Diphtheria toxin endocytosis and membrane translocation are dependent on the intact membrane-anchored receptor (HB-EGF precursor): studies on the cell-associated receptor cleaved by a metalloprotease in phorbol-ester-treated cells

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Preincubation of Vero cells with 1 μM phorbol 12-myristate 13-acetate (PMA) decreased the specific binding of diphtheria toxin by about 50%, whereas the toxic effect, endocytic uptake and membrane translocation were completely blocked. Toxin bound to PMA-treated cells was released upon incubation with heparinase. The effect of PMA was abrogated in the presence of EDTA or *N*-{DL-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}-L-3-(2′-naphthyl)-alanyl-L-alanine 2-aminoethylamide (TAPI), a specific inhibitor of matrix metalloproteases. The results indicate that PMA induces proteolytic cleavage of the diphtheria-toxin receptor [heparin-binding EGF-like growth factor (HB-EGF)-precursor] outside the membrane anchor, and that about 50% of the growth-factor ecto-domain remains associated with the cells, due to binding to surface proteoglycans containing heparan sulphates. Although the cleaved cell-associated HB-EGF binds diphtheria toxin, it does not serve as a functional receptor, since neither toxin internalization nor translocation occurs. Thus the intact HB-EGF precursor is of crucial importance for its function as the diphtheria-toxin receptor.

INTRODUCTION

Diphtheria toxin (DT) entry into cells is a well-characterized model system to study protein translocation across biological membranes. The toxin is secreted from *Corynebacterium diphtheriae* as a polypeptide of 58 kDa [1], which is proteolytically cleaved in an arginine-rich region to yield fragments A (21 kDa) and B (37 kDa), held together by an inter-fragment disulphide bridge [2]. Fragment A is the enzymically active part that ADP-ribosylates elongation factor 2 [3]. Fragment B is bifunctional, with a receptor-binding domain and a translocation domain [4].

The entry pathway of diphtheria toxin into cells is initiated by the binding of DT to its cell-surface receptor. The receptor has recently been identified as the heparin-binding EGF (epidermal growth factor)-like growth factor-precursor (HB-EGF-precursor) [5]. Binding of DT to this growth-factor precursor is followed by internalization of the toxin–receptor complex via endocytosis [6]. The low intracompartmental pH triggers the translocation of the enzymically active A-fragment through the membrane of the endocytic organelle [7,8]. Finally, the A-fragment is released into the cytosol, where it inhibits cellular protein synthesis.

It has previously been shown that Vero cells become resistant to diphtheria toxin upon pretreatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) [9,10]. PMA is known to stimulate protein kinase C, thereby activating a large set of cellular proteins by phosphorylation. High concentrations of PMA ($\geqslant 10$ μM) strongly decreased the binding of DT to the cell surface, whereas DT toxicity was already blocked at nanomolar concentrations [10]. Similarly, formation of cation channels by DT B-fragment was completely blocked by PMA at nanomolar concentrations [11]. Hence, since binding is only partially decreased at low concentrations [10], the block of toxicity and channel formation does not seem to be due to decreased binding only. In this report, we have analysed the effect of PMA in more detail. We show that treatment with the phorbol ester blocks endocytosis and translocation of DT, presumably by inducing cleavage of the DT receptor. We also demonstrate that the effect of PMA is blocked by a metalloprotease inhibitor, indicating that a matrix metalloprotease (MMP) could be involved in PMA-regulated processing of HB-EGF. While this work was in progress, another group reported that PMA induced release of mature HB-EGF from human MDA MB 231 cells [12]. We show that, in Vero cells, the cleaved receptor is still cell-associated, due to binding to cell-surface heparans, but it is no longer functional in the sense that it does not mediate poisoning by DT. Thus the intact receptor molecule seems to be required for translocation of toxin in cellular membranes.

EXPERIMENTAL

Materials

[^{3}H]Leucine and ^{125}I were obtained from Amersham. Heparinase was purchased from Sigma (heparinase III; Sigma H 8891). *N*-{DL-[2-(Hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}-L-3-(2′-naphthyl)-alanyl-L-alanine 2-aminoethylamide (TAPI) was generously given by Immunex Research and Development Corp., Seattle, WA, U.S.A. All other chemicals were of the highest purity commercially available.

Buffers

Hepes medium consisted of bicarbonate- and serum-free Eagle's minimal essential medium buffered with 20 mM Hepes to pH 7.4. Lysis buffer consisted of 0.1 M NaCl, 20 mM NaH_2PO_4, 10 mM

Abbreviations used: DT, diphtheria toxin; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; MMP, matrix metalloprotease; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; TAPI, *N*-{DL-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl}-L-3-(2′-naphthyl)-alanyl-L-alanine 2-aminoethylamide.

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EDTA, 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 1 mM *N*-ethylmaleimide (NEM), pH 7.4. Mes/gluconate buffer consisted of 140 mM NaCl, 20 mM Mes and 5 mM sodium gluconate, pH 4.8.

Cell culture

Vero cells were propagated as described previously [7]. For binding and toxicity experiments, cells were seeded into 12-well Costar (Cambridge, MA, U.S.A.) microtitre plates at a density of 2×10^5 cells/well on the day preceding the experiments.

Radiolabelling of DT

DT was labelled as described by Fraker and Speck [13] to a specific radioactivity of 15000–50000 c.p.m./ng of toxin.

Nicking of DT

DT at a concentration of 1.1 mg/ml was nicked with trypsin at 0.5 μg/ml for 30 min at 37 °C. The reaction was stopped by adding 25 μg/ml soybean trypsin inhibitor.

Toxin binding

Vero cells in 12-well plates were incubated for 20 min at room temperature with ^{125}I-labelled toxin in Hepes medium in the absence or presence of 10 μg/ml unlabelled toxin. The cells were then washed four times with Hepes medium, lysed in 0.1 M KOH, and the radioactivity was measured.

Measurement of cytotoxicity of endocytosed toxin

Vero cells were incubated with increasing amounts of DT for 20 min at room temperature. Unbound toxin was washed away and the cells were further incubated for at least 1 h at 37 °C. Thereafter, the cells were allowed to incorporate [^{3}H]leucine for 30 min at 37 °C in leucine-free Hepes medium supplemented with 1 μCi/ml [^{3}H]leucine. After a 10 min incubation in 5% trichloroacetic acid, followed by a brief wash in the same solution, the cells were dissolved in 0.1 M KOH and the radioactivity was measured.

Measurement of cytotoxicity of toxin translocated from the plasma membrane by low-pH pulse

Vero cells in 12-well plates were incubated for 20 min at room temperature with increasing concentrations of nicked toxin in Hepes medium containing 5 μM monensin. The cells were then washed four times with Hepes medium and incubated for 5 min in Mes/gluconate buffer, pH 4.8 at 37 °C. After a brief wash with Hepes medium containing 5 μM monensin, the cells were incubated in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum and 5 μM monensin for at least 2 h at 37 °C. The cells were allowed to incorporate [^{3}H]leucine for 30 min at 37 °C in leucine-free Hepes medium supplemented with 1 μCi/ml [^{3}H]leucine. After a 10 min incubation, followed by a brief wash in 5% trichloroacetic acid, the cells were dissolved in 0.1 M KOH and the radioactivity was measured.

Pronase-protection experiments

^{125}I-labelled DT (40 ng/10^5 cells in 0.5 ml) was bound to cells in Hepes medium containing 5 μM monensin for 20 min at room temperature. The unbound toxin was washed away, and surface-bound DT was nicked by incubation with 0.5 μg/ml trypsin for 5 min at room temperature, followed by washing three times with Hepes medium. After a 5 min exposure to pH 4.8 at 37 °C in Mes/gluconate buffer, the cells were incubated for 10 min at 37 °C with 5 mg/ml Pronase E in Hepes medium containing 5 μM monensin. The cells, which by now were detached from the plastic, were transferred to an Eppendorf tube, and pelleted by centrifugation. After washing with Hepes medium containing 1 mM NEM and 1 mM PMSF, the cells were lysed for 10 min in lysis buffer on ice, and nuclei were removed by centrifugation. Protein was precipitated with 5% trichloroacetic acid for 30 min on ice, and pelleted by centrifugation. The pellet was washed with ether and subjected to SDS/PAGE under non-reducing conditions.

Endocytosis assay

^{125}I-labelled DT (40 ng/10^5 cells in 0.5 ml) was bound to Vero cells for 1 h on ice. The cells were then extensively washed to remove unbound toxin and incubated for various periods of time in Hepes medium at 37 °C. Thereafter, the cells were treated with 10 mg/ml Pronase E for 20 min at room temperature, transferred to an Eppendorf tube and pelleted by centrifugation. The radioactivity in both pellet and supernatant was measured.

RESULTS AND DISCUSSION

PMA inhibits toxicity of DT only if the toxin is bound after PMA treatment

We first examined the effect of pretreating cells with 1 μM PMA for 30 min at room temperature. These conditions were used in all the following experiments unless otherwise indicated. In the PMA-treated cells, the total binding of ^{125}I-labelled DT was decreased to $51.4 \pm 15.9\%$ ($n = 9$) compared with untreated control cells. A 100-fold excess of unlabelled DT decreased the cell-associated radioactivity to background levels in both PMA-treated and untreated cells (results not shown), indicating that the portion of DT that binds to cells after PMA treatment is specifically bound to its receptor. In contrast with the moderate decrease in toxin binding, the toxic effect on the cells was completely blocked by PMA under the same conditions (Figure 1), confirming earlier results [10]. Interestingly, the toxic effect was not affected when the toxin was bound to the cells before the PMA treatment.

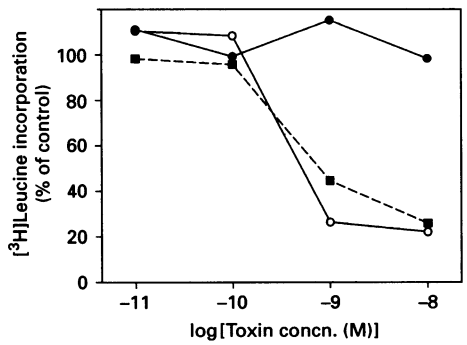

Figure 1 Effect of PMA on the cytotoxic effect of DT in Vero cells

DT at increasing concentrations was added to Vero cells that had been exposed to 1 μM PMA for 30 min at 20 °C. After 20 min incubation at 20 °C, unbound toxin was washed away and the cells were incubated further for 1 h, and then the ability of the cells to incorporate [^{3}H]leucine during 30 min was measured. ●, Cells were exposed to PMA before addition of DT. ○, Control cells not exposed to PMA. ■, DT was allowed to bind before the PMA treatment for 20 min at 20 °C. The results shown are representative for three independent experiments.

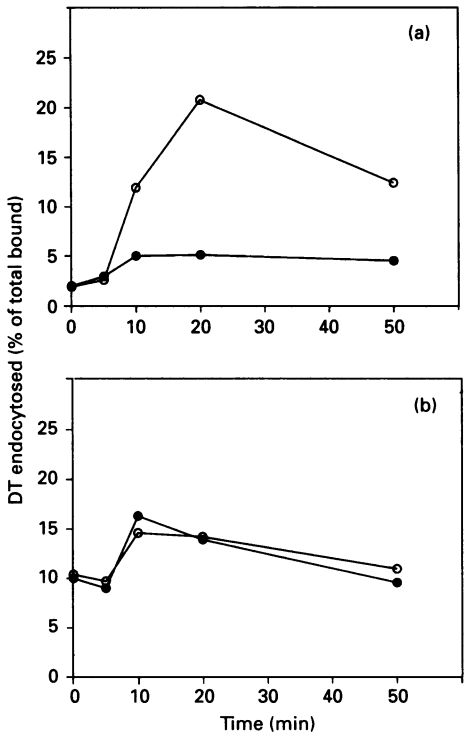

Figure 2 Effect of PMA on endocytosis of DT

Vero cells were treated with (●) or without (○) 1 μM PMA for 30 min at room temperature before (**a**) or after (**b**) ^{125}I-labelled DT was allowed to bind to the cells on ice for 1 h. The cells were then incubated for various periods at 37 °C to allow endocytosis of the bound toxin. Next, the cells were treated with 10 mg/ml Pronase E to remove surface-bound toxin and the cell-associated radioactivity was measured. The results shown are representative for three independent experiments.

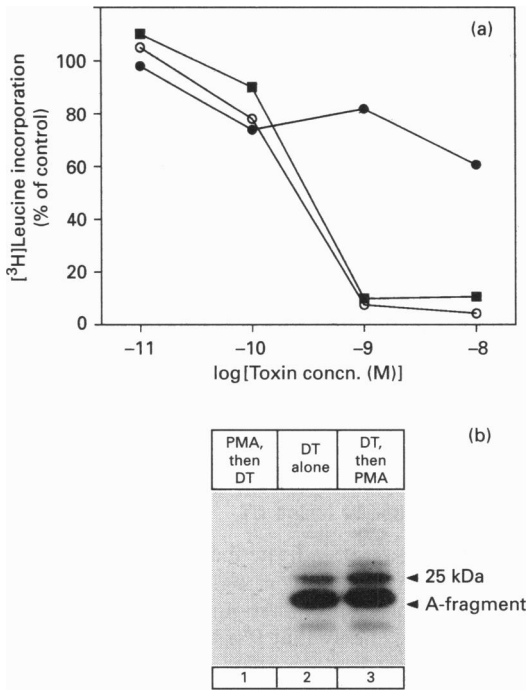

Figure 3 Effect of PMA on membrane translocation of DT

(**a**) Vero cells were incubated with increasing amounts of nicked toxin in the presence of 5 μM monensin for 20 min at room temperature. The cells were then washed and briefly exposed to pH 4.8. The cells were further incubated for 2 h in the presence of 5 μM monensin, and then their ability to incorporate [^{3}H]leucine during 30 min was measured. ●, Cells were exposed to PMA before DT was added. ○, Control cells that were not exposed to PMA. ■, DT binding was performed before the PMA treatment. The results shown are representative for three independent experiments. (**b**) ^{125}I-labelled DT was bound to cells for 20 min at room temperature in the presence of 5 μM monensin. The unbound toxin was washed away and surface-bound DT was nicked by incubation with trypsin. After a 5 min exposure to pH 4.8, the surface-bound toxin was digested with Pronase E and the cells were lysed. Proteins were precipitated with 5% trichloroacetic acid and analysed by SDS/PAGE under non-reducing conditions. Lane 1: cells were pretreated with 1 μM PMA. Lane 2: control cells not treated with PMA. Lane 3: toxin was bound before PMA treatment. The results shown are representative for five independent experiments.

PMA blocks endocytosis and translocation of DT

The above finding that the PMA-induced decrease in toxin binding to cell-surface receptors cannot account for the decrease in toxicity suggests that entry of DT must be blocked at a later step in PMA-treated cells. Therefore, we explored whether the endocytosis of DT was affected by the PMA treatment. For this purpose, ^{125}I-labelled DT was bound at 0 °C to Vero cells pretreated with or without PMA, and endocytosis was allowed to proceed at 37 °C for various time periods. Then the surface-bound toxin was removed by treatment with Pronase E, the cells were lysed and cell-associated radioactivity was measured. Figure 2(a) shows that, in cells not treated with PMA, the cell-associated radioactivity reached a maximum after 20 min. Thereafter, it declined, possibly due to degradation and extrusion of degraded material. In cells that had been PMA-treated, uptake of DT was blocked. In our assay, we also monitored the release of radioactivity to the incubation medium during the 37 °C incubation. There was no major difference between PMA-treated and non-treated cells, indicating that the observed difference in cell-associated radioactivity was not due to enhanced dissociation of bound toxin (results not shown). If the toxin was bound at 0 °C before the PMA treatment, the endocytic uptake was not inhibited (Figure 2b). In our experimental set-up, the cells that were PMA-treated after toxin binding at 0 °C were incubated with bound toxin at room temperature in the presence of PMA for 30 min before uptake was assayed. Slow endocytosis has probably occurred during this time, and therefore the initial cell-associated radioactivity in the experiment in Figure 2(b) is higher than in Figure 2(a).

During entry of DT, endocytic uptake is followed by translocation of the enzymically active fragment A through the endosomal membrane. We also examined whether this step is affected by the PMA treatment. The translocation in the endosomes can be mimicked at the level of the plasma membrane by exposing cells with surface-bound toxin to low pH [7,8]. The extent of translocation is then reflected in the magnitude of inhibition of protein synthesis. Figure 3(a) shows that PMA pretreatment protected the cells against DT poisoning, indicating that translocation of the DT A-fragment was inhibited. DT that was bound before the PMA treatment was fully able to poison the cells. It should be noted that cells that were pretreated with PMA were exposed to the drug for 20 min longer than cells that were exposed to DT first. But, even if the PMA exposure of cells with prebound DT was extended to up to 80 min, the translocation was not affected (results not shown). The PMA pretreatment did not induce an enhanced release of bound DT from cells during the low-pH exposure (results not shown).

Translocation of DT from the plasma membrane can also be assayed by removing surface-bound toxin by treating the cells

with Pronase E after the low-pH pulse [14]. Successful translocation yields mainly two protected polypeptides, the A-fragment (21 kDa) translocated to the cytosol and a 25 kDa polypeptide, representing the B-fragment part that is inserted into the membrane and therefore shielded from the Pronase [14]. Under certain conditions, where the translocation of the A-fragment is prevented, only the 25 kDa band is obtained [15]. In contrast, upon pretreatment with PMA, neither of the two fragments could be detected (Figure 3b, lane 3), suggesting that neither translocation of the A-fragment nor membrane insertion of the B-fragment has occurred. In accordance with the experiments above, DT that was bound before the PMA treatment was still able to translocate and yield the two protected fragments (Figure 3b, lane 2).

Treatment with PMA and heparinase abolishes specific binding of DT and causes release of bound DT

The data presented above indicate that pretreatment of cells with PMA inhibits DT endocytosis as well as translocation, whereas uptake and translocation of prebound DT are not affected by PMA. Thus it seems likely that PMA induces a modification of the DT receptor that impedes these processes, and that bound DT protects the HB-EGF-precursor from this modification. Several membrane-bound growth-factor precursors are cleaved in a region close to the membrane anchor [16]. Such proteolytic processing releases soluble growth factors into the extracellular fluid. It has been shown for transforming growth factor α, which like HB-EGF is a member of the EGF family of growth factors, that the proteolytic cleavage is regulated by protein kinase C [17] and by other signalling pathways [18]. Very recently, it was reported that, in human MDA MB 231 cells, biologically active HB-EGF is released upon phorbol ester treatment [12]. It is thus very likely that PMA induces a similar proteolytic processing of the HB-EGF-precursor in Vero cells. The possibility was therefore considered that PMA treatment could induce proteolytic cleavage of the HB-EGF-precursor, the DT receptor, and that the cleaved HB-EGF could remain associated with the cell surface, due to binding to surface proteoglycans containing heparan sulphates. It has been demonstrated that DT binds to mature HB-EGF immobilized on heparin–Sepharose, indicating that heparin binding does not compete with DT binding [19]. To test this possibility, we treated the cells with heparinase to remove surface heparans, exposed the cells to 1 μM PMA and then tested the cells for binding of DT. As shown in Table 1, the heparinase digestion combined with PMA pretreatment completely abolished the specific binding of DT to the cells. Treatment with heparinase alone did not affect the binding. Neither did heparinase treatment affect the toxic effect of DT (results not shown).

If the DT receptor is cleaved, but remains associated with the cell surface via surface heparans, it might be possible to compete out this association with free heparin. The results in Table 1 show that this is indeed the case. The presence of 10 units/ml heparin in the binding medium abolished the specific DT binding in PMA-pretreated cells, whereas it had no effect on the binding to cells not treated with PMA.

We also tested whether bound DT in PMA-pretreated cells is released upon heparinase treatment. ^{125}I-labelled DT was bound to PMA-treated and control cells. The cells were then washed, incubated in Hepes medium containing heparinase for 30 min at room temperature, and the radioactivity in the medium was counted. As shown in Table 1, the amount of released DT is drastically increased upon heparinase treatment. In agreement with the above findings, the release remained at a basal level when the toxin was bound before the phorbol ester treatment.

Table 1 Ability of heparinase and heparin to inhibit the binding of DT in PMA-treated cells and of heparinase to induce release of bound toxin

Binding: Vero cells were incubated for 30 min at room temperature in Hepes medium containing either 0.5 unit/ml heparinase or 10 units/ml heparin and/or 1 μM PMA or no addition (No treatment). Then the cells' ability to bind ^{125}I-labelled DT during 20 min at room temperature was measured. In a parallel series 10 μg/ml unlabelled DT was included during the binding, to measure the non-specific binding. The values for the specific binding were obtained by subtracting the non-specific binding from the total binding. The binding was then calculated as a percentage of the control (No treatment). Results are expressed as means ± S.D. from three independent experiments (n = 5–7). Release: Vero cells were either treated with 1 μM PMA for 30 min at room temperature or incubated in Hepes medium only. Then ^{125}I-labelled DT was allowed to bind to Vero cells for 30 min at room temperature. In one case (DT, then PMA + heparinase), cells were first exposed to ^{125}I-labelled DT and subsequently to PMA. Cells were then washed and incubated in Hepes medium with or without 0.5 unit/ml heparinase for 30 min at room temperature. Finally, the radioactivity released from the cells during the last 30 min of incubation was measured. Means ± S.D. from three independent experiments are shown (n = 7). Abbreviation: n.d., not determined.

	Binding (% of control)	Release (% of ^{125}I-DT bound originally)
No treatment (control)	100	29.0 ± 4.9
PMA	41.7 ± 9.8	39.6 ± 5.7
PMA + heparinase (0.5 unit/ml)	9.1 ± 7.1	67.4 ± 5.8
PMA + heparin (10 units/ml)	2.6 ± 8.5	n.d.
Heparinase (0.5 unit/ml)	97.4 ± 8.8	28.9 ± 3.7
Heparin (10 units/ml)	85.9 ± 35.3	n.d.
DT, then PMA + heparinase	n.d.	22.6 ± 1.4

A specific inhibitor of matrix metalloproteases (MMPs) blocks PMA-induced desensitization

To verify further the hypothesis of proteolytic cleavage also in Vero cells, we tested a number of protease inhibitors on their potential ability to abolish the effect of PMA on DT translocation. Of the various inhibitors tested, pepstatin (10 μM), PMSF (1 mM), iodoacetate (1 mM), p-chloromercuribenzoate (200 μM), captopril (10 mM), leucineamide (10 mM), calpain inhibitor (50 μg/ml), tryptophanamide (2 mM), bestatin (0.5 mM) and a furin inhibitor (50 μM) [20] were not able to block the PMA effect. However, as shown in Figure 4, 1 mM EDTA diminished the toxin protection exerted by PMA, indicating that a metalloprotease could be involved in the PMA-induced cleavage. Recently, three groups [21–23] reported inhibition of processing of tumour necrosis factor by hydroxamic acid derivatives, specific inhibitors of MMPs. We tested one of those compounds, TAPI, which was identical with compound 2 reported by Mohler et al. [21], except that the naphthylalanine side chain was replaced by a t-butyl group. Figure 4 shows that TAPI was a potent inhibitor of the PMA effect on DT translocation. This compound had an IC_{50} of 30 nM (results not shown) and therefore seemed to be highly specific. The family of MMPs is thought to be involved in connective-tissue remodelling, and aberrant regulation is associated with metastasis and several pathologies [24–26]. Further studies are required to clarify whether the HB-EGF-precursor is in fact a substrate for MMPs, or whether HB-EGF-precursor processing involves a cascade of several proteolytic events, including one mediated by a MMP. The C-terminal amino acid of mature HB-EGF is leucine, and a

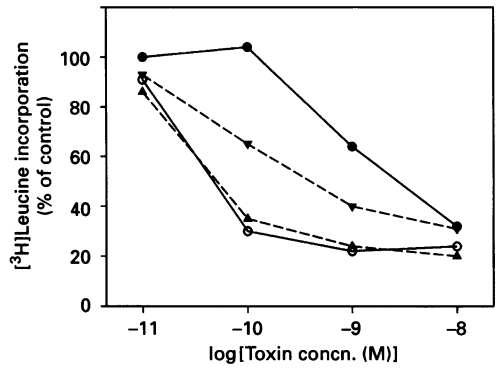

Figure 4 Inhibition of the effect of PMA

Vero cells were incubated with and without EDTA or TAPI for 10 min at 37 °C in presence of 5 μM monensin. To some of the cells 1 μM PMA was then added and the incubation was continued for 30 min more. Then, increasing amounts of nicked toxin were added and the cells were incubated for 20 min at room temperature. The cells were then washed and briefly exposed to pH 4.8. The cells were further incubated for 2 h in presence of 5 μM monensin, and then their ability to incorporate [^{3}H]leucine during 30 min was measured. ○, Control cells that were not exposed to PMA. ●, Cells pretreated with 1 μM PMA for 30 min. In some experiments 1 mM EDTA (▼) or 0.1 μM TAPI (▲) was added to cells 10 min before, and remained present during, the PMA treatment. The results shown are representative for three independent experiments.

valine residue is at P_2' [27]. This might indicate that HB-EGF-precursor is a potential MMP substrate [24–26]. Moreover, it is known that MMPs can be induced by various factors, among them phorbol esters and calcium ionophores [26].

Summarizing, our data suggest that protein kinase C activation induces cleavage of the HB-EGF-precursor also in Vero cells. This is in agreement with the recent results obtained in human MDA MB 231 cells [12]. The effects of EDTA and TAPI indicate that the proteolytic enzyme activated by PMA is a metalloprotease. After PMA-induced cleavage, a large part of the cleaved receptor appears to remain bound to the surface via surface heparan sulphates. We have previously shown that DT attached to cells via biotin–avidin was unable to poison cells or translocate from the plasma membrane [28]. Here, we show that toxin specifically bound to its receptor lacking the transmembrane anchor is neither internalized nor translocated. Thus, not only is the right receptor molecule required for internalization and translocation, but it appears that a tight membrane attachment of the natural receptor is mandatory for successful translocation of the A-fragment to the cytosol.

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