Role of the Ca\(^{2+}\)/Phosphatidylserine Binding Region of the C2 Domain in the Translocation of Protein Kinase C\(\alpha\) to the Plasma Membrane*  

Stephen R. Bolsover‡, Juan C. Gomez-Fernandez§, and Senena Corbalan-Garcia§§

From the ‡Department of Physiology, University College London, Gower St., London WC1E 6BT, United Kingdom and §Departamento de Bioquímica y Biología Molecular (A), Facultad de Veterinaria, Universidad de Murcia, Apartado 4021, E-30100 Murcia, Spain

Received for publication, December 1, 2002, and in revised form, January 9, 2003

Protein kinase C family (PKC) is known to control many cellular processes including metabolism regulation, receptor signal transduction, cell growth and differentiation, and hormone and neurotransmitter secretion (1, 2). This family consists of 10 closely related isoenzymes that can be divided into three groups according to the type of activator they need. For example, conventional PKCs (\(\alpha\), \(\beta\), \(\beta\)II, and \(\gamma\)) require the full complement of negatively charged phospholipids, Ca\(^{2+}\), and diacylglycerol or phorbol esters before they are activated. The C1 domain in the translocation of PKC\(/H11545\) is closely regulated by its subcellular localization. To map the molecular determinants mediating the C2 domain-dependent translocation of PKC\(\alpha\) to the plasma membrane, full-length native protein and several point mutants in the Ca\(^{2+}\)/phosphatidylserine-binding site were tagged with green fluorescent protein and transiently expressed in rat basophilic leukemia cells (RBL-2H3). Substitution of several aspartate residues by asparagine completely abolished Ca\(^{2+}\)-dependent membrane targeting of PKC\(\alpha\). Strikingly, these mutations enabled the mutant proteins to translocate in a diacylglycerol-dependent manner, suggesting that neutralization of charges in the Ca\(^{2+}\) binding region enables the C1 domain to bind diacylglycerol. In addition, it was demonstrated that the protein residues involved in direct interactions with acidic phospholipids play differential and pivotal roles in the membrane targeting of the enzyme. These findings provide new information on how the C2 domain-dependent membrane targeting of PKC\(\alpha\) occurs in the presence of physiological stimuli.

Conventional and novel PKCs share the property of using two membrane-targeting modules for the sensitive, specific, and reversible regulation of their function. Thus, the C1 and the C2 domains are involved in membrane translocation and subsequent enzyme activation (4). Many studies performed to date suggest a sequential mechanism in the activation of PKC\(\alpha\) by Ca\(^{2+}\) in which membrane association is followed by increased catalytic activity (5–9). However, it is still not clear whether these domains function in a concerted way or as independent modules.

The first step of this process is probably the Ca\(^{2+}\)-dependent contact of the C2 domain with negatively charged phospholipids at the plasma membrane (6, 8, 10–12). Recent studies on different C2 domains reveal that, despite their sequential and architectural similarity, they are functionally specialized modules that exhibit distinct equilibrium and kinetic behaviors that are probably optimized for different Ca\(^{2+}\)-signaling applications (13–14). Most of these studies have been performed with isolated C2 domains and, in the case of PKC\(\alpha\), the precise molecular mechanism driving the above interaction and the consequences for the general functioning of the enzyme are still not well defined.

Our previous crystallographic studies of the isolated C2 domain of PKC\(\alpha\) bound to Ca\(^{2+}\) and the short chain lipid 1,2-dicaproyl-sn-phosphatidyl-1-serine show that two or three Ca\(^{2+}\) ions bind to the domain, with one of them (Ca1) bridging the protein directly to phosphatidylserine (PS). Moreover, several residues in the domain seem to be involved in a direct interaction with PS (15–16), and further studies show that both Ca\(^{2+}\) and PS binding sites are important for in vitro enzyme activation (9, 17, 18). Whether two or three calcium ions are needed for plasma membrane translocation of PKC\(\alpha\) and how PS and diacylglycerol interact with the regulatory domain to orchestrate membrane targeting and activation of PKC\(\alpha\) in vivo are key questions that remain to be answered.

To investigate the molecular determinants mediating the C2 domain-dependent plasma membrane localization of PKC\(\alpha\), we generated a construct containing full-length PKC\(\alpha\) tagged to green fluorescent protein (PKC\(\alpha\)-GFP). Additionally, different constructs containing point mutations at the Ca\(^{2+}\) and PS binding sites in the C2 domain of PKC\(\alpha\) were produced and transfected into RBL-2H3 cells (a mast cell line that provides a useful model for studying signal transduction mechanisms). The cells are activated by antigens via the tyrosine kinase-dependent high affinity receptor for IgE (FeR\(\alpha\)) to induce the release of secretory granules and the generation of cytokines. Activation of the receptor leads to phospholipase C\(\gamma\) activation and the consequent generation of inositol 1,4,5-trisphosphate/ Ca\(^{2+}\) and diacylglycerol, both of which are regarded as PKC

* This work was supported by from Dirección General de Enseñanza Superior e Investigación Científica (Spain) Grant PB98-0389, Dirección General de Investigación (Spain) Grant BM/2002-00119, a grant from Fundación Séneca (Comunidad Autónoma de Murcia), Programa Ramón y Cajal from Ministerio de Ciencia y Tecnología (Spain) (to S. C.-G.), a short-term fellowship from The Wellcome Trust (to S. C.-G.), and The Wellcome Trust and Medical Research Council (UK) grants (to S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 34-968-364775; Fax: 34-968-364766; E-mail: senena@um.es.

§ The abbreviations used are: PKC, protein kinase C; PS, phosphatidylserine; GFP, green fluorescent protein; EGFP, enhanced GFP, RBL cells, rat basophilic leukemia cells; DNP, 2,4-dinitrophenyl; HSA, human serum albumin;
activators (19). Using confocal microscopy combined with measurements of intracellular Ca^{2+} concentration in time-lapse experiments, we have found that both Ca^{2+} and PS binding sites in the C2 domain play important and differential roles in the spatio-temporal localization of PKCa, suggesting a very accurate and controlled mechanism driven primarily by the C2 domain.

**EXPERIMENTAL PROCEDURES**

cDNA Constructions—N-terminal fusions of rat PKCa and the different mutants to GFP were generated by inserting cDNAs into the multiple cloning site of the pEGFP-N3 (Clontech Laboratories, Inc., Palo Alto, CA) mammalian expression vector. Briefly, cDNAs encoding PKCs and its mutants D187N, D246N/D248N, D187N/D246N/D248N, and the catalytic domain were cloned into the XhoI and KpnI sites of the pEGFP-N3 plasmid. PKCa and mutated constructs were ligated with the Xhol/KpnI-digested vector, thus generating the different fusion constructs. Further details on site-directed mutagenesis to generate the different mutants are reported by Conesa-Zamora et al. (17). All constructs were confirmed by DNA sequencing. The stability and viability of the mutated proteins were studied by using specific activity measurements. It was demonstrated that the mutated proteins could be activated in a PS-dependent manner, although not to the same extent as the wild-type protein. Earlier studies have shown that a C-terminal GFP tag does not affect the catalytic activity or the cofactor dependence of PKCa (20–22).

Cell Culture—Rat basophilic leukemia (RBL-2H3) cells were cultured at 37 °C in a humidified atmosphere of 5% CO_{2} in a growth medium of Dulbecco’s modified Eagle’s medium supplemented with 15% (v/v) fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 4 mM glutamine. Cells were prepared for confocal microscopy as described by Boscov et al. (23). Basically, harvested cells were resuspended in electroporation buffer (120 mM NaCl, 5.5 mM KCl, 2.5 mM MgCl_{2}, 25 mM glucose, 20 mM Hepes, pH 7.2) and 30 μg of cDNA. Cells were electroporated in a Bio-Rad GenePulser with two 500-V pulses.

RESULTS

Mutagenesis Rationale—Fig. 1A illustrates a scheme based on the structure of the C2 domain of PKCa bound to three Ca^{2+} and to the short chain lipid 1,2-dicapryloyl-sn-phosphatidyl-L-serine (15–16). The side chains of the five aspartates (Asp-187, Asp-189, Asp-246, Asp-248, and Asp-254) that directly coordinate Ca1 and Ca2 are shown. Simple (D187N), double (D246N/D248N) and triple (D187N/D246N/D248N) mutants were generated to study the role of these residues in the plasma membrane targeting of the enzyme. D187N was chosen because the crystallographic study showed that this aspartate residue forms a bidentate interaction with Ca1, which is the ion involved in bridging the protein residues with the phosphate group of the phospholipid molecule (Fig. 1A). In the case of the double mutant containing Asp-246 and Asp-248 substituted by Asn, key coordinations with Ca1 and specially with Ca2 were abolished. Double (D246N/D248N) and triple (D187N/D246N/D248N) substitutions of the aspartate residues by Asn neutralized the highly negative charge exhibited by this region, and it was thought that this might help understand whether this neutralization of charges is the only mechanism required for membrane targeting of the domain or a different mechanism operates this interaction.

Fig. 1B shows the side chains of the residues involved in the PS-binding site: Asn-189 (located in loop 1), Arg-216 (located in loop 2), Arg-249 and Thr-251 (both located in loop 3). Thr-251 is a special residue since it has been proposed to be involved in two types of interaction. When the C2 domain only binds two Ca^{2+}, this residue interacts with the sn-1 acyl group oxygen of the PS (15). However, when three Ca^{2+} are bound to the domain, the side-chain oxygen of Thr-251 also coordinates the third Ca^{2+}, which seems to bind to the C2 domain only in the presence of a very short phosphatidylserine analogue (16). Whether this third Ca^{2+} was necessary for PKCa plasma mem-
intracellular Ca\(^{2+}\) spikes were seen to be highly correlated with the translocation of PKCa-GFP. Maximum protein translocation occurred between 2 and 26 s after the first Ca\(^{2+}\) spike was generated. It is interesting to note that the first Ca\(^{2+}\) spike was more effective in producing the translocation of the protein to the plasma membrane, whereas the second and third pulses, which showed the same Ca\(^{2+}\) concentrations, were only capable of partially anchoring the protein. The addition of ionomycin in the same experiment produced a further increase in cytosolic Ca\(^{2+}\) and a parallel translocation of PKCa-GFP that started to revert to the cytosol 79 ± 23 s after ionomycin addition. It is interesting to note that protein started to revert to the cytosol when the intracellular Ca\(^{2+}\) concentration was still compatible with protein translocation. Similar translocation profiles were obtained in control experiments, in which ionomycin was added directly to the cells without previous antigen stimulation (data not shown).

To investigate how the aspartate residues coordinate the different Ca\(^{2+}\) ions and produce the membrane targeting of PKCa, we generated several mutants that were also fused to GFP, as stated above. As shown in Fig. 3A, all the mutants were localized homogeneously in the cytosol in unstimulated cells. When the cells were stimulated by the addition of 40 ng/ml DNP-HSA, no changes in plasma membrane translocation of the mutant proteins were detected; similar results were obtained when ionomycin was added. A comparison of Fig. 3, B and C, clearly illustrates that although Ca\(^{2+}\) spikes were generated in the cells transfected with the different mutant proteins, these mutations strongly affected the Ca\(^{2+}\)-dependent translocation of the enzyme (Fig. 3C). Additionally, none of the sustained proteins translocated to the plasma membrane, although sustained cytosolic Ca\(^{2+}\) concentrations ranging from 900 to 1400 nM were produced in the cells analyzed by the addition of ionomycin. Taken together these data suggest that the initial physiological translocation of PKCa to the plasma membrane is driven primarily in a Ca\(^{2+}\)-dependent manner by the C2 domain and that the residues coordinating Ca1 and Ca2 are critical in the in vivo protein-plasma membrane interaction.

**Ca\(^{2+}\) Binding Sites Are Involved in the C1 Domain-dependent Translocation of PKCa to the Plasma Membrane**—To determine the role of the C1 domain in the Ca\(^{2+}\)-dependent membrane targeting of PKCa, we examined the direct effect of increasing concentrations of the membrane-permeant diacylglycerol (DiC8) on both cytosolic Ca\(^{2+}\) and the kinetics of PKCa-GFP translocation. Neither the Ca\(^{2+}\) signal nor PKCa-GFP targeting of the plasma membrane was produced by increasing the concentrations of DiC8 alone (Fig. 4, A and B). Only when ionomycin was added to the medium did the protein translocate to the membrane in a DiC8 concentration-dependent manner (Fig. 4B). In fact, the higher the DiC8 concentration, the longer the residence time of PKCa at the plasma membrane. It is interesting to note that the protein translocation profile obtained after the addition of ionomycin to the medium containing 10 μg/ml DiC8 was very similar to that obtained when the cells were stimulated previously with 40 ng/ml antigen (Fig. 2C), suggesting that the concentration of diacylglycerol generated under physiological conditions might also be similar but probably only sufficient to anchor the protein to the plasma membrane for a very short length of time through the C1 domain, as has been suggested in previous reports (8, 26).

Because the C1 and C2 domains have always been considered as independent modules, it is striking that the effect of DiC8 is only observed when the concentration of intracytosolic Ca\(^{2+}\) increases. The results obtained with the Ca\(^{2+}\) binding mutants shed light on this issue. When the cells transfected...
with PKCα D187N mutant were stimulated with increasing concentrations of DiC8, no protein translocation was observed (n = 20 cells). However, when ionomycin was added (Fig. 5A) the mutant interacted with the plasma membrane only in 50% of the cells analyzed with a rate (R) of 0.6. Note, also, that after ionomycin stimulation the translocation profiles reflected a slower and more transient process compared with that of the wild-type protein that lasted at least 186 s with the same concentration of DiC8 (100 μg/ml). Moreover, maximum protein translocation of PKCα D187N mutant was obtained 123 ± 11 s after ionomycin addition, whereas wild-type protein showed maximum translocation after 55 ± 10 s.

Surprisingly, when the cells were transfected with the PKCα D246N/D248N mutant (Fig. 5B), the protein started to translocate slowly to the plasma membrane after the addition of 50 or 100 μg/ml DiC8. This effect was clearly concentration-dependent since 25 μg/ml DiC8 only produced a transient translocation after ionomycin stimulation, whereas 50 and 100 μg/ml DiC8 promoted the irreversible plasma membrane localization of the protein that was detectable after 115 ± 18 s (n = 8 cells). Note also that measurable levels of PKCα D246N/D248N mutant were recorded after 58 ± 4 s in the presence of ionomycin when the cells were previously stimulated with 25 μg/ml DiC8 compared with the 86 ± 4 s needed in the case of PKCα D187N mutant. Furthermore, the translocation of PKCα D246N/D248N to the plasma membrane became almost independent of ionomycin addition when 50 and 100 μg/ml DiC8 were used to stimulate the cells.
When PKCα D187N/D246N/D248N mutant (Fig. 5C) was tested, it was translocated to the plasma membrane at only 25 μg/ml DiC8, and it showed a faster kinetics (68 ± 5 s; n = 13 cells) of translocations than PKCα D246N/D248N mutant.

These results demonstrate a strong correlation between the degree of neutralization of the aspartate residues that coordinate the Ca\(^{2+}\) ions and the accessibility of DiC8 to the C1 domain.

**Fig. 4.** A, example of the time course of the Ca\(^{2+}\) signal generated after the addition of the plasma membrane-permeant diacylglycerol DiC8 (83 s) and ionomycin (381 s). B, examples of the time course of the plasma membrane translocation of PKCα after the addition of 10, 25, 50, and 100 μg/ml DiC8. The results are representative experiments of 4–14 cells obtained from at least four different experiments. R, relative plasma membrane translocation.

---

**FIG. 3.** A, confocal fluorescence images of RBL-2H3 cells expressing the different Ca\(^{2+}\)-binding site mutants, D187N, D246N/D248N, and D187N/D246N/D248N. The images were recorded at the different times stated. Antigen (40 ng/ml DNP-HSA) was added after 83 s of recording, and 1 μM ionomycin was added after 381 s of recording. B, examples of the time courses of the Ca\(^{2+}\) signals after antigen (Ag) and ionomycin additions. C, examples of the time course of plasma membrane localization of the different mutants. As shown, no translocation to the plasma membrane was observed for any of them. R, relative plasma membrane translocation.

---

**Role of the Ca\(^{2+}\)/PS Binding Region of the C2 Domain**
PKCa R249A and PKCa T251A mutant did not translocate to the plasma membrane even after ionomycin addition (Fig. 6C). Because both these proteins interact with one of the carbonyl group of the fatty acyl chains of the phospholipid (15), these results suggest that the protein might use these residues to anchor to the membrane interface, resulting in greater stabilization of the protein-membrane complex.

When RBL-2H3 cells transfected with these phospholipid binding mutants were stimulated with increasing concentrations of DiC8, no plasma membrane translocation was observed until ionomycin was added to the stimulation medium (Fig. 7). The fact that both PKCa N189A and PKCa R216A mutants translocated to a similar extent to the wild-type protein suggests that the C1 domain can overcome the slight lack of function of these C2 domain mutants (Fig. 7, A and B). In contrast, the PKCa R249A mutant did not translocate to the plasma membrane either in the presence of DiC8 or ionomycin (Fig. 7C). The PKCa T251A mutant targeted the plasma membrane in a transient manner (Fig. 7D), and the half-maximal plasma membrane dissociation time of 114 ± 14 s (n = 10 cells) was much shorter than that exhibited by wild-type PKCa, PKCa N189A, and PKCa R216A. These results suggest that the role played by Arg-249 and Thr-251 in the C2 domain membrane anchorage cannot be overcome by DiC8 acting through the C1 domain and that the interactions existing between these residues and the plasma membrane might be essential for the membrane targeting of PKCa.

**DISCUSSION**

**Role of Ca\(^{2+}\) Ions in the C2 Domain-dependent Translocation of PKCa**—The results obtained in this work suggest that a very sensitive mechanism exists whereby the Ca\(^{2+}\) binding region of the C2 domain drives PKCa membrane targeting in *vivo*. Substitutions of Asp-187 and Asp-246/248 by Asn had a dramatic effect on the Ca\(^{2+}\)-dependent membrane targeting of the enzyme, confirming previous biochemical studies in which similar substitutions produced a severe impairment of Ca\(^{2+}\)-dependent phospholipid binding (8, 9, 28). It is important to note that Asp-187 mainly coordinates Ca1, and the single substitution of an aspartate residue by asparagine was not expected to produce such a damaging effect, since the other four aspartate residues involved remain intact and could coordinate at least one Ca\(^{2+}\) ion. Thus, these results confirm the hypothesis proposed in the crystallographic model whereby Ca1 serves as a bridge between some of the aspartate residues located in the protein and the phosphate group of the PS (15, 16).

Similar function has been attributed to Ca1 in the case of synaptotagmin I (C2A domain) (29, 30). In contrast, Ca1 seems not to be essential for the membrane binding of cytosolic phospholipase A\(_2\) (31). Thus, the varied results obtained with the different proteins suggest that the subtle structural differences existing among C2 domains are probably the basis of the different functional mechanisms exhibited by the proteins that hold these domains.

Additionally, it was observed that the Ca\(^{2+}\) binding mutant proteins associated with the plasma membrane in a DiC8-dependent manner was strongly correlated with the number of aspartate residues neutralized in each mutant (Fig. 5). This effect could well correspond to the neutralization of charges that occurs after Ca2 binds to the C2 domain (13, 32, 33) and which probably produces a conformational change that in turn allows diacylglycerol access to the C1 domain. This hypothesis is supported by several studies that have shown through different techniques that the full-length enzyme undergoes a general conformational change upon Ca\(^{2+}\) binding (6, 12, 34–36). Whether or not this conformational change also produces the liberation of the pseudosubstrate region from the catalytic domain before C1 domain binding to diacylglycerol cannot be easily answered, but previous works in our lab have shown that a significant activation of the enzyme can occur in the only presence of PS and Ca\(^{2+}\), further supporting this hypothesis (9, 17).

The need of a third Ca\(^{2+}\) ion (Ca3) to anchor PKCa to the plasma membrane is still under debate. In this work, no Ca\(^{2+}\)-dependent translocation of PKCa T251A was observed, suggesting that if there is a need for a third Ca\(^{2+}\) to anchor the enzyme to the plasma membrane, this ion has to bind to the domain before PKCa interacts with the phospholipid bilayer. In addition, the reduced plasma membrane association exhib-
Edited by PKC.

T251A mutant in the presence of DiC8 implies that this residue might be involved in stabilizing C2 domain membrane docking before diacylglycerol binds to the C1 domain. Taking into account the low affinity exhibited by this C2 domain for a third Ca$^{2+}$/PS in solution (14, 16), it is more likely that this stabilization might be due to a direct inter-

FIG. 6. A, confocal fluorescence images of RBL cells expressing the different lipid-binding site mutants N189A, R216A, R249A, and T251A. The images were recorded at the different times stated. Antigen (40 ng/ml DNP-HSA) was added after 83 s, and 1 μM ionomycin was added after 381 s of recording. B, examples of the time course of the Ca$^{2+}$ signals after antigen (Ag) and ionomycin addition. C, examples of the time course of plasma membrane translocation of the different mutants. R, relative plasma membrane translocation.

FIG. 7. Comparison of the time course of DiC8-induced plasma membrane translocation of the different lipid-binding mutants, N189A (A), R216A (B), R249A (C), and T251A (D). The relative plasma membrane translocation (R) is represented as a function of time. DiC8 was added after 83 s, and ionomycin was added after 381 s. The DiC8 concentrations used in each experiment were 25 (■), 50 (▲), and 100 (●) μg/ml. Ag, antigen.
action between Thr-251 and the phospholipid bilayer, but the mediation of a third Ca$^{2+}$ in this process cannot be completely ruled out.

**Role of the Phosphatidylserine Binding Region in the C2 Domain-dependent Translocation of PKCα**—Another important issue addressed in this work was the role of the amino acidic residues at the Ca$^{2+}$-binding region of the C2 domain of PKCα involved in direct binding with the phosphatidylserine at the membrane surface. In this case, we observed that mutation to Ala of the side chains of Asn-189 and Arg-216 had a different effect on the Ca$^{2+}$-dependent translocation of PKCα than did the same substitutions in Arg-249 and Thr-251. In the first two cases, the mutations resulted in a weaker membrane association of the proteins. However, after the addition of diacylglycerol and ionomycin, these mutants bound to the membrane in a similar way to the wild-type protein, suggesting that the interactions exerted by these two residues located in loops 1 and 2 are important for stabilizing the protein-membrane complex, although they are not crucial for the protein to anchor to the membrane.

In contrast, interactions established by the side chain of Arg-249 were very critical since its mutation inhibited the ability of the protein to interact with the plasma membrane even in the presence of saturating Ca$^{2+}$ and diacylglycerol. This effect might be because of the loss of certain interactions with the phospholipid moieties since this mutant exhibited very similar Ca$^{2+}$-dependent activation to wild-type PKCα, as demonstrated in previous work (17). Moreover, PKCα is not the only case that has been reported since it has been shown recently (37) that the Ca$^{2+}$ binding affinity of a homologue residue mutated in the C2A domain of synaptotagmin I does not differ from observations made in wild-type protein. In the crystallographic model of the C2 domain of PKCα (15), the guanidinium group of Arg-249 has been proposed to interact through hydrogen bonding to the sn-2 acyl group oxygen and additionally to the ester oxygen of the sn-1 acyl chain. Because the Arg-216 side chain establishes hydrogen bonding with the carbonyl group of the sn-2 acyl chain, it seems that the interactions occurring between Arg-249 and the phospholipid in the C2 domain membrane anchorage are more critical than the corresponding interaction of Arg-216. It should also be mentioned that in the crystallographic model, extra hydrophobic interactions were established between the aliphatic carbons of Arg-249 and the acyl chains of 1,2-dicaproyl-sn-phosphatidyl-L-serine, and this is supported by the drastic effect observed in the membrane targeting of the enzyme when Arg-249 was substituted by Ala. Although it is not clear that the location and orientation of these acyl chains of the phospholipid shown in the crystallographic study are the same as their corresponding location in a bilayer, it is possible that hydrophobic interactions further increase the importance of Arg-249 for the C2 domain membrane docking and orientation. It is important to take into account that there are hydrophobic residues (Trp-245 and Trp-247) surrounding Arg-249 in loop 3 that have been implicated in the partial membrane penetration of PKCα (6). Similar evidence has been proffered in the case of the C2A domain of synaptotagmin I, where direct interactions of residues located in loop 3 and loop 2 with a soluble phosphatidylserine were reported (38–39).

**Conclusions**—Together, these results provide a deeper molecular understanding for the sequential mechanism of PKCα activation driven by the C2 domain (Fig. 8). In our model, increase in intracellular Ca$^{2+}$ produces the binding of Ca1 and Ca2 when the protein is still in the cytosol, leading to the membrane targeting of the enzyme through the C2 domain (Fig. 8b). Ca1 is responsible for bridging the protein to the specific phospholipid molecules, which are also recognized with the help of Asn-189 and Arg-216, whereas Ca2 is responsible for keeping Ca1 in its proper location and for inducing a conformational change in PKCα, which partially penetrates and orientates in the phospholipid bilayer through loop 3 (Arg-249 and Thr-251). Only after this has occurred can the C1 domain find the diacylglycerol generated in the membrane surface, enabling the protein to become fully activated (Fig. 8c). Whether or not the C2 domain-dependent translocation of PKCα itself is able to transduce signals downstream, representing a distinct, diacylglycerol-independent signaling mode is still not known and will have to be further studied.

The present study provides new insights into the molecular mechanism underlying the dynamic nature of PKCα redistribution in vivo. Our results have dissected a sequential mechanism that controls the function of the C2 domain of PKCα. Because the different PKC isoenzymes have been implicated in the malignant transformation and proliferation of cells, they...
have become a very attractive target for anticancer drug design (40). One of the main problems in using this strategy is the ubiquity and the lack of specific inhibitors/activators for each isoenzyme. For this reason, an accurate definition of the residues involved in the Ca$^{2+}$/lipid-dependent translocation is a very useful tool for the design of new drugs that can act specifically in this critical activation step.

Acknowledgments—We thank Professor Shamshad Cockcroft for the gift of RBL cells and access to the electroporation system, Victoria Allen-Baume for assays of cell signaling efficiency and help with electroporation, and Claudia Wiedemann for help with electroporation.

REFERENCES

1. Mellor, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
2. Ron, D., and Kazanietz, M. (1999) FASEB J. 13, 1658–1676
3. Newton, A., and Johnson, J. E. (1998) Biochim. Biophys. Acta 1376, 155–172
4. Cho, W. (2001) J. Biol. Chem. 276, 32407–32410
5. Bazzi, M. D., and Nelsestuen, G. L. (1990) J. Biol. Chem. 265, 17544–17552
6. Medkova, M., and Cho, W. (1999) J. Biol. Chem. 274, 17544–17552
7. Keranen, L. M., and Newton, A. C. (1997) J. Biol. Chem. 272, 25959–25967
8. Oancea, E., and Meyer, T. (1998) Cell 95, 307–318
9. Conesa-Zamora, P., Gomez-Fernandez, J. C., and Corbalan-Garcia, S. (2000) Biochim. Biophys. Acta 1487, 246–254
10. Edwards, A. S., and Newton, A. C. (1997) Biochemistry 36, 15615–15623
11. Corbalan-Garcia, S., Rodriguez-Alfaro, J. A., and Gomez-Fernandez, J. C. (1999) Biochem. J. 337, 513–521
12. Nalefski, E. A., and Newton, A. C. (2001) Biochemistry 40, 13216–13229
13. Nalefski, E. A., Wisner, M. A., Chen, J. Z., Sprang, S. R., Fukuda, M., Mikoshiba, K., and Falke, J. J. (2001) Biochemistry 40, 3089–4100
14. Kohout, S. C., Corbalan-Garcia, S., Torrecillas, A., Gomez-Fernandez, J. C., and Falke, J. J. (2002) Biochemistry 41, 11411–11424
15. Verdaguer, N., Corbalan-Garcia, S., Ochoa, W. F., Fita, I., and Gomez-Fernandez, J. C. (1999) EMBO J. 18, 6329–6338
16. Ochoa, W. F., Corbalan-Garcia, S., Eritja, R., Rodriguez-Alfaro, J. A., Gomez-Fernandez, J. C., Fita, I., and Verdaguer, N. (2002) J. Mol. Biol. 320, 277–291
17. Conesa-Zamora, P., Lopez-Andreo, M. J., Gomez-Fernandez, J. C., and Corbalan-Garcia, S. (2001) Biochemistry 40, 13988–13995
18. Corbalan-Garcia, S., Garcia-Garcia, J., Rodriguez-Alfaro, J. A., and Gomez-Fernandez, J. C. (2002) J. Biol. Chem. 278, 4971–4980
19. Beaver, M. A. (1996) Curr. Biol. 6, 798–801
20. Algmo, K., Arthammar, P. G., Thastrup, O., and Tulin, S. (1999) Biochem. J. 337, 211–218
21. Maasch, C., Wagner, S., Lindschau, C., Alexander, G., Burchner, K., Gollash, M., Luft, F. C., and Haller, H. (2000) FASEB J. 14, 1653–1663
22. Vallentin, A., Prevostel, C., Fauquier, T., Bonnefont, X., and Joubert, D. (2000) J. Biol. Chem. 275, 6014–6021
23. Bolsover, S., Ibrahim, O., O’Lunaigh, N., Williams, H., and Cockcroft, S. (2001) Biochem. J. 356, 345–352
24. Meyer, T., and Oancea, E. (2000) Methods Enzymol. 327, 500–513
25. Guex, N., and Peitsch, M. C. (1997) Electrophoresis 18, 2714–2723
26. Tanimura, A., Nieuw, A. M., Morita, T., Hashimoto, N., and Tajiya, Y. (2002) J. Biol. Chem. 278, 29854–29862
27. Medkova, M., and Cho, W. (1999) J. Biol. Chem. 274, 19862–19861
28. Tersel, M. N., and Meyer, T. (2002) Science 295, 1910–1912
29. Ubach, J., Zhang, X., Shao, X., Sudhof, T. C., and Rizo, J. (1998) EMBO J. 17, 3921–3930
30. Fernandez-Chacon, R., Shin, O. H., Konigstorfer, A., Matos, M. F., Meyer, A. C., Garcia, J., Gerber, S. H., Rizo, J., Sudhof, T. C., and Rosenmund, C. (2002) J. Neurosci. 22, 8438–8446
31. Bittova, L., Sumandea, M., and Cho, W. (1999) J. Biol. Chem. 274, 9665–9672
32. Zhang, X., Rizo, J., and Sudhof, T. C. (1998) Biochemistry 37, 12395–12403
33. Murray, D., and Honig, B. (2002) Mol. Cell 9, 145–154
34. Shah, N., and Shapley, G. G. (1992) Biochim. Biophys. Acta 1119, 19–26
35. Bosca, L., and Moran, F. (1993) Biochem. J. 290, 827–832
36. Stahelin, R. V., and Cho, W. (2001) Biochem. J. 359, 679–685
37. Fernandez-Chacon, R., Konigstorfer, A., Gerber, S. H., Garcia, J., Matos, M. F., Stevens, C. P., Brose, N., Rizo, J., Rosenmund, C., and Sudhof, T. C. (2001) Nature 410, 41–49
38. Chapman, R. E., and Davis, A. F. (1998) J. Biol. Chem. 273, 13995–14001
39. Chae, Y. K., Ahlggaard, F., Chapman, E. B., and Markley, J. L. (1998) J. Biol. Chem. 273, 25659–25663
40. Swannie, H. C., and Kaye, S. B. (2002) Curr. Oncol. Rep. 4, 37–46
