Biochem. J. (1988) 252, 269–273 (Printed in Great Britain)

Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle

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Phospholamban of isolated sarcoplasmic reticulum of cardiac and smooth muscle is phosphorylated by cyclic GMP-dependent protein kinase (G-kinase). Concomitantly, the affinity of the Ca^{2+} pump for Ca^{2+} is increased. These effects are very similar to those seen with cyclic AMP-dependent protein kinase (A-kinase). The phosphate incorporation into phospholamban and the stimulatory effects of both kinases on the Ca^{2+} pump are not additive, suggesting that G-kinase phosphorylates the same serine residue as A-kinase. A possible physiological role for phosphorylation of phospholamban by G-kinase is discussed.

INTRODUCTION

Phospholamban was first identified as the major substrate for cyclic AMP-dependent protein kinase (A-kinase) in isolated sarcoplasmic reticulum (SR) of cardiac muscle (LaRaia & Morkin, 1974; Tada *et al.*, 1975). Since the Ca^{2+} uptake by the SR membranes is increased, concomitantly with the phosphorylation of phospholamban, this process may mediate part of the mechanical response of the heart to catecholamines and other agents that elevate cyclic AMP concentrations. More recently, it has been shown that phospholamban is also a substrate for a Ca^{2+}+calmodulin-dependent protein kinase (Le Peuch *et al.*, 1979) and for protein kinase C (Iwasa & Hosey, 1984; Movsenian *et al.*, 1984). Phospholamban has also been identified in the SR of slow skeletal muscle (Jorgensen & Jones, 1986) and in the SR(ER) of smooth muscle (Raeymaekers & Jones, 1986).

It has been shown that agents which increase intracellular cyclic GMP may have profound effects on the cytoplasmic Ca^{2+} concentration, thereby mediating muscle relaxation (Kobayashi *et al.*, 1985; Hassid, 1986; Kai *et al.*, 1987). It is conceivable that the Ca^{2+}-transport system that removes Ca^{2+} from the cytoplasm is one of the targets of cyclic GMP-dependent protein kinase (G-kinase). Evidence has been presented indicating that cyclic GMP activates the plasmalemmal Ca^{2+}-extrusion ATPase (Suematsu *et al.*, 1984; Popescu *et al.*, 1985; Furukawa & Nakamura, 1987). In the present study we report evidence that G-kinase phosphorylates phospholamban and stimulates the Ca^{2+} uptake in isolated SR vesicles from cardiac and smooth muscle.

METHODS

Preparation of membranes

Crude SR preparations were prepared from dog and pig ventricles obtained immediately after killing of the animals, as described by Kirchberger & Antonetz (1982). Smooth-muscle ER was purified from bovine main pulmonary artery as described by Raeymaekers *et al.* (1985) for pig gastric smooth muscle. This ER fraction from bovine pulmonary artery is described in detail by Eggermont *et al.* (1988).

Phosphorylation of membranes

Membranes (600 μg/ml) were incubated for 2 min at 30 °C in a medium containing 150 mM-$MgCl_2$, 5 mM-NaN_3, 50 mM-imidazole (pH 6.9), 5 mM-$MgCl_2$, 0.5 mM-EGTA, 100 μM-[γ-^{32}P]ATP and various concentrations of protein kinase as indicated. Modifications of these conditions are indicated in the Results section.

$^{45}Ca^{2+}$ uptake

After phosphorylation under the conditions described above, SR or ER fractions were diluted 20-fold in Ca^{2+}-uptake medium at 30 °C containing 100 mM-KCl, 5 mM-NaN_3 5 mM-Tris/ATP, 6 mM-$MgCl_2$, 30 mM-imidazole/HCl (pH 6.9), 5 mM-potassium oxalate, traces of $^{45}CaCl_2$, and the specified free Ca^{2+} concentration buffered by $CaCl_2$–EGTA. The free Ca^{2+} concentration was calculated for the relevant pH from an absolute stability constant of 10^{11} M^{-1} (Portzehl *et al.*, 1964). Samples were removed at 2 and 4 min and filtered through nitrocellulose filters (Schleicher and Schull, 0.45 μm pore). The filters were rinsed with 2 × 2 ml of 0.25 M-sucrose/2 mM-EGTA, dried and counted for radioactivity. Corrections were made for $^{45}Ca^{2+}$ bound in the absence of ATP.

Detection of ^{32}P-labelled polypeptides

SR or ER fractions were phosphorylated as described above. The reaction was stopped at the indicated time by the addition of equal volume of SDS sample buffer. The mixture was applied to 10%- or 12%-acrylamide Laemmli-type slab gels (Laemmli, 1970). ^{32}P-labelled polypeptides were localized by autoradiography. To identify phosphorylated amino acids, samples of cardiac

Abbreviations used: A-kinase, cyclic AMP-dependent protein kinase; G-kinase, cyclic GMP-dependent protein kinase; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

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SR were hydrolysed in 6 M-HCl and processed as described by Agostinis *et al.* (1986).

Materials

The kinase preparations used were G-kinase prepared from bovine lung as described by Hofmann & Flockerzi (1983), a catalytically active fragment of G-kinase prepared as described by Heil *et al.* (1987), and the catalytic subunit of A-kinase purified from bovine cardiac muscle as described by Hofmann *et al.* (1977). The heat-stable inhibitor of A-kinase was prepared from rabbit skeletal muscle and kindly supplied by Dr. M. Bollen. 8-Bromo cyclic GMP was obtained from Sigma.

RESULTS

The Ca^{2+} uptake in cardiac SR is enhanced by G-kinase to a similar degree as by A-kinase (Fig. 1). The combination of maximally effective amounts of both kinases does not have an additive effect. The stimulation of the Ca^{2+} uptake is not due to the activation of an endogenous kinase by cyclic GMP, since cyclic GMP without added kinase did not exert any effect (Fig. 1*a*), and because the stimulation was also obtained with the catalytically active fragment of G-kinase (Fig. 1*b*). The stimulatory effect of G-kinase is not due to the presence of a contaminant A-kinase in the G-kinase preparation, since it was not inhibited by the heat-stable protein kinase inhibitor, added at a concentration that fully inhibited a 5-fold higher concentration of purified A-kinase (Fig. 1*b*). Figs. 1(*a*) and 1(*c*) show results on dog SR, and the results shown in Fig. 1(*b*) were obtained on SR from pig heart. Although the rate of Ca^{2+} uptake by these preparations differs by a factor of about 6, the effects of the kinases (expressed as percentage stimulation of the Ca^{2+} uptake) were very similar.

Phospholamban is also a substrate for a Ca^{2+} + calmodulin-dependent protein kinase associated with the SR (Le Peuch *et al.*, 1979). Phosphorylation occurs at a threonine residue that is adjacent to the serine residue phosphorylated by A-kinase (Simmerman *et al.*, 1986). The stimulation of the Ca^{2+} uptake by this endogenous Ca^{2+} + calmodulin-dependent protein kinase and by added A-kinase is additive (Le Peuch *et al.*, 1979), as shown in Fig. 1(*c*). A similar additivity was observed when G-kinase was added instead of A-kinase (Fig. 1*c*).

The phosphorylation of membrane proteins by A-kinase and by G-kinase is shown in Fig. 2. As expected, the major phosphorylated protein in dog cardiac SR is

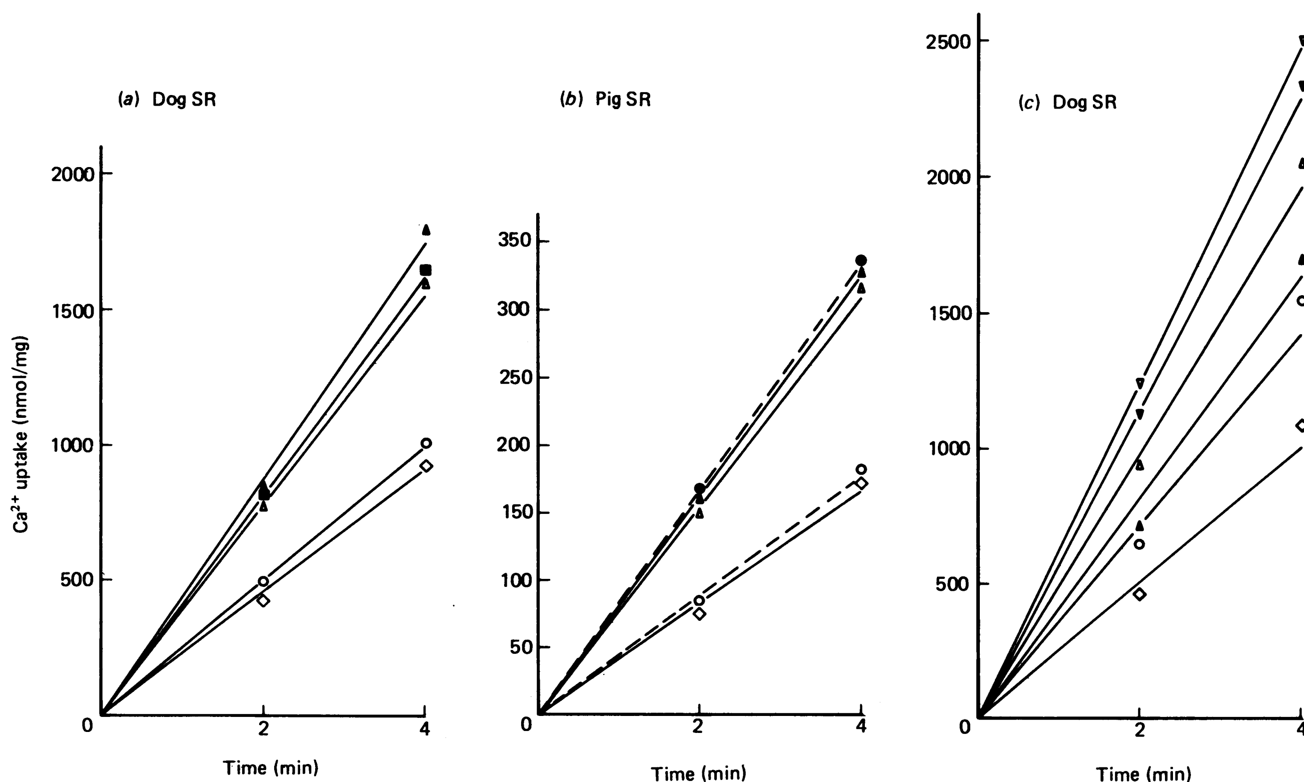

Fig. 1. Stimulation of the Ca^{2+} uptake in dog cardiac SR by protein kinases

(*a*) SR vesicles were preincubated in control conditions without ATP (◇) or in the presence of 100 μM-ATP and different protein kinases: △, 90 nM of the catalytic subunit of A-kinase; ○, 4 μM-8-bromo cyclic GMP; ▲, 90 nM-G-kinase and 4 μM-8-bromo cyclic GMP; ■, 8-bromo cyclic GMP and both A-kinase and G-kinase. The Ca^{2+} uptake was started by adding preincubated membranes to the Ca^{2+}-uptake medium, which contained 5 mM-ATP and Ca^{2+} buffered at 0.4 μM. (*b*) Effect of protein kinase inhibitor (PKI) on the stimulation of Ca^{2+} uptake by proein kinases in pig cardiac SR: ◇, control; △, catalytic subunit of A-kinase (150 nM); ▲, catalytically active fragment of G-kinase (30 nM); ○, A kinase (150 nM) in the presence of 240 μg of PKI/ml; ●, G-kinase (30 nM) in the presence of 240 μg of PKI. $[Ca^{2+}]_{free}$ was 0.4 μM. (*c*) Additivity of the stimulation of the Ca^{2+} uptake by the endogenous Ca^{2+}–calmodulin-dependent protein kinase and either added A-kinase or G-kinase: ◇, control; △, catalytic subunit of A-kinase (150 nM); ▲, catalytically active fragment of G-kinase (30 nM); ○, 10 μM-Ca^{2+} + 10 μg of calmodulin/ml; ▽, A-kinase + Ca^{2+}–calmodulin; ▼, G-kinase + Ca^{2+}–calmodulin.

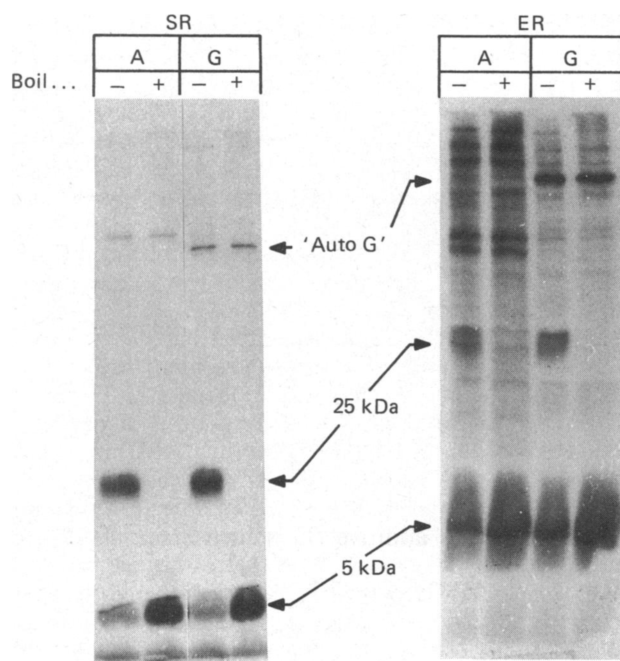

Fig. 2. Phosphorylation of phospholamban by A-kinase and by G-kinase

Autoradiograms of 10%- (left) and 12%- (right) acrylamide slab gels showing polypeptides phosphorylated by the catalytic subunit of A-kinase (A) or by the catalytically active fragment of G-kinase (G) in dog cardiac SR and in ER from pulmonary artery. The kinase concentrations were the same as in Fig. 1. The phosphorylation reaction was stopped after 2 min by addition of SDS sample buffer. This mixture was warmed at 37 °C for 10 min or heated at 100 °C ('Boil') as indicated. Monomeric phospholamban is visible at 5 kDa and pentameric phospholamban is visible at 25 kDa after warming at 37 °C (Kirchberger & Antonetz, 1982). 'Auto G' is autophosphorylated G-kinase. In the absence of added kinase, no phosphorylation was detected (results not shown).

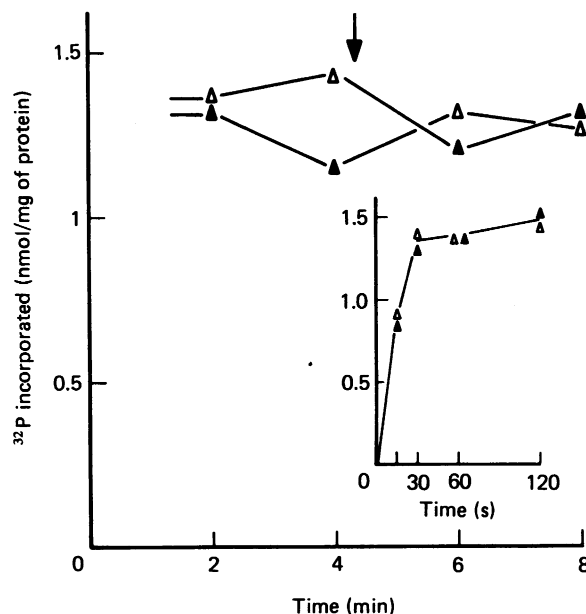

Fig. 3. Measurement of the amount of ^{32}P incorporated in pentameric phospholamban in the presence of A-kinase and G-kinase

At zero time, phosphorylation medium containing 300 μM-[γ-^{32}P]ATP and 90 nM-A-kinase (△) or 60 nM-G-kinase (catalytically active fragment; ▲) was added to dog cardiac SR (300 μg/ml). At the indicated times, samples were removed and quenched with SDS sample buffer for gel electrophoresis. At the time indicated by the arrow, A-kinase was added to the sample containing G-kinase, and vice versa, at the same final concentrations as specified above. Together with the kinase, an additional 100 μM-[γ-^{32}P]ATP of the same specific radioactivity was added, to ensure that the phosphorylation had not reached an apparent maximum owing to depletion of ATP. The dried gels were autoradiographed, the radioactive spots corresponding to pentameric phospholamban were cut out and their radioactivity was determined by counting the Čerenkov radiation. The inset shows the time course of ^{32}P incorporation at lower kinase concentrations (△, 35 nM-A-kinase; 35 nM-intact G-kinase).

phospholamban, a protein of M_r 25000 that decomposes to M_r-5000 subunits when the sample is boiled in the solubilization buffer (Tada *et al.*, 1975; Kirchberger & Antonetz, 1982). Fig. 2 shows that phospholamban is phosphorylated to a similar degree by A-kinase and by G-kinase. Also, phospholamban present in smooth-muscle ER is phosphorylated by both kinases (Fig. 2). However, in addition to phospholamban, other substrates are present in this preparation. In contrast with phospholamban, these substrates are better phosphorylated by A-kinase than by G-kinase. Also, in the isolated ER of smooth muscle, A-kinase and G-kinase has a similar stimulatory effect on the Ca^{2+} uptake, although to a lesser degree than in cardiac SR (L. Raeymaekers & R. Casteels, unpublished work).

To find out whether the phosphorylation of phospholamban by A-kinase and by G-kinase is additive, the ^{32}P incorporation in phospholamban was compared after addition of A-kinase or G-kinase or after sequential addition of both kinases. As shown in Fig. 3, ^{32}P incorporation in the presence of A-kinase or G-kinase reaches the same value, and it is not increased by the further addition of the other kinase. Acid hydrolysis of

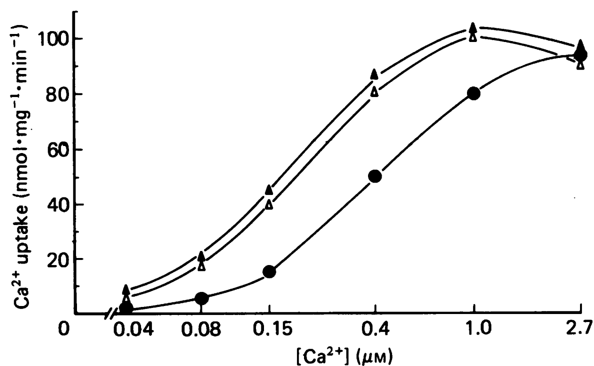

Fig. 4. [Ca^{2+}]-dependence of the Ca^{2+} uptake in pig cardiac SR, in control conditions (○) and after phosphorylation in the presence of 45 nM-A-kinase (△) or 45 nM-G-kinase (catalytically active fragment) (▲)

phospholamban phosphorylated by G-kinase revealed that ^{32}P was incorporated only in serine and not in threonine residues (results not shown). The time course of the phosphorylation of phospholamban at lower kinase concentrations, shown in the inset of Fig. 3, shows a similar rate of phosphorylation by both kinases.

Fig. 4 shows that, in pig cardiac SR, the stimulation of the rate of Ca^{2+} uptake by A-kinase or by G-kinase was very similar at all Ca^{2+} concentrations tested. The $V_{max.}$ was not affected, but the Ca^{2+} concentration for half-maximal activation decreased from 0.4 to 0.18 μM. Similar results were obtained with dog SR.

DISCUSSION

Phospholamban, an important modulator of Ca^{2+} transport of internal membranes, is present in cardiac and slow-skeletal muscle (Kirchberger & Tada, 1976; Jorgensen & Jones, 1976) and in smooth muscle (Raeymaekers & Jones, 1986). It has been shown that it is a substrate for A-kinase (Tada *et al.*, 1975), Ca^{2+}+calmodulin-dependent protein kinase (Le Peuch *et al.*, 1979) and protein kinase C (Iwasa & Hosey, 1984; Movsenian *et al.*, 1984). Each of these kinases phosphorylates a different amino acid. A-kinase and protein kinase C each phosphorylate a different serine residue, whereas Ca^{2+}+calmodulin-dependent protein kinase and protein kinase C phosphorylate a threonine residue (Simmerman *et al.*, 1986; Wegener *et al.*, 1986). The present experiments show that phospholamban is also phosphorylated by G-kinase at about the same rate as by A-kinase, and that in parallel the rate of Ca^{2+} uptake by the SR vesicles is increased. Since the amount of ^{32}P incorporation and the stimulation of the Ca^{2+} uptake by both kinases is not additive, it can be proposed that the site phosphorylated by G-kinase is the same as that phosphorylated by A-kinase. This conclusion is supported by the observation that G-kinase phosphorylates only serine and not threonine. Furthermore, the additional stimulation of the Ca^{2+} uptake by Ca^{2+}+calmodulin-dependent protein kinase was the same in the presence of either A-kinase or G-kinase.

The stimulating effect of cyclic AMP on the Ca^{2+} uptake by cardiac SR is generally considered to mediate the positive inotropic and chronotropic effect of β-adrenergic stimulation. It is at present not clear whether in cardiac muscle phosphorylation of phospholamban by G-kinase also occurs *in vivo*. The cytoplasmic cyclic GMP concentration is often, but not always, increased by agents that have a negative inotropic effect. In addition, it remains to be determined whether the action of cyclic GMP, besides its activation of a cyclic AMP phosphodiesterase, is also mediated by activation of G-kinase (for review, see Lincoln & Corbin, 1983; Walter, 1984). In intact guinea-pig ventricles, Watanabe *et al.* (1984) and Lindemann & Watanabe (1985) have shown that the cyclic-AMP-induced increase in the tension development and the increase in phosphorylation of phospholamban are decreased by muscarinic agonists. This observation argues against a phosphorylation of phospholamban by G-kinase in hearts under the stimulatory influence of β-adrenergic agonists. Although muscarinic agonists seem to have little contractile effect in non-stimulated ventricles, it cannot be excluded that under basal conditions, or in some non-ventricular parts, phosphorylation of phospholamban by G-kinase would occur *in vivo*. If so, one possibility for a physiological function could be that G-kinase, by increasing the affinity of the Ca^{2+} pump of the SR for Ca^{2+}, would prevent excessive depletion of the SR. Such depletion could otherwise be caused by the decreased availability of Ca^{2+} in the cytoplasm, owing to a decrease in the Ca^{2+} influx during negative inotropic and chronotropic conditions.

Smooth muscle contains a relatively high concentration of G-kinase, and it is a target organ for several agents that increase intracellular cyclic GMP, such as endothelium-derived relaxing factor, atrial natriuretic factor and nitro compounds (for review see Ignarro & Kadowitz, 1985; Murad *et al.*, 1985; Winquist, 1986). In contrast with cardiac muscle, there is no evidence for a very active cyclic-GMP-activated cyclic AMP phosphodiesterase, implying that cyclic GMP may mainly act through activation of G-kinase. In addition, several substrates for G-kinase have been detected in vascular smooth muscle (Ives *et al.*, 1980). An increase in cyclic AMP or cyclic GMP induces a decrease in the cytoplasmic Ca^{2+} concentration and muscle relaxation (Parker *et al.*, 1987; Rashatwar *et al.*, 1987). As has been proposed for cyclic AMP (Raeymaekers & Jones, 1986), the relaxation induced by cyclic GMP may also be mediated by stimulation of the Ca^{2+} uptake in the ER via phosphorylation of phospholamban. This process may act synergistically with the activation of other systems, such as the stimulation by cyclic GMP of the Ca^{2+} extrusion via the plasmalemmal (Ca^{2+} + Mg^{2+})-ATPase (Furukawa & Nakamura, 1987; Rashatwar *et al.*, 1987; M. Vrolix, L. Raeymaekers, F. Wuytack, F. Hofmann & R. Casteels, unpublished work).

We thank Dr. M. Bollen for the gift of protein kinase inhibitor and Dr. P. Agostinis for help with the phosphoamino acid analysis.

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Received 14 December 1987/22 February 1988; accepted 8 March 1988