Phosphorylation of the purified cardiac ryanodine receptor by exogenous and endogenous protein kinases

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The ryanodine receptor is the main Ca^{2+}-release structure in skeletal and cardiac sarcoplasmic reticulum. In both tissues, phosphorylation of the ryanodine receptor has been proposed to be involved in the regulation of Ca^{2+} release. In the present study, we have examined the ability of the purified cardiac ryanodine receptor to serve as a substrate for phosphorylation by exogenously added catalytic subunit of the cyclic AMP (cAMP)-dependent protein kinase (PK-A), cyclic GMP (cGMP)-dependent protein kinase (PK-G), or calmodulin-dependent protein kinase (PK-CaM). A large amount of phosphate incorporation was observed for PK-CaM (938 ± 48 pmol of P_i/mg of purified channel protein), whereas the level of phosphorylation was considerably lower with PK-A or PK-G (345 ± 139 and 96 ± 6 pmol/mg respectively). In addition, endogenous PK-CaM activity co-migrates with the ryanodine receptor through several steps of purification, suggesting a strong association of the two proteins. This endogenous PK-CaM activity is abolished by a PK-CaM-specific synthetic peptide inhibitor. Endogenous cAMP- and cGMP-dependent phosphorylation was not observed in the purified ryanodine-receptor preparation. Taken together, these observations imply that PK-CaM is the physiologically relevant protein kinase, capable of phosphorylating the channel protein to a minimum stoichiometry of 2 mol of P_i per mol of tetramer.

INTRODUCTION

Depolarization of muscle cells triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via a specialized channel, which displays high-affinity binding for the plant alkaloid ryanodine (for reviews see [1,2]). The so-called 'foot structure' of the terminal cisternae represents the morphological correlate of the ryanodine receptor [3–5]. The ryanodine-receptor–Ca^{2+}-channel complex was purified from the SR of cardiac and skeletal muscle and consists of a single polypeptide chain, which is believed to associate as a tetramer to form a channel [3–8]. Both cardiac and skeletal muscle ryanodine receptors show a wide spectrum of similarities, such as activation by Ca^{2+}, caffeine and adenine nucleotides, and inhibition by Mg^{2+}, Ruthenium Red and calmodulin (CaM) [3,5,9–12]. Reconstitution of the purified cardiac ryanodine receptor in lipid bilayers yields a channel activity with properties comparable with those of SR Ca^{2+}-release channel *in situ* [5,8,9]. Similarly, a typical ryanodine-receptor-channel activity is observed following heterologous expression of the cDNA coding for the ryanodine receptor [13].

Since the ryanodine receptor plays a pivotal role in excitation–contraction coupling, this protein is a candidate for direct regulation by intracellular signalling pathways. Phosphorylation by various protein kinases, in particular, has been proposed as an important control mechanism [14–16]; however, direct evidence for stoichiometric phosphorylation of the purified cardiac ryanodine receptor has not yet been obtained. In the present study, we have therefore assessed the ability of the purified cardiac ryanodine receptor to serve as a substrate for cyclic AMP (cAMP)-, cyclic GMP (cGMP)- and CaM-dependent protein kinases (PK-A, PK-G and PK-CaM). Our findings show that PK-CaM is capable of phosphorylating the cardiac ryanodine receptor to a minimum stoichiometry of 2 mol/mol of tetramer and suggest that this pathway is involved in the physiological modulation of ryanodine-receptor-channel activity.

MATERIALS AND METHODS

Materials

PK-G prepared from bovine lung [17] was generously given by Dr. F. Hofmann and Dr. W. Landgraf (Munich, Germany). Aprotinin was a gift from Bayer-Pharma (Vienna, Austria). Interferon-γ was kindly provided by Dr. G. Adolf (Bender, Vienna, Austria). [γ-^{32}P]ATP and [^{3}H]ryanodine were purchased from DuPont/New England Nuclear (Boston, MA, U.S.A.); unlabelled ryanodine was from Agrisystems International (Wind Gap, PA, U.S.A.); ATP, CHAPS, MOPS, phosphatidylcholine (from soybean, type IV-S), catalytic subunit of PK-A (from bovine heart), leupeptin, pepstatin, phenylmethanesulphonyl fluoride (PMSF), heparin–agarose and *p*-aminobenzamidine–agarose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); CaM–Sepharose, phenyl-Sepharose, Sephadex G-50 and G-25 (fine grade), electrophoresis calibration kits for high- and low-molecular-mass standards were from Pharmacia (Uppsala, Sweden); EGTA and Nonidet P40 were from Fluka Chemical AG (Buchs, Switzerland). All other compounds were of analytical grade.

Preparation of SR

SR from bovine heart was prepared as described previously [18] with slight modifications. Briefly, bovine heart, obtained from the slaughter-house, was homogenized in a Waring blender for 30 s in a medium containing 40 mM Tris/HCl (pH 7.0), 300 mM sucrose and 2 mM ascorbic acid. The homogenate was centrifuged at 4000 *g* for 30 min, and the supernatant was

Abbreviations used: SR, sarcoplasmic reticulum; CaM, calmodulin; cAMP, cyclic AMP; cGMP, cyclic GMP; PK-CaM, CaM-dependent protein kinase; PK-A, catalytic subunit of the cAMP-dependent protein kinase; PK-G, cGMP-dependent protein kinase; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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filtered through cheesecloth and again centrifuged for 1 h at 44000 *g*. The pellets were resuspended in a medium containing 10 mM histidine (pH 7.0), 600 mM KCl, 300 mM sucrose, 2.25 mM $MgCl_2$ and 2 mM ATP and centrifuged at 100000 *g* for 1 h. The pellets were washed in 10 mM histidine (pH 7.0) and 300 mM sucrose, centrifuged again at 100000 *g* for 35 min, resuspended in the same medium and stored at −80 °C. To limit proteolytic activity, the following protease inhibitors were added to all media used during preparation: 0.1 mM PMSF, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin and 1 μM pepstatin. All steps were carried out at 4 °C.

Preparation of the ryanodine receptor

The ryanodine receptor was purified from bovine cardiac SR fraction as described for skeletal muscle [19]. Briefly, the sarcoplasmic reticulum (15 mg/ml) was solubilized in medium A [50 mM Mops, pH 7.0, 5 mM NaN_3, 1 M NaCl, 2 mM dithiothreitol (DTT), 1.6% CHAPS, 2.5 mg/ml phosphatidylcholine, 0.5 μg/ml leupeptin, 0.1 mM PMSF, 1.4 μg/ml aprotinin, 1 μM pepstatin] and 300 nM [^{3}H]ryanodine for 30 min at room temperature. Subsequently, the solubilized protein was centrifuged for 60 min at 40 000 *g* (Beckman 65 rotor). The supernatant was centrifuged through a linear sucrose gradient (5–20% in the solubilization medium A, containing only 1% CHAPS) at 26000 rev./min for 16 h at 3 °C (Beckman SW 28 rotor, 38 ml tubes). The fractions of the sucrose gradient containing the [^{3}H]ryanodine-labelled peak were collected, and applied to a heparin–agarose column (15 cm × 1 cm) equilibrated in medium B (20 mM Tris/HCl, pH 7.4), 100 mM NaCl, 300 mM sucrose, 2 mM DTT, 1% CHAPS, 2.5 mg/ml phosphatidylcholine, 5 mM NaN_3, 0.5 μg/ml leupeptin, 0.1 mM PMSF, 1.4 μg/ml aprotinin and 1 μM pepstatin). The column was washed with medium B, and the ryanodine receptor was eluted with a linear 0.1–0.8 M NaCl gradient in medium B. The [^{3}H]ryanodine-labelled fractions were collected and dialysed for 2 h in medium B without NaCl. Subsequently, the protein was applied to a benzamidine–agarose column (3 cm × 0.5 cm) equilibrated in medium B and eluted with the same medium, containing 0.8 M NaCl. To remove detergent, the [^{3}H]ryanodine-labelled peak was dialysed for 48 h in 20 mM Mops (pH 7.0)/5 mM NaN_3/2 mM DTT/300 mM sucrose containing 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin and 0.1 mM PMSF and stored finally at −80 °C. All preparation steps were carried out at 4 °C. The overall yield of the preparation was about 0.1 mg of purified ryanodine receptor per 200 mg of cardiac SR.

For [^{3}H]ryanodine binding, the ryanodine receptor (0.15–0.7 μg) was incubated in a final volume of 50 μl of medium C (50 mM Mops, pH 7.0, 1 M NaCl, 5 mM NaN_3, 2 mM DTT, 30 μM $CaCl_2$, 0.125% CHAPS, 0.0625% phosphatidylcholine) and 300 nM [^{3}H]ryanodine. The incubation was carried out for 90–120 min at room temperature, which resulted in equilibrium binding. Subsequently, the samples were diluted 4-fold with medium C. Portions (100 μl) were applied on to a 1 ml Sephadex G-25 (G-50 for SR) column equilibrated in medium C and centrifuged at 100 *g* for 9 min. Samples of the eluate were counted for radioactivity in a liquid-scintillation counter. Non-specific binding was determined in the presence of unlabelled ryanodine at a concentration of 30 μM. These binding values were comparable with the blank observed in the absence of purified ryanodine receptor. [^{3}H]Ryanodine binding to the purified ryanodine receptor was 146 ± 55 pmol/mg (mean ± S.E.M.; $n = 9$), which corresponds to the levels observed by others [2].

[^{3}H]Ryanodine binding to SR (1 mg/ml) was performed under identical conditions, but without CHAPS and phosphatidylcholine.

Preparation of CaM and PK-CaM

CaM was prepared from bovine brain (obtained from the slaughter-house) by phenyl-Sepharose affinity chromatography as described previously [20]. The purification of PK-CaM was carried out as described previously [19,21]. Heavy SR fractions from skeletal muscle, which had been prepared in the absence of ATP and depleted of CaM, were used as starting material.

Phosphorylation reactions

Phosphorylation was carried out on SR (1 mg/ml) or the purified ryanodine receptor (30–80 μg/ml) with the following exogenous protein kinases in a final volume of 25 or 50 μl. The phosphorylation with PK-CaM (0.6–0.8 μg) was performed in Ca^{2+}-medium (50 mM Mops, pH 7.0, 5 mM NaN_3, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM $CaCl_2$) and 5 μM CaM.

Phosphorylation with the catalytic subunit of PK-A (0.7–1.4 μg) was assayed in EGTA-medium (50 mM Mops, pH 7.0), 5 mM NaN_3, 5 mM $MgCl_2$, 2 mM EGTA). Phosphorylation by PK-G (0.5–2 μg) was measured under the conditions described for PK-A, in the presence of 100 μM cGMP. Assay blanks were determined in the absence of SR or purified ryanodine receptor. Endogenous phosphorylation was assessed under conditions identical with those described for PK-CaM, PK-A or PK-G in the presence of 2.5 μM CaM, 100 μM cAMP or 100 μM cGMP respectively, but in the absence of exogenous protein kinase. Blank phosphorylation for endogenous phosphorylation was done in the absence of CaM, cAMP or cGMP, respectively. After subtracting the level of blank phosphorylation, the amount of phosphorylation was expressed as pmol/mg of SR or purified ryanodine receptor. Additive phosphorylation was performed in Ca^{2+}-medium with PK-A (1.4 μg) and PK-CaM (0.7 μg; including 5 μM CaM) from the beginning of the reaction. Sequential phosphorylation was performed in the same medium by preincubating for 10 min with PK-A (1.4 μg) and then adding PK-CaM (0.7 μg; including 5 μM CaM) for an additional 10 min. The sequence of kinase additions was also reversed. The phosphorylation reactions were carried out at 25 °C and started by addition of 3–750 μM [γ-^{32}P]ATP. Due to Ca^{2+}-ATPase activity in the SR, in particular in Ca^{2+}-medium, high concentrations of ATP have to be present when the phosphorylation reactions are carried out with these membranes. Incubation times (0–20 min) are indicated in the legends of the respective Figures and Tables. Phosphorylation was terminated by addition of Laemmli sample buffer [22]. [^{32}P]P_i incorporation was quantified by excision of the ryanodine-receptor band after SDS/PAGE [22] and autoradiography. The gel pieces were treated with H_2O_2/water (1:1, v/v) and counted for radioactivity by liquid-scintillation spectrometry.

PK-A activity in the presence of CHAPS and the inhibitor peptide was tested with interferon-γ as an artificial substrate [23]. We have preferred to use interferon-γ rather than the small peptide kemptide, since the molecular mass of interferon-γ makes it possible to assay phosphorylation by SDS/PAGE in a manner analogous to the conditions for the ryanodine receptor. Phosphorylation of interferon-γ with PK-A (0.29 μg) was carried out in the presence of 20 mM Tris/HCl (pH 7.2), 100 mM NaCl, 12 mM $MgCl_2$, 6 μM [γ-^{32}P]ATP and various agents as indicated in the Figure legends.

Protein concentration was measured by the method of Lowry

[24] or Kaplan [25] (for samples containing phospholipids) using bovine serum albumin as a standard.

RESULTS

Phosphorylation of the cardiac ryanodine receptor in SR

Initially, experiments were designed to identify endogenous kinases capable of phosphorylating the ryanodine receptor in the cardiac SR. Following autoradiography, the phosphorylated ryanodine receptor was excised from the gel and quantified. In Figure 1 the time course of the phosphorylation of the ryanodine receptor under various conditions is given. Addition of CaM in the Ca^{2+}-medium promotes the phosphorylation of a high-molecular-mass protein band, which co-migrates with the purified ryanodine receptor, to about 3.5 pmol/mg of SR. In contrast, sole addition of cAMP and cGMP results in the incorporation of 0.4–1 pmol/mg (results not shown), a value that is comparable with the phosphorylation in the presence of CaM in EGTA-medium (Figure 1). Endogenous phosphorylation by sole addition of CaM, cAMP or cGMP was drastically increased by the addition of exogenous protein kinases, namely PK-CAM, PK-A and PK-G (Figure 1, Table 1). The reaction reached equilibrium within 5–10 min, irrespective of whether endogenous or ex-

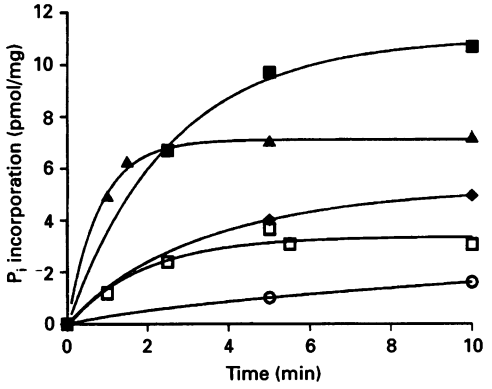

Figure 1 Time course of phosphorylation of the ryanodine receptor in SR

SR (1 mg/ml) was incubated in the presence of 500 μM [γ-^{32}P]ATP at 25 °C in 50 μl. PK-CaM (0.8 μg)-dependent phosphorylation (■) was carried out in Ca^{2+}-medium and 5 μM CaM. Endogenous phosphorylation was carried out in the presence of 5 μM CaM in Ca^{2+}-medium (□) or EGTA-medium (○) in the absence of exogenous kinases. PK-A (1.4 μg; ▲) and PK-G (2 μg; ◆)-dependent phosphorylation was carried out in EGTA-medium; 100 μM cGMP was present in the PK-G assay. Reactions were stopped by the addition of Laemmli sample buffer, and the samples were subjected to SDS/PAGE. The ryanodine-receptor band was excised and counted for radioactivity in a liquid-scintillation counter. Blank phosphorylation was subtracted from the respective data points generated in the presence of kinases or CaM as described in the Materials and methods section.

Table 1 Quantification of phosphorylation of the ryanodine receptor by various protein kinases

Phosphorylation conditions were as described in the legends to Figure 1 (for SR) and Figure 3 (for purified ryanodine receptor); the reaction mixture was resolved by SDS/PAGE and the ryanodine-receptor band was excised from the gel and counted for radioactivity. Blanks without ryanodine receptor (or SR), but with the kinase, were subtracted. Blanks for endogenous CaM-dependent phosphorylation were obtained in the absence of CaM. Endogenous phosphorylation of the purified ryanodine receptor (RyR; 0.6 μg) was performed in Ca^{2+}-medium containing 2.5 μM CaM and 8 μM [γ-^{32}P]ATP. SR (1 mg/ml) was phosphorylated in the presence of 500 μM [γ-^{32}P]ATP under the conditions given in Figure 1. Data represent means ± S.D. of at least three experiments.

	Phosphorylation			
	PK-CaM	PK-A	PK-G	CaM
SR	12.8 ± 4.0	7.1 ± 1.0	3.5 ± 1.7	3.5 ± 1.4
RyR	938 ± 48.3	345 ± 139	96 ± 6	76 ± 27

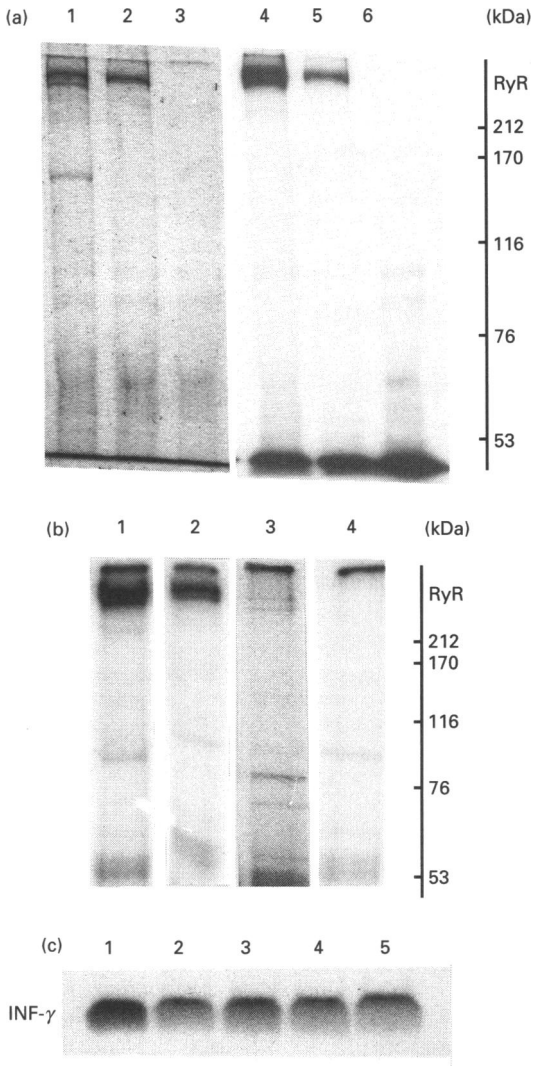

Figure 2 Phosphorylation of the ryanodine receptor and of interferon-γ

(**a**) Phosphorylation of the purified ryanodine receptor before and after dialysis. Coomassie-Blue-stained SDS/PAGE (lanes 1–3) and the autoradiography (lanes 4–6) are shown. Phosphorylation was carried out for 20 min at 25 °C in the presence of EGTA-medium, 8 μM [γ-^{32}P]ATP, 0.7 μg of the catalytic subunit of PK-A (lanes 1–6) and 0.4 μg of ryanodine receptor (RyR) before (lanes 2 and 5) and after (lanes 1 and 4) dialysis. Lanes 3 and 6 contained no added ryanodine receptor. (**b**) Autoradiogram of the phosphorylation of the dialysed ryanodine receptor (RyR) with PK-A (lane 1), after exogenous re-addition of 0.5% or 1% CHAPS (lanes 2 and 3 respectively) and after heat inactivation (15 min exposure to 80 °C; lane 4) were carried out under the above conditions. (c) Phosphorylation of interferon-γ. Interferon-γ (INF-γ; 200 ng) was phosphorylated for 45 min at 25 °C in the presence of 20 mM Tris/HCl (pH 7.2), 100 mM NaCl, 12 mM $MgCl_2$, 10 units of PK-A (0.29 μg) and 6 μM [γ-^{32}P]ATP (lane 1). Increasing concentrations of CHAPS (0.1%, 0.5%, 1%, 3%; lanes 2–5 respectively) were added to the incubation. The X-ray exposure of an SDS/15%-acrylamide gel within the molecular-mass range 14–22 kDa is shown.

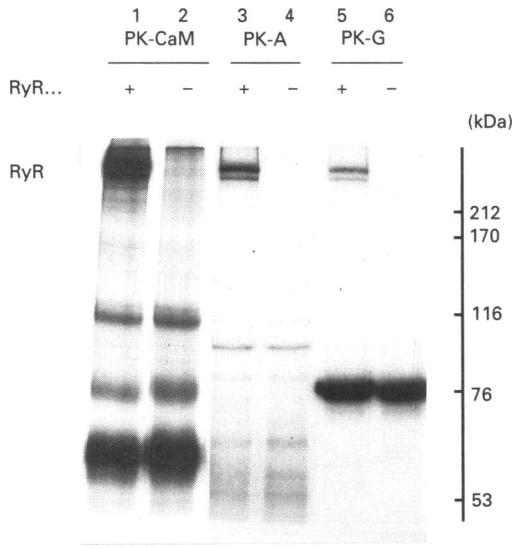

Figure 3 Phosphorylation of the purified ryanodine receptor by PK-CaM, PK-A or PK-G

Phosphorylation with 10 μM [γ-^{32}P]ATP was carried out for 25 min (25 °C) in the presence (lanes 1, 3 and 5) or absence (lanes 2, 4 and 6) of the purified ryanodine receptor (RyR; 0.7 μg). PK-CaM (0.6 μg)-dependent phosphorylation (lanes 1 and 2) was done in Ca^{2+}-medium and 5 μM CaM. PK-A (1.4 μg; lanes 3 and 4) and PK-G (2 μg; lanes 5 and 6) were incubated in EGTA-medium; for PK-G 100 μM cGMP was added. The reaction was terminated with Laemmli sample buffer. The autoradiograph of an SDS/7%-acrylamide gel is shown.

ogenous kinases were assayed. The highest level of phosphorylation was obtained by exogenous addition of PK-CaM (12.8 pmol/mg) and to an lesser extent with PK-A (7.1 pmol/mg). The number of ryanodine-binding sites determined from experiments with 300 nM [^{3}H]ryanodine was 6 ± 0.8 pmol/mg of SR (mean $\pm$ S.D., $n = 2$). Hence the stoichiometry of P_i incorporation for PK-CaM and PK-A corresponds to 2 and 1 phosphorylation sites per ryanodine-binding site respectively. This suggests that the ryanodine receptor may be phosphorylated by exogenous protein kinases to stoichiometric levels, in particular by PK-CaM and PK-A.

Phosphorylation of the purified cardiac ryanodine receptor

In order to investigate the phosphorylation of the ryanodine receptor, the ability of the purified ryanodine receptor to serve as a substrate for stoichiometric phosphorylation by protein kinases has been examined. Throughout the purification procedure, the ryanodine receptor was kept in CHAPS and phosphatidylcholine. In the final step of the purification, the protein was dialysed to remove CHAPS. Before dialysis, the purified ryanodine receptor was detected after gel electrophoresis as a single band (Figure 2a, lane 2), whereas, after dialysis, a doublet appeared with an additional fragment migrating at 170 kDa (Figure 2a, lane 1). Proteolysis is the most likely explanation for this phenomenon, which has been noted previously [4]. However, phosphorylation by exogenously added PK-A and PK-CaM (results not shown) requires removal of detergent. Only low levels of phosphate incorporation into the ryanodine receptor are detected before dialysis (Figure 2a). We rule out the possibility that the phosphorylation following dialysis is an artifact, e.g. arising from the exposure of the phosphorylation sites by denaturation or proteolytic cleavage during dialysis, for the following two reasons. (i) Exogenous re-addition of 1% CHAPS to the dialysed receptor results in drastically decreased phosphorylation (< 90%; Figure 2b). In order to rule out an inhibitory effect of the detergent on PK-A activity, interferon-γ was used as an artificial substrate for PK-A [23]. At 0.1% CHAPS, a concentration well below the critical micellar concentration, a modest inhibition of PK-A is observed (20–30%). However, concentrations of CHAPS of up to 3% had no additional inhibitory effect (Figure 2c). (ii) Heat inactivation of the ryanodine receptor completely abolished phosphorylation (Figure 2b). Taken together, these observations suggest that CHAPS primarily effects the conformation of the ryanodine receptor and to a lesser extent the protein kinase directly. Hence, only the dialysed ryanodine receptor was used in all the following experiments.

All three protein kinases tested, namely PK-CaM, PK-A and PK-G, were capable of phosphorylating the purified ryanodine receptor (Figure 3). The highest level of phosphate incorporation was obtained with exogenous PK-CaM (Figure 3 and Table 1). Comparable with SR, PK-A phosphorylated the purified ryanodine receptor to about half of the level obtained with PK-CaM. In contrast, the level of phosphorylation with PK-G compared with PK-CaM was about 25% in SR, but only 10% for the purified receptor (Table 1). This difference did not arise from insufficient levels of added protein kinases or a different nucleotide requirement; the stoichiometries of phosphorylation remained virtually unchanged when the amount of PK-A, PK-G or PK-CaM was increased in the range of 0.5–2 μg. Nevertheless, a high protein kinase concentration was used to optimize the phosphorylation reaction. Optimal phosphorylation with PK-CaM was observed at concentrations of CaM up to 2.5–5 μM (results not shown).

Taking a molecular mass of 564 kDa for one ryanodine receptor subunit [26], a minimum stoichiometry of PK-CaM-dependent phosphorylation of 0.5 mol/mol or two phosphorylation sites per tetramer was obtained (938 pmol of P_i incorporated per mg of purified ryanodine receptor).

As noted above, the ryanodine receptor was phosphorylated in the absence of any exogenous protein kinase (Table 1). We have identified this kinase activity as PK-CaM by the following criteria (Figure 4). (i) The endogenous phosphorylation was strictly dependent on the addition of CaM; 76 ± 27 pmol of P_i was incorporated per mg of purified ryanodine receptor; sole addition of cAMP or cGMP resulted in the detection of only 4.5–6.3 pmol of P_i/mg, irrespective of whether phosphorylation was carried out in Ca^{2+}-containing medium or EGTA-medium (see Figure 4). (ii) Addition of CaM also resulted in the observation of a phosphoprotein band at 58 kDa, which corresponds to the PK-CaM subunit (Figure 4a) [21]. (iii) A synthetic peptide, capable of specifically inhibiting CaM binding to the PK-CaM II from brain [27], decreased the endogenous phosphorylation of the purified ryanodine receptor to a value observed in the absence of CaM (2.5 pmol/mg; Figure 4a). In similar experiments carried out on SR membranes, the endogenous CaM-dependent phosphorylation was also inhibited by this synthetic peptide (results not shown).

In order to rule out the possibility that exogenous PK-A phosphorylates the endogenous PK-CaM, which then phosphorylates the ryanodine receptor, we have used the inhibitor peptide against the PK-CaM II. Inclusion or omission of Ca^{2+} had no effect on the PK-A-catalysed incorporation of P_i into the ryanodine receptor. In Ca^{2+}-medium, the inhibitor peptide had no effect on PK-A-dependent phosphorylation of the ryanodine receptor (Figure 4b). In EGTA-medium, the inhibitor peptide appears to produce a small increase in P_i incorporation (Figure 4b). To summarize, these data show that

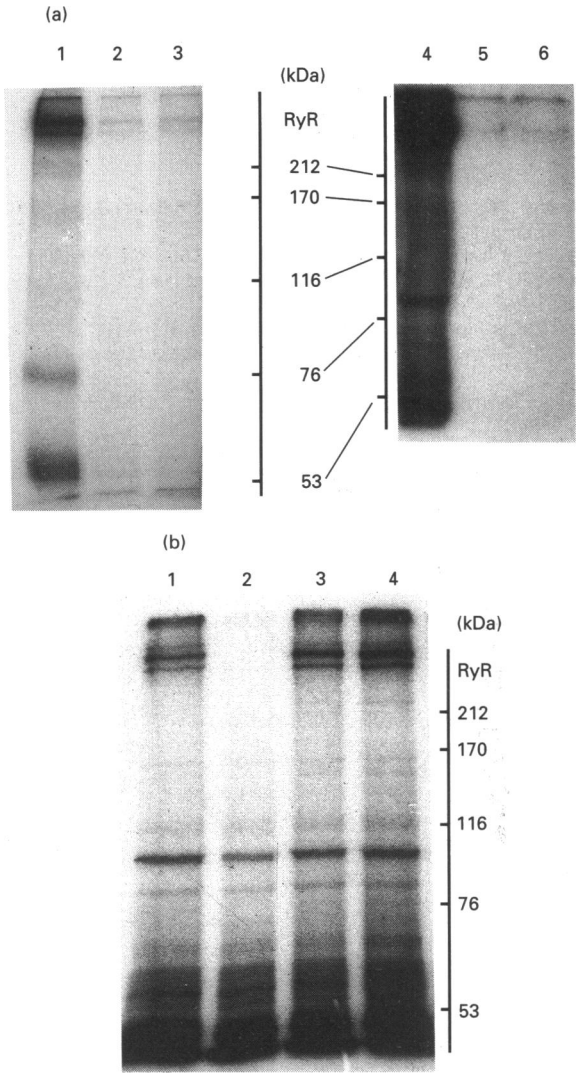

Figure 4 (a) Endogenous phosphorylation of the purified ryanodine receptor; (b) effect of the inhibitor peptide on the phosphorylation of the ryanodine receptor by PK-A

(**a**) Purified ryanodine receptor (RyR; 0.6 μg) was incubated for 25 min at 25 °C in 8 μM [γ-^{32}P]ATP, Ca^{2+}-medium (lanes 1–3) or EGTA-medium (lanes 4–6). Lanes: 1, 2.5 μM CaM; 2, no CaM; 3, 2.5 μM CaM and 30 μM inhibitor peptide of the PK-CaM II [23]; 4, PK-A (1.4 μg); 5, 100 μM cAMP; 6, 100 μM cGMP. The autoradiography of an SDS/7%-acrylamide gel is shown. (**b**) The ryanodine receptor (RyR; 0.35 μg) was phosphorylated by PK-A (1.4 μg) in 8 μM [γ-^{32}P]ATP, Ca^{2+}-medium (lanes 1 and 3) or EGTA-medium (lane 4) for 20 min at 25 °C; 30 μM inhibitor peptide was added in lanes 3 and 4. Lane 2 demonstrates the PK-A under phosphorylation conditions of lane 1 without added ryanodine receptor. The autoradiograph of an SDS/7%-acrylamide gel is shown.

PK-A does not phosphorylate the ryanodine receptor via activation of the endogenous PK-CaM. Control experiments were carried out with the artificial substrate interferon-γ in order to verify that the peptide did not affect PK-A activity; we failed to detect any effect of the inhibitor peptide over the concentration range 1–30 μM (results not shown).

The data presented so far suggest that PK-CaM and also PK-A are capable of phosphorylating the ryanodine receptor, both in SR membranes and after purification. We tested whether this reaction occurs on the same site by determining the stoichiometries after addition of both enzymes. The reactions were carried out in the presence of Ca^{2+}-medium, which results in no difference for PK-A activity (320 ± 126 pmol/mg; mean $\pm$ S.D., $n = 3$) compared with EGTA-medium (see Table 1). Irrespective of whether PK-A and PK-CaM were added simultaneously or sequentially, this combination of kinases did not result in phosphorylation exceeding the value for PK-CaM alone. In fact, the level of phosphorylation was consistently lower (721 ± 168 pmol/mg; mean $\pm$ S.D., $n = 4$). If PK-CaM was added first (10 min preincubation), no further increment in phosphorylation could be obtained by addition of PK-A. These observations suggest that the two kinases compete for a common phosphorylation site and that maximum phosphorylation of the purified ryanodine receptor is obtained with PK-CaM alone.

DISCUSSION

Electrophysiological experiments, where addition of PK-CaM has been shown to alter the gating characteristics of the SR Ca^{2+}-release channel [28,30], suggest an important role for phosphorylation in the regulation of ryanodine-receptor function. However, previous attempts to phosphorylate the purified cardiac ryanodine receptor have resulted in poor stoichiometries [28,31], thus casting doubt on the functional relevance of phosphorylation. The present work demonstrates that the purified ryanodine receptor is phosphorylated by exogenous PK-CaM to a minimum stoichiometry of 2 mol of P_i/mol of receptor tetramer. The stoichiometry is probably higher, since no correction has been made for protein losses during SDS/PAGE. Furthermore, partial denaturation of the ryanodine receptor during purification cannot be excluded. A possible explanation for the earlier failures to obtain stoichiometric P_i incorporation into the purified ryanodine receptor is provided by the observation that optimal reaction conditions require the removal of detergent. The purified ryanodine receptor was obtained by a combination of sucrose-density centrifugation and affinity chromatography. In the final step of this preparation, extensive dialysis minimized the concentration of detergent, leading to a higher yield in phosphorylation. This finding was confirmed by the observation that re-addition of CHAPS diminished the level of phosphorylation (Figure 2b), which was mainly due to the interaction of the detergent with the ryanodine receptor. Furthermore, during our preparation the ryanodine receptor was labelled with [^{3}H]ryanodine. It is possible that under these conditions the protein is more stable, and functional losses are therefore decreased.

The stoichiometry of phosphate incorporation obtained with PK-CaM is consistent with the involvement of this kinase in the regulation of the ryanodine receptor. This interpretation is substantiated by two additional observations. (i) Sole addition of CaM to the SR results in the phosphorylation of the ryanodine receptor via an endogenous PK-CaM. In contrast, sole addition of cAMP or cGMP to SR membranes resulted in only low levels of phosphorylation of the ryanodine receptor. (ii) A CaM-dependent protein kinase activity co-purifies with the ryanodine receptor over several purification steps (Figure 4a). The presence of a PK-CaM in the purified ryanodine-receptor preparation indicates close physical association between the two proteins, strong enough to persist in the presence of high salt concentrations and detergent.

The ryanodine receptor is also phosphorylated by PK-A and to a lesser extent by PK-G. PK-A-dependent phosphorylation has been reported by Takasago et al. [32] to amount to 150 pmol/mg of purified cardiac ryanodine receptor. The level of 354 ± 139 pmol/mg of purified ryanodine receptor observed in the present work still represents a sub-stoichiometric value

corresponding to about 1 mol/mol of tetramer; nevertheless, PK-A-dependent phosphorylation may be of functional relevance in cardiac muscle [28].

A phosphorylation site has been identified in both the skeletal (serine-2843; [19]) and cardiac (serine-2809 for PK-CaM; [28]) ryanodine receptor. We have shown previously that PK-CaM, PK-A and PK-G phosphorylate the ryanodine receptor from skeletal muscle on serine-2843 [19]. In the cardiac ryanodine receptor the phosphorylation site for PK-A and PK-G is not yet identified. The observation in the present study, that phosphorylation by PK-CaM and PK-A together is not additive, suggests a common phosphorylation site on the cardiac ryanodine receptor. An arginine residue is found three amino acids N-terminal to the phosphorylation site serine-2809 [28], thus yielding the consensus site (R-X-X-S/T) for PK-CaM [33]. Both cardiac and skeletal muscle ryanodine receptors exhibit a common motif, namely four highly repetitive sequences in the myoplasmic domain of the protein. Fourfold conserved repeats are also found in a variety of ion channels and are thought to play a crucial role in determining ion conductance and ion selectivity [34]. The residues serine-2809 in the cardiac and serine-2843 in the skeletal muscle ryanodine receptor are situated in the loop connecting the third and fourth repetitive sequences [26]. Based on this localization, a regulatory role of the phosphorylation site is highly probable.

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