Substrates of cGMP Kinase in Vascular Smooth Muscle and Their Role in the Relaxation Process

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ABSTRACT: G_1 is a hitherto unidentified substrate (molecular mass about 120 kDa) of the cGMP-dependent kinase, although its presence in vascular smooth muscle sarcolemma has been known for many years. Since it represents the major target of the G-kinase in smooth muscle, its physicochemical and biochemical properties were investigated. Solubilization of G_1 required a detergent: with Triton X-100, however, its extraction only occurred in the presence of high salt concentrations or millimolar ATP. These properties are typical for a membrane protein interacting with a nonmembraneous sedimentable moiety. Cupric phenanthroline-catalyzed oxidation revealed that the G_1 phosphoprotein could be oxidatively cross-linked to a sedimentable moiety. The latter was identified by two-dimensional (nonreduced/reduced) gel electrophoresis as actin which is attached to the sarcolemma. Furthermore, DNase I affinity chromatography demonstrated an interaction of solubilized G_1 with actin. The results suggest a role of G_1 in the plasma membrane–cytoskeleton interaction in smooth muscle cells.

Contracted smooth muscle cells relax upon stimulation of guanylate cyclase activity. Although the molecular events involved in the process are mostly unknown, it is reasonable to assume that a crucial step is the specific phosphorylation of key proteins by the activated G-kinase[1] (Walter, 1984; Lincoln & Johnson, 1984). In vascular smooth muscle, three proteins (named G_0, G_1, and G_2) which coisolate with the microsomal fraction (Casnellie & Greengard, 1974; Casnellie et al., 1980; Parks et al., 1987) are the main substrates of the G-kinase. Their nature is unknown: its clarification would shed light on the mechanism of the relaxation process. It has been proposed that G_1 (the major G-kinase substrate) is the Ca pump of the plasma membrane (Popescu et al., 1985; Furukawa & Nakamura, 1987). Alternatively, it has been proposed that the cGMP-dependent phosphorylation of plasma membrane proteins might regulate the Ca pump (Vrolix et al., 1988; Suematzu et al., 1984). However, previous work in our laboratory has clearly shown that G_1 is distinct from the Ca-ATPase (Baltensperger et al., 1988). The possibility of indirect coupling of G_1 phosphorylation to the stimulation of the Ca pump was also excluded since the Ca pumping activity of isolated plasma membranes was found to be independent of the phosphorylation level of G_1 (Baltensperger et al., 1988). Naturally, it is still possible that cGMP-dependent processes would improve Ca extrusion from the smooth muscle cell; this, however, would have to depend on the presence of hitherto unidentified cytosolic factors and would be independent of the phosphorylation of G_1. The present study was undertaken to understand the nature and, possibly, the function of the latter. Even if not involved in the mechanism of Ca extrusion, it was felt that the major substrate of G-kinase phosphorylation would deserve a detailed investigation.

Materials and Methods

Materials. [γ-^{32}P]ATP (triethylammonium salt, 3000 Ci/mmol) and [γ-^{35}S]ATP (600 Ci/mmol) were from Amersham International, England; adenosine 5′-[γ-thio]triphosphate tetralithium salt and leupeptin–hemisulfate were from Fluka, Buchs Switzerland; Triton X-100, DNase I (bovine pancreas, type IV), and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma Chemical Co., St. Louis, MO; SDS–polyacrylamide gel electrophoresis (PAGE) molecular weight standards (high range) were from Bio-Rad, Richmond, CA; CNBr-activated Sepharose 4B was from Pharmacia AB, Uppsala, Sweden; 1,10-phenanthroline was from Merck, Darmstadt, FRG. All other reagents were of the highest purity grade available.

Affinity-purified polyclonal anti-MLCK antibody was kindly provided by Dr. P. K. Ngai, Ciba-Geigy Ltd., Basel; antisera against chicken gizzard vinculin and integrain β-subunit were kindly provided by Drs. V. Niggli, University of Berne, Switzerland, and S. Johannson, University of Uppsala, Sweden, respectively.

Preparation of Sarcolemmal Fractions from Pig Aorta. Extracted microsomes were prepared as described (Baltensperger et al., 1988). A low ionic strength sucrose step gradient was prepared according to Kwan (1983). Briefly, 3 mL of a buffer containing 10 mM Hepes–KOH (pH 7.4), 1 mM DTT, and 60%, 40%, 30%, 15%, and 8% sucrose, respectively, was placed in Beckman SW 28.1 Ultra-Clear centrifuge tubes. Extracted microsomes (up to 10 mg or 1.5 mL/tube) were loaded on the gradient at the 8%/15% interphase and centrifuged overnight at 140000g_{max}. Fractions were collected from the 15%/30% interphase (F2), the 30%/40% interphase (F3), the 40% phase (F4), and the 40%/60% interphase (F5). The fractions were diluted with storage buffer and centrifuged for 45 min at 150000g. The pellets were then resuspended

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[1] Abbreviations: CuPh, cupric phenanthroline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PP_i, pyrophosphate; SDS, sodium dodecyl sulfate; TX-100, Triton X-100; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CTAB, cetyltriethylammonium bromide; DOC, deoxycholic acid sodium salt; DTT, dithiothreitol; A-kinase, cAMP-dependent protein kinase; G-kinase, cGMP-dependent protein kinase; eMS, extracted microsomes; MLCK, myosin light chain kinase; DNase I, deoxyribonuclease I.

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in the same buffer and stored at −70 °C.

Preparation of MLCK. A crude fraction of smooth muscle MLCK was prepared from pig aorta smooth muscle essentially as described (Ngai et al., 1984). MLCK was extracted from the detergent-treated pellet using high $MgCl_2$ concentrations (25 mM).

5′-Nucleotidase Activity. 5′-Nucleotidase activity was measured colorimetrically by determining the inorganic P_i released from Na-AMP (Aronson & Touster, 1974).

Phosphorylation of eMS. Cyclic GMP dependent phosphorylation was carried out according to a modification of the method described by Casnellie et al. (1980). eMS were phosphorylated with [γ-^{35}S]ATP and stored in aliquots at −70 °C. Six milligrams of eMS was diluted into a buffer containing 100 mM NaCl, 4 mM $MgCl_2$, 1 mM EGTA, 50 mM Hepes–NaOH (pH 7.0), 1 mM IBMX, 1 μM cGMP, and 1 mM GTP to obtain a final protein concentration of 1 mg/mL. The phosphorylation reaction was started by the addition of 4 μM [γ-^{35}S] containing 1 mCi of [γ-^{35}S]ATP (specific activity 200–400 mCi/mmol). After 15 min at 4 °C, the phosphorylation of the membranes was completed by the addition of 0.5 mM [γ-^{35}S]ATP and an additional incubation for 15 min. Radioactive [^{35}S]thiophosphate was removed by centrifugation. The membranes were resuspended in 0.75 mL of storage buffer/mg of eMS and stored at −70 °C.

Solubilization of Sarcolemmal Proteins. The solubility properties of the G-substrates were examined by an ultracentrifugation assay using a Beckman airfuge. Phosphorylated eMS were diluted into storage buffer (2 mg/mL with 250 mM sucrose/10 mM imidazole hydrochloride, pH 7.0) and solubilized by the addition of 0.65% Triton X-100 (or other detergents). Solubilized proteins were then separated from nonsoluble material by ultracentrifugation (105000*g* for 7 min). For the preparation of the TX-100/PP_i extract, 50 mM sodium pyrophosphate (pH 7.0) was added prior to the detergent.

Sequential Extraction Procedure. Phosphorylated eMS (4–5 mg/mL) were diluted into an equal volume of IF buffer containing 60 mM NaF, 60 mM imidazole hydrochloride (pH 7.8), 1 mM DTT, and 4 mM $MgCl_2$. To solubilize the membrane proteins, 0.65% TX-100 was added, and the solution (T1) was gently stirred for 15 min at 4 °C. The TX-100 soluble proteins were then separated from the insoluble material by ultracentrifugation in a Beckman TL 100 ultracentrifuge using the TL 100.3 rotor (250000*g* for 15 min). The supernatant fraction (S1) was discarded, and the pellet fraction (P1) was gently resuspended in IF buffer containing 4 mM EDTA instead of $MgCl_2$. P1 was then extracted by 10 mM ATP and 0.65% TX-100 to obtain the soluble fraction S2 which contains the G-substrates. S2 was centrifuged again at 40000*g* for 60 min to finally obtain the soluble fraction S400 and the particulate fraction P400.

Acetic Acid Extraction. An acetone powder from pig aorta smooth muscle sarcolemma was prepared basically according to Hubbard and Lazarides (1979). Cytoskeletal proteins were extracted from the acetone powder by incubating the powder in 1 M acetic acid for 1 h at 4 °C. Alternatively, acetic acid extraction was performed using a TX-100-insoluble residue from sarcolemma, which was prepared by solubilizing phosphorylated eMS with 0.65% TX-100 in a buffer containing 250 mM sucrose/10 mM imidazole hydrochloride (pH 7).

KI Extraction. KI extraction (1 M) of the cytoskeleton was performed as described (Gimona et al., 1987) using ^{35}S-thiophosphorylated eMS. The extract was dialyzed overnight against 5 mM Tris-HCl (pH 7.6), 0.1% (w/v) β-mercaptoethanol, and 1 mM PMSF and then processed for SDS–PAGE.

Oxidative Cross-Linking of Sarcolemmal Proteins. Oxidative cross-linking was essentially carried out as described by Murphy (1976) and Chiesi (1984). A stock solution of 6 mM $CuSO_4$ and 20 mM 1,10-phenanthroline (CuPh) was prepared and stored at 4 °C. Phosphorylated eMS (4 mg/mL) were diluted 4-fold into a buffer containing 100 mM NaCl/10 mM Hepes–NaOH, pH 7.0, and equilibrated at 25 °C. CuPh was then added to obtain a final concentration of 150 μM or as indicated in the figures (the concentrations given for CuPh refer to $CuSO_4$). The oxidation was stopped after 10 s by the addition of 10 mM EDTA. The TX-100/PP extract of phosphorylated membranes was cross-linked as eMS but for 5 min at 4 °C.

DNase I Affinity Chromatography. Sepharose 4B bound DNase I (2.5 mg of DNase I/g of resin) was prepared according to the manufacturer's instructions. A control resin was prepared identically but without DNase I.

The TX-100/PP_i extract from 2 mg of ^{35}S-thiophosphorylated eMS (0.6 mL) was diluted and dialyzed against a modified actin monomerization buffer containing 10 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.03% TX-100, 10 μg/mL soybean trypsin inhibitor, and 0.5 mM PMSF (CT-10 buffer) and dialyzed for 5 h at 4 °C against the same buffer. The dialyzate was then subjected to oxidation using the CuPh procedure (see above). Two identical DNase I columns (1.5-mL bed volume) were loaded (7.2 mL/h) with the oxidized TX-100/PP_i extract. The columns were washed with 10 volumes of CT-10 buffer and 10 volumes of the same buffer containing 50 mM PP_i (pH 8.0) until the radioactivity in the fractions reached background levels. Column 1 was then eluted at a flow rate of 4 mL/h with CT-10 buffer containing 50 mM PP_i and 10 mM DTT, whereas column 2 was treated identically but without DTT. Actin was eluted from both columns with 0.5 M sodium acetate, 30% (w/w) glycerol, 1 mM $CaCl_2$, 0.5 mM PMSF, 0.05% TX-100, and 3 M guanidine hydrochloride.

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out according to Laemmli (1970). If not stated otherwise, 8% separating gels were used. The analysis of samples under nonreducing conditions was carried out in the absence of DTT.

2-D (Nonreduced/Reduced) SDS–PAGE. After completion of the first (nonreduced) run, the gel was dissected as described in the figures. The gel slices were placed into Eppendorf tubes containing 50 μL of 3-fold-concentrated sample buffer (supplemented with 10 mM DTT) and incubated for 5 min at 95 °C. The gel slices were then fitted into the sample chambers of a second gel and overlayed with the incubation buffer. The second (reduced) run was carried out under the same conditions as the nonreduced run. After protein staining, the gels were dried and exposed overnight to Kodak X-Omat AR 5 films (Kodak) with an intensifying screen. When ^{35}S-thiophosphorylated samples were analyzed, the stained gel was immersed for 30 min in Amplify (Amersham International, England) before drying. The dry gel was then exposed as described above but without an intensifying screen. For the quantification of radioactivity, the radioactive bands were excised, immersed in 400 μL of perhydrol, and incubated for 15 min at 95 °C. Radioactivity was counted after the addition of 4 mL of scintillation cocktail (Beckman Ready SafeTM).

Electrophoretic Transfer and Immunodecoration. After electrophoresis, proteins were electrophoretically transferred to nitrocellulose. Immunodecoration was carried out by using

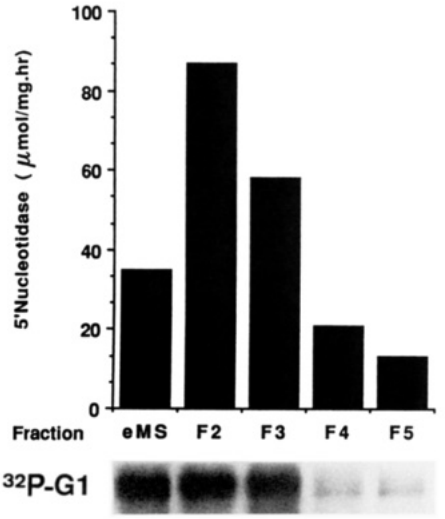

FIGURE 1: Codistribution of 5′-nucleotidase and the G_1-phosphoprotein. Extracted microsomes (eMS) and the sucrose step gradient fractions (F2–F5) were assayed for the activity of the sarcolemmal marker enzyme 5′-nucleotidase. A sample of each fraction containing 60 μg of protein was phosphorylated by the addition of 0.5 μM cGMP and 0.3 μM [γ-^{32}P]ATP and separated by SDS–PAGE. The inset shows autoradiography of the 120-kDa region of the dried gel. (For details, see Materials and Methods.)

the affinity-purified anti-MLCK antibody (raised in rabbits) at a dilution of 1:100. For the secondary antibody reaction, goat anti-rabbit conjugated horseradish peroxidase was used (dilution 1:1000), and cross-reacting bands were visualized with 0.5 mg/mL HRP color development reagent (Bio-Rad). The same protocol was used for the detection of vinculin, and the integrin b-subunit.

Protein Determination. Membrane protein was determined as described by Markwell et al. (1981) using bovine serum albumin as standard.

Results

Localization of the Major G-Kinase Substrates in Vascular Smooth Muscle. Three protein substrates of the cGMP-dependent kinase coisolate with the microsomal fraction of vascular smooth muscle (Casnellie et al., 1980; Baltensperger et al., 1988). These proteins represent a minor proportion of the total protein of the fraction and display apparent molecular masses of 230, 120, and 86 kDa. They have been named G_0, G_1, and G_2, respectively (Casnellie & Greengard, 1974). To clearly demonstrate their sarcolemmal origin, the KCl-free sucrose step gradient described by Kwan et al. (1983), which does not extract the membrane-bound cGMP-dependent kinase, was adopted to subfractionate the microsomal fraction. The amount of G-kinase substrates associated with the various membrane fractions produced by the procedure was then determined. Figure 1 shows that the distribution of the plasma membrane marker 5′-nucleotidase paralleled that of the G_1 phosphoprotein (apparent molecular mass 120 kDa), both being localized in the light gradient fractions F2 and F3. G_0 and G_2 (apparent molecular masses 230 and 86 kDa) followed the same distribution pattern (not shown).

Solubility Properties and Enrichment of the G-Substrates. The characterization of the major G-substrates began with a study of their solubility properties. For this purpose, in all the experiments reported in this study extracted microsomes (eMS) were phosphorylated in the presence of labeled [γ-thio]ATP. Under these conditions, G-kinase substrates phosphorylated to maximal levels (Baltensberger et al., 1988) are resistant to the action of phosphatases which were found to become activated during the solubilization procedures. The

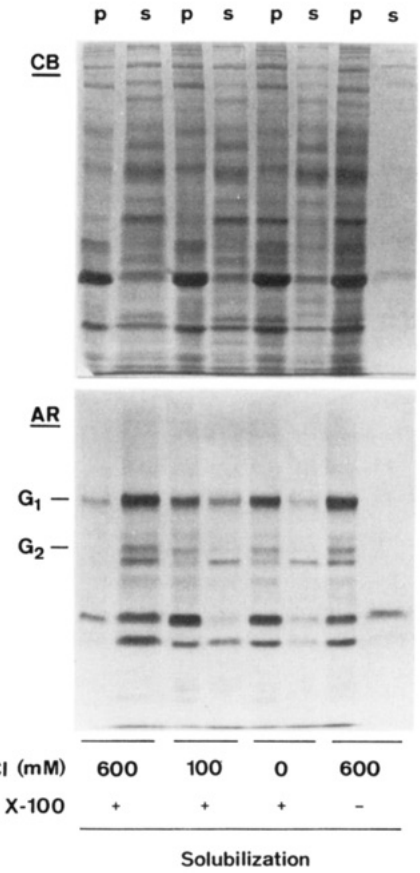

FIGURE 2: Dependence of the solubilization of the G-substrates by Triton X-100 on NaCl concentration. eMS were phosphorylated with [γ-^{35}S]ATP in the presence of 0.5 μM cGMP. Aliquots of 70 μg were solubilized by 0.65% Triton X-100 (+TX-100) in the presence of 600 mM NaCl, 100 mM NaCl, or 250 mM sucrose (0 mM NaCl). In a control experiment, incubation was carried out in the presence of 600 mM NaCl only. The pellet (p) and soluble (s) fractions obtained by ultracentrifugation were separated on a 8–15% Laemmli-type gel, which was stained with Coomassie blue (CB) and processed for autoradiography (AR).

G-kinase substrates had been previously found to be integral membrane proteins since they could not be extracted from microsomal membranes by high salt concentrations (Casnellie et al., 1980). Figure 2 shows that indeed none of the G-kinase substrates could be extracted by high salt in the absence of detergents. It was also found that G_1 could not be extracted from phosphorylated eMS with chaotropic agents such as NaSCN (1 M) or by extracting an acetone powder prepared from eMS with 1 M acetic acid; 6 M urea was also ineffective in extracting the phosphorylated proteins from the eMS. Only actin and other nonlabeled proteins were partially extracted. Interestingly, also the nonionic detergent Triton X-100 had a limited ability to extract the G-kinase substrates when no salt was present (Figure 2). Even the addition of 100 mM salt in 0.6% Triton X-100, which completely solubilizes many integral membrane proteins including the Ca-ATPase (not shown), solubilized only a very small portion of G_1. Solubilization of G_1 only occurred in Triton X-100 in the presence of high salt concentrations (0.6 M NaCl) (Figure 2). In addition to the G-kinase substrates, other phosphoproteins were also solubilized under these conditions. However, the extraction of most other proteins by Triton X-100 did not depend on the ionic strength of the medium, indicating a specific effect of the salt on the solubility properties of the G-kinase substrates; 50 mM sodium pyrophosphate or 10 mM ATP (Figure 3) were found to be as efficient as the high salt concentrations in solubilizing the G-kinase substrates: other nucleotides and

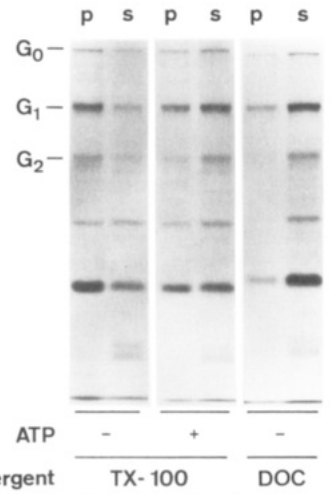

FIGURE 3: Solubilization of phosphorylated eMS by Triton X-100 in the presence of ATP or by deoxycholate. Extracted microsomes were phosphorylated with [γ-^{35}S]ATP in the presence of 0.5 μM cGMP. Seventy microgram aliquots were then solubilized by 0.65% Triton X-100 (TX-100) or deoxycholate (DOC) in the absence (−) or presence (+) of 10 mM ATP. The pellet (p) and soluble (s) fractions were separated and analyzed on a 10% Laemmli-type gel. Autoradiography of the dried gel is presented.

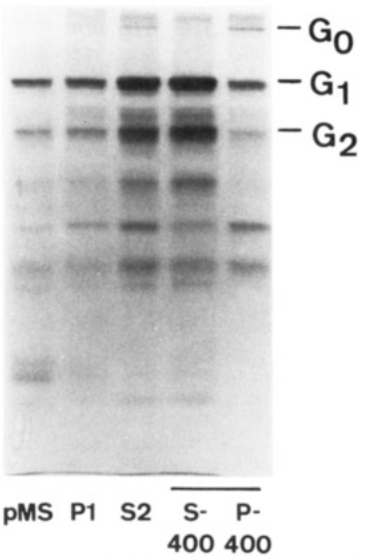

FIGURE 4: Enrichment of G_1 by sequential extraction. eMS were phosphorylated with [γ-^{35}S]ATP in the presence of 0.5 μM cGMP (pMS) and then solubilized by Triton X-100 in the absence of ATP. The insoluble material (P1) was separated from soluble proteins by ultracentrifugation and again solubilized in the same medium but with the addition of ATP. The soluble fraction (S2) of this step was collected by a second ultracentrifugation. The S2 fraction was subsequently centrifuged at 400000*g* for 1 h to obtain the soluble (S400) and particulate (P400) fractions. Samples containing equal amounts (75 μg) of proteins from each fraction were analyzed on a 10% Laemmli-type gel which was then processed for autoradiography.

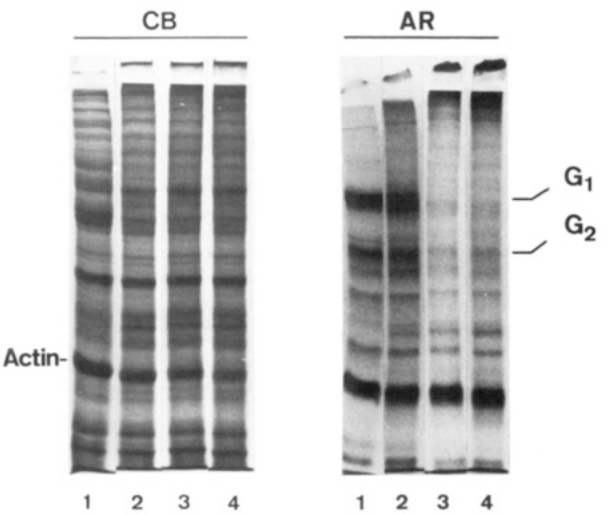

FIGURE 5: Oxidative cross-linking of the G-substrates by cupric phenanthroline. Extracted microsomes were phosphorylated with [γ-^{35}S]ATP in the presence of 0.5 μM cGMP. One hundered microgram aliquots were then analyzed by 8% SDS–PAGE under reducing (lane 1) or nonreducing (lanes 2–4) conditions after treatment without CuPh (lanes 1 and 2) or with 30 or 500 μM CuPh (lanes 3 and 4, respectively). CB, Coomassie blue stained gel; AR, autoradiography of the dried gel.

nonhydrolyzable ATP analogues were also effective (not shown). Inclusion of Ca or Mg (4 mM) in the solubilizing mixture slightly reduced the solubility of the G-kinase substrates, whereas EGTA or EDTA had no effect. A number of other detergents, such as CHAPS (1%), octyl glucoside (up to 2%), DOC (0.5%), and CTAB (0.5%), were also tested in the absence of ATP or of high salt concentrations, but only deoxycholate was able to solubilize the G-kinase substrates (Figure 3).

The particular solubility properties of G_1 from phosphorylated eMS were exploited to improve its extraction. Figure 4 shows the extraction of the G-kinase substrates when a two-step sequential solubilization procedure was applied to ^{35}S-thiophosphorylated eMS (pMS). In the first step, the membranes were solubilized by 0.65% Triton X-100 in a low ionic strength buffer containing Mg. This resulted in the solubilization of the bulk of the membrane proteins but not of the G-kinase substrates. The pellet (P1) was then resuspended in the same buffer, to which ATP (10 mM) and EDTA (1 mM) were added. The soluble fraction (S2) obtained from this step contained all of the phosphoproteins (including the G-kinase substrates) present in the pellet from the first solubilization step. The enrichment of G_1 in the various fractions obtained by this procedure was quantified as described under Materials and Methods and found to be 4-fold with respect to phosphorylated eMS. The enrichment could be further improved by ultracentrifugation (1 h, 40000*g*) of the S2 fraction. This step removed about 30% of the proteins, leading to an additional 1.5-fold enrichment of G_1 (Figure 4).

Oxidative Cross-Linking of G_1 with CuPh. Gel electrophoresis of phosphorylated eMS under nonreducing conditions frequently resulted in the broadening of the G_1 band and in a slight increase of its relative mobility (see Figure 5). In some cases, radioactivity appeared in the high molecular mass range of the gel. This observation was considered indicative of the formation of disulfide bridges by air oxidation among G_1 molecules or between G_1 and other proteins. Cross-linking of proteins can be catalyzed by cupric phenanthroline (CuPh), which promotes the air oxidation of spatially close (0.2 nm) sulfhydryl groups to disulfides (Kobashi, 1968). The reaction is fully reversed by reducing agents such as DTT or mercaptoethanol and provides a possibility for the mild and very specific cross-linking of proteins without affecting their activity. When thiophosphorylated eMS were treated at room temperature with low concentrations of CuPh, oxidation of G_1 and G_2 occurred within seconds. Radioactivity appeared in the high molecular mass region, whereas the G_1 and G_2 band rapidly disappeared (Figure 5). A considerable amount of the label did not penetrate into the stacking gel, indicating the formation of high molecular mass complexes. Cross-linking of G_1 and G_2 seemed to occur very specifically, since no other phosphoprotein shifted relative mobility, even when the concentration of CuPh was increased 15-fold. The Coomassie blue

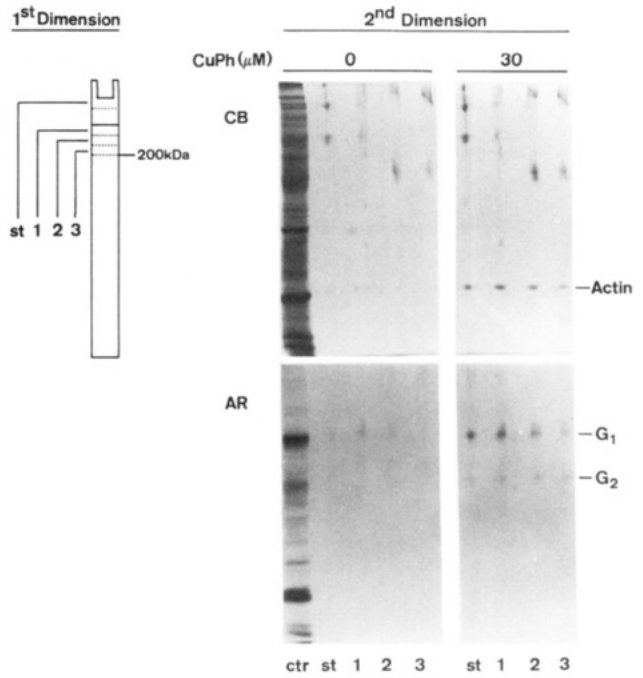

FIGURE 6: Two-dimensional (nonreduced/reduced) gel electrophoresis of phosphorylated eMS after cross-linking by CuPh. eMS were phosphorylated with [γ-^{35}S]ATP in the presence of 0.5 μM cGMP and subjected to oxidative cross-linking. Aliquots of 100 μg were separated on an 8% Laemmli-type gel under nonreducing conditions. Segments were then cut from the high molecular mass regions of the nonreduced gel, as indicated in the figure (first dimension). The gel slices from the stacking gel (st) and from the running gel (lanes 1, 2, and 3) were prepared for the second (reduced) dimension. In addition, an equal amount of the starting material used for the first dimension was prepared for the second dimension under reduced conditions (ctr). After completion of the second-dimensional run, the gel was stained for proteins and then processed for autoradiography. The gel (CB) and the autoradiography (AR) of the second dimension show the analysis for a sample, which was cross-linked with 30 μM CuPh, and for a sample which was treated identically but in the absence of CuPh.

stained gel showed that a single major protein of 42 kDa was affected by the CuPh treatment. This protein was identified as actin, because of its molecular mass (42 kDa) and of its extractability by the actomyosin extraction procedure. Thus, the G_1 molecules interacted either with themselves to form a homooligomer and/or with some other protein to form an heterooligomeric structure.

Actin Binding Property of G_1. The specificity of CuPh cross-linking for G_1 and actin suggested that the two proteins could interact in situ, thus favoring their heterologous cross-linking. In preliminary experiments, it was indeed observed that the amount of cross-linked actin in the high molecular mass region of the gel (including the stacking gel) paralleled that of G_1. Figure 6 shows the two-dimensional (nonreduced/reduced) gel separation of the proteins of cross-linked thiophosphorylated eMS. Gel slices in the molecular mass range higher than 200 kDa were cut from the first (nonreduced) dimension and run in the second (reduced) dimension. Radioactive G-kinase substrates and actin were not found in the high molecular mass region of the control eMS, i.e., in the sample which was not oxidized prior to the first dimension. In the second dimension, only a few proteins could be seen with apparent molecular masses of 200, 150, and 90 kDa. However, when the sample was oxidized with CuPh, the G_1, G_2, and actin bands appeared. Interestingly, the distribution of the G_1 band paralleled that of actin in the various gel slices. No other protein was cross-linked by CuPh, confirming the specificity of the method.

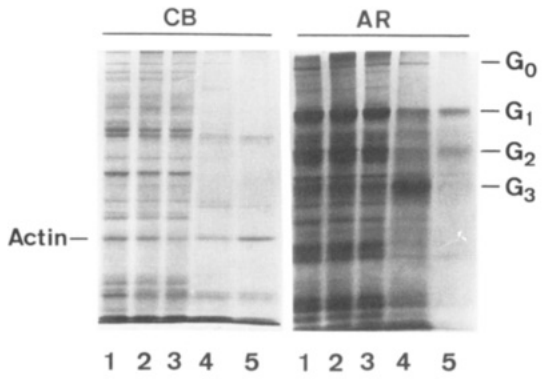

FIGURE 7: DNase I affinity chromatography of the TX-100/PP_i extract under high ionic strength conditions. The TX-100/PP_i-soluble fraction was diluted 20-fold into the high ionic strength column buffer and incubated for 2 h at 4 °C before being loaded onto a DNase I column. The fractions obtained from the column were then analyzed by 10% SDS–PAGE. The Coomassie blue stained gel (CB) and autoradiography of the dried gel (AR) show the TX-100/PP_i extract (lane 1), the diluted TX-100/PP_i extract loaded onto the column after preincubation (lane 2), the nonbound fraction (lane 3), the 0.75 M guanidinium chloride eluate (lane 4), and the 3 M guanidinium chloride eluate (lane 5). Lanes 4 and 5 contain 30 times more material than lanes 1–3.

The cross-linking experiments and the solubility properties of G_1 were thus highly indicative of an interaction between G_1 and actin. This possibility was further examined by using DNase I affinity chromatography. Since DNase I interacts with high affinity with actin, it is possible to bind indirectly actin binding proteins to the column. Solubilized G_1, obtained after the TX-100/PP_i extraction of phosphorylated eMS, was thus applied to a DNase I–Sepharose resin. Initial experiments were carried out by using a standard protocol for the affinity purification of actin under high ionic strength conditions (Figure 7): A fraction of G_1 bound to the column and coeluted with actin. The interaction of the G_1 portion with the column-bound actin was specific, because in the eluate of a control column (not coupled to DNase) a much smaller amount of G_1 was detected. The interaction, however, was very weak under the high ionic strength conditions required by the elution protocol, such that only 1–2% of the total G_1 applied was retained by the column. At low ionic strength, other proteins interacted with the column in addition to the G-substrates which were retained quantitatively (not shown). High ionic strength is thus required to avoid nonspecific binding to the column; on the other hand, the solubilization data show that the interaction between actin and G_1 was affected by high salt concentration. To overcome this problem, extracted G_1 was diluted in a low ionic strength medium to allow its association with actin, which, under these conditions, should have been depolymerized. Then, equal aliquots of the phosphorylated extract were oxidatively cross-linked and applied to two identical DNase I columns (Figure 8). The oxidation step was expected to induce the covalent binding of G_1 to actin, before the latter interacted with the DNase column. The interaction was then resistant to the necessary washing procedures. Both columns were washed extensively with the loading buffer containing 50 mM PP_i to remove nonspecifically adsorbed material; then column 1 was eluted with the PP_i buffer supplemented with 10 mM DTT, to reduce putative disulfide bonds: A fraction containing up to 10% of the total G_1 applied to the column was obtained together with other G-substrates (see Figure 8, PP_i + DTT). On the other hand, no additional material could be eluted under nonreducing conditions from column 2. The amount of total protein recovered in the PP_i + DDT fraction was less than 0.5% of that

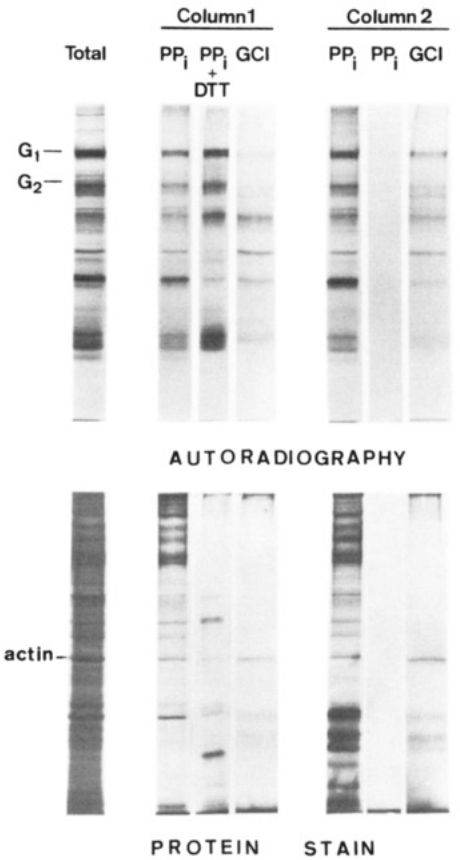

FIGURE 8: DNase I affinity chromatography of the TX-100/PP_i extract after oxidative cross-linking. The Triton X-100/PP_i-soluble fraction was cross-linked under low ionic strength conditions as described under Materials and Methods. Two identical DNase I columns (1 and 2) were then loaded with the cross-linked material (Total) and extensively washed with the loading buffer containing 50 mM PP_i. Subsequently, column 1 was eluted by the addition of 10 mM DTT to the wash buffer (PP_i + DTT), whereas column 2 was identically treated but without DTT (PP_i). The columns were then eluted with 3 M guanidinium chloride (GCl) to obtain the actin fraction. Aliquots were processed for 10% SDS–PAGE. The figure shows protein staining and autoradiography of the fractions obtained from columns 1 and 2.

applied to column 1. Thus, a considerable enrichment of G_1 (10–20-fold) was achieved even though, on gels stained with Coomassie Blue, no protein band corresponding to G_1 was visible. Subsequent elution of the columns with 3 M guanidine hydrochloride yielded a fraction containing mainly actin (see Figure 8, protein stain). A substantial quantity of G-substrates (including G_1) coeluted with actin from column 2 only.

Comparison of G_1 with Other Plasma Membrane Proteins of Similar Molecular Mass. The observations reported in this paper indicate that G_1 is a membrane-bound protein which interacts with actin. The properties of G_1 were thus compared with those of other proteins with similar molecular mass and characteristics (in particular, membrane-anchoring proteins). It was shown above that G_1 underwent rapid and specific cross-linking to a high molecular mass species by CuPh-catalyzed oxidation. This property was taken as a criterion to compare G_1 with vinculin and integrin. Using specific polyclonal antibodies, it was possible to detect integrin and vinculin in the eMS fraction. However, no CuPH-induced cross-linking of these proteins was observed, while G_1 monomers completely disappeared from the gel (not shown).

The recent proposal that G_1 corresponds to membrane-bound MLCK (Vrolix et al., 1989) was also investigated. A partially purified MLCK fraction was prepared from porcine aorta according to the method of Ngai et al. (1984). The

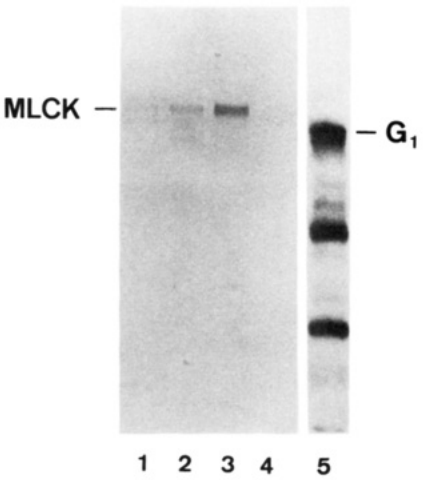

FIGURE 9: Immunological demonstration of MLCK in the $MgCl_2$ extract of porcine smooth muscle. A crude fraction of MLCK was prepared from porcine aortic smooth muscle by $MgCl_2$ extraction of the washed homogenized tissue slurry as described under Materials and Methods. Fifty micrograms of protein from the different fractions obtained from the MLCK preparation and 50 μg of eMS phosphorylated in the presence of cGMP were analyzed by 8% SDS–PAGE. The immunodecoration of a nitrocellulose replicate is shown. Lane 1, 17000*g* supernatant of the tissue slurry, corresponding to the starting material for the preparation of crude microsomes; lane 2, 17000*g* supernatant of the same fraction after two wash cycles; lane 3, $MgCl_2$ extract of the low-speed pellet containing a crude fraction of MLCK; lane 4, phosphorylated eMS. The G_1-phosphoprotein was visualized by autoradiography of the immunoreplicate (lane 5).

fractions obtained by this procedure were then analyzed by SDS–PAGE and Western blot (Figure 9) using affinity-purified polyclonal antibodies. As expected, MLCK was enriched only in the $MgCl_2$ extract (lane 3) of the low-speed pellet. No MLCK could be visualized in the eMS fraction (lane 4) enriched in the G-kinase substrates. Moreover, the immunodecoration of the nitrocellulose replicate of the gel showed a clear difference in the relative mobility of the MLCK, as recognized by the antibodies, and of G_1, as estimated by autoradiographing the dried blot (Figure 9). G_1 and MLCK are thus distinct entities.

Discussion

In principle, the suggestion of a linkage between G_1 phosphorylation and stimulation of Ca extrusion would be appealing, since it would provide a neat explanation for the well-known effect of cGMP on the relaxation of the smooth muscle cell. The data presented here show that G_1 is indeed associated with sarcolemmal membranes (see Figure 1). However, the properties demonstrate conclusively that the ATPase and G_1 are distinct entities. This is in agreement with previous results (Baltensperger et al., 1988) which also demonstrated that G_1 phosphorylation is not correlated with the stimulation of Ca transport. In this study, new interesting aspects of G_1 have been discovered which might be important for the understanding of its role. It should be noted that most of the properties of G_1, which is the major G-kinase substrate in smooth muscle, are shared by G_2, which probably represents a degradation product of the former (Parks et al., 1987), and by G_0. These three G-kinase substrates clearly belong to the same class of proteins and could thus play a similar role.

This study has shown that G_1 and actin in the eMS fraction can be rapidly and reversibly cross-linked by low concentrations of cupric phenanthroline, actin being present in the cross-linked material in large excess with respect to G_1. The cross-linked actin obviously derives from membrane-bound actin that was not removed by the actomyosin extraction procedure used for

the purification of the eMS. The presence of a nonextractable (membrane-bound) form of actin was observed also by Sharma and Bhalla (1986). The cupric phenanthroline-catalyzed cross-linking is highly specific and results in the formation of covalently linked G_1 complexes with molecular masses of several hundred kilodaltons, raising the possibility of the interaction of F-actin with the G_1 phosphoprotein in the sarcolemmal membrane. After two-dimensional gel electrophoresis separation of cross-linked membranes, actin and G_1 were found in similar relative proportions in the high molecular mass regions of the gel. This indeed indicated interaction between F-actin and the G-kinase substrate complexes.

The solubility characteristics of the G-kinase substrates deserve a comment. No extraction of G_1 from membranes by high salt concentrations or chaotropic agents could be observed in the absence of detergents, showing that G_1 is an integral membrane protein (Casnellie et al., 1980). However, the solubility properties of the G-kinase substrates differ from those of the majority of the other integral plasma membrane proteins, such as, for instance, the Ca-ATPase. While the latter enzyme is completely solubilized by 1% TX-100 in 100 mM NaCl (Niggli et al., 1979), under these conditions most of G_1 was still associated with the insoluble membrane material. Although the solubilization of the G-substrates in detergents was strongly dependent on the presence of a high salt concentration, the latter was not exclusive in determining solubilization: Pyrophosphate (50 mM) or ATP (10 mM) solutions (ionic strength of 0.3 and 0.08, respectively, at pH 7) were also very effective. Since the hydrolysis of ATP was not required for solubilization, a conformational change of G_1 upon saturation of an ATP (or PP_i) binding site, leading to its release from an insoluble matrix, appears probable. One should note, in this connection, that only the cytoskeleton and other filamentous material remain insoluble in the presence of 1% Triton X-100 and that the binding of ATP to F-actin at low ionic strength induces its depolymerization. This may be one of the possible conditions under which solubilization of the G-substrates occurs. In fact, if phosphorylated membranes were first cross-linked and then subjected to the Triton X-100/PP_i extraction procedure, no solubilization of G_1 occurred suggesting that G_1 binds to sedimentable, cross-linked actin filaments. Only deoxycholate was able to solubilize G_1 in the absence of ATP, in line with the observation that bile salts dissociate protein complexes with higher efficiencies than nonionic detergents (Hjelmeland & Chrambach, 1984). Microvilli from intestinal epithelial cells contain a complex of a 110-kDa protein with calmodulin (110K–CaM; Verner & Bretscher, 1985) which was also solubilized from the isolated cytoskeleton (prepared by Triton X-100 extraction) by physiological levels of ATP. The protein complex was shown to be involved in the interaction of the microfilament bundles with the plasma membrane. Although several criteria would rule out a relationship between this complex and G_1, the similarity in the solubility characteristics suggests that G_1 may also be involved in anchoring cytoskeletal elements (such as actin) to the plasma membrane.

The interaction between G_1 and actin was demonstrated by a DNase I affinity chromatography procedure. The method had already been applied to the purification of the cytoskeletal protein villin, which binds actin in a Ca-dependent manner (Bretscher & Weber, 1980). The data had clearly shown that the interaction of G_1 with the column was selective and occurred via actin. The experiments do not exclude the possibility that the G-substrates, rather than interacting directly with actin, are coupled to it via a minor protein component not detected by protein staining.

On the basis of the information available, an attempt to identify possible candidates for G_1 among known proteins could be made. Attempts to obtain partial sequences of the protein have so far been unsuccessful, due to the paucity of the material available and to other technical difficulties most likely related to the extraction procedure. MLCK was recently proposed as a candidate (Vrolix et al., 1989), since it can also be phosphorylated by a G-kinase, and has a molecular mass close to 130 kDa. However, the subcellular distribution and the solubility properties of G_1 and MLCK are completely different. In addition, the molecular masses of the two proteins from pig aorta smooth muscle differ by about 20 kDa.

Vinculin and the β-subunit of the integrin complex were also considered as likely G_1 candidates. Integrin is a membrane-spanning protein capable of interacting with extracellular matrix proteins (fibronectin, vitronectin, laminin) as well as with actin and other cytoskeletal components. Vinculin is a 130-kDa protein involved in the anchoring of actin to the plasma membrane. Vinculin itself is not an integral membrane protein but has been shown to be associated with adhesion plaques (Geiger, 1979). Both proteins are present in smooth muscle sarcolemma and share some properties wigh G_1, but immunodecoration experiments with specific antibodies have shown that they are different. Interestingly, however, integrin was coisolated with G_1 and could be enriched, together with G_1, by using the extraction protocol developed for G_1. The similarity in physicochemical properties thus suggests a similar functional role for the two proteins.

In conclusion, the information provided in this paper has shown that previous suggestions that the major substrate of the cGMP-dependent phosphorylation in smooth muscle is either the Ca pump or the membrane-bound MLCK are untenable. The results presented would support the following hypothesis: In sarcolemmal membranes, G_1 occurs as a membrane-bound structure which interacts with sedimentable actin filaments; G_1 could thus be a membrane attachment protein (Niggli & Burger, 1987; Pollard & Cooper, 1986). Several actin binding proteins have been shown to undergo phosphorylation. Although in most cases the physiological effect of the phosphorylation is not known, a regulatory role in cytoskeleton reorganization is possible. The specific phosphorylation of G_1 by the G-kinase could thus be involved in the control of cell morphology and in the reorganization of the cytoskeletal network. In this way, it would contribute to the relaxing effect of cGMP in smooth muscles.

Registry No. MLCK, 51845-53-5; G-kinase, 9026-43-1.

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CORRECTION

Partial Purification of the 5-Hydroxytryptamine-Reuptake System from Human Blood Platelets Using a Citalopram-Derived Affinity Resin, by E. A. L. Biessen, A. S. Horn, and G. T. Robillard*, Volume 29, Number 13, April 3, 1990, pages 3349–3354.

Page 3349. In the title, 5-hydroxytryptophan should read 5-hydroxytryptamine.