Characterization of calreticulin as a protein interacting with protein kinase C

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A protein kinase C (PKC)-binding protein was purified to homogeneity from the Triton-insoluble fraction from rat hepatocytes homogenates. The protein was identified as the mature calreticulin chain by N-terminal amino acid sequencing and by its immunoreactivity with anti-calreticulin antibody raised against the C-terminal KDEL (single-letter code) sequence. The calculated molecular mass was 46.6 kDa but the protein migrates in SDS/PAGE as a doublet with apparent molecular masses of 60 and 55 kDa. Studies *in vitro* with purified calreticulin with the use of an overlay assay approach demonstrated that it binds to activated PKC isoenzymes expressed in rat hepatocytes. Phosphorylation of purified calreticulin with a PKC isoenzyme-specific immune complex kinase assay showed that it is also a

very good substrate for all PKC isoforms *in vitro*. The treatment of intact cells with phorbol ester or with adrenaline (epinephrine) plus propranolol increased calreticulin phosphorylation, which was blocked by the pretreatment of cells with the PKC-specific inhibitor Ro 31-8220. The analysis of calreticulin immunoprecipitates from control or treated cells indicated that PKC α , PKC β , PKC θ , PKC ζ and PKC μ , but not PKC δ or PKC ϵ , co-immunoprecipitated with calreticulin. Taken together, our results indicate that PKC interacts *in vivo* with calreticulin and suggest that they can operate in common signalling pathways.

Key words: calcium-binding proteins, protein kinase C substrates, rat hepatocytes, RACKS.

INTRODUCTION

Protein kinase C (PKC) is a family of 12 lipid-dependent serine/threonine kinases that have key regulatory roles in a wide spectrum of signal transduction. These kinases were among the first signalling proteins to be shown to translocate to new cellular sites on activation, including the plasma membrane [1,2], cytoskeletal elements [3,4], the nucleus [5,6] and other subcellular compartments [7]. The study of PKC function is complicated by the presence of multiple PKC isoenzymes in individual cells. Precision of placement is particularly critical in signal transduction, in which background noise must be kept to a minimum. Invaluable insight into how specific motifs mediate precise targeting has come from the identification, biochemical characterization and structural elucidation of different protein modules that mediate protein–protein interactions that concentrate the enzymes at discrete locations within the cell, thus favouring the phosphorylation of substrate proteins that are found at these sites [8,9]. In contrast, the sharing of kinases between pathways also makes it critical that the cell route the various signals properly to prevent cross-talk. Cells seem to have solved this problem with the use of scaffolding/adaptor proteins by forming multienzyme complexes with kinases and phosphatases that are used by more than one pathway and are therefore shared.

A rapidly growing number of proteins that bind to PKC are being identified by overlay assays (a modification of Western immunoblotting that uses PKC rather than antibody to probe protein bands), by interaction cloning (in which a cDNA expression library is probed with PKC) and by using the yeast two-hybrid genetic screen for protein–protein interactions. By these methods it has been found that PKC binds to a multienzyme scaffold protein that belongs to the AKAP family [named as a result of their original discovery as cAMP-dependent (A-kinase)-anchoring proteins]: the AKAP79 family member, which localizes PKA, PKC and the type 2B phosphatase calcineurin to

postsynaptic densities [10]. Other binding proteins identified by such methods include several that also serve as direct substrates for PKC [substrates that interact with C-kinase ('STICKS')] [11] as well as proteins containing interaction motifs such as pleckstrin homology ('PH') domains and a class of proteins referred to as RACKs [receptors for activated C-kinase (PKC)] [12–15]. So far, two RACKs have been cloned, showing that they belong to a superfamily that includes the β subunit of G-proteins and other proteins that have an internally repeating element termed the WD-40 motif [16]. We have also identified at least seven proteins that behave like RACKs in the Triton-insoluble fraction of rat hepatocytes [15,17] and purified several of them to apparent homogeneity [18]. Here we present evidence that one of these proteins that we purified as a RACK is calreticulin. This protein was originally identified as a Ca²⁺-binding protein in the endoplasmic reticulum; however, since then, other functions of calreticulin have been characterized, including chaperone and lectin properties and the regulation of integrin and nuclear hormone receptor activity [19]. However, although calreticulin has been implicated in so many cellular functions, its precise task and the reason for its extremely high conservation during evolution remain elusive. So far, there has been no report about the interaction of calreticulin with PKC. Here we demonstrate that calreticulin is a target of PKC phosphorylation and associates with several PKC isoforms *in vivo*.

EXPERIMENTAL

Materials

Isoenzyme-specific polyclonal antibodies against the C-terminus of PKC were purchased from Santa Cruz Biotechnology (anti-PKC α , anti-PKC β , anti-PKC δ , anti-PKC θ , anti-PKC ζ and anti-PKC μ) or from Gibco (anti-PKC ϵ). Polyclonal anti-(human

Abbreviations used: PKC, protein kinase C (C-kinase); RACKs, receptors for activated C-kinase.

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calreticulin) antibodies were obtained from Upstate Biotechnology. Alkaline-phosphatase-conjugated goat anti-rabbit IgG was from Bio-Rad. PMA, Histone H1-IIIS, phosphatidylserine, Triton X-100 and 1,2-diolein were from Sigma. Protein A-Sepharose and Ro 31-8220 were from Calbiochem; DEAE-cellulose (DE-52) was from Whatman. [^{32}P]P_i and [γ - ^{32}P]ATP (6000 Ci/mmol) were from DuPont-NEN.

Isolation of rat hepatocytes

Male Wistar rats (200–250 g) fed *ad libitum* were used. Hepatocytes were isolated by the method of Berry and Friend [20].

Purification of calreticulin

Calreticulin was purified to homogeneity from the Triton X-100-insoluble fraction of rat hepatocyte homogenates as described by Robles-Flores et al. [18], or from perfused rat livers by the method of Milner et al. [21].

Protein sequencing

N-terminal sequence analysis was performed on purified protein electroblotted on PVDF membrane with a model LF3000 Protein Sequencer (Beckman) by using chemicals and software supplied by the manufacturer.

Immunoblot analysis

Purified protein (10 μg) was separated by SDS/PAGE [10% (w/v) gel] and transblotted to nitrocellulose membrane as described by Towbin et al. [22]. Protein was immunodetected with 1 $\mu\text{g}/\text{ml}$ rabbit polyclonal anti-(human calreticulin) antibodies and developed with a goat anti-rabbit IgG second antibody coupled to alkaline phosphatase.

Partial purification of PKC

PKC was purified as described previously [23]. In brief, hepatocytes were homogenized in ice-cold buffer [20 mM Tris/HCl (pH 7.5)/10 mM EGTA/2 mM EDTA/0.5% (v/v) Triton X-100/50 mM 2-mercaptoethanol/1 mM PMSF/10 $\mu\text{g}/\text{ml}$ leupeptin/0.1 mg/ml trypsin inhibitor], incubated at 4 °C for 30 min and then centrifuged. Portions of the supernatant were applied to DEAE-cellulose columns that had been equilibrated with column buffer [20 mM Tris/HCl (pH 7.5)/50 mM 2-mercaptoethanol] at 4 °C. After the column had been washed with column buffer, total PKC was eluted with column buffer containing 0.25 M NaCl, 2 mM EDTA and 0.1 mg/ml trypsin inhibitor. The eluate was concentrated with an Amicon device (YM-30 membrane) and used for overlay assays.

Determination of PKC binding activity by an overlay assay

PKC binding was determined as described previously [15]. In brief, sample protein was separated by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose membranes, which were then blocked with 3% (w/v) BSA. Partly purified and concentrated PKC (100–200 units; one unit is defined as 1 pmol of ^{32}P transferred from [γ - ^{32}P]ATP to histone/min per mg of protein; the final protein concentration was 0.5 mg/ml) was incubated for 1 h at 30 °C with the nitrocellulose strips in blotting buffer [50 mM Tris/HCl (pH 7.5)/1% poly(ethylene glycol)/0.2 M NaCl] in the presence of phosphatidylserine (20 $\mu\text{g}/\text{ml}$), 1,2-diolein (0.8 $\mu\text{g}/\text{ml}$) and CaCl₂ (1 mM). The PKC/blotting buffer mixture was removed and the membranes were washed three

times with blotting buffer. Bound PKC was detected with isoenzyme-specific anti-PKC antibodies and goat anti-mouse or goat anti-rabbit IgG second antibodies conjugated with alkaline phosphatase.

PKC immune complex kinase assay

Aliquots (1 ml) of partly purified and concentrated PKC (1 mg/ml) as described above but in the presence of phosphatase inhibitors (10 mM β -glycerophosphate, 1 mM Na₃VO₄, 11 mM NaF, 10 mM sodium pyrophosphate and 0.2 mg/ml phosphoserine) and in the absence of 2-mercaptoethanol were incubated at 4 °C with 1 $\mu\text{g}/\text{ml}$ isoenzyme-specific PKC antibody for 1 h with gentle shaking. Then 20 μl of Protein A-Sepharose [30% (w/v), Calbiochem] was added and incubation continued for 1 h. Immune complexes were then washed three times with buffer A [50 mM Tris/HCl/0.6 M NaCl/1% (v/v) Triton X-100/0.5% (v/v) Nonidet P40 (pH 8.3)] containing 0.1 mg/ml trypsin inhibitor and 1 mM PMSF, and once with kinase buffer [20 mM Tris/HCl (pH 7.5)/10 mM MgCl₂/0.5 mM CaCl₂/50 mM 2-mercaptoethanol]. Kinase activity was initiated by resuspending the immunoprecipitates in 50 μl of assay mixture, consisting of kinase buffer plus 20 $\mu\text{g}/\text{ml}$ phosphatidylserine, 0.8 $\mu\text{g}/\text{ml}$ 1,2-diolein, 10 μM [γ - ^{32}P]ATP (6000 Ci/mmol) and 200 $\mu\text{g}/\text{ml}$ of protein substrate (histone H1-IIIS or calreticulin). Reactions proceeded for 20 min at 30 °C, then were terminated by the addition of 50 μl of SDS/PAGE sample buffer, boiled for 5 min and analysed by SDS/PAGE [12.5% (w/v) gel] and autoradiography.

Metabolic labelling and determination of specific phosphorylation

Freshly isolated rat hepatocytes were incubated with 500 $\mu\text{Ci}/\text{ml}$ [^{32}P]P_i (carrier-free, 10 mCi/ml) in phosphate-free Krebs–Ringer buffer supplemented with 10 mM Hepes and 10 mM glucose, pH 7.4, for 90 min at 37 °C with shaking. When Ro 31-8220 (300 nM), PMA (1 μM) or adrenaline (epinephrine) (10 μM) plus propranolol (10 μM) were used, they were added to the incubation medium for the last 5 or 10 min of the incubation as indicated in the figure legends. Cell lysates were prepared and immunoprecipitated with anti-calreticulin antibody (1 $\mu\text{g}/\text{ml}$) as described above for PKC and then resolved by SDS/PAGE [12.5% (w/v) gel]. Phosphorylated calreticulin was detected by autoradiography and total protein was determined in the same membrane by immunoblotting with the enhanced chemiluminescence (ECL[®]) Western blotting detection system (Amersham). Autoradiographs were quantified with an Image Densitometer (Bio-Rad GS-670). The total specific phosphorylation was calculated as the ratio of ^{32}P -labelled protein to total protein.

RESULTS

Previously we reported the existence of at least seven proteins that behave like RACKs in rat hepatocytes [15]. We also developed a rapid and simple purification method of several RACKs, taking advantage of the ability of these proteins to be precipitated with Triton X-100 [18]. The method can be used for the isolation of other proteins that share this property, although not all proteins are precipitated with the same efficiency [18]. To isolate new proteins that behave like RACKs, we employed this protocol to isolate a protein from the Triton-insoluble fraction of rat hepatocyte homogenates by DEAE-cellulose chromatography followed by a second precipitation step with Triton X-100 and by electroelution of the desired protein. The aim of the present study was to identify the isolated PKC-binding protein by N-terminal sequencing and to characterize its interaction with PKC.

Figure 1 Identification of the purified protein as calreticulin

(A) SDS/PAGE analysis of purified PKC-binding protein. Protein was purified from the Triton X-100-insoluble fraction from rat hepatocyte homogenates by DEAE-cellulose chromatography (lane 2) followed by a second precipitation with Triton X-100 and electroelution of the 60 kDa band (lane 1). Sample protein (10 μ g) was separated by SDS/PAGE [12.5% (w/v) gel] followed by staining with Coomassie Blue. The positions of molecular mass markers are indicated (in kDa) at the left. (B) Analysis of the purified protein by N-terminal sequencing. The results obtained from a search of the SwissProt database are shown. The one-letter amino acid notation is used. (C) Immunoblot analysis. Purified calreticulin from rat hepatocytes (10 μ g) was separated by SDS/PAGE [10% (w/v) gel] and transferred to nitrocellulose membrane. Immunoblot analysis was performed with anti-calreticulin polyclonal antibody (Upstate Biotechnology) and developed with alkaline-phosphatase-conjugated anti-rabbit IgG second antibody. The positions of molecular mass markers are indicated (in kDa) at the right.

Figure 2 Overlay assay to determine the binding of PKC isoenzymes to purified calreticulin

Samples (12 μ g) of purified calreticulin (CR) or BSA were subjected to SDS/PAGE [10% (w/v) gel] and blotted to nitrocellulose. PKC binding was determined by incubating the membranes in the absence or presence of partly purified PKC and in the presence of phosphatidylserine, 1,2-diolein and Ca^{2+} for 1 h. The mixture was removed, membranes were washed and bound PKC was detected with isoenzyme-specific anti-PKC antibodies (which in all cases recognized the C-terminus) or with consensus anti-PKC (PKC_{con}) (which recognized all isoforms) as indicated. Membranes were developed with an alkaline-phosphatase-conjugated second antibody. The positions of molecular mass markers are indicated (in kDa) at the right. Results are representative of four independent experiments.

As shown in Figure 1(A) (lane 1), we purified to homogeneity a protein with an apparent molecular mass of 60 kDa from the Triton X-100-insoluble fraction of rat hepatocytes.

The purified protein was electroblotted to PVDF membranes and subjected to N-terminal sequence analysis. The sequence DPAIFYKEQFLDGDGDA was obtained (single-letter codes), which was aligned with non-redundant SwissProt database sequences. To our surprise, we found a perfect match with the N-terminus of the mature chain of rat calreticulin, as shown in Figure 1(B). To confirm the identity of the purified protein, we made use of a commercially available antibody raised against the C-terminus of human calreticulin (peptide QAKDEL), which cross-reacts with rat calreticulin, and performed an immunoblot analysis. As can be seen in Figure 1(C), the antibody specifically recognized the 60 kDa purified protein, clearly confirming its identity as calreticulin. This protein, first characterized as an abundant high-capacity Ca^{2+} -binding protein, has been found in every cell (with the exception of erythrocytes) of higher organisms including plants [24] but remarkably, until now, it has not been connected with PKC in any way.

As reported by Murthy et al. [25], the protein has a 17-residue hydrophobic signal sequence at its N-terminus that is removed post-translationally by proteolytic cleavage. The mature protein (residues 18–416) is highly charged and is very acidic, with a pI of 4. It can be divided into at least three structural domains with distinct functional properties: the N, P and C domains [19,26]. The first one-third of the protein is referred to the N domain, within which there are six putative PKC phosphorylation sites, a binding site for rubella virus RNA, a segment that binds to steroid hormone receptor and the cytoplasmic domains of the integrin α subunits [26].

In agreement with other reports, our results in Figure 1 indicate an apparent molecular mass of 60 kDa of calreticulin by SDS/PAGE, which is much larger than the calculated molecular mass from the primary sequence (46.6 kDa); this can be explained partly by a potential N-glycosylation site and partly by its highly acidic nature. When the protein was purified not by Triton X-100 precipitation but by the method of Milner et al. [21] or by immunoprecipitation, it was occasionally found to form doublets (Figure 2) with molecular masses of 60 and 55 kDa. This has been reported previously by other authors [27], who proposed that the behaviour might be due to the reversible oxidation of cysteine residues. Calreticulin has three cysteine residues, all located in the N-terminal domain, two of which most probably form a disulphide bridge that helps to stabilize this domain. Additionally, it has been reported in some systems that calreticulin exists in two molecular forms: one 'endocalreticulin' of 52 kDa probably bound to α integrin cytoplasmic tails, and the other 'ectocalreticulin' of 62 kDa anchored to the cell surface [28].

Once we had established the identity of our PKC-binding protein as calreticulin, we next explored its specificity to bind PKC isoforms in the presence of the kinase activators by using the overlay assay. Rat hepatocytes express seven PKC isoforms [29], and M. Robles-Flores, H. Hernández-Aguilar and E. Rendón-Huerta, unpublished work) that were co-eluted by DEAE-cellulose chromatography as described in the Experimental section: two conventional isoforms (α and β II), four novel isoforms (δ , ϵ , θ and μ) and one atypical isoform (ζ). We made use of this partly purified PKC extract as a probe in the overlay assay to explore which of them calreticulin binds to. Figure 2 shows clearly that all PKC isoforms tested bound to purified calreticulin in the presence of phosphatidylserine, 1,2-diolein and CaCl_2 . The interaction was not due to secondary antibody interactions and it was specific for calreticulin because

Figure 3 Phosphorylation *in vitro* of calreticulin by immunoprecipitated PKC isoenzymes

(A, B) Kinase activity was measured with the immunoprecipitates (IPP) as described in the Experimental section, in the absence or presence of substrate added to the immunoprecipitates as indicated. The arrows indicate the position of 60 kDa phosphorylated calreticulin (CR) and the open arrowheads the position of 31 kDa phosphorylated histone H1 (H-H1). The positions of molecular mass markers are indicated (in kDa) at the left. (C) Kinase activity towards calreticulin was determined with PKC immunoprecipitates in the absence or presence of 20 $\mu\text{g}/\text{ml}$ phosphatidylserine, 0.8 $\mu\text{g}/\text{ml}$ 1,2-diolein and 0.5 mM CaCl_2 as indicated. The arrow marks the position of calreticulin. Results are representative of three independent experiments.

no PKC was detected bound to other proteins such as BSA (see Figure 2) under the conditions employed. Interestingly, whereas PKC α was the only activated isoform that interacted strongly with the two molecular forms of calreticulin (i.e. the 60 and 55 kDa bands), PKC β , PKC δ and PKC ϵ bound only to the 55 kDa form and PKC θ , PKC ζ and PKC μ interacted preferentially with the 60 kDa form.

To examine whether calreticulin can also be a substrate *in vitro* for some of these PKC isoforms, we used PKC-isoenzyme-specific immune complexes to measure the phosphorylation activity of each towards the typical PKC substrate, histone H-1

Figure 4 Association of calreticulin and PKC *in vivo*

Freshly isolated rat hepatocytes were incubated for 5 min in the absence (C) or presence of 1 μ M PMA (P) or 10 μ M adrenaline plus 10 μ M propranolol (E) at 37 °C with shaking. Proteins were immunoprecipitated (Ip) with preimmune serum (**A**) or with rabbit anti-calreticulin antibodies (**B**) from cell lysates of rat hepatocytes containing 1 mg of total cellular protein and subjected to Western blot analysis (WB) with rabbit anti-calreticulin (CR) antibodies, with the anti-PKC consensus antibodies (PKCcon) or isoenzyme-specific antibodies as indicated. In lanes marked Cb, the immunoblot analysis of control precipitates was performed in the presence of the antigen peptide to show the specificity of each antibody. Results are representative of four independent experiments.

III-S (0.2 mg/ml) in comparison with calreticulin (0.2 mg/ml). Figure 3(A) shows the results obtained with PKC α , PKC β II and PKC δ , and Figure 3(B) shows those with PKC ϵ , PKC θ , PKC ζ and PKC μ . In each panel only one negative and one positive control are shown because the patterns obtained for them with each immunoprecipitated isoenzyme were identical. As can be seen, calreticulin was a very good substrate for all PKC isoforms *in vitro*, including PKC μ , which has been reported to differ in some structural and enzymic features from the other PKC isoforms known so far [30]. Phosphatidylserine, 1,2-diolein and Ca²⁺ were required PKC cofactors because the phosphorylation of calreticulin was greatly diminished in their absence, as shown in Figure 3(C). Remarkably, histone H1-III-S (0.2 mg/ml), which appeared as a doublet at 31 kDa (Figures 3A and 3B, second lanes) was phosphorylated less efficiently than calreticulin (0.2 mg/ml), which appeared as a prominent band at 60 kDa in all gels to which it was added. In addition to the band corresponding to autophosphorylated PKC, it can also be seen that several proteins that immunoprecipitate with PKC were also phosphorylated. We analysed the immunoprecipitates with anti-PKC antibodies and also with anti-histone antibodies (results not shown) and observed that two bands at 45 and 47 kDa corresponded to the catalytic fragment of PKC and that the faint bands at approx. 31 kDa cross-reacted with histones. The

identity of the band at 43 kDa is unknown. Taken together, these results indicate clearly that calreticulin interacts with all PKC isoforms *in vitro*.

To determine whether calreticulin associates with PKC *in vivo* in response to the activation of PKC, we incubated intact rat hepatocytes at 37 °C for 5 min in the absence or presence of 1 μ M PMA to activate PKC directly or in the absence or presence of 10 μ M adrenaline plus 10 μ M propranolol (β -adrenergic antagonist) to activate PKC via α_1 -adrenergic receptors. The analysis of calreticulin immunoprecipitates of cell lysates by Western blotting with specific antibodies against each PKC isoenzyme showed that calreticulin immunoprecipitated with PKC α , PKC β , PKC θ , PKC ζ and PKC μ but not with PKC δ and PKC ϵ (Figure 4B). No immunoreactive bands were observed in the precipitates obtained with non-immune serum (Figure 4A). Interestingly, it seems that calreticulin and PKC isoforms are constitutively associated *in vivo*, because they were detected in the immunoprecipitates from both unstimulated and PMA- or adrenaline-stimulated cells.

To examine whether the activation of PKC induces the phosphorylation of calreticulin in intact cells, freshly isolated rat hepatocytes were metabolically labelled with [³²P]P_i and stimulated with 1 μ M PMA or 10 μ M adrenaline plus 10 μ M propranolol in the absence or presence of the selective and potent

Figure 5 Induction of phosphorylation of calreticulin by activation of PKC *in vivo*

Freshly isolated rat hepatocytes were metabolically labelled with [32 P] P_i for a total period of 90 min. Vehicle (—) or Ro 31-8220 (+) were added 20 minutes before the end of the incubation period and, in addition to the basal (B) samples, either PMA (P) or adrenaline (epinephrine) plus propranolol (E) was also present during the last 5 min of incubation, as described in the Experimental section. Calreticulin was immunoprecipitated from the corresponding cell lysates, subjected to SDS/PAGE [10% (w/v) gel], transferred to nitrocellulose, dried and autoradiographed. The determination of protein content was done after autoradiography in the same membrane by immunoblotting. An example of phosphorylated 60 kDa and 52 kDa calreticulin forms (arrows) is shown in the autoradiogram (A). (B) Autoradiograms and immunoblots were quantified with an image densitometer and the specific phosphorylation was determined as the ratio of phosphorylated protein to the total protein content. Values plotted are means $\pm$ S.E.M. for three experiments with different cell preparations. Filled columns show values for the 60 kDa calreticulin; open columns show those for the 52 kDa calreticulin.

PKC-specific inhibitor Ro 31-8220. Cell lysates from control and treated cells were immunoprecipitated with antibodies directed against calreticulin and then resolved by SDS/PAGE [10% (w/v) gel]; the gels were subjected to autoradiography. The results shown in Figure 5 demonstrated that there was a significant increase in the phosphorylation of both the 60 kDa and the 52 kDa calreticulin forms during treatment with PMA or with adrenaline plus propranolol. Such stimulations were abolished by the pretreatment of labelled cells with 300 nM Ro 31-8220. Taken together, these results demonstrate that calreticulin and several PKC isoforms interact *in vivo* and suggest that calreticulin function might be regulated by PKC phosphorylation.

DISCUSSION

In this study we have purified a PKC-binding protein with an apparent molecular mass of 55–60 kDa and identified it as calreticulin. Furthermore, we provide evidence that this protein and PKC interact together both *in vitro* and *in vivo*. *In vitro*,

calreticulin was capable of binding to any activated PKC isoform tested (Figure 2); however, in intact cells it seemed to be constitutively associated with PKC α , PKC β , PKC θ , PKC ζ and PKC μ but not with PKC δ and PKC ϵ (Figure 4), suggesting that they operate as signalling complexes.

Remarkably, it was also found that calreticulin is phosphorylated in response to PMA or α_1 -adrenergic stimulation of PKC *in vivo* and that it is an excellent substrate for all PKC isoforms *in vitro*, even better than histone H1-III. Consistent with this is the fact that the PROSITE pattern motifs program used to search the calreticulin primary sequence for PKC putative phosphorylation sites gave six possible ones. In this regard, it is noteworthy that all putative PKC phosphorylation sites found were all located in the calreticulin N-domain, which has been involved in diverse protein–protein interactions [26]. The physiological effects that derive from calreticulin phosphorylation by PKC or from their interaction remain to be elucidated.

The fact that calreticulin was a PKC substrate does not exclude the possibility that it might also interact with PKC through a domain located outside the PKC catalytic site: GAP-43 and AKAP-79 are examples of PKC-binding proteins that are phosphorylated by PKC and are also capable of interacting with the kinase through other domains [10,31].

Calreticulin is an ancient and highly conserved protein. The similarities between calreticulins from diverse species, particularly in domains of the protein that are functionally important, suggest that it has had important biological functions for over 350 million years. That notwithstanding, the precise biological functions of calreticulin have been the subject of much debate and are only now becoming more clearly understood. Since the description of its Ca^{2+} -binding properties, calreticulin has been characterized as a molecular chaperone, an extracellular lectin, an intracellular mediator of integrin function, an inhibitor of steroid-hormone-regulated gene expression and a C1q-binding protein [19,26,28]. Nevertheless, until now, and in spite of the scope and variety of its many assigned functions, it had not been connected with the key signal transducer, PKC. In this respect, the findings presented here were unexpected and emphasize that the characterization of the calreticulin–PKC interaction might shed light on undefined physiological roles of these two key proteins.

Consistent with our results, there is experimental evidence that indicates some similarities, both in function and in structure, between calreticulin and the RACKs. So far, two RACKs, RACK1 and β' -COP, have been cloned [12,32]. Both RACKs have been shown to be members of the WD-40 family of regulatory proteins, which are made up of four to eight repeating units containing a conserved core of 27–45 residues bracketed by two characteristic dipeptide sequences, GH (Gly-His) and WD (Trp-Asp). Additionally, RACK1 is a member of the β subunit of the G-protein superfamily, composed exclusively of seven WD-40 repeats forming an overall β -propeller structure [16]. Several WD-repeat proteins belong to the structural group of ' β -propeller' polypeptides; however, the previously known propeller proteins have no obvious sequence similarity to WD-repeat proteins but have an almost identical fold. Interestingly, the N-domain of calreticulin, with also no obvious sequence similarity to WD-40, is predicted to form eight anti-parallel β -sheets [26]. Within this N-domain are located the putative PKC phosphorylation sites, a segment that binds to steroid hormone receptor and the cytoplasmic domains of integrin α subunits.

Integrins have been proposed as crucial $\alpha\beta$ heterodimeric transmembrane proteins linking the cytoskeleton to the extracellular matrix. The adhesion-dependent clustering of integrins leads to the activation of non-receptor tyrosine kinase focal

adhesion kinase and the Ras/mitogen-activated-protein-kinase pathway, the stimulation of inositol lipid metabolism, an increase in intracellular $[Ca^{2+}]$ and pH and the activation of PKC [33,34]. A direct association of RACK1 with the integrin β subunit cytoplasmic domain through its WD repeats five to seven has recently been reported, suggesting a direct linkage between integrins and PKC through RACK1 and further implicating PKC in integrin-mediated cell signalling [35]. As mentioned above, calreticulin has also been involved in integrin function and cell spreading: the functional knock-out of calreticulin by introducing anti-calreticulin antibody into cells or by gene disruption interferes with integrin-dependent cell adhesion [36]. Moreover, it has been reported that a cell-surface complex immunocaptured from B16 mouse melanoma cells contains $\alpha 6 \beta 1$ integrin, two molecular forms of calreticulin and KDEL (Lys-Asp-Glu-Leu) docking protein [28]; one of the calreticulins, 'endocalreticulin', a 52 kDa protein, is probably bound to α integrin cytoplasmic tails; the other calreticulin, 'ectocalreticulin', a 62 kDa protein, is probably anchored to surface KDEL receptor and co-operates with $\alpha 6 \beta 1$ integrin, triggering cell spreading [28].

In view of the evidence presented here, it is tempting to speculate that the presence of multiple WD domains for putative protein-protein interaction might allow RACKs to function as scaffold proteins to recruit PKC and other proteins such as calreticulin to the membrane at the site of the membrane cytoskeletal junction to mediate important cellular functions.

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