Annexin V inhibits protein kinase C activity via a mechanism of phospholipid sequestration

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In this study, we assessed the role of annexin V, a Ca^{2+} -dependent phospholipid-binding protein, as a regulator of protein kinase C (PKC) and characterized its mechanism of inhibition. Several mutants obtained by oligonucleotide site-directed mutagenesis were tested *in vitro* on PKC activity in cytosolic fractions from Jurkat cells and on purified PKC α . Annexin V inhibited phosphorylation of annexin II by endogenous PKC and phosphorylation of myelin basic protein by PKC α . In both systems, the use of single Ca^{2+} -binding-site mutants of annexin V led to a partial reversal of inhibition, and the Ca^{2+} -binding site located in the first domain of annexin V was found to have the most important role. An increase in the number of mutated Ca^{2+} -

binding sites led to a greater loss of inhibition. These results corroborated those showing the progressive loss of binding of these mutants to phospholipid liposomes. In conclusion, we show that PKC inhibition by annexin V is the consequence of a mechanism involving phospholipid sequestration by annexin V, and that the Ca^{2+} -binding site located in domain 1 of annexin V plays a predominant role in this process. In addition, we show that the R¹²²AIK site, which may act analogously to a PKC-inhibitory pseudosubstrate site, is not involved in PKC inhibition, and that a peptide corresponding to the C-terminal tail of annexin V inhibits PKC activity but to a lesser extent than annexin V itself.

INTRODUCTION

Annexins are a group of at least 13 structurally related proteins that share a core domain containing four repeats (eight for annexin VI) of 70 conserved amino acids [1]. Each of these repeats contains Ca^{2+} - and phospholipid-binding sites. The N-terminus, specific for each annexin, contains phosphorylation sites for protein kinase C (PKC) as well as for other kinases. In contrast with others, annexin V has a very short N-terminus which is not phosphorylated. Although annexin V is widely distributed, its precise biological function has not yet been established but it may be an intracellular inhibitor of PKC [2].

PKC belongs to a large family of Ser/Thr protein kinases containing at least 11 members identified as single polypeptides of molecular mass ranging from 67 to 84 kDa [3]. Activation of the conventional group of PKC (cPKC: α , β I, β II and γ) is dependent on the presence of Ca^{2+} , phosphatidylserine (PS) and diacylglycerol whereas Ca^{2+} is not necessary for the two other groups. Once activated, the cellular effects of PKC can be controlled in several ways: specific substrates are dephosphorylated by phosphatases like calcineurin [4]; PKC activity is turned off by a proteolysis mechanism involving proteases such as calpain [5]; some proteins would limit the kinase activity [6]. Among them, 14-3-3 proteins [7] and annexin V [2] have been shown to act as potent PKC inhibitors.

Annexin V inhibits the *in vitro* phosphorylation by PKC of annexin I [8–11], annexin II [12] and histone H1S [8,11]. Furthermore our group has found that annexin V can act preferentially on cPKC [11,12]. So far, the mechanism of PKC inhibition by annexin V is not clearly understood, but the following hypotheses are put forward. (1) As annexins interact with PKC in the presence of Ca^{2+} and phospholipids [13–15], the inhibition could be the consequence of a direct interaction. The sequence R¹²²AIK within domain 2 of annexin V has been proposed to be

a pseudosubstrate site maintaining PKC in an inactive state [9]. The C-terminal tail of annexins, which is highly conserved [2] and similar to a region in 14-3-3 proteins [16], was found to associate with PKC. (2) Competition between annexin V and PKC for phospholipids could occur and be responsible for the inhibition [10]. PS sequestration by annexin V in a Ca^{2+} -dependent manner may lead to a reduction in the amount of PS available for PKC activation.

In this study, we tested these hypotheses using annexin V mutated in the Ca^{2+} -binding sites or in the putative PKC-interacting domains.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, penicillin/streptomycin, glutamine, lysozyme and PKC inhibitor peptide [PKC-(19–36)] were purchased from Gibco-BRL. L- α -Phosphatidyl-L-serine, 1,3-diolein, glutathione, glutathione-agarose, thrombin, isopropyl thio- β -D-galactoside, Protein A-Sepharose, BSA and myelin basic protein (MBP) were from Sigma. Fetal calf serum and Nonidet P40 were respectively obtained from Boehringer-Mannheim and Fluka. The pGEX-2T plasmid was purchased from Pharmacia, and recombinant rabbit brain PKC α expressed in a baculovirus system was obtained from Upstate Biotechnology Inc. Human annexin V cDNA was kindly provided by Biogen. [γ -³²P]ATP was from Amersham International. The peptides AVc (SGDYKKALLLLCGEDD), AVc inv. (DDEGCLLLAKK-YDGS) and AVc sc. (LGSLDKDLICYDEGAKI) were synthesized by Chiron Mimotopes. Polyclonal antibody against annexin II has already been described [12]. 1-Palmitoyl-1,2-oleoyl-*sn*-glycero-3-phosphocholine (PC) and PS were from Avanti Polar Lipids.

Abbreviations used: AVc, peptide corresponding to the C-terminus of AV; PS, phosphatidylserine; PC, phosphatidylcholine; PKC, protein kinase C; cPKC, conventional protein kinase C; MBP, myelin basic protein; RACK, receptor for activated kinase C; GST, glutathione S-transferase; cPLA₂, cytosolic phospholipase A₂.

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Cloning and expression of human annexin V

The cDNA encoding human annexin V was inserted into the pGEX2T vector at *Bam*HI–*Hind*III sites and electroporated in the *Escherichia coli* strain NB42. Bacteria were grown overnight at 37 °C in liquid broth medium containing ampicillin (50 µg/ml). Culture was diluted (1:10) the day after and continued at 30 °C until $A_{600} = 0.8$ and then at 25 °C. The expression of the glutathione S-transferase–annexin V fusion protein (GST–annexin V) was induced over 6–8 h with isopropyl thio- β -D-galactoside (0.1 mM).

Site-directed mutagenesis

Mutations were introduced into annexin V cDNA by oligonucleotide-directed mutagenesis by the method of Kunkel [17] and checked by dideoxy sequencing. All constructs were expressed as GST–annexin V proteins as described above. Site R¹²²AIK (R¹²²-A-I-K¹²⁵) located in domain 2 of annexin V was changed into AAIA as the positive amino acids R and K were replaced by the neutral residue A, and a second mutant RSIK was also made (see Figure 1). High-affinity Ca²⁺-binding sites in domains 1, 2 and 4 were mutated separately on residues E72, D144 and D303. These residues, shown to be involved in the coordination of Ca²⁺, were changed from D to N and E to Q (Figure 1). Residue E228 of a fourth site recently shown in domain 3 was mutated to A228 (Figure 1). Single Ca²⁺-binding-site mutants of each domain were designated respectively M1, M2, M3 and M4 (Figure 1). The multiple Ca²⁺-binding-site mutants M124, M123 and M1234 were obtained from a combination of single mutants (Figure 1).

Purification of recombinant wild-type and mutated annexin V

Purification of GST–annexin V was carried out essentially as described [18]. Bacteria were resuspended in 50 mM Tris/HCl, pH 7.5, lysis buffer containing 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Nonidet P40, 10% glycerol, 1 mM PMSF and 1 µg/ml each of leupeptin, aprotinin and pepstatin. All steps were performed at 4 °C. After incubation for 1 h with lysozyme (0.5 mg/ml) followed by sonication, lysed bacteria were centrifuged at 10000 g for 15 min and the supernatant was loaded on to a glutathione–agarose column. Columns were washed with 1 M NaCl and with 50 mM Tris/HCl, pH 8. GST–annexin V was cleaved by thrombin (10 units/mg of fusion protein) in 50 mM Tris/HCl, pH 8, buffer containing 150 mM NaCl. Wild-type and mutated annexin V were further purified by chromatography on a Mono Q column (FPLC system; Pharmacia). Purity and identification of all these proteins were checked by SDS/PAGE and Western-blot analysis. Proteins were concentrated in Tris buffer (10 mM) containing 0.1 mM EDTA using Centricon-10 (Amicon). Their concentrations were determined by the method of Bradford [19] or Thomas and Nelsestuen [20] using the absorption coefficient of annexin V at 280 nm ($0.59 \pm 0.03 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$). Structural integrity of mutated annexin V was checked by CD.

Cell culture

A Jurkat T-lymphocyte cell line derived from clone J77-6.8 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin/streptomycin, 2 mM glutamine and 1 mM Hepes.

Effects of annexin V on annexin II phosphorylation by PKC from Jurkat cells

Jurkat cells were washed with PBS and homogenized in a buffer containing 20 mM Tris/HCl, pH 7.5, 250 mM sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM PMSF and 20 µg/ml leupeptin. The homogenates were centrifuged for 1 h at 105000 g at 4 °C. The supernatants represented the cytosolic fractions. Cytosols from 2.5×10^6 cells (100 µl) were incubated with a mixture of PS (86 µg/ml), diolein (2.6 µg/ml) and an excess of CaCl₂ (0.2 mM) in 300 µl final volume. Basal activity was measured in the absence of Ca²⁺, PS and diolein. The effects of wild-type and mutated annexin V (5 µM) were studied by adding them to the incubation medium. The reaction was initiated by adding [γ -³²P]ATP with a specific radioactivity of 6000–7000 c.p.m./pmol and performed for 10 min at 30 °C. Immunoprecipitation of cytosolic annexin II was performed as described [12]. Immunoprecipitated annexin II was analysed by SDS/PAGE (12.5% gel), and phosphorylated annexin II was detected by the PhosphorImager SITM system (Molecular Dynamics).

Effects of annexin V on MBP phosphorylation by purified PKC α

The phosphorylation of MBP in the presence of Ca²⁺, PS, diolein and PKC α (dilution 1:40) was performed as described above, except that the final volume was 50 µl and the [γ -³²P]ATP specific radioactivity was 3000–4500 c.p.m./pmol. After 10 min of incubation at 30 °C, MBP was separated by SDS/PAGE (15% gel). Radioactivity was detected using the PhosphorImager SITM system.

The effects of a 16-amino acid peptide (AVc) corresponding to the C-terminus of annexin V, its inverse (AVc inv.) and a scrambled (AVc sc.) peptide, used as a control, were also examined.

Phospholipid-binding study

Ca²⁺-dependent phospholipid-binding properties of mutants were compared with wild-type annexin V using a Ca²⁺-dependent liposome-pelleting assay [21]. A mixture of PS and PC (20%/80%) in chloroform/ethanol (95:5, v/v) was dried in a speed-vac for 24 h and then resuspended in 50 mM Tris/HCl, pH 8, containing 0.1 mM EDTA. Annexin V binding on liposomes was carried out for 15 min at room temperature in 50 mM Tris/HCl, pH 8, buffer containing 1 mM MgCl₂, 1 mM EGTA and an excess of 0.1 mM CaCl₂. Wild-type or mutated annexin V (2.5 µM) was then added. After a 47000 g centrifugation for 15 min, the supernatant was removed and annexin V bound to liposomes was released by incubation for 15 min with 50 mM Tris/HCl, pH 8, containing 10 mM EDTA. Equal volumes from each experiment were then subjected to SDS/PAGE (12.5% gel). Densitometric analysis of Coomassie Blue staining was used to quantify annexin V bound to the PS/PC liposomes.

Expression of results

PKC activity measured in the absence or presence of wild-type or mutated annexin V was estimated by quantification of ³²P-labelled annexin II or MBP by the PhosphorImager SITM system coupled with the software Image densitometric analysis IP Lab gel program. For all data, control values corresponding to basal phosphorylation of annexin II or MBP were subtracted. The results were then normalized and expressed as the percentage inhibition of total PKC activity measured in the absence of annexin V.

Wild-type and mutated annexin V bound to liposomes were also determined using the software Image densitometric analysis

IP Lab gel program. Results for mutated annexin V were expressed as a percentage of total annexin V bound to the liposomes. All experiments were repeated at least three times and means $\pm$ S.E.M. are given for each series of experiments.

RESULTS

Ca²⁺-binding-site mutants are less inhibitory to cytosolic PKC activity than wild-type annexin V

We have previously shown that annexin V inhibits the phosphorylation of several proteins, including annexin II, by PKC in cytosol from Jurkat cells [12]. To study the importance of each high-affinity Ca²⁺-binding site of annexin V in PKC inhibition, the effects of single Ca²⁺-binding-site mutants M1, M2, M3 and M4 (Figure 1) on annexin II phosphorylation by endogenous PKC were compared with wild-type annexin V. Immunoprecipitation of endogenous annexin II revealed that its phosphorylation was less inhibited in the presence of M1, M2, M3 and M4 than in the presence of wild-type annexin V (Table 1). Therefore mutation of each high-affinity Ca²⁺-binding site of annexin V led to partial reversal of PKC inhibition. M1 appeared

Figure 1 Schematic representation of wild-type and mutated annexin V

Regions mutated on Ca²⁺-binding-site domains 1, 2, 3 and 4 of annexin V are indicated within black rectangles. The mutations of the potential pseudosubstrate site R¹²²AIK in the second domain of annexin V in AAIK and RSIK are also indicated. Mutations were performed as described in the Materials and methods section.

to be the least potent inhibitor, but there was no significant difference when compared with other single mutants (Table 1). The importance of increasing the numbers of mutated Ca²⁺-binding sites was then studied by testing the effects of M124, M123 and M1234 on cytosolic PKC activity. Mutation of several Ca²⁺-binding sites resulted in almost complete reversal of PKC inhibition by annexin V (Table 1).

Effects of annexin V mutants on MBP phosphorylation by purified PKC α

As different PKC isoforms are expressed in Jurkat cells [22], we tested whether annexin V can inhibit purified PKC α . The choice of this isoform was justified by the fact that we have previously shown that annexin V can preferentially act on cPKC [11,12]. MBP phosphorylation by PKC α was inhibited in the presence of annexin V (Table 1). However, when mutants M1, M2, M3 and M4 were used, some phosphorylation of MBP was maintained. This effect on PKC α was more pronounced with M1 than with other single mutants (Table 1), indicating a predominant role for the Ca²⁺-binding site located in domain 1 of annexin V in the mechanism of PKC inhibition. The effects of multiple Ca²⁺-binding-site mutants were also compared with wild-type annexin V and showed an almost complete reversal of PKC inhibition by annexin V (Table 1).

Loss of phospholipid binding of annexin V mutants

Numerous studies have shown that annexin V binds in a Ca²⁺-dependent manner to anionic phospholipids. Quantitative phospholipid binding in the presence of an amount of Ca²⁺ comparable with that required for PKC activity, revealed that mutation of the Ca²⁺-binding site in domain 1 resulted in a higher loss of binding capacity than those in domains 2, 3 and 4 (Table 2). Increasing the number of mutations of Ca²⁺-binding sites led to a complete lack of binding in mutants M123, M124, M1234 (Table 2), but the results were no significantly different from those for M1, indicating the crucial role of the Ca²⁺-binding site located in domain 1 in the Ca²⁺-dependent phospholipid-binding property of annexin V.

The potential pseudosubstrate site R¹²²AIK in annexin V is not involved in PKC inhibition

To test whether annexin V could regulate PKC via the potential pseudosubstrate site R¹²²AIK located in domain 2, we tested the

Table 1 Comparative effects of wild-type annexin V and its single and multiple Ca²⁺-binding-site mutants on the phosphorylation of annexin II by cytosolic PKC from Jurkat cells and on the phosphorylation of MBP by purified PKC α

Results are expressed as percentage of the inhibition of total PKC activity measured in the absence of annexin V or mutants. Means $\pm$ S.E.M. are given for at least three experiments. Statistical analysis using Student's *t* test was used for comparison between annexin V and mutants (A) and between M1 and other mutants (B). *P* < 0.05 was considered significant (***P* < 0.01, **P* < 0.05; NS, not significant).

		Inhibition of PKC activity (%)							
		Annexin V	M1	M2	M3	M4	M123	M124	M1234
Phosphorylation of annexin II by cytosolic PKCs from Jurkat cells	A		*	*	NS	*	**	**	**
	B	95 $\pm$ 2	58 $\pm$ 15	78 $\pm$ 4	81 $\pm$ 10	77 $\pm$ 5	24 $\pm$ 13	24 $\pm$ 15	22 $\pm$ 16
Phosphorylation of MBP by purified PKC α	A		**	*	NS	*	**	**	**
	B	96 $\pm$ 3	40 $\pm$ 3	73 $\pm$ 8	87 $\pm$ 5	75 $\pm$ 4	30 $\pm$ 3	46 $\pm$ 3	37 $\pm$ 3
				*	**	**	NS	NS	NS

Table 2 Binding study of annexin V and mutants

It was performed as described in the Materials and methods section. Protein bound to liposomes was quantified and expressed as the percentage of wild-type annexin V binding. Results are given as means $\pm$ S.E.M. for at least three experiments. Statistical analysis using Student's *t* test was performed to compare M1 with other mutants; ***P* < 0.01; **P* < 0.05.

Decrease in phospholipid binding/wild-type annexin V (%)							
Annexin V	M1	M2	M3	M4	M123	M124	M1234
0	79 $\pm$ 6	38 $\pm$ 4**	47 $\pm$ 9**	38 $\pm$ 6*	98 $\pm$ 1	85 $\pm$ 7	93 $\pm$ 6

Figure 2 Effect of the peptide AVc corresponding to the 16 C-terminal residues of annexin V on MBP phosphorylation by PKC α

Peptides AVc (○), AVc inv. (△) and peptide AVc sc. (□) were added at increasing concentrations from 1 to 200 μ M and tested on MBP phosphorylation by PKC α . These data represent means $\pm$ S.E.M. for three different experiments.

effects on MBP phosphorylation by PKC α of two mutants with the modified sequences AAIA and RSIK. Annexin V and both mutants inhibited MBP phosphorylation by PKC α to a similar extent (annexin V, 84 $\pm$ 4%; AAIA, 77 $\pm$ 14%; RSIK, 77 $\pm$ 14%). In addition, use of the RSIK mutant did not lead to a PKC phosphorylatable sequence, since no 32 P could be detected in the protein (results not shown). Our results demonstrate that the R¹²²AIK sequence of annexin V is not involved in PKC inhibition, in contrast with the suggestion of others [9].

A peptide corresponding to the C-terminus of annexin V partially inhibits MBP phosphorylation by PKC α

As the C-terminal domain of annexin I was reported to interact with PKC [15,23], we analysed the effect of a peptide corresponding to the last 16 C-residues of annexin V (AVc) on PKC α activity. Increasing AVc concentrations led to a progressive inhibition of MBP phosphorylation by PKC α (Figure 2). At the highest concentration tested (200 μ M), PKC activity was inhibited by 70%. In contrast, control peptides (AVc inv. and AVc sc.) did not alter MBP phosphorylation. These results showed a specific inhibitory effect of peptide AVc on PKC α activity. However, at the highest concentration tested (200 μ M),

this peptide appeared to be 55-fold less potent (70% inhibition) than annexin V itself at 5 μ M (96% inhibition).

DISCUSSION

Partial but not complete reduction of PKC inhibition was found when single Ca²⁺-binding site mutants M1, M2, M3 and M4 were used instead of wild-type annexin V. Interestingly, mutant M1 was found to be the least inhibitory. This partial reversal of PKC inhibition could be explained by partial loss of Ca²⁺ binding and consequently loss of phospholipid binding by annexin V, as reported for annexins II [21,24] and IV [25] using a similar approach. Indeed, loss of Ca²⁺-dependent phospholipid binding is observed with the mutants and, interestingly, mutant M1 has the lowest ability to bind to phospholipids when compared with other single Ca²⁺-binding-site mutants. The observation that domain 1 of annexin V is more important than the three others suggests that each Ca²⁺-binding site exhibits different affinities for Ca²⁺ and that the first domain has a predominant role in governing the overall Ca²⁺-dependent phospholipid-binding capacity of annexin V. In fact, it has been shown that Ca²⁺ apparently binds most strongly to domain 1, this domain exhibiting the highest affinity for Ca²⁺ [26,27].

The triple and quadruple mutants lost their ability to bind to

Figure 3 Correlation of PKC inhibition of annexin V with its ability to bind to phospholipids in a Ca²⁺-dependent manner

PKC inhibition of wild-type annexin V (●) and mutants (□, M1; ■, M2, M3, M4; ▲, M123, M124; ◆, M1234) is plotted against their ability to bind to liposomes in the presence of Ca²⁺. (A) Jurkat cells; (B) purified PKC α .

phospholipids completely in the presence of Ca^{2+} as well as their ability to inhibit the phosphorylation of annexin II by PKC, strongly indicating that annexin V inhibits PKC via a mechanism of phospholipid sequestration. However, these mutants still continue to partially inhibit MBP phosphorylation by PKC α , suggesting that other sites in annexin V may also be involved in PKC inhibition. However, when we plot PKC inhibition against binding to liposomes for annexin V and all mutants, we observe a good relationship between these two parameters, for both annexin II (Figure 3A) and MBP (Figure 3B) phosphorylation. Therefore our results strongly support the model of phospholipid sequestration as the mechanism of PKC inhibition by annexin V.

The direct interaction hypothesis was also studied by testing the effects of mutants of the sequence R¹²²AIK in domain 2 of annexin V. This region is exposed to solvent and is possibly accessible to PKC in the cytoplasm, but from our results, the pseudosubstrate hypothesis was eliminated. This is in agreement with the presence of a variant form of annexin V that differs from annexin V by only two residues; one is located in the R¹²²AIK sequence (R¹²²AIE) [28]. In that study, this particular variant was found to inhibit PKC to the same extent as annexin V, in agreement with the present study showing no effect of the RAIK site on the inhibition of PKC.

The C-terminal tail of annexin I has been reported to interact with PKC in the presence of Ca^{2+} and phospholipids [15,23]. As the C-terminus of annexins is highly conserved [2], we analysed its potential role in PKC inhibition. We first tried to obtain a mutant of annexin V with the C-terminal 16 residues deleted. However, possibly because of incorrect folding, this mutant was not soluble as a GST fusion protein (results not shown). As an alternative approach, we used a peptide corresponding to the last 16 C-terminal residues of annexin V (AVC) and found that it inhibited PKC α in a dose-dependent manner. However, much higher concentrations of the peptide than annexin V were required for PKC inhibition, indicating that the C-terminal tail has a much weaker potential to inhibit PKC activity than annexin V. Binding of peptide AVC to PKC could impair the interaction of PKC with its substrates, and this may explain its inhibitory effect. A receptor for activated PKC, RACK1, contains a sequence that is similar in annexins and 14-3-3 proteins [29]. 14-3-3 proteins were also shown to interact with [30] and to inhibit [7] PKC. A synthetic peptide corresponding to the C-terminus of annexin I prevented the binding of PKC to RACK [15], blocked both the insulin-induced translocation of this kinase in oocytes [31] and the phosphorylation of histone by PKC from rat hepatocytes [32]. It seems that this domain may be involved in translocation and regulation of PKC. In general, the C-terminal tail of annexins probably acts as an anchor for PKC enabling it to target a precise location on the membrane after cell activation.

cPKCs are the only members of the kinase C family that possess a Ca^{2+} - and phospholipid-binding domain (named C2) which is also found in synaptotagmin, cytosolic phospholipase A₂ (cPLA₂), p120^{GAP}, phospholipase C and phosphatidylinositol 3-kinase. Of these proteins, PKC [13–15], cPLA₂ [33] and p120^{GAP} [34] have been shown to interact with annexins, and the association with p120^{GAP} is mediated by its C2 domain [34]. Therefore it is possible that the C2 domain represents a novel binding motif for the association with annexins. In addition, our group has shown that annexin V inhibits cPLA₂ activity in a permeabilized HL60 mammalian cell model [35]. The inhibitory mechanism also involves the Ca^{2+} -dependent phospholipid-binding property of annexin V with a predominant role for the Ca^{2+} -binding site located in domain 1 [35]. Therefore the results from both studies suggest a general process for the modulation by annexin V of the activity of a number of proteins containing a C2

domain in which Ca^{2+} -dependent phospholipid-binding properties play a crucial role.

These C2 domain proteins translocate from the cytosol to the membranes on cellular activation in the presence of Ca^{2+} (and diacylglycerol for cPKC), and the C2 region appears to be required for their translocation and function. Translocation of PKC at the plasma membrane was thought to involve direct binding of the isoenzyme to lipids in the membrane. However, recent studies indicate that PKC may interact at the site of translocation with proteins such as annexins, RACK and cytoskeletal proteins [36]. Annexin V also translocates to the plasma membrane on cell activation via elevation of the intracellular Ca^{2+} concentration [37–40]. The similar intracellular localizations of cPKC and annexin V as well as their ability to translocate to the plasma membrane after cell activation supports the physiological relevance of our results, and suggests that annexin V may be an intracellular regulator of cPKC activity.

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