Activated protein kinase C α associates with annexin VI from skeletal muscle

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We have previously detected a number of protein kinase C (PKC) α -binding proteins in skeletal muscle cytosol by blot overlay assay, and now identify the major, 69 kDa binding protein as annexin VI by immunoblotting and overlay assay of hydroxyapatite chromatography fractions. Annexin VI was also detected in immunoprecipitates of PKC α . Annexin VI and PKC α are both calcium-dependent phospholipid-binding proteins, and detection of the interaction was dependent on the presence of calcium and phosphatidylserine (PS). The association probably involves specific protein–protein interactions rather than mere bridging by lipid molecules: firstly, detection of PKC α -annexin VI complexes by overlay assay was not diminished when PS concentrations were increased over a 10-fold range, while that of other PKC α -binding protein complexes was reduced or

abolished; secondly, the presence in the overlay assay of a PKC pseudosubstrate peptide, analogous to a PKC sequence previously found to be involved in PKC binding activity, reduced complex formation; thirdly, we were also able to detect annexin VI interaction with PKC β by overlay of skeletal muscle cytosol, but not with PKC θ , the major novel PKC in this tissue, suggesting sequences specific to calcium-dependent PKC isoenzymes are involved. While other annexin isoforms may be PKC substrates or inhibitors, annexin VI phosphorylation by PKC α could not be detected after co-purification, while phosphorylation of subsequently-added histone H1S was readily observed. Annexin VI is a major skeletal muscle protein and our data are consistent with a role for this isoform in the control of calcium-dependent PKC.

INTRODUCTION

The protein kinase C (PKC) family consists of several lipid-activated isoenzymes playing key roles in many signal transduction pathways [1]. Three groups of PKC have been distinguished: the conventional, calcium-, phospholipid- and diacylglycerol (DAG)-dependent isoenzymes α , β and γ , the novel forms δ , ϵ , θ and η , which are calcium-independent and the atypical isoenzymes ζ and ι which are both calcium- and DAG-independent and whose activation is less well understood [2,3]. As the isoenzymes exhibit different tissue distributions and activator requirements, it is likely that they play individual roles in intracellular signalling. However, the *in vitro* substrate specificities of some isoenzymes do not appear to differ greatly [4], suggesting additional determinants of the target proteins which they phosphorylate. Activation of PKCs often involves translocation from cytosolic to membrane compartments or between different intracellular locations, and when a number of isoenzymes are translocated upon cell stimulation, different redistributions have been observed [5,6]. This suggests isoenzyme-specific interaction with membrane and cytoskeletal proteins both before and upon PKC activation, and such divergence may enable phosphorylation of different proteins, already compartmentalized at the various sites.

A number of proteins which interact with PKCs have been reported. Recent evidence indicates that inactive PKC may be localized by scaffolding proteins such as AKAP 79, and released upon lipid hydrolysis to phosphorylate co-localized proteins [7,8]. Such scaffolding proteins may also co-ordinate the actions

of other kinases and phosphatases to promote cross-talk and signal termination. In addition, proteins which bind PKC in a phospholipid-dependent manner have been described, suggesting that they will only associate with PKCs in an active conformation. These include the receptors for activated C-kinase (RACKs) [9] and cytoskeletal proteins such as vinculin and talin [4]. Some RACKs have been found to be isoenzyme-specific, and use of RACK-derived peptides to block individual isoenzyme relocalization interferes with specific cell functions [10,11]. These findings suggest that regulation of PKC isoenzyme activity may be determined not only by calcium and lipid activators, but also by association with binding proteins.

We have previously demonstrated the presence of several PKC-binding proteins in skeletal muscle [12]. Here we identify the major protein as annexin VI, and characterize its interaction with PKC α . Our data suggest annexin VI could play a role in the localization of calcium-dependent PKC activity in this tissue.

EXPERIMENTAL

Materials

Mouse monoclonal antibody against an 18 kDa fragment of rat brain PKC α (P16520) was from Transduction Laboratories, Lexington, KY, U.S.A. Mouse monoclonal antibody against purified rabbit brain PKC β (MC-2a) was from Seikagaku Corporation, Tokyo, Japan. Rabbit anti-peptide antibody against PKC θ (SC-1090) was from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A. Rabbit anti-annexin VI serum 7770 was prepared as described previously [13]. Other bio-

Abbreviations used: PKC, protein kinase C; nPKC, novel PKC; DAG, diacylglycerol; DOG, 1,2-dioctanoyl-*sn*-glycerol; DTT, dithiothreitol; PS, L- α -phosphatidyl-L-serine; RACKs, receptors for activated C-kinase.

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chemicals were obtained as previously [12] or from Sigma Chemical Co. or BDH Laboratory Supplies, Poole, Dorset, U.K.

General methods

Rat red skeletal muscles were extracted and a 100000 *g* cytosolic fraction prepared as previously [12]. Hydroxyapatite chromatography using a linear 80–140 mM KH_2PO_4 gradient, SDS/PAGE, immunoblotting, and densitometry were also carried out as previously [12].

Immunoprecipitation

Skeletal muscle cytosolic fractions (210 μl) were incubated on ice in a final volume of 250 μl , after addition of 2 mM CaCl_2 , 125 $\mu\text{g}/\text{ml}$ PS, 2.5 $\mu\text{g}/\text{ml}$ 1,2-dioctanoyl-*sn*-glycerol (DOG), and either 16 μl of anti-PKC α mouse monoclonal antibody, or 5 μl anti-annexin VI rabbit serum. After 2 h, either goat anti-mouse IgG-sepharose or protein A-sepharose conjugate respectively was added, as 80 μl of a 1:1 (v/v) suspension in wash buffer [100 mM Mops, pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 1 mM CaCl_2 , 20 $\mu\text{g}/\text{ml}$ PS, 0.4 $\mu\text{g}/\text{ml}$ DOG, 20 $\mu\text{g}/\text{ml}$ leupeptin and 2 mM benzamidine], followed by gentle rocking for 1 h at 4 °C. Incubations were then centrifuged for 30 s at 20000 *g* at room temperature, and resulting immunoprecipitates washed twice by centrifugation with 600 μl wash buffer, resuspended in 40 μl of Laemmli sample buffer, boiled for 2 min, subjected to SDS/PAGE on 10% gels and immunoblotted for PKC α and annexin VI. The procedure was also carried out without the addition of the specific antibodies to determine the amount of PKC α and annexin VI which was precipitated non-specifically: no PKC α could be detected in these cases, while some annexin VI was non-specifically precipitated by either goat anti-mouse IgG-sepharose or protein A-sepharose, found by densitometry to be less than 20% of the annexin VI in the PKC α immunoprecipitates.

Overlay assay

Cytosolic and hydroxyapatite fractions were assayed for PKC-binding proteins using an overlay assay modified from Chapline et al. [14] as described [12]. Briefly, samples were subjected to SDS/PAGE followed by electroblotting, and membranes pre-incubated for 16 h at 4 °C in three changes of Tris-buffered saline containing 0.05% (v/v) Tween 20, 10 mg/ml BSA and 1 mM dithiothreitol (DTT), then incubated with partly purified PKC α for 2 h at room temperature, in the presence of 2 mM CaCl_2 , 125 $\mu\text{g}/\text{ml}$ PS and 2.5 $\mu\text{g}/\text{ml}$ DOG unless stated. Membranes were then washed five times with phosphate-buffered saline containing 1 mM CaCl_2 , 20 $\mu\text{g}/\text{ml}$ PS, 0.4 $\mu\text{g}/\text{ml}$ DOG for 5 min, once with 0.75% (v/v) formaldehyde in phosphate-buffered saline for 40 min, once with 10% (w/v) glycine for 20 min and twice in Tris-buffered saline containing 0.05% (v/v) Tween 20. Bound PKC α was then detected by immunoblotting. Where stated, skeletal muscle or brain cytosolic fractions, prepared identically, were used in the overlay instead of partly purified PKC α .

Partial purification of PKC α and annexin VI

Rat red skeletal muscle (4.5 g of soleus, red gastrocnemius and red quadriceps muscle) was homogenized in 18 ml ice-cold buffer A (20 mM Mops, pH 7.5, 250 mM mannitol, 1 mM CaCl_2 , 1 mM DTT, 200 $\mu\text{g}/\text{ml}$ leupeptin, 2 mM PMSF and 2 mM benzamidine) using a Janke and Kunkel Ultra Turrax tissue homogenizer. The following procedures were all carried out at 4 °C. The extract was centrifuged at 100000 *g* for 45 min, and the

pellet washed twice by resuspension in 18 ml buffer A containing only 0.1 mM CaCl_2 and recentrifugation. The washed pellet was resuspended in 18 ml ice-cold buffer A containing 2 mM EDTA and 2 mM EGTA in place of CaCl_2 , and the resuspension stirred for 45 min before centrifugation at 100000 *g* for 45 min. The supernatant was applied to a 3 ml Bio-Rad DEAE Macrorep column which had been equilibrated with buffer B (20 mM MOPS, pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 20 $\mu\text{g}/\text{ml}$ leupeptin, 0.2 mM PMSF and 2 mM benzamidine). The column was washed with 15 ml buffer B followed by 15 ml buffer B containing 100 mM NaCl. Finally the column was eluted with 9 ml buffer B containing 300 mM NaCl, and the resulting eluate applied to a 1 ml Bio-Rad DNA-grade Biogel HTP hydroxyapatite column which had been equilibrated with buffer C (20 mM KH_2PO_4 , pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 20 $\mu\text{g}/\text{ml}$ leupeptin, 0.2 mM PMSF and 2 mM benzamidine). The column was washed with 5 ml buffer C, and then with 5 ml buffer C containing 80 mM KH_2PO_4 , and finally eluted with 3 ml buffer C containing 180 mM KH_2PO_4 . Fractions of 0.2 ml were collected and 10 μl samples used for immunoblotting, while the remainder was made up to 0.05% Triton X-100 and 10% glycerol in a final volume of 250 μl , before freezing in liquid nitrogen and storage at -80 °C.

In attempts to phosphorylate annexin VI by co-purified PKC α , 20 μl samples of peak annexin VI/PKC α -containing fractions were incubated at 30 °C for 30 min in a volume of 50 μl , containing in addition 20 mM MOPS, pH 7.5, 0.04% (v/v) Triton X-100, 1 mM CaCl_2 , 120 nM cyclic AMP-dependent protein kinase inhibitor peptide, 100 μM [γ - ^{32}P]ATP (7.5–10 μCi per incubation; 1500–2000 c.p.m./pmol) and 5 mM $\text{Mg}(\text{CH}_3\text{COO})_2$. Where stated, 125 $\mu\text{g}/\text{ml}$ PS and 2.5 $\mu\text{g}/\text{ml}$ DOG, 0.5 mg/ml histone H1S or 100 μM PKC pseudosubstrate inhibitor peptide (19–31) were also present. Reactions were terminated by the addition of 10 μl Laemmli sample buffer and heating for 2 min at 95 °C. 20 μl samples of the reaction mixture were subjected to SDS/PAGE on 10% gels, and the gels dried and exposed for 3 h to X-OMAT AR film for autoradiography.

RESULTS

Identification of a 69 kDa PKC α -binding protein in skeletal muscle cytosol

In a recent study of the PKC activities present in skeletal muscle we demonstrated, by use of a blot overlay assay, that a number of PKC α -binding proteins are present in the cytosolic fraction, ranging from 30 to 140 kDa, the most prominent of which was a 69 kDa protein [12]. A PKC-binding protein of similar molecular weight has been observed in rat embryonic fibroblast Ref52 cells, which was found to be heat-soluble [15]. Boiling of a muscle cytosolic fraction, followed by 20000 *g* centrifugation and overlay assay of the supernatant and pellet, indicated that the 69 kDa protein we observe is heat-labile and probably not related to the 70 kDa protein in Ref52 cells (data not shown).

On the basis of several observations we hypothesized that the skeletal muscle 69 kDa binding protein might be the calcium-dependent phospholipid-binding protein annexin VI: its molecular weight upon SDS/PAGE corresponded with that of annexin VI; it was found to be present in the cytosolic fraction upon tissue homogenization in the presence of EGTA, but in the particulate fraction when homogenization was in the presence of calcium (not shown), as expected for annexin VI [16]; other annexin isoforms have previously been shown to exhibit PKC-binding activity [17,18]; and annexin VI is highly abundant in skeletal muscle [16]. We were able to make a positive identification

Figure 1 Identification of a 69 kDa PKC α -binding protein as annexin VI

(A) After hydroxyapatite chromatography of skeletal muscle cytosol, fractions were subjected to PKC α overlay assay and also to immunoblotting, using PKC α - or annexin VI-specific antibodies, as indicated. The molecular masses of PKC α and of the binding protein are indicated. Similar results were obtained for two other chromatographic separations. (B) Immunoprecipitates of PKC α from skeletal muscle cytosol were subjected to SDS/PAGE and immunoblotting using PKC α - and annexin VI-specific antibodies as indicated. The migrations of molecular mass markers, given in kDa, are also indicated. The lower band in the PKC α immunoblot represents IgG heavy chain. Similar results were observed for two other immunoprecipitates. All procedures were carried out as described in the Experimental section.

of the 69 kDa protein as annexin VI using an annexin VI-specific antibody (Figure 1). Annexin VI, detected by immunoblotting, was shown to co-elute precisely with the binding protein, detected by overlay assay, upon hydroxyapatite chromatography of skeletal muscle cytosol (Figure 1A). The overlay assay also indicates the elution of endogenous PKC α present in chromatography fractions, which matches that detected by immunoblotting with PKC α -specific antibody without prior overlay (Figure 1A). In addition, immunoprecipitation using the PKC α -specific antibody was able to co-precipitate annexin VI (Figure 1B). While the anti-annexin VI serum used here was able to immunoprecipitate annexin VI from rat fibroblasts [19], we were unable to do so from the skeletal muscle cytosol under the conditions employed here (data not shown). However, taken together, the results shown in Figure 1 provide strong evidence that the 69 kDa PKC-binding protein is annexin VI.

Characterization of the association between PKC α and annexin VI

Since both PKC α and annexin VI are calcium-dependent phospholipid-binding proteins, it was necessary to determine whether the observed association of the two proteins involved

Figure 2 Dependence of PKC α -annexin VI interaction on calcium and phospholipid

(A) Skeletal muscle cytosol was subjected to overlay assay in the absence and presence of calcium and PS-DOG micelles as indicated. Similar results were obtained in three experiments using different PKC α and cytosol preparations. (B) Skeletal muscle cytosol was subjected to overlay assay using increasing amounts of PS-DOG micelles as indicated. The ratio of PS to DOG in the micelle preparation was kept constant at 50:1. (C) Densitometric analysis of the overlay assays shown in (B). The results from separate incubations were normalized to the endogenous PKC α peak. The migrations of molecular mass markers, given in kDa, and several PKC α -binding proteins are indicated. For clarity, data from only the incubations carried out with 10 μ g/ml PS (solid line) and 100 μ g/ml PS (broken line) are shown.

specific protein–protein interactions, or bridging by phospholipid micelles. While detection of annexin VI by overlay assay was dependent on the presence of both calcium and mixed micelles (Figure 2A), the amount of the PKC α -annexin VI complex was not reduced as the amount of micelles present was increased over a 10-fold range, in contrast to complexes of PKC α with 55 kDa, 120 kDa and 150 kDa proteins (Figure 2B). Detection of the complexes was quantified by densitometry and assays normalized using the density of the PKC band, since this is expected to remain constant (Figure 2C). In this way, the binding of PKC α to the 55 kDa, 120 kDa and 150 kDa proteins is shown to be highly sensitive to PS concentration, whereas that to annexin VI and, to a lesser extent, a 100 kDa protein, is not (Figure 2C). These data suggest that the complexes of PKC α with annexin VI and the 100 kDa protein involve protein–protein interactions while those with the other proteins may involve only phospholipid or micelle bridging which is reduced at higher phospholipid concentrations when the proteins bind separate micelles. However, since the detection of annexin VI by overlay assay does require the presence of both calcium and phospholipid (Figure 2A), it appears that PKC α must be in an active conformation before any interaction with annexin VI is possible.

Since substrates for PKC can be detected by overlay assay ([14] and C. Schmitz-Peiffer, unpublished), and some annexins are PKC substrates *in vitro* [20–22], it was important to determine whether annexin VI was detected in the overlay assay by acting as a PKC substrate. To test this, annexin VI was co-purified with PKC α (Figure 3A), and could be distinguished as a major 69 kDa band in Coomassie Blue-stained polyacrylamide gels (Figure 3B). We were unable to demonstrate subsequent phosphorylation of a 69 kDa protein under conditions of detectable calcium- and phospholipid-dependent phosphorylation of added histone IIIS (Figure 3C, lane 4). Autoradiographs exposed for longer than that shown also enabled detection of an 82 kDa species, suggestive of PKC autophosphorylation [23]. The PKC β pseudosubstrate peptide inhibited both histone IIIS phosphorylation (Figure 3C, lane 8) and that of the 82 kDa band (not shown). Similarly, phosphorylation of annexin VI could not be detected in immunoprecipitates of PKC α from skeletal muscle cytosol, in contrast to that of subsequently-added histone IIIS, even though the presence of annexin VI in the immunoprecipitates had been confirmed by immunoblotting (not shown). Our results indicate that PKC α does not readily recognize annexin VI as a substrate.

Further information concerning the interaction of PKC α with annexin VI was obtained by addition of a peptide derived from the PKC pseudosubstrate sequence to the overlay assay buffer. It has been reported that the pseudosubstrate region of PKC is involved in the interaction with binding proteins [15]. In agreement, detection of annexin VI was greatly reduced in overlay assays when carried out in the presence of the PKC β pseudosubstrate peptide (Figure 4). Since, from the results shown in Figure 3, annexin VI is not a substrate for PKC α , this result is unlikely to be due to competition for the active site of the kinase.

Finally, we determined whether the association between PKC α and annexin VI was specific for this kinase, or whether complexes with other PKC isoenzymes could be detected in the overlay assay. Skeletal muscle cytosolic fractions, electroblotted onto nitrocellulose membranes, were incubated with crude skeletal muscle or brain cytosolic fractions, which contained a mixture of free PKC isoenzymes. Bound PKCs were detected with isoenzyme-specific antibodies directed against PKC α , PKC β or novel PKC θ (nPKC θ). We were able to detect annexin VI interaction with both PKC α (Figure 5A) and PKC β (Figure 5B), but not with nPKC θ (Figure 5C), a major isoenzyme

Figure 3 Annexin VI is not a substrate for co-purified PKC α

(A) Fractions obtained from hydroxyapatite chromatography prepared as described in the Experimental section were subjected to SDS/PAGE and immunoblotting for PKC α and annexin VI. (B) A peak fraction for PKC α and annexin VI (fraction 11), without and with addition of histone IIIS as indicated, was also Coomassie-stained after SDS/PAGE. Arrows indicate protein bands present due to addition of histone IIIS. (C) Samples of fraction 11 were incubated as described in the Experimental section with [γ - 32 P]ATP and the combinations of PKC activators (PS-DOG), histone IIIS (HIIS) and PKC pseudosubstrate peptide inhibitor (PSP) as indicated, and subjected to SDS/PAGE and autoradiography. Arrows indicate phosphorylation of proteins present upon addition of histone IIIS. The migrations of molecular mass markers, given in kDa, are indicated in (B) and (C).

expressed in skeletal muscle [24,25]. We have also been unable to detect an annexin VI complex with PKC ϵ in this way (not shown). These results indicate that annexin VI may only associate with the calcium-dependent but not the nPKC isoenzymes. As

Figure 4 The effects of addition of PKC pseudosubstrate peptide to the overlay assay

Overlay assays were carried out on skeletal muscle cytosolic fractions as detailed in the Experimental section, with the addition of 100 μ M PKC β pseudosubstrate peptide (19–31) as indicated. The migration of a 69 kDa molecular mass marker is indicated.

Figure 5 Investigation of PKC isoenzymes which associate with annexin VI

Overlay assays of skeletal muscle cytosolic fractions, subjected to SDS/PAGE and electroblotting, were carried out using cytosolic fractions prepared from either brain (Br) or skeletal muscle (SkM) as sources of free PKC isoenzymes. Control assays (Con) were incubated in the absence of cytosolic fractions. Membranes were then subjected to immunoblotting using specific antibodies for either (A) PKC α , (B) PKC β or (C) PKC θ . The migrations of 69 kDa molecular mass markers, PKC isoenzymes and annexin VI are indicated.

expected, endogenous muscle PKC α and nPKC θ were detected in the overlay assays (Figures 5A and 5C), while PKC β was not (Figure 5B), possibly due to its low level of expression in this tissue [12,24,26].

In these experiments, the annexin VI signals are relatively weaker than those observed when purified PKC α is used as the PKC source, and the annexin VI band is resolved into a doublet. This may be due to alternative splicing of annexin VI mRNA [27], or perhaps limited proteolysis during preparation of skeletal muscle cytosolic fractions, despite the presence of protease inhibitors. We have also occasionally observed such a doublet both in overlay assays similar to those shown in Figure 1A and in immunoprecipitations similar to that shown in Figure 1B, suggesting that its appearance may also depend on optimum resolution during SDS/PAGE and lower signal strength. It is unclear why the signal of the upper doublet band appears

greater when brain rather than muscle cytosol is used in the overlay assay (Figures 5A and 5B), but it is possible that muscle cytosol contains factors not present in brain that compete against the interaction between PKC and the larger of the two annexin VI species, giving rise to relatively different amounts of PKC binding.

DISCUSSION

We have identified annexin VI as a major PKC α -binding protein in skeletal muscle cytosol, by use of overlay assays and specific annexin VI antibodies. While certain isoforms of the annexin family have been found to exhibit this binding activity, there has been conflicting evidence concerning the ability of annexin VI to interact with PKC. In one study, only annexin I and to a lesser extent annexins II and VII bound PKC in an overlay assay [17], while in another, annexins I, II, V and VI did bind PKC, although annexin VI interacted weakly if transferred from a denaturing gel [18]. Here we were able to detect annexin VI easily by overlay assay, which may be explained both by the renaturing step employed after transfer, and also by the abundance of annexin VI in skeletal muscle [16].

In addition to skeletal muscle, where it constitutes 0.2 % of total cellular protein, annexin VI is also highly expressed in several other tissues [16]. The annexin family consists of at least 13 structurally related, calcium-dependent, phospholipid-binding proteins, sharing homology in a 34 kDa C-terminal domain. This domain is composed of four repeats, or in the case of annexin VI, eight repeats of an approx. 70 amino acid sequence known as the endonexin fold [28]. As with the other members of the annexin family, the precise physiological role of annexin VI is undefined, although it has been variously suggested to be involved in tumour suppression [29] and the regulation of endocytosis [30], calcium release channels [31] and plasma membrane Ca^{2+} -ATPase [32]. Indeed, overexpression of annexin VI in the hearts of transgenic mice led to disruption of calcium homeostasis [33]. In this context it is interesting to note that phosphorylation by PKC has been shown to stimulate Ca^{2+} -ATPase activity [34].

The data presented here suggest that activated PKC α would be co-localized with annexin VI in skeletal muscle. The intracellular sites to which the isoenzyme translocates in this tissue upon stimulation have not been determined; however, in heart, it was found to translocate to the sarcolemma [35], while in fibroblasts it was found at both the cell margin and the endoplasmic reticulum [36]. These locations are in agreement with the distribution of annexin VI in skeletal muscle, where it is found at the sarcolemma ([37] and J. H. Walker and T. L. Luckuck, unpublished results), and at the sarcoplasmic reticulum [37], further supporting the existence of a complex between the two proteins in intact cells.

We have shown that annexin VI is not a PKC α substrate under conditions where histone H1S is highly phosphorylated (Figure 3). In previous studies, annexins I, II and IV have been found to be good substrates for PKC *in vitro* [20–22], whereas annexins V and VI are very poor substrates [38,39]. This may be related to the fact that these annexins lack the consensus PKC phosphorylation site present at the N-termini of annexins I, II and IV [22]. Annexin V, however, has been shown to inhibit the *in vitro* phosphorylation of annexin II by PKC, which may involve either direct interaction between the two proteins [40] or competition for phospholipid [41].

Detection of an annexin VI interaction with PKC β , but not with PKC θ (Figure 5), suggests that the binding is specific for PKC subfamilies, but is not isoenzyme-specific. Specific binding of calcium-dependent PKCs has also been observed for

28–36 kDa RACKs present in rat hepatocytes, while 12–14 kDa RACKs bound nPKCs in a calcium-independent manner [42]. However, some RACKs may be isoenzyme-specific, since the V1 regions of PKC δ and PKC ϵ , suggested to contain RACK-binding sites, only inhibited translocation of the isoenzymes from which they were derived [11]. It is likely that annexin VI and RACKs specific for calcium-dependent PKCs bind to the C2 region which is present only in these isoenzymes. Indeed, a fragment of the vesicle protein p64, which contains a homologous C2 domain, can compete with PKC for RACK binding [43]. Recently, annexin VI has been shown to associate with the CaLB domain of P120^{GAP} [19], also consistent with a role for the C2 region of PKC in the association with annexin VI.

In addition, the results shown in Figure 4, obtained using a PKC pseudosubstrate peptide to inhibit PKC α -annexin VI interaction, suggest that the pseudosubstrate region of PKCs may also be involved in the binding. In an earlier investigation of PKC-binding proteins in Ref52 cells [15], a PKC pseudosubstrate peptide was able to bind PKC-binding proteins, while PKC mutants lacking this region exhibited greatly diminished although still detectable binding, and it was concluded that the pseudosubstrate region contributes to binding but is not the sole determinant of PKC binding activity. Taken together with the data shown in Figure 2, these observations suggest that although annexin VI associates with cell membranes in a calcium-dependent manner [16,44], and would be expected to associate with phospholipid micelles together with calcium-dependent PKC, it is likely that the PKC α -annexin VI complex involves protein–protein interactions. However, our data do not exclude the possibility that bridging between the proteins by phospholipid also contributes to the formation of the complex.

A peptide derived from the annexin I C-terminal sequence, peptide I, showing 73% homology with amino acids 127–142 of another PKC-binding protein, the 14-3-3 isoform PKCI [45], inhibits PKC binding to RACKs [42,46]. This suggests that the C-terminus of annexin I is responsible for its RACK activity. Since the C-terminal region to which peptide I corresponds is 80% conserved between annexin I and annexin VI [28], it is likely this region might also be involved in the annexin VI-PKC α binding.

In addition to P120^{GAP}, a number of other proteins have been found to associate with annexin VI, including a 56 kDa protein kinase in activated Jurkat cells [47], a 67 kDa protein tyrosine phosphatase in pig lung extract [48] and several proteins in rat forebrain including 240 kDa caldesmon and the synaptic vesicle protein synapsin I [49,50]. Our results suggest that annexin VI might regulate the localization of calcium-dependent PKC upon activation, perhaps ensuring phosphorylation of specific substrate pools. Alternatively, annexin VI may bring together PKC and synapsin I to regulate kinase activity negatively, since cytosolic synapsin I exhibits DAG kinase activity [51], which may be important in the termination of PKC signalling. A third possibility is that by virtue of its preference for acidic phospholipids [52], annexin VI may help create high density PS domains in cell membranes that are required for PKC activity [53].

In summary, we have identified the major PKC α -binding protein present in skeletal muscle cytosol as the calcium-dependent phospholipid-binding protein annexin VI, and have provided evidence that the association between the two proteins involves specific protein–protein interactions. While annexin VI does not appear to be a substrate for the kinase, it may act to regulate calcium-dependent PKC activity, either by targeting the PKC to specific intracellular locations, inducing a membrane environment beneficial for substrate phosphorylation or perhaps

by locally enhancing signal termination. Regulation of PKC activity may contribute to some of the functions previously suggested for annexin VI, although its precise role in skeletal muscle remains to be determined.

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