Evidence for a role of conventional protein kinase-C α in the control of homotypic contacts and cell scattering of HT-29 human intestinal cells

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Incubation of HT-29 M6 cells with the phorbol ester phorbol 12-myristate 13-acetate (PMA) induces cell scattering, loss of cellular contacts and inactivation of E-cadherin. We have investigated the involvement of different protein kinase C (PK-C) isoforms in these processes using specific activators. Thymeleatoxin, a derivative of mezerein that activates conventional PK-Cs (cPK-Cs) but not novel PK-Cs (nPK-Cs), promoted effects that were similar to those of PMA, i.e. at concentrations of 200 nM it induced scattering of HT-29 M6 colonies, loss of homotypic contacts and dissociation of E-cadherin from the cytoskeleton. Among the isoforms activated by this compound, only cPK-C α was detected in HT-29 M6 cells by Western blot. The specificity

of this compound with respect to the rest of the PK-C isoforms present in these cells was determined; thymeleatoxin induced, as did PMA, the translocation of cPK-C α from the cytosol to the membrane and the cytoskeleton, and its partial down-regulation. On the other hand, thymeleatoxin did not modify the cellular levels or localization of nPK-C ϵ or atypical PK-C ζ . 'In vitro' assays also showed that thymeleatoxin did not activate nPK-C ϵ at the concentrations added to the cell cultures. These results indicate that thymeleatoxin is selective for cPK-C α over nPK-C ϵ and show a role for the former enzyme in the regulation of cell-cell contacts and the inactivation of E-cadherin in HT-29 M6 cells.

INTRODUCTION

Protein kinase C (PK-C) is a key enzyme in the processes of signal transduction triggered by growth factors, hormones and neurotransmitters [1,2]. Several members of this extended family have been described so far; these protein kinases have been classified into three different subfamilies: (1) the conventional PK-C (cPK-C) subfamily which comprises four members α , β I, β II and γ that require Ca²⁺, phospholipid and diacylglycerol or phorbol ester for activation; (2) the novel PK-C (nPK-C) subfamily, composed of four members (δ , ϵ , η and θ), which is independent of Ca²⁺; and (3) the atypical PK-Cs (aPK-Cs), ζ and λ , which are insensitive to diacylglycerol and Ca²⁺ [2,3]. Recently, a new transmembrane form of PK-C, denominated PK-C μ , has been shown to be also activable by phorbol 12-myristate 13-acetate (PMA) [4,5].

The phorbol ester PMA has been commonly used to activate PK-C in cells as it is membrane-permeable and causes a maintained stimulation of most members of this family (except aPK-Cs) [1]. The characterization of compounds with structural similarity to PMA, but with selective activity towards certain PK-C isotypes or, at least, subfamilies has been the subject of recent research. Recent progress in this area has been achieved by the characterization of the activation of cPK-Cs but not nPK-Cs by the mezerein-analogous thymeleatoxin (TMTA) [6,7]. This specificity has prompted several groups to use this compound to ascribe effects of PMA to a specific cPK-C isoform [8,9].

Activation of PK-C by the phorbol ester PMA exerts a remarkable effect over the phenotype of HT-29 M6 cells, a cell line that after confluence differentiates to a mucus-secreting phenotype [10,11]. Results from our laboratory have shown that PMA causes the scattering of HT-29 M6 cell colonies and the acquisition of a 'fibroblastic' morphology [12]. As one of the initial steps in this process, a decrease in the cellular adhesive

properties of these cells is induced by PMA [12]. Our aim in this study has been to identify the PK-C isoform involved in the loss of cell-cell contacts and scattering of HT-29 M6 cells.

EXPERIMENTAL

Materials

Phosphatidylserine, myelin basic protein (MBP), PMA, phenylarsine oxide (PAO) and PMSF were supplied by Sigma (St. Louis, MO, U.S.A.). TMTA was from LC Laboratories (Woburn, MA, U.S.A.). Both PMA and TMTA were dissolved in DMSO at 100 μ M and stored at -40°C . Leupeptin and the PK-C inhibitor GF109203X (GF) were provided by Boehringer (Mannheim, Germany). DEAE-Sephacel was from Pharmacia (Uppsala, Sweden). [γ -³²P]ATP was purchased from New England Nuclear. Prestained SDS/PAGE molecular-mass (MW) markers were from Bio-Rad (Richmond, CA, U.S.A.). All the other chemicals were commercial products of the highest grade available.

Antibodies

The monoclonal antibodies (mAbs) anti-cPK-C α , anti-cPK-C β and anti-nPK-C ϵ were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). These antibodies were raised against an 18 kDa fragment (amino acid residues 270–427) of cPK-C α , a 23 kDa fragment (residues 126–324) of cPK-C β , and a 20 kDa fragment (residues 1–175) of nPK-C ϵ , respectively. The mAb anti-cPK-C α MC5 (Amersham), which mapped to residues 312–323, was kindly provided by Dr. D. Colomer (Hospital Clínic, Barcelona, Spain). Anti-peptide antibodies anti-cPK-C γ , raised against peptide NYPLELYERVRTG (residues 306–318),

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GF, PK-C inhibitor GF109203X; mAb, monoclonal antibody; MBP, myelin basic protein; PAO, phenylarsine oxide; PK-C, protein kinase C; cPK-C, nPK-C, or aPK-C, conventional, novel or atypical PK-C; TMTA, thymeleatoxin; PMA, phorbol 12-myristate 13-acetate.

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anti-nPK-C ϵ , raised against peptide DGFSYFGEDLMP (residues 726–737), and anti-aPK-C ζ , raised against peptide GFEYINPLLLSAEESV (sequence 577–592), were purchased from Gibco (Gaithersburg, MD, U.S.A.). A polyclonal antibody directed against the same peptide of aPK-C ζ was prepared in our laboratory [13]. In order to validate our conclusions, in this study Western-blot analysis of cPK-C α and nPK-C ϵ were always repeated with the two different antibodies recognizing each one of these isoforms; the results were always identical. In our hands, all the antibodies listed here, broadly used in many studies, showed an absolute specificity for the isoform indicated; only the anti-peptide antibody anti-aPK-C ζ reacted weakly with a 77 kDa cPK-C [13] that we have identified as cPK-C α . Our conclusions with respect to aPK-C ζ are restricted to the major 72 kDa band only.

Cell culture

The HT-29 M6 cell line, originally described and characterized with the name HT-29 (10⁻⁶ methotrexate) [10,11] was provided by Dr. Alain Zweibaum (INSERM, Villejuif, France). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) in a humidified atmosphere of 5% CO₂/95% air. Culture medium was changed every other day to avoid nutrient depletion. Experiments were performed with cells at days 4–5 of culture, when they were 40–60% confluent.

Cell dissociation assay

This assay was performed as described [14] with some minor modifications. Cells (approx. 1 × 10⁶ cells per 6-cm-diam. dish) were treated with 2 ml of trypsin (0.05%) in Puck's EDTA medium (NaHCO₃, 4 mM; NaCl, 136 mM; KCl, 4 mM; EDTA, 1 mM; glucose, 1 mg/ml; Phenol Red, 0.005 mg/ml) (TE treatment) or in Puck's EDTA medium supplemented with CaCl₂ (5 mM) (TC treatment) for 20 min at 37 °C, and dissociated by pipetting 20 times. The extent of dissociation of the cells was represented by the index N_{TC}/N_{TE} , where N_{TC} and N_{TE} are the total particle number after the TC and TE treatments respectively.

Cell fractionation

Cells were washed twice in buffer A [25 mM Tris/HCl, pH 7.6, 1 mM EGTA, 10 mM NaCl, 1 mM dithiothreitol] and homogenized on ice by 40 strokes in a Dounce homogenizer in the same buffer containing 1 mM PMSF and 20 µg/ml leupeptin. Homogenate was centrifuged at 15000 *g* for 15 min at 4 °C; supernatant constituted the soluble cytosolic fraction. Pellet was resuspended in buffer A–Triton (buffer A plus PMSF and leupeptin and made to 1% in Triton X-100) by ten strokes in a Dounce homogenizer and left to stand on ice for 30 min. After centrifugation under the same conditions as before, the supernatant constituted the detergent-extracted, membrane fraction. Pellets were triturated in SDS-buffer (25 mM Tris/HCl, pH 7.6, 5 mM EDTA, 2.5 mM EGTA, 1% SDS) and boiled for 10 min at 100 °C. When the presence of PK-C isoforms was analysed by Western blot using anti-peptide antibodies, the cytosolic and membrane fractions were further purified by DEAE-Sephacel; samples were applied to a DEAE-Sephacel column pre-equilibrated in buffer A and PK-Cs were eluted in the same buffer made to 150 mM NaCl.

In order to analyse E-cadherin association to the cytoskeleton, Triton-soluble and -insoluble fractions were prepared as described by Nelson and co-workers [15]. Cells were rinsed in PBS plus 1 mM CaCl₂ and homogenized in CSK buffer (50 mM

NaCl, 10 mM Pipes, pH 6.8, 3 mM MgCl₂, 0.5% Triton X-100, 300 mM sucrose, 1 mM PMSF, 10 µg/ml leupeptin, 1 mM orthovanadate, 20 µM PAO) for 10 min at 4 °C with gentle rocking. After centrifugation in a microfuge for 10 min at 4 °C, the supernatant constituted the Triton-soluble fraction. Pellet was triturated in SDS buffer and boiled at 100 °C for 10 min (Triton-insoluble fraction).

Western-blot analysis

Proteins were separated by SDS/PAGE, transferred electrophoretically to nitrocellulose and incubated for 2 h with the different antibodies. After washing with TBSt (20 mM Tris/HCl, pH 7.6, 140 mM NaCl, 0.2% Triton X-100), membranes were incubated with peroxidase-conjugated goat anti-(rabbit IgG) or goat anti-(mouse IgG) antibodies (Dakopatts, Copenhagen, Denmark) and reacting antigens were visualized using enhanced chemiluminescence (ECL) detection reagents (Amersham).

PK-C activity

PK-C activity was assayed using MBP (0.5 mg/ml) as substrate as described in [16] with 10 or 3.2 µg of partially purified cPK-C α or nPK-C ϵ , respectively, as the source of enzyme. In order to separate these two enzymes, the procedure described in [13] was used. Briefly, cells were washed and homogenized in a Dounce homogenizer in buffer B (25 mM Tris/HCl, pH 7.6, 10 mM NaCl, 3 mM CaCl₂) supplemented with protease inhibitors as above. After 30 min on ice, the homogenate was centrifuged for 15 min at 15000 *g*, the pellet washed again with the same buffer and spun under the same conditions. After this second centrifugation, the pellet was resuspended in buffer C (25 mM Tris/HCl, pH 7.6, 10 mM NaCl, 4 mM EGTA) plus protease inhibitors by ten strokes in a Dounce homogenizer and allowed to stand on ice for 30 min. After centrifugation, the supernatant (EGTA wash) only contained cPK-Cs (in these cells cPK-C α) and was devoid of any nPK-C ϵ detectable by Western blot. nPK-C ϵ was present in the pellet of this last centrifugation; it was solubilized in buffer A–Triton X-100 as indicated above. Both fractions (EGTA wash and Triton-solubilized) were purified by chromatography on DEAE-Sephacel as mentioned.

RESULTS

We have previously reported that PMA induces remarkable morphological changes in HT-29 M6 cultures [12]. After addition of this phorbol ester, HT-29 M6 colonies scatter and cells acquire a fibroblastic aspect. This effect of PMA is blocked or reversed by PK-C inhibitors such as GF [12], which suggests that the persistent activation of some PMA-responsive PK-C isoform is triggering this event. As we have mentioned previously, TMTA is a compound that activates selectively cPK-Cs but not nPK-Cs. Addition of TMTA to HT-29 M6 cultures promoted scattering of these cells; after 15 h the resultant phenotype was identical to that obtained after incubation with PMA (Figure 1). The kinetics of cell scattering were similar as well, although slightly slower in the case of TMTA. At the same concentration (200 nM), PMA induced the appearance of morphological changes after 1 h of incubation, whereas TMTA required 2 h. TMTA showed a 5-fold lower potency than PMA; the minimal dose required to induce cell scattering after 15 h of incubation was approximately 20 nM and 4 nM, respectively, for TMTA and PMA. As for PMA, morphological changes induced by TMTA were prevented by the PK-C inhibitor GF (2 µM) (Figure 1).

One of the events necessary for cell scattering is the loss of cell-to-cell contacts. To evaluate homotypic contacts we have used

Figure 1 PMA and TMTA induce scattering of HT-29 M6 intestinal cells

Cells, grown in complete medium (DMEM supplemented with 10% FBS) were incubated with PMA (panels **B** and **C**) or TMTA (panels **D**, **E** and **F**) (200 nM both) in the same medium. Pictures were taken under the phase-contrast microscope at $\times 200$ magnification after 0 (panel **A**), 3 (panels **B** and **D**) or 15 h (panels **C**, **E** and **F**). In the experiment shown in panel **F**, cells were incubated in the presence of TMTA for 3 h; the medium was then supplemented with the PK-C inhibitor GF (2 μ M) and cells were incubated for other 12 h. No morphological differences were observed from the control when GF, GF plus PMA, or GF plus TMTA were added to the cells at the beginning of the experiment (results not shown).

Figure 2 Phorbol esters increase dissociation of HT-29 M6 cells

Preconfluent cells, grown in complete medium, were incubated with PMA (TPA) or TMTA (200 nM both) for 9 h; cells were then treated with trypsin (0.05%) in the presence of Ca^{2+} (TC treatment) or EDTA (TE treatment). The cell dissociation index M_{TC}/M_{TE} (see the Experimental section) is shown at the bottom. The Figure displays the results of one experiment of five performed; the mean values ($\pm$ S.D.) were 0.084 ($\pm$ 0.053), 0.344 ($\pm$ 0.087) and 0.244 ($\pm$ 0.098), for control cells, cells treated with PMA or TMTA respectively. Representative photographs of cells after TC or TE treatments are shown; it can be observed that the size of aggregates after TC treatment were always much smaller when the cells had been incubated with the phorbol esters.

the assay described by Nagafuchi and co-workers with minor modifications. Cells, incubated with PMA or TMTA, were treated with trypsin in the presence or absence of Ca^{2+} (TC or TE

treatment respectively) and the number of particles (clusters or single cells) was counted. A representative experiment of five performed is shown in Figure 2. In control HT-29 M6 cells the

Figure 3 Phorbol esters solubilize E-cadherin

Triton-soluble or -insoluble fractions were prepared from control cells or cells treated for 9 h with PMA (TPA) or TMTA (200 nM both) as described in the Experimental section. Equivalent amounts of both fractions (approx. 40 μ g of Triton-soluble and 8 μ g of Triton-insoluble) were subjected to SDS/PAGE and analysed by Western blot with the anti-E-cadherin mAb HECD-1 [a kind gift from Dr. A. Cano (Universidad Autónoma, Madrid)]. Only a band of 120 kDa, corresponding to E-cadherin, was detected. The Figure shows a representative experiment of three performed.

Figure 4 HT-29 M6 cells contain cPK-C α but not cPK-Cs β or γ

HT-29 M6 cells (40 μ g of cytosol plus membrane fractions) were analysed by Western blot with mAbs anti-cPK-C α , anti-cPK-C β , and with anti-peptide antibody anti-cPK-C γ (see the Experimental section). As a control, the Ewing sarcoma cells IARC EW-1 were used (20 μ g of extract).

mean N_{TC}/N_{TE} ratio was determined to be 0.084 (± 0.053 , S.D.), reflecting the fact that, since molecules required for homotypic contacts are preserved after TC but not after TE treatment, most cells are in contact in these conditions. This ratio is increased up to 0.344 (± 0.087) or to 0.244 (± 0.098) by PMA or TMTA respectively, indicating the presence of a lower number of cells in contact (Figure 2). The increase in the index was higher when PMA was added (4.1-fold) than in the case of TMTA (2.8-fold), reflecting the fact that, as in the case of cell scattering, TMTA showed a slightly lower capability to break the homotypic contacts.

In our laboratory we have characterized that, concomitantly with cell scattering, PMA induces the inactivation of E-cadherin, a protein that plays a major role in the establishment of homotypic contacts in epithelial cells [17]. PMA causes the dissociation of E-cadherin from the cytoskeleton and, later on, its down-modulation [18]. As shown in Figure 3, TMTA induced a decrease in the amount of E-cadherin present in the Triton-resistant, cytoskeletal fraction, although to a lower extent than PMA.

The analysis of PK-C isoforms in HT-29 M6 cells have revealed the presence of cPK-Cs, nPK-C ϵ and aPK-C ζ , and not of nPK-Cs δ and η [13]. Our results using TMTA suggested that activation of a cPK-C isoform was responsible for cell scattering.

Figure 5 TMTA activates cPK-C α but not nPK-C ϵ in 'in vitro' protein kinase assays

cPK-C α and nPK-C ϵ were purified as described in the Experimental section. PK-C assays were performed using MBP as substrate under the conditions indicated. (A) cPK-C α ; (B) nPK-C ϵ . The mean $\pm$ S.E.M. (bars) of three experiments is shown. When indicated $-Ca^{2+}$, the assay was performed in the presence of 2 mM EGTA; $+Ca^{2+}$, corresponded to a final concentration of Ca^{2+} in the assay of 50 μ M. PMA and TMTA were used at a final concentration of 200 nM.

To find out which isoenzyme was involved in such effects several experiments were performed.

First, the presence of the different cPK-Cs was studied. Expression of both cPK-C α and β has been described in intestinal cells [19]; on the other hand, cPK-C γ seems to be exclusively expressed in brain [1]. However, as expression of cPK-C γ has been reported in a HT-29 clone [20], we analysed the presence of the three cPK-Cs in HT-29 M6 extracts using specific mAbs. cPK-C α was detected but not cPK-Cs β or γ (Figure 4). The presence of cPK-C α as the only cPK-C in these cells was supported by two other results. First, only a peak of PK-C activity was detected after purification of HT-29 M6 cPK-Cs by chromatography on hydroxyapatite, a column that separates these three isoforms; this peak of activity co-purified with immunoreactive cPK-C α (results not shown). Moreover, reverse-transcription PCR analysis of the cPK-Cs expressed in HT-29 M6 cells only showed the presence of cPK-C α (E. Batlle and A. G. de Herreros, unpublished work).

The specificity of TMTA towards c and nPK-Cs was also determined. *In vitro* PK-C assays using partially purified preparations of cPK-C α and nPK-C ϵ revealed that, at the concentrations used in our assays of cell scattering (200 nM), TMTA activated cPK-C α but not nPK-C ϵ (Figure 5). As expected, this activation required the presence of Ca^{2+} . At the same concentration PMA stimulated both PK-C isoforms (Figure 5).

In cell cultures, addition of PMA (200 nM) induced the rapid loss of cPK-C α from the cytosol and its appearance in the membrane and cytoskeletal fraction (Figure 6A). After 24 h, a high proportion of this protein was down-regulated (Figure 6A);

Figure 6 TMTA translocated and down-regulated cPK-C α but not nPK-C ϵ or aPK-C ζ in HT-29 M6 cells

Cytosolic, membrane and cytoskeletal fractions, or total extracts were prepared from cells treated with the two phorbol esters (200 nM) for the indicated times. Samples were analysed by SDS/PAGE and Western blot with antibodies anti-PK-C α (A), anti-nPK-C ϵ (B), or anti-aPK-C ζ (C). The major bands detected with the three antibodies corresponded to molecular masses of 80, 90 or 68 kDa, for cPK-C α , nPK-C ϵ or aPK-C ζ respectively. The time of exposition of the film was not the same in the different panels; therefore it should not be compared. The Figure shows the result of a representative experiment of five performed. m, min.

low levels were detected only in the cytoskeletal and membrane fractions (results not shown). In control cells, nPK-C ϵ was detected in both cytosolic and membrane fractions. After 30 min, PMA induced the disappearance of the cytosolic nPK-C ϵ , a modest increase in the level of this isoenzyme in the membrane, and a remarkable augmentation in the cytoskeleton (Figure 6B). A long-term incubation (24 h) with the phorbol ester induced the down-regulation of this PK-C isoenzyme in the cytosolic and cytoskeleton fractions, and minor changes in the membrane (Figure 6B).

Regarding cPK-C α , TMTA exerted the same effects as PMA, although with slower kinetics; it also induced the loss of cytosolic enzyme and its appearance in the membrane and cytoskeleton fractions (Figure 6A). These slower kinetics can be observed in Figure 6(A) (membrane panels), the appearance of cPK-C α in the membrane fraction was observed after 30 min of incubation of HT-29 M6 cells with PMA but required 120 min when TMTA was added. This translocation of cPK-C α to the membrane was slightly preceded by its appearance in the cytoskeleton. TMTA also caused an important down-regulation of cPK-C α (Figure 6A). Contrarily to these effects on cPK-C α , the cellular levels or distribution of nPK-C ϵ were not altered by short- or long-term treatments with TMTA (Figure 6B). The lack of effect of this compound on this isoenzyme is particularly remarkable in the cytosol and cytoskeleton. Levels in the cytosol were decreased after 30 min of incubation with PMA but remained unmodified even after 24 h of incubation with TMTA. Moreover, this phorbol ester, contrarily to PMA, did not induce any significant increase in the cytoskeleton nPK-C ϵ level (Figure 6B).

The cellular distribution of aPK-C ζ was also determined after addition of the two phorbol esters to HT-29 M6 cells. This isoform was detected exclusively in the cytosolic fraction. As expected, incubation of the cells with PMA or TMTA did not alter the cellular distribution or the total levels of this enzyme (Figure 6C).

DISCUSSION

Selective activators of individual PK-C isoforms provide a powerful tool to study the role of various PK-C isoforms in physiological functions. Several compounds, chemically related to the phorbol ester PMA, have been isolated [21]. Among them, TMTA (an analogue of mezerein) has received great attention since it reportedly stimulates specifically cPK-Cs and not nPK-Cs [6]. Using an *in vitro* assay with purified enzymes, these authors reported that this compound activated cPK-Cs with ED₅₀ values of 100 nM or less, but was inactive on nPK-Cs at 1 mM. The specificity of TMTA has been confirmed recently by binding assays [7], although the difference in selectivity was lower than reported; in this study TMTA was 5- to 20-fold more potent on cPK-Cs α or γ than on nPK-Cs δ , ϵ or θ . Owing to these properties TMTA has been used recently by several groups to establish the involvement of specific PK-Cs in several effects [8,9,22].

Using this compound we have been able to demonstrate that activation of cPK-C α induces cell scattering. Cell scattering is the result of the combined effects of PMA on cell attachment to the matrix and cell-cell adhesion [12]; both a decrease in cell-cell adhesion and an increase in cell motility are necessary for the cells to be able to disperse. TMTA induced cell scattering, loss of cellular contacts and dissociation of E-cadherin from the cytoskeleton, although with a lower potency than PMA. Studies of the translocation and down-regulation of the different PK-C isoforms detected in HT-29 M6 cells revealed that, at the same doses: (1) TMTA induced the translocation of cPK-C α from a soluble cytosolic fraction to the membrane fraction, although with a slower time-course than when induced by PMA; (2) TMTA also caused the down-regulation of this enzyme to a lower extent than PMA; (3) the presence of cPK-C α in the membrane fraction was preceded by its appearance in a Triton-insoluble, cytoskeletal fraction; and more remarkably, (4) TMTA

did not affect either the localization or the levels of nPK-C ϵ or aPK-C ζ . All together, these results indicate that activation of cPK-C α can induce cell scattering and loss of cell contacts.

Previous results of other groups suggested that, among the different PK-C isoforms, cPK-C α was the best candidate to be involved in cell scattering since: (1) this enzyme has been located in the points of association to the matrix (focal adhesions) and in cell-cell adhesion sites [23,24]; and (2), in REF52 cells, activation of cPK-C α has been shown to be necessary for migration [25], a phenomenon related to scattering in HT-29 M6 cells.

Several groups have recently questioned the specificity of TMTA for cPK-Cs [26,27]. These two groups found that TMTA induced a modest translocation but also the total down-modulation of nPK-C ϵ in PC-12 cells and in mesengial cells, suggesting that this compound might be activating *in vivo* nPK-Cs as well as cPK-Cs. These results are contradictory to ours since we did not detect any decrease in the total cellular levels of nPK-C ϵ after 24 h of incubation with TMTA at concentrations that promoted a marked morphological effect. We do not have a definitive response to this different sensitivity to TMTA of nPK-Cs in PC-12 or mesengial cells compared with HT-29 M6 cells. However, it should be remembered that translocation or down-regulation of a PK-C isoform by a compound does not always correlate with direct activation of this isoform by the compound; for instance several reports have shown changes in the cellular distribution and levels of aPK-C ζ by PMA [28,29], although the phorbol ester does not stimulate directly this enzyme [30]. An increase in the activity of a particular PK-C isoform can promote the indirect activation of additional forms of PK-C, via the stimulation of phospholipases C and D and the subsequent production of diacylglycerol [1,31]. In this respect it should be mentioned that Parker and co-workers have recently described that nPK-C ϵ down-regulation can be induced by activation of other PK-Cs; these authors suggested that down-regulation could be the consequence of the activation of certain PK-C isotypes, with other isotypes affected in *trans* [32]. It is then possible that the effects of TMTA on nPK-C ϵ observed by the mentioned authors [26,27] might be due to the indirect activation of this enzyme through cPK-C α stimulation.

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