Stimulation of cleavage of membrane proteins by calmodulin inhibitors

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The ectodomain of several membrane-bound proteins can be shed by proteolytic cleavage. The activity of the proteases involved in shedding is highly regulated by several intracellular second messenger pathways, such as protein kinase C (PKC) and intracellular Ca^{2+}. Recently, the shedding of the adhesion molecule L-selectin has been shown to be regulated by the interaction of calmodulin (CaM) with the cytosolic tail of L-selectin. Prevention of CaM–L-selectin interaction by CaM inhibitors or mutation of a CaM binding site in L-selectin induced L-selectin ectodomain shedding. Whether this action of CaM inhibitors also affects other membrane-bound proteins is not known. In the present paper we show that CaM inhibitors also stimulate the cleavage of several other transmembrane proteins, such

as the membrane-bound growth factor precursors pro-transforming growth factor-α and pro-neuregulin-α2c, the receptor tyrosine kinase, TrkA, and the β-amyloid precursor protein. Cleavage induced by CaM inhibitors was a rapid event, and resulted from the activation of a mechanism that was independent of PKC or intracellular Ca^{2+} increases, but was highly sensitive to hydroxamic acid-based metalloprotease inhibitors. Mutational analysis of the intracellular domain of the TrkA receptor indicated that CaM inhibitors may stimulate membrane-protein ectodomain cleavage by mechanisms independent of CaM–substrate interaction.

Key words: CaM, metalloproteases, shedding.

INTRODUCTION

The ectodomains of a number of transmembrane proteins can be released as soluble fragments by the action of cell surface proteases [1–3]. Physiologically relevant molecules whose ectodomains are shed include membrane-anchored growth factors [1], some of their receptors [4–9], cell adhesion proteins [10], ectoenzymes [11], molecules involved in immune responses [2] and proteins, such as the β-amyloid precursor protein (βAPP), whose function has not been fully elucidated [2]. Alterations in the cleavage of some of these membrane proteins may lead to disease. Mutations in the ectodomain of the p75 tumour necrosis factor receptor (p75^{TNFR}) that decrease the shedding of its ectodomain have been linked to an autosomal dominant disease characterized by fever and severe local inflammation [12]. The pathogenesis of these disease signs probably involves impaired clearance of the p75^{TNFR} resulting both in a decreased buffering activity of a soluble receptor, together with an increased level of the membrane holo-receptor that factilitates tumour necrosis factor α (TNFα) responses. Another example of the importance of membrane sheddases has been obtained in animal models of pseudoinfectious or cachectic states. Injection of rodents with inhibitors of the shedding of membrane proTNFα prevented the release of soluble TNFα into the serum and protected these animals against a lethal dose of endotoxin [13,14]. Thus control of the activity of the membrane-protein-processing proteases may have therapeutic implications.

Pharmacological experiments using inhibitors of the different protease families have pointed to metalloproteases as the enzymes responsible for membrane protein ectodomain cleavage [13–15]. Later, by the use of these inhibitors [16] and in vitro peptide cleavage assays [17], an enzyme, the proTNFα converting enzyme (TACE) that participates in the solubilization of TNFα from its

precursor, has been isolated. Structurally, TACE is a type I membrane protein that contains several domains in the extracellular region, including disintegrin and metalloprotease-like (ADAM) domains, characteristic of the ADAM subfamily of metalloproteases [18,19]. TACE may also participate in the cleavage of other transmembrane proteins, since cells from TACE-deficient animals fail to efficiently cleave pro-transforming growth factor α (proTGFα), L-selectin, the p75^{TNFR} [20] or βAPP [21]. It is possible that other proteases may be involved in the regulation of membrane-protein ectodomain cleavage. In fact, solubilization of the ectodomain of the angiotensin converting enzyme is unaffected in fibroblasts derived from TACE-deficient animals [11], and studies on the solubilization of pro-heparin binding epidermal growth factor (EGF)-like growth factor (proHB-EGF) have indicated that other ADAM family members, such as MDC9/ADAM9/Meltrin-γ, may also participate in the release of soluble forms of membrane-bound molecules [22].

A characteristic of the activity of membrane secretases is their highly regulated nature. In fact, activation of intracellular second messenger systems, such as the protein kinase C (PKC) or intracellular Ca^{2+} pathways, up-regulates the activity of membrane protein secretases [1,2]. Experiments performed on proTGFα have shown that these intracellular pathways may act independently of each other [23]. Thus treatments that block PKC activation are unable to prevent Ca^{2+} ionophore-induced cleavage of proTGFα. On the other hand, chelation of extracellular Ca^{2+}, that prevents Ca^{2+} ionophore-induced cleavage of proTGFα, does not affect PKC-mediated cleavage of proTGFα.

An important intracellular mediator in the actions of Ca^{2+} is calmodulin (CaM) [24–26]. Recently, CaM was shown to be involved in the regulation of the shedding of the adhesion molecule L-selectin [27]. CaM was found to bind to the endodomain of L-selectin, and prevention of such interaction by CaM

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inhibitors stimulated the shedding of L-selectin [27]. In the present study, we evaluated the possible role of CaM as a generic regulator of the shedding of membrane proteins. We report that CaM inhibitors trigger the shedding of a number of trans-membrane proteins by a novel PKC- and Ca^{2+}-independent mechanism. We show that cleavage of the receptor tyrosine kinase, TrkA, induced by CaM inhibitors is independent of the association of CaM with the cytosolic domain of TrkA, indicating that shed proteins may use different mechanisms for their regulated release by CaM inhibitors.

EXPERIMENTAL

Reagents and immunochemicals

Cell culture media, sera and G418 were purchased from Gibco BRL. Horseradish peroxidase-conjugates of anti-rabbit or anti-mouse IgGs and nitrocellulose membranes were from Bio-Rad laboratories. Immobilon-P membranes were from Millipore (Bedford, MA, U.S.A.), and the Fuji Medical X-ray film from Fuji Photo Film Co. (Tokyo, Japan). Luminol, p-iodophenol, phorbol 12-myristate, 13-acetate (PMA), trifluoperazine (TFP), wortmannin, W7 (a CaM inhibitor), calmidazolium and A23187 (a Ca^{2+} ionophore) were purchased from Sigma Chemical Co.; and rapamycin and bisindolylmaleimide (BIM) were from Calbiochem. EGF was from Collaborative Research (Lexington, MA, U.S.A.). The inhibitor compound BB3103 was generously provided by British Biotech (Oxford, U.K.). All other chemicals were reagent grade, and were purchased from Sigma Chemical Co., Boehringer Mannheim Biochemicals, or Merck.

The monoclonal antibody (mAb) MGR12 against the ecto-domain of TrkA was obtained from Dr. S. Ménard (Istituto Nazionale dei Tumori, Milán, Italy); the polyclonal antiserum against the βAPP endodomain was generously provided by Dr. Sam Gandy (Cornell University, Ithaca, NY, U.S.A.); and the anti-EGFR mAb 225 was from Dr. J. Mendelsohn (Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.). The mAb against the ectodomain of TGFα was from Santa Cruz Biotechnology. The anti-phosphotyrosine mAb 4G10 and the anti-CaM antibodies were from UBI (Lake Placid, NY, U.S.A.). The polyclonal antiserum (R2086) raised against the endodomain of TGFα has been described [28]. Two polyclonal anti-TrkA antisera against peptides corresponding to different domains (ectodomain and endodomain) of human TrkA were generated in rabbits injected with the respective peptides coupled to keyhole limpet haemocyanin. The anti-panTrk (anti-endodomain) anti-serum was raised against a peptide that corresponded to the 14 C-terminal residues of human TrkA [29], while the anti-ecto-domain antiserum was raised against the peptide CWAENDV-GRAEVSV. The anti-neuregulin (NRG) antiserum was raised in rabbits injected with the peptide CETPDSYRDSPHSER.

Cell culture

All cells were cultured at 37 °C in a humidified atmosphere in the presence of air/CO_2 (19:1). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose (4500 mg/l) and antibiotics (penicillin, 100 units/ml and strep-tomycin, 100 μg/ml), and supplemented with fetal bovine serum at 5% (v/v) [for HEK-293, Chinese hamster ovary (CHO) and derived clones or HeLa cells] or 10% (v/v) (PC12^{TrkA} cells).

Metabolic labelling, immunoprecipitation and Western blotting

Detailed description of these protocols can be found in Cabrera et al. [8]. For immunoprecipitations, cells were washed with PBS and lysed in ice-cold lysis buffer [140 mM NaCl, 10 mM EDTA, 10% (v/v) glycerol, 1% Nonidet P-40, 20 mM Tris/HCl, pH 8.0, 1 mM PMSF, 1 mM sodium orthovanadate]. Samples were centrifuged at 10000 g at 4 °C for 10 min and supernatants transferred to new tubes with the corresponding antibody and protein A–Sepharose. Immunoprecipitations were performed at 4 °C for 2 h and the immune complexes were washed in lysis buffer four times before boiling in electrophoresis sample buffer [4% (v/v) SDS, 20% (v/v) glycerol, 100 mM Tris/HCl (pH 6.8), 2% (v/v) β-mercaptoethanol]. Samples were loaded on SDS/PAGE gels, electrophoresed and transferred to PVDF membranes. Filters were blocked for 1 h in TBST [20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween 20, 1% (w/v) BSA] and then incubated for 2–16 h with the corresponding antibody. After washing with TBST, filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min, and bands visualized by chemiluminescence as previously described [8]. For the CaM blots the method of Van Eldik and Wolchok [30] was used. Briefly, after transferring to nitrocellulose membranes, filters were incubated in 0.2% (v/v) glutaraldehyde in PBS for 45 min at room temperature, followed by blocking in TBST/2% (w/v) BSA. The rest of the steps were as above.

For metabolic labelling, cells were washed twice with methionine- and cysteine-free DMEM and incubated for 4 h in this medium supplemented with 75 μCi/ml of a mixture of [^{35}S]methionine and [^{35}S]cysteine. Monolayers were then washed and treated with the different agents. Media and cells were im-munoprecipitated with the MGR12 or anti-panTrk antibodies.

Immunofluorescence

The immunofluorescence assay to evaluate the expression of mutant TrkA receptors was performed as previously described [29]. Briefly, cells plated on glass coverslips were washed with PBS, fixed in 2% p-formaldehyde and permeabilized in 1% Triton X-100. Monolayers were incubated with a 1:100 dilution of the MGR12 antibody for 2 h at 4 °C. After washing, the coverslips were incubated with Cyanine-3-conjugated secondary antibody, and mounted with antifading medium.

EGF receptor (EGFR) tyrosine phosphorylation

HeLa cells were plated in 60 mm tissue culture dishes, and used at 80% confluency. To avoid EGFR desensitization by PKC activation, cells were treated overnight with 1 μM PMA. Monolayers were incubated with either EGF (10 nM), or the conditioned media from PMA- or TFP-treated CHO$^{TGF\alpha}$ cells, for 10 min, and then cells were washed and lysed in lysis buffer. EGFR was immunoprecipitated with the 225 mAb, and the phosphotyrosine content analysed by Western blotting with anti-phosphotyrosine antibodies.

Construction of mutants and transfections

The cDNA coding for the non-neuronal form of human TrkA was subcloned into the expression plasmid pCDNA3 (Invitrogen). The different mutants were generated by oligonucleotide-directed mutagenesis, following standard methods [31]. For the deletion of the intracellular domain of TrkA, the complementary oligonucleotide 5′-CAAATGTGGA-CGGAGACTCGAGTTTGGGATCAACC-3′ was used. The resulting truncated receptor, termed TrkA$^{\Delta Intra}$ mutant, lacks 349 of the 356 amino acids of the TrkA endodomain. For the construction of the TrkA$^{NK \rightarrow EE}$ the original residues Asn^{466} and Lys^{467} were substituted with Glu residues by using the oligo-

nucleotide 5′-GGTTGATCCCAAA<u>CTCCTC</u>TCTCCGTCCA-CATTTG-3′ (the codons for the Glu residues are underlined). Verification of the mutant sequences was done by automated sequencing.

Transfections in HEK-293 or CHO cells were made by the calcium phosphate technique [31]. Stable clones of CHO cells expressing the mutant TrkA receptors were obtained by geneticin ('G418') selection (500 μg/ml), followed by ring cloning of single colonies. Mutant TrkA expression in individual clones was analysed by immunoprecipitation and Western blotting, and immunofluorescence.

RESULTS

Calmodulin inhibitors induce cleavage of several membrane proteins

In neutrophils and lymphocytes, CaM inhibitors trigger a rapid shedding of L-selectin [27]. To investigate whether an analogous mechanism regulated the release of other membrane proteins, we first analysed the effect of CaM inhibitors on the cleavage of TrkA. In CHO cells expressing TrkA (CHO$^{\text{TrkA}}$ cells, Figure 1A), the receptor is initially synthesized as a 110 kDa form that is

A

— PMA TFP

125 —   — gp140$^{\text{TrkA}}$
            — gp110$^{\text{TrkA}}$
88 —
65 —
55 —   — Ig

            = p41
35 —   p40

B

— PMA TFP

125 —
            — s-TrkA
88 —

C

— PMA TFP W7 Clm

            — p41/40
            ← (arrow)

Figure 1 CaM inhibitors trigger the cleavage of TrkA

(**A**) CHO$^{\text{TrkA}}$ cells were treated with PMA (1 μM) or TFP (100 μM) for 30 min, and then lysates were subjected to immunoprecipitation with the anti-panTrk antiserum, followed by SDS/PAGE [10% (w/v) gel] and Western blotting with the same antiserum. Ig, heavy chain of IgG. The molecular mass markers (kDa) are shown at the left. (**B**) CHO$^{\text{TrkA}}$ cells were metabolically labelled with [^{35}S]Met and [^{35}S]Cys for 4 h and then chased in serum-free culture media containing PMA or TFP. Media were collected and immunoprecipitated with the anti-ectodomain antibody MGR12, followed by SDS/PAGE and autoradiography. The position of the soluble form of TrkA (s-TrkA) is indicated. (**C**) Action of different CaM inhibitors on TrkA cleavage. CHO$^{\text{TrkA}}$ cells were treated with PMA (1 μM), TFP (100 μM), W7 (25 μM), or calmidazolium (Clm, 25 μM) for 30 min, and then samples analysed as described in (**A**). The position of a low M_r fragment of TrkA (p38) is indicated by an arrow.

converted into a 140 kDa mature form [8,29]. The latter can be cleaved upon stimulation of PKC with phorbol esters, such as PMA, and results in the generation of cell-bound fragments of 41 and 40 kDa (p41 and p40, respectively; Figure 1A and [8,29]). Addition of the CaM inhibitor TFP also induced TrkA cleavage, as indicated by both the generation of cell-bound fragments (Figure 1A), and the release of soluble TrkA to the culture media (Figure 1B). Other CaM inhibitors structurally unrelated to TFP, such as W7 or calmidazolium, were also able to induce TrkA cleavage, but with a lower potency than TFP (Figure 1C). In contrast to PMA, the CaM inhibitors induced the generation of a lower M_r form of TrkA in addition to p41/p40 (Figure 1A and arrow in Figure 1C) that represented a cleavage of TrkA at the endodomain (E. Díaz-Rodríguez and A. Pandiella, unpublished work).

Since TFP was found to be the most efficient CaM inhibitor in terms of TrkA cleavage, we used this agent to further investigate the effect of CaM inhibition on the cleavage of the ectodomain of several other membrane proteins. The ectodomain of the membrane-bound growth factor proTGFα has been shown to be released from the cell surface in a regulated manner [32]. ProTGFα is biosynthesized as a membrane precursor of 17 kDa that accumulates at the cell surface and contains the active growth factor domain [33]. Upon cleavage, the ectodomain is released leaving a 15 kDa cell-bound form that contains the transmembrane and cytosolic domains [28]. Immunoprecipitation and Western blotting of CHO$^{\text{TGF}\alpha}$ cells with an antibody that recognized a C-terminal intracellular proTGFα epitope showed that under steady-state conditions both the 15 and 17 kDa forms were present at levels favouring the 17 kDa form (Figure 2A, left hand panel). Treatment of CHO$^{\text{TGF}\alpha}$ cells with PMA or TFP induced the conversion of the 17 kDa form into the 15 kDa tail fragment (Figure 2A, left hand panel). The effect of these drugs on proTGFα cleavage was also observed by using an antibody directed to the ectodomain of proTGFα that identified only the 17 kDa form (Figure 2A, right hand panel). Treatment with PMA resulted in a complete loss of the 17 kDa form, while TFP had a less pronounced effect, leaving some 17 kDa proTGFα at the cell surface.

To assess whether cleavage of the ectodomain of proTGFα by both PMA and TFP resulted in the release of soluble bioactive forms of TGFα, culture supernatants from cells treated with these drugs were analysed for their ability to induce activation of the EGF/TGFα receptor (EGFR) in HeLa cells. Serum-starved HeLa cells were incubated with the culture media from TFP- or PMA-treated CHO$^{\text{TGF}\alpha}$ cells, and tyrosine phosphorylation of the EGFR was analysed on Western blots from anti-EGFR immunoprecipitates. As shown in Figure 2(B), the conditioned media from TFP- or PMA-treated CHO$^{\text{TGF}\alpha}$ cells stimulated tyrosine phosphorylation of the EGFR in HeLa cells. Other lower M_r proteins that likely represent co-precipitating EGFR substrates were also found to be tyrosine phosphorylated in conditioned media-treated cells, although to a much lesser extent than the EGFR.

The release of other membrane proteins in different cell types was also investigated. In HEK-293 cells transfected with the α2c version of the NRG1 precursor (proNRGα2c), treatment with phorbol esters or TFP induced cleavage of the 65 kDa proNRGα2c into several cell-bound lower M_r fragments (tail in Figure 2C). Cleavage of endogenously expressed βAPP was also induced by PMA or TFP treatment of PC12 cells (Figure 2D). Taken together these data indicated that CaM inhibitors induced cleavage of multiple membrane proteins in different cell types, and thus pointed to a general effect of CaM inhibitors in the regulation of membrane-protein ectodomain cleavage.
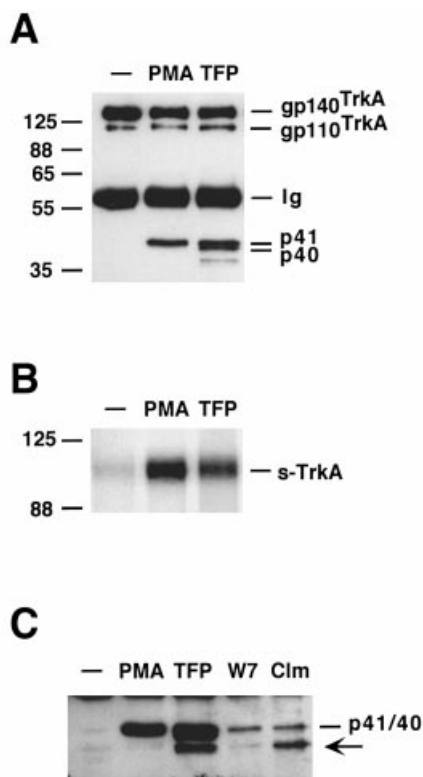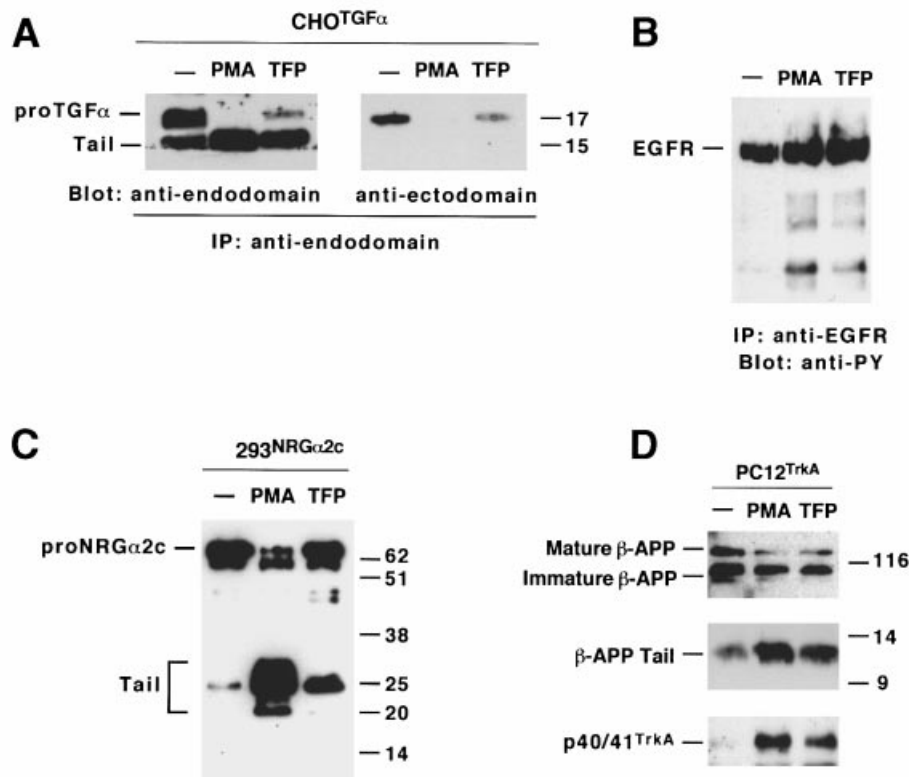
Figure 2   TFP induces the shedding of multiple membrane proteins

(**A**) Effect of TFP on proTGFα processing. CHO$^{TGF\alpha}$ cells were treated with PMA or TFP, and cell lysates were immunoprecipitated (IP) with the R2086 affinity-purified antiserum. The blots were probed with R2086 (left panel) or an anti-ectodomain monoclonal antibody (right panel). (**B**) Release of bioactive TGFα from PMA- and TFP-treated CHO$^{TGF\alpha}$ cells. HeLa cells were incubated for 10 min with conditioned media from untreated (−), PMA-, or TFP-treated CHO$^{TGF\alpha}$ cells. HeLa cell lysates were immunoprecipitated with an anti-EGFR mAb and blots were probed with anti-phosphotyrosine antibodies. (**C**) Cleavage of proNRGα2c induced by TFP. HEK-293 cells transfected with the proNRGα2c isoform were treated for 30 min with PMA (1 μM) or TFP (100 μM), and then lysates immunoprecipitated with the 312 anti-NRG antiserum. Western blotting was performed with the same antiserum. (**D**) Cleavage of βAPP induced by TFP. PC12^{TrkA} cells were incubated with PMA (1 μM) or TFP (100 μM), and then lysates immunoprecipitated with the 369 anti-βAPP antiserum. Western blotting was performed with the same antiserum. Mature βAPP is the only form that is efficiently processed by PMA or TFP. Processing is more evident by analysis of the low molecular mass tail fragments of βAPP. As a control for the cleavage, the bottom panel shows TrkA processing induced by the different treatments in PC12^{TrkA} cells. The positions of molecular mass markers (kDa) are shown on the right side of panels (**A**), (**C**) and (**D**).

Time-course and dose-response of the effect of CaM inhibitors on membrane protein cleavage

The effect of TFP was found to be time- and dose-dependent. Cleavage of TrkA with generation of p41/p40 was already detected at the earliest time point analysed (1 min) after treatment with TFP (Figure 3A, top panel). The effect on p41/p40 generation increased with time, reaching a peak between 20–30 min of treatment (Figure 3B). A concomitant decrease in the holo-receptor gp140^{TrkA}, indicative of a precursor–product relationship, was also detected at later time points. The time course for generation of TrkA fragments in cells treated with PMA followed similar kinetics, peaking at 20–30 min of treatment with the phorbol ester (Figure 3A, bottom panel). However, cleavage of TrkA induced by PMA was of a larger magnitude than that induced by TFP, and at later time points the amount of the fragments decreased, probably due to PKC-induced degradation of these fragments (results not shown).

The dose-dependence of the TFP effect on proTGFα and TrkA was next investigated. Cleavage of proTGFα was maximal at 100 μM, and half maximal at concentrations between 25 and 50 μM (Figures 3C and 3E), which fall within the expected range of CaM inhibition by this compound [34]. The dose dependence of TrkA cleavage induced by this CaM inhibitor was slightly

shifted to the right with respect to proTGFα (Figures 3D and 3E).

CaM inhibitors act on TrkA cleavage by a Ca²⁺- and PKC-independent mechanism

In addition to PKC, intracellular Ca²⁺ increases have been reported to induce membrane-protein ectodomain cleavage. Since a direct relationship between these pathways and CaM exists [26], the question of whether the action of CaM inhibitors could be mediated by any of the above second messenger routes was analysed. As a preliminary step, and since TrkA cleavage in response to Ca²⁺ agonists has not been reported, the action of agents that increase intracellular free Ca²⁺ concentration on TrkA cleavage was investigated. In CHO^{TrkA} cells treatment with the Ca²⁺ ionophore A23187 induced cleavage of TrkA, which was prevented by the chelation of extracellular Ca²⁺ with EGTA (Figure 4). Extracellular Ca²⁺ chelation did not, however, affect PMA- or TFP-induced TrkA cleavage, indicating that these treatments operated in a Ca²⁺-independent fashion. Ca²⁺ chelation decreased the effect of vanadate, another stimulator of the cleavage of TrkA [29] and other membrane proteins [35,36]. Thus the action of vanadate on membrane-protein ectodomain
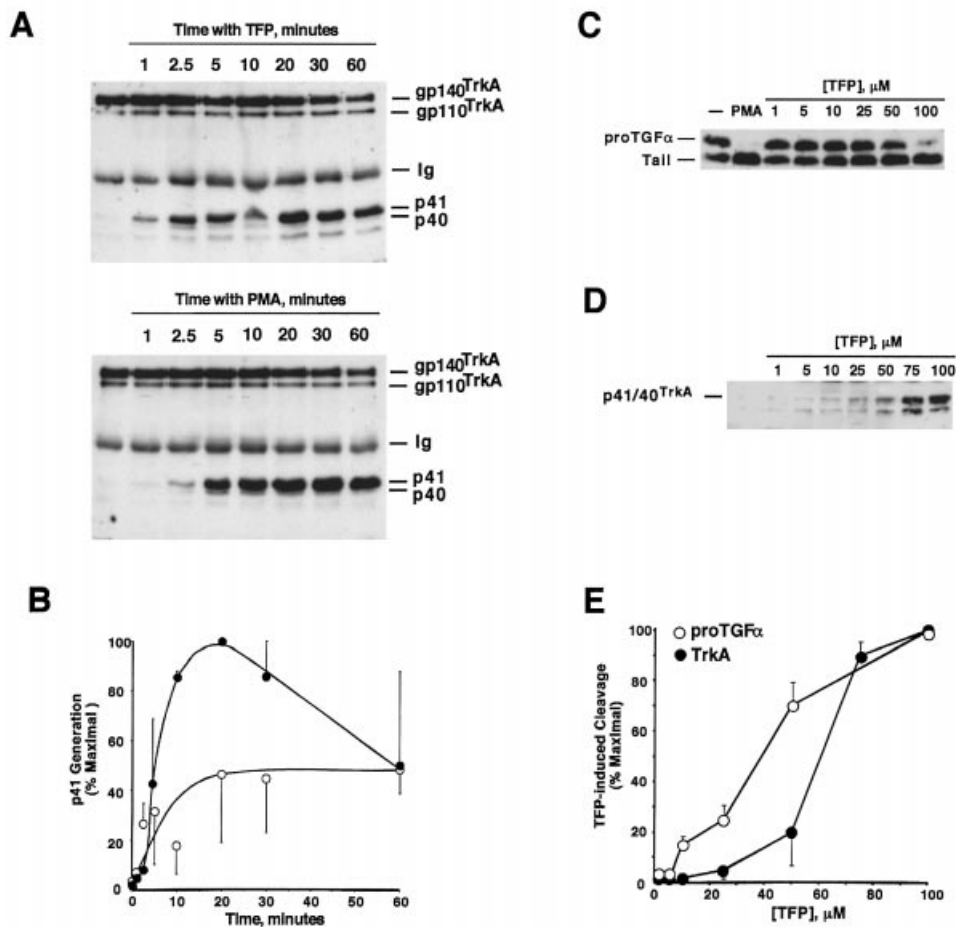
Figure 3   Time course and dose-response effect of PMA and TFP on TrkA and proTGFα cleavage

(**A**) Time-course of the effect of TFP and PMA on TrkA cleavage. CHO^{TrkA} cells were treated with 100 μM TFP or 1 μM PMA for the indicated times, and TrkA was precipitated from cell lysates with the anti-panTrkA antiserum. (**B**) Representation of the time-course of TrkA cleavage induced by PMA (●) and TFP (○). Data shown are the means ± S.D. of three independent experiments. (**C**) Dose–response of the effect of TFP on proTGFα cleavage. CHO$^{TGF\alpha}$ cells were treated for 30 min with the indicated concentrations of TFP. Then the lysates were immunoprecipitated with the R2086 antiserum, and the blots were probed with the same antiserum. (**D**) Dose–response of the effect of TFP on TrkA cleavage. Cells were treated for 30 min with the indicated concentrations of TFP, and p41 generation analysed by Western blotting with the anti-panTrk antiserum. (**E**) Graphical representation of the dose–response curves of TFP-induced proTGFα (○) and TrkA (●) cleavage. The data (means ± S.D.) are from at least three independent experiments.

cleavage may partially be due to influx of Ca^{2+} from the extracellular medium.

To analyse a possible intermediate role of PKC in the action of CaM inhibitors, cells were preincubated with the PKC antagonistic drug BIM prior to treatment with activators of TrkA cleavage. Treatment with BIM had a potent inhibitory effect on phorbol ester-induced TrkA cleavage, but was unable to prevent TFP-, vanadate- or Ca^{2+} ionophore-induced cleavage, indicating that these pathways act on TrkA cleavage by mechanisms independent of PKC activity. Another treatment that affects membrane-protein ectodomain cleavage, i.e. phosphatidylinositol 3-kinase inhibition [37], did not substantially affect the PKC, Ca^{2+} or TFP pathways (Figure 4). Taken together, the above results indicated that several independent intracellular pathways mediated cleavage of TrkA, and that TFP acted by a novel mechanism.

Metalloprotease activities mediate TFP-induced generation of truncated forms of TrkA and proTGFα

Even though distinct intracellular pathways may stimulate membrane-protein ectodomain cleavage [23], shedding is usually highly sensitive to metalloprotease inhibitors of the hydroxamic acid family [18]. To analyse whether TFP-induced release of TrkA and TGFα had a similar inhibitory spectrum, cells expressing these proteins were preincubated with the hydroxamic acid derivative BB3103, and then treated with TFP. This inhibitor, was found to potently inhibit the cleavage of both TrkA (Figure 5A) and TGFα (Figure 5B) in response to TFP. This protease inhibitor did not, however, affect the generation of the lower M_r p38 TrkA fragment, indicating that the generation of the latter occurred by the action of a TFP-activated protease distinct from those responsible for the generation of truncations at the ectodomain of TrkA.

TFP-induced cleavage of TrkA results in the generation of tyrosine-phosphorylated cell-bound truncated fragments

Phorbol ester-induced cleavage of TrkA has been reported to result in the generation of cell-bound truncated fragments that are tyrosine-phosphorylated [8] and associate with signalling intermediates of the neurotrophin pathway of signal transduction [29]. To determine whether TFP-induced cleavage resulted in the generation of tyrosine-phosphorylated truncated TrkA frag-
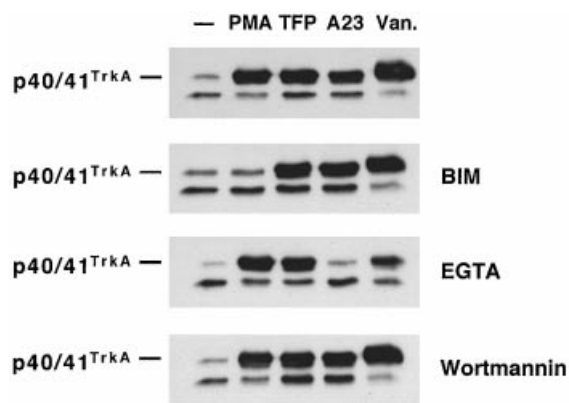
Figure 4   TFP acts on membrane protein ectodomain cleavage by a PKC- and Ca²⁺-independent pathway

CHO^TrkA cells were treated with inhibitors (BIM, 2 μM; EGTA, 2 mM; Wortmannin, 100 nM) for 30 min before treatment with the activators [1 μM PMA, 100 μM TFP, 1 μM A23187 (A23), or 1 mM vanadate (Van.)] for 60 min. Immunoprecipitation and Western analyses were performed with the anti-panTrk antiserum.

ments, CHO^TrkA cells were treated with TFP, and then samples of anti-TrkA immunoprecipitates were analysed for their phospho-tyrosine content by Western blotting with anti-phosphotyrosine antibodies. As previously reported [8,29] PMA treatment resulted in the generation of tyrosine-phosphorylated TrkA fragments (Figure 5C, top panel). Analogously, TFP treatment resulted in the generation of tyrosine-phosphorylated fragments. *In vitro* treatment of the precipitates with calf intestinal phosphatase substantially reduced anti-phosphotyrosine staining of the blots (Figure 5C, top panel), without affecting anti-panTrk recognition of the truncated fragments (Figure 5C, bottom panel).

TFP-induced cleavage of TrkA is independent of the cytosolic region of the receptor

Association of CaM with the cytosolic tail of L-selectin has been proposed to mediate a regulatory role in L-selectin shedding [27]. Experiments aimed at the identification of a possible direct CaM–TrkA interaction by precipitation with the anti-panTrk antibody, followed by Western blotting with an anti-CaM antibody, failed to detect any CaM that could be co-precipitated with TrkA (results not shown). To investigate whether TrkA cleavage induced by CaM inhibitors could be regulated by a weak, or otherwise difficult to detect, interaction of CaM with the cytosolic domain of the receptor, mutants of the cytosolic domain of TrkA were analysed for their regulated cleavage in response to TFP. CaM binding to proteins depends on small interaction modules in target proteins consisting of basic and hydrophobic amino acids [24,25]. In L-selectin such a binding site, located in the juxtamembrane region, contains the RRLK sequence essential for CaM regulation of L-selectin shedding [27]. Analysis of the juxtamembrane intracellular region of TrkA indicated that this receptor contained the sequence RRNK analogous to the one involved in L-selectin–CaM interaction. Since mutants of L-selectin in which amino acids C-terminal to the dibasic RR were substituted by Glu (L^{370} or K$^{371} \rightarrow$ E) prevented CaM association to L-selectin [27], a TrkA mutant was created that changed the sequence RRNK to RREE. As shown in Figure 6(A), treatment of CHO^TrkA-EE cells that express the mutant receptor with PMA or TFP induced proteolytic cleavage of TrkA. Quantitative
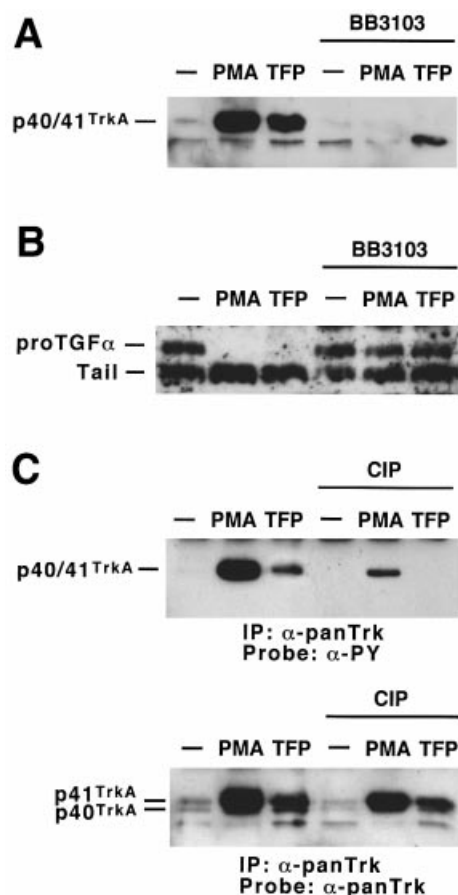

Figure 5   TFP-induced truncation of TrkA and proTGFα is sensitive to hydroxamic acid-based metalloprotease inhibitors

(**A**) Effect of the protease inhibitor BB3103 on TrkA cleavage. BB3103 (30 μM) was added to cells 15 min before activation with PMA or TFP. Immunoprecipitates of TrkA were analysed as described in the legend of Figure 1. (**B**) Effect of BB3103 on proTGFα cleavage. Cells were treated as above, and the anti-proTGFα immunoprecipitates analysed as described in the legend of Figure 2. (**C**) Generation of tyrosine-phosphorylated fragments of TrkA. CHO^TrkA cells were treated with 1 μM PMA or 100 μM TFP for 30 min, and then anti-panTrk immunoprecipitates (IP) were analysed for their tyrosine phosphorylation by probing of the blots with either anti-phosphotyrosine (α-PY) or anti-panTrk (α-panTrk) antibodies. Where indicated, samples were incubated for 30 min with calf intestinal phosphatase (CIP).

analyses indicated that this processing was of similar magnitude to that observed with the wild-type TrkA receptor (results not shown).

Because the large intracellular domain of TrkA could contain other potential CaM binding sites, another TrkA mutant receptor in which the entire intracellular domain was deleted, was transfected into CHO cells (CHO^TrkAΔIntra), and evaluated for its sensitivity to stimuli that induced holoreceptor cleavage. Since intracellular deletion of proteins, such as proTGFα, may cause resistance to endoproteolytic cleavage [38,39] due to retention in intracellular compartments [40], the expression pattern of the mutated receptor was first analysed. The subcellular distribution of the TrkA^Δintra mutant was essentially indistinguishable from the wild-type TrkA receptor, as assessed by indirect immuno-fluorescence experiments (results not shown). Since the mutated TrkA^Δintra receptor lacked the intracellular epitope recognized by the anti-panTrk antibody, the release of soluble forms of the
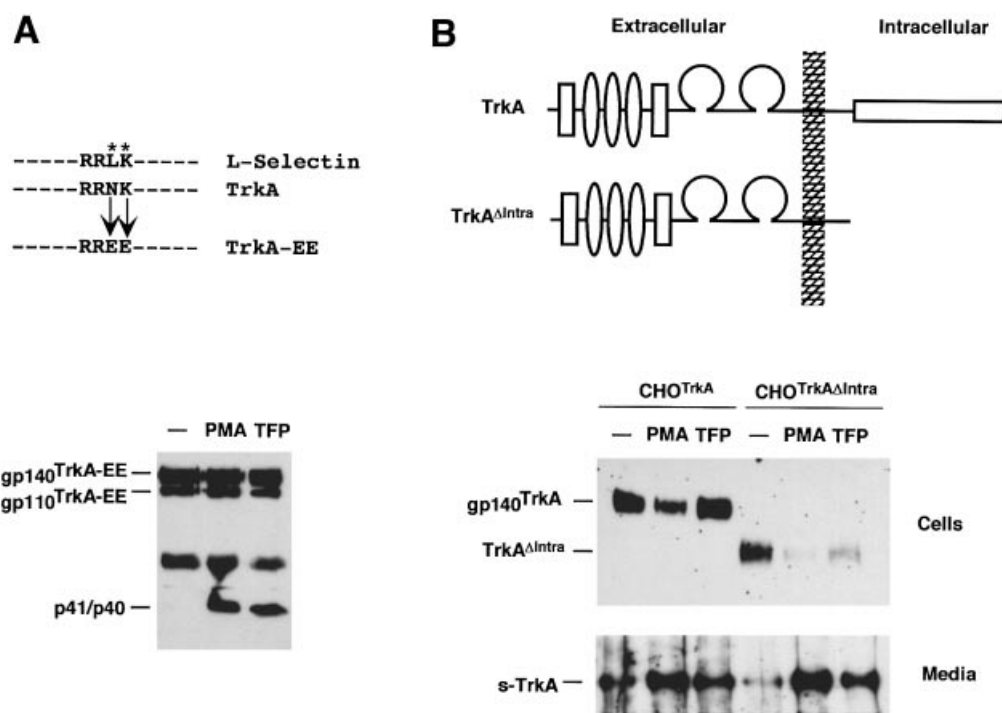
Figure 6   Effect of cytosolic tail mutations on PMA- and TFP-induced TrkA cleavage

(**A**) The motif (RRLK) in the juxtamembrane region of L-selectin that is responsible for L-selectin–CaM interaction is shown, and a similar juxtamembrane sequence (RRNK) in the TrkA receptor that may act as a potential CaM-binding site. The two amino acids residues marked by an asterisk have been mutated to glutamic acid to mimic the mutants in L-selectin that prevent CaM–L-selectin interaction. The bottom panel shows the effect of PMA and TFP on the cleavage of the mutated TrkA-EE receptor. Immunoprecipitation and Western analysis of the mutant receptor expressed in CHO cells was performed as described in the legend to Figure 1. (**B**) Deletion of the cytoplasmic domain of TrkA does not affect PMA- or TFP-induced cleavage. A schematic representation of the different TrkA domains and the deleted receptor is shown at the top, and the experimental data obtained by treatment of CHO^{TrkA} or CHO$^{TrkA\Delta Intra}$ cells with TFP or PMA is shown at the bottom. Immunoprecipitates of cell lysates or culture supernatants were performed with the MGR12 antibody, followed by Western analysis with the anti-ectodomain antipeptide antibody.

receptor was analysed by Western blot of the culture media or cell lysates with an antibody that recognized a peptide located in the ectodomain of TrkA. Western blotting of cell lysates from CHO$^{TrkA\Delta Intra}$ cells treated with PMA or TFP showed that both agents induced a decrease in the amount of the holoreceptor present in the cells (Figure 6B). In addition, treatment with these drugs was able to induce shedding of the TrkA ectodomain to the culture media in both CHO^{TrkA} and CHO$^{TrkA\Delta Intra}$ cells.

DISCUSSION

Several reasons led us to investigate a potential role of CaM in membrane-protein ectodomain cleavage. First, CaM acts as a cellular intermediate of multiple Ca^{2+} actions, and changes in intracellular Ca^{2+} have been reported to regulate the cleavage of several membrane proteins [1]. Secondly, CaM has been shown to interfere with the PKC pathway by embedding of PKC phosphorylation sites on substrates in CaM crypts [26]. Finally, a recent report suggested a role of CaM in the release of soluble L-selectin, probably by direct interaction of CaM with the cytosolic domain of the adhesion molecule [27].

In the present study we provide evidence indicating that a novel pathway, triggered by CaM inhibitors, may control the shedding of the ectodomain of several proteins. In CHO cells transfected with TrkA, treatment with structurally different CaM inhibitors triggered the release of the ectodomain of the receptor, with concomitant accumulation of several cell-bound fragments (p41, p40, and p38). In addition to TrkA, CaM inhibitors were

also able to induce the cleavage of other membrane-bound molecules such as the precursors for the growth factors TGFα and NRGα2c, and the βAPP, indicating a general role for CaM in the regulation of membrane-protein ectodomain cleavage.

Cleavage induced by both PMA and TFP was highly sensitive to the metalloprotease inhibitor BB3103, indicating that the final components of the processing machinery may be under the control of metalloproteases. A candidate metalloprotease could be TACE/ADAM17. This metalloprotease, which was initially isolated as a proTNFα processing enzyme [17], has also been shown to participate in the shedding of proTGFα, L-selectin, and the p75^{TNFR} [20]. If TACE is the proTGFα shedding protease, PMA and TFP may up-regulate its activity to shed proTGFα as well as other membrane proteins. How this potential activation of TACE by CaM inhibitors might occur is not known, but it probably does not involve a direct CaM–protease interaction, since attempts to co-immunoprecipitate CaM with TACE have been unsuccessful (E. Díaz-Rodríguez, and A. Pandiella, unpublished work).

While PMA and TFP share several properties such as rapid time course and protease inhibitor sensitivity, differences exist in their action. PMA was more potent in the induction of membrane-protein ectodomain cleavage. In addition, differences were also observed with respect to the spectrum of truncated TrkA forms generated by both agents. Although the major TrkA product that results from PKC activation is a p41 cell-bound fragment [8], a small fraction of the holoreceptor is cleaved to a p40 fragment [29]. TFP and PMA both stimulated the production

of p41 and p40; the former, however, stimulated p40 generation in a more potent way than PMA. This may indicate a preference for activation of the enzyme that generates p41 in the case of PKC-triggered cleavage of TrkA with respect to the CaM inhibitor. TFP may have less selectivity and may thus activate both protease activities in a more balanced way. Another interesting difference between PMA and TFP was the effect of the latter on the generation of a 38 kDa TrkA fragment. TFP stimulated the generation of this smaller TrkA fragment, whereas PMA did not.

Another conclusion of the present work is that TFP acts on membrane-protein ectodomain cleavage by a novel mechanism that is independent of intracellular Ca^{2+} rises or PKC activity. In CHO^{TrkA} cells intracellular Ca^{2+} increases activated the cleavage of TrkA. The action of Ca^{2+} ionophores on intracellular Ca^{2+} consists of two phases, an initial, rapid and large increase in intracellular Ca^{2+} mainly due to redistribution of Ca^{2+} from intracellular stores, followed by a longer lasting plateau phase due to influx from the extracellular medium [41,42]. Treatment with extracellular Ca^{2+} chelators prevents the second phase, affecting only marginally the burst due to redistribution from stores. It is the second phase that appears to be more critically involved in the regulation of TrkA cleavage. In fact, treatment with EGTA completely prevented A23187-induced TrkA cleavage. Thus proteolytic cleavage of TrkA, induced by intracellular Ca^{2+} increases, requires a sustained and discrete, rather than transient and elevated, intracellular Ca^{2+} rise. Whereas Ca^{2+} influx appears to be essential for Ca^{2+} ionophore-induced TrkA cleavage, it is completely dispensable for PMA- or TFP-induced cleavage. Even though intracellular Ca^{2+} rises may also increase PKC activity [43], the use of treatments that prevent or down-regulate PKC activity has indicated that Ca^{2+} acts on membrane-protein ectodomain cleavage by a mechanism that is independent of PKC activity [23]. In the case of TrkA, intracellular Ca^{2+} rises had a substantial effect on the generation of p41, even in the presence of PKC inhibitors, indicating that the effect of Ca^{2+} was not indirectly mediated by PKC activation.

Evidence accumulated during the last few years indicates that CaM and PKC have opposite actions because of the embedding of PKC phosphorylation sites in CaM-binding domains [26]. Thus it was of interest to elucidate whether CaM inhibitors acted by releasing a substrate that participated in the regulation of membrane-protein ectodomain cleavage, and was activated by PKC. However, the results with the PKC inhibitor BIM indicated that the action of TFP was not mediated through a PKC pathway. Therefore the effect of CaM inhibitors on membrane-protein ectodomain cleavage appears to be mediated by a novel PKC- and Ca^{2+}-independent pathway.

Several Ca^{2+}-dependent and -independent motifs have been described in CaM-interacting proteins [25]. Amphipathic helices rich in basic residues have been reported to mediate CaM binding to several proteins [24]. In L-selectin a CaM binding motif has been identified in the juxtamembrane intracellular region, and includes the RRLK sequence [27]. Mutational analysis of this motif indicated that this region has an essential role in CaM binding to L-selectin. Interestingly, the juxtamembrane cytosolic region of TrkA also contains an amphipathic domain that includes an RRNK sequence. However, attempts to co-immunoprecipitate CaM with TrkA have been unsuccessful (E. Díaz-Rodríguez and A. Pandiella, unpublished work), and mutation of this putative CaM binding site in the juxtamembrane region of TrkA did not affect PMA- or TFP-induced TrkA cleavage. Furthermore, deletion of the cytoplasmic domain of TrkA also had no effect on PMA- or TFP-induced TrkA cleavage. This also indicates that the endodomain of TrkA is dispensable

for cleavage, in contrast to the essential role of the endodomain of proTGFα in the targeting [40] and cleavage [38,39] of the membrane-bound factor.

Regulation of the cleavage of membrane-bound proteins is a complex phenomenon that has an essential role in animal homoeostasis [20]. A previous finding that CaM inhibitors were able to induce L-selectin shedding has been extended in the present work to several other membrane proteins, suggesting a generic role of CaM inhibitors in the regulation of membrane-protein ectodomain cleavage. Interestingly this mechanism acts by a pathway that is independent of other well known and universal stimulators of cleavage, the PKC and Ca^{2+} pathways. Whereas cleavage of L-selectin may be regulated by direct CaM–L-selectin interactions, cleavage of other membrane proteins, such as TrkA, does not apparently depend on CaM–substrate interaction. Therefore intermediate steps must be required by CaM inhibitors to induce membrane-protein ecto-domain cleavage. Identification of the molecular components implicated in this pathway may allow us to better understand the regulation of the proteases that participate in this important biological function.

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