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Biochemical and Biophysical Research Communications 300 (2003) 155–160
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Association of phospholamban with a cGMP kinase signaling complex☆

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Received 18 November 2002

Abstract

The cGMP kinase signaling complex identified previously in tracheal smooth muscle membranes contains a number of cGMP kinase substrates termed G0 through G4. G0, G1, and G2 were identified as IP_3 receptor I (IP_3RI), IRAG, and cGMP kinase I. Sequencing of purified G3 and G4 showed that these proteins were proteolytic cleavage products of IRAG. However, the purified cGMP kinase signaling complex contained following additional proteins: α-actin, calponin H1, and phospholamban (PLB) as verified by MALDI-TOF as well as MS/MS sequencing and immune detection. The complex of these six proteins was immune precipitated by antibodies to each protein. The proteins were phosphorylated by the endogenous cGMP kinase I with the exception of α-actin and calponin H1. The complex did not contain the Ca^{2+}-ATPase SERCA II. PLB, IP_3RI, and cGMP kinase Iβ were co-immune precipitated after expression in COS-7 cells. These results suggest that PLB may have additional functions to regulate the activity of SERCA II.
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Keywords: Phospholamban; cGMP kinase I; IRAG; IP_3 receptor I; α-Actin; Calponin H1; SERCA II; Co-immune precipitation; Signaling complex; Smooth muscle

Phospholamban (PLB) is a 5-kDa membrane protein that forms a pentamer and associates with the sarcoplasmatic Ca^{2+}-ATPase II (SERCA II) in heart, smooth muscle, and other tissues [1,2]. PLB is phosphorylated by cAMP kinase at Ser^{16}, by protein kinase C at Ser^{10}, and by Ca^{2+}/calmodulin kinase at Thr^{17}. Phosphorylation results in dissociation of the protein from the Ca^{2+}-ATPase [3] relieving inhibition of Ca^{2+} transport from the cytosol to the sarcoplasmatic reticulum. Phosphorylation of PLB has been detected in vitro by cGMP kinase I (cGKI) [4] and after stimulation with atrial natriuretic peptide (ANP) [5] in smooth muscle tissue. In intact aortic smooth muscle cells, PLB is phosphorylated after treatment with sodium nitroprussid or ANP [6]. Deletion of PLB in mice increased vascular tone, but decreased the sensitivity of several smooth muscle preparations against potassium chloride, phenylephrine, and carbachol [7,8]. It was even reported that phosphorylation of PLB played only a minor role, if any, in cyclic nucleotide-mediated relaxation [9].

Cassnellie and Greengard [10] identified a number of cGMP kinase substrates in membrane fractions from smooth muscle, termed G0 to G4. Several of the substrates have been identified. G0 is the IP_3 receptor I, G1 is IRAG, and G2 is cGMP kinase Iβ (see [11] for references). These three proteins are associated in a tight complex and form a cGMP kinase signaling complex in bovine tracheal smooth muscle membranes [12]. Analysis of this complex revealed that it contained additional unknown proteins termed G3 and G4 that were phosphorylated in the presence of cGMP. The purpose of this study was to identify the nature of G3 and G4. Sequencing of the purified G3 and G4 proteins identified

☆ *Abbreviations:* RIα, regulatory subunit Iα of cAMP kinase; NDKB, nucleoside diphosphate kinase B; MALDI, matrix-assisted laser desorption ionisation; ES, nano-electrospray mass spectrometry.

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PII: S0006-291X(02)02799-7

them as proteolytic peptides of IRAG. However, this study also shows that PLB is associated with the cGMP kinase signaling complex. The complex does not contain the Ca^{2+}-ATPase SERCA II.

Materials and methods

Unless indicated otherwise, all procedures including buffers used for the purification of the membranes and the protein complex were carried out at 0–4 °C. All buffers contained following protease inhibitors: 1 mM benzamidine, 0.5 μg/μl leupeptin, and 0.3 mM phenylmethylsulfonyl fluoride.

Preparation of smooth muscle membranes. Bovine tracheal smooth muscle was prepared and phosphorylated as described previously [12]. The preparation of microsomal membranes was modified. EDTA (5 mM) and the above protease inhibitors were added to the homogenization buffer (20 mM MOPS, pH 7.4, 8% sucrose). After centrifugation, the pellet was re-suspended in the same buffer without adding EDTA.

Purification of cGKI substrates and associated proteins for protein sequencing. Microsomal membranes (5–10 mg) were purified using 8-AET-cGMP-agarose beads (Biolog) [12]. Separated proteins stained by Coomassie blue were excised and analyzed by MALDI-TOF and nano-electrospray mass spectrometry.

Immune precipitation. COS-7 cell lysate or bovine tracheal smooth muscle microsomes (500 μg) were subjected to immune precipitation with specific antibodies against IP_3RI, IRAG, cGKI, α-actin, calponin H1, NDKB, PLB, and a pre-immune serum. The antibodies were pre-bound to 15 μl protein-A or protein-G Sepharose (Sigma) for 1 h in 400 μl Lubrol-buffer (20 mM Tris, pH 8.0, 80 mM NaCl, 0.1% Lubrol PX, and 5 mM EDTA). Samples were solubilized in 200 μl of 2% Lubrol PX containing 5 mM EDTA and centrifuged (10 min, 11,000*g*) and the supernatant was incubated with the immobilized antibody for 2 h adding 800 μl Lubrol-buffer. Beads were washed three times, phosphorylated in the presence of 3 μM of 8-pCPT-cGMP and 0.1 mM [γ-^{32}P]ATP (2000 cpm/pmol, Amersham Pharmacia Biotech), and eluted by Laemmli buffer as described [12].

Immune precipitation of cGMP–agarose purified proteins was performed after elution of the complex by 2 ml of 20 mM cGMP-Na in 20 mM Tris, pH 8.0, 60 mM NaCl, 0.1% Lubrol PX, and 5 mM EDTA, for 1 h at room temperature. cGMP was removed through dialysis against Lubrol-buffer (2 h, 12 h, and 2 h) and the proteins were then immune precipitated as described above.

Cloning of PLB. Poly(A) mRNA was prepared from mouse heart and PLB coding sequence was amplified by PCR using following primer pairs: 5′-CCG GAA TTC ACC GCC ATG GAA AAA GTG CAA TAC-3′ and 5′-GCG GGA TCC TCA CAG AAG CAT CAC AAT-3′. The resulting PCR product was identical to PLB mouse cDNA published by Ganim et al. [13] and cloned into pcDNA 3.1 (Invitrogen) by *Eco*RI/*Bam*HI restriction sites.

COS-7 cell expression and lysate preparation. IP_3RI, IRAGa, cGKIα/β [14], and PLB coding DNA was inserted into pcDNA 3.1 (Invitrogen) and transiently expressed in COS-7 cells using calcium phosphate transfection method. Three days after transfection, cells were scraped in ice-cold phosphate-buffered saline (PBS) and harvested by centrifugation (720*g*, 10 min, 4 °C). After washing the cells twice in PBS, they were resuspended in a hypotonic lysis buffer (20 mM KPO_4, pH 7.4, 2 mM EDTA). The resulting suspension was subjected to two freeze–thawing cycles and pressed through a 23-gauge needle by a 1-ml syringe. Protein concentration was determined by the method of Bradford, using BSA as a standard. Protein expression was analyzed by Western blotting.

Polyacrylamide gel electrophoresis and immunoblot analysis. SDS–polyacrylamide gel electrophoresis (PAGE) was performed by the methods of Laemmli (12.5%, range: 240–35 kDa) and Schägger (Tricine–PAGE, 16.5%, range: 35–5 kDa) [15]. Immune detection used secondary antibodies conjugated with horseradish peroxidase (Dianova) or alkaline phosphatase (Dianova) visualized by enhanced chemiluminescence system (NEN Life Science Products) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT, Sigma), respectively.

Antibodies. The following antibodies were commercially available: IP_3RI (ABR), SERCA II (Dianova), PKA RIα (Transduction Laboratories), α-smooth muscle actin (Sigma), smooth muscle calponin H1 (Sigma), NDKB (Dianova), and PLB (ABR). Antibodies against IRAG [14] and cGKI [16] have been described. The pre-immune serum was from non-immunized rabbits.

Results

Characterization of the cGK signaling complex

Several previous studies showed that smooth muscle membranes contain a number of cGKI substrates termed G0, G1, G2, G3, and G4 [10,12,17]. Recently, G0,

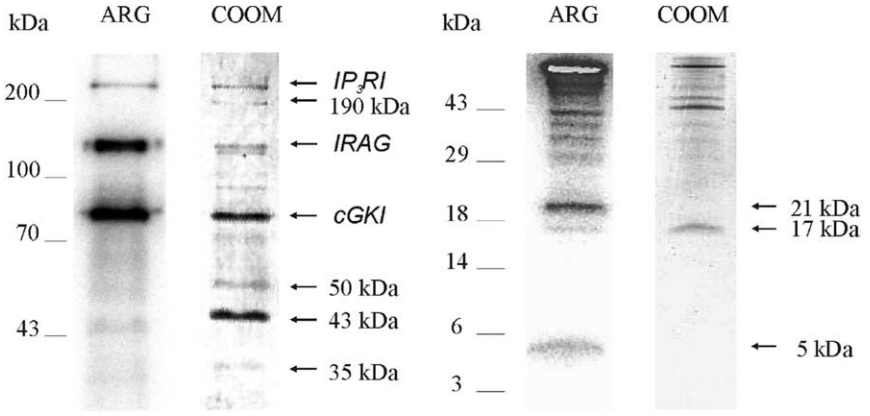

Fig. 1. The purified microsomal cGKI signaling complex. Solubilized bovine tracheal microsomal membrane proteins were purified by the cGMP–agarose method. Proteins were phosphorylated and separated by SDS–PAGE (12.5%; left panels) and Tricine–SDS–PAGE (16.5%; right panels). The left lane shows the autoradiogram (ARG), the right lane the Coomassie stain (COOM).

identified as IP_3 receptor type I (IP_3RI) [18], G1, a new protein termed IRAG (IP_3 receptor associated cGMP kinase substrate), and G2, cGMP kinase Iβ, were demonstrated to form a multimeric complex [12]. The nature of the other two substrates remained unclear. MALDI-TOF analysis of the purified putative substrates G3 (65 kDa) and G4 (30 kDa) showed that both proteins were proteolytic products of the IRAG protein. The generation of these proteolytic products was prevented by the inclusion of 5 mM EDTA in all purification buffers. Purification in the presence of 5 mM EDTA yielded only three cGMP-stimulated phosphoproteins in the 250 to 30 kDa region: G0 (migrating at an apparent M_r 240 kDa; IP_3RI), G1 (M_r 130 kDa; IRAG), and G2 (M_r 78 kDa; cGKIβ) (Fig. 1). However, additional copurifying proteins were identified by MALDI-TOF, nano-electrospray mass spectrometry, and immune decoration using the improved purification buffer and the cGMP–agarose procedure: myosin heavy chain (M_r 190 kDa), cAMP kinase regulatory subunit Iα (M_r 50 kDa), smooth muscle α-actin (M_r 43 kDa), smooth muscle calponin H1 (M_r 35 kDa), Rho A (M_r 21 kDa), nucleoside diphosphate kinase B (M_r 17 kDa), and PLB (M_r 5 kDa) (Table 1). From these proteins only the M_r 21 kDa band (Rho A) and the M_r 5 kDa band (PLB) were phosphorylated in the presence of cGMP. The regulatory subunit of cAMP kinase and the nucleoside diphosphate kinase B were contaminations caused by binding to the cGMP–agarose, since they were not immune precipitated by an anti-cGKI antibody. However, PLB, Rho A, α-actin, and calponin H1 were immune precipitated together with G0, G1, and G2 by this antibody. The specificity of the apparent interaction was further analyzed for PLB, α-actin, and calponin H1.

Table 1
Proteins purified with the cGKI signaling complex

M_r (kDa)	Identity	Method
190	Myosin heavy chain	MALDI
50	cAMP-kinase RIα	MALDI
43	Smooth muscle α-actin	MALDI
35	Smooth muscle calponin H1	MALDI/ES
21	Rho A	Western blot
17	NDKB	MALDI/ES
5	Phospholamban	Western blot

Solubilized bovine tracheal microsomal membrane proteins were purified by the cGMP–agarose method and were separated on SDS–PAGE as described in legend to Fig. 1.

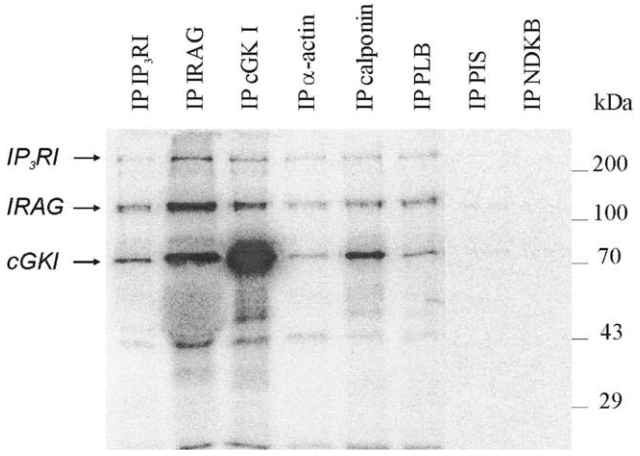

Fig. 2. Immune precipitation of the complex with specific antibodies. Solubilized microsomal membranes were purified by cGMP–agarose and phosphorylated in presence of 8-pCPT-cGMP. The autoradiogram shows phosphorylated IP_3RI, IRAG, and cGKI after immune precipitation (IP) with anti-IP_3RI, anti-IRAG, anti-cGKI, anti-α-actin, anti-calponin H1, anti-PLB, anti-NDKB antibodies or pre-immune serum (PIS).

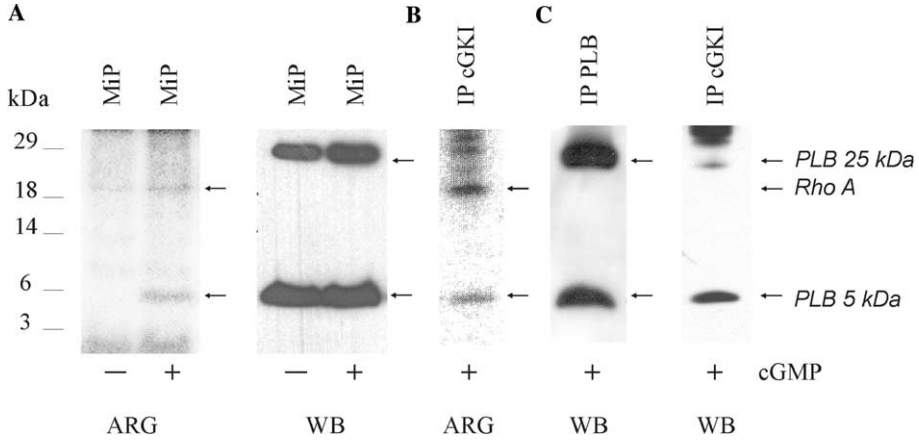

Fig. 3. Phosphorylation and immune precipitation of phosphorylated PLB. (A) Solubilized microsomal membranes (MiP) were phosphorylated in the presence (+) and absence (−) of 8-pCPT-cGMP for 2 min at 30 °C (ARG). Immune decoration of the identical blot with anti-PLB antibody shows an equal amount of PLB in the two lanes (WB). (B) Immune precipitation of solubilized bovine tracheal membranes with anti-cGKI antibody. The proteins were phosphorylated in the presence of 8-pCPT-cGMP, seperated by Tricine–SDS–PAGE and detected by autoradiography (ARG). (C) Immune precipitation of 500 μg and 2.5 mg solubilized bovine tracheal membranes with anti-PLB and anti-cGKI antibody, respectively. The precipitated proteins were separated by Tricine–SDS–PAGE and PLB was detected by an anti-PLB antibody.

The phosphorylation of Rho A by cGMP kinase has been reported previously [19,20]. From these reports it was obvious that Rho A associates with membranes and cGKI. Therefore, this interaction was not analyzed further.

In contrast, the interaction of PLB and the cytoskeletal proteins with the cGMP kinase signaling complex suggested that these proteins interacted specifically with the sarcoplasmic reticulum. The cGMP kinase signaling complex was immune precipitated from solubilized and cGMP–agarose purified bovine tracheal membrane proteins by antibodies specific for the IP_3RI, IRAG, cGKI, α-actin, calponin, and PLB (Fig. 2). These proteins were not precipitated by a pre-immune serum or by an antibody against the nucleoside diphosphate kinase B, suggesting the interaction is specific.

Phosphorylation of PLB in the cGK complex

PLB was phosphorylated by cGKI in microsomal membranes (Fig. 3A) as described [4–6] and after precipitation of solubilized membranes with anti-cGKI antibodies (Fig. 3B). Phosphorylated PLB runs mainly as a monomer (5 kDa) on the SDS–PAGE. These results suggested but did not prove the fact that PLB was a member of the cGMP kinase signaling complex. Comparison of the amount of immune stained PLB after precipitation from solubilized membranes suggested that the anti-cGKI antibody precipitated about 5–10% of the amount precipitated by the anti-PLB antibody (Fig. 3C). This number suggested that a small amount of PLB may be a member of the smooth muscle cGMP kinase signaling complex.

Interaction of heterologously expressed PLB with cGKI

To support this hypothesis, we expressed murine PLB together with other members of the cGMP kinase signaling complex in COS-7 cells. Native COS-7 cells express PLB that runs as a pentamer (25 kDa) on the SDS–PAGE (Fig. 4), whereas a monomer (5 kDa) could be identified after transfection with the PLB-cDNA. The anti-PLB antibody precipitated IP_3RI and cGKIβ (Fig. 5). cGKIβ was not precipitated by PLB antibodies from extracts containing only PLB and cGKIβ. Furthermore, replacement of cGKIβ by cGKIα prevented precipitation of the cGKIα and IP_3RI (data not shown), suggesting that PLB forms a complex with IP_3RI and cGKIβ in COS-7 cells. Control experiments with solubilized tracheal membranes indicated that the same anti-PLB antibody immune precipitated IP_3RI, IRAG, and cGKIβ in this tissue (Fig. 6). This apparent difference could be due to the presence of additional proteins, i.e., the cytoskeletal proteins, in the tracheal smooth muscle. It has

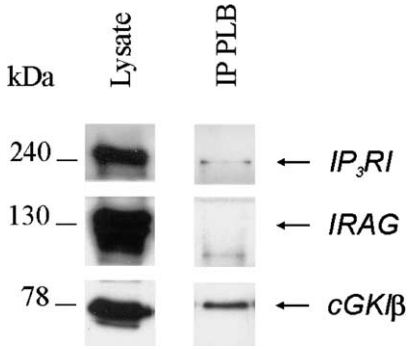

Fig. 5. Expression and immune precipitation of the signaling complex in COS-7 cells. IP_3RI, IRAGa, cGKIβ, and PLB were co-expressed in COS-7 cells. Left lane: Lysate (30 μg protein) of transfected cells. Right lane: Proteins immune precipitated from a cell lysate (500 μg protein) with anti-PLB antibody. The proteins were separated by 12.5% SDS–PAGE and detected by anti-IP_3RI, IRAG, and cGKI antibodies.

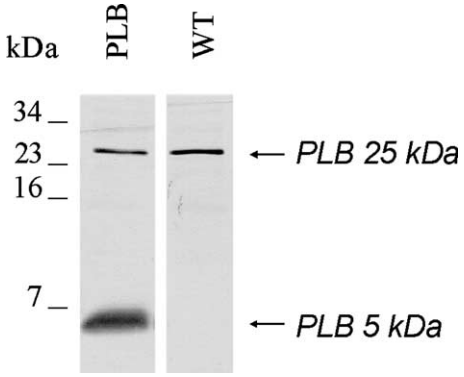

Fig. 4. Expression of PLB in COS-7 cells. COS-7 cells were transfected with control and PLB-cDNA containing vector. Lysates (30 μg protein) of PLB overexpressing (PLB, left lane) and wild type (WT, right lane) cells were separated and immune decorated by anti-PLB antibody.

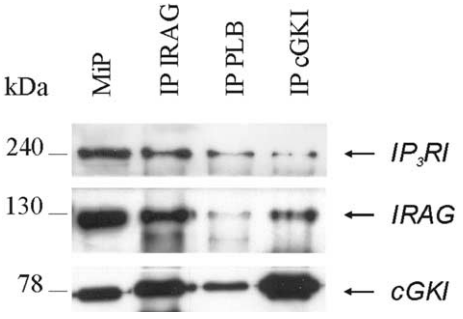

Fig. 6. Co-immune precipitation of the microsomal cGKI complex proteins from bovine tracheal smooth muscle membranes. Solubilized microsomal membranes (500 μg) were precipitated with antibodies against IRAG, PLB, or cGKI and were separated by 12.5% SDS–PAGE together with the 30 μg microsomal membranes (MiP). Proteins were detected by anti-IP_3RI, -IRAG, and -cGKI antibodies.

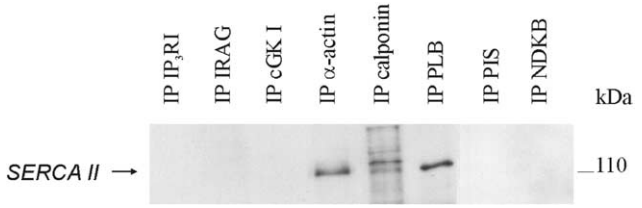

Fig. 7. Non-association of SERCA II with the cGKI signaling complex. Solubilized microsomal membranes (2.5 mg) were purified using cGMP–agarose prior immune precipitation with antibodies against IP_3RI, IRAG, cGKI, α-actin, calponin H1, PLB, NDKB or a preimmune serum. Precipitates were separated on a 12.5% SDS–PAGE. SERCA II was detected by a specific monoclonal antibody.

been reported that G1, i.e., IRAG, interacts with actin [21].

PLB-cGKI association is independent of SERCA II

The results described so far suggest that PLB is a member of the cGMP kinase signaling complex. On the other hand, non-phosphorylated PLB is associated with the Ca^{2+}-ATPase SERCA II in heart, smooth muscle, and other tissues [1,2]. We asked therefore whether or not the cGMP signaling complex contained SERCA II. Solubilized tracheal membrane proteins were purified on cGMP–agarose and the eluate was immune precipitated with a number of antibodies (Fig. 7). SERCA II immune reactivity was not observed in the complex precipitated with antibodies against IP_3RI, IRAG, and cGKI. SERCA II was precipitated with antibodies against α-actin, calponin H1, and PLB. This result could be confirmed, because immune precipitation with antibodies against SERCA isoforms IIa and IIb yielded PLB, SERCA II, and further bands, but never IP_3RI, IRAG, and cGKIβ immune reactive bands.

Discussion

This study analyzed the composition of a cGMP kinase signaling complex present in the microsomal membranes of tracheal smooth muscle. The analysis of the cGMP kinase substrates G3 and G4 showed that these proteins were derived proteolytically from the recently identified cGMP kinase substrate IRAG. However, we identified by sequencing, immune decoration, and immune precipitation additional members of the signaling complex. Calponin and α-actin are cytoskeletal proteins. Presumably, they anchor the IP_3-sensitive stores to other parts of the smooth muscle cell, such as the plasma membrane as described in neurons [22]. Previously, it was suggested that emptying of the IP_3-sensitive stores brings the IP_3 receptor in close contact with the plasma membrane localized trp ion channels [23]. It is therefore not surprising that these cytoskeletal proteins were found in the cGMP kinase signaling complex.

To our surprise a small, but significant amount of PLB precipitated with the cGMP kinase signaling complex. This association was seen after immune precipitation with antibodies against PLB and cGKI and after coexpression of IP_3RI, IRAG, cGKIβ, and PLB. PLB was associated in a tighter complex in the native membrane than after expression in COS cells, suggesting that additional proteins may stabilize the interaction. The functional significance of PLB in the cGMP kinase signaling complex is unclear since the Ca^{2+}-ATPase SERCA II was not associated with this fraction of PLB. Deletion of PLB in mice suggested that the lack of PLB decreased the sensitivity of agonists to contract smooth muscle preparations [7]. This finding was not in line with the known function of PLB, i.e., regulation of the SERCA II activity. Removal of PLB should have resulted in an increased calcium load of the calcium vesicles and an increased calcium release after agonist stimulation. This should have resulted in an increased sensitivity of agonist induced contraction. It is possible that the finding in the knockout animals was caused by the absence of PLB in the cGMP kinase signaling complex. If this speculation is correct, PLB may facilitate IP_3 induced calcium release as discussed by Lalli and colleagues [9]. Phosphorylation of PLB in smooth muscle [4–6,24] may therefore be associated with modulation of IP_3 receptor activity and a decrease in calcium release supporting the well-established finding that cGMP kinase suppresses agonist stimulated calcium levels in smooth muscle [25]. In support of this interpretation is the report that cGMP did not affect the intracellular calcium uptake in intact megakaryocytes [26].

The majority of PLB is associated with SERCA II. Antibodies to calponin and α-actin precipitated also the Ca^{2+}-ATPase SERCA II that again is part of the microsomal Ca^{2+} store vesicles. However, SERCA II was not found in the cGMP kinase complex, suggesting that (a) the immune precipitation procedure was specific and did not precipitate just microsomal membranes and (b) that the ATPase and the IP_3 receptor are not interacting directly with each other, although most likely they are localized on the same vesicle membrane. The dual localization of PLB could also explain the phenomenon that an increase in cGMP does not increase intracellular IP_3-stimulated calcium release [16]. The immune precipitation did not yield any hint that cGMP kinase I and SERCA II are colocalized in tracheal smooth muscle. The different localization would not allow cGMP kinase I to phosphorylate PLB associated with SERCA II. In conclusion, this report raises the possibility that PLB has multiple functions in smooth muscle. It points further the direction to understand some of the

controversial findings in the smooth muscle physiology of PLB.

Acknowledgments

We thank Dr. F. Wuytack for SERCA IIa and SERCA IIb polyclonal antibodies and Dr. M. Aepfelbacher for the Rho A monoclonal antibody. This work was supported by grants from Deutsche Forschungsgemeinschaft, Sanderstiftung, Fond der Chemischen Industrie, and stipend by the TU München to A.K.

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