The cardiac sarcoplasmic reticulum phospholamban kinase is a distinct δ-CaM kinase isozyme

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Abstract **Phospholamban is the regulator of the Ca^{2+}-ATPase in cardiac sarcoplasmic reticulum (SR). It is phosphorylated by a Ca^{2+}/calmodulin-dependent protein kinase (SRCaM kinase) which is closely associated with cardiac SR membrane preparations. We found that, upon renaturation of pig cardiac SR proteins, blotted onto PVDF membrane, two polypeptides of 54 and 52 kDa showed Ca^{2+}/calmodulin-dependent autophosphorylation. In Western blots of SR proteins, the 54/52 kDa polypeptides were recognized by an antibody specific for the δ-CaM kinase isoforms, but not by an anti-α-CaM kinase. The two polypeptides were selectively immunoprecipitated from solubilized SR vesicles with the anti-δ-CaM kinase. The CaM kinase inhibitors KN-62 and peptide CaMK-(281–302) inhibited the activity of the SRCaM kinase with IC_{50} values in the same range with those obtained for the brain isozyme. In addition, initial autophosphorylation (Ca^{2+}-dependent) produced a partially Ca^{2+}-independent enzyme while further autophosphorylation (Ca^{2+}-independent) made the enzyme completely Ca^{2+}-independent. Based on these results we suggest that the SRCaM kinase is a distinct δ-CaM kinase isozyme.**

Key words: CaM kinase; Phospholamban; Membrane phosphorylation; Renaturation (in situ); Sarcoplasmic reticulum; Pig heart

1. Introduction

Phospholamban (PLB) is a small pentameric protein complex [1] composed of identical subunits, each 52 amino acids long. PLB is proposed to be the key phosphoprotein in mediating the heart's contractile responses to β-adrenergic agonists, by regulating the activity of SERCA2a [2]. Dephosphorylated PLB is an inhibitor of SERCA2a while phosphorylation relieves the inhibitory effect. PLB has been shown to be phosphorylated, in vivo, during β-adrenergic stimulation at Ser-16 and Thr-17 [3]. Phosphorylations of these two residues, in vitro, are being catalyzed by cAMP-dependent protein kinase and CaM kinase, respectively [4].

The CaM kinase is the major multisubstrate protein kinase that is activated by increases in intracellular Ca^{2+} ion concentration (for review [5]). The CaM kinase is composed of a family of isoforms derived from four (α, β, γ and δ) closely related genes. Isoforms range in size from 50–60 kDa (SDS-PAGE) and form homomultimers or heteromultimers of 6–12 subunits. Each CaM kinase isoform contains an amino terminal catalytic domain, a central regulatory domain and a carboxy terminal association domain. The predominant difference between the isoforms is their variable domain between the regulatory and association domains. A hallmark of CaM kinase is autophosphorylation which converts the kinase to a Ca^{2+}-independent enzyme.

For a long time it was known that the cardiac SR contains an endogenous Ca^{2+}/CaM-dependent kinase which phosphorylates PLB [6]. Recently it was shown that this SRCaM kinase directly phosphorylates and activates SERCA2a, in addition to PLB [7,8]. Also, the CRC has been shown to be phosphorylated by the SRCaM kinase [7,9], although the physiological function of this later phosphorylation is not yet established. In skeletal muscle, phosphorylation of the CRC by the cytosolic CaM kinase leads to its activation while phosphorylation by the membrane bound CaM kinase leads to inhibition [10,11]. Attempts to characterize this SRCaM kinase have been limited [12,13]. In view of the central role of SRCaM kinase in the regulation of Ca^{2+} uptake and release by cardiac SR and the fact that the CaM kinase is a growing isozyme family we examined whether the cardiac SRCaM kinase is a definite member of this family and of which subtype. Our results suggest that it is a distinct δ-CaM kinase isozyme with subunits two polypeptides of 54 and 52 kDa, in approximately a 1:1 ratio.

2. Materials and methods

2.1. Materials

Chemicals were obtained from the following sources: protein-A Sepharose CL-4B, Triton X-100, Syntide-2, anti-rabbit IgG-POD, cholic acid sodium salt, Sigma; Phosphocellulose (P11), P81 filter paper, Whatman International; Leupeptin, soybean trypsin inhibitor, PMSF, DTT, Immobilon-P membrane, BSA (fraction V), Serva; Anti-mouse IgG-POD (fab fragments), anti-Ca^{2+}/Calmodulin-dependent protein kinase II (anti-α), Boehringer-Mannheim; calmodulin-Sepharose 4B, Pharmacia; KN-62, Biomol; CaM kinase II inhibitor (281–302), RBI; anti-mouse IgG + IgA + IgM (H + L), Zymed; ECL Western blotting detection kit, [γ-^{32}P]ATP (spec.act. 3000 Ci/mmol), Amersham; Protein Phosphatase-2A was prepared according to [14]. Calmodulin prepared according to [15] was a gift from Dr. I. Morano. CK2-DELTA (anti-δ) affinity purified polyclonal antibody [16] was kindly provided by Dr. H. Singer (Geisinger Clinic, Danville, PA, USA).

2.2. Purification of CaM kinase

Rat brain kinase was purified according to [17] with minor modifications. All subsequent steps were carried out at 4°C. Forebrains from 25 Wistar male rats were quickly removed and homogenized in a Teflon/glass homogenizer in Buffer A (1:10 w/v) containing 50 mM

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Abbreviations: CaM, calmodulin; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase II; SERCA2a, cardiac sarcoplasmic reticulum calcium pump; CaMK-(281–302), synthetic peptide analog of residues 281–302 of the α-subunit of the rat brain CaM kinase; CRC, calcium release channel of SR; PP-2A, protein phosphatase-2A; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride.

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HEPES (pH 7.5), 5 mM EGTA, 5 mM EDTA, 0.5 mM DTT, 0.3 mM PMSF, 10 mg/liter leupeptin and 40 mg/liter soybean trypsin inhibitor. The homogenate was centrifuged at 35,000 × *g* for 25 min. The pellet was rehomogenized (1:5 weight of original tissue/volume) in Buffer B and stirred slowly for 20 min. Buffer B was identical to Buffer A except for the following substitutions: 4 mM HEPES (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA. The rehomogenized material was then centrifuged at 35,000 × *g* for 25 min. The two 35,000 × *g* supernatants were pooled and centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was filtered twice through glasswool, adjusted to 50 mM HEPES (pH 7.5) and then applied to a phosphocellulose column (20 g) that had been equilibrated in Buffer C: 50 mM HEPES (pH 7.5), 1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 0.3 mM PMSF, 5 mg/liter leupeptin and 20 mg/liter soybean trypsin inhibitor. The column was washed with 2 column volumes of Buffer C and then 10 column volumes of Buffer C containing 0.2 M NaCl. The kinase was eluted with Buffer C containing 0.45 M NaCl. The active fractions (coincide with the protein peak) were pooled, adjusted to 50 mM HEPES (pH 7.5), 1 mM DTT, 0.6 mM $CaCl_2$, 3 mM magnesium acetate, 10% (v/v) glycerol, 0.2 M NaCl and loaded to a CaM-Sepharose affinity column (10 ml) which had been previously equilibrated in Buffer D: 50 mM HEPES (pH 7.5), 0.2 M NaCl, 1 mM DTT, 0.6 mM $CaCl_2$ and 3 mM magnesium acetate. After washing the column with 10 volumes of Buffer D containing 0.8 M NaCl the kinase was eluted with 50 mM HEPES (pH 7.5), 0.2 M NaCl, 2 mM EGTA, 1 mM DTT and 1 mM magnesium acetate. The active fractions were pooled, dialyzed in 50 mM HEPES (pH 7.5), 50% (v/v) glycerol, 10% (v/v) ethylene glycol, 0.5 mM EDTA, 1 mM DTT and stored at −25°C.

2.3. Preparation of SR membranes

A microsomal fraction enriched with SR vesicles from pig ventricular muscle was prepared according to [18] with the following modifications: (i) the homogenization buffer contained additionally 1 mg/liter leupeptin, 4 mg/liter soybean trypsin inhibitor and 0.2 mM PMSF; (ii) the final resulting pellet containing SR vesicles was suspended in buffer containing 10 mM HEPES (pH 7.2), 0.29 M sucrose, 0.1 M KCl and stored frozen (in small aliquots) in liquid N_2. Under these conditions the SRCaM kinase activity was stable up to nine months.

2.4. Synthetic peptide

The PLB-peptide, corresponding to amino acids 2–25 of PLB (cytoplasmic domain) according to the nomenclature given in [4,19] was synthesized by the solid phase method [20] and purified by reverse-phase HPLC on a Bischoft Polyencap 300 (10 mm particle size) column. The identity of the purified peptide was verified by amino acid analysis. Using the PLB-peptide as substrate for the SRCaM kinase, we obtained a specific activity of approximately 0.15 μmol P_i/min/mg of SR protein. The Ca^{2+}-independent activity was minimal (0.5% of the total activity).

2.5. Activity assay

The standard assay mixture contained, 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mg/ml BSA, 35 μM PLB-peptide, 0.1 mM [γ-^{32}P]ATP (50–100 μCi/μmol) with 1 mM EGTA (Ca^{2+}-independent activity) or with 0.4 mM $CaCl_2$ and 0.8 μM CaM (total activity) in a volume of 50 μl. Reactions were initiated by addition of SR membranes (0.5–1 μg), incubated 1–2 min at 30°C and terminated by addition of TCA to final concentration of 2.5% (w/v). Precipitated protein was pelleted by centrifugation and 40 μl of the supernatant were spotted onto Whatman P81 phosphocellulose paper, followed by washing in 75 mM phosphoric acid and counting Cerenkov radiation in a liquid scintillation counter [21]. The inhibition experiments with KN-62 were performed as described above except that 0.15 μM CaM was used, and the reaction was initiated by addition of [γ-^{32}P]ATP and CaM.

2.6. Autophosphorylation

5 μg of cardiac SR membranes were preincubated in 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 1 mg/ml BSA, 0.2 mM $CaCl_2$ and 0.4 μM CaM in the presence or absence of 0.1 mM ATP at 18°C. After 2 min the reaction was terminated by 4-fold dilution with ice cold STOP buffer: 50 mM HEPES (pH 7.5), 1 mg/ml BSA, 20% ethylene glycol and 10 mM EDTA. The activity of the autophosphorylated kinase was assayed immediately as described above. Ca^{2+}-independent autophosphorylation, was performed in the same manner, except that after 2 min of incubation a burst of Ca^{2+}-independent autophosphorylation was initiated by addition of excess EGTA (3.3 mM final) and the reaction was allowed to proceed for 1 min.

2.7. Immunoprecipitation

50 μg of SR membranes were autophosphorylated as described above (without BSA, at 30°C for 2 min) and the reaction was terminated by the addition of excess EDTA. Membrane proteins were solubilized with Buffer E (final concentrations: 25 mM HEPES, pH 7.5, 0.1 M NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 30 mM NaF, 1% Triton X-100, 4 mM sodium cholate, 0.5 mM PMSF and 0.5 mM DTT) for 1 h at 4°C. Insoluble material was pelleted by centrifugation at 100,000 × *g* for 30 min. The supernatant was diluted 10-fold with Buffer F (Buffer E diluted 10-fold including 0.1% BSA) and incubated overnight at 4°C with CK2-DELTA (or anti-α). The samples were further processed as described in [22]. When anti-α was used the protein-A Sepharose CL-4B was pretreated with anti-mouse IgG + IgA + IgM.

2.8. Electrophoresis, immunoblotting and autoradiography

SDS-PAGE was performed according to [23] using 10% polyacrylamide gels. For measurement of [^{32}P]P_i incorporation into native PLB, SR proteins were separated on Urea/SDS gels [24]. Autoradiograms were quantitated with a PDI (NY, USA) densitometer or bands of interest were cut out and counted by liquid scintillation. Immunoblotting was performed according to [25], using peroxidase conjugated secondary antibodies. Blots were developed using the ECL chemiluminescene kit.

2.9. Other methods

Protein concentration was determined by the Bio-Rad protein binding assay using bovine serum albumin as standard. In situ renaturation was performed as described in [26], including 5 μM of unlabelled ATP in the phosphorylation buffer.

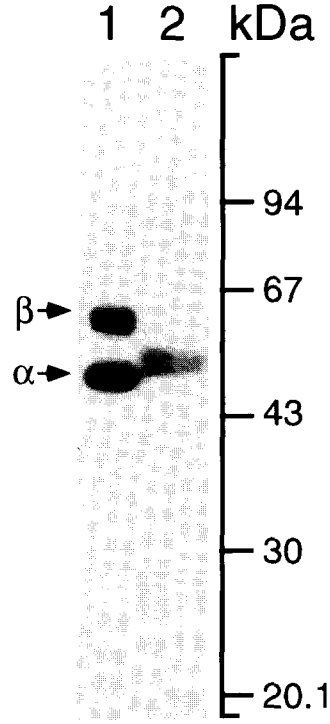


Fig. 1. Detection of CaM kinase activity, following in situ renaturation. Cardiac SR proteins were resolved on SDS-PAGE (10%), electroblotted onto Immobilon-P, and then renatured and phosphorylated in situ (in presence of Ca^{2+}/CaM). Lane 1: 2.5 μg of purified brain CaM kinase (α and β subunits) and lane 2: 50 μg of cardiac SR proteins. Molecular weight markers are given in kDa.

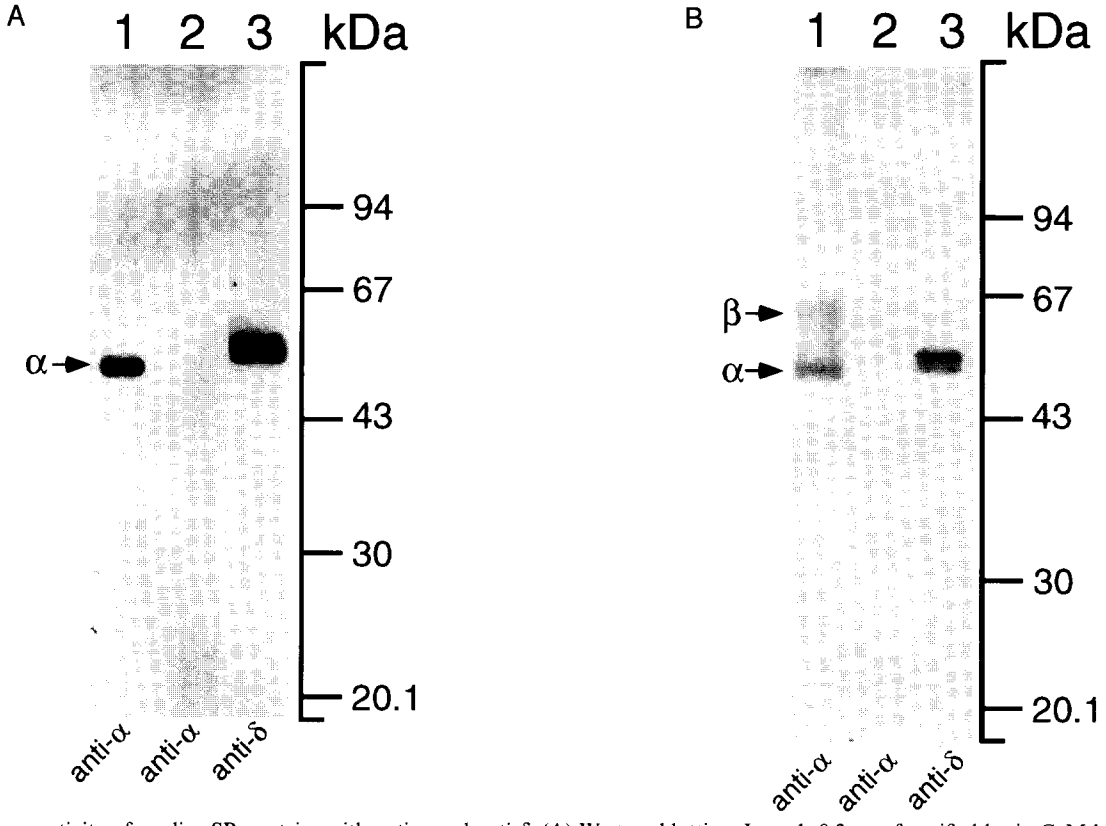


Fig. 2. Immunoreactivity of cardiac SR proteins with anti-α and anti-δ. (A) Western blotting. Lane 1: 0.2 μg of purified brain CaM kinase; lane 2 and lane 3, 10 μg of cardiac SR proteins. (B) Immunoprecipitation. Lane 1, 5 μg of purified brain CaM kinase; lane 2 and lane 3, 50 μg of cardiac SR proteins.

3. Results

In order to identify polypeptides exhibiting Ca^{2+}/CaM-dependent autophosphorylation activity in SR cardiac membranes, we adopted the in situ renaturation method, which detects CaM kinase activity after renaturation of proteins transferred from SDS-PAGE to PVDF membranes [26]. When autophosphorylation was performed in the presence of Ca^{2+}/CaM two polypeptides were detected, of 54 and 52 kDa (Fig. 1, lane 2). Under the same experimental conditions the brain CaM kinase showed two bands (Fig. 1, lane 1), of 50 kDa (α-subunit) and 60 kDa (β-subunits). In the absence of Ca^{2+}/CaM no signal was detected (not shown).

When SR proteins were immunoblotted using anti-δ, two highly cross-reactive bands were observed (Fig. 2A, lane 3), corresponding to the above identified polypeptides of 54/52 kDa. Also, in immunoprecipitation of solubilized SR proteins the anti-δ precipitated specifically the 54/52 kDa polypeptides (Fig. 2B, lane 3). A monoclonal antibody for the α-subunit of the brain CaM kinase (Fig. 2A and B, lanes 1) did not cross-react with SR proteins in immunoblotting (Fig. 2A, lane 2) and did not recognize solubilized SR proteins in immunoprecipitation (Fig. 2B, lane 2). Quantitation of the signals (integrated peak area) corresponding to the 54/52 kDa polypeptides from Western blotting, immunoprecipitation and in situ renaturation gives a relative ratio 1:1, suggesting that the two polypeptides are present in cardiac SR membranes in equal molar ratio.

Next we examined the effects of two CaM kinase inhibitors, KN-62 and peptide CaMK-(281–302), on the SRCaM kinase activity. The activity was measured by assaying PLB-peptide phosphorylation. The IC_{50} values obtained were 7 and 10 μM for KN-62 and peptide CaMK-(281–302), respectively. Similar IC_{50} values were obtained when syntide-2 or endogenous PLB were used as substrates.

The activity of the SRCaM kinase in its dephosphorylated state (Fig. 3, control) ranges from 0.5% (Ca^{2+}-independent activity) to 100% (total activity). Autophosphorylation (in presence of Ca^{2+}/CaM) generates a partially Ca^{2+}-independent form, with Ca^{2+}-independent activity of 25% (Fig. 3, partial). Additional autophosphorylation (in presence of excess EGTA) creates a second form of SRCaM kinase with suppressed total activity of 23% (Fig. 3, complete). Treatment of the two autophosphorylated forms with PP-2A (i) results in reduction of the Ca^{2+}-independent activity to 7% and 4.5% (Fig. 4, partial and complete, respectively), and (ii) partially restores the suppressed total activity to 55% (Fig. 4, complete).

4. Discussion

It is known that the selective inhibitor of CaM kinase, KN-62, inhibits competitively with Ca^{2+}/CaM by directly binding to the CaM binding site of CaM kinase [27]. We obtained an IC_{50} value of 7 μM for the SRCaM kinase which is in the same range with the IC_{50} value obtained for the brain CaM kinase 0.5 μM [28]. We can assume that the SRCaM kinase contains a CaM binding site similar to that of the CaM kinase.

The autoinhibitory domain of brain CaM kinase (residues 281–309) interacts with elements of the catalytic domain to suppress the phosphotransferase activity of the kinase [29]. In order to examine if the SRCaM kinase contains a similar autoinhibitory segment, we used the peptide CaMK-(281–302), which represents the minimum autoinhibitory segment of α-CaM kinase and does not significantly bind Ca^{2+}/CaM. We obtained an IC_{50} value of 10 μM which is similar to that obtained for the brain CaM kinase, 8 μM [30]. This result indicates that the SRCaM kinase does have an autoinhibitory domain in the same region as the α-subunit.

Autophosphorylation is likely the most important mechanism regulating CaM kinase activity, following stimulation by Ca^{2+}/CaM. Initial autophosphorylation of SRCaM kinase exhibits a requirement for Ca^{2+}/CaM and generates a partially Ca^{2+}-independent form of the kinase (Fig. 3, partial) with little loss in total activity, probably due to some thermal instability [31]. Once some Ca^{2+}-independent activity has been generated by initial autophosphorylation additional autophosphorylation proceeds in the absence of Ca^{2+}/CaM giving rise to inhibition of total activity and a completely Ca^{2+}-independent form (Fig. 4, complete). Addition of excess EGTA causes CaM to dissociate from the SRCaM kinase, thereby unmasking new autophosphorylation site(s). Therefore, the site(s) responsible for the suppression of the total activity should lie within the CaM binding domain of SRCaM kinase. PP-2A reduces the Ca^{2+}-independent activity and partially restores the inhibited total activity (Fig. 4).

Our results are consistent with the proposed model for the regulation of the brain CaM kinase by autophosphorylation [5]. It states that autophosphorylation results in multisite phosphorylation and separate autophosphorylation sites are responsible for (i) generation of Ca^{2+}-independent activity, and (ii) suppression of total activity. Dephosphorylation by protein phosphatases regenerates the native enzyme. The sites responsible for inhibition of the total activity of CaM kinase lie within its CaM binding domain [32].

The polypeptides of 54 and 52 kDa are the only cardiac SR proteins exhibiting Ca^{2+}/CaM-dependent autophosphorylation and high cross-reactivity with the anti-δ. So far, cDNA clones for four isoforms (δ_2, δ_3, δ_6 and δ_7) all splice variants of the δ-subunit have been detected in cardiac tissue [16,33]. The CK2-DELTA antibody is made to a 12 amino acid peptide from the unique C-terminus of the δ-subunit [16]. It recognizes all δ-subunit splice variants which have this C-terminus. Thus, we have to exclude δ_6 and δ_7 as candidate subunits for the SRCaM kinase, since they have a deleted C-terminus [33]. Recombinant δ_3 and δ_2 CaM kinaes isoforms purified from transfected COS-7 cells exhibit relative molecular masses of 54 and 52 kDa (from SDS-PAGE), respectively [34]. These values coincide with the relative molecular masses of the two polypeptides we have identified in our study, as the subunits of the SRCaM kinase.

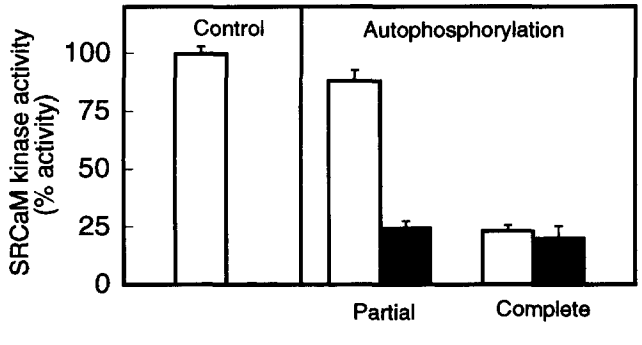


Fig. 3. Effects of autophosphorylation of SRCaM kinase on activity. Preincubation of cardiac SR membranes (5 μg) to allow autophosphorylation was performed with unlabelled ATP (100 μM) and the effect of autophosphorylation assessed by substrate (PLB-peptide) phosphorylation. Open bars represent total activity and dark bars Ca^{2+}-independent activity. Control, preincubation without ATP; partial, Ca^{2+}-dependent autophosphorylation; complete, Ca^{2+}-independent autophosphorylation (as described in section 2). Kinase activity ± S.D. of triplicate determinations is represented as % activity. 100% activity is defined as that determined in the presence of Ca^{2+}/CaM for the control (−ATP).

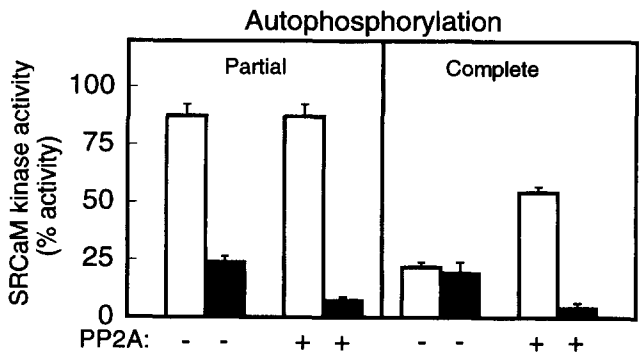


Fig. 4. Reversal of the effects of autophosphorylation by PP-2A. Phosphatase storage buffer (−) or PP-2A (+), were added to both forms (partial and complete) of autophosphorylated SRCaM kinase. Incubation was for 2 min at 30°C, aliquots were removed, diluted with ice-cold STOP buffer and kinase activities assayed as described in legend to Fig. 3. Open bars represent total activity and dark bars Ca^{2+}-independent activity. Kinase activity ± S.D. of triplicate determinations is represented as % activity.

Subcellular targeting of CaM kinase heteromultimers is believed to be determined by the ratio of the subunits. Excess of δ_3-isoform targets the holoenzyme to the nucleus, while excess of δ_2-isoform targets the holoenzyme to the cytosol [36]. Additional evidence in support of this hypothesis, is provided from studies on the subcellular distribution of the brain CaM kinase, where the relative ratio of its subunits ($\alpha:\beta/\beta'$) determines the subcellular localization of the holoenzyme [37]. Therefore, we propose that the CaM kinase heteromultimer containing an equal ratio of δ_3 and δ_2 subunits is targeted to cardiac SR membranes and is the SRCaM kinase identified in the present study.

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References

[1] Arkin, I.T., Adams, P.D., MacKenzie, K.R., Lemman, M.A., Brunger, A.T. and Engelman, D.M. (1994) EMBO J. 13, 4757–4764.

[2] Luo, W., Grupp, I.L., Harrer, J., Ponniah, S., Grupp, G., Duffy, J.J., Doetschman, T. and Kranias, E.G. (1994) Circ. Res. 75, 401–409.

[3] Wegener, A.D., Simmerman, H.K., Lindemann, J.P. and Jones, L.R. (1989) J. Biol. Chem. 264, 11468–11474.

[4] Simmerman, H.K.B., Collins, J.H., Theibert, J.L., Wegener, A.D. and Jones, L.R. (1986) J. Biol. Chem. 261, 13333–13341.
[5] Braun, A.P. and Schulman, H. (1995) Annu. Rev. Physiol. 57, 417–445.
[6] Le Peuch, C.J., Haiech, J. and Demaille, J.G. (1979) Biochemistry 18, 5150–5157.
[7] Xu, A., Hawkins, C. and Narayanan, N. (1993) J. Biol. Chem. 268, 8394–8397.
[8] Toyofuku, T., Curotto-Kurzydlowski, K., Narayanan, N. and MacLennan, D.H. (1994) J. Biol. Chem. 269, 26492–26496.
[9] Takasago, T., Imagawa, T., Furukawa, K., Ogurusu, T. and Shigekawa, M. (1991) J. Biochem. 109, 163–170.
[10] Wang, J. and Best, P.M. (1992) Nature 359, 739–741.
[11] Hain, J., Nath, S., Mayrleitner, M., Fleischer, S. and Schindler, H. (1994) Biophys. J. 67, 1823–1833.
[12] Molla, A. and Demaille, J.G. (1986) Biochemistry 25, 3415–3424.
[13] Jett, M.F., Schworer, C.M., Bass, M. and Soderling, T.R. (1987) Arch. Biochem. Biophys. 255, 354–360.
[14] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.J., Stralfors, P. and Tung, H.Y. (1988) Methods Enzymol. 159, 390–408.
[15] Gopalakrishna, R. and Anderson, A.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[16] Schworer, C.M., Rothblum, L.I., Thekkumkara, T.J. and Singer, H.A. (1993) J. Biol. Chem. 268, 14443–14449.
[17] Hashimoto, Y., Schworer, C.M., Colbran, R.J. and Soderling, T.R. (1987) J. Biol. Chem. 262, 8051–8055.
[18] Pegg, W. and Michalak, M. (1987) Am. J. Physiol. 252, H22–H31.
[19] Fujii, J., Ueno, A., Kitano, K., Tanaka, S., Kadoma, M. and Tada, M. (1987) J. Clin. Invest. 79, 301–304.
[20] Schnorrenberg, G. and Gerhardt, H. (1989) Tetrahedron 45, 7759–7762.
[21] Roskoski, R. Jr. (1983) Methods Enzymol. 99, 3–6.
[22] Haase, H., Karczewski, P., Beckert, R. and Krause, E.G. (1993) FEBS Lett. 335, 217–222.
[23] Laemmli, U.K. (1970) Nature 227, 680–685.
[24] Karczewski, P., Bartel, S. and Krause, E.G. (1990) Biochem. J. 266, 115–122.
[25] Haase, H., Wallukat, G., Flockerzi, V., Nastainczyk, W. and Hofmann, F. (1994) Receptors Channels 2, 41–52.
[26] Shackelford, D.A. and Zivin, J.A. (1993) Anal. Biochem. 211, 131–138.
[27] Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H. (1990) J. Biol. Chem. 265, 4315–4320.
[28] Enslen, H., Sun, P., Brickey, D., Soderling, S.H., Klamo, E. and Soderling, T.R. (1994) J. Biol. Chem. 269, 15520–15527.
[29] Smith, M.K., Colbran, R.J., Brickey, D.A. and Soderling, T.R. (1992) J. Biol. Chem. 267, 1761–1768.
[30] Tokumitsu, H., Brickey, D.A., Glod, J., Hidaka, H., Sikela, J. and Soderling, T.R. (1994) J. Biol. Chem. 269, 28640–28647.
[31] Lai, Y., Nairn, A.C. and Greengard, P. (1986) Proc. Natl. Acad. Sci. USA 83, 4253–4257.
[32] Hanson, P.I. and Schulman, H. (1992) J. Biol. Chem. 267, 17216–17224.
[33] Mayer, P., Mohlig, M., Schatz, H. and Pfeiffer, A. (1994) Biochem. J. 298, 757–758.
[34] Edman, C.F. and Schulman, H. (1994) Biochim. Biophys. Acta 1221, 89–101.
[37] Vallano, M.L. (1989) J. Neurosci. Methods 30, 1–9.