Biochem. J. (1988) 255, 855–863 (Printed in Great Britain)

Cyclic GMP-dependent protein kinase stimulates the plasmalemmal Ca^{2+} pump of smooth muscle via phosphorylation of phosphatidylinositol

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The effect of phosphorylation by cyclic GMP-dependent protein kinase (G-kinase) on the activity of the plasmalemmal Ca^{2+}-transport ATPase was studied on isolated plasma membranes and on the ATPase purified from pig erythrocytes and from the smooth muscle of pig stomach and pig aorta. Incubation with G-kinase resulted, in both smooth-muscle preparations, but not in the erythrocyte ATPase, in a higher Ca^{2+} affinity and in an increase in the maximal rate of Ca^{2+} uptake. Cyclic AMP-dependent protein kinase (A-kinase) did not exert such an effect. The stimulation of the $(Ca^{2+}+Mg^{2+})$-dependent ATPase activity of the purified Ca^{2+} pump reconstituted in liposomes depended on the phospholipid used for reconstitution. The stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity by G-kinase was only observed in the presence of phosphatidylinositol (PI). G-kinase, but not A-kinase, stimulated the phosphorylation of PI to phosphatidylinositol phosphate (PIP) in a preparation of $(Ca^{2+}+Mg^{2+})$-ATPase obtained by calmodulin affinity chromatography from smooth muscle, but not in a similar preparation from erythrocytes. Adenosine inhibited both the phosphorylation of PI and the stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase by G-kinase. In the absence of G-kinase the $(Ca^{2+}+Mg^{2+})$-ATPase was stimulated by the addition of PIP, but not by PI. In contrast with previous results of Furukawa & Nakamura [(1987) J. Biochem (Tokyo) **101**, 287–290], no convincing evidence for a phosphorylation of the $(Ca^{2+}+Mg^{2+})$-ATPase was found. Evidence is presented showing that the apparent phosphorylation occurs in a contaminant protein, possibly myosin light-chain kinase. It is proposed that G-kinase stimulates the plasmalemmal Ca^{2+} pump of smooth-muscle cells indirectly via the phosphorylation of an associated PI kinase.

INTRODUCTION

The relaxation of smooth-muscle cells by nitro vasodilators, atrial natriuretic factor and endothelium-derived relaxing factor is mediated by the activation of the guanylate cyclase system and the concomitant increase in cyclic GMP (see [1–6] for reviews). It has been proposed that this effect is mediated by the phosphorylation of specific proteins by cyclic GMP-dependent protein kinase (G-kinase) [7–11]. Recently, considerable progress has been made in elucidating the link between the activation of G-kinase and relaxation. Several investigators have shown that one major action of cyclic GMP is to decrease the cytoplasmic Ca^{2+} concentration [12–16]. In principle, several regulatory pathways for cytoplasmic $[Ca^{2+}]$ may contribute to this effect. Cyclic GMP could cause a decrease in the Ca^{2+} influx, or increase the Ca^{2+} extrusion, or modify the Ca^{2+} release or the Ca^{2+} sequestration in intracellular organelles. The last effect has been demonstrated by the observation that G-kinase phosphorylates phospholamban and thereby stimulates the Ca^{2+} uptake by isolated sarcoplasmic reticulum of cardiac and smooth muscle [17]. Strong evidence in favour of a stimulation by G-kinase of the Ca^{2+} extrusion from smooth muscle cells via the ATP-dependent Ca^{2+} pump of the plasmalemma has also been presented [12,15]. Furthermore, a stimulation of the Ca^{2+} uptake or of the $(Ca^{2+}+Mg^{2+})$-dependent ATPase activity by G-kinase has been observed in crude membrane fractions [15], in plasma-membrane-enriched fractions [18,19] and in the solubilized plasmalemmal Ca^{2+} pump purified by calmodulin affinity chromatography [20]. It was recently proposed by Furukawa & Nakamura [20] that the mechanism responsible for the stimulation of the plasmalemmal Ca^{2+} pump is a phosphorylation by G-kinase of the Ca^{2+}-transport protein itself. In the present paper we show that this apparent phosphorylation can be explained by the presence of a contaminant protein having a similar mobility in SDS/polyacrylamide gels to the Ca^{2+} pump. Evidence is presented that G-kinase stimulates the plasmalemmal Ca^{2+} pump indirectly by increasing phosphatidylinositol phosphate, probably via the phosphorylation of an phosphatidylinositol kinase that co-purifies with the Ca^{2+}-transport ATPase. Preliminary accounts of this work have been presented [21,22].

EXPERIMENTAL

Materials

Calmodulin was prepared from bovine brain as

Abbreviations used: A-kinase, catalytic subunit of the cyclic AMP-dependent protein kinase (EC 2.7.1.37); G-kinase, cyclic GMP-dependent protein kinase (EC 2.7.1.37); PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; PS, phosphatidylserine; PC, phosphatidylcholine; $(Ca^{2+}+Mg^{2+})$-ATPase, $(Ca^{2+}+Mg^{2+})$-dependent ATPase.

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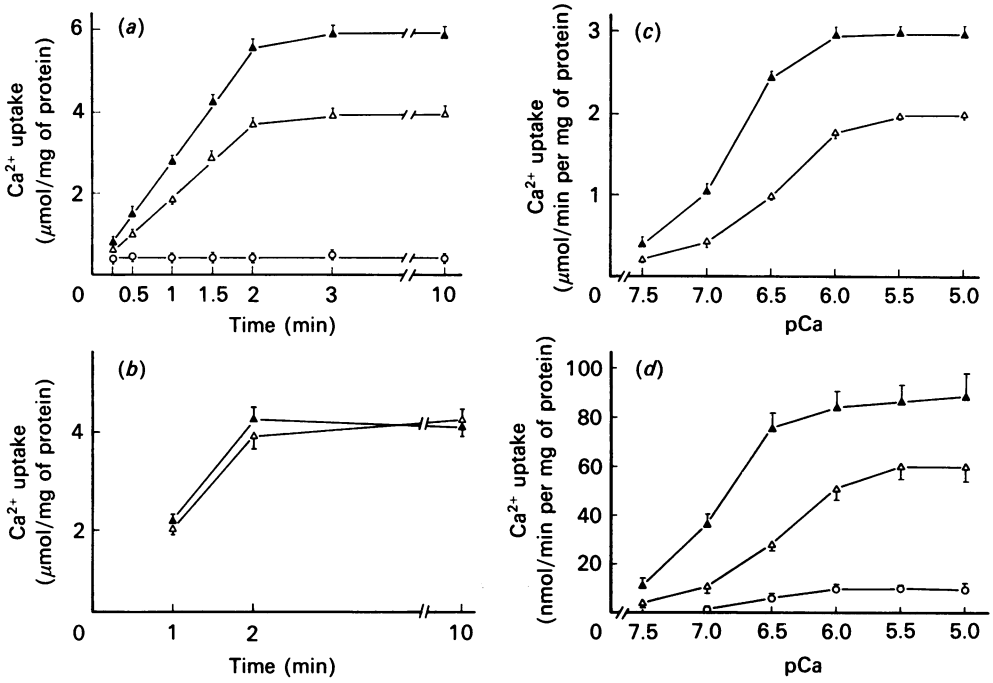

Fig. 1. Time course and Ca^{2+}-dependence of the ^{45}Ca uptake by reconstituted proteoliposomes containing the purified plasmalemmal ($Ca^{2+}+Mg^{2+}$)-ATPase from pig aortic smooth muscle (*a–c*) and by a plasma-membrane-enriched fraction from pig stomach smooth muscle (*d*)

The ATPase was prepared and reconstituted in the presence of the indicated phospholipids. The plasma membranes were isolated from pig stomach smooth muscle. The symbols represent ^{45}Ca uptake: ○, in the absence of ATP; △, in the presence of ATP; ▲, in the presence of ATP after a preincubation with G-kinase at 37 °C for 10 min (*a–c*) or 5 min (*d*). (*a*) Time course of ^{45}Ca uptake in asolectin-reconstituted ($Ca^{2+}+Mg^{2+}$)-ATPase (1 μg/ml). (*b*) Time course of ^{45}Ca uptake in PS proteoliposomes. (*c*) Ca^{2+}-dependence of the rate of Ca^{2+} uptake in asolectin-reconstituted proteoliposomes. (*d*) Ca^{2+}-dependence of the rate of ^{45}Ca uptake by the plasma-membrane enriched fraction (25 μg/ml). In (*a*), (*c*) and (*d*) cyclic GMP was used at 1 μM and G-kinase at 33 nM. In (*b*) the G-kinase concentration was 70 nM. In (*a*) and (*b*) free [Ca^{2+}] was 10 μM. The data shown in (*c*) and (*d*) represent the amounts of Ca taken up after 1 min incubation. The vertical bars show the S.E.M. for three experiments.

described previously [23]. Cyclic GMP and molecular-mass standards for the gel electrophoresis were obtained from Sigma. A23187 (from Calbiochem) was dissolved in ethanol at 1 mg/ml. The following phospholipid preparations were used: phosphatidylserine (PS) from bovine brain (99% pure, Sigma); asolectin from soya bean (Associate Concentrates); phosphoinositide mixture from bovine brain, type I (Sigma); L-α-phosphatidylinositol (PI) from soya bean (99% pure, Sigma); L-α-phosphatidylinositol 4-monophosphate (PIP) from bovine brain (98% pure, Sigma); L-α-phoshatidylinositol 4,5-bisphosphate (PIP_2) from bovine brain (98% pure, Sigma). Antibodies to myosin light-chain kinase from chicken gizzard (raised in goat) were given by Dr. P. de Lanerolle (University of Illinois, Chicago, IL, U.S.A.), and those to myosin light-chain kinase from bovine trachea (raised in rabbits) by Dr. J. Stull and Dr. M. Nunnally (University of Texas, Dallas, TX, U.S.A.). G-kinase was prepared as described by Hofmann & Flockerzi [24] and cyclic nucleotides were removed by the method of Hofmann *et al.* [25]. A catalytically active fragment of G-kinase was prepared as described by Heil *et al.* [26]. Cyclic AMP-dependent protein kinase (A-kinase) was prepared as described by Walter [27]. SDS/polyacrylamide-gel electrophoresis indicated that the G-kinase and A-kinase enzymes were 95–100% pure.

Isolation of membrane fractions

Starting from 300 g of tissue (pig aorta or stomach), freshly obtained from a local slaughterhouse, a crude membrane fraction was prepared as described previously [28]. This fraction was extracted for 1 h at 4 °C in 0.6 M- or 1.2 M-KCl, as indicated. The KCl-extracted pellet was resuspended in 0.25 M-sucrose at a protein concentration of about 5 mg/ml. A membrane fraction enriched in plasma membranes was prepared from pig stomach as described previously [29]. Pig erythrocyte ghosts were prepared as described by Gietzen & Kolandt [30]. Protein was measured by the method of Lowry *et al.* [31], with bovine serum albumin as a standard.

Purification of the ($Ca^{2+}+Mg^{2+}$)-ATPase and reconstitution in phospholipid vesicles

The ($Ca^{2+}+Mg^{2+}$)-ATPase was solubilized from the KCl (0.6 M or 1.2 M)-extracted smooth-muscle membranes or from pig erythrocyte ghosts as described previously [29]. The ($Ca^{2+}+Mg^{2+}$)-ATPase was purified by calmodulin affinity chromatography by two different methods: in a first series of experiments, the enzyme was purified in the presence of the indicated phospholipids (PS or asolectin) [32]. Immediately after purification, the isolated enzyme was reconstituted by the cholate-dialysis

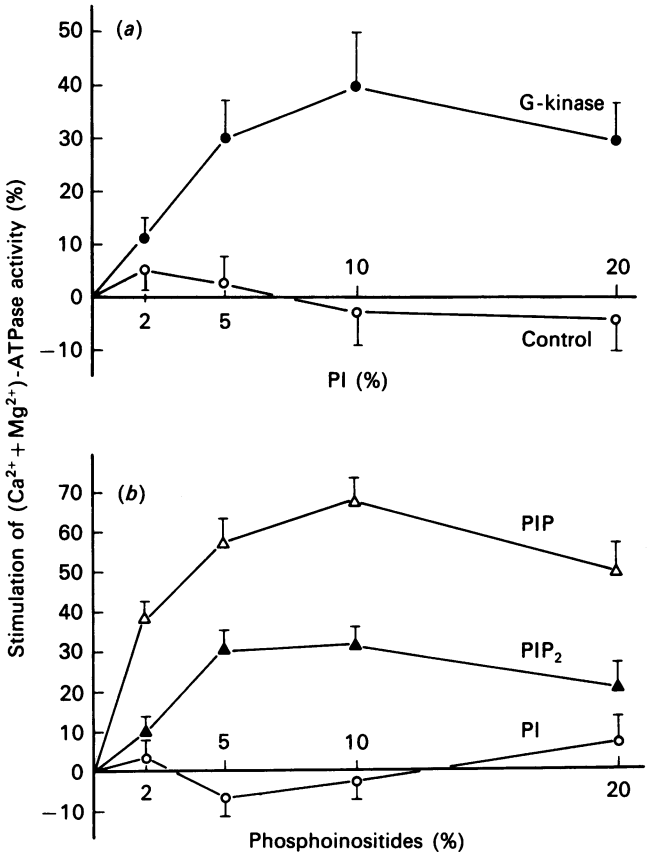

Fig. 2. Effect of phosphoinositides on the plasmalemmal Ca^{2+} pump from pig stomach and on its stimulation by G-kinase

The $(Ca^{2+}+Mg^{2+})$-ATPase was prepared in the absence of phospholipids and re-activated as described in the Experimental section by using 200 μg of PS/ml and various amounts of phosphoinositides (indicated on the abscissa as a percentage of the amount of PS). The $(Ca^{2+}+Mg^{2+})$-ATPase activity was measured at a protein concentration of 1 μg/ml and at a free $[Ca^{2+}]$ of 0.3 μM. The vertical bars show the S.E.M. of four experiments. (*a*) Stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity by G-kinase in the presence of different amounts of PI: ○, control; ●, 12.5 nM-G-kinase and 1 μM-cyclic GMP. (*b*) Stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase by different amounts of phosphoinositides in the absence of G-kinase: ○, PI; △, PIP; ▲, PIP_2.

method as described previously [33]. For the experiments with added phosphoinositides a modification of the purification procedure described by Kosk-Kosicka & Inesi [34] and Kosk-Kosicka *et al.* [35] was used. This method allows purification in the absence of phospholipids. Briefly, the enzyme was solubilized and purified in the same buffers as described in ref. [32], but in the presence of 20% (v/v) glycerol. The yield of purified protein amounted to 0.08–0.1% of the KCl-extracted membrane protein. After purification, the enzyme preparation was supplemented with a mixture of different relative amounts of phosphoinositides and PS. Routinely, each 1 ml of eluted enzyme was supplemented with 20 μl of a sonicated 1% phospholipid mixture in the elution medium, vortex-mixed and stored at −70 °C.

Determination of the Ca^{2+} uptake

Membrane protein was preincubated for 5 min at 37 °C, in 200 μl of a medium containing 0.1 mM-ATP, 6 mM-$MgCl_2$, 150 mM-KCl, 30 mM-imidazole/HCl, pH 6.9, 1 mM-EGTA, and with or without cyclic GMP/G-kinase. Then 25 μg of membrane vesicles or 1 μg of the reconstituted enzyme prepared in the presence of phospholipids was added. The uptake reaction was started by the addition of ATP up to a final concentration of 5 mM and of $^{45}CaCl_2$ up to the desired free Ca^{2+} concentration [36]. The reaction was stopped by diluting the uptake medium 10-fold with ice-cold stop solution containing 1 mM-$LaCl_3$, 100 mM-KCl, 20 mM-Hepes, pH 7.4, followed by rapid filtration and rinsing the filters with 2 × 2 ml of the same solution. Filters were dried and counted for radioactivity. Almost all the $^{45}Ca^{2+}$ taken up could be released by addition of 1 μg of A23187 (results not shown).

Determination of $(Ca^{2+}+Mg^{2+})$-ATPase activity

$(Ca^{2+}+Mg^{2+})$-ATPase activity was measured spectrophotometrically as described previously [29], by using the coupled enzyme system, in a medium similar to that used for ^{45}Ca uptake, except that EGTA was 0.5 mM. To obtain the maximum $(Ca^{2+}+Mg^{2+})$-ATPase activity, 0.6 μM-calmodulin was added. For measurement of the activity of the ATPase prepared in the presence of phospholipids, the accumulation of inhibitory high Ca^{2+} concentrations in sealed vesicles was prevented by the addition of the Ca^{2+} ionophore A23187 (3 μg/ml). The $(Ca^{2+}+Mg^{2+})$-ATPase activity of the Ca^{2+} pump prepared without phospholipids proceeded linearly with time, and addition of the ionophore was not required. In each of the 1 ml cuvettes of the double-beam spectrophotometer, 3 μg of reconstituted proteoliposomes was incubated at 37 °C. Since the solution in both cuvettes was identical, the absorbance difference did not change with time. In one cuvette $CaCl_2$ was then added to obtain the desired free Ca^{2+} concentration. The $(Ca^{2+}+Mg^{2+})$-ATPase activity was recorded for 30 min from the slope of the curve describing the change of the absorbance difference with time. Afterwards, A-kinase or 1 mM-cyclic GMP and G-kinase at the indicated concentrations were added to both cuvettes. The stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity by the kinase was measured from the increase in the slope of the curve.

Phosphoprotein detection and immunoblotting

Membrane protein was incubated in a medium containing 130 mM-KCl, 10 mM-$MgCl_2$, 0.5 mM-EGTA, 20 mM-Hepes, pH 7.4, and 0.1 mM-[γ-^{32}P]ATP at the temperature and for the time indicated in the Figure legends. Kinases were added as indicated. The reaction was stopped by the addition of 40 μl of SDS-sample buffer and heating to 60 °C for 5 min. A 60 μl portion of this diluted sample was applied on 7.5%- or 10%-polyacrylamide slab gels (thickness 0.75 mm) of the Laemmli [37] type. Gels were stained with Coomassie Brilliant Blue R-250, quick-dried and exposed to Kodak X-Omat R film (XR-1) at −70 °C. For immunoblotting, the proteins were electrophoretically transferred on to Immobilon (Millipore) sheets. Binding of polyclonal antibodies and their detection by peroxidase-conjugated secondary antibodies were carried out by methods previously described [38].

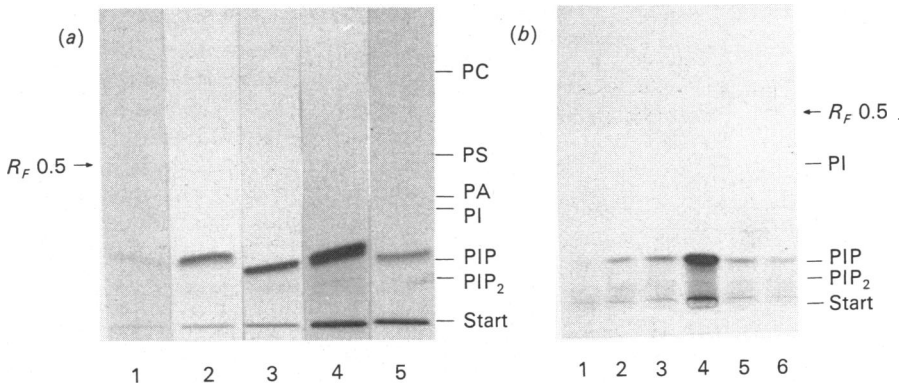

Fig. 3. Autoradiograms of thin-layer chromatograms showing ^{32}P-labelled lipids extracted from reconstituted proteoliposomes containing the (Ca^{2+} + Mg^{2+})-ATPase

The (Ca^{2+} + Mg^{2+})-ATPase preparations were prepared in the absence of added phospholipids. (*a*) PIP labelling in the ATPase preparation from pig stomach, incubated in a final volume of 200 μl at 37 °C for 10 min, unless indicated otherwise. The catalytically active fragment of G-kinase was present at 17 nM. Lanes: 1, 40 μl of (Ca^{2+} + Mg^{2+})-ATPase (2 μg) reconstituted in pure PS (200 μg/ml) was added; 2–4, as in lane 1, but ATPase reconstituted in PS + PI (2% of the amount of PS) and phosphorylated for 3, 5 and 10 min respectively; 5, as lane 4 but with heat-inactivated G-kinase. (*b*) ATPase (1 μg/200 μl) was added. The same medium as in (*a*) was used, except that 2% Triton X-100 was present. Lanes: 1, erythrocyte ATPase in the presence of 1 μM-cyclic GMP and 20 nM-G-kinase; 2, pig stomach ATPase; 3, pig stomach ATPase in the presence of 50 nM-A-kinase; 4, pig stomach ATPase in the presence of 1 μM-cyclic GMP and 20 mM-G-kinase; 5 and 6, as lane 4, but in the presence of 20 μM- and 500 μM-adenosine respectively. Abbreviation: PA, phosphatidic acid.

Detection of ^{32}P incorporation in phospholipids of the reconstituted proteoliposomes

(Ca^{2+} + Mg^{2+})-ATPase prepared by the modified procedure of Kosk-Kosicka *et al.* [34,35] was used. The phosphorylation reaction was performed in the medium described above. After the indicated times the reaction was stopped by diluting the sample 10-fold in methanol/HCl (50:3, v/v). Extraction of the lipids and their separation by t.l.c. were performed as described by Andrews & Conn [39].

RESULTS

Dependence of the stimulation of the plasmalemmal Ca^{2+}-pump by G-kinase on phospholipid

The plasmalemmal (Ca^{2+} + Mg^{2+})-ATPase, purified by Triton X-100 extraction and calmodulin affinity chromatography, was reconstituted in liposomes of different phospholipid composition. As shown in Fig. 1 for the (Ca^{2+} + Mg^{2+})-ATPase from pig aorta, the stimulation by G-kinase of the ATP-dependent ^{45}Ca uptake depended on the phospholipids used for reconstitution. A stimulation of the Ca^{2+} uptake by G-kinase was observed in proteoliposomes consisting of crude asolectin (Figs. 1*a* and 1*c*), but not in liposomes consisting of pure phosphatidylcholine (PC) (results not shown) or pure phosphatidylserine (PS) (Fig. 1*b*). The $V_{max.}$ was increased by 48%, and the $K_{0.5}$ for Ca^{2+} was decreased from 0.8 μM to 0.5 μM. The stimulation of the Ca^{2+} uptake by G-kinase could also be seen in a fraction specifically enriched in plasma membrane (Fig. 1*d*). A similar phospholipid-dependency of the G-kinase effect was seen when the activity of the reconstituted Ca^{2+} pump from pig stomach was assessed by measuring the Ca^{2+}-stimulated ATPase activity in the presence of Mg^{2+}. This ATPase activity, which amounted to 1.05 and 4.1 μmol/min per mg at 10 μM-Ca^{2+} in the presence of PC or PS respectively, was not affected by G-kinase (added to a final concentration of 100 nM). The ATPase activity in asolectin-reconstituted liposomes was increased by the addition of 12.5 nM-G-kinase, from 3.9 to 5.9 μmol/min per mg. The effect was maximal within 3 min of incubation. Increasing the concentration of G-kinase did not augment or accelerate the effect (results not shown).

Since the asolectin preparation contains several lipids besides PC (results not shown), this result could indicate that some phospholipids other than PS or PC were required for the G-kinase-induced stimulation of the Ca^{2+} pump. Therefore we searched for such a component.

Effect of phosphoinositides on the Ca^{2+} pump

The (Ca^{2+} + Mg^{2+})-ATPase prepared from pig stomach by the modified procedure of Kosk-Kosicka *et al.* [34,35] was reconstituted in pure PS, and the effect of addition of known amounts of various pure phosphoinositides was studied. As shown in Fig. 2(*a*), G-kinase exerted a stimulatory effect when PI was added to the PS used for reconstitution. Its maximal effect was observed at a PI/PS ratio of about 0.1. No stimulation by G-kinase was observed when the ATPase was reconstituted in PS and various amounts of PIP or PIP_2. No significant stimulation of the Ca^{2+} pump was observed if A-kinase (up to 100 nM) was used instead of G-kinase (results not shown). The ATPase was stimulated in the absence of G-kinase by the addition of small relative amounts of PIP to the PS-reconstituted liposomes (Fig. 2*b*). The effect of PIP_2 was smaller than that of PIP, and PI had no effect. The degree of stimulation was of the same order of magnitude as that seen with G-kinase. When the corresponding (Ca^{2+} + Mg^{2+})-ATPase from pig erythrocytes was used, a stimulation by phosphoinositides was also seen, in agreement with other reports [40–44].

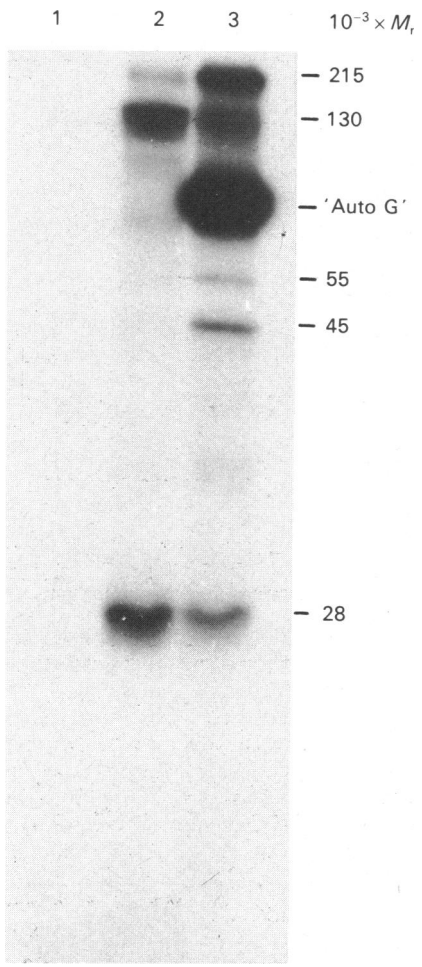

Fig. 4. Autoradiogram of a SDS/polyacrylamide (12%) gel of the $(Ca^{2+} + Mg^{2+})$-ATPase preparation from pig stomach, phosphorylated for 2 min at 37 °C

Lanes: 1, control; 2, 20 nM-A-kinase added; 3, in the presence of 1 μM-cyclic GMP and 20 nM-G-kinase; 1 μg of ATPase was applied per lane. 'Auto G' is autophosphorylated G-kinase.

However, this $(Ca^{2+} + Mg^{2+})$-ATPase was not stimulated by G-kinase under similar conditions to those used for the $(Ca^{2+} + Mg^{2+})$-ATPase preparation from smooth muscle (results not shown).

The findings that PIP was the most effective species in stimulating the Ca^{2+} pump of smooth muscle, that PI did not have any effect, and that the stimulation of the Ca^{2+} pump by G-kinase required PI, suggested that the action of G-kinase could be mediated by a phosphorylation of PI to PIP. Therefore the phosphorylation of PI was studied.

Effect of G-kinase on PI phosphorylation

The $(Ca^{2+} + Mg^{2+})$-ATPase was reconstituted in PS with or without PI and incubated in the presence of [γ-^{32}P]ATP with or without G-kinase. The phospholipids were then extracted and separated. In the $(Ca^{2+} + Mg^{2+})$-ATPase preparation from pig stomach, ^{32}P was incorporated time-dependently into a band that co-migrated with PIP if the medium was supplemented with PI and G-kinase (Fig. 3*a*, lanes 2–4). If heat-inactivated G-kinase was used (lane 5), the formation of radioactive PIP was much decreased. Almost no ^{32}P was incorporated into phospholipids if either PI (Fig. 3*a*, lane 1) or the ATPase preparation from smooth muscle (Fig. 3*b*, lane 1) was omitted from the incubation medium. This observation indicates that PIP is not formed from endogenous PI and that G-kinase itself does not phosphorylate PI. A-kinase (50 nM) did not stimulate the phosphorylation of PI (Fig. 3*b*, lane 3).

Since the Ca^{2+}-pump preparation from erythrocytes did not phosphorylate PI (Fig. 3*b*) and was not sensitive to G-kinase (see above), the phosphorylation of PI observed in the presence of the smooth-muscle ATPase is most likely not catalysed by G-kinase itself, but by a PI kinase that is stimulated by G-kinase and that is associated with the $(Ca^{2+} + Mg^{2+})$-ATPase from smooth muscle and not with that from erythrocytes. Further evidence in favour of the participation of a PI kinase in the stimulation of the Ca^{2+} pump by G-kinase comes from the observation that the phosphorylation of PI was inhibited by adenosine in the micromolar range (Fig. 3*b*). Adenosine has been reported to be an inhibitor of PI kinase, more specifically of the type 2 PI kinase described by Whitman *et al.* [45]. We also observed that 100 μM-adenosine inhibited the stimulatory effect of G-kinase on the $(Ca^{2+} + Mg^{2+})$-ATPase activity by about 75%. However, adenosine did not inhibit the G-kinase, as determined from the phosphorylation of histones, nor did it inhibit the $(Ca^{2+} + Mg^{2+})$-ATPase in the absence of G-kinase (results not shown).

Our conclusion that the effect of G-kinase on the plasmalemmal Ca^{2+} pump from smooth muscle is mediated by a PI kinase contrasts with the mechanism proposed by Furukawa & Nakamura [20]. According to these authors, G-kinase would directly phosphorylate the Ca^{2+}-pump protein. In order to elucidate the discrepancy, the effect of G-kinase on the protein phosphorylation in the $(Ca^{2+} + Mg^{2+})$-ATPase preparation was studied.

Effect of G-kinase on protein phosphorylation

Several phosphorylated polypeptides can be detected in the $(Ca^{2+} + Mg^{2+})$-ATPase preparation from smooth muscle (Fig. 4). One of these polypeptides co-migrates in SDS/polyacrylamide gels with the $(Ca^{2+} + Mg^{2+})$-ATPase at the 130 kDa position and could be the Ca^{2+}-pump protein, as proposed by Furukawa & Nakamura [20]. However, in contrast with the result of Furukawa & Nakamura [20], the ^{32}P incorporation in our preparation was also seen in other bands than that at the position of the $(Ca^{2+} + Mg^{2+})$-ATPase (Fig. 4). Although the ATPase studied by Furukawa & Nakamura [20] was also prepared by calmodulin affinity chromatography, as was done in our laboratory, the starting material was obtained by a different procedure. We solubilized the ATPase starting from membranes extracted with 0.6 M-KCl, whereas Furukawa & Nakamura [20] used 1.2 M-KCl for extraction. We therefore compared membranes extracted in 0.6 M- or 1.2 M-KCl and also compared the ATPase preparations prepared from these membranes. When membranes were extracted in 0.6 M-KCl, most of the ^{32}P label at 130 kDa was present in the supernatant, whereas in 1.2 M-KCl this phosphoprotein appeared in the membrane pellet (Fig. 5*a*). As shown in Fig. 5(*b*), the ATPase preparation from 1.2 M-KCl-extracted membranes (lane 2) showed much more ^{32}P labelling at 130 kDa than did that prepared from 0.6 M-KCl-

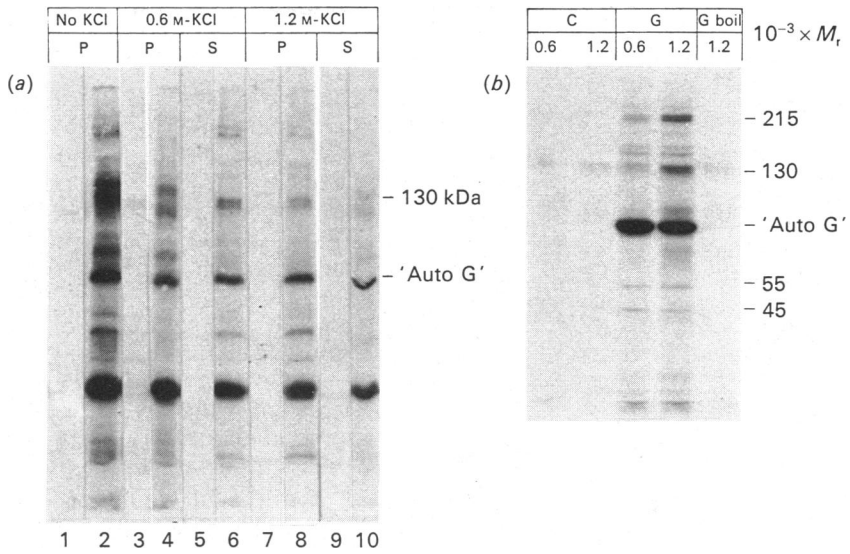

Fig. 5. Autoradiographed dried polyacrylamide slab gels showing the effect of salt extraction on ^{32}P-labelled polypeptides

(*a*) Crude membranes from pig stomach (lanes 1 and 2) were extracted in 0.6 M-KCl (lanes 3–6) or in 1.2 M-KCl (lanes 7–10) and centrifuged as described in the Experimental section. The pellet (P) of insoluble material (lanes 3, 4, 7, 8) and the supernatant (S) (lanes 5, 6, 9, 10) were phosphorylated for 5 min at 37 °C. Lanes 1, 3, 5, 7 and 9: control. Lanes 2, 4, 6, 8 and 10: + 1 μM-cyclic GMP and 20 nM-G-kinase. (*b*) Influence of the salt concentration used for membrane extraction on the subsequently isolated ($Ca^{2+} + Mg^{2+}$)-ATPase preparation. The plasmalemmal Ca^{2+} pump was isolated by the modified Kosk-Kosicka [34,35] procedure, starting from pig stomach membranes extracted in 0.6 M- or 1.2 M-KCl, as indicated. In each lane, equal amounts of ATPase were applied, as determined from the ($Ca^{2+} + Mg^{2+}$)-ATPase activity (about 1 μg). Phosphorylation was carried out at 37 °C for 2 min. Key: C, control; G, in the presence of 1 μM-cyclic GMP and 20 nM-G-kinase; G boil, as G, but with heat-inactivated G-kinase.

extracted membranes (lane 1). This result indicated that most or all of the ^{32}P was not incorporated in the ATPase but in a contaminant protein that co-migrated with the ATPase. A likely candidate was myosin light-chain kinase, since this enzyme has molecular mass of about 130 kDa, is phosphorylatable by A-kinase and by G-kinase [46,47], and is expected to bind to a calmodulin affinity column. We have investigated this possibility by using antibodies to myosin light-chain kinase. As shown in Fig. 6(*a*), a more pronounced antibody labelling was observed at 130 kDa in the 1.2 M-KCl preparation than in that prepared in 0.6 M-KCl. In addition, Ca^{2+} and calmodulin decreased the ^{32}P labelling at 130 kDa in the presence of both A-kinase (Fig. 6*b*) and G-kinase (results not shown), a characteristic of myosin light-chain kinase [38,39]. Further support for the view that the 130 kDa phosphoprotein is not the Ca^{2+} pump is the observation that only G-kinase and not A-kinase stimulated the ($Ca^{2+} + Mg^{2+}$)-ATPase, whereas the 130 kDa substrate is phosphorylated by both A-kinase and G-kinase (Fig. 4).

Besides the 130 kDa protein, several other phosphorylatable polypeptides were present in the ATPase preparations. A substrate of 28 kDa present in some preparations (e.g. Fig. 4) and not in others (Figs. 5*b* and 6*b*) is a substrate for both A-kinase and G-kinase. One or several of the other substrates (215, 55 and 45 kDa) could represent a PI kinase, since they are better substrates for G-kinase than for A-kinase (Fig. 4). The ($Ca^{2+} + Mg^{2+}$)-ATPase preparation from erythrocytes differed from the ATPase of smooth muscle mainly by the absence of phosphorylation of the 45 kDa and 55 kDa bands (Fig. 7). Since the ATPase from erythrocytes was insensitive to G-kinase, these observations provide indirect evidence that the 45 kDa and/or 55 kDa polypeptides could represent the PI kinase that co-purifies with the Ca^{2+} pump from smooth muscle.

DISCUSSION

The effect of purified exogenously added G-kinase on the plasmalemmal Ca^{2+}-transport ATPase of the smooth muscle of the pig stomach and aorta has been investigated. As previously observed by Furukawa & Nakamura [20], the ATPase purified by calmodulin affinity chromatography was stimulated by G-kinase. Furukawa & Nakamura [20] ascribed this effect to a direct phosphorylation of the ATPase by G-kinase. However, we did not find convincing evidence for a phosphorylation of the Ca^{2+}-transport enzyme. The ^{32}P incorporation observed in SDS/polyacrylamide gels at the 130 kDa position could be explained by the presence of a contaminant protein that shows the properties of myosin light-chain kinase. It was also demonstrated that the ATPase as prepared by Furukawa & Nakamura [20] contains more of this contaminant protein than does the preparation used in the present study. An alternative explanation for the stimulation of the Ca^{2+} pump by G-kinase was suggested by the observation that this stimulatory effect of G-kinase depended on the phospholipid used for reconstitution of the ATPase. The stimulatory effect of G-kinase was observed if crude asolectin was used, but not if pure PC or pure PS was the only constituent. In the experiments of Furukawa & Nakamura [20], only crude soya-bean phospholipids were used. It was found that the stimulatory effect of G-kinase selectively required the

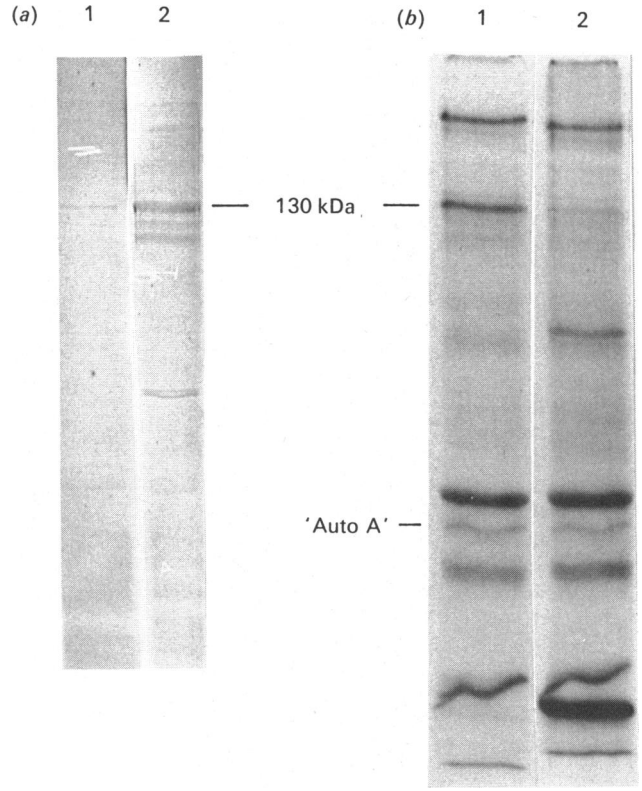

Fig. 6. Characterization of the 130 kDa phosphoprotein

(*a*) Immunoblotting with polyclonal antibodies to chicken gizzard MLCK (1:1000 dilution): SDS/polyacrylamide-gel electrophoresis (7.5% slab gel) of the $(Ca^{2+}+Mg^{2+})$-ATPase prepared in the presence of phospholipids from membranes extracted in 0.6 M-KCl (lane 1) or 1.2 M-KCl (lane 2). Each lane contained the same amount of $(Ca^{2+}+Mg^{2+})$-ATPase (about 2 μg) as determined from the $(Ca^{2+}+Mg^{2+})$-ATPase activity. Similar results (not shown) were obtained with antibodies to MLCK from bovine trachea. (*b*) Effect of Ca^{2+} and calmodulin on the 130 kDa phosphoprotein. The autoradiogram of a 7.5%-acrylamide gel shows the ^{32}P-labelled proteins in a membrane pellet extracted in 1.2 M-KCl, phosphorylated by 62 nM-A-kinase for 1 min at 4 °C: (1) control, (2) in the presence of 0.1 mM Ca^{2+} and 0.6 μM-calmodulin (20 μg of protein was applied per lane). 'Auto A' is auto-phosphorylated A-kinase.

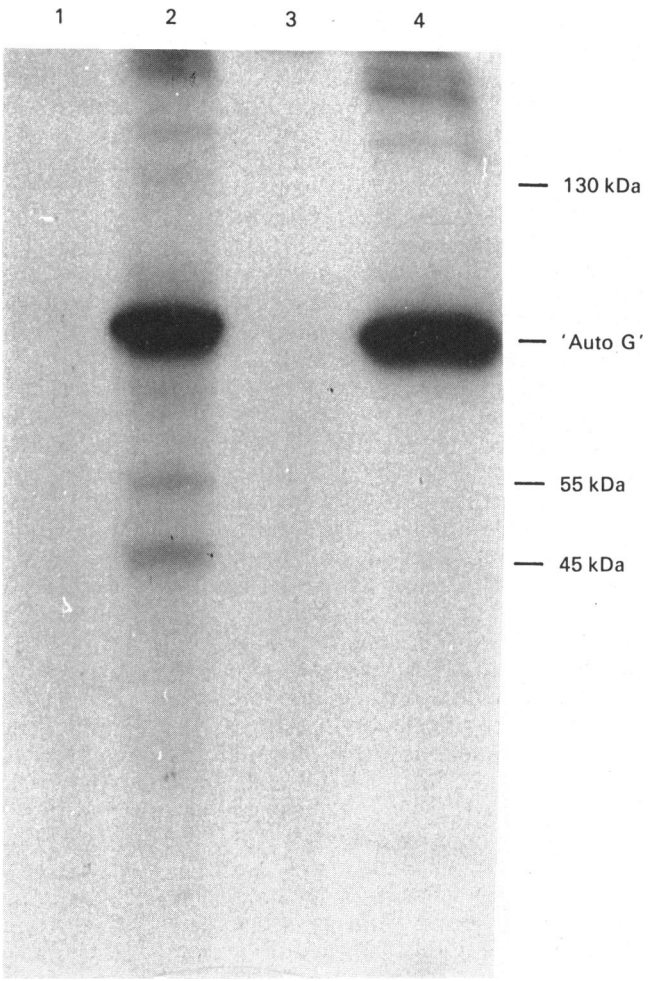

Fig. 7. Comparison of the phosphorylation of the $(Ca^{2+}+Mg^{2+})$-ATPase preparations from pig stomach and from pig erythrocytes

Autoradiographed 10%-acrylamide gel of $(Ca^{2+}+Mg^{2+})$-ATPases phosphorylated for 5 min at 37 °C. Lanes: 1, 2, 0.4 μg of pig stomach ATPase; 3, 4, 1 μg of pig erythrocyte ATPase; 1 and 3, control; 2 and 4, in the presence of 1 μM-cyclic GMP and 20 nM-G-kinase.

presence of PI, whereas PIP and PIP_2 were ineffective. The requirement for PI could in principle be explained in at least two ways: (1) PI may be required for protein phosphorylation or for the transmission of the signal of protein phosphorylation to the Ca^{2+}-transport machinery; (2) PI could be phosphorylated to PIP, and PIP itself would directly stimulate the Ca^{2+}-transport enzyme or act as described in (1). The simplest explanation is that the ATPase is directly stimulated by PIP formed under the influence of the action of G-kinase. The following observations support this view. The ATPase preparation catalyses the phosphorylation of PI to PIP, and this process is enhanced by G-kinase; the addition of PIP to the ATPase in the absence of G-kinase, and therefore of protein phosphorylation, mimics the effect of G-kinase. If this hypothesis is correct, the Ca^{2+}-transport ATPase should be more effectively stimulated by PIP than by PI, which was indeed observed. PIP_2 was also effective, but to a lesser extent than PIP. Although several reports have appeared on the stimulation of the corresponding Ca^{2+}-transport ATPase of erythrocytes by negatively charged phospholipids [40–44], no direct comparison of the effectiveness of PI and PIP has been published. It was reported that PI stimulates the Ca^{2+}-transport ATPase reconstituted in PC, which is in apparent contradiction with our results [41,42]. However, this discrepancy can be explained by the difference in the bulk lipid used for reconstitution. In the presence of PC, the enzyme is in the basal unstimulated state, whereas in the presence of the negatively charged PS it is already activated, although only partially, as shown by its further stimulation by the addition of PIP. Furthermore, the reported minimum concentration required for the stimulatory effect was higher for PI than for PIP_2 [42]. These observations indicate that, among the negatively charged phospholipids that are able to stimulate the Ca^{2+}-transport ATPase, PIP has unique properties. The ability of PIP to

stimulate the Ca^{2+} pump even in the presence of an excess of PS is important, because *in vivo* PS in the membrane is also in excess over PIP. This observation therefore lends further support to the idea that the regulation of the PIP concentration in the neighbourhood of the ATPase could be a physiologically relevant mechanism.

Phosphorylation of PI associated with an ATPase has previously been described for the $(Ca^{2+}+Mg^{2+})$-ATPase of the sarcoplasmic reticulum of skeletal muscle [48]. This inositol lipid remained associated with the Ca^{2+}-pump protein after SDS/polyacrylamide-gel electrophoresis, whereas in the plasmalemmal enzyme such tight association could not be observed.

A further question that arises from our results concerns the mechanism of the stimulation of the phosphorylation of PI to PIP by G-kinase. A first possibility is that G-kinase would directly phosphorylate PI to PIP. This hypothesis is rather unlikely, since pure G-kinase without added $(Ca^{2+}+Mg^{2+})$-ATPase did not catalyse this process. However, it could still be argued that the Ca^{2+} pump would be required for the binding of PI and for presenting PI in a suitable environment to G-kinase. The second possibility is that the phosphorylation of PI to PIP is catalysed by a specific PI kinase that is associated with the ATPase and stimulated by G-kinase. Our results strongly favour the second possibility, for the following reasons: (1) adenosine, a known inhibitor of some types of PI kinase [45,49,50], inhibited the phosphorylation of PI by the $(Ca^{2+}+Mg^{2+})$-ATPase preparation and inhibited the stimulation of the $(Ca^{2+}+Mg^{2+})$-ATPase activity by G-kinase; (2) the $(Ca^{2+}+Mg^{2+})$-ATPase from erythrocytes, which has all the properties of the plasmalemmal Ca^{2+} pump from smooth muscle [51], did not catalyse the phosphorylation of PI and was insensitive to G-kinase.

It has been reported that A-kinase may phosphorylate the plasmalemmal $(Ca^{2+}+Mg^{2+})$-ATPase from erythrocytes, although only after prolonged incubation [52]. The relation of this phenomenon to the proposed indirect mechanism via an associated PI kinase remains to be investigated. The proposed indirect mechanism via PI kinase is specific for G-kinase, but it does not exclude an additional action of A-kinase via other pathways. This PI kinase/$(Ca^{2+}+Mg^{2+})$-ATPase system is the first characterized target that is selective for G-kinase. Possible candidates for this PI kinase are polypeptides of 55 and 45 kDa which were better substrates for G-kinase than for A-kinase and which were not observed in the Ca^{2+}-transport ATPase preparation from erythrocytes (Fig. 7). The further identification of the G-kinase-regulated PI kinase requires further research. It also remains to be determined whether this G-kinase-regulated PI kinase affects other systems besides the plasmalemmal Ca^{2+} pump.

In conclusion, G-kinase stimulates the plasmalemmal ATP-dependent Ca^{2+}-extrusion pump of vascular and non-vascular smooth muscle. This stimulation does not involve a direct phosphorylation of the Ca^{2+}-transporting enzyme, but G-kinase might affect the activity of the $(Ca^{2+}+Mg^{2+})$-ATPase via the activation of an associated PI kinase. Together with the observation that phospholamban of the sarcoplasmic reticulum of cardiac and smooth muscle is a substrate for G-kinase [17], the present results further support the idea that the modulation of ATP-dependent Ca^{2+} transport may be a major mode of action of the cyclic GMP/G-kinase system.

We thank Dr. P. de Lanerolle (University of Illinois, Chicago) for the gift of antibodies to myosin light-chain kinase from chicken gizzard, and Dr. M. Nunnally and Dr. J. Stull for the gift of antibodies to myosin light-chain kinase from bovine trachea.

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Received 10 November 1987/9 June 1988; accepted 4 July 1988