PKC plays a critical role in diseases such as cancer, stroke and cardiac ischemia, and participates in a variety of signal transduction pathways such as apoptosis, cell proliferation, and tumor suppression. Though much is known about PKC downstream signaling events, the mechanisms of regulation of PKC activation and subsequent translocation have not been elucidated. Protein-protein interactions regulate and determine the specificity of many cellular signaling events. Such a specific protein-protein interaction is described here between δPKC and annexin V.

We demonstrate, at physiologically relevant conditions, that a transient interaction between annexin V and δPKC occurs in cells after δPKC stimulation, but before δPKC translocates to the particulate fraction. Evidence of δPKC/annexin V binding is provided also by FRET and by in vitro binding studies. Dissociation of the δPKC/annexin V complex requires ATP and microtubule integrity. Furthermore, depletion of endogenous annexin V, but not annexin IV, with siRNA inhibits δPKC translocation following PKC stimulation. A rationally designed eight amino acid peptide, corresponding to the interaction site for δPKC on annexin V, inhibits δPKC translocation and δPKC-mediated function as evidenced by its protective effect in a model of myocardial infarction. Our data indicate that translocation of δPKC is not simply a diffusion-driven process, but is instead a multi-step event regulated by protein-protein interactions. We show that following cell activation, δPKC/annexin V binding is a transient and an essential step in the function of δPKC, thus identifying a new role for annexin V in PKC signaling and a new step in PKC activation.

Protein-protein interactions determine the specificity of many cellular signaling events. One set of such interactions is mediated by RACKs (Receptors for Activated C-Kinase) that localize different activated protein kinase C (PKC) isozymes to distinct subcellular sites and determine isozyme-specific roles by anchoring each active PKC next to its corresponding substrates (1). Other examples of anchoring proteins include AKAPs (2), and STICKs (3). The importance of anchoring for downstream signaling is well recognized (4,5).

PKC isozymes are usually found in the cell cytosol when inactive. Following the generation of the second messenger diacylglycerol, active PKCs translocate to the cell particulate fraction (6). Although the molecular basis of PKC translocation has not been elucidated, the critical role of PKC/RACK interaction in mediating specific PKC functions both in vitro and in vivo has been demonstrated using peptides designed to inhibit or increase PKC-RACK binding (7-9).

We previously found that both of the isozyme-specific RACKs identified to date (βIIIRACK and εRACK) share a short sequence of homology with their corresponding PKCs (10,11); (e.g., SVEIWD in βIIIPKC241-246 is homologous to SIKIWD in βIIIRACK255-260). The RACK homologous sequences in the corresponding PKCs were termed pseudoRACK sequences (ψRACKs) (8,10). Peptides corresponding to the ψRACK-site act as allosteric agonists by interfering with the auto-inhibitory intramolecular interaction between the ψRACK-site and the RACK-binding site within PKC, thus stabilizing a state in PKC in which the RACK-binding site is available for protein-protein interaction (8,12,13).

δPKC plays a critical role in diseases such as cancer (14), stroke and cardiac ischemia (9,15-
and participates in a variety of signal transduction pathways such as apoptosis (18,19), cell proliferation (20-22), and tumor suppression (23). Though much is known about its downstream signaling events, the process of \(\delta PKC\) translocation and activation and the proteins that regulate it have not been identified.

A potential insight into the \(\delta PKC\) translocation process came from the observation that the small molecule JTV-519 (also known as K201) has been shown to protect hearts from ischemia and reperfusion damage (24) and to modulate the translocation of \(\delta PKC\) (25). There is no evidence that JTV-519 binds to \(\delta PKC\) directly, and therefore it is a possibility that JTV-519 modulates \(\delta PKC\) translocation indirectly through a change in protein-protein interaction during the activation process of \(\delta PKC\). In a separate study, JTV-519 has been reported to modulate annexin V function (26,27). Although we do not have access to JTV-519 for this study, based on the observations described above, we hypothesized that annexin V plays a role in the \(\delta PKC\) translocation process.

Indirect evidence made this hypothesis plausible: members of the annexin family of proteins, like the PKC family, bind phospholipids, participate in a variety of similar cellular functions and co-localize with some PKC isozymes in cells (28-30). Members of the annexin family are also substrates of select PKC isozymes and their cellular activity can be regulated by this PKC-mediated phosphorylation (31-33). However, no direct evidence for a role of annexins in PKC function has been suggested. Here, we determined that annexin V interacts with \(\delta PKC\), and studied the consequences of this protein-protein interaction on \(\delta PKC\) translocation and function. Our results have led us to propose that transient \(\delta PKC/annexin V\) interaction is an essential step in \(\delta PKC\) translocation and function.

**EXPERIMENTAL PROCEDURES**

*Materials:* Anti-MBP antibodies were obtained from New England Biolabs. Other antibodies, siRNA for annexins, and protein-G agarose beads were obtained from Santa Cruz biotechnology. Secondary HRP-conjugated antibodies were purchased from Amersham. pAnxV and pEAnxV peptides were synthesized and conjugated by a Cys S-S bond to TAT_{57-57} (34) by SynPep.

*Sequence alignments:* Sequences of the human annexin family members (accession numbers: P04083,NP_001002858, P12429, P09525,P08758, P08133, P20073, P13928, O76027, NP_665876, P50995, P27216) and various annexin V species homologs (accession number: AAF25883, BAA07708, AAB60648, BAA11012, NP_00102670) were aligned using MULTALIN (35). \(\delta PKC/annexin V\) fragment alignment (accession numbers: NP_579841,NP_579841) was conducted with LALIGN (36).

*Protein purification:* Annexin V, a kind gift from Joel Ernst, was purified from E. coli as described (37). Briefly, transformed E. coli were grown to OD 0.8, then induced with 0.5mM IPTG for 4 hours, pellets lysed in homogenization buffer (20mM Tris pH 7.4, 10mM EGTA, 2mM EDTA, 12mM \(\beta\)-mercaptoethanol, protease inhibitor cocktail (Sigma)). The bacterial lysate was incubated with a liposome column (phospholipids were prepared as described (38), then conjugated to activated HZ beads (BioRad) according to manufacturer’s instructions. After washing with 20mM Tris pH 7.4, annexin V was eluted in wash buffer containing 10\(\mu\)M CaCl\(_2\). MBP-\(\delta V1\), MBP-\(\epsilon V1\) and GST-\(\delta V5\) lysates were induced as described for annexin V, and purified using amylose (New England Biolabs) and glutathione (Amersham) beads, respectively. A partial PKC purification was conducted as described (38).

*ELISA:* 0.1\(\mu\)g of pure annexin V was bound to an ELISA plate in carbonate buffer at 4°C overnight, and blocked with 1\% BSA. Bacterial lysate expressing \(\delta PKC\) was incubated in 100mM HEPES (pH 7.4) in the presence of 10\(\mu\)M CaCl\(_2\) for 1h at 37°C with 0.5\(\mu\)M peptides where appropriate, and washed with 100mM HEPES. Amount of \(\delta PKC\) bound was then determined with anti-\(\delta PKC\) antibodies, and followed with a secondary antibody conjugated to alkaline phosphatase, and developed for 3h using PNPP (Pierce).

*Overlay:* All bacterial lysates were prepared as describe above. Binding of \(\delta\) and \(\epsilon PKC\) fragments and full-length enzyme to annexin V was determined as described (39). Briefly, annexin V bacterial lysate was chromatographed on 12% SDS-PAGE, transferred to nitrocellulose, and
incubated with recombinant PKC isoforms or fragments in the presence of 10µM Ca2+ and phospholipids where necessary. After washing as described (39), the strips were probed with anti-MBP, anti-GST, or anti-PKC antibodies and visualized by ECL. 

Co-Immunoprecipitation of δPKC and annexin V complex: CHO-K1 cells (ATCC) were grown in F-12 (HAM) nutrient mixture supplemented with Glutamax, 10% fetal bovine serum, and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin sulfate, all from Invitrogen). Cells were cultured at 37 °C 5% CO2. 48 hours prior to experiments, cells were serum starved. They were stimulated with 10nM phorbol 12-myristate 13-acetate (PMA) (LC Laboratories) or with 5mM H2O2 (Sigma) for the indicated duration, as described by Konishi et al. (40). Cells were washed with cold PBS, and homogenized on ice with trituration in homogenization buffer (20mM Tris pH 7.4, 2mM EDTA, 10mM EGTA, 0.25M sucrose, 12mM β-mercaptoethanol, 0.1% Triton X-100, protease inhibitor cocktail (Sigma), and 1% formaldehyde). We found that 0.1% Triton extracts all the translocatable PKC from the cell. After 30 min at 4°C, the lysates were quenched with 0.14M glycine for 20min at 4°C, and samples spun at 14K rpm at 4°C.  The supernatant was incubated with 1µg anti-δPKC antibody (Santa Cruz) for 1hour, followed by protein-G beads (Invitrogen) for 3 hours at 4°C. The beads were then washed with wash buffer (20mM Tris pH 7.5, 2mM EDTA, 100mM NaCl, 12mM β-mercaptoethanol, 0.1% Triton), and separated on 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with anti-annexin V (Santa Cruz), followed by visualization with ECL.

Translocation of δPKC: Cells were fractionated as described in (41). Briefly, after stimulation, cells were washed with cold PBS, scraped in homogenization buffer as described above (but without crosslinker), and spun at 100,000g for 30 min at 4°C, resulting in the soluble fraction. The pellet was then resuspended in homogenization buffer with 1% Triton X-100, and spun under the same conditions. Where applicable, the cells were pre-incubated with 1µM peptide for 15min prior to stimulation. The samples were then analyzed by Western blot, and loading corrected based on protein concentration, using an internal control such as actin or by Bradford.

siRNA knockdown of annexin protein level: 40% confluent CHO (as described above) or HeLa cells (ATCC), maintained in DMEM supplemented with 10% fetal bovine serum, and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin sulfate, Invitrogen). Cells were transfected using GeneSilencer (Gene Therapy Systems, Inc) according to manufacturer’s instructions with 20nM siRNA for annexin family members (Santa Cruz Biotechnology). 24 hours post-transfection, cells were serum starved for an additional 24 hours. CHO cells were then stimulated with 10nM PMA and HeLa cells with 100µM UDP (Sigma) for the indicated times and fractionated as described above.

Microscopy and Analysis—CHO cells were grown on chambered #1 Borosilicate coverglass (Lab-Tek), transfected at 50% confluence using Fugene 6 (Roche) according to manufacturer’s instructions with CFP-annexin V and YFP-δPKC cloned into the pECFP-C1 and pEYFP-C1 vectors, respectively (Clontech), and serum starved for 24 hours. Real-time confocal imaging was conducted on a spinning disk Nipkow confocal microscope. Cells were viewed using an inverted Olympus IX70 microscope with a 40x oil immersion Olympus objective (1.35 NA) and images were acquired with a CCD camera (Hamamatsu) and 2X2 pixel binning. CFP was excited with the 442 nm laser line of a helium-cadmium laser (Kimmon), whereas YFP was imaged with the 514 nm line of an argon ion laser (Melles-Griot). Images were acquired at 27°C every 5-10sec for 10 min. 100nM PMA was added to the cell chamber after the 10th image in each time series. Where indicated, the cells were pretreated with nocodazole (10µM, 30min). Exposure time was adjusted for photobleaching levels lower than 10%. Fluorescence intensity was measured using Metamorph® data analysis software (Universal Imaging). To monitor the translocation of PKC and increases in FRET signal, a small region of interest was selected from each cell and fluorescence intensity values graphed against time and normalized to the initial fluorescence. FRET was calculated using the formula that corrects for bleed-through and donor concentration as previously described (42):
FRET=(I(442-530)-αI(442-480)-βI(515-530))/I(442-480). α and β coefficients were calculated from singly transfected cells, where α=I(442-530)/I(442-480) =47.6% of CFP bleed-through into the YFP channel, and β=I(442-530)/I(515-530)=14.4% of YFP excitation from the CFP channel.

Ex vivo model of ischemia and reperfusion: Wistar rats (300 to 350 g) were heparinized (2000 U/kg IP) and then anesthetized with sodium pentobarbital (100 mg/kg IP). The hearts were rapidly excised and then perfused with an oxygenated Krebs-Henseleit solution containing (in mmol/L) NaCl 120, KCl 5.8, NaHCO₃ 25, NaH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 1.0, and dextrose 10, pH 7.4, at 37°C in a Langendorff coronary perfusion system (43). The coronary flow rate was kept constant during the experiment at 10 mL/min. Hearts were submerged into a heat-jacketed organ bath set at 37°C. Coronary effluent was collected to determine creatine phosphokinase (CPK) release from necrotic cells. After 10 minutes of equilibration, the hearts were subjected to a 30-minute global ischemia and a 60-minute reperfusion to measure ischemic damage and CPK release during reperfusion. The hearts were perfused with 1µM TAT 47-57 carrier peptide conjugated to pAnxV, TAT47-57 peptide conjugated to EpAnxV, TAT47-57 peptide alone, or vehicle for either 10 minutes prior to ischemia and/or during the first 10 minutes of reperfusion.

Infarct size measurement: At the end of the reperfusion period, hearts were sliced into 1-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4) at 37°C for 15 minutes, as reported previously (44). Infarct size was expressed as a percentage of the total LV muscle mass.

Statistical Analysis: For quantitative analysis, autoradiographs were scanned and quantified using NIH Image software. Statistical significance for all analyses was calculated using 2-tail type 2 Student’s t test (Microsoft Excel).

RESULTS
A short homologous sequence indicative of protein-protein interaction is found in annexin V and δPKC.

As mentioned above, PKCs and their corresponding PKC-binding proteins, RACKs, share a short sequence of homology [6-8 amino acids long (8)]. The RACK-like sequence on the PKC was termed ψRACK, as it participates in an inhibitory intramolecular interaction with the RACK binding site on the PKC. This intramolecular interaction is broken upon PKC activation, allowing PKC binding to its RACK. We reasoned that if annexin V binds selectively to δPKC during its activation process, annexin V might also have a short δPKC homologous sequence. Using LALIGN (36), we searched for a short homology between annexin V and δPKC. We focused on the V1/C2 domain of δPKC (amino acids 1-123), because that domain is critical for δPKC anchoring and mediation of many critical intracellular processes (45-47). A portion of the highest ranked region of homology from the LALIGN output features the characteristic charge difference (glutamate, E₇₈ in δPKC to arginine, R₁₆₁ in annexin V) (red in Fig 1A), which we have previously found to be indicative of a potential protein-protein interaction site for PKC (8,10). Importantly, as expected for a selective protein-protein interaction, the δPKC homologous sequence in annexin V is unique and not found in other members of the annexin family (Fig 1B), and is conserved among different species (Fig 1C).

Surprisingly, the annexin V-like homology sequence in δPKC is located within the previously identified ψδRACK sequence [δPKC₇₄-₈₁; (7)], further suggesting that that annexin V might be a δPKC-binding protein. In addition, the ψδRACK-like sequence in annexin V is in close proximity (3.2Å; Fig. 1E) to the binding site on annexin V for JTV-519 (27), a compound that regulates δPKC function (25), indicating the potential importance of the ψδRACK-like sequence on annexin V for δPKC binding. We therefore hypothesized that there is a specific protein-protein interaction between δPKC and annexin V that is mediated, at least in part, via the region of annexin V that is homologous between the two proteins.

δPKC binds annexin V, in vitro.

We first set out to determine whether δPKC binds annexin V in vitro, using immobilized annexin V and recombinant full-length δPKC as
well as the δV1 (the C2 domain of δPKC), δV5 and εV1 fragments. All these domains have been previously shown to participate in important protein-protein interactions (45,46,48). Domains V1/C2 and V5 of δPKC (Fig. 1F, red), as well as full-length δPKC bound to annexin V (Fig. 1G). In contrast, the V1 region of another member of the novel PKC isozymes, εPKC, did not bind to annexin V (Fig. 1G), indicating selectivity of this interaction. Phosphatidylserine and diacylglycerol micelles, to which both annexin V and δPKC can bind independently, did not affect δPKC/annexin V association in vitro (data not shown), suggesting that annexin V binding to δPKC does not depend on a lipid bridge between these two lipid-binding proteins (49).

**Association of δPKC/annexin V precedes δPKC translocation, in cells.**

We next determined whether, in the absence of over-expression of any of the proteins in question, δPKC and annexin V interact in cells. The complexes formed with δPKC were then immunoprecipitated with anti-δPKC antibodies and probed for the presence of annexin V at the combined molecular weight of ~110kD. Stimulation of CHO cells with phorbol 12-myristate 13-acetate (PMA, 10nM) caused about a 4-fold increase in the amount of annexin V/δPKC complex within 0.5 minute of treatment (Fig. 2A, left). The interaction was short-lived, and after 1 min of stimulation the amount of annexin V/δPKC complex was reduced often below the levels obtained prior to PMA treatment. Transient association between annexin V and δPKC was also observed following activation of δPKC with H2O2 (5mM, 0.5-1 min treatment, Fig. 2A, right). Therefore, annexin V/δPKC interaction occurs early after cell stimulation, regardless of the means used to stimulate δPKC.

Activation of PKC results in its translocation from the cell soluble to the cell particulate fraction (6). We therefore determined the timing of annexin V/δPKC complex formation relative to the timing of δPKC translocation to the particulate fraction. While maximum annexin V/δPKC complex accumulation was found between 0.5-1min (Fig 2A, C), translocation of δPKC from the soluble to the cell particulate fraction occurred later; it began only after 1min of PMA stimulation and reached maximum after 5 minutes of stimulation (Fig. 2B, C). Based on the above observations, we concluded that formation of the δPKC/annexin V complex preceded δPKC translocation from the cytosol to the cell particulate fraction.

**ATP and microtubules are important for δPKC/annexin V complex disassociation.**

Because of the fast and transient PMA-induced association/dissociation of δPKC with annexin V, we next determined whether δPKC/annexin V complex formation requires energy. Whereas in native conditions (37°C), the disassociation of the complex was completed by 1 min of treatment with 10nM PMA (Fig. 2D left top panel), substantial amounts of δPKC in complex with annexin V were found even after 5 min of PMA stimulation, when the cells were treated with 2-deoxyglucose to deplete cellular ATP (50) (Fig. 2D, bottom left). ATP might be required for enzymatically-mediated disassociation of the complex or the actual movement of the complex to its site of disassociation, but determination of the exact role of ATP was beyond the scope of this study. As annexins are well known to bind to actin cytoskeleton and to affect the dynamics of actin fibers (51), we tested different cytoskeleton perturbation agents. Whereas disrupting actin elements with cytochalasin D did not affect kinetics of the δPKC/annexin V complex (Fig 2D, top right), pretreatment with the microtubule-destabilizing agent, nocodazole, greatly delayed complex dissociation (Fig. 2D bottom right). Based on this observation, we tested the role of microtubules in δPKC translocation, and found that nocodazole blocks PMA-induced translocation of δPKC (Fig. 2E), thus confirming the importance of microtubules in δPKC translocation process. Together, our data suggest that an initial step of δPKC translocation to the cell particulate fraction involves its association with annexin V, and that dissociation of the complex is an energy-dependent process that requires intact microtubule filaments, but is independent of actin polymerization.
δPKC/annexin V interaction as observed by FRET

Both δPKC and annexin V are cytosolic proteins in inactive cells, and co-localization studies by immuno-histochemistry can be misleading, as they may be interpreted to show that the two proteins interact in unstimulated cells. Therefore, real-time fluorescent imaging was conducted with CFP-annexin V and YFP-δPKC, and FRET was measured upon stimulation of CHO cells with PMA. Whereas annexin V remains cytosolic throughout the experiment (Fig. 3A, top panel), δPKC translocation from the cytosol to the membrane can be observed starting from 2 minutes of stimulation and reaches its maximal translocation by 9 min (Fig. 3A, middle panel). FRET signal increased predominantly in the cytosolic region of the stimulated cells (Fig. 3A, bottom panel, quantitated in Fig. 3B). The basal FRET level at the cell membrane was higher than found in the cytosol, but did not increase after cell stimulation. Interestingly, the timecourse of FRET increase followed the timecourse of δPKC translocation (Fig. 3C). Importantly, the fold increase of the FRET signal was comparable to the fold increase of complex association as seen in the co-immunoprecipitation study (Fig. 2B). It is important to note that while physiological expression of annexin V and δPKC resulted in transient interaction upon stimulation (Fig 2), the overexpressed proteins interacted throughout the presence of stimulus, suggesting impairment of the proper translocation machinery. In fact, pretreatment of cells overexpressing δPKC with nocodazol did not inhibit δPKC translocation, unlike that seen in the non-overexpressing cells (Fig 2E vs Fig 3C). These results suggest that while δPKC and annexin V interact in the overexpressing conditions upon δPKC stimulation, the cellular machinery involved in translocation and anchoring may be limited in its amounts and only functions properly when the proteins are present at endogenous levels.

A peptide designed to block δPKC/annexin V complex formation blocks δPKC translocation.

Because δPKC/annexin V binding occurs prior to accumulation of δPKC at the particulate fraction (Fig 2C), we tested the hypothesis that annexin V is required for δPKC translocation by designing a peptide to specifically block this interaction. We have previously shown that peptides derived from the PKC-binding proteins inhibit PKC translocation and function (10,52). Therefore, we tested whether a peptide corresponding to the δPKC homology site on annexin V will inhibit δPKC translocation and function. We have extended the length of the annexin-derived peptide (AnxV157-164) by one amino acid at each side, to correspond to the length of the ΨδRACK peptide (7) (Fig. 1A) and to prevent potential effects of the free carboxyl and amino groups at the ends of the peptide.] The annexin V-derived peptide was synthesized and conjugated to TAT47-57 carrier to enable intracellular delivery of the peptide across biological membranes (34). If annexin V interaction with δPKC is required for δPKC translocation, we predicted that the annexin V-derived peptide (pAnxV) should block stimulation-induced translocation of δPKC by binding to δPKC and preventing its association to annexin V. When used in CHO cells, pretreatment with 1μM pAnxV blocked PMA-induced translocation of δPKC to the particulate fraction (Fig. 4A; measured after 5 minutes of PMA stimulation). As discussed earlier, there is a charge difference in the homologous sequence between annexin V and δPKC (R161 to E78, respectively; see Fig. 1A), similar to that observed in all the homologous sequences between PKCs and their binding proteins (8). As expected, when the arginine in annexin V was replaced with the glutamate found in δPKC (EpAnxV), the peptide lost its inhibitory effect on δPKC translocation (Fig. 4A, left panel). Importantly, neither peptide affected PMA-induced translocation of εPKC (Fig. 4A, right panel), indicating the selectivity of the annexin V-derived peptide effect on δPKC translocation. The pAnxV peptide was also tested in the co-immunoprecipitation studies as described in Fig 2A, and was seen to block δPKC/annexin V complex formation (Fig. 4B). In addition, pAnxV inhibited δPKC/annexin V binding in an ELISA assay, whereas EpAnxV did not affect it significantly (Fig. 4C). Together, these data suggest that pAnxV peptide corresponds to at least a part of the δPKC-binding site on annexin V, and that annexin V/δPKC interaction is required for
stimulation-induced δPKC translocation from the cell soluble to the cell particulate fraction.

**Annexin V knockdown prevents δPKC translocation.**

To further demonstrate that the interaction of δPKC with annexin V is necessary for δPKC translocation to the particulate fraction, we used an alternative approach; annexin V was knocked down using a pool of siRNA (Santa Cruz). When annexin V levels were reduced by ~80%, PMA-induced translocation of δPKC was similarly inhibited, whereas εPKC translocation was unaffected (Fig. 5A). We confirmed that the same selective inhibition of δPKC translocation by annexin V knockdown is observed when cells are treated with a more physiologically relevant hormone stimulation. Using HeLa cells, the purinergic receptor, P2Y, was stimulated by UDP and the role of annexin V in δPKC translocation was determined. Treatment with siRNA of these cells reduced annexin V levels by 80% without affecting the levels of other annexins (e.g. annexin IV, Fig. 5B). Importantly, δPKC translocation was lost in the annexin V siRNA-treated cells, but not in the annexin IV siRNA-treated cells (Fig. 5C). Therefore, the presence of annexin V in cells is specifically required for activation-induced translocation of δPKC to the particulate fraction in at least two cell types and as determined by at least two modes of PKC activation.

**δPKC/annexin V complex formation is required for δPKC function.**

We next determined if δPKC/annexin V complex formation is required for δPKC-mediated function, in vivo. We previously found that, in models of cardiac ischemia, inhibition of δPKC translocation with δV1-1 inhibits cardiac damage (9,44). Since we suggest that interaction of δPKC with annexin prior to translocation is essential in the process of translocation, we hypothesized that the annexin V-derived peptide would also be cardioprotective when administered prior to δPKC activation (Fig 6A). When hearts were treated with the annexin V-derived peptide, pAnxV, prior to ischemia, there was a 70% reduction in infarct size (Fig 6 B,C) and ~50% reduction in cell necrosis as measured by the leakage of the cardiac enzyme, creatine phosphokinase (CPK), into the heart perfusate (Fig. 6D). The extent of protection was similar to the effect observed with the δPKC/RACK interaction inhibitor, δV1-1, in the same model (7,9). Note that EpAnxV, which did not inhibit δPKC translocation (Fig. 3A), also did not inhibit ischemia-induced δPKC-mediated damage to the heart (Fig. 6B,C,D). These data indicate that δPKC-annexin V interaction is an essential step in δPKC function, and confirm the importance of δPKC-annexin V complex formation as an initial step for δPKC function in vivo.

**DISCUSSION**

In this study, we provide evidence that translocation of δPKC to the cell particulate fraction is not simply a diffusion-driven process. Our study provides evidence that δPKC translocation is a complex process involving selective and transient binding of the activated isozyme to annexin V. We found that δPKC/annexin V interaction precedes δPKC translocation to the cell particulate fraction and that microtubule integrity and energy are required for the dissociation of the complex. We showed that δPKC binding to annexin V is an essential step in δPKC translocation; annexin V knockdown with siRNA selectively inhibited stimulation-induced δPKC translocation, but not translocation of other PKC isozymes. In addition, a peptide, derived from annexin V and corresponding to the interaction site of δPKC on annexin V, acted as a selective inhibitor of δPKC translocation. This peptide also selectively inhibited δPKC function in vivo; treating hearts with the annexin-derived peptide prior to ischemic insult inhibited δPKC-mediated cardiac damage. Together, our data demonstrate that δPKC/annexin V interaction precedes PKC translocation, and is an essential step in the function of δPKC, thus modifying the paradigm for PKC activation (Fig. 7). The exact role of annexin V in δPKC translocation is still not clear, but this work suggests that annexin V may serve as a shuttle protein on microtubules, moving δPKC to its subcellular destination. Since no direct interaction of annexins has been reported with microtubules, we hypothesize that this movement might be facilitated through annexin V-mediated membrane vesicle binding that is transported on
the microtubules. The suggestion that microtubules transport activated PKC to the particulate fraction appears plausible in view of data that translocation of another PKC isozyme, αPKC, is also dependent on microtubule integrity (53). As annexin V translocates to membranes upon increase in intracellular calcium levels (54,55), annexin V may act as an early sensor in the δPKC activation cascade, thus initiating δPKC translocation and activation (Fig. 7). Though the RACK for δPKC has yet to be identified, unique δPKC signaling suggests the presence of an isozyme-specific anchoring protein (δRACK) that determines proper subcellular location and access to specific substrate for phosphorylation. However, the details of the final steps of the translocation process for δPKC remain to be elucidated.

There are multiple reports that PKC translocation is independent of cytoskeletal elements (56,57). However, most of this work was conducted with over-expressed PKCs. Here we demonstrate that over-expressed δPKC no longer requires microtubules for translocation to the cellular membrane, thus emphasizing the importance of studies at endogenous levels for identification of physiologically relevant cellular mechanisms. In addition, unlike the endogenous δPKC-annexin V association, the interaction of the over-expressed δPKC and annexin V was not transient, likely due to the fact that the levels of these proteins exceed the endogenous “final” δPKC binding partner, the δRACK. This observation is not surprising. We have observed that over-expressed activated GFP-εPKC is not localized to the same cell compartment as endogenous εPKC (unpublished data), and Ping and collaborators showed that when εPKC is over-expressed, it binds not only to εRACK following activation, but also to the βIIRACK (58). Together, these discrepancies emphasize the importance of studies at the endogenous levels when PKC activation mechanisms are investigated.

It was previously suggested that PMA is not necessarily sufficient to induce proper translocation of PKCs. For example, Vallentin et al. (59) showed that PMA did not include translocation of αPKC in single, isolated cells, whereas it caused translocation to cell-cell contacts when cells were in a cluster. This result is an example that PMA is an initiator of PKC activation, but does not itself determine translocation of PKC to the proper physiological locations. Though the mechanism underlying this observation is still not fully understood, our work adds to the hypothesis that additional steps involving annexin V, energy and microtubules are required for δPKC translocation.

The effect of annexin V-derived peptide described above merits further discussion. It represents an example for rational design of a pharmacological agent with interesting therapeutic potential, which we show here to prevent ischemic damage to hearts in a model of myocardial infarction. The annexin V-derived peptide was identified by a simple sequence homology search between two unrelated gene products, δPKC and annexin V, that we suspected and now confirmed, to interact in cells in a stimulation-dependent manner. The homology between the two proteins was on a short stretch of six consecutive amino acids (Fig. 1A) and was rather limited: only three amino acids were identical and two other represent relatively conservative substitutions. However, one amino acid of these six represents a difference in charge – from glutamate in δPKC to arginine in annexin V (Fig. 1A). It is because of this charge change that we focused our attention on this sequence; we have previously found that sequence homologies between two other PKC-binding proteins and their corresponding PKCs have such a charge change (8). We suggested that the charge present on the PKC-binding protein, in this case arginine₁₆₁ in annexin V, is critical for a high affinity interaction with δPKC (13). As predicted, when a single amino acid substitution in the annexin V-derived peptide (pAnxV) from arginine to glutamate was made, the new peptide (EpAnxV) was inactive as a δPKC inhibitor (Figs. 4,6). Therefore, this study demonstrates a potential method to rationally identify sites of protein-protein interactions without extensive mutagenesis analysis or structural analysis of the complex. Furthermore, this method provides an easy and fast approach to make pharmacological agents to determine the role of protein-protein interactions of interest.

A role for annexins in PKC activation may be a common theme in PKC signaling. Previous
reports suggest that individual PKC isozymes interact with unique members of the annexin family (e.g., βPKC/annexin I, εPKC/annexin II and αPKC/annexin VI) (30,52,60-62). It remains to be determined whether, as for δPKC and annexin V, the biological activity of other PKC isozymes requires a step of association with other members of the annexin family and whether association with these annexins also precedes translocation of the corresponding PKC isozymes. Regardless of whether the other annexins have a role in regulating PKC activation and function, our finding that the δPKC/annexin V complex is required for δPKC function alters the paradigm of δPKC activation process. This study further illustrates the importance of understanding protein-protein interactions in signal transduction for the development of potential pharmacological modulators.

REFERENCES

1. Mochly-Rosen, D. (1995) Science 268, 247-251
2. Scott, J. D. (1997) Soc Gen Physiol Ser 52, 227-239
3. Chapline, C., Cottom, J., Tobin, H., Hulmes, J., Crabb, J., and Jaken, S. (1998) J Biol Chem 273, 19482-19489
4. Pawson, T., and Scott, J. D. (1997) Science 278, 2075-2080
5. Moscat, J., and Diaz-Meco, M. T. (2000) EMBO Rep 1, 399-403
6. Kraft, A. S., and Anderson, W. B. (1983) Nature 301, 621-623
7. Chen, L., Hahn, H., Wu, G., Chen, C. H., Liron, T., Schechterman, D., Cavallaro, G., Banci, L., Guo, Y., Bolli, R., Dorn, G. W., 2nd, and Mochly-Rosen, D. (2001) Proc Natl Acad Sci U S A 98, 11114-11119
8. Souroujon, M. C., and Mochly-Rosen, D. (1998) Nat Biotechnol 16, 919-924
9. Inagaki, K., Chen, L., Ikeno, F., Lee, F. H., Imahashi, K., Bouley, D. M., Rezaee, M., Yock, P. G., Murphy, E., and Mochly-Rosen, D. (2003) Circulation 108, 2304-2307
10. Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Proc Natl Acad Sci U S A 91, 839-843
11. Mochly-Rosen, D., Wu, G., Hahn, H., Osinska, H., Liron, T., Lorenz, J. N., Yatani, A., Robbins, J., and Dorn, G. W., 2nd. (2000) Circ Res 86, 1173-1179
12. Dorn, G. W., 2nd, Souroujon, M. C., Liron, T., Chen, C. H., Gray, M. O., Zhou, H. Z., Csukai, M., Wu, G., Lorenz, J. N., and Mochly-Rosen, D. (1999) Proc Natl Acad Sci U S A 96, 12798-12803
13. Schechterman, D., Craske, M. L., Kheifets, V., Meyer, T., Schechterman, J., and Mochly-Rosen, D. (2004) J Biol Chem 279, 15831-15840
14. Weinstein, I. B. (1991) Princess Takamatsu Symp 22, 277-283
15. Miettinen, S., Roivainen, R., Keinanen, R., Hokfelt, T., and Koistinaho, J. (1996) J Neurosci 16, 6236-6245
16. Yoshida, K., Kawamura, S., Mizukami, Y., and Kitakaze, M. (1997) J Biochem (Tokyo) 122, 506-511
17. Bright, R., Raval, A. P., Dembner, J. M., Perez-Pinzon, M. A., Steinberg, G. K., Yenari, M. A., and Mochly-Rosen, D. (2004) J Neurosci 24, 6880-6888
18. Brodie, C., and Blumberg, P. M. (2003) Apoptosis 8, 19-27
19. Murriel, C. L., Churchill, E., Inagaki, K., Szweda, L. I., and Mochly-Rosen, D. (2004) J Biol Chem 279, 47985-47991
20. Li, W., Jiang, Y. X., Zhang, J., Soon, L., Flechner, L., Kapoor, V., Pierce, J. H., and Wang, L. H. (1998) *Mol Cell Biol* **18**, 5888-5898
21. Kiley, S. C., Clark, K. J., Duddy, S. K., Welch, D. R., and Jaken, S. (1999) *Oncogene* **18**, 6748-6757
22. Braun, M. U., and Mochly-Rosen, D. (2003) *J Mol Cell Cardiol* **35**, 895-903
23. Lu, Z., Hornia, A., Jiang, Y. W., Zang, Q., Ohno, S., and Foster, D. A. (1997) *Mol Cell Biol* **17**, 3418-3428
24. Inagaki, K., Kihara, Y., Izumi, T., and Sasayama, S. (2000) *Cardiovasc Drugs Ther* **14**, 489-495
25. Inagaki, K., Kihara, Y., Hayashida, W., Izumi, T., Iwanaga, Y., Yoneda, T., Takeuchi, Y., Suyama, K., Muso, E., and Sasayama, S. (2000) *Circulation* **101**, 797-804
26. Kaneko, N., Matsuda, R., Toda, M., and Shimamoto, K. (1997) *Biochim Biophys Acta* **1330**, 1-7
27. Kaneko, N., Ago, H., Matsuda, R., Inagaki, E., and Miyano, M. (1997) *J Mol Biol* **274**, 16-20
28. John, C., Cover, P., Solito, E., Morris, J., Christian, H., Flower, R., and Buckingham, J. (2002) *Endocrinology* **143**, 3060-3070
29. Xu, T. R., and Rumsby, M. G. (2004) *FEBS Lett* **570**, 20-24
30. Antonicelli, F., Omri, B., Breton, M. F., Martiny, L., Rothrut, B., Russo-Marie, F., Lambert, B., Pavlovic-Hournac, M., and Haye, B. (1990) *Reprod Nutr Dev* **30**, 297-307
31. Barnes, J. A., Michiel, D., and Holberg, M. D. (1991) *Biochem Cell Biol* **69**, 163-169
32. Stoehr, S. J., Smolen, J. E., and Suchard, S. J. (1990) *J Immunol* **144**, 3936-3945
33. Chen, L., Wright, L. R., Chen, C. H., Oliver, S. F., Wender, P. A., and Mochly-Rosen, D. (2001) *Chem Biol* **8**, 1123-1129
34. Corpet, F. (1988) *Nucleic Acids Res* **16**, 10881-10890
35. X. Huang, W. M. (1991) *Adv. Appl. Math.* **12**, 337-357
36. Burger, A., Berendes, R., Voges, D., Huber, R., and Demange, P. (1993) *FEBS Lett* **329**, 25-28
37. Schectman, D., Murriel, C., Bright, R., and Mochly-Rosen, D. (2003) *Methods Mol Biol* **233**, 351-357
38. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) *Proc Natl Acad Sci U S A* **94**, 11233-11237
39. Schectman, D., and Mochly-Rosen, D. (2002) *Methods Enzymol* **345**, 470-489
40. Zal, T., and Gascoigne, N. R. (2004) *Biophys J* **86**, 3923-3939
41. Hondeghem, L. M., and Cotner, C. L. (1978) *Am J Physiol* **235**, H574-580
42. Inagaki, K., Hahn, H. S., Dorn, G. W., 2nd, and Mochly-Rosen, D. (2003) *Circulation* **108**, 869-875
43. Johnson, J. A., Gray, M. O., Chen, C. H., and Mochly-Rosen, D. (1996) *J Biol Chem* **271**, 24962-24966
44. Mochly-Rosen, D., Miller, K. G., Scheller, R. H., Khaner, H., Lopez, J., and Smith, B. L. (1992) *Biochemistry* **31**, 8120-8124
45. Nalefski, E. A., and Falke, J. J. (1996) *Protein Sci* **5**, 2375-2390
48. Stebbins, E. G., and Mochly-Rosen, D. (2001) J Biol Chem 276, 29644-29650
49. Mochly-Rosen, D., Khaner, H., and Lopez, J. (1991) Proc Natl Acad Sci U S A 88, 3997-4000
50. Kondo, T., and Beutler, E. (1979) J Lab Clin Med 94, 617-623
51. Hayes, M. J., Rescher, U., Gerke, V., and Moss, S. E. (2004) Traffic 5, 571-576
52. Mochly-Rosen, D., Khaner, H., Lopez, J., and Smith, B. L. (1991) J Biol Chem 266, 14866-14868
53. Battistella-Patterson, A. S., Fultz, M. E., Li, C., Geng, W., Norton, M., and Wright, G. L. (2000) Acta Physiol Scand 170, 87-97
54. Moss, S. E., and Morgan, R. O. (2004) Genome Biol 5, 219
55. Gerke, V., Creutz, C. E., and Moss, S. E. (2005) Nat Rev Mol Cell Biol 6, 449-461
56. Schaefer, M., Albrecht, N., Hofmann, T., Rudermann, T., and Schultz, G. (2001) Faseb J 15, 1634-1636
57. Shirai, Y., Sakai, N., and Saito, N. (1998) Jpn J Pharmacol 78, 411-417
58. Pass, J. M., Gao, J., Jones, W. K., Wead, W. B., Wu, X., Zhang, J., Baines, C. P., Bolli, R., Zheng, Y. T., Joshua, I. G., and Ping, P. (2001) Am J Physiol Heart Circ Physiol 281, H2500-2510
59. Vallentin, A., Prevostel, C., Fauquier, T., Bonnefont, X., and Joubert, D. (2000) J Biol Chem 275, 6014-6021
60. Ron, D., and Mochly-Rosen, D. (1994) J Biol Chem 269, 21395-21398
61. Schmitz-Peiffer, C., Browne, C. L., Walker, J. H., and Biden, T. J. (1998) Biochem J 330 (Pt 2), 675-681
62. Orito, A., Kumanogoh, H., Yasaka, K., Sokawa, J., Hidaka, H., Sokawa, Y., and Maekawa, S. (2001) J Neurosci Res 64, 235-241

FOOTNOTES

This research was supported by an NIH grant HL52141. The authors greatly appreciate important discussions and insight provided by Dr. Adrienne Gordon and Dr. Yasuki Kihara, and the FRET expertise provided by Dr. Tobias Meyer and Dr. Marc Fivaz. The authors also thank Melissa Wong and Philip Vitorino for assistance with the binding studies. Dr. Mochly-Rosen is the founder of KAI Pharmaceuticals, Inc, a company that plans to bring PKC regulators to the clinic. However, none of the work described here is based on or supported by the company.

Abbreviations used: PKC: protein kinase C; RACK: receptor for activated C kinase; PMA: phorbol 12-myristate 13-acetate; pAnxV: peptide designed from annexin V sequence; siRNA: small interfering RNA; CHO: Chinese hamster ovary cells.

FIGURE LEGENDS

Fig 1. Selective binding of δPKC to annexin V, in vitro.
A. Sequence similarity between annexin V and δPKC sequences, found within the ψδRACK sequence ((7) blue box), with an amino-acid charge difference characteristic of a PKC-RACK relationship (highlighted in red).
B. The ψδRACK-like sequence in annexin V is not found in other members of the annexin family. In the “consensus” bottom line, + indicates no homology, capital letters indicate >90% identity, lower case letters indicate >50% identity.
C. Alignment of the ψδRACK-like region in rat annexin V with annexin V from other species was obtained as in B. In the “consensus” bottom line, + indicates no homology, capital letters indicate >90% identity, lower case letters indicate >50% identity.

D. Structure of annexin V with the ψδRACK-like site highlighted in light blue and JTV-519 in yellow, shown in stick representation (27).

E. Closer view of the boxed region in D (rotated slightly, relative to the image in D). JTV-519 binds adjacent to the ψδRACK-like site on annexin V.

F. Schematic of δPKC domains structure. δPKC is a member of the calcium independent novel family of PKC isozymes. The V1 and the V5 domains (in red) have been reported to participate in protein-protein interactions.

G. Recombinant δPKC domains, MBP-δV1 and GST-δV5, bind to annexin V; partially purified rat brain δPKC full-length, but not εPKC, binds to annexin V. Western blots were probed for the respective PKC isozymes. A representative figure of four independent experiments.

Fig 2. Binding of δPKC and annexin V in cells.

A. A representative blot of co-immunoprecipitation of δPKC/annexin V complex with anti-δPKC antibodies from cross-linked CHO cell lysates, probed with anti-annexin V and then with anti-δPKC. Molecular weight of the complex corresponds to ~110kD, the sum of 73kD δPKC and 35kD annexin V. Comparable amounts of δPKC are immunoprecipitated in each condition. Maximum complex formation is observed at 0.5-1 min of 10nM PMA stimulation (left panel). Stimulation with 5mM H2O2 resulted in a similar time course of complex association (right panel).

B. δPKC translocation of PMA stimulated CHO cells – time course. δPKC translocation to the membrane begins only after 1 min of PMA stimulation and is completed only after 5 minutes. A representative Western blot.

C. δPKC/annexin V complex formation precedes δPKC translocation. Quantitation of 4 independent co-immunoprecipitation experiments as described in A (*p<0.02 relative to basal level of complex prior to stimulation) and quantitation of 4 independent translocation experiments as described in B. (*p<0.02 relative to the basal level prior to stimulation.)

D. Disassociation of the δPKC-annexin V complex. Whereas during PMA stimulation, the complex disassociates to the basal level after 1min of stimulation, ATP depletion using 2-deoxyglucose (20mM, 4h pretreatment in glucose-free buffer) slows down the disassociation. Pretreatment of cells with nocodazole (10 μM, 30 min pretreatment) blocked complex disassociation, whereas cytochalasin D (2μM, 1h pretreatment) had no effect. A representative blot of three independent experiments.

E. Nocodazole prevents δPKC translocation. δPKC translocates from the cytosolic fraction upon PMA stimulation (10nM for indicated times). Pretreatment of cells for 30 min with 10 μM nocodazole prevents the disappearance of δPKC from the cytosolic fraction regardless of the duration of PMA stimulation. Actin loading control confirms equal loading. A representative blot of four independent experiments.

Fig 3. Interaction of δPKC with annexin V as determined by FRET.

A. Snapshots of real-time imaging after 100nM PMA stimulation. Whereas annexin V (top panel) remains cytosolic, δPKC (middle panel) translocates from cytosol to the cell membrane. FRET (bottom panel) in pseudocolor indicates protein-protein interaction. Regions chosen for quantitation are shown in the first panel of FRET. The calibration bar (bottom left) shows fold increase of the FRET signal. A representative set of frames from 10 independent experiments.

B. Quantitation of FRET. Pixel intensity was monitored in the selected regions as a function of time after 100nM PMA stimulation. FRET signal indicates the interaction of δPKC and annexin V, and is seen to increase upon PMA stimulation in a time-dependent manner. Representative of 30 cells from 10 independent experiments.
C. Quantitation of δPKC translocation. Drawing a region in the cytosolic region of the cell and monitoring decline in pixel intensity allows the measurement of δPKC translocation. δPKC begins translocation from the cytosol after 1min of PMA stimulation. 10µM nocodazole pretreatment for 30min did not alter the translocation profile of overexpressed δPKC. Average of 6 cells from 3 independent experiments.

Fig 4. pAnxV blocks binding of δPKC to annexin V and δPKC translocation.
A. pAnxV (1µM, 15 min pretreatment) inhibits δPKC (left panel), but not εPKC (right panel), translocation in CHO cells. Data are percent of PKC present in the membrane fraction over total PKC after 10nM PMA stimulation (5min). Mutant peptide R161E (EpAnxV) does not inhibit translocation (n=6, *p<0.001 – vs. PMA, #p<0.001 PMA vs. pAnxV+PMA). Representative blot of δPKC translocation from soluble (S) to the particulate (P) fraction is also shown (bottom panel).
B. pAnxV blocks δPKC/annexin V complex formation in cells. 1µM pAnxV (15min pretreatment) blocks the association of δPKC with annexin V as observed by cross-linking immuno-precipitation. Representative of 3 experiments.
C. pAnxV blocks association of δPKC with annexin V in vitro. 0.5mM pAnxV inhibited binding of δPKC to immobilized annexin V, whereas EpAnxV did not show a significant inhibition of binding, n=3, *p<0.006 pAnxV vs. -, #p<0.007 pAnxV vs. EpAnxV.

Fig 5. Binding of δPKC to annexin V is critical for δPKC translocation.
A. δPKC translocation is inhibited in cells treated with annexin V siRNA, but not mock-transfected cells (left panel). εPKC translocation was not affected by annexin V knock-down (right panel; n=3, *p<0.0005 - vs. PMA, #p<0.001 PMA vs. pAnxV+PMA).
B. Knock-down levels of annexin V and annexin IV in HeLa cells using siRNA. Annexin IV and V total levels in cell lysate are shown following 48hour transfection with 20nM siRNA. Shown is a representative blot (bottom panel) and quantitation of three independent experiments (upper panel). *p<0.005 - vs. anxIV siRNA, # p<0.001 - vs. anxV siRNA.
C. δPKC translocation is inhibited in cells treated with annexin V siRNA (solid line), but not in cells treated with annexin IV siRNA (dashed line). HeLa cells transfected for 48hours with 20nM annexin V or annexin IV siRNA were serum-starved for 24hour post-transfection, and stimulated by 100µM UDP for times indicated. (n=3, *^# p<0.005 for all time points except 0 and 5min stimulation)

Fig 6. Association of δPKC with annexin V is critical for downstream signaling in an ex vivo model of ischemia/reperfusion.
A. Isolated rat hearts were perfused with pAnxV or EpAnxV (1µM) for 10 min prior to a 30-min no-flow ischemia and for 10 min at the onset of 60min reperfusion.
B. Upon completion of experiment, hearts were sliced and stained with TTC to visualize live tissue red and dead tissue white. Representative heart slices showing TTC staining.
C. Infarct size was measured by taking the average of the white area on both sides of the tissue slices. pAnxV treatment reduced infarct size by ~70%, whereas EpAnxV had no effect (n=3,*p<0.05).
D. Creatine phophokinase (CPK) release was assessed in the heart perfusate during the duration of reperfusion. Treatment with pAnxV resulted in a >50% reduction in necrosis, whereas treatment with EpAnxV did not have statistical difference from control heart (n=3, *p<0.05).

Fig 7. Scheme of δPKC activation and translocation.
Inactive cytoplasmic δPKC (Step 1) binds to annexin V (in red) as the initial step after δPKC activation (Step 2) through, at least in part, the ψδRACK-like site on annexin V (highlighted in blue), thus displacing the inhibitory intramolecular interaction within δPKC mediated by the ψRACK site (dark gray
Annexin V/δPKC complex then translocates in microtubule- and ATP-dependent manner, possibly on vesicles carried by kinesin motor (Step 3). Disassociation of the complex allows complete translocation and anchoring of the active δPKC to the particulate fraction, where it binds to its isozyme specific anchoring RACK (Step 4), thus facilitating substrate (S) phosphorylation and downstream signaling (Step 5). Inactivation of the PKC (Step 6) may return the system to the basal state.
Annexin family alignment

\[ \psi \delta RACK \text{-like sequence} \]

Regulatory domain

Catalytic domain

V1/C2

C1

C3

C4

V5

V1

\( \delta V1 \)

\( \delta V5 \)

\( \delta PKC \)

AnxI

RNALLSLAKGDRSEDFGVNE-DL

AnxII

RKLMVALAKGRGAEDGSVIDYEL

AnxIII

RKALLTLADGRDE-SLKVEDHL

AnxIV

QRMLVVLLQANRDPDGTEAQ

AnxV

RRILSLAPGEERQ-GL-DQ

AnxVI

ERLLSVMQGNRNFQSHQMP

AnxVII

ERILVCILQSGDPPVSNFVDPUL

AnxVIII

QDRLIAKGRGDSYSGIDYNL

AnxIX

RTILMLVQGTREE--GYTDPA

AnxX

QRMLVSVQGNRDESTVOM-SL

AnxXI

KRILVSLQANRQGGDDVK-DL

AnxXII

+++L++L++g+Rde+++++d+l

Annexin V species alignment

newt

ERLLVSLVQANRDPVGKVDEGQVE

chicken

QRLLVVLLQANRDPDGRVDEALVE

rat

QRMLVVLLQANRDPDAGIDEAQ

human

QRMLVVLLQANRDPDAGIDEAQ

bovine

qRmLVvLlQANRDPdg+iDeaqVE

\( \psi \delta RACK \)-like sequence

JTV

\[ \text{newt} \]

\[ \text{chicken} \]

\[ \text{rat} \]

\[ \text{human} \]

\[ \text{bovine} \]

\( \psi \delta RACK \)-like sequence

JTV

\( \psi \delta RACK \)-like sequence

JTV

\( \psi \delta RACK \)-like sequence

JTV

\( \psi \delta RACK \)-like sequence

JTV

\( \psi \delta RACK \)-like sequence
A

- Ischemia
- Reperfusion

-10 0 30 90

B

- pAnxV
- EpAnxV

C

Infarct size (%)

D

CPK release (U)

- pAnxV
- EpAnxV
Step 1: Inactive PKC

Step 2: Active non-anchored

Step 3: Translocation ATP/microtubule dependent

Step 4: Active Anchored

Step 5: Substrate phosphorylation

Step 6: Inactivation

Activation signal

pAnxV

δV1-1

ATP/microtubule dependent
δPKC-annexin V interaction; a required step in δPKC translocation and function
Viktoria Kheifets, Rachel Bright, Koichi Inagaki, Deborah Schechtman and Daria Mochly-Rosen

J. Biol. Chem. published online June 18, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602075200

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