different isoforms in chicken muscle. *J Muscle Res Cell Motil* **10**: 369–378, 1989.
23. Neumann J, Bokník P, Herzig S, Schmitz W, Scholz H, Wiechen K and Zimmermann N, Biochemical and electrophysiological mechanisms of the positive inotropic effects of calyculin A, a protein phosphatase inhibitor. *J Pharmacol Exp Ther* **271**: 535–541, 1994.