Protein kinase C δ and η isoenzymes control the shedding of the interleukin 6 receptor α in myeloma cells

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The soluble interleukin 6 receptor α is an agonistic molecule of interleukin 6 (IL-6) and is important in the biology of multiple myeloma. More precisely, it potentiates the deleterious effects of IL-6 during tumour progression, facilitating angiogenesis and bone resorption. Because the mechanisms involved in the shedding of the interleukin 6 receptor α (IL-6R α) in multiple myeloma are not known, we have investigated them in the XG-6 human myeloma cell line. Here we provide evidence that PMA-induced IL-6R α shedding is controlled by a metalloproteinase and by protein kinase C (PKC) isoenzymes that do not require Ca²⁺ for their activation. We show that XG-6 cells express PKC- δ , - η and - ζ isoenzymes. However, after stimulation with PMA, only PKC- δ and PKC- η are activated, as shown by their translocation to the membrane. Treatment with PMA induces an increase in PKC- δ phosphorylation in its active loop. In addition, by using rottlerin,

a specific inhibitor of PKC- δ , we demonstrate that PKC- δ is involved in the PMA-induced shedding of IL-6R α . With the use of UO126, a specific inhibitor of the mitogen-activated protein kinase (MAPK) pathway, we show that the PMA-induced IL-6R α shedding is mediated in part by the MAPK pathway. Finally, whereas GF109203X, a general PKC inhibitor, inhibits the activation of ERK1/2 (extracellular signal-regulated protein kinase 1/2), rottlerin has no inhibitory effect, indicating that the Ras/MAPK activation is PKC-dependent but PKC- δ -independent. Taken together, these results suggest that the PMA-induced shedding of IL-6R α is mediated by a PKC isoenzyme network.

Key words: ERK/MAP kinase, phorbol ester, PKC inhibitors, metalloproteinase.

INTRODUCTION

Multiple myeloma (MM) is a plasma-cell malignancy characterized by the accumulation of slowly growing malignant plasma cells within the bone marrow. Interleukin 6 (IL-6) is the major growth and survival factor of myeloma cells [1,2]. Its biological activity results from the interactions between the cytokine and a receptor complex consisting of gp130, the signal-transducing subunit and a specific ligand-binding protein (interleukin 6 receptor α , gp80) which occurs in both membrane-anchored (mIL-6R α) and soluble (sIL-6R α) forms [3,4]. Elevated sIL-6R α serum levels have been found in patients with MM in correlation with a poor prognosis [5]. We have shown that sIL-6R α levels released by myeloma cells are correlated with disease progression, explaining in part the strong prognostic value of sIL-6R α serum levels in patients with MM [6]. Of major interest is the fact that the sIL-6R α is an agonist of IL-6 able to potentiate its activity [7,8]. Furthermore, we and others have shown that sIL-6R α facilitates tumour progression and bone resorption in MM [9–11]. The release of the sIL-6R α is the result of two non-exclusive mechanisms, namely a proteolytic cleavage of IL-6R α at the cell surface and the production of a form lacking the transmembrane domain [6,12]. The proteolytic cleavage, also named shedding, is inhibited by synthetic hydroxamate-based metalloproteinase inhibitors, indicating the involvement of a metalloproteinase in this process [13]. Among the metalloproteinases, the ADAM ('a disintegrin and metalloproteinase') family has recently been implicated in the shedding of several molecules [14,15] and is probably involved in the release of the sIL-6R α . The constitutive release of the sIL-6R α by myeloma cells is strongly up-regulated

by PMA, suggesting the implication of a cellular signalling pathway involving protein kinase C (PKC) [16]. The PKC family consists of 11 isoenzymes, which can be subdivided into three groups [17,18]. The conventional PKCs (α , β_1 , β_{II} and γ) depend on Ca²⁺ and are activated by diacylglycerol or PMA. The novel PKCs (δ , ϵ , η and θ) are also activated by diacylglycerol but are Ca²⁺-independent. Finally, the atypical PKCs (ζ and λ) are Ca²⁺-independent and do not respond to diacylglycerol. The expression of PKC isoenzymes seems to be cell-specific and each isoenzyme has specific functions. Protein phosphorylation seems to be a general mechanism involved in the regulation of shedding [19], and recent progress has been made in the characterization of some signalling events regulating the shedding. Indeed, different mitogen-activated protein kinase (MAPK) activation pathways have been involved in the shedding: the extracellular signal-regulated protein kinase/MAPK (ERK/MAPK) pathway in the shedding induced by PMA, serum and growth factors [20,21], and the p38 MAPK pathway in basal shedding [21]. Although evidence supports the notion that PKCs are involved in the activation of the ERK/MAPK pathway, it is not clear whether PKCs activate it directly [22–25].

The purpose of this study is to identify the PKC isotypes expressed by myeloma cells and to define their respective roles in the regulation of IL-6R α shedding. We now report that the human myeloma cell line XG-6 express the PKC- δ , - η and - ζ isoenzymes and we demonstrate the implication of PKC- δ in the regulation of the PMA-induced shedding of IL-6R α . Furthermore, we provide evidence that the ERK/MAPK pathway regulates the PMA-induced IL-6R α shedding. Finally, we show that PKC is implicated in the activation of this pathway.

Abbreviations used: ADAM, a disintegrin and metalloproteinase; ERK, extracellular signal-regulated protein kinase; FCS, fetal calf serum; IL, interleukin; MAPK, mitogen-activated protein kinase; MFR, mean fluorescence ratio; MM, multiple myeloma; PKC, protein kinase C; sIL-6R α , soluble interleukin 6 receptor α .

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Figure 1 PMA-induced shedding of IL-6R α on XG-6 myeloma cells involves a Ca²⁺-independent PKC isotype

(A) XG-6 cells were plated at 10^6 cells/ml in RPMI 1640 containing 2% (v/v) FCS without or with 25 nM PMA in the presence of the following reagents [$1 \mu\text{M}$ ionomycin, $25 \mu\text{M}$ BB-94, $8 \mu\text{M}$ GF109203X (GFX) or 5 mM EDTA] as indicated. After incubation for 4 h at 37 °C, supernatants were collected and used for sIL-6R α determination by ELISA as described in the Materials and methods section. Results are means $\pm$ S.D. ($n = 5$). * $P < 0.0001$ compared with control. (B) XG-6 cells (10^6 cells/ml) were stimulated for 4 h with either PMA (25 nM) (thick line) or PMA plus BB-94 (25 μM) (dotted line). Thin line, untreated control. Cells were then stained for cell-surface expression of IL-6R α and analysed by flow cytometry. Abbreviation: PE, phycoerythrin.

EXPERIMENTAL

Monoclonal antibodies and reagents

PMA and ionomycin were from Sigma (St Louis, MO, U.S.A.). The MAP kinase inhibitor UO126 and PKC inhibitors (rottlerin and bisindolmaleimide I, i.e. GF109203X) were obtained from Alexis (San Diego, CA, U.S.A.). The anti-(phospho-Thr⁵⁰⁵-PKC- δ), anti-(phospho-p44/42 MAPK) and anti-(p44/42 MAPK) were purchased from New England Biolabs (Beverly, MA, U.S.A.). The polyclonal antibodies against the PKC isoenzymes α , β , δ , γ , ϵ and ζ and also anti-actin were purchased from Boehringer Mannheim (Germany). The anti-(PKC- η) and anti-(PKC- θ) antibodies were obtained from Transduction Laboratories (Tebu, France). Phycoerythrin-conjugated anti-IL-6R α and control phycoerythrin-conjugated IgG1 were obtained from Immunotech (Marseille, France).

Human cell-line culture conditions

The IL-6-dependent human myeloma cell line XG-6 has been described previously [26]. XG-6 is cultured in the presence of 3 ng/ml recombinant IL-6. The monocytic human cell line THP-1 was obtained from the American Type Culture Collection (A.T.C.C., Manassas, VA, U.S.A.). XG-6 and THP-1 were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 2 mM glutamine, antibiotics and 10 mM 2-mercaptoethanol. The cells were incubated at 37 °C in a humidified air/CO₂ (19:1) atmosphere. Before all experiments, cells were cultured for 24 h under conditions of low serum [2% (v/v) FCS].

Determination of sIL-6R α production

After a 24 h period of low-serum culture conditions, myeloma cells were seeded in 96-well plates at 10^6 cells/ml in RPMI 1640 medium with 2% (v/v) FCS for 4 h; supernatants were then collected. The concentration of the sIL-6R α in cell supernatants was determined with a sandwich ELISA kit (Pelikine Compact; Tebu, CLB, Amsterdam, The Netherlands). The sensitivity of the test was 10 pg/ml. Assays were performed in accordance with the manufacturer's protocol.

Flow cytometry analysis

For immunofluorescence staining, 10^6 cells were incubated with the various phycoerythrin-conjugated monoclonal antibodies (20 min, 4 °C). Cells were fixed in 1% (v/v) formaldehyde and analysed on a FACScalibur flow cytometer with Cell Quest Software (Becton Dickinson). The mean fluorescence ratio (MFR) was obtained by dividing the MFR of each marker by the MFR of the control staining.

Subcellular fragmentation

Cells (10^7) were washed first with cold PBS and then with a cold buffer containing Mg²⁺ [25 mM Hepes (pH 7.4)/2.5 mM MgCl₂/250 mM sucrose/1 mM dithiothreitol/50 mM KCl/1 $\mu\text{g/ml}$ aprotinin/1 $\mu\text{g/ml}$ leupeptin/1 mM PMSF/1 mM NaF] [27]. Treated cells were then resuspended in this Mg²⁺-containing buffer and passed several times through a 26-gauge needle. The resulting homogenate was centrifuged at 1000 g for 10 min to obtain supernatants collected as postnuclear cell lysates. This supernatant was further centrifuged at 100000 g for 30 min and the resulting supernatant was saved as the cytosolic fraction. The pellet was resuspended in a lysis buffer [10 mM Tris/HCl (pH 7.6)/150 mM NaCl/5 mM EDTA/0.5% (v/v) Nonidet P40, containing 2 mM PMSF, 2 mM Na₃VO₄, 1 mM NaF and 2 mg/ml aprotinin] and incubated with agitation for 30 min at 4 °C. The membrane fraction was collected as the supernatant after centrifugation (12000 g for 30 min at 4 °C). Protein concentrations in each fraction were quantified with bicinchoninic acid (BCA protein assay; Pierce, Rockford, IL, U.S.A.), and equal amounts of protein were separated by SDS/PAGE.

Western blotting

Cells (4×10^6) were washed twice in cold PBS and resuspended in lysis buffer [10 mM Tris/HCl (pH 7.6)/150 mM NaCl/5 mM EDTA/0.5% (v/v) Nonidet P40, containing 2 mM PMSF, 2 mM Na₃VO₄, 1 mM NaF and 2 mg/ml aprotinin] and were kept on ice. Lysates were cleared by centrifugation at 12000 g for

Figure 2 XG-6 cells express PKC- δ , - η and - ζ isoenzymes

XG-6 or THP-1 cells (4×10^6) were lysed; equal protein aliquots (80 μ g) were then resolved by SDS/PAGE [10% (w/v) gel]. Samples were immunoblotted with antibodies against the PKC isoenzymes α , β , γ , δ , ϵ , η , θ or ζ . Actin was used as an internal control.

30 min at 4 °C. Protein concentration of lysates was determined with the BCA protein assay. Equal quantities of proteins were separated by 10% (w/v) PAGE, then transferred to Hybond PVDF membranes (Amersham Pharmacia Biotech). Membranes were incubated with 10% (v/v) blocking reagent (Boehringer Mannheim) in TBS [10 mM Tris/HCl (pH 8)/150 mM NaCl] for 1 h at room temperature and incubated with the appropriate primary antibody at 4 °C overnight. The membranes were then probed with horseradish-peroxidase-conjugated anti-mouse/rabbit secondary antibody at room temperature for 30 min. Membranes were detected with a chemiluminescent detection system, namely the Supersignal West Dura Extended Duration

Substrate (Pierce). Quantification of the blot was performed with 1D Image Analysis Software (Eastman Kodak Company, Rochester, NY, U.S.A.).

RESULTS

PMA-induced IL-6R α shedding is controlled by a Ca^{2+} -independent PKC in XG-6 myeloma cells

Phorbol ester, a well-known activator of PKC, has been shown to facilitate the shedding of several cell-surface receptors [28,29]. PMA increased the release of the sIL-6R α by 6.7-fold in XG-6 cells (Figure 1A). In contrast, this PMA-induced release of sIL-6R α was strongly decreased by BB-94, a hydroxamate-based compound (70% inhibition), demonstrating that this PMA-induced shedding was mediated by a metalloproteinase. The PMA-induced IL-6R α shedding was monitored by cell-surface fluorescence. After 4 h of treatment with PMA, a strong decrease in the cell-surface expression of IL-6R α was found: MFR 1.7 compared with 4.7 (Figure 1B). It is noteworthy that this cell-surface down-regulation of IL-6R α was strongly inhibited in the presence of BB-94: MFR 1.7 compared with 3.8. As shown in Figure 1(A), the general PKC inhibitor GF109203X [30,31] inhibited release of the sIL-6R α by 50%, demonstrating that PKC isoenzymes were involved in the PMA-induced sIL-6R α shedding. To determine whether or not PKC activity required Ca^{2+} , we analysed the effects of the Ca^{2+} ionophore ionomycin and those of EDTA on the sIL-6R α release induced by PMA. Neither ionomycin nor the depletion of Ca^{2+} by 5 mM EDTA had an effect on the release of the sIL-6R α (Figure 1A). Taken together, these results suggest that PMA-induced IL-6R α shedding on myeloma cells implies PKC isotypes that do not require Ca^{2+} for their activation.

Involvement of PKC- δ in the PMA-induced IL-6R α shedding

To investigate more directly the involvement of a specific isoenzyme of PKC in the shedding of IL-6R α , we further examined the PKC isoenzymes expressed on XG-6 cells by Western blot analysis with isoenzyme-specific anti-PKC antibodies. As shown in Figure 2, XG-6 cells expressed both novel PKCs (δ , η) and atypical PKC (ζ), whereas conventional PKCs were not expressed (in contrast with THP-1 cells, which also expressed conventional PKC- α and PKC- γ). We next analysed

Figure 3 Subcellular distribution of PKC isoenzymes in response to PMA

XG-6 cells (10^7) were stimulated with 25 nM PMA for 15 min at 37 °C. Then cells were homogenized in Mg^{2+} -containing buffer, and fractioned into cytosol (C) and total membranes (M). Each fraction was analysed by immunoblotting with antibodies against PKC- δ (A), PKC- η (B) and PKC- ζ (C). Band intensities were quantified by densitometric scanning and the percentage of PKC- δ , - η and - ζ isoenzymes in each cellular fraction is indicated before and after treatment with PMA in the respective panels. The blots represent one example of experiments replicated three times.

Figure 4 Analysis of phosphorylation of PKC- δ in its active loop in response to PMA

XG-6 cells (4×10^6 /ml) were incubated with 25 nM PMA for various durations. Cells were lysed and equal amounts of proteins were resolved by SDS/PAGE [10% (w/v) gel] then subjected to immunoblotting with anti-(phospho-Thr⁵⁰⁵ PKC- δ) antibody.

the subcellular distribution of these PKC isoenzymes under basal conditions and also their subcellular redistribution on stimulation with PMA. In the absence of any stimulus, PKC- η and PKC- ζ were located mainly in the cytosolic fraction, whereas 46% of PKC- δ was associated with the membrane (Figure 3). After 15 min of treatment of the cells with PMA we observed an activation of both PKC- δ and PKC- η , as shown by a significant translocation from the cytosol to the membrane (Figures 3A and 3B). Indeed, the proportions of PKC- δ and PKC- η in the membrane fraction were 89% and 81% respectively after treatment with PMA (Figure 3). In contrast, only a weak membrane translocation of PKC- ζ (approx. 10%) was observed, indicating the probable absence of PKC- ζ activation by PMA. This was in agreement with the absence of a PMA-binding site on PKC- ζ [32]. Because the phosphorylation of PKC- δ on Thr-505 is essential for PKC- δ activation [33], we analysed it by Western blotting with an anti-(phospho-PKC- δ) antibody. As shown in Figure 4, PMA strongly induced the phosphorylation

Figure 6 Effects of PKC inhibitors on PMA-induced IL-6R α shedding in XG-6 cells

XG-6 cells (10^6 /ml) were preincubated or not with 6 μ M rottlerin in RPMI containing 2% (v/v) FCS for 1 h, then stimulated for 4 h at 37 °C with 25 nM PMA in the presence of PKC inhibitors as indicated. Then supernatants were collected and used for the determination of sIL-6R α concentration. Results are means $\pm$ S.D. ($n = 4$). * $P < 0.0001$ compared with control.

of PKC- δ in a time-dependent manner, with a maximum increase at 30 min. Because prolonged treatment with PMA has been shown to deplete cells of PKC expression [34], we analysed the expression of PKC- δ , - η and - ζ after 4 and 24 h of treatment with PMA (Figure 5). After 4 h we observed a marked decrease in PKC- δ level and a total disappearance after 24 h of treatment, whereas the level of PKC- η was affected only weakly by prolonged treatment with PMA (Figures 5A and 5B). Although PKC- ζ was weakly activated by PMA, as shown by the analysis of subcellular localization, we nevertheless observed decreases in the PKC- ζ level of 25% and 48% after 4 and 24 h of PMA treatment respectively. Because rottlerin specifically inhibits PKC- δ [35], we next examined the effect of this PKC inhibitor on the PMA-induced release of the sIL-6R α (Figure 6). Rottlerin brought about a 42% inhibition of PMA-induced sIL-6R α release without affecting cell viability, indicating that PKC- δ was involved in the cleavage of IL-6R α (Figure 6). Taken together, these results indicate that the active form of PKC- δ was involved at least in the regulation of PMA-induced shedding of IL-6R α on XG-6 cells.

Figure 5 Down-regulation of PKC isoenzymes after long-term exposure to PMA

XG-6 cells (10^7) were incubated at 37 °C for 4 and 24 h in the presence of 25 nM PMA. Cells were lysed and equal amounts of protein were resolved by SDS/PAGE [10% (w/v) gel] then subjected to immunoblotting with antibodies against PKC- δ (A), PKC- η (B) and PKC- ζ (C). Band intensities were quantified by densitometric scanning and the percentage of PKC- δ , - η and - ζ isoenzymes was calculated by setting the control values of PKC isoenzyme expression under basal conditions to 100%.

Figure 7 Ras/MAPK pathway is involved in PMA-induced IL-6R α shedding in XG-6 cells

(A) Cells (4×10^6) were preincubated or not with 5 μ M UO126 for 1 h, then stimulated with 25 nM PMA for 15 min at 37 °C in the presence or absence of UO126. Cells were lysed and equal amounts of protein were resolved by SDS/PAGE [10% (w/v) gel] then immunoblotted with anti-(phospho-p44/p42) and anti-(p44/p42) antibodies. The blots represent one example of experiments replicated three times. (B) XG-6 cells (10^6 /ml) were preincubated or not with UO126, then stimulated with PMA (25 nM) or UO126 (5 μ M) for 4 h at 37 °C. Supernatants were collected and used for sIL-6R α determination by ELISA as indicated in the Materials and methods section. Results are mean $\pm$ S.D. ($n = 5$). * $P < 0.0001$ compared with control.

Figure 8 Ras/MAPK pathway activation on stimulation with PMA is dependent on PKC in XG-6 cells

XG-6 cells (10^6 /ml) were preincubated or not with 6 μ M rottlerin or 8 μ M GF109203X for 1 h, then stimulated for 15 min at 37 °C with 25 nM PMA in the presence of PKC inhibitors as indicated. Cells were recovered and lysed; equal amounts of protein were then separated by SDS/PAGE [10% (w/v) gel] and immunoblotted with anti-(phospho-p44/p42) antibody. As a control, the blot was stripped and reprobed with anti-(p44/p42) antibody. The blots are typical examples of experiments replicated three times.

Activation of the MAPK pathway is involved in shedding of IL-6R α

Several studies have demonstrated that PMA activates the Ras/MAPK pathway and that this pathway regulates the shedding process [20,21]. Thus, to evaluate whether or not MAPK activation was involved in the PMA-induced cleavage of IL-6R α , we prevented the activation of ERKs by using UO126, an inhibitor of MAPK kinase. Treatment of XG-6 cells with PMA resulted in the activation of p42 and p44 MAPK (Figure 7A); their pretreatment with 5 μ M UO126 totally inhibited ERK1/2 activation and decreased the production of sIL-6R α by 59% (Figures 7A and 7B). Finally, the combination of UO126 and rottlerin strongly decreased shedding of IL-6R α to the basal cleavage level observed in the absence of PMA. These results indicate that the PMA-induced IL-6R α shedding was regulated both by the ERK/MAPK activation pathway and by PKC- δ activation.

Implication of PKC isoenzymes in ERK activation by PMA

Because it has been well established that PKCs regulate the activation of the ERK/MAPK pathway [36,37], we examined the requirement for PKCs in the PMA-induced activation of ERKs by using different PKC inhibitors. Treatment with GF109203X resulted in a total inhibition of ERKs activation, whereas rottlerin had no effect (Figure 8). Because rottlerin is unable to block the activation of ERKs by PMA, PKC- δ is not implicated in this process. Given that atypical PKCs were not activated by PMA on the XG-6 cells, these results suggest that PKC- ζ was not involved. This is also underlined by the fact that 1 μ M GF109203X strongly inhibited the activation of ERKs (results not shown), whereas at this concentration PKC- ζ is not affected by the GF109203X. Finally, because XG-6 cells express PKC- η and PKC- δ as novel PKC isoforms, we can assume that the novel PKC, namely PKC- η , is involved in the activation of ERKs by PMA.

DISCUSSION

In the present study we have demonstrated that several PKC isotypes control the PMA-induced shedding of IL-6R α on human myeloma cells. On the one hand, we have found that the activity of PKC- δ is directly involved in the shedding of IL-6R α . On the other hand, we have found that this proteolytic cleavage is also regulated by the activation of the Ras/MAPK pathway, which requires PKC activity, but not PKC- δ activity, for its activation.

We have shown that XG-6 cells express three different PKC isoforms: two novel PKCs, namely PKC- δ and PKC- η , and the atypical PKC- ζ . The expression of PKC- δ and PKC- ζ on myeloma cells is in agreement with the results of Mischak et al. [38] obtained in mouse and showing that PKC- δ and PKC- ζ are uniformly expressed in both murine B cells and plasmacytomas. Curiously, no expression of either PKC- α or PKC- β was detected in XG-6 cells. Indeed, PKC- α and PKC- β have been shown to be expressed in most of the cell lines investigated until now, and there is no cell line from which both are missing [34]. Moreover, it has been shown that the differentiation of murine B cells into antibody-secreting plasma cells is accompanied by an increase in

PKC- α expression and by a loss of PKC- β expression [38]. However, the expression of PKC isotypes on human malignant plasma cells has never been evaluated until now.

The cellular distribution of PKC isotypes in a specific cell type and their redistribution and degradation on stimulation with PMA lead to a better understanding of the activation process. In myeloma cells, we found that a large portion of PKC- δ is already associated with the membrane fraction, whereas PKC- η and PKC- ζ are mainly cytosolic. Depending on the cell type, PKC- δ seems to be differentially distributed within the cell. Although totally located in the cytosol of human platelets [39], most PKC- δ is found associated with the membrane fraction in rat fibroblasts [40]. Because PKC- η is mainly cytosolic, in contrast with PKC- δ , which is associated with the membrane fraction, the fact that these PKCs belong to the same group as the novel PKC does not imply a similar subcellular distribution. Furthermore, as previously observed in other cell types, we have also found that PKC- ζ is present in the cytosolic fraction [40,41]. Finally, the physiological function of PKC isoenzymes in the particular fraction of unstimulated cells is still unclear, although it has been proposed that it could reflect constitutive activation of these isoforms [42]. After activation of the cells by PMA, we have observed a total translocation of PKC- δ and PKC- η to the cellular membrane, in contrast with PKC- ζ . The translocation of PKC- δ and PKC- η seems to reflect their degree of activation and is consistent with the presence in these PKCs of the C1 domain (a tandem repeat of cysteine-rich motifs), which is the binding site for PMA [18]. In many cell types, prolonged treatment with PMA results in the depletion of cellular PKCs. After long-term exposure of myeloma cells to PMA we observed a complete down-regulation of PKC- δ and a partial down-regulation of PKC- ζ ; PKC- η expression was not affected. This total depletion of PKC- δ is consistent with previous reports [40]. Although both PKC- δ and PKC- η possess a PMA-binding site, they are regulated in different ways after long-term exposure to PMA. Indeed, there is now a trend of evidence for an absence of PKC- η down-regulation induced by PMA in many other cell types [43–45]. An up-regulation in both mouse thymoma cells and human astrocyte cells has even been shown [44,45]. Finally, given that PKC- ζ lacks a PMA-binding site, it is surprising to observe a partial down-regulation of PKC- ζ on long-term exposure to PMA. An explanation for this observation is currently lacking, but we hypothesize that PMA-responsive PKCs trigger a cascade of phosphorylation events leading to cross-talk between PKC isoenzymes. Taken together, these results support the idea that PKC- δ and PKC- η are involved in PMA-induced IL-6R α shedding. More direct evidence of the implication of PKC- δ was supported by observations that rottlerin significantly decreased the effect of PMA on the release of the sIL-6R α , and PMA induced the phosphorylation of Thr⁵⁰⁵ in the active loop of PKC- δ . The involvement of PKC- δ in other cell-surface receptor shedding has already been reported. Izumi et al. [46] demonstrated that overexpression of a kinase knock-out mutant of PKC- δ in Vero-H cells suppresses the PMA-induced shedding of the pro-heparin-binding epidermal growth factor-like growth factor (pro-HB-EGF), demonstrating the role of PKC- δ in this process. Furthermore, it was shown that the processing of pro-HB-EGF requires MDC9 (ADAM9), a member of the ADAM family of metalloproteases, and involves a direct interaction between PKC- δ and the cytoplasmic domain of ADAM9. Given that (1) PKC- δ is directly involved in the PMA-induced shedding of IL-6R α and (2) this process is sensitive to BB-94, it would be interesting to investigate further whether ADAM9 interacts with PKC- δ in myeloma cells and is implicated in the proteolytic cleavage of IL-6R α .

Several recent studies have defined the signalling pathways responsible for ectodomain shedding. The ERK/MAPK pathway has been involved in the PMA-induced shedding of both proHB-EGF and L1 adhesion molecule [20,47] and in the shedding of transforming growth factor α and syndecan 1 induced through the activation of receptor protein tyrosine kinase [21,48]. In the same way, our results implicate the ERK/MAPK pathway in the PMA-induced cleavage of IL-6R α as well as in basal IL-6 shedding (results not shown). Finally, activation of the other MAPK cascades, namely p38 MAPK, has been demonstrated to regulate the basal cleavage of transforming growth factor α , tumour necrosis factor α and L-selectin [21]. Taken together, these results support the notion that MAPK activation and more specifically ERK/MAPK activation might represent a general mechanism involved in shedding.

Because PKC is the major receptor for phorbol esters, the latter have been implicated in the activation of the ERK/MAPK pathway [24,36]. We confirm here the implication of PKC in ERK/MAPK activation on stimulation with PMA. Thus these findings suggest that PMA-induced shedding is not a direct consequence of PKC activity but instead results from PKC-induced ERK/MAPK signalling. However, because PKC- δ activity is involved in IL-6R α shedding, but not in the activation of ERK1/2, we hypothesize that the activity and phosphorylation of PKC- δ are directly responsible for IL-6R α shedding. The absence of implication of PKC- δ in the activation of ERK1/2 on treatment with PMA contrasts with the result of Ueda et al. [23] showing that among the constitutively active mutants of PKC- α , - ϵ and - δ only a constitutively active mutant of PKC- δ causes the activation of MEK1 (MAP kinase/ERK kinase 1) and ERK1. However, Schönwasser et al. [37] have shown that all PKC isotypes are potentially able to induce ERK1/2 activation. The reason for these different results is unclear, but the use of activated PKC isotypes in investigations of cell functions do not necessarily reflect a physiological role. Moreover, on the basis of the data of Schönwasser et al. [37], it is believed that among the novel PKC isotypes the most potent MAPK activator is PKC- η ; thus our results are compatible with this idea. Indeed, the role of PKC- η in ERK1/2 activation on stimulation with PMA is suggested by the observations that (1) PKC- δ is not responsible for this activation and (2) 1 μ M GF109203X totally inhibits ERK1/2 activation without affecting PKC- ζ . Despite the fact that the metalloproteinase responsible for IL-6R α shedding has not yet been identified, our results suggest that PKC- δ could interact with a metalloproteinase and consequently activate it by phosphorylation. Further investigation will be necessary to determine whether the activation of ADAM metalloproteinase by PKC activity could represent a general and physiological mechanism of this metalloproteinase family. Finally, activation of the MAPK pathway is a common feature of shedding induced by PMA and might be explained by the ubiquitous expression in many cell types of PKC isotypes containing a PMA-binding site. This study has underlined the central role of PKC isotypes in PMA-induced shedding of IL-6R α ; thus it is conceivable that such a PKC network could be also involved in the shedding of many protein ectodomains known to be shed in response to PMA.

In MM, IL-6 is the essential factor for sustaining the survival and expansion of tumour cells within the bone marrow. Attempts to inhibit IL-6 with neutralizing anti-(IL-6) monoclonal antibody have been shown to improve the efficacy of high-dose chemotherapy in patients with advanced MM [49]. However, the effects of IL-6 in terms of survival and proliferation are amplified by sIL-6R α , which is a potent agonist of IL-6 on human myeloma cells [50]. Thus the IL-6-sIL-6R α complex rather than IL-6 alone

is responsible for MM expansion within the bone marrow, and emerges as a better therapeutic target in MM. For these reasons, the capacity of some PKC inhibitors to eliminate the release of sIL-6R α would be of great therapeutic interest, considering that these inhibitors are already available for clinical trials [51].

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