A Critical Intramolecular Interaction for Protein Kinase Cε Translocation

Disruption of intramolecular interactions, translocation from one intracellular compartment to another, and binding to isozyme-specific anchoring proteins termed RACKs, accompany protein kinase C (PKC) activation. We hypothesized that in inactive εPKC, the RACK-binding site is engaged in an intramolecular interaction with a sequence resembling its RACK, termed ψεRACK. An amino acid difference between the ψεRACK sequence in εPKC and its homologous sequence in RACK constitutes a change from a polar non-charged amino acid (asparagine) in εRACK to a polar charged amino acid (aspartate) in εPKC. Here we show that mutating the asparagine to aspartate in εPKC increased intramolecular interaction as indicated by increased resistance to proteinolysis, and slower hormone- or PMA-induced translocation in cells. Substituting aspartate for a non-polar amino acid (alanine) resulted in binding to εRACK without activators, in vitro, and increased translocation rate upon activation in cells. Mathematical modeling suggests that translocation is at least a two-step process. Together our data suggest that intramolecular interaction between the ψεRACK site and RACK-binding site within εPKC is critical and rate limiting in the process of PKC translocation.

The protein kinase C (PKC) family of phospholipid (PL)-dependent serine/threonine kinases undergoes a conformational change and translocation, or movement, from the cytosolic to the cell particulate fraction upon activation (1, 2). Conformational changes in PKC from an inactive to an active state result in exposure of domains required for PKC anchoring to the particulate fraction and in increased sensitivity of the enzyme to proteases (1–3, 41). Therefore, the inactive state exists in a closed conformation, with the proteolytic sites protected, whereas the active state is in an open conformation with exposed proteolytic sites. Structural alterations from the closed to open states involve disruption of intramolecular interactions within the enzyme.

An intramolecular interaction in inactive PKC between the catalytic site and a site in the regulatory domain that resembles a substrate phosphorylation site but lacks a serine or threonine phosphoacceptor (pseudosubstrate site) has been previously identified (3, 6). Deletion of the pseudosubstrate (ψ-substrate) site generated a constitutively active enzyme (6) and mutations of the basic residues in the ψ-substrate site reduced the affinity of the catalytic site to the ψ-substrate site generating a constitutively active enzyme, preferentially localized to the cell particulate fraction (6). Furthermore, conversion of the alanine in the ψ-substrate site to a glutamic acid, mimicking a phosphorylated amino acid, resulted in loss of binding of the ψ-substrate site to the catalytic site, creating a constitutively active enzyme (6). Finally, a peptide corresponding to the ψ-substrate site is a competitive inhibitor of PKC catalytic activity (6).

We previously demonstrated that translocation of PKC is associated with binding of each activated PKC isozyme to a corresponding anchoring protein, termed RACK, for receptor for activated C-kinase (7). RACKs also function as molecular scaffolds, binding and regulating other signaling proteins. For example, RACK1 binds dynamin-1 (8), Src (9), phospholipase Cγ (10), protein-tyrosine phosphatase µ (11), cyclic nucleotide phosphodiesterase (PDE4E5) (12), Fyn kinase and the N-methyl d-aspartate receptor NR2B subunit (13). RACK2 (εRACK), also known as b’cop, is a member of the coatomer complex, COPI, and binds several coatomer proteins and the small G protein ARF (14).

Since isozyme-specific PKC binding to respective RACKs occurs upon enzyme activation, we predicted that PKC activation involves a conformational change that induces the unmasking of the RACK-binding site in PKC (15–17). Therefore, we proposed the existence of a second intramolecular interaction in inactive PKC. This intramolecular interaction forms between the RACK-binding site and another site in PKC, termed the pseudo-RACK (ψRACK), which resembles and mimics a sequence in the RACK (15–17). It is likely that even in unstimulated cells, PKC exists in at least two conformations: an active open conformation, that has the RACK- and PL-binding sites exposed, and an inactive closed conformation, with these sites unavailable for binding to RACK and PL. In the presence of activators, the open conformation would be stabilized, shifting the equilibrium between the two conformations toward the active state.

ψRACK sequences were identified by searching for regions of homology between each PKC isozyme and its RACK. In εPKC, the sequence HDAPIGYD (ψPKC 85–92), named ψεRACK, has ~75% homology with a sequence in εRACK consisting of amino
Intramolecular Interaction in ePKC

acids NNVALGyd (eRACK 285–292). A peptide corresponding to the ϕεRACK sequence functions as a ePKC-selective agonist. We predicted that the agonist activity occurs because the ϕεRACK peptide binds the εRACK-binding site in ePKC, interfering with the intramolecular interaction between the εRACK and the εRACK-binding site, and thus stabilizing the open form. Since, after treating cells with a ϕεRACK peptide, ePKC was localized with eRACK (17), we suggested that the ϕεRACK peptide is eventually replaced by the εRACK, possibly because the affinity of the εRACK-binding site to the εRACK peptide is lower than the affinity of the εRACK-binding site to the RACK (16). A notable difference between the ϕεRACK sequence in ePKC and the homologous sequence in eRACK involves an amino acid change from a polar charged amino acid (aspartate (Asp-86)) in ePKC to a polar non-charged amino acid (asparagine (Asn-86)) in eRACK. Such a change in charge fits the prediction that the proposed intramolecular interaction mediated by the ϕεRACK site and the RACK-binding site in ePKC lacks a crucial interaction and is subsequently competitively replaced by the εRACK upon enzyme activation (18).

The aim of this study was to test the importance of the intramolecular interaction between the ϕεRACK site and the RACK-binding site and its influence in the process of ePKC translocation and function. If the ϕεRACK site is engaged in an intramolecular interaction with the RACK-binding site, we expected that a mutation in the ϕεRACK site, which increases the resemblance of the ϕεRACK site to the εRACK, should stabilize this intramolecular interaction and yield a more closed conformation. To test this hypothesis, we mutated Asp-86 in the ϕεRACK site to an Asn-86, so that the ϕεRACK site would more closely resemble the εRACK or to a non-polar amino acid, alanine (Ala). We then determined the sensitivity of the mutants and wild-type enzyme to protease digestion and their dependence on activators for binding to εRACK in vitro. These mutants were also expressed in mammalian cells as GFP fusion proteins and the effect of the mutation on translocation rates was examined.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes were from New England Biolabs. Anti-ePKC V5 antibodies were from Santa Cruz Biotechnology.

Cell Cultures—CHO-Hir cells (kindly provided by Bio Image A/S, Soeborg, Denmark) were kept in culture in F-12(HAM) nutrient mixture (Invitrogen), and antibiotics. Cells were grown in 2% CO₂. Insect cells were grown at 26 °C in the presence of PL in homogenization buffer, beads were washed four times in 20 mM Tris-HCl, pH 7.5, 0.1% Triton X-100. The supernatant was then incubated with the soluble RACK peptide, (phosphatidylyserine 12 μg/reaction and, sn-1,2 dioleoylglucero 400 ng/reaction) for 1 h at 4 °C. Following a thorough washing with wash buffer, bound proteins were eluted in SDS-PAGE sample buffer and proteins separated on 8% SDS-polyacrylamide gels. The amount of ePKC interacting with εRACK was determined by Western blot using anti-ePKC antibodies.

RACK Binding Assays—Insect cells expressing either ePKC or GST-εRACK were lysed as described above. GST–εRACK, 10 ng was immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences), and washed thoroughly with wash buffer (20 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 mM NaCl, 12 mM β-mercaptoethanol, and 0.1% Triton X-100). Immobilized GST–εRACK was then incubated with the soluble fraction of insect cell lysates containing 100 ng of wild type, D86A, or D68N ePKC protein, in the presence or absence of phospholipid activators (PL) (phosphatidylserine 12 μg/reaction and, sn-1,2 dioleoylglucero 400 ng/reaction) for 1 h at 4 °C. Following a thorough washing with wash buffer, bound proteins were eluted in SDS-PAGE sample buffer and proteins separated on 8% SDS-polyacrylamide gels. The amount of ePKC interacting with εRACK was determined by Western blot probing with anti-ePKC antibodies. Blots were then probed against anti-GST (Santa Cruz Biotechnology) to verify that the same amount of GST–εRACK was present in each binding assay. Alternatively, GST–εRACK was incubated with 200 ng of soluble GFP-εPKC and GFP–εPKC mutants. Soluble GFP–εPKC and GFP–εPKC mutants were obtained from 48-h-transfected CHO-Hir cells that were serum-starved 24 h after transfection. Cells were then lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM EGTA, 0.25 mM sucrose, 12 mM β-mercaptoethanol, and 0.1% Triton X-100). Immobilized GST–εRACK was then incubated with the soluble fraction of insect cell lysates containing 100 ng of wild type, D86A, or D68N εPKC protein, in the presence or absence of phospholipid activators (PL) (phosphatidylserine 12 μg/reaction and, sn-1,2 dioleoylglucero 400 ng/reaction) for 1 h at 4 °C. Following a thorough washing with wash buffer, bound proteins were eluted in SDS-PAGE sample buffer and proteins separated on 8% SDS-PAGE gels, as described above.

Immunoprecipitation—CHO-Hir cells were washed twice in serum-free medium and serum-starved 12–24 h after transfection. Cells were then kept in serum-free medium for ~12 h before the experiment. Cells were lysed in homogenization buffer for ~1 h at 4 °C. Following a thorough washing with wash buffer, bound proteins were eluted in SDS-PAGE sample buffer and proteins separated on 8% SDS-PAGE gels, as described above.

Kinase Assays—The ability of the different ePKC mutants to phosphorylate substrates was assayed by following the incorporation of [γ-32P]ATP into myelin basic protein according to a method modified from Kikkawa et al. (20) myelin basic protein phosphorylation was measured either by liquid scintillation or for immunoprecipitation experiments, by autoradiography. For the kinase reaction of immunoprecipitated ePKC, inter-
cipated enzyme, heads were resuspended in 20 μl of a kinase reaction buffer composed of 20 mM Tris, pH 7.5, [γ-32P]ATP (Amersham Biosciences) 0.3 μCi/reaction, ATP (Sigma) 9 μCi/reaction, myelin basic protein (Sigma) 12 μg/reaction, and MgCl2 50 mM/reaction. 4 μl of phospholipids were added per reaction when needed. Phospholipids were prepared as described (20). Kinase reactions were stopped with SDS sample buffer and boiling. Samples were then run on a 12% SDS-PAGE and transferred to nitrocellulose exposed for autoradiography. The same nitrocellulose was then developed with anti ePKC (V5) antibodies (Santa Cruz Biotechnology) to verify the amount of fusion protein immunoprecipitated.

Analysis of PKC Translocation by Western Blot—After 24 h of transfection cells were serum-starved for an additional 24 h and incubated with phorbol 12-myristate 13-acetate (PMA) (LC Laboratories) for the indicated times and concentrations at room temperature and subsequently fractionated as previously described (21). To assess PKC distribution the different cell fractions were run on SDS-PAGE, transferred for Western blot analysis and probed with anti-ePKC V5 antibodies. Lysates of overexpressed GFP-ePKC were diluted to ~20 ng/μl. This concentration endogenous ePKC was not detected.

Microscopy and Analysis—CHO cells were grown on glass cover slips, and serum-starved as described above. For each experiment, cells were transferred to a commercially available metal coverslip holder (Molecular Probes) in which the coverslip formed the bottom of a 1 ml bath. The media was then replaced with extracellular buffer (120 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, 1.5 mM MgCl2, 20 mM Hepes, and 30 mM glucose). Cells were stimulated by either PMA (100 nM), or with ATP (1 mM) in extracellular buffer. Fluorescence images of GFP-tagged constructs were obtained using the 488 nm excitation line of a laser scanning confocal microscope (Pascal, Zeiss), and emission was collected through a 505–550 nm band pass filter. Cells were imaged on the stage of an inverted microscope (Axiovert 100M) using a 40–550 nm oil immersion objective (1.35 NA) and images were acquired with a CCD camera (Hamamatsu and 2K2K pixel binning). GFP was excited with the 442 nm laser line of a helium-cadmium laser (Kinmon) whereas YFP was imaged with the 514 nm line of an argon ion laser (Melles-Griot). To selectively photobleach YFP labeled proteins in local regions of the cytoplasm for FRAP experiments, the 514 nm line of an Enterprise laser (Coherent) at maximal power (~400 milliwatts) was used. For these experiments, we used a 60× oil immersion objective (1.4 NA). Under these conditions, and by placing an iris in the beam path, it was possible to bleach 80% of the YFP fluorescence in 500 ms in an area of the cytosol measuring ~35 μm². Real-time confocal images were acquired every 10–15 s for 20 min for experiments utilizing PMA, and every 5 s for a total duration of 3 min in the case of cells stimulated with ATP. Reagents were added to the cell chamber after the fifth image in each time series. Control time lapses were acquired using the same imaging conditions used in the experiments to check that the level of general dye photobleaching did not exceed 10–30%. All images were acquired at room temperature. Images were exported as 12 or 16 bit files and changes in fluorescence intensity were measured using Metamorph® data analysis software (Universal Imaging). To monitor the translocation of PKC, a small region of interest was selected in the cytosol of each cell and fluorescence intensity values graphed against time after subtraction of background values and normalized so that the initial fluorescence was 100%. Averages of 10–15 cells from three independent experiments were used.

RESULTS
Sensitivity to Protease Degradation of the Different ϕeRACK ePKC Mutants—If the Asp-86 in the ϕeRACK site is engaged in an intramolecular interaction with the RACK-binding site, we expected the D86N mutant to reside more in the closed state, and therefore be more resistant to proteolysis. In contrast, the D86A mutant should favor an open conformation and therefore be more susceptible to proteolysis. To test this hypothesis, ePKC WT and mutants expressed in insect cells were subjected to proteolysis by the protease, Arg C, detected by Western blot analysis with anti-ePKCV5 antibodies. B, average of five independent experiments showing the rate of degradation of the ϕeRACK ePKC mutants by Arg C. Data were normalized to the initial amount of enzyme, and are expressed as percent of full-length ePKC (WT, D86A, D86N, D86N). Average of four independent experiments) for binding of ϕeRACK ePKC mutants to GST-eRACK in the presence and absence of PL as determined by Western blot with anti-ePKC V5. D, quantitative data (average of four independent experiments) for binding of ϕeRACK ePKC mutants to GST-eRACK, in the absence (plain bars) or presence of PL (filled bars), *p < 0.05 using Student’s t test. E, in vitro binding of ϕeRACK ePKC mutants to GST-eRACK mutants expressed in insect cells were subjected to proteolysis by the endopeptidase, Arg C as previously shown for ϕPKC (19). Degradation by Arg C was monitored by the decrease of full-length ePKC. Although under these experimental conditions D86A mutation did not alter susceptibility of the enzyme to degradation by Arg C when compared with the wild-type enzyme, the D86N mutant was significantly more resistant to proteolysis than either D86A or wild type (Fig. 1, A and B).

Binding of the ePKC Mutants to eRACK—RACKs bind active ePKC (22, 23). If the intramolecular interaction between ϕeRACK and the RACK-binding site stabilizes the inactive closed form, increasing or decreasing the affinity of this intramolecular interaction should cause a corresponding decrease or increase in the ability of the enzyme to bind to its...
RACK. To test this prediction, we determined the binding of insect cell-expressed \( PKC_{(Wt \text{ and } D\,(86)N}) \) to immobilized GST-\( RACK \) in the presence and absence of phospholipid (PL) activators.

Binding of D86A \( ePKC \) mutant to \( eRACK \) in the absence of PL activators was at least \( 2\)-fold greater than binding of either D86N or wild-type enzymes (Fig. 1, \( C \) and \( D \)). Binding of D86N and of wild-type enzyme to \( eRACK \) was significantly increased in the presence of PL, whereas binding of the D86A mutant to \( eRACK \) was not increased (Fig. 1, \( C \) and \( D \)). In the presence of an equal concentration of PL, there was less \( eRACK \) binding of D86N \( ePKC \) than either D86A or wild-type \( ePKCs \). These results are consistent with the prediction that the \( eRACK \)-binding site in the D86A mutant is already available for binding to \( eRACK \), whereas this site is masked in both wild-type or D86N \( ePKCs \) and becomes accessible for binding only upon activation.

**Rate of Translocation of \( ePKC \) and \( ePKC \) Mutants in Cells**—

The above in vitro studies of \( \psi\)eRACK mutants and wild-type \( ePKC \) support our hypothesis that \( \psi\)eRACK is involved in an intramolecular interaction that stabilizes the inactive closed

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**Fig. 2.** GFP-\( ePKC \) mutants expressed in CHO cells are catalytically active, bind to \( eRACK \), and translocate upon PMA activation. A, immunoprecipitated GFP-\( ePKC \) mutants detected by Western blot with anti-\( ePKC \) V5 antibodies (upper panel) and their catalytic activity measured by autoradiography of \( \gamma\)-\( ^{-32} \)P-labeled myelin basic protein (a representative experiment; upper panel). Average of three independent kinase reactions (lower panel) show equal activity of the \( ePKC \) mutants upon activation, as seen by myelin basic protein phosphorylation in the presence of PL (reactions were carried out for 3 and 15 min). B, a representative experiment of four independent experiments showing that the GFP fusion to the \( ePKC \) mutants expressed in CHO cells did not interfere with their selective binding to GST-\( eRACK \) in the presence of PL as determined by Western blot with anti-\( ePKC \) V5 (upper panel). Quantitative data (average of four independent experiments) for binding of GFP-\( \psi\)eRACK \( ePKC \) mutants to GST-\( eRACK \) (lower panel). C, a representative experiment of translocation of \( ePKC \) in CHO cells transfected with GFP-\( ePKC \) \( \psi\)eRACK mutants treated with 100 \( \text{nM} \) PMA for 10 min. GFP-\( ePKC \) was detected by Western blot with anti-\( ePKC \) V5 antibodies. D, translocation of GFP-\( ePKC \) mutants in MCF-7 cells following stimulation with 10 \( \text{nM} \) PMA for 10 min (upper panel). Average of four independent experiments (lower panel) of translocation of GFP-\( ePKC \) \( \psi\)eRACK in MCF-7 control (unfilled bars), and cells stimulation with 10 \( \text{nM} \) PMA for 10 min (filled bars). *, \( p < 0.05 \) using Student’s \( t \) test.
state. To further test this hypothesis, we examined the rate of translocation of ϕeRACK mutants and wild-type ePKC in cells in response to stimulation. We proposed that part of the process of translocation requires disruption of the intramolecular interaction between the ϕeRACK site and the eRACK-binding site. Therefore, we expected that modulation of this intramolecular interaction should affect translocation rates of ePKC. To investigate this hypothesis, ePKC ϕeRACK mutants were fused at their N termini to GFP, CFP, or YFP proteins and translocation was analyzed both by cell fractionation and by real-time imaging. In an in vitro kinase assay, we first confirmed that the GFP fusion proteins had similar catalytic activity. As seen in Fig. 2A, immunoprecipitated GFP-ePKC mutants phosphorylated myelin basic protein to a similar extent upon activation. We next confirmed that fusion of GFP to the ePKC mutants does not interfere with the binding of the enzymes to eRACK in vitro. Fig. 2B demonstrated a selective binding of wild type and the two GFP-ePKC mutants to GST-eRACK.

We also confirmed that the wild-type and ϕeRACK ePKC mutants were sensitive to activation by phorbol ester and that they translocated from the soluble to the particulate fraction of the cell upon activation. Translocation of the ePKC mutants was determined using two different cell lines, MCF-7 and CHO. MCF-7 cells were stimulated for 10 min with 100 nM PMA (Fig. 2C). CHO cells were stimulated with 10 nM PMA for 10 min (Fig. 2D), since stimulation of MCF-7 cells with higher doses of PMA caused a detachment of the cells. After treatment with PMA, cells were fractionated into soluble and particulate fractions, and GFP-ePKC was detected and quantified by Western blot analysis using anti-ePKC. Only GFP-ePKC fusion proteins (~110 kDa) and not the endogenous ePKC (~80 kDa) were detected when 20 ng of protein was loaded per lane, and all the mutants had a similar level of expression (Fig. 2, C and D). On PMA treatment, there was a greater increase in D86A ePKC in the particulate fraction than either D86N or wild-type ePKCs (Fig. 2, C and D). This is not due to PKC degradation; the total amount of ePKC was not changed upon activation. Therefore, GFP-ePKC ϕeRACK mutants were catalytically active and responded to activation by PMA by translocating from the soluble to the particulate fraction of the cell. Using two different cell lines, we found that after 10 min of treatment with PMA, more D86A ePKC compared with wild-type or D86N ePKCs translocated to the particulate fraction.

Since cell fractionation experiments are not suitable for dynamic studies, we used the GFP tag to follow the rate of translocation of the different ePKCs by real-time microscopy in CHO cells (Fig. 3). Translocation of ePKC was seen as a decrease of fluorescence intensity in the cytoplasm concomitant with an increase in fluorescence intensity at the cell periphery. All of the ePKC mutants translocated to the same place (cell periphery) when stimulated by PMA (Fig. 3A). Translocation of the D86A mutant to the cell periphery began within 1 min of PMA stimulation, whereas translocation of the wild-type enzyme became apparent only after 2–3 min of PMA stimulation (Fig. 3A, arrows). In contrast, translocation of the D86N mutant became apparent only after 5 min of stimulation. A typical line intensity profile showing the distribution of each ePKC between the cell periphery and cytosol is shown for a representative cell at different time points (Fig. 3B).

The amount of fluorescence decrease in the cytoplasm was proportional to the amount of fluorescence increase at the cell periphery (24), since the total fluorescence did not change. To quantitatively monitor translocation of the different ePKC enzymes, we measured the decrease of fluorescence intensity in a region of the cytoplasm upon PMA stimulation using Meta- morph software (Universal Imaging). A graphical comparison of ePKC translocation obtained by the decrease of fluorescence in the cytoplasm indicated that the rates of translocation were significantly different between the two mutants and wild-type ePKC (p < 0.0001; Fig. 3C). The D86A mutant translocated at
a faster rate than either the wild-type or D86N ePKC mutant. The wild-type enzyme reaches a similar steady state level (the level at which there was no further accumulation of ePKC at the cell periphery) as the D86A mutant, but did so at a slower rate. In contrast, the D86N mutant achieved a steady state at a higher level than either the wild type or D86A mutant. Therefore, the D86A mutant translocated at a faster rate than either the D86N or wild-type ePKCs, and the amount of D86N ePKC mutant that reached the cell periphery was lower than the amounts of either D86A or wild-type ePKCs.

Mathematical Modeling of ePKC Translocation Suggests That ePKC Translocation Is at Least a Two Step Process—To further characterize the initial process of translocation of the different ePKC εεRACK mutants, we fitted the decrease of fluorescence in the cytoplasm to a mathematical model. Fluorescence time courses were analyzed by a non-linear regression analysis with single and bi-exponential equations (1 and 2) as previously done by Nalefski and Newton (25), where \( I(t) \) is the concentration of observed molecules in the cytosol at time \( t \), and \( C_{1-5} \) are constants in Equations 1 and 2.

\[
I(t) = C_1 + C_2 e^{-k_1 t} (Eq. 1)
\]
\[
I(t) = C_1 + C_2 e^{-k_1 t} + C_3 e^{-k_2 t} (Eq. 2)
\]

Residual error graphs obtained using single exponential equations (Fig. 4A) and using bi-exponential equations (Fig. 4B) for the D86A ePKC mutant translocation process is shown in Fig. 4. Residual error analysis for D86N and wild-type ePKCs showed similar results (data not shown). Since a superior fit, with a smaller and more equally distributed error was obtained with a bi-exponential equation, we adopted a two-step model to illustrate the initial process of ePKC translocation where the first step is the opening of ePKC and disruption of the intramolecular interaction between the εεRACK and the εεRACK-binding site, and the second step is ePKC binding to the membrane (binding to the membrane in this case may be binding to either lipids or proteins). This process can be described as follows in Scheme 1,

\[
\text{Closed ePKC in the cytosol} \xrightarrow{k_1} \text{Open ePKC in the cytosol} \xrightarrow{k_2} \text{Membrane-bound ePKC}
\]

Scheme 1

where \( k_1 \) is the rate of ePKC opening in the cytosol, \( k_{-1} \) is the rate of closing in the cytosol, \( k_2 \) is the rate of ePKC binding to the membrane, and \( k_{-2} \) is the rate of detachment from the membrane. Using the bi-exponential equation, we estimated the values for the constants \( C_1 \), \( C_3 \), and \( C_5 \) (Table I). The value of \( C_1 \) corresponds to the level of ePKC in the cytosol at steady state. We next determined whether the hypothesis that the values obtained for \( C_1 \) and \( C_3 \) of D86A and wild-type ePKCs are statistically equivalent. Using WALD’s parameter test, and using F-Test and \( \chi^2 \)-Squared test, we obtained \( p \) values of 0.9 and 0.7, respectively. We therefore assumed that the steady state level for D86A and WT, \( C_1 \), is the same. We also assumed that the second step (binding to the membrane) should not differ between the εεRACK ePKC mutants, since \( C_3 \) was equal for both wild-type and D86A ePKC. Therefore, if the steady state level (\( C_1 \)) was the same for D86A and wild-type ePKCs and the second step (binding to the membrane) was also the same, it is plausible that \( k_{-1} \) (rate of closing of ePKC) for both D86A and D86N ePKCs would be negligible. When \( k_{-1} \) is negligible, the following equations (3–5) hold and can be used to calculate \( k_{-2} \) (Equation 6) and \( k_2 \) (Table I).

\[
C_1 / 100 = k_{-2} (k_2 + k_{-2}) \tag{Eq. 3}
\]
\[
C_3 = (k_2 + k_{-2}) \tag{Eq. 4}
\]
\[
C_5 = k_1 \tag{Eq. 5}
\]

Fig. 4. Mathematical modeling analysis of D86A is represented in this figure. Similar results were obtained with D86N and wild-type ePKC. A, non-linear regression analysis using the single or bi-exponential equation. C, fit between curves of the raw data for D86A. The curves were obtained by nonlinear regression with a bi-exponential equation. D, fit between curves of the raw data for D86A. The curves were obtained by a differential equation using the values for \( k_1, k_{-1}, k_2, \) and \( k_{-2} \) provided in Table II. The residual error for all curves fitted data was similar to the one obtained with a non-linear regression using a bi-exponential equation.


If \( I_c(t) \) equals the concentration of closed ePKC in the cytosol at time \( t \), \( I_o(t) \) equals the concentration of open ePKC in the cytosol at time \( t \), \( I_d(t) \) equals the concentration of ePKC at the membrane at time \( t \) and \( I(t) \) equals the concentration of open and closed ePKC in the cytosol at time \( t \) \( (I_c(t) + I_o(t)) \) we can then use the following differential Equations (7-9) to describe our model:

\[
\frac{dI_c(t)}{dt} = -(K1)I_c(t) + (K-1)I_o(t) \quad (Eq. 7)
\]

\[
\frac{dI_o(t)}{dt} = -(K - 1 + K2)I_o(t) + (K1)I_c(t) + (K - 2)I_d(t) \quad (Eq. 8)
\]

\[
\frac{dI_d(t)}{dt} = -(K - 2)I_d(t) + (K2)I_c(t) \quad (Eq. 9)
\]

We next solved the differential equations for \( I(t) \) using the Runge-Kutta algorithm (Berkeley-Madonna) and compared the solutions to the experimental data (loss of fluorescence in the cytoplasm). We solved for \( I(t) \) and used the calculated parameters for \( k_1, k_{-1}, k_2, \) and \( k_{-2} \) (Table I) assuming that the initial amount of closed ePKC in the cytoplasm is equal to 100% \( I_c(0) = 100 \). Fig. 4C, shows the fit between curves of the raw data for D86A ePKC, and the curve obtained by nonlinear regression with a bi-exponential equation. Fig. 4D shows the fit between curves of the raw data for D86A and the curve obtained by the differential equations solving for \( I(t) \). Similar fitting results were obtained for D86N and wild-type ePKCs (data not shown). The residual error for all curve fitting data was similar to the one obtained with a non-linear regression, using a bi-exponential equation (Fig. 4B; data not shown).

The steady state level for D86N was higher than for either D86A or wild-type (Table I), and therefore it is not possible to assume that \( k_1 \) for this mutant was negligible. In this case, we also used the Runge-Kutta algorithm to solve differential equations. Because the rate of the second step, binding to the membrane, should not be altered for either of the ePKC mutants, we assumed that the values of \( k_2 \) and \( k_{-2} \) should be in the range of

\begin{table}
\centering
\caption{Estimated constants using a bi-exponential equation and calculated values for \( k_1, k_{-1}, k_2, \) and \( k_{-2} \)}
\begin{tabular}{|c|c|c|c|}
\hline
 & D86A & Wt & D86N \\
\hline
\( C_1 \) & 30.59 ± 0.286 & 30.46 ± 0.991 & 38.38 ± 0.254 \\
\( C_3 \) & 0.016 ± 0.001 & 0.016 ± 0.001 & 0.010 ± 0.0003 \\
\( k_{-2} = (k_2 + k_{-2}) = C_2/100 \) & 0.004 ± 0.004 & 0.004 ± 0.004 \\
\( k_{-2} = (k_2 + k_{-2}) = C_3 \) & 0.005 ± 0.0004 & 0.005 ± 0.0001 & 0.0052 \\
\( k_2 \) & 0.004 ± 0.0007 & 0.004 ± 0.0001 & 0.0153 \\
\( k_{-2} = C_5^a \) & 0.001 ± 0.0007 & 0.001 ± 0.0002 & 0.01138 \\
\( k_{-2} \) & 0.0004 ± 0.005 & 0.0001 ± 0.001 & 0.005 \\
\hline
\end{tabular}
\end{table}

\footnote{For D86N the relation \( k_1 = C_5 \) is not valid because the corresponding values for \( k_1, k_{-1}, k_2, \) and \( k_{-2} \) were obtained by simulations using differential equations.}

**Fig. 5.** D86A translocates faster than Wt upon PMA activation and faster than D86N upon stimulation with ATP. A, in cells transfected with both wild-type and the D86A ePKCs, the D86A ePKC mutant translocated faster than wild-type ePKC. Confocal image of a CHO cell transfected with both YFP-ePKC D86A and CFP-ePKC wild type (in pseudo-color) at different times upon stimulation with 100 nM PMA. B, quantitative analysis (CFP and YFP) of the decrease in fluorescence CFP-ePKC wild type (I) and YFP-ePKC D86A (○) in a region in the cytoplasm of the cell. Levels of the YFP-ePKC D86A mutant in the cytoplasm decrease faster than levels of CFP-ePKC wild type. When compared with wild type, the D86A mutant had a similar translocation rate and the eRACK D86N mutant had a slower translocation rate upon stimulation with 1 mM ATP. Confocal images of translocation of ePKC mutants at different time points after the addition of 1 mM ATP. The arrows indicate the time at which translocation to the cell periphery began to be apparent for each ePKC enzyme. C, translocation rates were analyzed by measuring the loss of fluorescence in the cytoplasm relative to time after addition of 1 mM ATP. Wt, II D86A, ○ D86N. Data are averages of at least three independent experiments with at least three cells in each experiment. The time course for the D86N mutant was statistically different from either D86A or wild-type ePKCs using a two-way ANOVA test with \( p < 0.001 \).
the ones obtained for D86A and wild type.

Together, our experimental data and mathematical modeling suggest a two-step translocation process of ePKC to the cell membrane upon activation. Whereas the second step was independent of intramolecular interactions, the first step, which we predicted involves opening of the enzyme, was greatly dependent on these intramolecular interactions.

Co-transfection of D86A ωRACK Mutant and Wild-type ePKC in the Same Cell—To further investigate the behavior of the ePKC mutants within a single cell, we co-transfected two different ePKC constructs into CHO cells, fused to either YFP or CFP. A representative cell that co-expresses a YFP-D86A and a CFP-Wt ePKC at similar levels is shown in Fig. 5A. Upon stimulation with PMA, the D86A mutant translocated faster than the wild-type enzyme (Fig. 5A). Quantitative analysis, expressing the decrease of fluorescence intensity in the cytoplasm in Fig. 5A (right panel). The D86A mutant was found at the cell periphery after 1.5 min after PMA stimulation whereas translocation of wild-type ePKC was still minimal even after 10 min (Fig. 5A). Therefore, even when the D86A ωRACK mutant and wild-type ePKC were in the same cell, D86A translocated faster than wild-type ePKC.

Translocation Rates of the ωRACK ePKC Mutants upon Cell Stimulation by a G Protein-coupled Receptor—We next determined whether differences in translocation rates of the different ePKCs were also observed when translocation was stimulated via receptor signaling rather than by PMA. ATP has been previously used to activate PKC in CHO cells by stimulating purinergic G protein-coupled receptors (26). We found that translocation of GFP-ePKC upon stimulation with ATP to the cell periphery was faster than PMA-induced translocation (Fig. 5B versus Fig. 3). Translocation of the D86A and wild-type ePKCs was already apparent 10 s after stimulation, whereas translocation of the D86N ePKC enzyme occurred after 40 s of stimulation reaching a steady state at significantly higher levels than either D86A or wild-type ePKCs (Fig. 5B).

Diffusion Rates of the ePKC ωRACK Mutants—Different translocation rates may reflect differences in overall mobility (diffusion) of the ePKCs in cells. Therefore, we measured fluorescence recovery after photo bleaching (FRAP) of the cytoplasmic enzyme; mobility of ePKCs was measured by monitoring the time required for the fluorescent to recover in a bleached region. Fifty percent FRAP was reached at similar times for all ePKCs. (The average of at least 10 cells/each ePKC was: Wt = 9.0 ± 1.2; D86A = 9.2 ± 1.1 and D86N = 9.1 ± 1.6 s.) By comparing the fluorescence in the bleached region after full recovery (F0) with that observed before bleaching (Ft) and just after bleaching (Ff), we determined the mobile fraction = (Ff - F0)/(F0 - Ft). This was important to determine, since the mobile fraction may be affected by differences in interactions of the wild type and the mutant ePKCs with other proteins and membranes. We found that the mobile fraction was the same for all ePKCs (63 ± 2% for Wt, 63 ± 4 for D86A and 64 ± 2.5% for D86N, averages of at least 10 cells/each). Therefore, differences in translocation rates were not due to differences in mobility of the inactive enzyme, but rather, to modulation of the intramolecular interaction between the ωRACK and RACK-binding site.

**DISCUSSION**

A key component in signal transduction is the inherent mechanism by which the enzymes remain inactive in the absence of extracellular stimuli. This mechanism involves the use of intramolecular interactions that stabilize a closed conformation with unexposed active site. Upon stimulation, the enzyme adopts an open conformation whereby intramolecular interactions are interrupted and binding sites for intermolecular interactions that stabilize the open form are exposed, resulting in a catalytically active enzyme. In the case of PKC, these intramolecular interaction sites are the binding sites for phospholipids and anchoring proteins (27, 28). We have previously suggested that one intramolecular interaction in ePKC that maintains the enzyme in a closed form is between an eRACK-binding site and a ωRACK site (17). Here, we demonstrated that alterations in this intramolecular interaction affected the translocation rate of the enzyme, further supporting a role of this intramolecular interaction in ePKC translocation and signaling.

As noted earlier, the ωRACK sequence in ePKC is ~25% different from the sequence in eRACK we suggested that the charge change (Asn-Asp) contributed to the difference in strength of the intramolecular interaction within ePKC as compared with the intermolecular interaction between ePKC and its RACK. eRACK (16, 17). By mutating the Asp-86 in the ωRACK sequence of ePKC to an Asn, we have created an enzyme that translocates slower than the wild-type enzyme, presumably because we have increased the intramolecular interaction between the ωRACK and the RACK-binding site in ePKC. Mutating Asp-86 to an Ala in ePKC abolished the intramolecular interaction between the ωRACK and the RACK-binding site, and resulted in an enzyme that translocated at a faster rate than the wild-type enzyme.

**Mutations in the ωRACK Site in ePKC Affect Intrinsic Properties of ePKC—**We used three criteria to demonstrate the role of the ωRACK site in the intramolecular interaction within ePKC and the role of Asp-86 in this interaction. The first criterion was the sensitivity of the enzyme to proteases; an open enzyme should be more susceptible to protease degradation after activation (19). We showed here that the D86N mutant was more resistant to proteolysis; D86N mutant required twice the time for the same extent of degradation of either the D86A or wild-type enzymes (Fig. 1). Therefore, D86N mutant is a more closed or inactive enzyme. Because sensitivity to proteolysis of the A mutant was the same as wild-type Asp-86 ePKC, we could not determine whether it was conformationally different from the wild-type enzyme using this method.

The second criterion examined PL-dependent binding of wild-type and mutant enzymes to eRACK. If Asp-86 is critical for an intramolecular interaction, we predicted that D86A would be less dependent on lipid activation for eRACK binding. Indeed, the single amino acid substitution modulated the intramolecular interaction between the ωRACK and the eRACK-binding site; the D86N mutant had a greater similarity to the eRACK sequence, and a reduced ability to bind to its eRACK, indicating that the D86N mutant is in a more closed conformation. In contrast, the D86A ePKC mutant was less dependent on activators for RACK binding, indicating that it is in a more open conformation.

**Mutations in the ωRACK Site and Translocation Rates of ePKC in Cells—**A third criterion demonstrating a critical role of the ωRACK site in intramolecular interactions examined the rate of translocation of the enzyme upon activation in cells. The D86A ePKC mutant translocated significantly faster than either D86N or wild-type ePKCs, as measured by cell fractionation studies. Using real-time confocal microscopy we demonstrated that the D86A mutant translocated at a faster rate than wild-type ePKC, which in turn translocated faster than the D86N mutant. Together, it appears likely that the ωRACK site mediates a critical intramolecular interaction that stabilizes the closed conformation in ePKC in the absence of stimulation.

A scheme for the mechanism of translocation of the different ePKC mutants is in Fig. 6. Mathematical modeling of our data
further elucidated the molecular events leading to translocation (see Fig. 4). Using non-linear regression analysis, an equation with two exponents gave a better fit than a single exponential equation, indicating that PMA-induced translocation involves at least two steps (Figs. 3 and 4). We proposed that the first step represents the opening and closing processes of the enzyme and the second step represents binding of the open enzyme to the cell membrane. Importantly, the steady state level of the D86N mutant was higher than that of either D86A or of wild-type ePKC, indicating that the amount of D86N that reached the cell periphery was lower than the amount of either wild-type or D86A ePKCs. The D86A mutant translocated significantly faster than either the D86N or wild-type ePKCs. Since the steady state level of D86A in the particulate fraction was similar to the steady state level of the wild-type enzyme, and the second step of translocation (binding to the membrane) was the same for all mutants, the rate of closing of an enzyme, once it was opened, could be considered negligible. Ochoa et al. (29) demonstrated that the binding of the V1 domain to PL is not altered by D86A mutation, supporting our hypothesis that the second step of translocation (binding to the membrane) is not altered. However, for the N mutant, the $k_i$ (rate of ePKC opening) was slower than that of D86A and similar to wild-type ePKC, and the $k_{-1}$ (rate of ePKC closing) was no longer negligible.

Similar to Shirai et al. (30), we found that ATP-induced translocation of ePKC was much faster than PMA-induced translocation (Figs. 5B versus 3). Since ATP-mediated translocation was a fast process, differences between the wild type and D86A mutants were not observed at the time intervals analyzed. However, the translocation of the D86N mutant was significantly slower than either the wild type or N mutant, further supporting the importance of the $\psi$RACK site and the disruption of intramolecular interaction for PKC translocation.

Schaefer et al. (31, 32) suggested that differences in translocation between classical and novel PKCs are due to differences in diffusion rates, and collision efficiencies with the membrane. Although diffusion and collision with the membrane are likely factors in the translocation rate, our data demonstrate that conformational changes in the enzyme also occur, leading to at least a two-step process.

There is evidence for a two-step process in the translocation of classical PKC isoforms. Nalefski and Newton (25) demonstrated that binding of $\beta$PKC to the membrane is a two step process, in which one of the steps involves a conformational change (25). Bolsover et al. (33) recently demonstrated that $\alpha$PKC undergoes a calcium-dependent conformational change exposing the lipid-binding sites, which precedes membrane binding. In the case of the novel PKC (calcium-independent) isoforms, it is still not clear what triggers the opening of the enzyme. Here, we showed that disrupting the intramolecular interaction between the $\psi$RACK and $\alpha$PKC-binding site is a critical step in activation that precedes translocation and anchoring to the cell periphery. Whether this anchoring is mainly mediated by binding to membranes, whether it involves anchoring proteins, and whether binding to lipids precedes binding to proteins could not be determined in cells overexpressing ePKC. However, we have previously demonstrated that translocation of endogenous ePKC results in its co-localization with its $\alpha$RACK (34) and that disruption of binding to $\alpha$RACK in cells with a peptide corresponding to one of the RACK-binding sites in ePKC (eV1-1 peptide) inhibits ePKC translocation and co-localization with $\alpha$RACK (35, 36). Importantly, we showed that a peptide corresponding to the $\psi$RACK sequence induces ePKC translocation and co-localization with $\alpha$RACK and triggers ePKC function (17).

Our data suggest that the process of ePKC translocation to the membrane that we followed in this study involves at least two steps and may occur independently of binding to RACK. An additional step involving RACK binding could not be detected in this system, since when the enzyme is overexpressed, as it is in this study, it is likely that the binding proteins, including RACKs, are no longer present in stoichiometric amounts relative to the enzyme (37). Indeed, the overexpressed wild-type ePKC and the endogenous $\alpha$PKC did not co-localize in the cells, even after activation with phorbol ester. In contrast, the endogenous ePKC co-localized with the $\alpha$RACK following activation in non-transfected cells (not shown). Because many attempts to co-express RACK with ePKC have failed, the translocation experiments in this study reflect mainly the interaction of the GFP enzyme with lipids in the cell membrane.

Where in ePKC is this RACK-binding site? The V1 domain of ePKC is homologous to the C2 domain of the $\beta$PKC (1). However, there is an additional RACK-binding site in the V5 region of $\beta$PKC (38) and molecular dynamics studies with the C2 region of $\beta$PKC showed that an intramolecular interaction between the $\psi$RACK and the RACK-binding site in the C2 region is not possible (39). Instead we suggest that the intramolecular interaction between the $\psi$RACK and the RACK-binding site in $\beta$PKC is likely to occur between the C2 and V5 regions in $\beta$PKC. This may also be the case for ePKC. Recently Stubbs and co-workers (40) have demonstrated that in ePKC there is also an additional intramolecular interaction between the C1 and C2 domains that maintains the enzyme in its inactive state. In addition, they have suggested that ePKC forms dimers through an intermolecular interaction between the C1 and C2 domains, which we cannot reject the hypothesis that this is also the case for the $\alpha$RACK-binding site and the $\psi$RACK (40).

CONCLUSIONS

By mutating the $\psi$RACK site in the intact ePKC, we have demonstrated the importance of interrupting the intramolecular interaction between the $\psi$RACK and the RACK-binding site in the process of ePKC translocation. To our knowledge, this is the first time that a single charge change made outside of the ($\psi$-substrate) site or the catalytic site, or of calcium-binding sites evoked a change in translocation rate. Together, we conclude that disruption of the interaction between the $\psi$RACK and the RACK-binding site is a critical and rate-limiting step in PKC translocation.

Acknowledgments—We thank Dr. Oleg Jardetzky and Dr. Adrienne Gordon for critical review and discussions, Dr. Leon Chen for discussions during the conception of the project, and Bio Image for the $\alpha$PKC and $\alpha$RACK clones and CHO-Hir cell line.
