

A Spatiotemporally Coordinated Cascade of Protein Kinase C Activation Controls Isoform-Selective Translocation†

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In pituitary GH3B6 cells, signaling involving the protein kinase C (PKC) multigene family can self-organize into a spatiotemporally coordinated cascade of isoform activation. Indeed, thyrotropin-releasing hormone (TRH) receptor activation sequentially activated green fluorescent protein (GFP)-tagged or endogenous PKC β 1, PKC α , PKC ϵ , and PKC δ , resulting in their accumulation at the entire plasma membrane (PKC β and δ) or selectively at the cell-cell contacts (PKC α and ϵ). The duration of activation ranged from 20 s for PKC α to 20 min for PKC ϵ . PKC α and ϵ selective localization was lost in the presence of Gö6976, suggesting that accumulation at cell-cell contacts is dependent on the activity of a conventional PKC. Constitutively active, dominant-negative PKCs and small interfering RNAs showed that PKC α localization is controlled by PKC β 1 activity and is calcium independent, while PKC ϵ localization is dependent on PKC α activity. PKC δ was independent of the cascade linking PKC β 1, α , and ϵ . Furthermore, PKC α , but not PKC ϵ , is involved in the TRH-induced β -catenin relocation at cell-cell contacts, suggesting that PKC ϵ is not the unique functional effector of the cascade. Thus, TRH receptor activation results in PKC β 1 activation, which in turn initiates a calcium-independent but PKC β 1 activity-dependent sequential translocation of PKC α and ϵ . These results challenge the current understanding of PKC signaling and raise the question of a functional dependence between isoforms.

Space and time parameters are intimately linked to the biological function of proteins and are pivotal in the organization and functioning of living matter. Particularly important in the case of intracellular signaling networks, this spatiotemporal constraint determines the modalities by which a given protein interacts with its partners in order to exchange information. Efforts are currently being made to translate the experimental evidence of this plasticity into a visualization concept of dynamic signaling networks (4, 22). A degree of complexity is added when different isoforms of a protein coexist within the same cell and when they all potentially respond to the same stimulus, which is the case of the protein kinase C (PKC) family.

The PKC family is composed of at least 11 isoforms. Several isoforms usually coexist within a given cell type, and each isoform is thought to mediate distinct cellular functions leading to proliferation, differentiation, apoptosis, or secretion. PKC function requires its translocation to a membrane compartment. The current understanding of PKC translocation/activation has been largely based on the work of Oancea and

Meyer (34), who presented conventional PKCs (cPKCs) and novel PKCs (nPKCs) as molecular machines responsible for decoding calcium and/or diacylglycerol (DAG)-mediated signals. Several observations suggest, however, that other, as yet unknown, parameters are involved in the temporal organization of PKC signaling. Indeed, despite similarities in sequence and cofactor regulation by DAG and Ca²⁺, the conventional PKC β I and PKC β II were shown to exhibit isoform-specific oscillation patterns following metabotropic glutamate receptor 1a (mGluR1) activation (3). This behavior was interpreted by the authors as a differential decoding of second messengers, although the key amino acids involved were located in the V5 region of PKC β II, which is not involved in binding to cofactors. Also, during mammalian egg activation, Eliyahu and Shalgi have shown the translocation of PKC α , PKC β 1, and PKC β 2 but noted that only PKC α translocation did not occur in the presence of ionomycin, a calcium ionophore known to elicit cPKC translocation in many systems (14). In a recent work, Braun et al. provided evidence that PKC α translocation was determined primarily by factors other than the localization of phorbol esters, unlike PKC δ translocation (5). Raghunath et al. (44) analyzed the translocation of PKC α , β 2, δ , and ϵ in neuroblastoma cells upon exposure to the muscarinic receptor agonist carbachol. These authors observed sustained translocations of PKC ϵ , while PKC δ translocated on rare occasions. Similar translocation kinetics were observed for PKC α and β 2, and PKC α was translocated a few seconds after an individual C2 domain, the translocation of which coincided exactly with calcium variations (44). Concerning the work we per-

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formed with the GH3B6 cell line, several observations also question the general organization of PKC signaling. We have shown that upon stimulation by phorbol myristate acetate (PMA), PKC α (a cPKC) and PKC ϵ (an nPKC) were selectively translocated to the cell-cell contact, whereas PKC δ (an nPKC) is recruited to the entire plasma membrane (43). Hence, PKC isoforms that belong to the same PKC subclass (PKC ϵ and δ), and are supposedly regulated similarly, are targeted differently when cells are stimulated by PMA (43). Another interesting finding was the lack of correlation between the rise in the intracellular concentration of Ca²⁺ ([Ca²⁺]_i) and the subcellular localization of the PKC α isoform. Indeed, [Ca²⁺]_i increased similarly in single cells and cells in contact upon thyrotropin-releasing hormone (TRH) stimulation, but PKC α did not translocate to the plasma membranes of isolated cells (52). In GH3B6 cells, the fact that PKC α relocated to the cytoplasm before the [Ca²⁺]_i decreased to baseline levels (52) also indicates that cPKC activation is not always phase locked with variations in calcium concentration as suggested in many studies, such as that of Violin et al., who also showed the coincidence of PKC substrate phosphorylation with calcium oscillations (54). In addition, we have shown that the selective targeting of PKC α to cell-cell contacts is probably finely regulated at the molecular level, since the natural D294G point mutation (located in the V3 region of the enzyme and thus not involved in calcium or DAG binding), previously identified in human pituitary and thyroid tumors (2, 40, 41), abolished the selective accumulation of PKC α at the cell-cell contact (51). The same result was obtained when this mutation was introduced in the PKC ϵ coding sequence (43). Together, these observations suggest the existence of a complex spatiotemporal organization of PKC signaling.

In order to understand the links between PKC signaling and the physiological roles of a given stimulus, one approach consists in first defining the functional relationships between the different PKC isoforms. The GH3B6 cell line is a suitable model system to study these links, since it has conserved essential characteristics of growth hormone- and prolactin-secreting cells. In particular, it is still physiologically regulated by hypothalamic hormones, such as TRH, and the cell-cell contact targeting of PKC α is similar to what is observed in pituitary tissue (43). The present study was aimed at clarifying the calcium dependence/independence of PKC α recruitment to the cell-cell contact and establishing the precise spatiotemporal dynamics of translocation of PKC α , β 1, ϵ , and δ (these isoforms are constitutively expressed in the GH3B6 cell line). Results show that PKC α translocation is indeed calcium independent in this cell line and that a coordinated cascade of

isoform activation exists that links PKC α and ϵ subcellular localization at cell-cell contacts to PKC β 1 activity. Furthermore, results on the TRH-induced β -catenin relocation at cell-cell contacts suggest that the PKC cascade does not have a unique functional effector.

MATERIALS AND METHODS

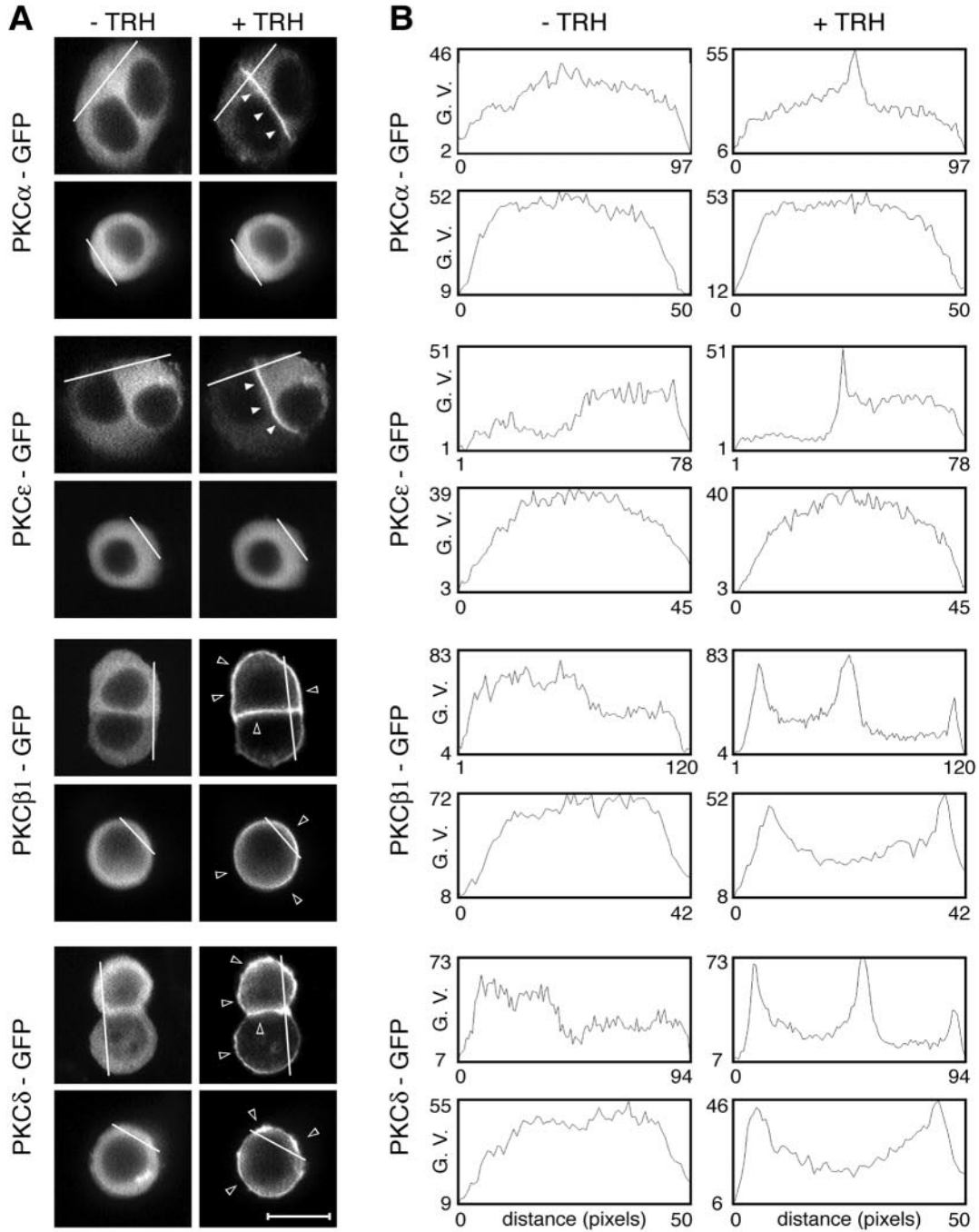
Materials. Ionomycin was purchased from Sigma (Saint Quentin Fallavier, France). Restriction enzymes were from Biolabs New England (Beverly, Mass.). DNA polymerase (DyNAzyme) was from Finnzyme (Espoo, Finland). Synthetic oligonucleotides were purchased from Sigma-Genosys (Haverhill, United Kingdom). pEGFP-N1 plasmid was purchased from Clontech (Palo Alto, Calif.). The plasmids containing the full-length PKC β 1, PKC ϵ , and PKC δ cDNAs were generously provided by P. J. Parker (Imperial Cancer Research Fund, London, United Kingdom). The pcDNA-PKC α -RFP (red fluorescent protein) plasmid was a gift of T. Ng (Randall Division of Cell and Molecular Biophysics, Guy's Campus, King's College London, London, United Kingdom). Plasmid containing dominant-negative (DN) human PKC α (hPKC α) (K368M) was kindly provided by B. Mari (INSERM U526, IFR50, Faculté de Médecine Pasteur, Nice, France). The QuikChange site-directed mutagenesis kit was purchased from Stratagene. ExGen 500 (linear ethylenimine polymer) was purchased from Euromedex (Souffelweyersheim, France). Fetal bovine serum was from Bio Media (Biossens, France). G66976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole] was from Calbiochem (Meudon, France). Thyrotropin-releasing hormone was purchased from Bachem Biochimie Sarl (Voisins-le-Bretonneux, France). Ham F-10 medium and horse serum were obtained from Eurobio (Les Ulis, France). Polyclonal anti-PKC δ was purchased from Sigma, monoclonal anti-PKC α and polyclonal anti-PKC ϵ were from Euromedex, and polyclonal anti- β -catenin and monoclonal anti-PKC β 1 were from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Construction of plasmids. Construction of hPKC α -pEGFP-N1, PKC α -pcDNA-RFP, mPKC ϵ -pEGFP-N1, and rPKC δ -pEGFP-N1 has been described previously (38, 43, 52). In the constitutively active (CA) PKC β 1 (CAPKC β 1-pEGFP-N1), alanine 25 (codon GCC) was replaced by glutamic acid (codon GAA). In the dominant-negative PKC β 1 (DNPKC β 1-pEGFP-N1), lysine 371 (codon AAG) was replaced by methionine (codon ATG). CA and DN PKC β 1 and DN PKC α were then subcloned into the pcDNA-RFP plasmid. PKC β 1 was also subcloned into the pcDNA-RFP and pEGFP-N1 plasmids. In the calcium-insensitive hPKC α and hPKC β 1, aspartic acids 187/246/248 (codons GAT/GAC/GAT) were replaced by asparagines (codons AAT/AAC/AAT). All constructs were checked by sequencing.

Site-directed mutagenesis. The point mutated CA PKC β 1 (A25E) and DN PKC β 1 (K371M) were created by using the QuikChange 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'-GCCCGC AAAGGCGAAGTCCGGCAGAAGAAGC-3' and reverse 5'-CGTTCCTCTGCC GGAGTTCGCCCTTTGCGGGC-3' for CA PKC β 1 (A25E); forward 5'-GCTCTA TGCTGTGATGATCCTGAAGAAGG-3' and reverse 5'-CCTTCTTCAGGA TCATCACAGCATAGAGC-3' for DN PKC β 1 (K371M).

For D187/246/248N hPKC α - and hPKC β 1-pEGFP-N1, aspartic acids 187/246/248 (codons GAT/GAC/GAT) were mutated to asparagines (codons AAT/AAC/AAT). The pairs of synthetic oligonucleotides used to obtain the mutated PKCs were as follows: forward 5'-CTAATCCCTATGAATCCAACGGGCTTTCA GATCC-3' and reverse 5'-GGATCTGAAAGCCGTTTGGATTTCATAGGG ATTAG-3' for D187N hPKC α ; forward 5'-CTGTAGAAATCTGGAATCGGA ATCGAACAAGG-3' and reverse 5'-CCTTGTGTTCCGATTCCAGTT

FIG. 1. Analysis of PKC α , β 1, ϵ , and δ targeting in the GH3B6 cell line. (A) Micrographs depicting what we define as a targeting to the entire plasma membrane or to cell-cell contacts. PKC α , β 1, ϵ , and δ were subcloned in frame with GFP at the C-terminal end of their sequence in the pEGFP-N1 vector (Clontech). PKC β 1 and δ accumulated at the entire plasma membrane in the presence (+) of 100 nM TRH, whereas PKC α and ϵ accumulated selectively at the cell-cell contact. In isolated cells, PKC β 1 and δ behaved as in cells in contact, whereas PKC α and ϵ remained in the cytoplasm. Observations were performed with a confocal microscope. In Fig. 1 to 10, the empty arrowheads show translocation at the entire plasma membrane, whereas the filled arrowheads show selective translocation to cell-cell contacts. Bar, 5 μ m. (B) Analysis with Image J 1.32j software (National Institutes of Health, Bethesda, Md.) was used to quantify PKC translocation and demonstrated that the pixel values increased only at the cell-cell contacts for PKC α and ϵ , which was not the case for PKC β 1 and δ . G. V., gray values. (C) Summary of the results as the percentages of cells where TRH-induced translocation was observed either selectively at cell-cell contacts or at the entire plasma membrane. The percentage of cells where no translocation was observed is also indicated.



C

PKC	n	% of translocation (number of cells)		
		cell-cell contact	entire plasma membrane	no translocation
α	52	51.9	21.2	26.9
ϵ	87	57.5	18.4	24.1
β 1	44	0	84.1	15.9
δ	57	0	59.7	40.3

CCAGATTTCTACAG-3' for D246/248N hPKC α ; forward 5'-CTTGTACCT ATGAACCCCAATGGCTGTACAGATCC-3' and reverse 5'-GGATCTGAC AGGCCATTGGGGTTCATAGGTACAAG-3' for D187N hPKC β 1; and forward 5'-CAGTAGAGATTTGGAATTGGAATTTGACCAGCAGG-3' and reverse 5'-CCTGCTGGTCAAATTCGAATTCGAATCTCTACTG-3' for D246/248N hPKC β 1.

Cell culture and transfection of fusion protein. GH3B6 cells were cultured in Ham F-10 medium supplemented with 2.5% (vol/vol) fetal bovine serum and 15% (vol/vol) horse serum both heat inactivated at 56°C for 1 hour. Transient transfection of GH3B6 cells was performed with ExGen as described previously (43). Briefly, the cells were seeded at 300,000 cells per well in 24-well dishes (Falcon) 18 h before transfection. Immediately before transfection, fresh culture medium (1 ml) was added to the cells. ExGen 500 stock solution (1.25 μ l) was diluted in 12.5 μ l of 150 mM NaCl. Plasmid DNA (250 ng/well) was diluted in 12.5 μ l of 150 mM NaCl. These solutions were then mixed. After 30 min, the transfection mixture was added to the cells. The 24-well dishes were then centrifuged for 5 min at 280 \times g and maintained for 48 h at 37°C. To coexpress PKCs, equal quantities of DNA plasmids (0.250 μ g) were cotransfected according to the manufacturer's instructions. In order to inhibit cPKC activity, GH3B6 cells were incubated with 100 nM Gö6976 for 15 min before and during TRH stimulation. Transfection by TRH was achieved by applying TRH to single cells.

siRNA transfection. Small interfering RNAs (siRNAs), targeting rat PKC α , PKC β , and PKC ϵ , were synthesized and purified by Eurogentec (Liège, Belgium). The sequences of each siRNA pair were as follows: for PKC α -siRNA, 5'-UCUUGCAAAGUGCAGUAUGt-3' and 5'-CAUACUGCACUUUGCAAAG Att-3'; for PKC β -siRNA, 5'-GUUUUAGAUAUCCACCUACt-3' and 5'-GUA GGUGUGGAUCUUAAACt-3'; and for PKC ϵ -siRNA, 5'-CUUGAAACAAC AUCCGGATT-3' and 5'-UCCGGAUGUUGUUUCAAGt-3'. A scrambled siRNA was used as a control. For PKC expression analyses, GH3B6 cells were plated at 7×10^5 per well in six-well plates. After 24 h, siRNAs (final concentrations, 50, 100, and 200 nM) were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 72 h, siRNA efficacy on PKC α , - β , and - ϵ expression was analyzed by Western blotting. For immunocytochemical experiments, GH3B6 cells were plated at 3×10^5 per well in 24-well plates. After 24 h, siRNAs targeting PKC β , - α , and - ϵ were transfected as described above and 72 h later, translocation of endogenous PKCs upon TRH stimulation for 15 s, 60 s, or 5 min was observed. The TRH-induced β -catenin relocalization was analyzed by immunocytochemistry after transfection of siRNAs against PKC α and - ϵ and stimulation with TRH for 1 h. Efficiency of siRNA transfection was higher than 80% as assessed by the transfection of a control nonsilencing siRNA labeled with rhodamine (QIAGEN).

Immunocytochemistry. GH3B6 cells were seeded on 12-mm round coverslips in 1 ml medium. They were washed three times with phosphate-buffered saline (PBS), pH 7.4, before being fixed for 10 min with 4% paraformaldehyde in PBS, pH 7.4 (vol/vol). The cells were then washed twice with PBS, permeabilized in 0.2% Triton X-100 for 5 min, washed in PBS, and incubated for 30 min in PBS supplemented with 1% bovine serum albumin. The coverslips were then incubated overnight at 4°C with the primary monoclonal anti-PKC α (diluted 1/250), polyclonal anti-PKC ϵ (diluted 1/500), polyclonal anti-PKC δ (diluted 1/500), polyclonal anti- β -catenin (diluted 1/100), or monoclonal anti-PKC β 1 (diluted 1/100) antibodies. They were then washed three times for 10 min with PBS supplemented with 1% bovine serum albumin and further incubated for 1 h at room temperature with the secondary antibodies, which were either a Cy3-conjugated goat anti-mouse (Fab')₂ antibody or an Alexa 488-conjugated goat anti-rabbit antibody (diluted 1/1,000). The coverslips were then mounted in Mowiol and examined by conventional microscopy (Zeiss) by using a 63 \times objective.

Real-time fluorescence microscopy. The localization of fusion proteins in living cells was examined by conventional or confocal fluorescence microscopy. Conventional microscopy was performed with an Axiophot 2.0 from Zeiss. Cells were viewed at room temperature with a 63 \times 0.9-numerical-aperture Achromplan water immersion objective lens (Zeiss). 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, 6 mM glucose, pH 7.4. The camera was a CoolSNAP camera from Photometrics, a division of Roper Scientific. The acquisition software was MetaVue version 6.1 from Universal Imaging Corporation. The confocal laser-scanning microscope was equipped with an Ar/Kr laser (Odyssey XL with InterVision 1.4.1 software; Noran Instruments, Inc., Middleton, WI) as described by Guerineau et al. (18). Cells were viewed with a 63 \times 0.9-numerical-aperture Achromplan water immersion objective lens (Zeiss). At the time of observation, the culture medium was replaced by a prewarmed buffer, at 37°C, identical to the one used for the conventional microscopic observations. Images were acquired continuously with intervals between frames of 0.533 s. The ImageJ 1.32j software (National Institutes of Health, Bethesda, Md.) was used to

quantify PKC translocation to plasma membrane. When movies were used for illustrations, five consecutive images were averaged in order to decrease pixelization.

See Fig. S1 and S2 in the supplemental material for the subcellular distribution of green fluorescent protein (GFP)-tagged PKC α , - β 1, - ϵ , and - δ upon PMA stimulation and the various subcellular distributions of PKC δ upon PMA stimulation, respectively. See Videos S1 to S4 in the supplemental material (they correspond to Fig. 3) for recordings of GFP-tagged isoforms before and after stimulation with TRH that starts at image 20. One image was taken every 0.533 s, with a Kallman average of 64 images. See Video S5 in the supplemental material for cells transfected with CA PKC β 1 fused to GFP. In this case, there is no TRH stimulation. Note that in this case, translocation is oscillatory.

RESULTS

Different spatial organizations of activated PKC α , - β 1, - ϵ , and - δ . We have previously described the spatial organization of TRH-activated PKC α and those of PMA-stimulated PKC α , - ϵ , and - δ in GH3B6 cells (43, 52). Figure 1A illustrates the spatial organization of PKC α , - ϵ , - β 1, and - δ in contacting and isolated cells upon TRH stimulation. While all isoforms were located in the cytoplasm in unstimulated cells, GFP-tagged PKC α and - ϵ accumulated selectively at the cell-cell contact in 52 and 57%, respectively, of the cells analyzed, whereas GFP-tagged PKC δ never did, accumulating instead at the entire plasma membrane in 60% of cases (Fig. 1A and C). In the case of PKC β 1, the native endogenous protein (see Fig. 8) as well as the GFP-tagged fusion protein accumulated without selectivity at the entire plasma membrane in 84% of cells. Furthermore, in TRH-stimulated isolated cells, PKC α and - ϵ remained in the cytoplasm, whereas PKC β 1 and - δ accumulated at the plasma membrane (Fig. 1A). Analysis of pixel values along a line that crosses the cell without going through the nucleus illustrates these various spatial patterns and confirms that the pixel values increased only at the cell-cell contacts for PKC α and - ϵ , which was not the case for PKC β 1 and δ (Fig. 1A, right panels). The same analyses performed with isolated cells show an increase in the pixel values at the plasma membrane only in the case of activated PKC β 1 and - δ , demonstrating that isoforms that selectively accumulated at cell-cell contacts do not translocate in isolated cells, in contrast to isoforms that accumulated without selectivity at the plasma membrane, which also translocated in isolated cells. Therefore, although belonging to different PKC subclasses, PKC α and - ϵ on the one hand and PKC β 1 and - δ on the other display identical subcellular distributions after activation. In many cases where a PKC isoform accumulated at the entire plasma membrane, the cell-cell contact appeared more fluorescent than the remainder of the plasma membrane (see Fig. 3A, PKC β 1, and 4A, C, and D, PKC α , + TRH + Gö-6976), probably due to the apposition of the plasma membranes of the two contacting cells.

Upon PMA stimulation, the distribution of activated enzymes was very similar to that in the presence of TRH (see Fig. S1 in the supplemental material) (43), except that, in some cells, PKC δ also accumulated in a perinuclear region as well as at the nuclear membrane as reported for other cellular systems (5) (see Fig. S2 in the supplemental material). It is noteworthy that such nuclear and perinuclear localizations have never been observed upon TRH stimulation.

Existence of a calcium-independent translocation mechanism for a cPKC. In order to analyze the calcium dependence of PKC α translocation to cell-cell contacts, we first studied the

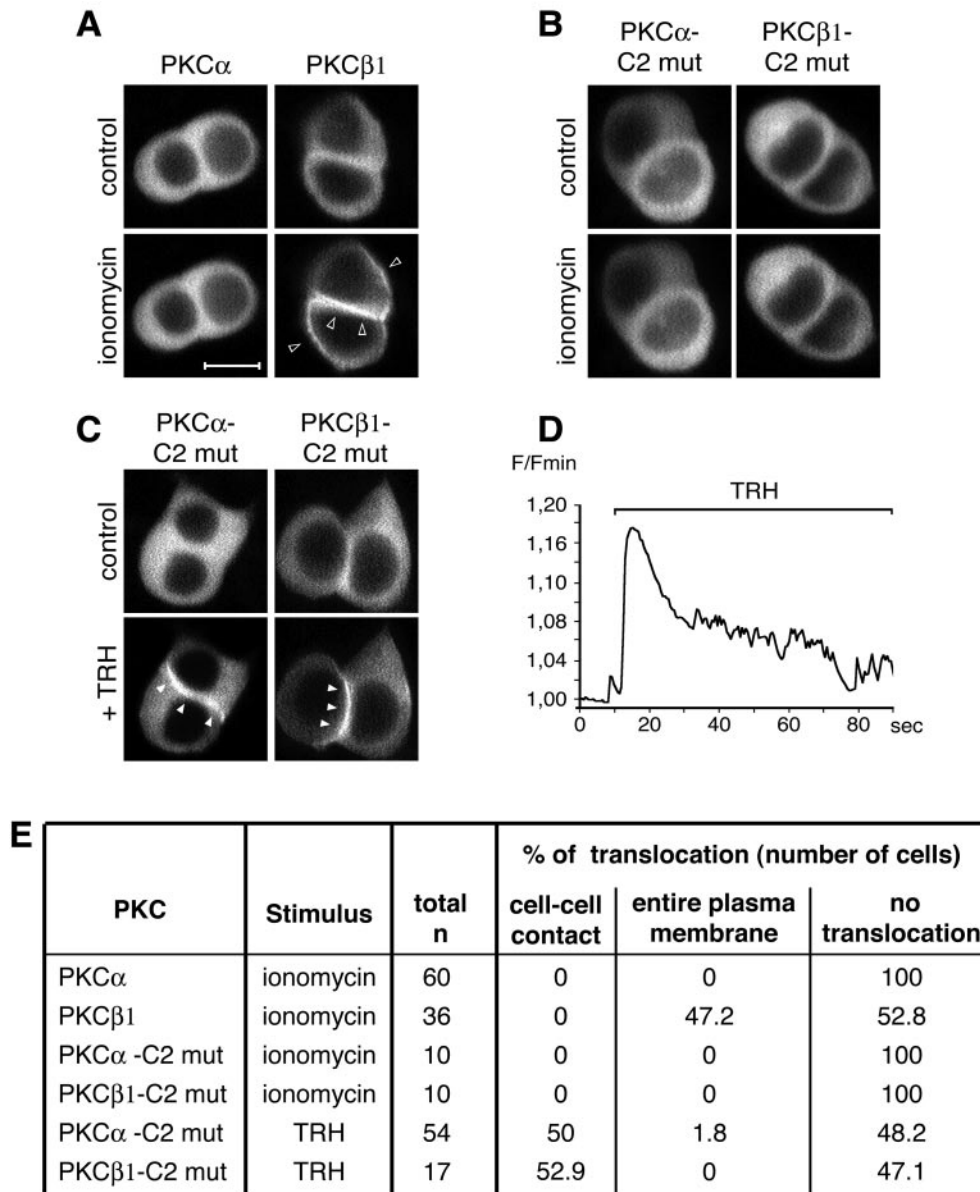


FIG. 2. Existence of a calcium-independent targeting of PKC α . (A) PKC α and β 1 behaved differently when GH3B6 cells were incubated with 2 μ M ionomycin. PKC β 1 is activated and accumulated at the entire plasma membrane similar to what is seen in the presence of 100 nM TRH. PKC α was not activated and remained in the cytoplasm. (B) PKC α and β bearing the triple point mutations D187/246/248N do not translocate in the presence of ionomycin. mut, mutant. (C) D187/246/248N PKC α , unable to bind calcium, was still targeted to the cell-cell contacts upon stimulation, indicating that its translocation can be calcium independent. The same mutations in the PKC β 1 sequence show that PKC β 1 behaved like PKC α when unable to bind calcium: it accumulated at cell-cell contacts. Bar, 5 μ m. (D) An example of intracellular calcium modification upon TRH stimulation. Image acquisition was performed on a confocal microscope, with 0.533-s intervals between frames. Calcium monitoring was described previously (48). F/Fmin, fluorescence/minimum fluorescence. (E) Summary of the results as described in the legend to Fig. 1C.

effects of a calcium ionophore on the subcellular localization of PKC α , while PKC β 1 was used for comparison. We found that whereas PKC β 1 was translocated and accumulated at the entire plasma membrane in the presence of 2 μ M ionomycin, PKC α remained in the cytoplasm (Fig. 2A). A similar lack of PKC α translocation was described before in eggs incubated in the presence of 2 μ M ionomycin (14). The differences of sensitivity of the two isoforms towards ionomycin cannot be explained by different calcium binding affinities, since it has been shown that the 50% inhibitory concentrations of PKC α and

β 1 for calcium are very similar (23). This calcium independence of PKC α in GH3B6 cells was further demonstrated by using a PKC α -GFP fusion protein bearing the D187/246/248N mutations, known to abolish calcium binding to the C2 domain (10). This calcium-insensitive PKC α -GFP was still able to accumulate selectively at the cell-cell contact in the presence of TRH (Fig. 2C), providing the first convincing evidence that a cPKC can be translocated by a different mechanism than the one currently accepted today, which is that an increase in the [Ca²⁺]_i is the leading event in cPKC translocation. For a con-

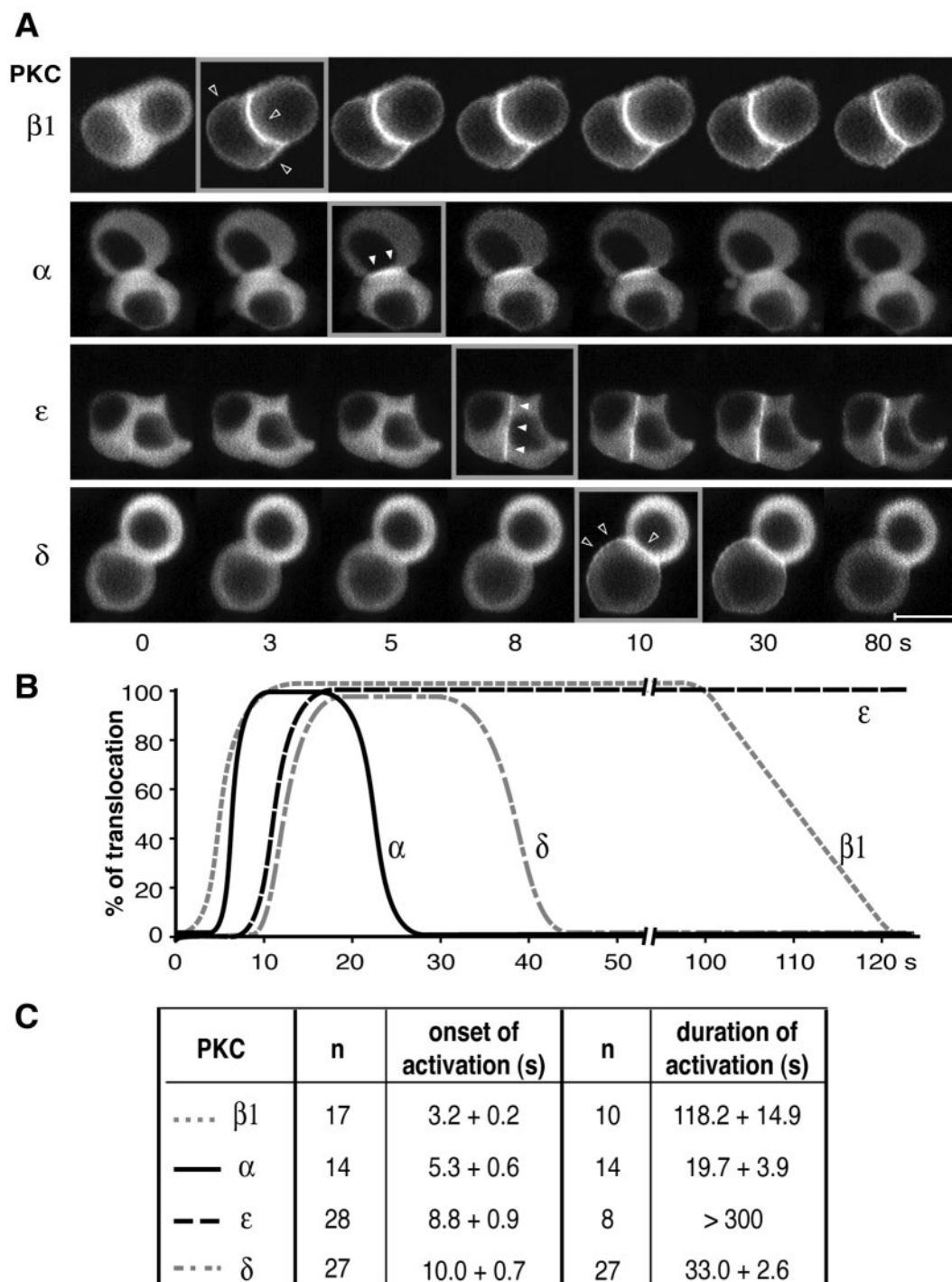


FIG. 3. Analysis of the translocation kinetics of PKC α , β 1, ϵ , and δ . (A) Examples of real-time recordings of spatiotemporal activation of PKC β 1, α , ϵ , and δ fused to GFP in GH3B6 cells stimulated with 100 nM TRH. Recordings were performed with a Noran confocal microscope. Images were acquired continuously, with intervals of 0.533 s between frames. Time zero is defined as the time of TRH application. Videos can be seen in the supplemental material. Bar, 5 μ m. (B) Schematic representation of the data showing the different onsets and durations of PKC β 1, α , ϵ , and δ translocations. (C) Statistical analysis of the data, with the number of cells analyzed. The *P* values were calculated with the Student *t* test.

tol, we introduced the same three point mutations in the C2 domain of PKC β 1. This mutant did not translocate in the presence of ionomycin, as expected (Fig. 2B). Surprisingly, we found that in the presence of TRH, the calcium-insensitive

PKC β 1 does not accumulate at the entire plasma membrane, but like native PKC α (Fig. 2C), accumulates selectively at the cell-cell contact. Conversely, we had shown before that D294G PKC α , like native PKC β 1, accumulates at the entire plasma

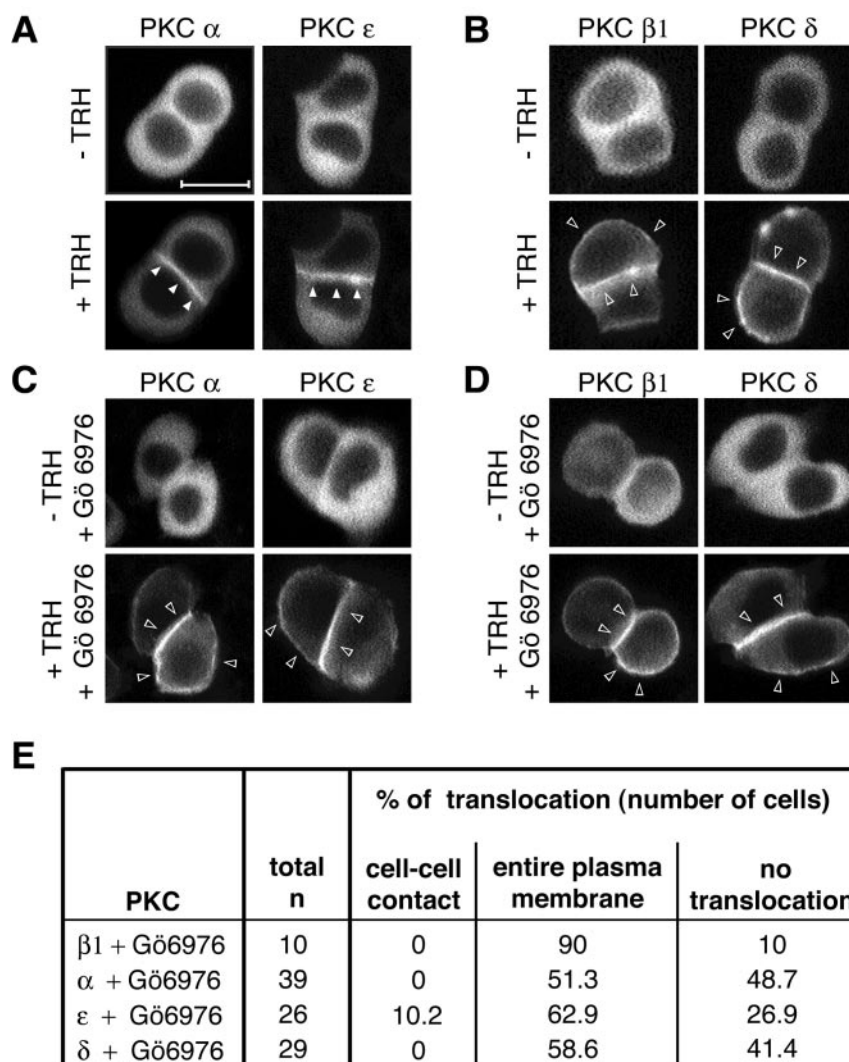


FIG. 4. Cell-cell contact targeting of PKC α and - ϵ is dependent on PKC activity, unlike the PKC β 1 and - δ targeting to the entire plasma membrane. (A to D) GH3B6 cells were preincubated (A and B) or not (C and D) with the cPKC activity inhibitor Gö6976 and then stimulated (+) with 100 nM TRH. Real-time recordings were performed as described in the legend to Fig. 2. (C) When preincubated with 100 nM Gö6976 for 15 min, PKC α and - ϵ behaved like PKC β 1 and - δ : they accumulated at the entire plasma membrane. Gö6976 had no effect on PKC β 1 and - δ targeting (D). Bar, 5 μ m. (E) Summary of the number of cells analyzed.

membrane instead of being selectively translocated to the cell-cell contact (51). Thus, affecting either amino acid 294 in PKC α or abolishing calcium binding in PKC β 1 switches the mode of translocation of PKC α to that of PKC β 1 and vice versa. Figure 2D confirms that TRH induced the expected variation in intracellular calcium under these experimental conditions. The unambiguous nature of these results is emphasized when they are expressed as percentages of cells where PKC translocation is observed or PKC translocation is not observed (Fig. 2E).

Existence of a cascade of isoform activation. Our second goal was to uncover a possible hierarchy in the kinetics of PKC isoform activation. Figure 3 shows a statistically relevant analysis of PKC isoform targeting kinetics. It demonstrates that upon TRH activation, PKC β 1 is the first isoform to be translocated to the plasma membrane (starting 3.2 ± 0.2 s [$n = 17$]

after TRH application), followed 2 seconds later by the selective translocation of PKC α to cell-cell contacts (starting 5.3 ± 0.6 s [$n = 14$]; statistically different from PKC β 1 onset of activation, $P < 0.05$). PKC ϵ was translocated another 3.5 s later, immediately followed by PKC δ . The duration of activation was also found to vary significantly between the different isoforms, independently of the subclass to which these isoforms belong (Fig. 3B and C): 118 s for PKC β 1, 20 s for PKC α , 15 to 20 min for PKC ϵ , and 33 s for PKC δ (see the videos in the supplemental material). Strikingly, the time during which PKC β 1 remained activated was identical to that of the D294G PKC α mutant (51). The fact that PKC α mutant activation is phase locked with $[Ca^{2+}]_i$ variations (51) suggests that the PKC α mutant has indeed switched from a Ca^{2+} -independent to a Ca^{2+} -dependent translocation as hypothesized above. Conversely, the D187/246/248N PKC β 1 mutant, which accumu-

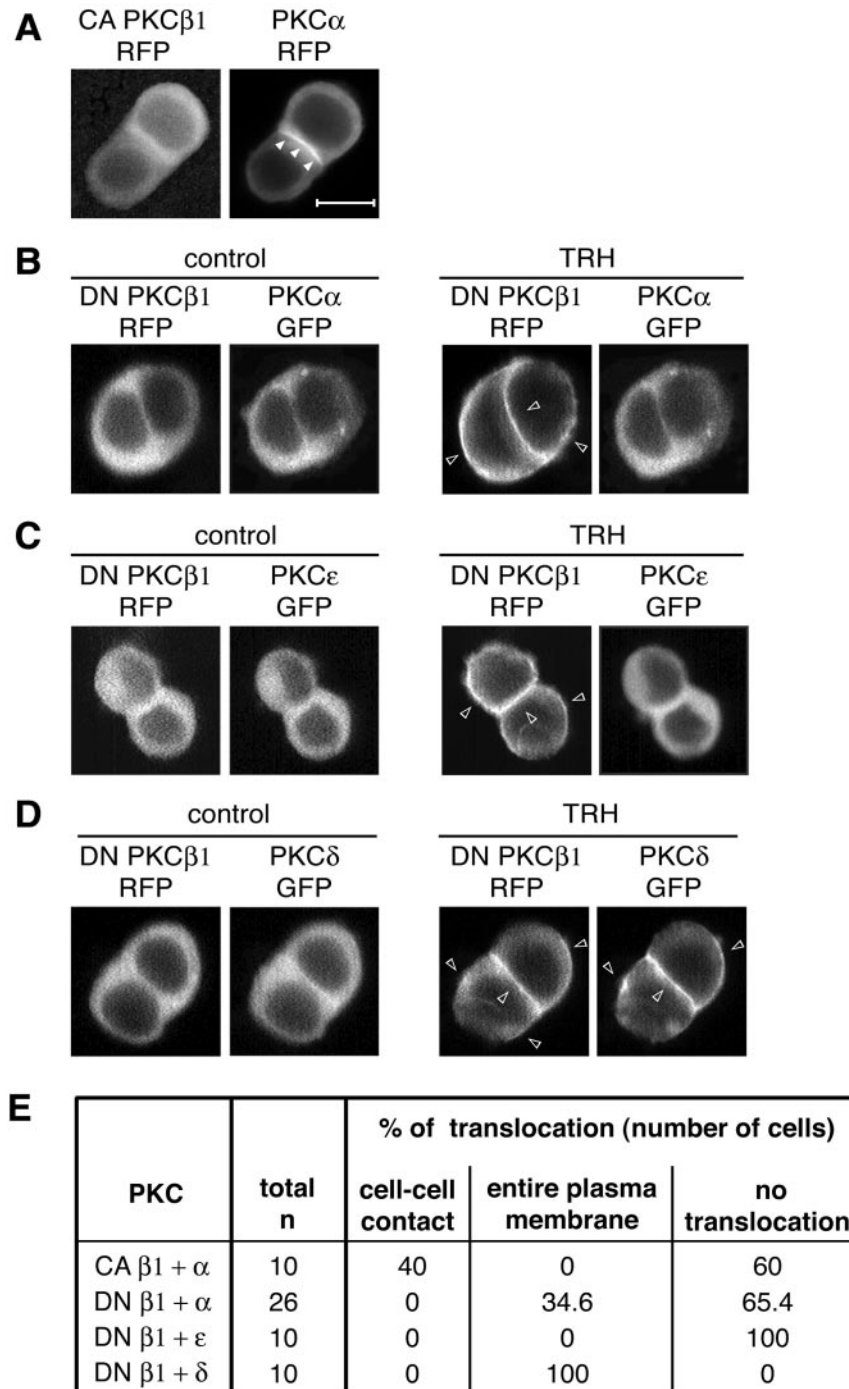


FIG. 5. PKC α and - ϵ translocation depends on PKC β 1 activity. CA PKC β 1-RFP was first cotransfected with PKC α -GFP in GH3B6 cells, and observations were performed 48 h later. (A) PKC α -GFP still accumulated at cell-cell contacts in the presence of CA PKC β 1, which bypasses receptor activation and therefore does not induce any increase in intracellular Ca²⁺ and DAG concentrations. Observations were performed with a conventional microscope. A video in the supplemental material shows that CA PKC β 1-GFP oscillates spontaneously, without TRH, between the cytoplasm and plasma membrane. In the presence of DN PKC β 1-RFP (B) and in the presence of 100 nM TRH, PKC α -GFP remained in the cytoplasm of most cells or accumulated at the entire plasma membrane in 9/26 cells, as did PKC ϵ (C). PKC δ translocation was not affected by the cotransfection of DN PKC β 1-RFP (D). All the data presented in panels A to D were obtained 48 h after the cotransfection by real-time recordings. (E) Summary of the total number of cells analyzed in each situation and the subcellular localizations of the different GFP fusion proteins. As previously shown by others for DN PKC γ and - δ (49), we found that in the presence of 100 nM TRH, DN PKC β 1 accumulated at the same site as the native enzyme, namely, at the entire plasma membrane (B, C, and D), while the CA PKC β 1 keeps oscillating between the cytoplasm and plasma membrane (see the video in the supplemental material). In each experiment, it was checked ($n = 5$ cells for each PKC) that PKC α , PKC ϵ , and PKC δ -GFP transfected alone were localized exclusively at cell-cell contacts.

lated at cell-cell contacts (see above), displayed kinetic characteristics that were different from those of PKC β 1 but close to those of PKC α (onset of activation, 5.8 ± 0.6 s; duration of activation, 46 ± 10 s [$n = 3$]), indicating that spatial and temporal dimensions are indeed undissociable clues for understanding PKC signaling.

The selective accumulation of PKC α and - ϵ at cell-cell contacts is dependent on PKC activity. Since there is a statistically significant difference between the onsets of activation of PKC β 1 and - α , we asked whether PKC β 1, which is the first to be activated, could play the role of "leader" in this cascade of sequential translocation/activation. We started by looking at the effect of Gö6976, an inhibitor of conventional PKCs at the dose used in our experiments (100 nM) (28), even though it can also inhibit other kinases but at much higher doses (>1 μ M) (42). As shown in Fig. 4A to D, when cells were incubated in the presence of Gö6976 and TRH, PKC α and - ϵ were translocated to the entire plasma membrane instead of accumulating at the cell-cell contact. This suggested that selective translocation of PKC α and - ϵ to cell-cell contacts was dependent on the activity of at least one conventional PKC isoform. PKC β 1 was a more obvious candidate than PKC α , since we had previously shown that deleting the catalytic domain of PKC α does not abolish its selective accumulation at cell-cell contacts (52). Although PKD is also inhibited by Gö6976 (17), it is probably not the isoform involved here, as it is not sensitive to calcium or DAG variations.

Coordination of the cascade of PKC isoform activation. (i) PKC α translocation depends on PKC β 1 activity. If PKC β 1 were the leader in the sequential PKC isoform activation cascade and if it conditions PKC α translocation, then bypassing activation of the TRH receptor by using a constitutively active PKC β 1 (resulting in no increases in Ca^{2+} and DAG concentrations within the cell) should elicit a spontaneous accumulation of PKC α at the cell-cell contact. Figure 5A shows that indeed, in the absence of TRH, PKC α accumulated at the cell-cell contact of cells transfected with CA PKC β 1-RFP. This does not mean, however, that CA PKC β 1-RFP is sufficient to fully activate PKC α at the cell-cell contact, only that PKC β 1 activity is sufficient to elicit PKC α translocation. By contrast, in cells transfected with a dominant-negative PKC β 1-RFP plasmid, PKC α -GFP remained in the cytoplasm, even in the presence of TRH (Fig. 5B). Figure 5B shows also that DN PKC β 1-RFP (as well as DN PKC β 1-GFP transfected alone [data not shown]) localizes at the entire plasma membrane upon TRH stimulation as did the native enzyme. These observations demonstrated that PKC β 1 plays an essential role in the initiation and regulation of PKC α translocation to the cell-cell contact.

(ii) PKC ϵ , but not PKC δ , translocation depends on PKC β 1 and PKC α activities. We then went on to investigate the roles of PKC β 1 and - α in the control of PKC ϵ localization. Similar to DN PKC β 1, which localized at the same subcellular sites as the native enzyme, DN PKC α -RFP (or DN PKC α -GFP alone [data not shown]) translocated, as did native PKC α , to the cell-cell contact upon TRH stimulation (Fig. 6A). In cells transfected with either DN PKC β 1-RFP or DN PKC α -RFP (Fig. 5C and 6B), the TRH-induced PKC ϵ targeting to the cell-cell contact was abolished and PKC ϵ remained in the cytoplasm. The fact that the DN PKC α was sufficient to induce this effect indicates that PKC α is essential to the control of

PKC ϵ localization at the cell-cell contact. However, in contrast to PKC ϵ , PKC δ was not found to be a part of the cascade controlled by PKC β 1, since neither the expression of DN PKC β 1-RFP nor that of DN PKC α -RFP prevented the TRH-induced PKC δ -GFP translocation (Fig. 5D and 6C).

If PKC α were indeed activated following activation of PKC β 1, abolishing its activity should not affect PKC β 1 translocation. This was analyzed by cotransfecting DN PKC α -RFP with PKC β 1-GFP. Figure 6A shows that indeed PKC β 1-GFP translocation was not affected by the presence of the DN PKC α .

Finally, cotransfection experiments demonstrated that PKC isoforms implicated in the cascade do accumulate at their expected subcellular localizations in the same cell. Figure 7 shows that indeed, upon TRH stimulation, both PKC α and - ϵ accumulate at the cell-cell contact within the same cells and that PKC β 1 accumulates at the entire plasma membrane in cells where PKC α and - ϵ are present at the cell-cell contact.

Endogenous PKCs are also organized as a coordinated cascade. In order to check that the organization of PKC signaling that we describe for the GFP-tagged enzymes reflects the physiological behavior of PKC isoforms in these cells and is not an artifact of the GFP tag or of the transient transfection, we checked that it also existed for the endogenous proteins. We performed immunocytochemistry experiments following stimulation of GH3B6 cells with TRH for 15 s, 60 s, or 5 min, these time points being chosen on the basis of the results of the time course experiments shown in Fig. 3. Figure 8 shows that indeed, after 15 s of stimulation, all endogenous PKCs are translocated either at the entire plasma membrane (PKC β 1 and - δ) or selectively at cell-cell contacts (PKC α and - ϵ). After 60 s of stimulation, only PKC β 1 and - ϵ remain translocated, and after 5 min, only PKC ϵ is detected at cell-cell contacts. Thus, what we have shown for the GFP-tagged enzymes in transient-transfection experiments does reflect the behavior of the endogenous proteins.

Then, RNA interference was used in order to knock down selectively the expression of PKC β 1, - α , and - ϵ , and the consequences were systematically analyzed on the translocation of endogenous PKCs. Figure 9A shows that all three siRNAs used were efficient in knocking down enzyme expression. Maximum efficacy was achieved at 200 nM, reaching 70%, 95%, and 97% inhibition for PKC β 1, - α , and - ϵ siRNAs, respectively. Figure 9B shows that knocking down PKC β 1 expression abolished the translocation of PKC α and - ϵ , but not that of PKC δ , after stimulation with TRH. In contrast, PKC α depletion affected PKC ϵ translocation, but not translocation of PKC β 1 and - δ , and finally, knocking down PKC ϵ expression did not have any effect on PKC β 1, PKC α , or PKC δ translocation. A scrambled siRNA was used as a control and did not affect translocation of any of the PKCs.

Thus, the data presented here argue in favor of a novel organization of endogenous PKC signaling into a coordinated cascade of activation in the pituitary GH3B6 cell line.

PKC α , but not - ϵ , is responsible for the TRH-induced β -catenin relocation. In order to clarify the functional significance of such a coordinated cascade of PKC activation, we tried to establish whether PKC isoforms accumulating in the same location after stimulation would induce the same biological effect, implying that only the isoform situated in the most downstream position would act as the effector of the cascade.

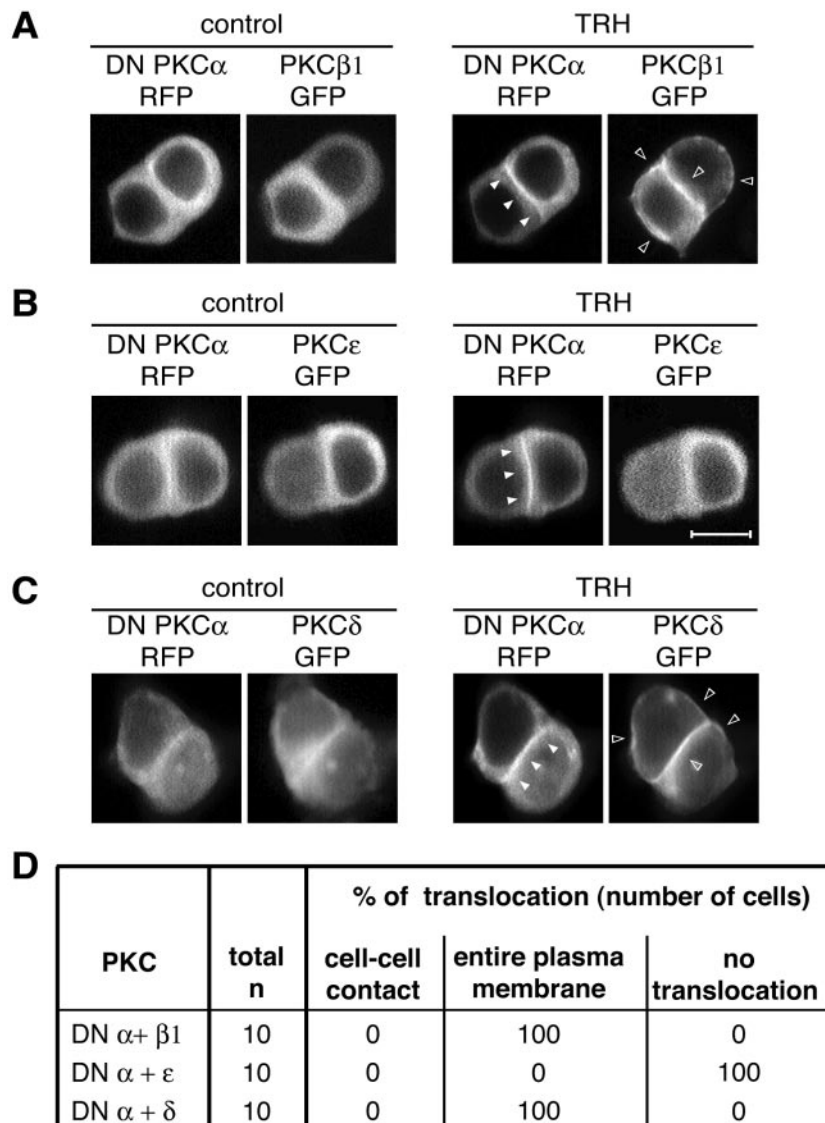


FIG. 6. PKC ϵ translocation depends on PKC α activities. When cotransfected with DN PKC α -RFP, PKC ϵ -GFP remained in the cytoplasm of all the TRH-stimulated cells analyzed (B). Under the same conditions, PKC β 1 and - δ translocation were not affected (A and C). (D) Summary of the total number of cells analyzed in each situation and the subcellular localizations of the different GFP fusion proteins. All the data presented in panels A, B, and C were obtained 48 h after the cotransfection by real-time recordings. In each experiment, it was checked ($n = 5$ cells for each PKC) that PKC β 1, PKC ϵ , and PKC δ -GFP, transfected alone, were localized at cell-cell contacts for PKC ϵ and at the entire membrane for PKC β 1 and - δ . Bar, 5 μ m.

In order to answer this question, we analyzed the involvement of PKC α and - ϵ on the TRH-induced β -catenin relocation at cell-cell contacts previously reported for this cell line (51).

Figure 10A shows the time course of β -catenin relocation at cell-cell contacts upon TRH stimulation. Reorganization of β -catenin distribution was detected from 10 min after stimulation. After 30 min of stimulation, a more intense staining started to be homogeneously distributed along the cell-cell contact, and after 1 h of stimulation, relocation of β -catenin was maximal, as shown by the very intense homogenous staining along cell-cell contacts. The involvement of PKC α and - ϵ in translocation was therefore studied after 1 h of TRH stimulation.

Figure 10B shows the incidence of siRNA-mediated deple-

tion of PKC α or PKC ϵ on β -catenin relocation at cell-cell contacts. While the siRNA targeted against PKC α prevented the β -catenin relocation at cell-cell contact, the siRNA targeted to PKC ϵ had no effect, showing that both isoforms are clearly capable of exerting independent biological functions despite their participation in a common activation cascade.

DISCUSSION

There are many examples of signaling cascades involving series of kinases, the canonical example of which are the mitogen-activated protein kinase cascades (7, 35). In these cascades, phosphorylation of one kinase by the other is mandatory

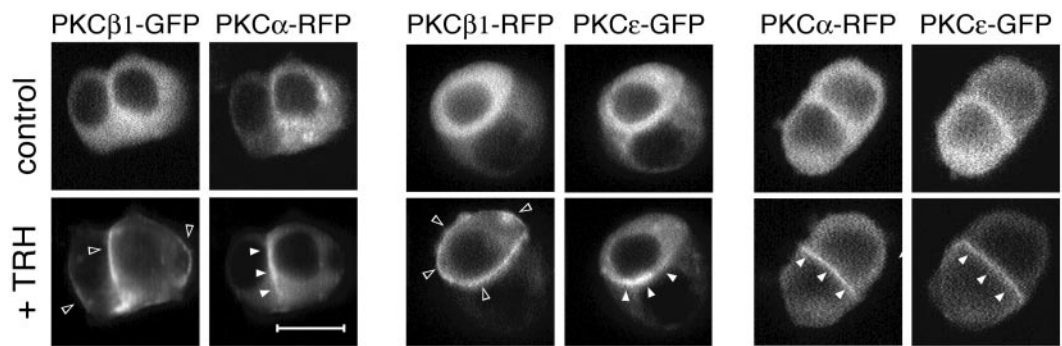


FIG. 7. The different subcellular compartment localizations of PKC α , - β 1, and - ϵ occur within the same cells. Cotransfection experiments of PKC β 1 and PKC α subcloned in the pcDNA-RFP vector with either PKC β - or PKC ϵ -GFP indicated that PKC β 1 accumulated at the entire plasma membrane in cells where PKC α and - ϵ were translocated to cell-cell contacts. Cotransfection with PKC α -RFP and PKC ϵ -GFP indicated that these two isoforms accumulated at the same subcellular location, the cell-cell contacts, within the same cells. Recordings were performed with a Noran confocal laser-scanning microscope equipped with an Ar/Kr laser (Odyssey XL with InterVision 1.4.1 software; Noran Instruments, Inc., Middleton, WI). Images were acquired with intervals of 17 s between the two frames. Bar, 5 μ m.

for the activation of the former and differences in terms of kinetics and localization have been reported; these differences depend on whether the cascade is activated via β -arrestin or via G proteins (1). The PKC family of isoforms has never been considered up to now as potentially organized into a cascade, although the sequential activation of several isoforms has been observed upon physiological stimulation of living cells (3, 19, 25, 26, 31, 44, 46, 47, 49, 50, 53, 55). The results of the present study demonstrate the existence of such an organization and unravels a new regulatory mechanism, a coordinated cascade

of isoform activation controlling localization at a specific subcellular location.

Today, translocation of a PKC (a cPKC or nPKC) is considered essential for its activation and is understood to be regulated by calcium and/or DAG. The two modules C1 and C2 are responsible for translocation events. Recognition of calcium by the C2 module, in the case of a cPKC, occurs in the cytoplasm, and an increase in calcium concentration seems sufficient to induce translocation. Activation of a cPKC is thus considered "phase-locked with calcium oscillations" (54). Concerning DAG dependence, PKCs are supposed to act as sensors for a concentration gradient, which is, for example, higher at the membrane after activation of a G-protein-coupled receptor. However, we have found some discordant data in this general scheme of calcium- and DAG-dependent PKC activation. The change in subcellular location of the mutated D294G PKC α enzyme was accompanied by phase locking with calcium, which was not the case for the wild-type enzyme. From that observation, we could already suspect that a calcium-independent translocation of PKC α was possible, in addition to the well-documented calcium-dependent one. The following results presented in the present study demonstrate that such a calcium-independent mechanism occurs. (i) PKC α does not translocate in the presence of ionomycin. (ii) PKC α D187/246/268N, which is calcium insensitive, still translocates selectively to cell-cell contacts. Furthermore, PKC β 1 D187/246/268N also translocates to the cell-cell contacts. The latter observation did puzzle us and suggests that a cPKC might be able to switch from a calcium-dependent to a calcium-independent mechanism and vice versa. Consistent with this, we observed a switch in the PKC α translocation site from cell-cell contacts to the entire plasma membrane in the presence of the cPKC inhibitor G δ 6976. Thus, an alternative control of PKC translocation regulation exists, independent of calcium and DAG, and associated with the cell-cell contact targeting. In favor of the hypothesis that two regulatory mechanisms can control PKC translocation is the fact that within a given cell population, not all cells behave identically. Indeed, upon stimulation with TRH, PKC α and PKC ϵ selectively accumulate at cell-cell contacts in 55% of the cells, but in a substantial fraction of the cells

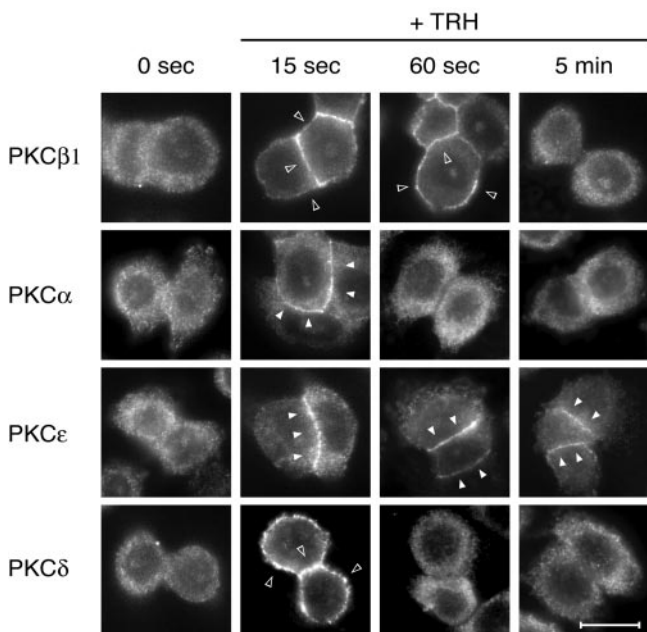


FIG. 8. Endogenous PKCs are also sequentially activated by TRH. GH3B6 cells were stimulated with TRH for the indicated times and then immediately fixed with paraformaldehyde. Immunocytochemistry performed with selective PKC antibodies showed that endogenous PKCs behave like the GFP-tagged ones. After 15 s of TRH stimulation, all PKCs were translocated. After 60 s, only PKC β 1 and - ϵ were still activated, whereas at 5 min of stimulation, only PKC ϵ was activated. Bar, 5 μ m.

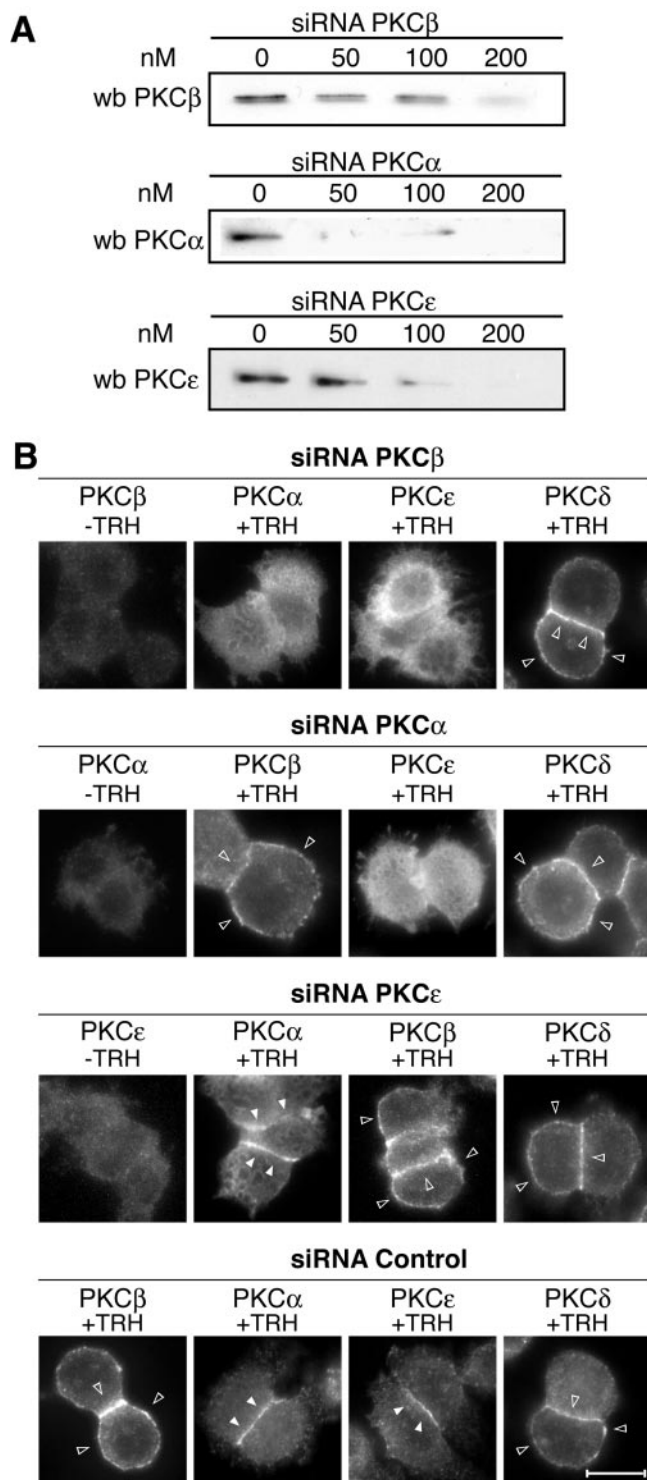


FIG. 9. The sequential endogenous PKC activation is coordinated. By using siRNAs targeted to PKC β 1, α , or ϵ , we show that the sequential activation of endogenous PKCs is coordinated in the same way as it is for the GFP-tagged enzymes. (A) The efficacy of the siRNAs is dose dependent, and it is maximal for 200 nM. At this concentration, the siRNA targeted to PKC β 1 abolished translocation of PKC α and ϵ but not that of PKC δ (B). The siRNA targeted to PKC α abolished translocation only of PKC ϵ , and the siRNA targeted to PKC ϵ did not affect the translocation of PKC β 1, α , or δ (B). wb, Western blot. Bar, 5 μ m.

(20%), PKC α and PKC ϵ are translocated to the entire plasma membrane like native PKC β 1 (Fig. 1C).

Several reports have documented the fact that activation of a membrane receptor, such as a G-protein-coupled receptor, may activate several PKC isoforms (3, 12, 44, 46, 50, 53). In these studies, the fact that all PKC isoforms involved translocated to the same subcellular location, the plasma membrane, did not suggest different, isoform-specific mechanisms of translocation. In the present work, we hypothesized that decoding the temporal pattern of activation, in addition to the spatial one, could be important to understand the organization of PKC signaling. Several reasons lead us to suggest that the cascade of activation could be coordinated. First, the fact that PKC α localization at cell-cell contacts was dependent on cPKC activity (the present study) but did not require its own kinase activity (52) and the fact that PKC β 1 was the first to be activated suggest that PKC α activation could be under PKC β 1 control. The results obtained in this work for both endogenous and GFP-tagged PKCs show that it is indeed the case and demonstrate that similarly, PKC ϵ is under the control of PKC α . Thus, there is a close association between the control of localization and that of activation. However, the mechanism by which PKC β 1 controls the location of PKC α and the mechanism by which PKC α controls the location of PKC ϵ are not known at present. Since an activated PKC isoform is not in the same subcellular compartment as an inactive PKC, the links between PKC β 1 and PKC α and between PKC α and PKC ϵ are probably indirect. Also, since it is the activity of PKC β 1 and α that controls cell-cell contact targeting of PKC α and ϵ , respectively, then a substrate of PKC β 1 may serve as the link between PKC β 1 and PKC α , and a substrate for PKC α may serve as the link between PKC α and PKC ϵ . These substrates would translocate from the membrane compartment to the cytoplasm upon phosphorylation. An example of such a substrate, able to translocate from the membrane to the cytoplasm upon phosphorylation by a PKC, was provided by the characterization of MARCKS (named MARCKS for myristoylated alanine-rich C-kinase substrate) (13, 31). These considerations led us to propose the hypothetical model presented in Fig. 11: upon stimulation of the Gq-coupled TRH receptor, intracellular Ca²⁺ and DAG concentrations increase and PKC β 1 is activated and accumulate at the plasma membrane, according to the currently accepted paradigm of PKC translocation/activation. Upon phosphorylation, a PKC β 1 substrate would then relocate to the cytoplasm. This unknown substrate would, alone or in association with other partners, play the role of cargo to elicit the selective translocation of PKC α to the cell-cell contact. PKC α would in turn phosphorylate a substrate, itself mediating directly or indirectly PKC ϵ translocation to the cell-cell contact. PKC δ does not belong to this cascade, since its translocation to the plasma membrane is not dependent on PKC activity. This hypothesis takes into account the current "dogma," since PKC β 1 is calcium and DAG regulated and is the isoform that initiates the cascade. However, it also introduces the concept that without this coordinated cascade of activation, and without the existence of a calcium-independent but PKC activity-dependent control of PKC translocation, PKC α and ϵ would not be targeted to their site of action. In a recent work, Denis and Cyert highlighted the complex regulation of the localization of the unique PKC isozyme present in

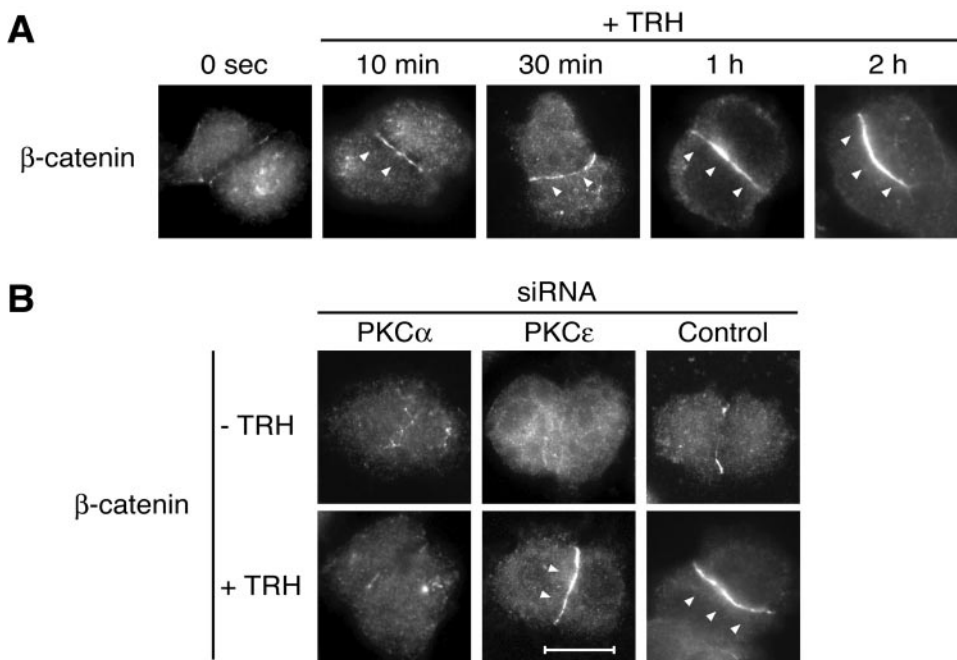


FIG. 10. PKC α , but not PKC ϵ , is responsible for the TRH-induced relocation of β -catenin at cell-cell contacts. In order to clarify whether PKC α and - ϵ had distinct functions at the cell-cell contact, we analyzed the previously described TRH-induced β -catenin relocation to cell-cell contact (48) in the presence or absence of siRNAs targeted either to PKC α or to PKC ϵ . (A) Relocation of β -catenin started as soon as 10 min after TRH stimulation, and the effect was maximal after 1 h of stimulation. The TRH-induced redistribution of β -catenin was abolished when cells were transfected with the siRNA targeted to PKC α , which was not the case in cells transfected with the siRNA targeted to PKC ϵ (B). Bar, 5 μ m.

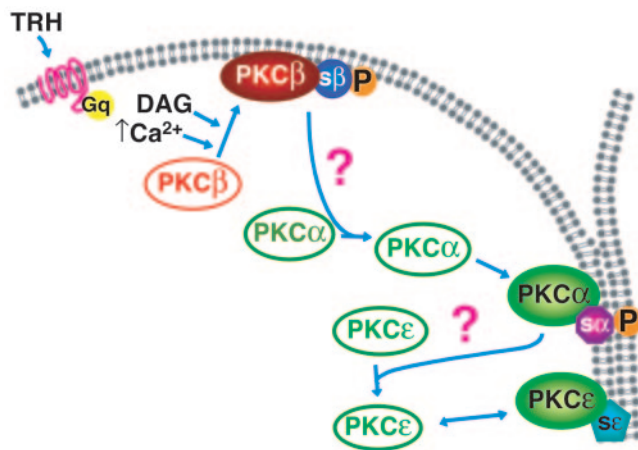


FIG. 11. Schematic representation of the coordinated cascade of PKC β 1, - α , and - ϵ activation. Upon stimulation of the Gq-coupled TRH receptor, intracellular Ca $^{2+}$ and DAG concentrations increase and PKC β 1 would be activated according to the currently accepted paradigm of PKC translocation/activation. It accumulates at the plasma membrane where it would phosphorylate a substrate that would relocate to the cytoplasm, as already shown for example in the case of MARCKS (31). The unknown PKC β 1 substrate would elicit directly or indirectly the selective translocation of PKC α to the cell-cell contact. PKC α would in turn phosphorylate a substrate that would mediate directly or indirectly PKC ϵ translocation to the cell-cell contact. PKC δ would not enter in this scheme, as its translocation to the plasma membrane is not dependent on PKC activity. s β , PKC β 1 substrate; s α , PKC α substrate; s ϵ , PKC ϵ substrate.

Saccharomyces cerevisiae, Pkc1p (11). In particular, it was shown that functional Pkc1p activity is essential for its proper targeting, an observation which is in line with our results demonstrating that the cell-cell contact selective targeting of PKC α and - ϵ is dependent on PKC activity.

Decoding the sequential and coordinated activation of PKCs has evidenced that PKCs display different activation profiles, in regards to the time of onset and the duration of activation, which are both unrelated to the subclass the isoform belongs to or to the subcellular targeting site. The various durations of activation could be interpreted in terms of different interaction modes at the membrane, either with a protein or with membrane phospholipids, and/or to the different phosphorylation status of each PKC isoform. Indeed, PKCs are known to interact with many partners (39), some of which are adaptor proteins able to anchor specific isoforms to selective subcellular locations and thus to maintain each PKC isoform next to a subset of proteins and away from the other substrates (29, 30, 48). A recent example of this has been provided by Park et al. who show that RACK1 (named RACK1 for receptor for activated C kinase I) anchors PKC β on melanosomes where it phosphorylates tyrosinase (36). In PKC ϵ -induced heart failure, PKC β 2-RACK1 interactions are enhanced (37). AKAPs (A-kinase anchoring proteins) are also important interacting proteins for PKC signaling (6), able for instance to associate with cadherin adhesion molecules (16). Other proteins also play the role of anchor for selective PKCs in selective subcellular sites, such as pericentrin for PKC β 2 in the centrosome (8). In GH3B6 cells, we know that RACK1 does not account for PKC α or PKC ϵ cell-cell contact location, since RACK1 immu-

noreactivity can be evidenced at the plasma membrane but is excluded from the cell-cell contact (51). A search for the PKC α and - ϵ partners expressed in the GH3B6 cell line is currently under way. The various onsets and durations of activation could also be due to the phosphorylation state of each isoform. Concerning the onset of activation, if the cPKC isoform phosphorylation status does not seem to be involved, this might not be the case for the nPKCs isoform status. Under the usual experimental conditions, i.e., in the presence of serum, cPKC isoforms already bear the three priming phosphorylations necessary for their activation. This is in agreement with our previous observations showing that the regulatory domain of PKC α , which does not contain any of the three amino acids involved in the priming phosphorylations, is still able to accumulate at cell-cell contacts at a rate similar to that of PKC α (52). In the case of the nPKCs, these phosphorylations are completed together with the activation process and they can involve cross regulation between the nPKCs themselves (45). Interestingly, it has been shown that integrin engagement can induce PKC ϵ phosphorylation (21). It is also known that the phosphorylation of the activation loop of nPKCs is involved in the duration of activation, since the concerned isoform remains activated for a longer time when phosphorylation is prevented by targeted mutation (15, 49).

The coordinated cascade of PKC isoform activation revealed in the present study could be a hallmark of differentiated secreting pituitary cells. Although its function is yet unknown, targeting of selective PKC isoforms at cell-cell contacts has been shown to be functionally important in other cellular systems. Indeed, in a coculture of tumoral epithelial cells and healthy fibroblasts, PKC α and - ϵ strictly accumulate at the heterologous cell-cell contact. In this case, the establishment of these cell-cell contacts and the activation of PKC α and - ϵ are essential for the production of matrix metalloproteinase stromelysin-3 by the fibroblasts (27). In the macrophage-like cell line RAW 264.7, PKC α is targeted selectively at the site of phagocytosis induced by contact with beads covered with immunoglobulin G (24). PKC θ has also been shown to selectively localize at the immunologic synapse, which is the heterologous contact between the T cell and the antigen-presenting cell (32, 33). This selective PKC θ localization is dependent upon CD28 expression and is involved in T-cell activation (20). In the GH3B6 cell line, TRH is known to regulate hormone secretion and proliferation, but it does not have any well-established functions at cell-cell contacts. PKC α and - ϵ at cell-cell contacts may not serve the hormonal secretion, since it has been shown in primary cultured isolated lactotrophs that TRH elicits secretion (9). Indeed, in these isolated cells, PKC α and - ϵ activation is not expected, since we show (Fig. 1) that, in the presence of TRH, PKC α and - ϵ are not translocated, and thus not activated, in isolated pituitary GH3B6 cells. Rather, results from our group suggest that the selective targeting of PKC α and - ϵ to the cell-cell contact may regulate adherent junctions, since TRH was found to induce the relocalization of β -catenin at the cell-cell contact (51). In the present study, we show that PKC α is involved in the relocalization of β -catenin at cell-cell contacts, whereas PKC ϵ is not. This suggests that although they are mechanistically associated into a cascade of activation and translocated to the same location after stimulation, each of these PKC isoforms might exert one or several specific func-

tions. Furthermore, these results demonstrate that PKC ϵ clearly does not behave as an "end-point" kinase and is not the unique functional effector of this activation cascade.

However, the fact that PKC signaling is organized into a coordinated cascade implies that knocking down PKC β 1 would affect the biological effects directly related to this isoform but also those connected to the isoforms located further downstream in the cascade, namely, PKC α and - ϵ . This also renders more complex the interpretation of the various phenotypes obtained in PKC isoform-specific knockout mice by questioning to which extent one can attribute a particular phenotype to a particular isoform.

In conclusion, signaling by the PKC family constitutes a good example of a space/time/isoform-determined process. It is capable of self-organization in order to achieve an appropriate spatiotemporal control of localization and activation of selective isoforms.

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REFERENCES

- Ahn, S., S. K. Shenoy, H. Wei, and R. J. Lefkowitz. 2004. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J. Biol. Chem.* **279**:35518–35525.
- Alvaro, V., L. Levy, C. Dubray, A. Roche, F. Peillon, B. Querat, and D. Joubert. 1993. Invasive human pituitary tumors express a point-mutated alpha-protein kinase-C. *J. Clin. Endocrinol. Metab.* **77**:1125–1129.
- Babwah, A. V., L. B. Dale, and S. S. Ferguson. 2003. Protein kinase C isoform-specific differences in the spatial-temporal regulation and decoding of metabotropic glutamate receptor 1a-stimulated second messenger responses. *J. Biol. Chem.* **278**:5419–5426.
- Brancho, D., J. J. Ventura, A. Jaeschke, B. Doran, R. A. Flavell, and R. J. Davis. 2005. Role of MLK3 in the regulation of mitogen-activated protein kinase signaling cascades. *Mol. Cell. Biol.* **25**:3670–3681.
- Braun, D. C., Y. Cao, S. Wang, S. H. Garfield, G. M. Hur, and P. M. Blumberg. 2005. Role of phorbol ester localization in determining protein kinase C or RasGRP3 translocation: real-time analysis using fluorescent ligands and proteins. *Mol. Cancer Ther.* **4**:141–150.
- Carnegie, G. K., F. D. Smith, G. McConnachie, L. K. Langeberg, and J. D. Scott. 2004. AKAP-Lbc nucleates a protein kinase D activation scaffold. *Mol. Cell* **15**:889–899.
- Chang, L., and M. Karin. 2001. Mammalian MAP kinase signalling cascades. *Nature* **410**:37–40.
- Chen, D., A. Purohit, E. Halilovic, S. J. Doxsey, and A. C. Newton. 2004. Centrosomal anchoring of protein kinase C betaII by pericentrin controls microtubule organization, spindle function, and cytokinesis. *J. Biol. Chem.* **279**:4829–4839.
- Cochilla, A. J., J. K. Angleson, and W. J. Betz. 2000. Differential regulation of granule-to-granule and granule-to-plasma membrane fusion during secretion from rat pituitary lactotrophs. *J. Cell Biol.* **150**:839–848.
- Corbalan-Garcia, S., J. A. Rodriguez-Alfaro, and J. C. Gomez-Fernandez. 1999. Determination of the calcium-binding sites of the C2 domain of protein kinase Alpha that are critical for its translocation to the plasma membrane. *Biochem. J.* **337**:513–521.
- Denis, V., and M. S. Cyert. 2005. Molecular analysis reveals localization of *Saccharomyces cerevisiae* protein kinase C to sites of polarized growth and Pkc1p targeting to the nucleus and mitotic spindle. *Eukaryot. Cell* **4**:36–45.
- Disatnik, M. H., S. C. Boutet, C. H. Lee, D. Mochly-Rosen, and T. A. Rando. 2002. Sequential activation of individual PKC isozymes in integrin-mediated muscle cell spreading: a role for MARCKS in an integrin signaling pathway. *J. Cell Sci.* **115**:2151–2163.
- Disatnik, M. H., S. C. Boutet, W. Pacio, A. Y. Chan, L. B. Ross, C. H. Lee, and T. A. Rando. 2004. The bi-directional translocation of MARCKS between membrane and cytosol regulates integrin-mediated muscle cell spreading. *J. Cell Sci.* **117**:4469–4479.

14. **Eliyahu, E., and R. Shalgi.** 2002. A role for protein kinase C during rat egg activation. *Biol. Reprod.* **67**:189–195.
15. **Feng, X., and Y. A. Hannun.** 1998. An essential role for autophosphorylation in the dissociation of activated protein kinase C from the plasma membrane. *J. Biol. Chem.* **273**:26870–26874.
16. **Gorski, J. A., L. L. Gomez, J. D. Scott, and M. L. Dell'acqua.** 2005. Association of an A-kinase-anchoring protein signaling scaffold with cadherin adhesion molecules in neurons and epithelial cells. *Mol. Biol. Cell.* **16**:3574–3590.
17. **Gschwendt, M., S. Dieterich, J. Rennecke, W. Kittstein, H. J. Mueller, and F. J. Johannes.** 1996. Inhibition of protein kinase C mu by various inhibitors. Differentiation from protein kinase c isoenzymes. *FEBS Lett.* **392**:77–80.
18. **Guerineau, N. C., X. Bonnefont, L. Stoeckel, and P. Mollard.** 1998. Synchronized spontaneous Ca^{2+} transients in acute anterior pituitary slices. *J. Biol. Chem.* **273**:10389–10395.
19. **Halet, G.** 2004. PKC signaling at fertilization in mammalian eggs. *Biochim. Biophys. Acta* **1742**:185–189.
20. **Huang, J., P. F. Lo, T. Zal, N. R. Gascoigne, B. A. Smith, S. D. Levin, and H. M. Grey.** 2002. CD28 plays a critical role in the segregation of PKC theta within the immunologic synapse. *Proc. Natl. Acad. Sci. USA* **99**:9369–9373.
21. **Ivaska, J., L. Bosca, and P. J. Parker.** 2003. PKCepsilon is a permissive link in integrin-dependent IFN-gamma signalling that facilitates JAK phosphorylation of STAT1. *Nat. Cell Biol.* **5**:363–369.
22. **Jenster, G.** 2004. A visualisation concept of dynamic signalling networks. *Mol. Cell. Endocrinol.* **218**:1–6.
23. **Kohout, S. C., S. Corbalan-Garcia, A. Torrecillas, J. C. Gomez-Fernandez, and J. J. Falke.** 2002. C2 domains of protein kinase C isoforms alpha, beta, and gamma: activation parameters and calcium stoichiometries of the membrane-bound state. *Biochemistry* **41**:11411–11424.
24. **Larsen, E. C., J. A. DiGennaro, N. Saito, S. Mehta, D. J. Loegering, J. E. Mazurkiewicz, and M. R. Lennartz.** 2000. Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. *J. Immunol.* **165**:2809–2817.
25. **Lenz, J. C., H. P. Reusch, N. Albrecht, G. Schultz, and M. Schaefer.** 2002. Ca^{2+} -controlled competitive diacylglycerol binding of protein kinase C isoenzymes in living cells. *J. Cell Biol.* **159**:291–302.
26. **Looby, E., A. Long, D. Kelleher, and Y. Volkov.** 2005. Bile acid deoxycholate induces differential subcellular localisation of the PKC isoenzymes beta 1, epsilon and delta in colonic epithelial cells in a sodium butyrate insensitive manner. *Int. J. Cancer* **114**:887–895.
27. **Louis, K., N. Guerineau, O. Fromigue, V. Defamie, A. Collazos, P. Anglard, M. A. Shipp, P. Auberger, D. Joubert, and B. Mari.** 2005. Tumor cell-mediated induction of the stromal factor stromelysin-3 requires heterotypic cell contact-dependent activation of specific protein kinase C isoforms. *J. Biol. Chem.* **280**:1272–1283.
28. **Martiny-Baron, G., M. G. Kazanietz, H. Mischak, P. M. Blumberg, G. Kochs, H. Hug, D. Marme, and C. Schachtele.** 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* **268**:9194–9197.
29. **Mochly-Rosen, D.** 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* **268**:247–251.
30. **Mochly-Rosen, D., B. L. Smith, C. H. Chen, M. H. Disatnik, and D. Ron.** 1995. Interaction of protein kinase C with RACK1, a receptor for activated C-kinase: a role in beta protein kinase C mediated signal transduction. *Biochem. Soc. Trans.* **23**:596–600.
31. **Mogami, H., H. Zhang, Y. Suzuki, T. Urano, N. Saito, I. Kojima, and O. H. Petersen.** 2003. Decoding of short-lived Ca^{2+} influx signals into long term substrate phosphorylation through activation of two distinct classes of protein kinase C. *J. Biol. Chem.* **278**:9896–9904.
32. **Monks, C. R., B. A. Freiberg, H. Kupfer, N. Siciak, and A. Kupfer.** 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature* **395**:82–86.
33. **Monks, C. R., H. Kupfer, I. Tamir, A. Barlow, and A. Kupfer.** 1997. Selective modulation of protein kinase C-theta during T-cell activation. *Nature* **385**:83–86.
34. **Oancea, E., and T. Meyer.** 1998. Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. *Cell* **95**:307–318.
35. **Olson, J. M., and A. R. Hallahan.** 2004. p38 MAP kinase: a convergence point in cancer therapy. *Trends Mol. Med.* **10**:125–129.
36. **Park, H. Y., H. Wu, C. E. Killoran, and B. A. Gilchrist.** 2004. The receptor for activated C-kinase-I (RACK-1) anchors activated PKC-beta on melanosomes. *J. Cell Sci.* **117**:3659–3668.
37. **Pass, J. M., J. Gao, W. K. Jones, W. B. Wead, X. Wu, J. Zhang, C. P. Baines, R. Bolli, Y. T. Zheng, I. G. Joshua, and P. Ping.** 2001. Enhanced PKC beta II translocation and PKC beta II-RACK1 interactions in PKC epsilon-induced heart failure: a role for RACK1. *Am. J. Physiol. Heart Circ. Physiol.* **281**:H2500–H2510.
38. **Peter, M., S. M. Ameer-Beg, M. K. Hughes, M. D. Keppler, S. Prag, M. Marsh, B. Vojnovic, and T. Ng.** 2005. Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophys. J.* **88**:1224–1237.
39. **Poole, A. W., G. Pula, I. Hers, D. Crosby, and M. L. Jones.** 2004. PKC-interacting proteins: from function to pharmacology. *Trends Pharmacol. Sci.* **25**:528–535.
40. **Prevostel, C., V. Alvaro, F. de Boisvilliers, A. Martin, C. Jaffiol, and D. Joubert.** 1995. The natural protein kinase C alpha mutant is present in human thyroid neoplasms. *Oncogene* **11**:669–674.
41. **Prevostel, C., V. Alvaro, A. Vallentin, A. Martin, S. Jaken, and D. Joubert.** 1998. Selective loss of substrate recognition induced by the tumour-associated D294G point mutation in protein kinase Calpha. *Biochem. J.* **334**:393–397.
42. **Qatsha, K. A., C. Rudolph, D. Marme, C. Schachtele, and W. S. May.** 1993. Go 6976, a selective inhibitor of protein kinase C, is a potent antagonist of human immunodeficiency virus 1 induction from latent/low-level-producing reservoir cells in vitro. *Proc. Natl. Acad. Sci. USA* **90**:4674–4678.
43. **Quittau-Prevostel, C., N. Delaunay, A. Collazos, A. Vallentin, and D. Joubert.** 2004. Targeting of PKCalpha and epsilon in the pituitary: a highly regulated mechanism involving a GD(E)E motif of the V3 region. *J. Cell Sci.* **117**:63–72.
44. **Raghunath, A., M. Ling, and C. Larsson.** 2003. The catalytic domain limits the translocation of protein kinase C alpha in response to increases in Ca^{2+} and diacylglycerol. *Biochem. J.* **370**:901–912.
45. **Rybin, V. O., A. Sabri, J. Short, J. C. Braz, J. D. Molkentin, and S. F. Steinberg.** 2003. Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations. *J. Biol. Chem.* **278**:14555–14564.
46. **Schaefer, M., N. Albrecht, T. Hofmann, T. Gudermann, and G. Schultz.** 2001. Diffusion-limited translocation mechanism of protein kinase C isotypes. *FASEB J.* **15**:1634–1636.
47. **Schaefer, M., H. Mischak, S. Schnell, A. Griese, R. Iakubov, G. Riepenhausen, and C. Schoff.** 2004. Mechanisms of arginine-vasopressin-induced Ca^{2+} oscillations in beta-cells (HIT-T15): a role for oscillating protein kinase C. *Endocrinology* **145**:4635–4644.
48. **Schechtman, D., and D. Mochly-Rosen.** 2001. Adaptor proteins in protein kinase C-mediated signal transduction. *Oncogene* **20**:6339–6347.
49. **Seki, T., H. Matsubayashi, T. Amano, Y. Shirai, N. Saito, and N. Sakai.** 2005. Phosphorylation of PKC activation loop plays an important role in receptor-mediated translocation of PKC. *Genes Cells* **10**:225–239.
50. **Uchino, M., N. Sakai, K. Kashiwagi, Y. Shirai, Y. Shinohara, K. Hirose, M. Iino, T. Yamamura, and N. Saito.** 2004. Isoform-specific phosphorylation of metabotropic glutamate receptor 5 by protein kinase C (PKC) blocks Ca^{2+} oscillation and oscillatory translocation of Ca^{2+} -dependent PKC. *J. Biol. Chem.* **279**:2254–2261.
51. **Vallentin, A., T. C. Lo, and D. Joubert.** 2001. A single point mutation in the V3 region affects protein kinase C α targeting and accumulation at cell-cell contacts. *Mol. Cell. Biol.* **21**:3351–3363.
52. **Vallentin, A., C. Prevostel, T. Fauquier, X. Bonnefont, and D. Joubert.** 2000. Membrane targeting and cytoplasmic sequestration in the spatiotemporal localization of human protein kinase C alpha. *J. Biol. Chem.* **275**:6014–6021.
53. **van Baal, J., J. de Widt, N. Divecha, and W. J. van Blitterswijk.** 2005. Translocation of diacylglycerol kinase theta from cytosol to plasma membrane in response to activation of G protein-coupled receptors and protein kinase C. *J. Biol. Chem.* **280**:9870–9878.
54. **Violin, J. D., J. Zhang, R. Y. Tsien, and A. C. Newton.** 2003. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J. Cell Biol.* **161**:899–909.
55. **Wang, Q. J., G. Lu, W. A. Schlupkohl, A. Goerke, C. Larsson, H. Mischak, P. M. Blumberg, and J. F. Mushinski.** 2004. The V5 domain of protein kinase C plays a critical role in determining the isoform-specific localization, translocation, and biological function of protein kinase C-delta and -epsilon. *Mol. Cancer Res.* **2**:129–140.