Membrane Targeting and Cytoplasmic Sequestration in the Spatiotemporal Localization of Human Protein Kinase C α*

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In order to map the molecular determinants that dictate the subcellular localization of human protein kinase C α (hPKCα), full-length and deletion mutants of hPKCα were tagged with the green fluorescent protein (GFP) and transiently expressed in GH3B6 cells. We found that upon thyropxin-releasing hormone (TRH) or phorbol 12-myristate 13-acetate stimulation, hPKCα-GFP was localized exclusively in regions of cell-cell contacts. Surprisingly, PKCα failed to translocate in single cells despite the presence of TRH receptors, as attested by the TRH-induced rise in intracellular calcium concentration in these cells. TRH-stimulated translocation of hPKCα-GFP from the cytoplasm to cell-cell contacts was a biphasic process: a fast (measured in seconds) and transient phase, followed by a slower (approximately 1 hour) and long lasting phase. The latter and the translocation induced by phorbol 12-myristate 13-acetate absolutely required the N-terminal V1 region. In contrast to the full-length hPKCα, the N-terminal regulatory domain alone or associated with the V3 hinge region was spontaneously and uniformly localized at the plasma membrane of single and apposed cells. However, treatment with the calcium chelator BAPTA/AM induced a differential cytoplasmic/nuclear redistribution of the regulatory domain, depending on its association with V3, which suggests the existence of a mechanism controlling the cytoplasmic sequestration of inactive hPKCα and involving the V3 region. By using other deletion mutants, we were able to map the sequence required for this sequestration to the C2+V3 regions. This work points to the existence of a complex interplay between membrane targeting and cytoplasmic sequestration in the control of the spatiotemporal localization of hPKCα.

Protein kinase C (PKC)† is a term coined to designate a family of isoforms that play key roles in the processes of proliferation/apoptosis, differentiation, or hormone release and the function of which is regulated at multiple levels: transcription, phosphorylation, and subcellular targeting.

Subcellular targeting of PKC, and particularly that of the conventional PKC α, β, and γ isoforms, is linked to enzyme activation. Indeed, inactive PKC is mostly cytoplasmic, whereas activated PKC translocates to various membrane compartments, such as the plasma membrane. Physiological activation of the conventional PKC is associated with an increase in diacylglycerol (DAG) and intracellular Ca2+ concentrations (1), which results from the activation of a seven transmembrane receptor coupled to phospholipase Cγ via a heterotrimeric G protein (2). The increase in Ca2+ is thought to be essential for translocation, although it can be bypassed by the phorbol ester phorbol 12-myristate 13-acetate (PMA) (3). The increase in DAG concentration at the plasma membrane allows additional conformational changes to achieve PKC activation at its targeting site. When inactive, PKC is in a “closed” conformation due to the interaction of the pseudosubstrate sequence with the catalytic site (4, 5). Upon activation, this interaction is disrupted, unmasking the regions of the enzyme involved in translocation and accumulation at the targeting site. Although numerous studies have been aimed at determining which region of the protein is involved in each step, it is not clear yet whether or not membrane sequestration results from an increase in the affinity of PKC for membrane phospholipids (essentially phosphatidylserine), or if it involves an interaction with a “shuttle” protein. Nevertheless, it appears that the NH2-terminal regulatory domain is essential for translocation, whereas the catalytic domain seems to be less important, even though it may influence PMA-induced PKC translocation (6). The amino acid sequences responsible for the interaction with phospholipids are located within the C1 and in the C2 regions (7). This has been demonstrated both by conventional biochemistry and direct microscopic observation in the living cell by use of the green fluorescent protein (GFP) tag (3, 8–11). Accumulation of the enzyme at its targeting site may also result from an interaction with anchoring proteins (12). Moehly-Rosen and co-workers (13, 14) have cloned the first cDNA encoding a receptor for activated protein kinase C (RACK1), recently described as an inhibitor of Src tyrosine kinase activity and NIH 3T3 cell growth (15). The interaction between PKC and RACK1 involves the C2 region (16), also known for its high affinity for Ca2+ (17, 18). More recently, using the V1 region of PKCα as a bait in the two hybrid system, β′-COP has been characterized as a RACK for this PKC isoform (19). It is noteworthy that although it does not bind Ca2+, the V1 region of PKCα shares homologies with the C2 region of conventional PKCs (20). Additional studies provide evidence for the involvement of V1 in PKC subcellular targeting; the V1 region of atypical PKC contains a protein-protein interacting motif that mediates its interaction with p62 (21), and the involvement of the V1 region of...
PKCγ in the translocation kinetics of PKCγ has recently been evidenced by Oancea and Meyer (22).

In the present study, human PKCα (hPKCα)-GFP and GFP-tagged deletion mutants were transiently expressed in the pituitary GH3B6 cell line, stimulated by PMA or by thyrotropin-releasing hormone (TRH), used as a physiological stimulus. We show here the following: 1) the exclusive localization of hPKCα-GFP at the interface between two apposed stimulated cells; and 2) the biphasic nature of translocation upon TRH stimulation (rapid and reversible versus slow and long lasting) and the dependence on V1 for the slow and long lasting phase; 3) the absence of hPKCα-GFP translocation in single cells despite the presence of TRH receptors as attested by the induced calcium increase in these cells; and 4) the key role played by the C2-V3 region in the control of the cytoplasmic sequestration of the hPKCα-GFP.

**EXPERIMENTAL PROCEDURES**

**Materials—**PMA, 1,2-bis(2-aminophenoxy)-ethane-N,N',N''-tetraacetic acid tetrasodium salt (BAPTA/AM) and phosphatidylinerine were purchased from Sigma. Restriction enzymes were from Promega (Charbonnières, France). T7 DNA polymerase, Ham's F-10 medium, and medium components were from Eurobio (Les Ulis, France). ExGen 500 (linear polyethyleneimine) and monomodal anti-PCKCα antibody were from Euromedex (Souffelweyersheim, France). Fetal bovine serum were from BioWhittaker (Walkersville, MD). Monoclonal antibody against GFP, anti-mouse IgG-peroxidase Fab fragments, and chemiluminescence detection kit were from Roche Molecular Biochemicals. pEGFP-N1 plasmid was from CLONTECH (Palo Alto, CA). The cDNA clone coding for hPKCα was provided by Drs. Alvaro and Weinstein from the Columbia Cancer Center (New York, NY). Protein A was from Bio-Rad (Ivy sur Seine, France). (g)(g+1)-Pi ATP and membrane Hybrid Cy-C-Extra were from Amersham Pharmacia Biotech. Goat anti-rabbit IgG, (H+L), horseradish peroxidase conjugated were from Pierce. TRH was from Calbiochem (Meudon, France). Goat anti-mouse IgG TRITC was from Jackson ImmunoResearch (Marseille, France).

**Construction of Plasmids Encoding Fusion Proteins—**The constructs used here are presented in Fig. 2. The full-length hPKCα cDNA and its various deletion mutants with an EcoRI site at their 5’-terminus and a KpnI site at their 3’-terminus were produced by polymerase chain reaction using hPKCα cDNA subcloned in the pBlue vector as a template.

The following synthetic oligonucleotides primers were used to generate the hPKCα constructs. Sense primers were 5’-1, GAAATTCGAGCAGAGTGTTT; 5’-2, GAGAATTCCATGACCAGCATGTCTC-TGTA; 5’-3, GGAATTCCATGTCACAGTACGAGATGCA; 5’-4, GGAATTCCATGGACCGACGACTGTCAG; 5’-5, GGAATTCCATGGTCACAGTACGAGATGCA; 5’-6, GGAATTCCATGGTCACAGTACGAG. The antisense primers without stop codon were 3’-1, GGGTCACTCCATCGTCATTAGATG; 3’-2, GGGGTACCCGGATGATTCTACCTGCT; 3’-3, GGGGTACCCGGATGGCTTACCTGATCT; and 3’-4, GGGTACCCGGATGGCTTACCTGATCT.

Oligonucleotides 5’-1 and 3’-1 were used as primers for the full-length enzyme; 5’-2 and 3’-1 for (ΔV1)hPKCα-GFP; 5’-1 and 3’-3 for RD-GFP; 5’-5 and 3’-2 for (ΔV1) (RD+V3)-GFP; 5’-4 and 3’-2 for (ΔV1)-PS (RD+V3)-GFP; 5’-3 and 3’-2 for (C2)+V3-GFP; 5’-2 and 3’-2 for (C2)+V3-GFP; 5’-3 and 3’-2 for V3-GFP; and 5’-6 and 3’-4 for V1-GFP. The cycle parameters were 94°C for 1 min, 54°C for 2 min, and 72°C for 3 min.

The various polymerase chain reaction fragments encoding full-length or deletion mutants of hPKCα were gel-purified, digested with EcoRI and KpnI, and then fused in-frame to GFP by ligation into EcoRI and KpnI digested pEGFP-N1 vector. The sequences of ligated polymerase chain reaction fragments were checked by DNA sequencing, and the desired amount of plasmid DNA was also diluted in 50 μl of NaCl 150 mM in order to have 10 linear polyethyleneimine nanograms per DNA phosphate. The two solutions were then mixed. After 10 min, the transfection mixture was added to the cells. The six-well dishes were then centrifuged for 5 min at 280 × g and maintained for 4 h at 37°C. The medium was then replaced with fresh medium. The fluorescence of fusion proteins was observed 48 h after transfection. The localization of fusion proteins in living cells was examined by conventional (long term treatment) or confocal (short term treatment) fluorescence microscopy. Confocal laser scanning microscope was equipped with an Ar/Kr laser ( Odyssey XL with InterVision 1.4.1 software; Rockafeller Instruments Inc., Middleton, WI) as described by Seri neau et al. (23). At the time of observation, the culture medium was replaced by a buffer composed of 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, glucose 6 mM, pH 7.4.

Localization of hPKCα fusion proteins was monitored under basic conditions and after application of 100 mM PMA or 100 mM TRH for the times indicated. Although GH3B6 cells are clonal cells, we observed that not all of the cells behaved identically concerning translocation. Absence of translocation of a particular fusion protein was assumed when it could not be observed in any cells; however, successful translocation was never observed in 100% of the cells. For long term treatments, at least 100 cells were observed in each experiment. For short term treatments, at least 10 cells were observed in each experiment. All experiments were repeated three and 10 times.

**PKCα Detection by Immunocytochemistry—**GH3B6 cells were seeded on 20 × 20-mm coverslips in 2.5 ml of Ham's F-10 medium and grown for 24 h. Cells were washed quickly three times with PBS (140 mM NaCl, 27 mM KCl, 8 mM NaHPO4, 1.5 mM KH2PO4) and fixed for 1 min with 3% formaldehyde (v/v) in PEM buffer (80 mM PIPES, 5 mM EDTA, 2 mM MgCl2, pH 6.5) and for 8 additional min with 3% formaldehyde (v/v) in 100 mM sodium borate at pH 11. Cells were incubated for 15 min in PBS containing 0.1% (v/w) sodium borohydride, washed, permeab lized by incubation in PBS supplemented with 0.2% Triton X-100, washed again, incubated overnight at 4°C with the anti-PCKCα antibody (diluted 1:100), washed, and further incubated for 60 min with the secondary antibody (goat anti-mouse IgG TRITC, diluted 1:40). After washing, cells were postfixed for 15 min with 5% formaldehyde in PBS and incubated in the presence of 50 mM NH4Cl for 10 min. Coverslips were mounted in 1,4-diazabicyclo[2.2.2]octane, 100 mg/ml in PBS containing 50% glycerol.

**Immunoprecipitation—**hPKCα-GFP, (ΔV1)hPKCα-GFP, and GFP constructs were transiently transfected into GH3B6 cells. Forty-eight hours after transfection, cells were washed in cold PBS and incubated in lysis buffer (50 mM NaCl, 1 mM Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM/ml aprotinin, 1 μM/ml leupeptin, 1 μM/ml pepstatin) at 4°C by gently rocking. Cells were then scrapped and collected in microcentrifuge tubes. Lysates were preclarified with 50 μl of protein A beads, incubated for 10 min at 4°C by gentle rocking, and centrifuged at 14,000 × g for 10 min at 4°C. Supernatants were collected. Two mg of EGTA were mixed with each 20 μl of GFP per G6GAG, and this reaction mixture was incubated at 4°C overnight. Immunocomplexes were captured by adding 50 μl of protein A beads. This mixture was gently rocked at 4°C overnight. After centrifugation and washing of the beads with 800 μl of 50 mM Tris, pH 7.4, supplemented with 1 μM/ml aprotinin, 1 μM/ml leupeptin, and 1 μM/ml pepstatin, immunocomplexes were resuspended with EGTA, 50 μl of 50 mM Tris, pH 7.4, for further kinase activity assay or in 50 μl of Laemmli buffer (24) for Western blot analysis.

**PKCα Catalytic Activity Measurement—**Catalytic activity of hPKCα purified from Sf9 cells and of immunoprecipitated hPKCα-GFP, (ΔV1)hPKCα-GFP, or GFP were measured with the S17R peptide as substrate (SLKRRSGFSFRKLASSIR). The S17R peptide corresponds to the PKC phosphorylation site of clone 35F (25). The amount of each protein used for catalytic activity assay was estimated by the assay by Western blot analysis with a PKCα antibody.

Activity was measured in the presence of 20 μM S17R, 1 μM EGTA, 10 μM PMA, 5 mM MgAc, 25 μM ATP, 1 mM dithiothreitol, 1 mM Na+ (g+1)-Pi ATP (specific activity, 30 C/mmole) (Amersham Pharmacia Biotech), 10 μM/ml PS (26). The reaction was prepared in the absence of calcium. Reaction was started by the addition of calcium by backfilling with both heat inactivated at 56°C for 1 h. Transient transfection of GH3B6 cells was performed with ExGen 500 according to the manufacturer's standard protocols. Briefly, cells were seeded at 25,000 cells per well in six-well dishes (Falcon) for 18 h before transfection. Immediately before transfection, fresh culture medium (2.5 ml) was added to the cells. Five μl of ExGen 500 stock solution were diluted in 50 μl of NaCl 150 mM.
Cell Fractionation and Western Blot Analysis—Transiently transfected HGB36 cells were separated into soluble and membrane fractions. Untreated or 100 nM PMA-treated cells were washed with cold PBS followed by scraping in homogenization buffer (10 mM Tris, 2 mM EDTA, 0.01% phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin). Cells were then homogenized in a glass Dounce homogenizer and centrifuged for 30 min at 14,000 rpm. Supernatants were collected; they correspond to soluble fractions. Pellets were resuspended in homogenization buffer supplemented with 1% (v/v) Nonidet P-40 and incubated for 45 min on ice. This corresponds to membrane fractions.

For immunoblotting, soluble and membrane fractions were subjected to 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred onto nitrocellulose membranes. Nonspecific binding sites were blocked by incubation with Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.4) containing 10% powdered milk for 1 h at room temperature. Membranes were then incubated with anti-PKCα (1:2000) or anti-GFP (1:1000) antibody overnight at 4 °C or for 1 h at room temperature, respectively. After washing with Tris-buffered saline containing 0.1% Tween, membranes were incubated with anti-mouse IgG-peroxidase antibody (1:4000) for 1 h at room temperature. Immunoreactive bands were visualized with chemiluminescence detection kit.

Production and Purification of hPKCa from Sf9 Cells—T-Flasks containing 1.5 × 10^6 Sf9 cells were incubated for 3 days at 27 °C with 5 × 10^6 pfu/ml of recombinant baculovirus encoding hPKCa (27). Infected cells were resuspended in medium, pelleted, and washed three times in PBS, pH 7.4. Cells were sonicated in homogenization buffer (10 mM Tris, pH 7.6, 2 mM EDTA, 0.01% phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin), and hPKCa was partially purified according to Birman et al. (28) using a DEAE-cellulose (DE-52) column. hPKCa was eluted with 100 mM NaCl and stored at −80 °C in a buffer containing 20 mM Tris, pH 7.4, 1 mM EGTA, 1 mM dithiothreitol, 50% glycerol (final concentrations).

Intracellular Calcium Concentration Changes—Cytoplasmic free calcium concentration ([Ca^{2+}]_i) was measured with a real-time confocal laser scanning microscope (23). Cells were visualized with a 63 × 0.9 numerical aperture achroplan water immersion objective lens (Zeiss). The larger slit (100 μm) was used, giving bright images with a 3.1 μm axial resolution. Cells were loaded with the Ca^{2+}-sensitive fluorescent probe fluo-3 by exposure to 50 μM fluo-3 acetoxymethyl ester (fluor-3/AM, Molecular Probes, Eugene, OR) by incubation for 30 min at 37 °C in a humidified incubator. Fluo-3 was excited through a 488-nm band pass filter, and the emitted fluorescence was collected through a 515-nm barrier filter. [Ca^{2+}i] changes were expressed as the ratio F/F_{min}, where F_{min} was the minimum fluorescent intensity measured during the recording (30 images/s). Acquired data were then processed for analysis using Igor 3.14 software (Wavemetrics Inc., Lake Oswego, OR). Three separate experiments were performed, and in each experiment, a minimum of 10 fields with both single and contacting cells were analyzed for [Ca^{2+}i] changes.

RESULTS

The Exclusive Localization of hPKCa at the Cell-Cell Contacts upon PMA Stimulation Is Not Affected by the GFP Tag—Fig. 1A shows that the transfected hPKCa-GFP, visualized in the living cell by fluorescent microscopy, was found in the same subcellular compartments as is the rat endogenous PKCa, as revealed by an anti-PKCα antibody. Both the rat endogenous PKCa and the hPKCa-GFP were cytoplasmic under basal con-
conditions, whereas under stimulation with 100 nM PMA for 60 min, both translocated to a restricted area of the plasma membrane, localized at the interface between apposed cells. Stimulation-induced translocation was never observed in isolated cells (at least 100 cells observed). In contrast, GFP was evenly distributed between the cytoplasmic and nuclear compartments (see Fig. 6B), and its localization was not affected by PMA treatment (data not shown).

The fact that PKCa localization was not affected by the GFP tag on localization correlated with the lack of effect of the GFP tag on catalytic activity. This was shown by comparing the properties of hPKCa extracted from Sf9 cells to those of hPKCa-GFP immunoprecipitated from transiently transfected GH3B6 cells. The contents of hPKCa, hPKCa-GFP and GFP in cell extracts and immunoprecipitates were first evaluated by Western blot analysis (Fig. 1B). The catalytic activities of hPKCa-GFP and hPKCa were then measured in the presence of PMA and phosphatidylserine, in the presence or absence of Ca2+. As shown in Fig. 1B, the catalytic activities of both hPKCa-GFP and hPKCa were increased upon Ca2+ addition, whereas GFP alone was, as expected, catalytically inactive.

The GFP fusion proteins used in transient transfection experiments are schematically represented in Fig. 2A, and the Western blot shown in Fig. 2B shows that the translated products are of the expected size. Transfection efficiency approximated 25% in GH3B6 cells, although the efficiency was higher for the smaller sized constructs.

**hPKCa Translocation to the Plasma Membrane Is Biphasic upon TRH Stimulation and Monophasic upon PMA Exposure**—Fig. 3 illustrates the time-dependent translocation of hPKCa-GFP to the plasma membrane of living contacting cells upon stimulation with 100 nM TRH. Translocation always occurred at cell-cell contacts but never in single cells, regardless of the duration of TRH stimulation. In agreement with recent publications (3, 8–11, 22, 29), the stimulation of a seven transmembrane domain receptor (the TRH receptor in the present study) induced a rapid (8 s in all of the cells observed; n > 20) but transient translocation of hPKCa-GFP (Fig. 3A). This first rapid phase was followed by a relocation of hPKCa-GFP to the cytoplasm within 60 s. Unexpectedly, however, a second phase occurred within 1 h of stimulation, and hPKCa-GFP was still located at the plasma membrane after 3 h (data not shown). Deletion of the V1 region (ΔV1) abolished PMA-induced hPKCa translocation. Soluble (s) and membrane (m) fractions of untreated or PMA-treated GH3B6 cells transiently transfected with hPKCa-GFP or ΔV1-hPKCa-GFP were subjected to SDS-polyacrylamide gel electrophoresis and Western blot (WB) using an anti-GFP antibody. Under basal conditions, both proteins were recovered in the soluble fraction. Under PMA treatment (100 nM, 1 h), hPKCa redistributed to the membrane fraction, whereas ΔV1-hPKCa-GFP remained cytosolic. Scale, 1 cm = 10 μm.

![Fig. 2. hPKCa-GFP fusion proteins. A, schematic representation of hPKCa-GFP fusion proteins. As described under “Experimental Procedures,” full-length hPKCa cDNA or truncated hPKCa cDNA fragments without the stop codon were subcloned in-frame at the 5’ end of the sequence encoding GFP with EcoRI and KpnI sites. Shown are the different fusion proteins that were generated and their expected molecular masses. B, Western blot analysis of hPKCa-GFP fusion proteins expressed in GH3B6 cells. GH3B6 cells that had been transiently transfected with overexpressing vectors were harvested and subjected to SDS-polyacrylamide gel electrophoresis and Western immunoblotting as described under “Experimental Procedures.” The membrane was probed with a polyclonal antibody directed against GFP. A positive immunoreactive band was evidenced for each fusion protein at the appropriate molecular sizes.](Image 51x461 to 295x729)
shown by Western blot analysis (Fig. 3C). These results suggest abolished, regardless of the duration of PMA treatment, as V1 was deleted, the PMA-induced translocation was totally (Fig. 3C) and membrane (C, s) after 1 h of stimulation. When lasting, as shown by the Western blot performed with cytosol stimulation, translocation started after 10 min and was long

**Rearrangement of PKC α**

In order to establish whether the absence of translocation of PKCα in isolated cells could be attributed to a deficiency in TRH receptors, we measured the variations in intracellular calcium concentration in both isolated and apposed cells upon TRH stimulation. As shown in Fig. 4, TRH elicited a rise in calcium concentration of similar amplitude in apposed cells (Fig. 4B) and in isolated cells (Fig. 4C). All of the cells observed showed this intracellular calcium rise, and in particular all of the isolated cells. This implies that TRH receptors are indeed present in isolated cells; their absence cannot account for the lack of PKCα translocation. Intracellular calcium rise, which is known to be necessary for translocation, thus may not be sufficient for hPKCα-GFP translocation in GH3B6 cells.

**Removal of the Catalytic Domain Results in the Constitutive and Uniform Localization of the Regulatory Domain + V3 Region at the Plasma Membrane**—When inactive, native PKCα localizes exclusively in the cytoplasm. Activation results in the “opening” of the enzyme due to disruption of the interaction between the N-terminal pseudosubstrate sequence and the C-terminal catalytic site. Deletion of the catalytic domain may therefore unmask regions of the RD involved in translocation. We found that the RD+V3-GFP (Fig. 5A) was spontaneously located at the plasma membrane of single as well as apposed cells, with no preferential localization at cell-cell contacts and no change in localization upon treatment with PMA (Fig. 5A) or TRH (data not shown). The results obtained by direct microscopic observation were confirmed by Western blot analysis (Fig. 5B).

**Inactivation of PKCα Is “Sequestered” in the Cytoplasm. Involvement of the C2-V3 Region**—Similarly to the RD+V3-GFP construct, the RD-GFP construct spontaneously located at the plasma membrane of single (data not shown) as well as apposed cells (Fig. 6A). Likewise, in single cells, the treatment with BAPTA/AM induced a dissociation of the RD-V3-GFP construct from the membrane. A similar phenomenon was observed in apposed cells, except that fluorescence persisted on cell-cell contacts (Fig. 6A). We concluded that accumulation of the RD+V3 at the plasma membrane may result from both Ca2+-dependent and Ca2+-independent mechanisms and that it contains all of the sequences involved in the accumulation of PKCα at the plasma membrane, including at cell-cell contacts.

**Inactive hPKCα Is “Sequestered” in the Cytoplasm. Involvement of the C2-V3 Region**—Similarly to the RD+V3-GFP construct, the RD-GFP construct spontaneously located at the plasma membrane of single (data not shown) as well as apposed cells (Fig. 6A). Likewise, in single cells, the treatment with BAPTA/AM induced a dissociation of the RD-GFP construct from the membrane, just as it did for the RD+V3-GFP construct (data not shown). The same thing happened in apposed cells at the exception of the cell-cell contacts (Fig. 6A). However, Fig. 6A also shows that in the absence of Ca2+, the subcellular relocalization of the RD-GFP is not identical to that of the RD+V3-GFP. In the presence of V3, the fluorescence relocates from the membrane to the cytoplasm whereas in the absence of V3, the fluorescence relocates both in the cytoplasm and the nucleus upon treatment of cells with BAPTA/AM. This prompted us to hypothesize that there is a sequestration of hPKCα in the cytoplasm and that this may involve the V3 region.
selective accumulation of hPKCα affects the first TRH-dependent phase of translocation or the final destination. Proteins such as PKCα that do not contain a peptide signal are cytoplasmic when inactive and require an active mechanism to be addressed to specific subcellular compartments upon stimulation. The present study aimed at understanding the structure-localization relationship of hPKCα. To this end, intracellular displacements of various hPKCα-GFP fusion proteins were assessed in living GH3B6 cells. Our main findings can be summarized as follows. 1) Upon physiological stimulation of the TRH receptor, the translocation of hPKCα to the plasma membrane displays two phases, each phase being controlled by different mechanisms. 2) Translocation is observed at cell-cell contacts, but not in isolated cells, although TRH induces a similar rise in intracellular calcium concentra-
tion in isolated and in apposed cells. 3) the catalytic domain is necessary neither for translocation nor for the selectivity of the subcellular targeting. 4) The cytoplasmic localization of hPKCa results from a sequestration involving specific regions of PKCa.

The Early Signals—In our rat pituitary-derived experimental model, the GH3B6 cells, we showed that translocation and accumulation of PKCa (both endogenous and hPKCa-GFP) only occurs in contacting cells, never in single cells. Furthermore, we showed that the absence of translocation in single cells is not due to the lack of functional TRH receptors, because intracellular Ca2+ concentration does rise similarly in single and in apposed cells. The increase is rapid and transient, as previously reported (30). Therefore, the fact that there is no translocation in single cells suggests that the rapid translocation induced by TRH may require another signal in addition to Ca2+. This result challenges the dogma recently proposed by Onaeea and Meyer (22), presenting “PKC as a molecular machine for decoding calcium and DAG signals.” The authors showed that repetitive calcium spikes triggered by stimulation of the FceRI receptor induce a parallel repetitive translocation of GFP-tagged PKC to the plasma membrane of rat basophilic leukemia 2H3 cells. The fact that we did not observe translocation of hPKCa at the plasma membrane of single GH3B6 cells, as it should be if the relationship between calcium and PKC translocation were sufficient, may be attributable to the different isoforms involved or to the different cell lines used. However, unlike PKCa, which is a ubiquitous isoform, PKCy is a strictly brain-specific isoform. The fact that PKCy was translocated in cells that normally do not express this isoform may be relevant: in the absence of isoform-specific and tissue-specific PKC-interacting proteins, translocation may strictly depend on variations in DAG and Ca2+ concentrations. The nature of the additional signal required for translocation of PKCa in GH3B6 contacting cells needs to be identified.

Because the RD alone spontaneously accumulates uniformly at the plasma membrane, the RD may contain the required membrane interacting sequences, possibly the C2 region that binds calcium (17, 18) and the C1 region that binds DAG. In addition, the spontaneous accumulation of the RD-GFP at the plasma membrane also suggests that the increase in DAG and intracellular Ca2+ concentrations required for disrupting the interaction between the pseudosubstrate and the catalytic site may not be required for “translocation.”

Which Route to Take?—The uniform accumulation of the RD at the plasma membrane, by contrast to the native protein accumulation at the interface between apposed cells, indicates that selectivity of translocation and sequestration in the cytoplasm have been lost. It is as if the RD alone ignored the signal telling which route to choose, despite the fact that the sequences capable of interacting with this unknown signal are most probably in the RD because (a) Δ(V1)RD-V3-GFP translocates at the interface of apposed cells upon stimulation with TRH, as does the endogenous PKCa; and (b) RD-GFP localized at the interface between cells upon treatment with BAPTA/AM.

We have seen that RD and RD + V3 are constitutively located at the plasma membrane. Upon Ca2+ removal, RD + V3 is shifted back to the cytoplasm, whereas RD alone is uniformly distributed between cytoplasm and nucleus (at the exclusion of the cell-cell contacts). This is compatible with V3 being involved in the cytoplasmic sequestration of hPKCa under basal conditions. This hypothesis is further supported by the finding that under basal conditions, Δ(V1)RD is uniformly distributed within the cell, whereas Δ(V1)RD + V3 is exclusively cytoplasmic (data not shown). The V3 region is, however, necessary but not sufficient because cytoplasmic sequestration requires the C2 region. What mechanism could account for cytoplasmic sequestration? One possible explanation is that the C2-V3 region of hPKCa is a site of specific interaction with a cytoplasmic protein. This would be in agreement with the hypothesis of Mochly-Rosen and Gordon (12) speculating that PKC, in its inactive conformation, may interact with a receptor for inactive kinase C.

The Final Destination—How to explain the observation that hPKCa accumulates at the interface between apposed cells? It could be mediated either by a direct interaction with the membrane or via anchoring proteins. However, the lack of BAPTA/AM effect on RD accumulation at cell-cell contacts makes a direct interaction of hPKCa with the plasma membrane rather unlikely. Indeed, previous studies have shown that the interaction of PKC with phospholipidic vesicles is rapidly disrupted in the presence of a calcium chelator (31). The interaction between PKCa and anchoring proteins, such as RACK1, has been evidenced in the MDA-MB-231 cell line (32). Such an interaction is supposed to maintain an active pool of enzyme in the vicinity of its substrates. RACK1 is known to interact with the integrin β subunit (33), which is part of the adhesion receptors that mediate attachment of cells to the extracellular matrix. The selective localization of PKCa at the cell interface and its absence from adhesion foci in GH3B6 cells is not in favor of the existence of a direct interaction between PKCa and RACK1. Thus, the presence of hPKCa at the inter-
face between cells is more likely mediated via an as yet unidentified protein. The membranes located at the contact between cells contain the multireceptor complexes (gap junctions and the catenin/cadherin system) involved in cell-cell communication. Localization of PKCα in the apposed membranes may indicate a privileged role for PKCα in this type of communication, as already suggested by different studies showing for instance the direct role of PKC in the control of gap junction permeability (34, 35).

Two Phases of Translocation—Up to now, PKC translocation has been widely accepted as being both monophasic and transient. Here, we report for the first time that PKCα can display a two phase kinetics upon chronic TRH stimulation: a fast (measured in seconds) and transient phase being followed by a slower (approximately 1 hour) and long lasting phase. Speculative mechanisms underlying the late translocation phase may include TRH receptor recovery from desensitization (36, 37), or alternatively, it may indicate that a new protein needs to be synthesized or matured. In addition, one major difference between the first and the second phase of translocation is the time that hPKCα-GFP remains at the targeting site: seconds for the first phase, hours for the second, implying that proteolytic-dependent down-regulation might occur only during the second phase. In our study, one prominent result is that deletion of V1 from PKCα abolishes both the second phase of TRH-induced translocation and the PMA-induced translocation in GH3B6 cells. The potential role of V1 in translocation/accumulation had previously been addressed for PKCγ transfected into rat basophilic leukemia 2H3 cells (22). The authors of that study showed that V1-deleted PKCγ translocates faster than the wild type enzyme upon phorbol 12,13-dibutyrate stimulation. These results could be explained by the different isoforms studied, which differ mainly in the variable regions (such as V1 or V3), the different cell lines used, or the absence of specific PKC-interacting proteins.

The results obtained with the Δ(V1)RD+V3 construct, that is, abolition of the spontaneous accumulation of the RD+V3 at the plasma membrane, suggests that V1 may play a direct role during translocation. However, not only is V1 not a membrane targeting sequence by itself (because it does not address GFP to the membrane) but, in contrast to PKCε (38), V1 overexpression does not prevent translocation of the endogeneous PKCα. Therefore, if the role of V1 in targeting is exerted through an interaction with a protein, this interaction is not rate-limiting.

In conclusion, these observations as a whole provide a new insight into the spatiotemporal localization of hPKCα, the complexity of this process reflecting the complexity of each step: sequestration, targeting, and accumulation. Our work provides arguments for 1) the existence of two phases of translocation upon stimulation by TRH, differently controlled and involving the V1 variable region; 2) the requirement of a signal other than calcium that together with calcium triggers translocation; and 3) the existence of a controlled cytoplasmic sequestration involving the C2 and V3 regions.

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REFERENCES

1. Liu, W. S., and Heckman, C. A. (1998) Cell. Signal. 10, 529–542
2. Berridge, M. J. (1987) Annu. Rev. Biochem. 56, 189–190
3. Almholte, K., Arkhammar, P., Thastrup, D., and Tullin, S. (1999) Biochem. J. 337, 211–218
4. House, C., and Kemp, B. (1987) Science. 238, 1726–1728
5. House, C., Robinson, P. J., and Kemp, B. E. (1989) FEBS Lett. 249, 243–247
6. Ac, P., Bogi, K., Lorenzo, P., Marques, A., Biro, T., Szallasi, Z., and Blumberg, P. (1997) J. Biol. Chem. 272, 22148–22153
7. Johnson, J., Edwards, A., and Newton, A. (1997) J. Biol. Chem. 272, 30767–30772
8. Feng, X., Zhang, J., Barak, L., Meyer, T., Caron, M., and Hannun, Y. (1998) J. Biol. Chem. 273, 10755–10762
9. Oancea, E., Teruel, M., Quest, A., and Meyer, T. (1998) J. Cell Biol. 140, 485–498
10. Sakai, N., Sasaki, K., Ikegaki, N., Shirai, Y., Ono, Y., and Saito, N. (1997) J. Cell Biol. 139, 1465–1476
11. Barak, L. S., Warabi, K., Feng, X., Caron, M. G., and Kwaitra, M. (1999) J. Biol. Chem. 274, 7565–7569
12. Mochly-Rosen, D., and Gordon, A. (1998) FASEB J. 12, 35–42
13. Mochly-Rosen, D., Khuner, H., and Lopez, J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3997–4000
14. Ron, D., Chen, C., Caldwell, J., Jamieson, L., Ore, E., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
15. Chang, B., Conroy, K., Machedler, E., and Cartwright, C. (1998) Mol. Cell. Biol. 18, 3245–3256
16. Ron, D., Luo, J., and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24180–24187
17. Sutton, R. B., Davletov, B. A., Berhuis, A. M., Sudhof, T. C., and Sprang, S. R. (1995) Cell 80, 929–938
18. Shao, X., Davletov, B. A., Sutton, R. B., Sudhof, T. C., and Rizo, J. (1996) Science 273, 248–251
19. Cravatk, M., Chen, C. H., De Matteis, M. A., and Mochly-Rosen, D. (1997) J. Biol. Chem. 272, 29200–29206
20. Newton, A. C. (1995) J. Biol. Chem. 270, 28495–28498
21. Sanchez, P., De Carre, G., Sanduval, I., Moscat, J., and Diaz-Meco, M. (1998) Mol. Cell. Biol. 18, 3069–3080
22. Oancea, E., and Meyer, T. (1998) Cell 95, 307–318
23. Greiner, N. C., Bonnefont, X., Stoeckel, L., and Mallard, P. (1998) J. Biol. Chem. 273, 10389–10395
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Liu, L., Hyatt, S., Chapline, C., and Jaken, S. (1994) Biochemistry 33, 1229–1233
26. Alvaro, V., Pruvostel, C., Joubert, D., Slosberg, E., and Weinstein, I. P. (1998) Oncogene 14, 677–685
27. Pruvostel, C., Alvaro, V., Valles, A., Martin, A., Chiotet, D., Jaken, S., and Joubert, D. (1996) Biochem. J. 334, 393–397
28. Birman, P., Touraine, P., Bai-Grenier, F., Dubray, C., Kaabache, T., Peillon, F., and Joubert, D. (1989) Acta Endocrinal 121, 489–494
29. Shirai, Y., Kashiwagi, K., Yagi, K., Sakai, N., and Saito, N. (1998) J. Cell Biol. 143, 511–521
30. Martin, T. F. J. (1983) J. Biol. Chem. 258, 1416–1422
31. Mosior, M., and Newton, A. C. (1995) J. Biol. Chem. 270, 25526–25533
32. Rotenberg, S., and Sun, X. (1999) J. Biol. Chem. 274, 2390–2395
33. Liliental, J., and Chang, D. (1998) J. Biol. Chem. 273, 2379–2383
34. Jansen, L. A. M., Mesnil, M., and Jongen, W. M. F. (1996) Carcinogenesis 17, 1527–1531
35. Leibold, E., Greim, H., and Schwartz, L. R. (1994) Carcinogenesis 15, 1265–1269
36. Yu, R., and Hinkle, P. M. (1997) J. Biol. Chem. 272, 28301–28307
37. Yu, R., and Hinkle, P. M. (1998) Mol. Endocrinol. 12, 737–749
38. Johnson, J., Gray, M., Chen, C. H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966
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