Targeting of PKCα and ε in the pituitary: a highly regulated mechanism involving a GD(E)E motif of the V3 region

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Summary

Protein kinase C (PKC) has been implicated in the control of intercellular adhesion. Our previous observation demonstrating that activated PKC alpha (PKCα) is selectively targeted to cell-cell contacts of pituitary GH3B6 cells supports these findings. The relevance of this observation is further strengthened by the present data establishing that this targeting selectivity also occurs in the pituitary gland. Moreover, a new mechanism involved in the control of PKC targeting is unravelled. We demonstrate that a three amino acid motif located in the V3 region of α and epsilon (ε) (GDE/GEE respectively) is essential for the targeting selectivity of these isoforms because: (1) this motif is absent in delta (δ) and mutated in the natural D294G-PKCα mutant, which do not exhibit such selectivity, and (2) a GEE to GGE mutation abolishes the selectivity of targeting to cell-cell contacts for ε, as it does for the D294G PKCα mutant. Thus the GD(E)E motif may be part of a consensus sequence able to interact with shuttle and/or anchoring proteins. GFP-tagged deletion mutants also reveal a new function for the pseudosubstrate in the cytoplasmic sequestration. Together, these data underline the complexity of PKC subcellular targeting in the pituitary, determined by the cell-cell contact, at least for α and ε.

Key words: Protein kinase C, Targeting, Pituitary, Cell-cell contact

Introduction

The assembly/disassembly of macromolecular complexes located at cell-cell contacts is highly regulated and involves a balance of phosphorylation/dephosphorylation of many proteins that determines their localisation at cell-cell contacts. Protein kinase C (PKC) activation is involved in this balance (Cowell and Garrod, 1999; Lewis et al., 1994; Sheu et al., 1989; van Hengel et al., 1997; Williams et al., 1993) and several components of these complexes such as vinculin, an actin binding protein of adherent junctions, are PKC substrates (Perez-Moreno et al., 1998). In the pituitary gland, contacts between endocrine cells contain adherent junctions, gap junctions and desmosomes. In a recent study, we have shown that the PKC α isoform is selectively targeted to cell-cell contacts of the rat pituitary GH3B6 cell line upon a physiological stimulation [thyrotropin releasing hormone (TRH), an activator of thyroid stimulating hormone (TSH) and prolactin secretions] or a pharmacological stimulation [phorbol 12-myristate 13-acetate (PMA)] (Vallentin et al., 2000). Moreover, our data suggest that cell-cell contacts determine the on/off signal of this targeting since PKCα is not translocated to the plasma membrane of isolated GH3B6 cells. To our knowledge, this is the only study reporting such a selective compartmentalisation within the plasma membrane for a PKC isoform in epithelial cells, even though the presence of PKCα at cell-cell contacts has already been observed during spontaneous or TPA-induced compaction of the embryo (Pauken and Capco, 1999). This observation raises the question of the relevance of PKCα at cell-cell contacts within the tissue: does this targeting really occur in the pituitary gland? This question is of potential importance in view of the involvement of both PKC and cell adhesion in oncogenic transformation.

Functional alterations of PKC frequently occur in human tumours including pituitary tumours: most of the tumours exhibit variable levels in PKC accumulation in comparison with normal tissue (Alvaro et al., 1992; Hagiwara et al., 1990; O'Brian et al., 1989; Prevostel et al., 1995; Shimizu et al., 1991) and a mutated form of PKCα (the D294G-PKCα mutant) has been discovered by our group in a subpopulation of human pituitary (Alvaro et al., 1993) and thyroid tumours (Prevostel et al., 1995) which in addition, exhibit an accumulation of PKCα. Consistent with this, Schiemann et al. (Schiemann et al., 1997) have shown that pituitary tumours with no increase in PKCα accumulation do not contain the D294G mutation. More recently, Fagin and colleagues (Knauf et al., 2002) failed to confirm the presence of the D294G mutation in human thyroid tumours by using single-strand conformation polymorphism (SSCP) and specific allele oligonucleotide hybridization (SAOH) analyses of genomic DNA. As proposed by Fagin and colleagues, this may reflect the highly heterogenous distribution of cells containing the mutated form of PKCα within the tumour. However, in addition to its potential interest in tumourigenesis, the D294G mutation turned out to be a powerful tool to investigate the mechanisms underlying the selectivity of PKCα subcellular targeting. Indeed, upon activation, PKCα is no longer

selectively targeted to cell-cell contacts (Vallentin et al., 2001), indicating that translocation to cell-cell contacts is a highly regulated process, probably mediated through a specific targeting motif(s).

The D294G mutation is located within the third PKCα variable region (V3), also called the hinge region. Very little is known about the function of this region. In a previous study, we have established that the D294G mutation induces a selective loss in the recognition of substrates with properties of anchoring proteins (Prevostel et al., 1998). Recently, Parsons et al. (Parsons et al., 2002) have identified a binding motif for β1 integrin within the PKCα V3 region that is critical for directed tumour cell migration. Thus, these observations are strong arguments in favour of a major role of the V3 region in PKC functions.

In the present study, we establish that the specificity of PKCα targeting to cell-cell contacts does exist in the pituitary gland and that it is neither cell-type-, nor isozyme-specific since PKC epsilon (PKCε) is, similarly to PKCα, selectively targeted to cell-cell contacts upon TRH or TPA stimulation. In contrast, activated PKC delta (PKCδ) exhibits no selectivity in plasma membrane compartmentalisation. The comparison of PKCα, ε and δ protein sequences, together with the transient transfection of GFP-tagged PKCα, ε and δ mutants clearly demonstrates the importance of the GD(E)E motif in the specificity of targeting to cell-cell junctions. This motif is located within the V3 region of both PKCα and ε; it is absent in PKCδ and mutated in the D294G PKCα mutant. The use of PKCα deletion mutants indicates, however, that the C2 domain is also required for the targeting to cell-cell contacts.

Materials and Methods

Materials

pEGFP-N1 plasmid was purchased from Clontech (Palo Alto, CA, USA). Restriction enzymes were from Biolabs New England (Beverly, Ma). DNA polymerase (DyNAzyme) was from Finnzyme (Espoo, Finland). Synthetic oligonucleotides were purchased from Genset SA (France). The cDNAs encoding wild-type (wt) or D294G-mutated (D294D) PKCα were provided by V. Alvaro and B. I. Weinstein, Colombia Cancer Center, New York, USA. The plasmids containing the full-length PKCδ and ε cDNAs were given by P. J. Parker, Imperial Cancer Research Fund, London. The QuickChange™ site-directed mutagenesis kit was purchased from Stratagene. The GH-GFP transgenic mice expressing GFP under the control of the growth hormone promoter were a generous gift from Iain Robinson, National Institute for Medical Research Mill Hill, London, UK. The pituitary GH3B6 cell line was provided by Danielle Gourdji, CNRS UMR 7631, Paris, France. HAM F-10 medium and horse serum were obtained from Eurobio (Les Ulis, France). Foetal bovine serum was from Bio Media (Boussens, France). ExGen 500 (linear ethylenimin polymer), mouse monoclonal IgG1 PKCα antibody and rabbit polyclonal anti-PKCε antibodies were purchased from Euromedex (Souffelweyersheim, France). PMA, rabbit polyclonal anti-PKCα and rabbit polyclonal anti-PKCδ antibodies were purchased from Sigma (St Quentin Fallavier, France). Membrane Hybond C-Extra was from Amersham Pharmacia Biotech (Les Ulis, France). The monoclonal antibody against green fluorescent protein and the chemiluminescence detection kit were from Roche Molecular Biochemicals (Indianapolis, USA). Goat anti-mouse horseradish peroxydase-conjugated antibody was purchased from Chemicon (Temecula, USA). The goat anti-rabbit horseradish peroxides-conjugated antibody was from Pierce (Rockford, Ill, USA). The goat anti-rabbit Cy3 and Cy5, the goat anti-mouse (Fab')2 antibody Cy3 and the goat anti-rabbit Alexa-conjugated antibodies, were purchased from Jackson Immunoresearch laboratories Inc (Marseille, France).

Analysis of endogenous PKC α, ε and δ subcellular localisations

In rat and transgenic mice pituitary glands

Pituitary glands from adult rats or mice were incubated for 1 hour at 37°C with HamF10 medium supplemented with foetal calf and horse serums (see GH3B6 medium) with or without 100 nM PMA or TRH. Pituitary glands were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4 for 30 minutes, washed 3 times with PBS pH 7.4 and cut sagittally with a vibratome into 40 μm thick sections. Theses sections were carefully rinsed in PBS and subsequently incubated overnight at 4°C with several antibodies diluted in PBS pH 7.4 containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA): a rabbit polyclonal anti-PKCα (mouse sections), a mouse monoclonal PKCα (dilution: 1/250) (rat sections), a rabbit polyclonal anti-PKCε (dilution: 1/500), and a rabbit polyclonal anti-laminin (dilution: 1/250). After 3 washes for 10 minutes at room temperature in PBS pH 7.4 containing 0.1% Triton X-100 and 1% bovine serum albumin (BSA), the sections were incubated for 1 hour at room temperature with the secondary goat anti-rabbit or anti-mouse antibodies conjugated to indocarbocyanine 3 (Cy3) or Alexa (dilution: 1/1000). Immunostained sections were then mounted in Mowiol (Calbiochem, La Jolla, CA) and examined by confocal microscopy. Controls consisted of omitting the primary antibodies.

In GH3B6 cells

the GH3B6 cells were seeded on 12 mm round coverslips in 1 ml medium and grown for 24 hours. They were washed three times with PBS pH 7.4 before being fixed for 15 minutes with 4% paraformaldehyde in PBS pH 7.4 (v/v). The cells were then washed twice with PBS, permeabilised in 0.2% Triton X-100 for 5 minutes, washed in PBS and incubated for 30 minutes in PBS supplemented with 1% BSA (PBSA). The coverslips were then incubated overnight at 4°C with the primary mouse anti-PKCα (dilution: 1/250), and rabbit anti-PKCε (dilution: 1/500) antibodies. They were then washed three times for 10 minutes with PBSA, and further incubated for 1 hour at room temperature with the secondary antibodies, which were respectively, a goat anti-mouse (Fab′)2 Cy3 conjugated for PKCα and a goat anti-rabbit Cy3-conjugated antibody for PKCε (dilution: 1/1000). The coverslips were then mounted in Mowiol and examined by conventional microscopy (Zeiss) by using a ×100 objective.

Construction of plasmids encoding GFP-tagged PKC

The GFP-tagged proteins transiently expressed in GH3B6 cells are schematically represented in Fig. 1A,B. The corresponding PKCα constructs were generated as previously described (Vallentin et al., 2000). Briefly, PKCα deletion mutants were amplified by polymerase chain reaction (PCR) using the full-length wt, or D294G, PKCα cDNAs subcloned in the pBabe vector as a template. For the GFP-tagged full-length wt and D294G PKCα constructs the synthetic oligonucleotides used have been described by Vallentin et al. (Vallentin et al., 2000). To generate the (V1-C1)+V3, (V1-C1)+$V3_{D294G}$, C1+V3, C1+$V3_{D294G}$ constructs, the PCR amplified (V1-C1) and C1 were ligated in frame to the wt and D294G mutated V3 domains by using a *Xba*I restriction site. The oligonucleotides used were as follows: *forward:* 5′-GGAATTCCGGAGCAA-GAGGTGGTT-3′ for (V1-C1) and (V1-C1) in both (V1-C1)+V3 and (V1-C1)+$V3_{D294G}$; 5′-GGAATTCCATGCGCTTCGCCCGCAAA-GGG-3′ for ΔV1, 5′-GGAATTCCATGAACGTGCACGAG-GTGAAG-3′ for Δ(V1+PS), Δ(V1+PS)-RD, Δ(V1+PS)-RD+V3, Δ(V1+PS)-RD+$V3_{D294G}$ and C1 in both C1+V3 and C1+$V3_{D294G}$;

5′-GGAATTCCATGATTCCGGAAGGGGACGAG-3′ for V3; 5′-GGAATTCCATGATTCCGGAAGGGGGCGAG-3′ for V3D294G; 5′-TGCTCTAGAGCAATTCCGGAAGGGGACGAG-3′ for V3 in (V1-C1)+V3 and C1+V3; 5′-TGCTCTAGAGCAATTCCGGAAGGGGGCGAG-3′; for V3D294G in (V1-C1)+$V3_{D294G}$ and C1+$V3_{D294G}$. *Reverse:* 5′-GGGGTACCCCTACTGCACTCTGTAAGATG-3′ for ΔV1 and Δ(V1+PS); 5′-GGGGTACCCCCCGCCCCCTCTTCTCAGT-3′ for (V1-C1) and C1; 5′-TGCTCTAGAGCACCGCCCCCTCTTCTCAGT-3′ for (V1-C1) in both (V1-C1)+V3 and (V1-C1)+$V3_{D294G}$, and C1 in both C1+V3 and C1+$V3_{D294G}$; 5′-GGGGTACCCCCGTGAGTTTCACTCGGTC-3′ for Δ(V1+PS)-RD+V3, Δ(V1+PS)-RD+$V3_{D294}$, V3, V3 in (V1-C1)+V3 and C1+V3, V3D294G, V3D294G in (V1-C1)+$V3_{D294G}$ and C1+$V3_{D294G}$. All PCR amplified DNA fragments were gel purified and subcloned in frame to GFP within the *Eco*RI and *Kpn*I restriction sites of the pEGFP-N1 plasmid.

The constructs encoding the GFP-tagged PKCδ and ε were obtained by using a similar procedure, with the exception that full-length PKCδ and ε cDNAs were subcloned within the *Xho*I and *Kpn*I restriction sites of the pEGFP-N1 plasmid. The synthetic oligonucleotides used in the PCR amplification were as follow: forward 5′-CCTCGAGGATGGCACCGTTCCTGCGC-3′ and reverse 5′-GGGGTACCCCTTCCAGGAATTGCTCATATTT-3′ for PKCδ; forward 5′-CCTCGAGGATGGTAGTGTTCAATGGCCT-3′ and reverse 5′-GGGGTACCCCGGGCATCAGGTCTTCACCA-3′ for PKCε. All constructs were sequenced.

Site directed mutagenesis

The point mutated PKCδ and ε were created by using the QuickChange™ site-directed mutagenesis kit according to the manufacturer's standard protocol. The pairs of synthetic oligonucleotides used to obtain the mutated PKCs were as follows: forward 5′-GACAACCGAGGAGGGGAGCACCGAGCC-3′ and reverse 5′-GGCTCGGTGCTCCCCTCCTCGGTTGTC-3′ for E374G-PKCε; forward 5′-GTCGGAATATACCAGGGAGAGGAGAAGAAGACA-3′ and reverse 5′-TGTCTTCTTCTCCTCTCCCTGGTATATTCCGAC-3′ for F314E-PKCδ. Mutated PKC were fully sequenced by Genome express (Meylan, France).

Analysis of GFP tagged PKC subcellular location in living cells

Cells were cultured as previously described (Vallentin et al., 2000). Transient transfection experiments were carried out with ExGen 500 according to the manufacturer's standard protocol. Briefly, the cells were seeded at 300,000 cells per well in a 24-well dishes (Falcon) 18 hours before transfection. Immediately before transfection, fresh culture medium (1 ml) was added to the cells. ExGen 500 stock solution (1.25 μl) was diluted in 12.5 μl NaCl 150 mM. The plasmid DNA (250 ng/well) was diluted in 12.5 μl of NaCl 150 mM. These solutions were then mixed together. After 30 minutes, the transfection mixture was added to the cells. The 24-well dishes were then centrifuged for 5 minutes at 280 ***g*** and maintained for 48 hours at 37°C. Subcellular localisation of GFP-tagged PKC was monitored under basal or TPA stimulated conditions (100 nM for 1 hour). At the time of the observation, the culture medium was replaced by a phosphate-buffered saline solution (pH 7.4). The localisation of the fusion proteins was examined by conventional microscopy. All experiments were performed at least three times.

Western blot analysis

Transfected GH3B6 cells were washed in PBS before being lysed and boiled in 150 μl of Laemmli buffer. Equal amounts of proteins were loaded for 12% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked with Tris-buffered saline (50 mM Tris, 150 mM NaCl) containing 10% milk powder for 1 hour at room temperature. The membrane was then incubated with a monoclonal anti-GFP antibody (1/1000) at 4°C overnight. After washing in Tris-buffered saline containing 0.1% Tween, membranes were incubated with a goat anti-mouse horseradish peroxydase-conjugated antibody. The immunoreactive bands were revealed using a chemiluminescence detection kit. Fig. 1C,D attest that the GFP-tagged PKC were expressed at the expected size.

Results

Activated PKCα is selectively targeted to cell-cell contacts in the pituitary gland

In the rat pituitary GH3B6 cell line, activated PKCα accumulates specifically at cell-cell contacts (Vallentin et al., 2000). In order to investigate the relevance of this observation in the tissue, we performed immunostaining experiments on sagittal slices of unstimulated and TRH- (Fig. 2) or TPA- (data not shown) stimulated rat pituitary glands. The concentration of 100 nM TRH was chosen to ensure a maximal effect of TRH, taking into account that TRH may be degraded and that it has to penetrate the tissue. In unstimulated glands, PKCα was mainly located in the cytoplasm. Upon TRH or TPA stimulation, the kinase was translocated to cell-cell contacts. Laminin immunostaining indicated that PKCα was completely excluded from the contacts between cells and the extracellular matrix (Fig. 2). The selective translocation of PKCα to cell-cell contacts was observed in a larger number of cells upon TPA stimulation than upon TRH stimulation. This was expected since not all pituitary endocrine cells express TRH receptors. In order to determine whether the selective targeting of PKCα occurs between homotypic and/or between heterotypic cells, we used pituitary glands of transgenic mice expressing GFP under the control of the GH promoter (Magoulas et al., 2000). As shown in Fig. 3A, PKCα selective targeting could indeed be observed between homotypic as well as between heterotypic cells upon TPA stimulation. Thus, the selectivity in PKCα targeting to cell-cell contacts does exist in the pituitary gland, is not restricted to the rat species, and is not cell type specific.

The targeting selectivity to cell-cell contacts is not restricted to the classical PKCα isoform

The distinct subcellular location of each PKC isoforms is supposed to be closely linked to their different biological functions (Csukai and Mochly-Rosen, 1999), raising the question of whether specific targeting to cell-cell contacts is restricted to the α isoform, and thus to its function in the pituitary. As shown in Fig. 3B, PKCε is, similarly to the α isoform, located in the cytoplasm in basal conditions and selectively translocated to cell-cell contacts upon TRH or TPA stimulation of pituitary glands of transgenic mice expressing GFP specifically in GH cells. Upon TPA stimulation, the presence of PKCε at cell-cell contacts was observed in GFP-positive as well as in GFP-negative cells. Upon TRH stimulation, translocation of PKCε to cell-cell contacts also occurred although to a lesser extent.

In TPA stimulated GH3B6 cells, PKCα and ε were both located at cell-cell contacts (Fig. 4), indicating that this cell line is a valuable model for investigating the molecular basis underlying PKC subcellular targeting.

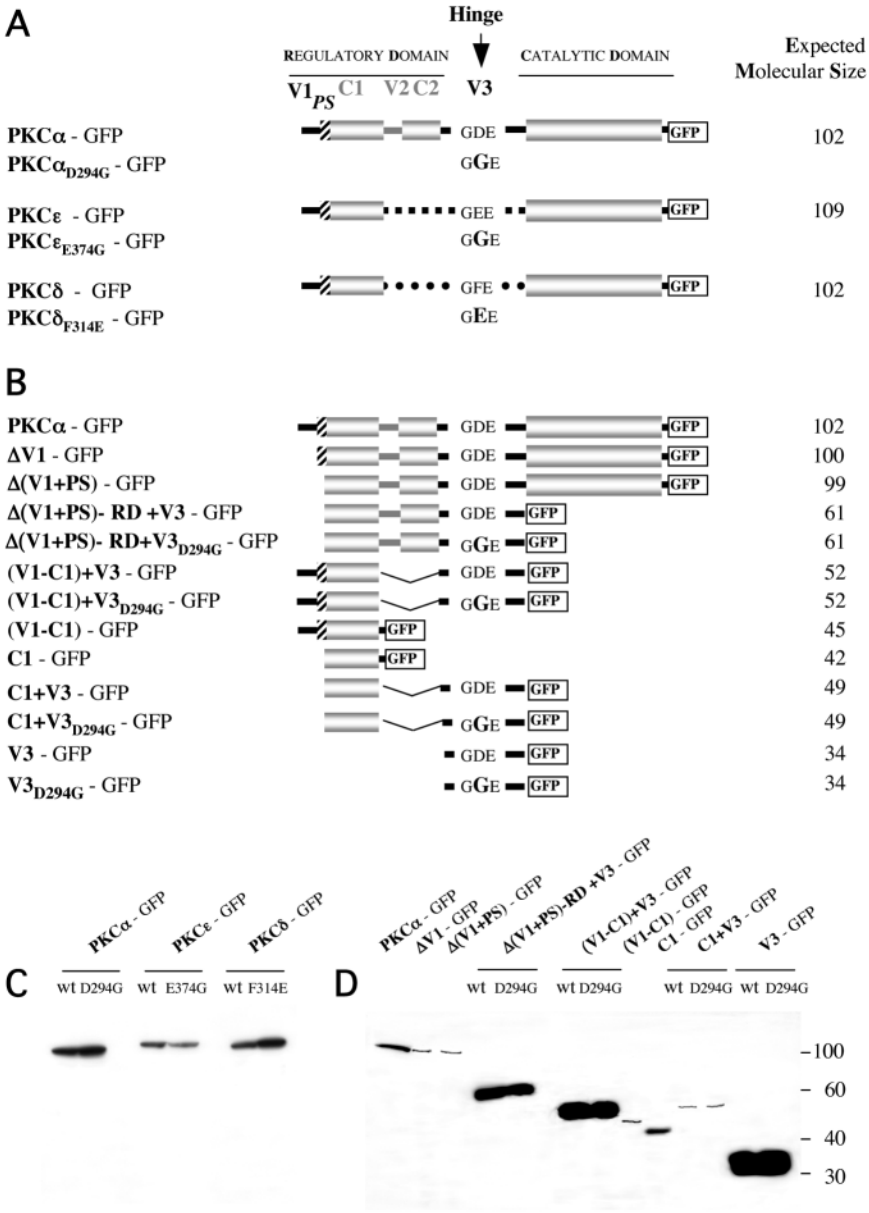

Fig. 1. Schematic view and expected molecular size (A,B) of the generated GFP-tagged wild-type and mutated PKC. (C,D) Western blot analysis of the fusion proteins transiently transfected in the GH3B6 cell line. A positive immunoreactive band was evidenced for each GFP tagged protein at the appropriate molecular size.

The observation that the selectivity of targeting to cell-cell contacts is not restricted to PKCα suggests that a common mechanism is involved in the targeting of both isoforms, despite distinct activation modes: calcium-dependent for α and calcium-independent for ε.

A critical role for the GD(E)E motif located within the V3 variable region of PKCα and ε in the selectivity of targeting to cell-cell contacts

The point mutation leading to the substitution of an aspartic acid by a glycine in the hinge region of PKCα (position 294) is sufficient to induce a loss in the specificity of targeting to cell-cell contacts (Vallentin et al., 2001). The sequence alignment of PKCα and ε reveals the presence of similar motifs (GDE, GEE) in their V3 regions (see Fig. 1A) If this motif is really involved in the selectivity of targeting, the GEE to GGE substitution in PKCε might have the same effect as the GDE to GGE substitution in the D294G mutant of PKCα, that is a loss of cell-cell contact targeting selectivity. We therefore introduced the E374G mutation in PKCε and tested its impact on the selectivity of targeting. Both GFP-tagged mutated and wt PKCε were transiently transfected into GH3B6 cells. As shown in Fig. 5, the E374G mutation of PKCε was, similarly to the D294G mutation of PKCα, sufficient to abolish the selectivity of targeting to cell-cell contacts upon TPA stimulation. This demonstrates that the GD(E)E motif plays a crucial role in the selectivity of PKC targeting to cell-cell contacts.

The F314E point mutation in PKCδ V3 region does not induce cell-cell contact targeting

Further comparison of the subcellular localisation and proteic sequences of PKC α, ε and δ indicated that the V3 region is not the only region responsible for cell-cell targeting. The subcellular location of GFP-tagged PKCδ in GH3B6 cells is shown in Fig. 5, in comparison to those of PKCα and ε. In contrast to the GFP-tagged PKCα and ε, the GFP-tagged PKCδ is targeted to the entire plasma membrane upon activation. An analysis of the proteic sequence of the V3 region of PKCδ indicated the presence of a GFE motif instead of GD(E)E (Fig. 1A). This, together with the observation that PKCδ is not selectively targeted to cell-cell contacts, argues for the importance of the GD(E)E motif in the selectivity of targeting. However, the F314E mutation introduced in the V3 region of PKCδ in order to restore the GD(E)E motif, was not able to target PKCδ to cell-cell contacts (Fig. 5). This therefore indicates that the GD(E)E motif is essential but is not on its own sufficient to confer the selectivity of targeting. This motif could therefore be part of a binding domain for an anchoring/cargo protein, the expression or post-transductional properties of which are determined by the cell-cell contact formation (see Discussion).

The PKCα V3 region does not contain the whole information for the selectivity of targeting

According to our previous data, the PKCα V3 region alone is not spontaneously located at cell-cell contacts (Vallentin et al., 2000). Thus, either V3 does not contain the whole targeting

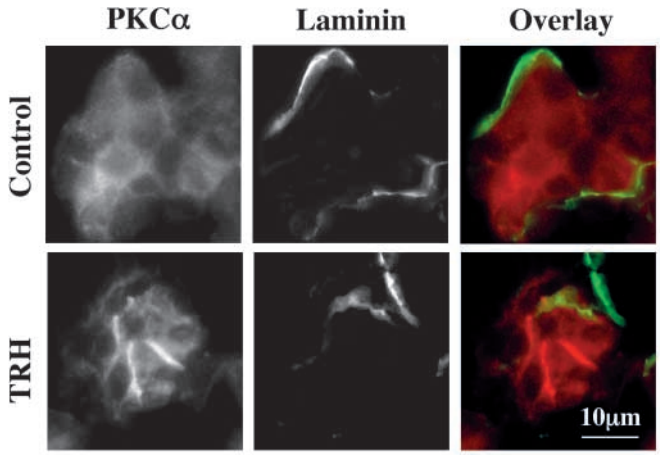

Fig. 2. PKCα and laminin immunostaining of sagittal sections (40 μm) of unstimulated and TRH-stimulated rat pituitary. Pituitary glands were removed from the animal and directly incubated in HamF10 culture medium containing 100 nM TRH for 1 hour. Pituitary glands were fixed in paraformaldehyde, cut into 40 μm sections and treated as described in the Materials and Methods. Note that PKCα is, as expected, located in the cytoplasm in unstimulated conditions and selectively translocated to cell-cell contacts upon stimulation. PKCα is excluded from cell-matrix contacts as attested by the laminin immunostaining.

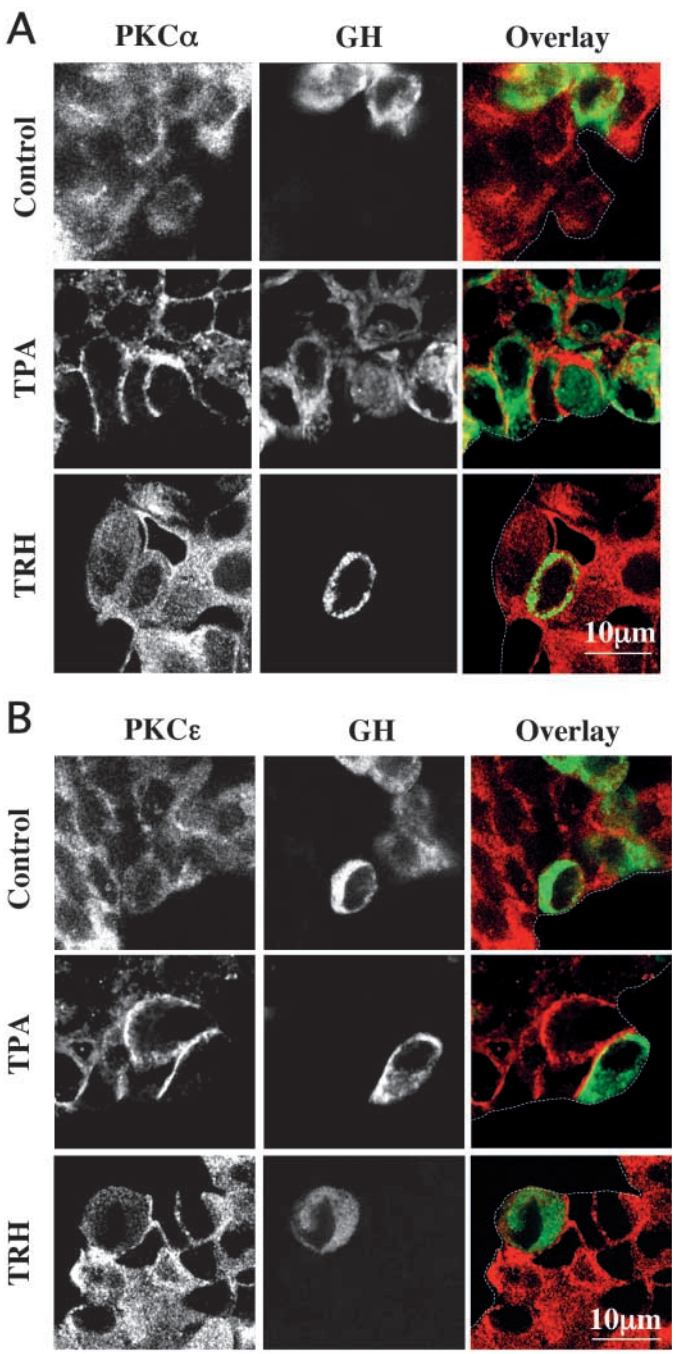

Fig. 3. Subcellular localisation of PKCα (A) and PKCε (B) in unstimulated and TPA or TRH stimulated pituitary glands of transgenic mice exhibiting GFP-GH expressing cells. Pituitary glands were removed from the animals, incubated in HamF10 culture medium containing or not 100 nM TPA or TRH for 1 hour, fixed in paraformaldehyde, cut into 40 μm sections and immunostained with antibodies raised against the α and ε PKC isoforms. Both enzymes are cytoplasmic under unstimulated conditions and selectively translocated to cell-cell contacts of green fluorescent GH cells as well as a subpopulation of GFP-GH-negative cells.

sequence or it does contain the targeting sequence but it is not able to accumulate at cell-cell contacts. Since the C1 domain alone can act as a plasma membrane-targeting module in response to TPA (Oancea et al., 1998), we postulated that its association with V3 might suffice for cell-cell contact specificity of targeting upon TPA stimulation. However, as shown in Fig. 6A, the C1 domain did not translocate when associated to the wt (or the D294G mutated) V3 region, although, consistent with the results of Oancea et al. (Oancea et al., 1998), C1 alone was efficiently translocated to the plasma membrane upon TPA stimulation. Therefore, the association of V3 to the C1 domain may induce conformational changes that may abolish the ability of C1 to interact with TPA.

Another possible explanation is that the V3 region inhibits the translocation of the C1 domain. Consistent with this, our previous data have established that the V3 region is involved in the cytoplasmic sequestration of PKCα. In the presence of V1 and the pseudosubstrate (PS), translocation of C1-V3 (or C1-$V3_{D294G}$) is restored but without any selectivity for cell-cell contacts, which was of course expected for C1-$V3_{D294G}$ but not for C1-V3 (Fig. 6B). This not only mean that V1-PS might play an important role in the translocation of C1 as it does for the entire protein (Vallentin et al., 2000), but that the V3 region does not contain the whole information to target PKCα to cell-cell contacts. Interestingly (Fig. 5B, bottom), when C1 was associated with V1, it did not translocate either. This suggests that the mechanisms by which V1 and V3 block translocation of C1 may be related to each other.

The PKCα cell-cell contact targeting motif is located within the C2-V3 region

The above results together with our previous data suggest that the V1 region may be important for translocation/localisation of PKCα to/at the plasma membrane but is not required for the cell-cell contact targeting selectivity. This is consistent with the

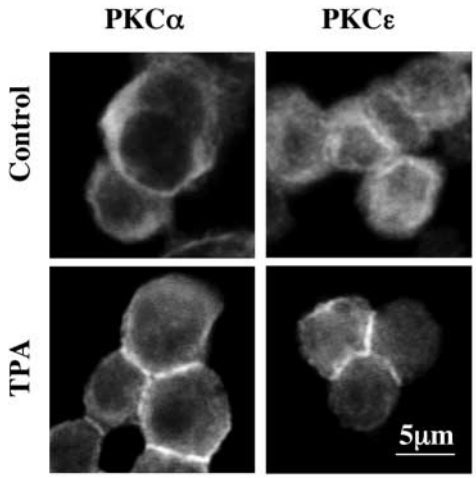

Fig. 4. Subcellular localisation of endogenous PKC α and ε in unstimulated and TPA-stimulated GH3B6 cells. Cells were incubated in the presence of 100 nM TPA for 1 hour and immunostained as described in Materials and methods. PKC α and ε are selectively accumulated at the cell-cell contacts in the presence of TPA.

fact that PKCα and ε are both selectively translocated to cell-cell contacts, despite the fact that they exhibit strong differences within their V1 regions. In agreement with this, Fig. 7A shows that although the removal of the V1 domain alone abolishes the TPA-induced translocation of PKCα to the plasma membrane, a further deletion of the pseudosubstrate is able to restore the selective translocation of PKCα to cell-cell contacts. Thus the N-terminal part of the protein, including both V1 and the pseudosubstrate, is not required for the selectivity of targeting to cell-cell contacts, even though it is involved in the control of translocation. Fig. 7B also establishes that the catalytic domain is also not required for the selectivity of PKCα to cell-cell contacts. Indeed, the PKCα region, including C1 to V3, translocates selectively to cell-cell contacts. As expected, the same region bearing the D294G mutation has lost targeting selectivity to cell-cell contact and accumulates at the entire plasma membrane. The amino acid sequence that determines the selectivity of PKCα targeting to cell-cell contacts is thus probably located within the C2-V3 region.

Discussion

In the present study, we show that PKCα is targeted to cell-cell contacts of TRH or TPA stimulated pituitary glands. This phenomenon, previously observed in monolayer cultures of stimulated pituitary GH3B6 cells may thus be relevant. Moreover, we provide evidence for the existence of a new mechanism involved in the control of PKC targeting to cell-cell contacts that not only concern PKCα but also PKCε. The selectivity of targeting of both isoforms to cell-cell contacts requires a three amino acid-motif located in the V3 hinge regions of PKCα and PKCε, although this motif is not sufficient by itself. These observations raise the question of the role of PKCα and PKCε at cell-cell contacts in the pituitary gland and of the molecular events involved in the targeting selectivity.

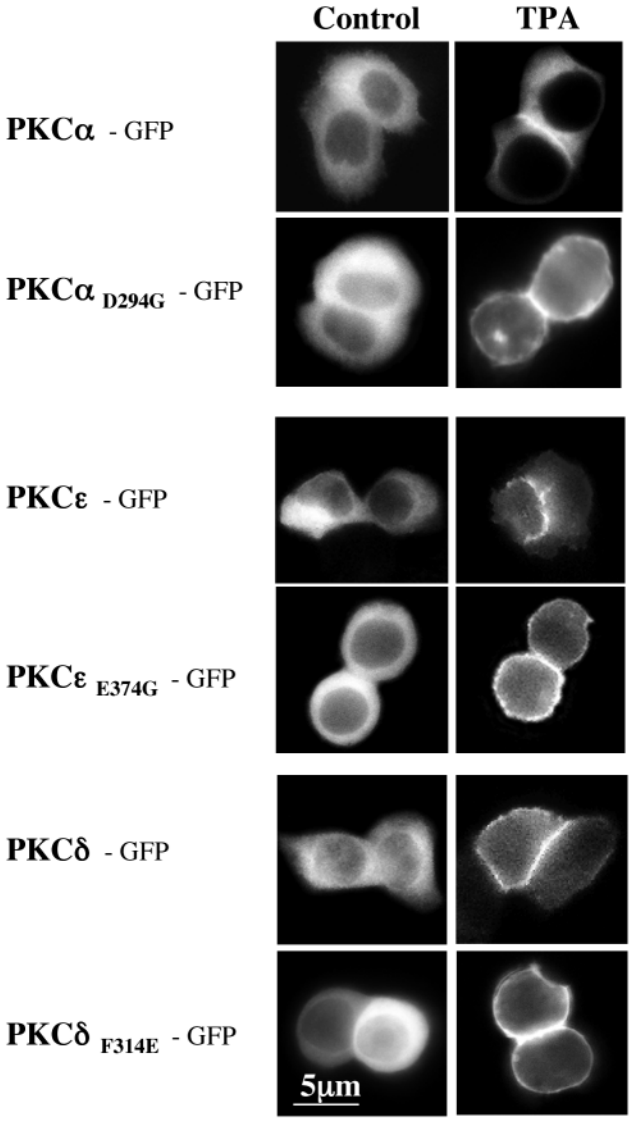

Fig. 5. The GD(E)E motif located in the V3 region of both PKCα and ε is critical but not sufficient for the targeting to cell-cell contacts. GFP-tagged wild-type and mutated PKCα, ε and δ were transiently transfected in GH3B6 cells. Cells were stimulated or not with 100 nM TPA. A selective translocation to cell-cell contacts was only observed for the wild-type PKCα and ε upon TPA stimulation. This targeting was abolished when the GD(E)E motif within the V3 region of both isoforms was mutated (mutants PKCα-D294G and PKCε-E374G). The presence of this motif is however not sufficient to target PKCδ to cell-cell contacts.

Possible role(s) for PKCα and PKCε at cell-cell contacts in the pituitary gland

To our knowledge, the only immunocytochemical analysis of the localisation of PKC subtypes in normal rat pituitary cells was performed on primary cultures, in a situation where cell-cell contacts as well as cell-extracellular matrix contacts were abolished (Naor, 1990). In the present study, we report for the first time that upon a physiological (TRH) or a pharmacological stimulation (TPA) of the pituitary gland, PKCα is selectively targeted to the contacts between GH-expressing or GH-non expressing cells. This indicates that the

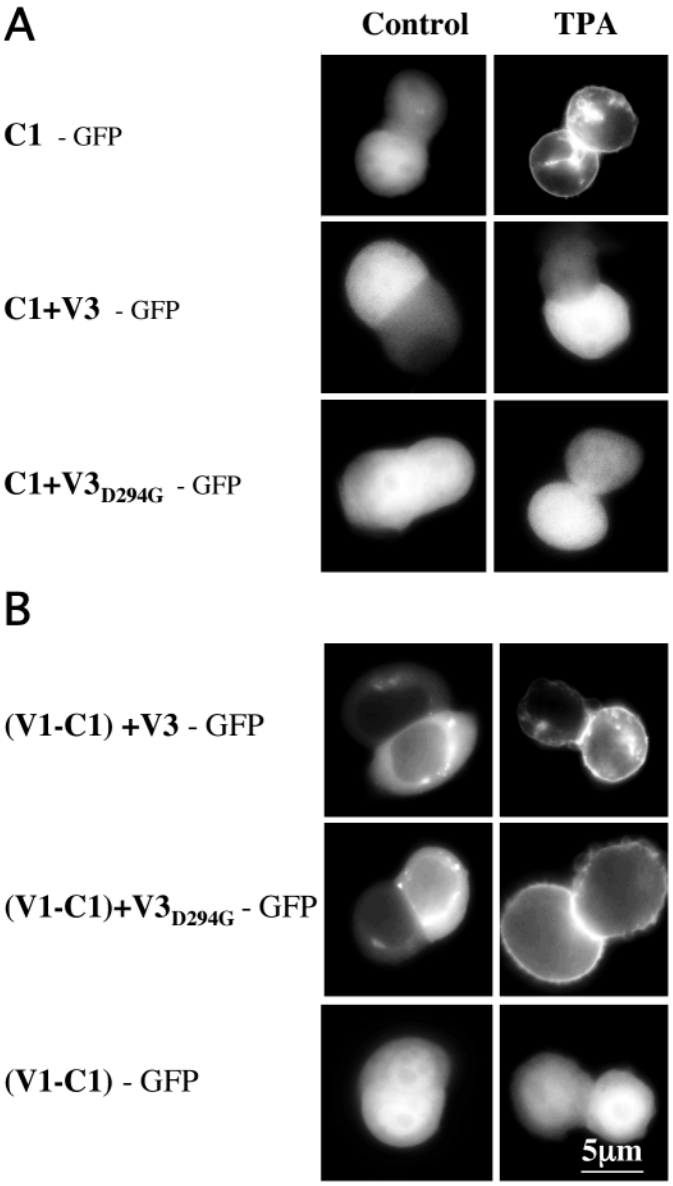

Fig. 6. The V3 variable region does not contain the full information to target PKCα to cell-cell contacts**. (**A) Although C1-GFP is, as expected (Oancea et al., 1998), translocated to the plasma membrane upon TPA stimulation, the (C1+V3)-GFP wild-type and mutated constructs do not translocate in similar conditions. (B) the N terminus of PKCα, including the variable V1 and the pseudosubstrate regions, restores targeting to the plasma membrane with no selectivity for cell-cell contacts.

molecular mechanisms that determine the selectivity of PKCα targeting to cell-cell contacts is not cell-type-specific. Interestingly, the selectivity of targeting to cell-cell contacts is isozyme-specific although it is not restricted to PKCα. Indeed, whereas PKCα and the novel PKCε are selectively translocated to cell-cell contacts upon activation, PKCδ is translocated to the entire plasma membrane. Together, these data raise the question of the physiological role(s) of both PKCα and ε at cell-cell contacts.

The role of a kinase at a particular subcellular site is dependent on the dynamics of its own accumulation at that location, as well as that of its substrates. In a previous study, we showed that a TRH stimulation induces two phases of PKCα translocation in GH3B6 cells (Vallentin et al., 2000): a short and transient phase occurring a few seconds after the beginning of the stimulation and a long lasting phase. The dynamics of PKCα and ε translocation have now to be further analysed in the intact pituitary gland in order to determine whether these two phases of translocation exist in the tissue and to clarify the respective roles of these two PKC isoforms at cell-cell contacts. A role in the regulation of hormonal secretion or cell proliferation is expected for PKCα and partly demonstrated for PKCε (Akita et al., 1994). Furthermore, according to Akita et al. (Akita et al., 2000), MARCKS, a regulatory component of the cytoskeletal architecture, is a major substrate of PKCε in vivo, and its phosphorylation may regulate TRH-stimulated hormonal secretion. It will, therefore, be important to know whether this phosphorylation occurs at cell-cell contacts or not. The fact that the release of hypothalamic hormones is pulsatile has also to be considered. For example, the interplay between somatostatin and growth hormone releasing hormone determines the release pattern of growth hormone (Tannenbaum, 1984). This means that the receptors for these hormones are not under a constant activation, and may explain the need for the alternation between the cytoplasmic and cell-cell contact localisation of PKCα and ε. The stimulation by TRH or PMA may amplify the accumulation of PKCα and ε at their physiological subcellular locations. Several reports also argue in favour of a specific role of PKCα and PKCε in regulating intercellular communication via cell-cell contacts. Indeed, according to Perez-Moreno et al. (Perez-Moreno et al., 1998) the PKC-mediated phosphorylation of vinculin is required for the redistribution of both vinculin and α-actinin from the cytoplasm to the cell periphery and may be a crucial step for the assembly of adherent junctions. To the same extent, the TRH-induced translocation of PKCα at contacts between GH3B6 cells is associated with a redistribution of β-catenin to the same site (Vallentin et al., 2001). Finally, Sheu et al. (Sheu et al., 1989) have reported that the desmosomal proteins desmoplakins are redistributed from the cytoplasm to the plasma membrane upon PKC stimulation and that this redistribution is accompanied by desmosomal formation. Desmoplakins are phosphorylated on both serine and threonine residues although it is not clear whether this phosphorylation is mediated by PKC (Pasdar et al., 1995).

What determines the selectivity of subcellular PKCα and ε targeting?

The presence of a PKC isoform at the plasma membrane has long been thought to depend on its interaction with phospholipids, such as phosphatidylserine. This was the dogma until the receptor for activated kinase C (RACK-1) was discovered (Mochly-Rosen et al., 1991a; Mochly-Rosen et al., 1991b). Since then, concepts have evolved towards the idea that accumulation of PKC at a particular cell location can be mediated by anchoring proteins (Mochly-Rosen and Gordon, 1998). In GH3B6 cells and in the pituitary gland, activated PKCα and ε are compartmentalised at cell-cell contacts. This compartmentalisation is determined by the cell-cell contact since PKCα and ε do not translocate in isolated cells, despite the activation of the TRH receptors (Vallentin et al., 2000).

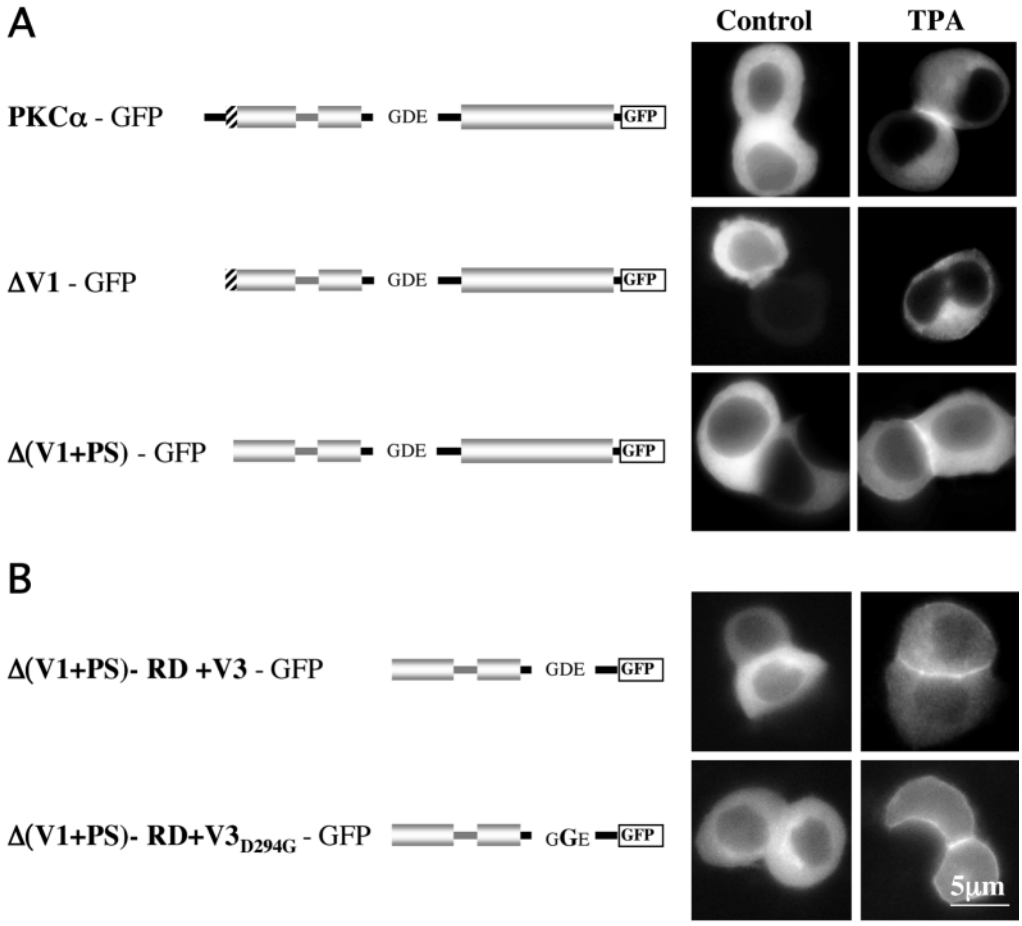

Fig. 7. Cell-cell contact targeting may be controlled through the PKCα C2-V3 region. (A) although the deletion of the V1 domain abolishes PKCα translocation, the selective targeting to cell-cell contacts is restored in the absence of both V1 and the pseudosubstrate domain. Thus, the PKCα N terminus including the V1 and the pseudosubstrate domains is not required for PKC targeting to cell-cell contacts. (B) A further deletion of the catalytic domain does not affect the targeting to cell-cell contacts. A comparison of the (C1-V3)-GFP and the (C1+V3)-GFP (see Fig. 6A) subcellular locations suggests that the C2 region may also be required for the selectivity of targeting to cell-cell contacts.

Two questions arise: is there a restricted amino acid sequence in PKC responsible for this selectivity and what is the cell-cell contact-induced signal responsible for the selectivity?

A restricted amino acid sequence involved in targeting selectivity

The simplest way to address this is to distinguish the different steps of what is called 'translocation'. Activation is a prerequisite for translocation and, at least for PKCα, calcium has been shown to be necessary: without an increase in the $[Ca]_i$, PKCα does not translocate. This process involves the C2 domain (Bolsover et al., 2003; Raghunath et al., 2003). Although PKCα and ε targeting to cell-cell contacts only occurs upon stimulation, the mechanism determining the selectivity of targeting observed in pituitary cells probably co-exists or pre-exists this activation process. This selectivity of targeting is however not calcium dependent since the novel PKCε is calcium independent. When PKCα and ε are mutated in their V3 region (D294G and E374G mutations respectively), the selectivity is abolished although translocation still occurs. The GDE/GEE motif, affected by these point mutations, may thus be involved in the recognition of a cell-cell contact-induced signal. These are not the first data demonstrating an involvement of the V3 region in the control of PKC targeting or in the interaction with anchoring proteins. Indeed, Parsons et al. (Parsons et al., 2002) have recently established that a 12 amino-acid motif within the PKCα V3 region (aa 313-325) is required for the direct association of PKCα with β-integrin. However, the cell-cell contacts targeting selectivity requires an additional sequence that is, for PKCα, located in the C2 region. Indeed, the C1 module requires the presence of C2 in addition to V3 in order to be targeted to cell-cell contacts. Our data suggest that a similar sequence exists in PKCε even though it has not yet been identified. In contrast this sequence is probably not present in PKCδ, another novel PKC isoform since the introduction of the GEE motif in PKCδ (which possesses a GFE motif instead of the GDE/GEE motif), is not sufficient to target PKCδ to cell-cell contacts.

The present work also suggests that the pseudosubstrate sequence is involved in the targeting through a mechanism distinct from the commonly accepted one implicating the binding to the catalytic core. Indeed, the deletion of the V1 region inhibits PKCα translocation but the translocation is recovered by further deleting the PS (Fig. 6). To the same extent, the C1 module accumulates uniformly at the plasma membrane upon TPA stimulation but no longer translocates a when fused to the PS (data not shown). This means that an additional level in the control of translocation may well exist, such as an off/on signal involving the PS/V1 sequence. The PS sequence is already known to be able to bind proteins, such as cytoskeletal proteins (Garcia-Rocha et al., 1997; Liao et al., 1994; Schmitz-Peiffer et al., 1998), lending support to our hypothesis.

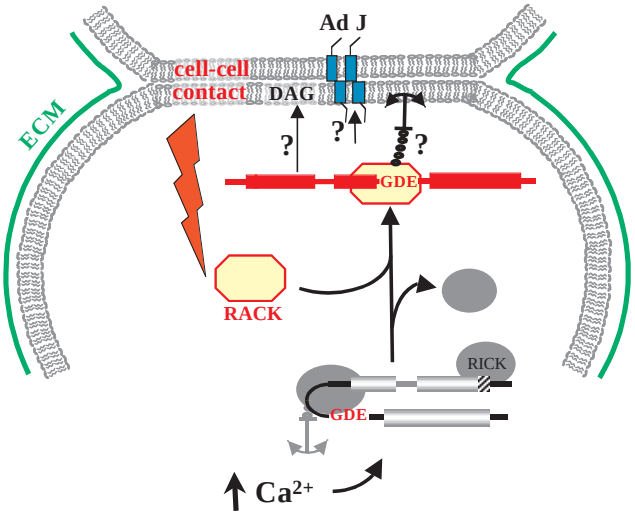

Fig. 8. Hypothetical model of the subcellular targeting of PKCα. PKCα translocation to the plasma membrane is regulated through at least two distinct mechanisms: a selective targeting to cell-cell contacts mediated through the C2-V3 region (and at least the V3 domain of ε) and a non-selective targeting to the entire plasma membrane involving the V1 and the pseudosubstrate domains. In both cases the translocation to the plasma membrane may require the release of PKCα from a cytoplasmic anchoring protein, which should be a RICK. This protein, involved in the cytoplasmic sequestration of PKCα, would interact with the C2-V3 region and possibly also with the pseudosubstrate region. Once released from binding to the cytoplasmic anchoring protein, C2-V3 would bind another protein, a RACK, whose expression or ability to bind to PKC is induced by cell-cell contacts. This protein may be either a shuttle or a cargo/anchoring protein that selectively targets PKC to cell-cell contacts upon stimulation. RICK, receptor for inactive c-kinase; RACK, receptor for active c-kinase; Ad J, adherens junctions; ECM, extracellular matrix; DAG, diacylglycerol.

A cell-cell contact-induced signal responsible for the selectivity

The D294G mutation induces a selective loss in the recognition of substrates exhibiting anchoring protein properties without affecting PKCα catalytic activity (Prevostel et al., 1998). This suggests that the selectivity of PKCα targeting to cell-cell contacts might be mediated through protein-protein interactions. The D294G mutant may not be able to interact with those proteins anymore, its translocation being probably governed only by the changes in $[Ca^{2+}]_i$ and DAG. As soon as two cells make contact, it generates a signal able to determine translocation and selectivity of translocation of PKCα and ε. This signal could be either the synthesis or the relocalisation of a PKC interacting protein. This cell-cell adhesion-dependent factor could play the role of a shuttle to target PKC at cell-cell contacts. This is not RACK-1 since we have previously shown that it is excluded from cell-cell contacts in the GH3B6 cell line and is not co-immunoprecipitated with PKCα upon PMA stimulation (Vallentin et al., 2001). The characterisation of this factor is under investigation.

According to our hypothetical model (Fig. 8), inactive PKCα is sequestered in the cytoplasm through the binding of its C2 and V3 regions with a cytoplasmic RICK (receptor for inactivated C kinase) protein. The pseudosubstrate sequence is bound to the catalytic core and to a protein that may or may not be distinct from the C2-V3 binding protein. The signal induced by intercellular adhesion, probably a RACK, is present in the cytoplasm. Upon activation (increase in $[Ca^{2+}]_i$), the affinity of C2-V3 for the cytoplasmic anchoring protein decreases whereas its affinity for the intercellular adhesion-induced RACK increases. The interaction of PKC with this protein induces its selective targeting to cell-cell contacts. The RACK protein may only be a shuttle that transports PKC to cell-cell contacts where it can interact either directly with DAG or with existing macromolecular complexes such as adherens junctions. Alternatively, the RACK protein may be a cargo that also mediates PKC anchoring at the cell-cell contact. This model proposes a new mechanism for the regulation of PKC activity based on a co-ordinated control of cytoplasmic sequestration and targeting to cell-cell contacts. It is based on the existence of a cell-cell contact determined signal(s).

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