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Regulation of Epidermal Growth Factor Receptor Activity by Crotoxin, a Snake Venom Phospholipase A_2 Toxin

A NOVEL GROWTH INHIBITORY MECHANISM

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ABSTRACT. Crotoxin (CT), a phospholipase A_2 (PLA_2) derived from the venom of *Crotalus durissus terrificus*, is a heterodimeric protein composed of subunit B with enzymatic activity and a binding regulatory subunit (A) without enzyme activity. Although the PLA_2 activity of CT may be important in its anti-proliferative activity, its cytostatic mechanism is unknown. In this study, we examined the cytostatic effect of PLA_2-associated CT activity on squamous carcinoma cells expressing distinct levels of epidermal growth factor receptor (EGFr). CT was most effective in suppressing growth on cells expressing high intrinsic levels of EGFr. Cardiotoxin, another membrane active toxin with no intrinsic PLA_2 activity, had no differential anti-proliferative activity on cells expressing high EGFr levels, suggesting a correlation between EGFr expression and CT-directed anti-proliferative activity. Both chemically modified CT (MCT) devoid of PLA_2 activity and covalently cross-linked CT (CCT), which is functionally unable to utilize cellular membranes as PLA_2 substrate, were also without growth inhibitory activity. No evidence for direct binding of CT to EGFr was found, although pretreatment with EGF was able to partially suppress the anti-proliferative activity of CT. Tyrosine phosphorylation of EGFr, however, was stimulated by CT in intact A431 cells. Tyrosine phosphorylation of EGFr was concentration-dependently stimulated (3- to 8-fold) in cellular membranes of A431 cells treated *in vitro* with CT but not with anti-proliferatively inactive MCT or CCT. The data provide evidence for transmembrane receptors involved in growth signaling (namely EGFr) as cellular targets and potential effectors of PLA_2-mediated anti-proliferative activity of snake venom. BIOCHEM PHARMACOL 51;11:1535–1543, 1996.

KEY WORDS. crotoxin; phospholipase A_2; epidermal growth factor; tyrosine phosphorylation; growth regulation

Enzymatic activity is critical to the action of many animal toxins. The ability of these toxins to differentially affect cell growth, however, may reside with target cell properties, which are largely unknown. Therefore, understanding the cellular mechanism(s) associated with enzymatic toxins will be valuable in describing their mechanism of action and in determining their clinical potential.

Animal toxins with intrinsic phospholipase activities have been shown to exist in the venom of various snakes, spiders, and insects [1–4]. While purified enzymatic preparations have been utilized as tools to investigate phospholipid metabolism, paradoxically the intrinsic cellular and biological properties of these toxins have not been investigated extensively. The venom of the snake *Crotalus durissus terrificus* contains a protein enzymatic complex, CT¶, with intrinsic PLA_2 activity which has been shown to inhibit tumor cell growth *in vitro* and *in vivo* [2, 5, 6]. CT exists as a 24-kDa heterodimeric complex where the B subunit expresses intrinsic PLA_2 activity and the A subunit encodes a binding regulatory protein that prevents the non-selective binding of subunit B to cell surfaces (phospholipids) [7–9]. Although both subunits share sequence homology to other type PLA_2s, only CT-B possesses phospholipase activity, while the CT-A subunit is believed to act as a chaperone [10]. Subunit dissociation may be essential for CT anti-proliferative activity, thus providing evidence that CT must be guided to a cellular target that allows subunit dissociation and PLA_2 activation [2, 11]. Other venoms containing single peptide cytotoxic products, such as cardiotoxin from *Naja naja atra* venom, also have an affinity

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¶ *Abbreviations:* CT, crotoxin; MCT, chemically modified crotoxin; CCT, covalently cross-linked crotoxin; CD, cardiotoxin; RIPA, radioimmunoprecipitation; EGF(r), epidermal growth factor (receptor); TNF, tumor necrosis factor; PLA_2, phospholipase A_2; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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for cellular membranes despite their deficiency in intrinsic phospholipase activity [6, 12, 13]. The distinct cytostatic and/or toxic mechanisms of venomous toxins may indicate that assessing cellular sensitivity to toxin based solely upon enzymatic characterization may not be forthcoming. The targeting of toxins to cell membranes suggests a possible involvement of changes in cell membrane integrity and/or composition in the anti-proliferative signaling process associated with animal toxins. Therefore, it was hypothesized that cell growth signaling events, including epidermal growth factor receptor activation, may be affected directly or indirectly by changes associated with toxin-mediated anti-proliferative action.

In the present report, the anti-proliferative activities of both CT and CD were investigated on squamous carcinoma cells expressing various levels of EGFr. The results suggest a previously undetected association between high level EGFr expression and enhanced cellular sensitivity to the PLA_2 activity of CT, but not on the cellular response to CD. In addition, although CT does not bind directly to the ligand binding domain of EGFr, the toxin enhances the state of EGFr activation through a mechanism involving its intrinsic PLA_2 activity.

MATERIALS AND METHODS

Cell Lines

The ME-180 parental cervical carcinoma cell line was obtained originally from the American Type Culture Collection (ATCC, Rockville, MD). The establishment and characterization of EGFr-expressing variants of the ME-180 cell line were described previously [14, 15]. A431 cells were provided by Dr. Gary Gallick, Department of Tumor Biology, M.D. Anderson Cancer Center. Colon carcinoma cell lines DiFi and HT-29 were supplied by Dr. Marsha Frazier, Department of Gastrointestinal Oncology and Digestive Diseases, M.D. Anderson Cancer Center. All cell lines were screened and found free of mycoplasma contamination. Cells were propagated in minimal essential medium (MEM; Gibco-BRL, Grand Island, NY) containing 5% calf serum and 5% fetal bovine serum (Hyclone, Logan, UT) and 50 μg/ml gentamycin (Tri-Bio Laboratories, State College, PA).

CD and CT Isolation and Modification

Native CT and its subunits (CT-A and CT-B) were purified from *C. durissus terrificus* venom as previously described [2, 16]. PLA_2 activity was measured by titration at 25° on egg yolk lipoprotein as previously described [17]. CT specific activity was 21 ± 6 μmol fatty acid released/min/mg protein, whereas that of CT-B was 60 ± 8. Inactivation of CT-PLA_2 activity was achieved by 1,4′-dibromoacetophenone alkylation, as previously described, until residual PLA_2 activity was <0.4% of the original preparation [18]. Amino acid analysis of alkylated CT-B (Beckman 7300 Amino Acid Analyzer) demonstrated the lack of a single histidine residue when compared with unmodified CT-B. Cross-linking of CT with dimethylsuberimidate and separation of unreacted subunits were performed as previously described [19], and the isolated CCT had a specific activity of 19 ± 4 μmol/min/mg protein. However, this preparation is functionally inactive since it is sterically unable to utilize plasma membranes as PLA_2 substrate. The CT preparation had no EGF-like contaminating activity based upon its inability to stimulate tyrosine phosphorylation of isolated EGFr in an immune-complex kinase assay.

CD was purified from the venom of *N. naja atra* as previously described [20, 21].

Epidermal Growth Factor and Other Reagents

Recombinant human EGF was purchased from R&D Systems (Minneapolis, MN). EGF radiolabeling for binding studies was performed with chloramine T as previously described [22]. [^{32}P]ATP (>3000 Ci/mmol) for protein phosphorylation was obtained from Dupont-NEN (Boston, MA).

Effect of CT on Cell Growth

EGFr-expressing cells (4000 cells/well) were plated into individual wells of 96-well plates and incubated overnight prior to their incubation with filter-sterilized toxin for 72 hr at 37° in normal cell growth medium. Cell survival was estimated by the crystal violet cell-staining assay as previously described [14, 22]. Cell number was directly proportional to crystal violet cell stainable and extracted dye as measured by light absorbance at 570 nm in a microtiter plate reader. Colon carcinoma cellular sensitivity to CT was measured by the MTT assay as previously described [23].

Preparation of Cellular Membranes

Cell membranes were prepared from A431 cells as previously described [24]. The effects of CT and other toxins on EGFr tyrosine phosphorylation were analyzed in these preparations after preincubation with toxin alone at 37° (30 min) and continued incubation in the presence of Mn^{2+} and [^{32}P]ATP (with high or low concentrations of sodium vanadate) for 5 min at 4° as previously described [22]. EGFr phosphorylation was detected by SDS–PAGE of the incubation mixture and autoradiography of the dried gels as described [22].

Phosphotyrosine Immunoblotting of Cellular Extracts

Subconfluent A431 cells were treated with 50 μg/mL toxin (37°, 30 min), rinsed three times in ice-cold PBS, and released with a cell scraper. Cells were collected in PBS containing 2.5 mM EDTA and 2.5 mM sodium vanadate and rapidly pelleted by centrifugation (5 min, 2000 g). Cell pellets were retained and proteins solubilized (RIPA buffer

containing 2.5 mM sodium vanadate) by sonication on ice (5 sec, microcell disruptor). Extracts were clarified by centrifugation (100,000 *g*, 60 min, 4°), and equal protein aliquots (50 μg) were subjected to SDS–PAGE and transferred to nitrocellulose. Phosphotyrosine was detected by incubation with monoclonal anti-phosphotyrosine (4G10 MAb; UBI, Lake Placid, NY) and ^{125}I-labeled anti-murine Ig (Dupont-NEN) [24].

Immune-Complex Kinase Assay

EGFr tyrosine kinase activity was analyzed in colon carcinoma cell lysates by an immune-complex kinase assay as previously described [14, 15] with minor modification. Subconfluent cultures of DiFi and HT-29 cells were rinsed in ice-cold PBS, released, lysed, and clarified by centrifugation as described above. Equal protein aliquots (600 μg) were incubated with 2 μg of anti-EGFr monoclonal antibody (A108 [25]), and immune-complexes were precipitated with pansorbin (Calbiochem, La Jolla, CA). After extensive washing, immune-complexes were incubated with [^{32}P]ATP (10 μCi) in the presence of Mn^{2+} and sodium vanadate as previously described [22]. EGFr tyrosine phosphorylation was determined by SDS–PAGE and autoradiography. EGFr tyrosine phosphorylation was used as an estimate of relative receptor expression, which was quantitated by excision of the EGFr bands and Cerenkov radiation measurements in a scintillation counter.

Studies of the Influence of Toxin on ^{125}I-Labeled EGF Binding in A431 Cells

A431 cells (2.5×10^4) were plated into individual wells of a 24-well plate and were incubated at 37° overnight prior to toxin treatment or washed in preparation for competitive binding studies. Cell monolayers were rinsed three times with PBS containing 0.5% BSA, incubated 30 min at 37° in this medium, and rinsed again on ice with PBS containing 0.1% BSA at 4°. ^{125}I-Labeled EGF (sp. act. = 1.8×10^5 cpm/ng) was incubated with control or toxin-treated cells at a concentration previously determined to be subsaturating (1 nM). Competition for EGFr binding by toxin was determined by incubation of ^{125}I-labeled EGF in the presence of toxin (10–100 μg/mL; 0.3 to 3 μM) or unlabeled EGF (1–100 nM) and measuring cell bound radioactivity after 90 min of incubation at 4° in extensively washed monolayers [22].

RESULTS

CT-Mediated Anti-proliferative Activity on EGFr-Expressing Tumor Cells

ME-180 cervical carcinoma cell variants were selected previously for differential expression of EGFr [14]; both the selected variants as well as the parental cell line possess similar doubling times (22–28 hr). As shown in Fig. 1, ME-180R cells expressed 3- to 4-fold greater levels of EGFr

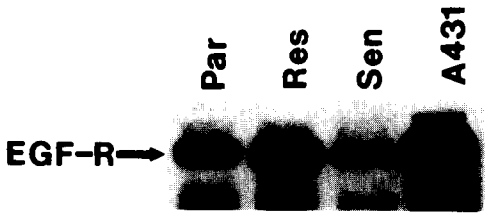

FIG. 1. Expression of EGFr in A431 and ME-180 squamous carcinoma cells. Equal protein lysates (50 μg) of ME-180 parental cells (Par), clonally derived variants expressing sensitivity (Sen) or resistance (Res) to TNF, and A431 cells were subjected to SDS–PAGE and transferred to nitrocellulose for immunodetection of EGFr as described in Materials and Methods. Similar distinctions in receptor levels could also be detected by receptor autophosphorylation studies in immune-complex kinase assays with A108 monoclonal antibody.

(both at the protein and mRNA level [14]) when compared with the parental or ME-180S population and approached a level of EGFr overexpression similar to that described for A431 cells [26]. Since EGF and TGF-α have been shown previously to affect PLA_2 activity and the production of arachidonic acid in both tumor and normal cells [27, 28], studies were initiated to determine the effect of toxins with intrinsic PLA_2 activity on the survival of cells expressing distinctions in their EGFr levels. As shown in Fig. 2, CT differentially suppressed cell growth with the greatest sensitivity in EGFr overexpressing cells (ME-180R and A431), suggesting a potential correlation. In support of this hypothesis, CT-mediated growth suppression could be re-

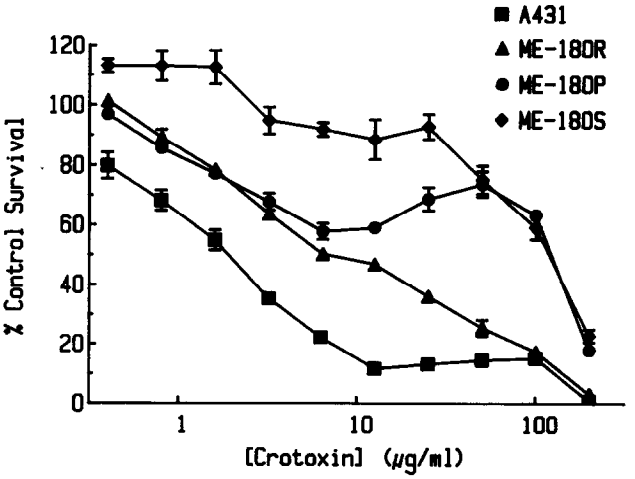

FIG. 2. Effect of CT on A431 and ME-180 cell survival. ME-180 parental (ME-180P), TNF-sensitive (ME-180S), TNF-resistant (ME-180R) or A431 cells, plated at an initial density of 5×10^3 per well of a 96-well plate, were incubated with various concentrations of CT (as indicated) for 72 hr at 37° and cell survival was estimated by crystal violet staining as described in Materials and Methods. The results represent the means ± SEM of 4 determinations, and the results are presented as percent control (untreated cell staining) incubated under the same conditions and in the same assay plate as treated cells.

duced in EGF-pretreated A431 cells (2 nM, 4 hr, 37°) but, as shown in Fig. 3, only at low concentrations of CT, suggesting that the influence of EGFr on CT growth suppression could be masked at high CT concentrations. Pretreatment of A431 cells with other cytokines, such as TNF, interferon-α or interleukin-1 did not influence CT anti-proliferative activity (data not shown).

PLA_2 Activity in CT Anti-proliferative Action

To determine the potential role of PLA_2 in CT cytostatic activity, cells were incubated with PLA_2-deficient CT (MCT) or covalently cross-linked non-dissociable CT (CCT), and cell growth was examined after 72 hr. As shown in Fig. 4, modified CT (or covalently cross-linked CT, data not shown) possessed no anti-proliferative activity, thus suggesting a role for either PLA_2 activity or the dissociation of CT subunits in CT-mediated growth suppression.

The regulatory effect on cell growth of CD, which is devoid of PLA_2 activity but expresses high cell-surface specific binding properties, was also examined. As shown in Fig. 5, treatment with CD affected the growth of all cell lines tested in a concentration-dependent manner, but no greater than 2-fold differential sensitivity between cell lines was observed (IC_{50} values = 10–20 μg/mL). Unlike CT, the anti-proliferative activity of CD demonstrated limited differential selectivity for EGFr-overexpressing ME-180R or A431 cells.

CT Activity on EGFr-Expressing Colon Carcinoma Cells

CT-mediated cytostasis was also examined on colon carcinoma cells differentially expressing EGFr. As shown in Fig. 6, DiFi cells expressed ~100-fold greater EGFr than HT-29

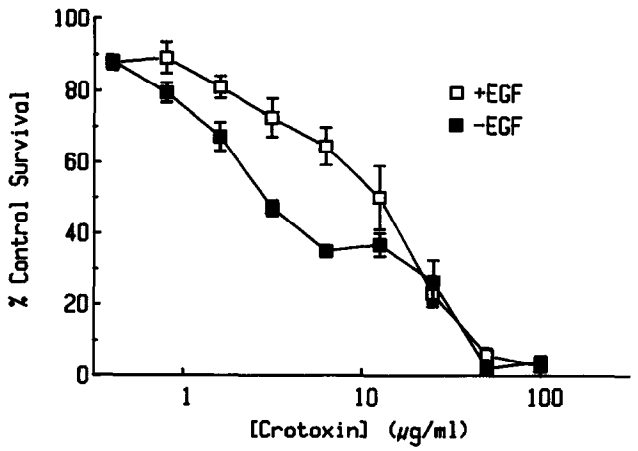

FIG. 3. Effect of EGF on CT growth suppression in A431 cells. A431 cells (plated at an initial density of 4 × 10^3 cells/well) were treated with 2 nM EGF (+EGF) or cell growth medium alone (-EGF) for 4 hr prior to their incubation with CT at various concentrations (as noted) for 72 hr at 37°. Cell survival was estimated as described in Fig. 2 and represents the means ± SEM of 4 determinations.

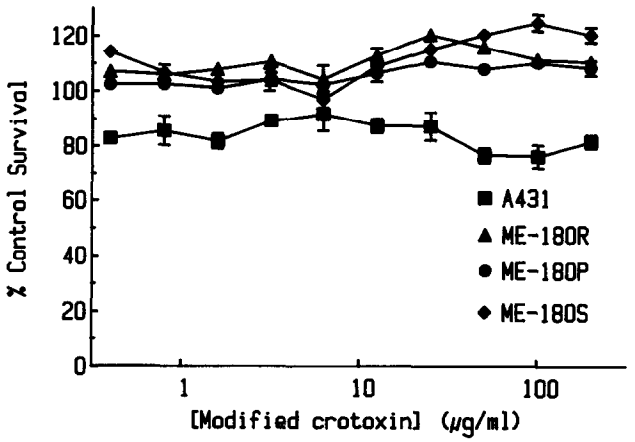

FIG. 4. Effect of modified CT on A431 and ME-180 cell survival. A431 and ME-180 cells (plated at an initial density of 5 × 10^3 cells/well) were incubated with concentrations of modified CT as noted, and cell survival was determined after 72 hr of incubation at 37°. The results represent the means ± SEM of 4 determinations.

cells based upon analysis of intrinsic EGFr tyrosine kinase activity in cell lysates. When cellular response to CT-mediated cytostasis was analyzed by IC_{50} measurements, ~20-fold greater sensitivity was detected in DiFi cells when compared with HT-29 cells (IC_{50} = 2.2 μg/mL in DiFi vs 42 μg/mL in HT-29), suggesting that differential CT sensitivity may be associated with EGFr expression in tumors of distinct origin. The mechanism of differential CT sensitivity on EGFr-overexpressing carcinoma cells and the role of PLA_2 activity in this response were explored further.

CT and EGF Binding to EGF Receptor

To determine the potential mechanism of enhanced cellular sensitivity to CT in EGFr-overexpressing cells, the di-

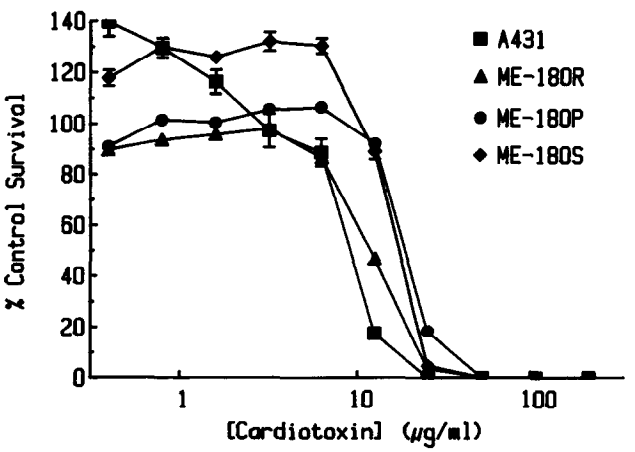

FIG. 5. Effect of CD of A431 and ME-180 cell survival. ME-180 and A431 cells (plated at an initial density of 5 × 10^3 cells/well of a 96-well plate) were incubated in the presence of CD (at the concentrations indicated) for 72 hr at 37°, and cell survival was estimated as described in Fig. 2. The results represent the means ± SEM of 4 determinations.

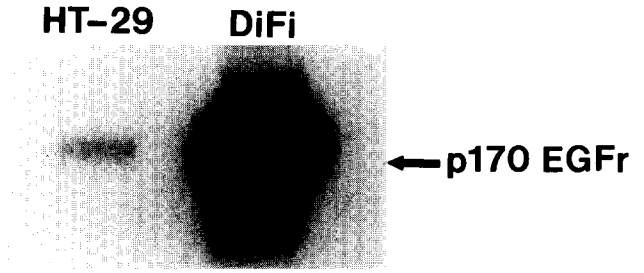

FIG. 6. EGFr tyrosine kinase activity and CT sensitivity in colon carcinoma cell lines. EGFr was immunoprecipitated from equal protein lysates of HT-29 and DiFi cells and analyzed for tyrosine kinase activity by autophosphorylation of immune-complexes as described in Materials and Methods. The results of one experiment are shown. Approximately 100-fold greater expression of EGFr was estimated in DiFi cells when compared with HT-29 cells in two independent assays. DiFi and HT-29 sensitivity to CT was determined by the MTT assay after incubation for 72 hr at 37°. The results of two independent assays are tabulated below.

Cell line	IC_{50} (μg/mL) ± SEM	
	Expt. 1	*Expt. 2*
HT-29	42.0 ± 2.2	44.0 ± 1.4
DiFi	2.2 ± 0.2	2.0 ± 0.1

rect binding of CT to EGFr was assessed by the ability of CT to inhibit ^{125}I-EGF binding to A431 cells. Binding of non-saturating concentrations of ^{125}I-EGF to A431 cells was examined in the presence or absence of CT and/or unlabeled EGF (Fig. 7). EGF inhibited ^{125}I-EGF binding at 10- and 100-fold molar excess. Some competition was also measurable at equal concentrations of labeled and unlabeled EGF (1 nM). In contrast, at concentration levels

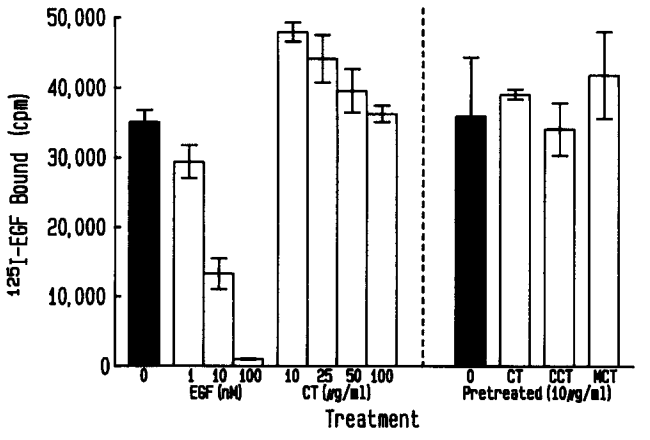

FIG. 7. Effect of CT on ^{125}I-EGF binding to A431 cells. Left: A431 cells, plated at a density of 2.5 × 10^4 in individual wells of a 24-well plate, were washed and incubated with 1 nM ^{125}I-EGF (~2.7 × 10^5 cpm) in the presence of 1-, 10-, or 100-fold molar excess of unlabeled EGF or 10–100 μg/mL CT at 4° for 90 min. After extensive washing, cell-associated radioactivity was solubilized (2% SDS in 50 mM NaOH) and quantitated. Right: A431 cells were pretreated for 1 hr at 37° with 10 μg/mL CT, CCT, or MCT (as noted), and after washing, 1 nM ^{125}I-EGF binding was measured and quantitated as described above. For all data points the results represent the means ± SEM of 3 determinations.

sufficient to mediate cell growth suppression, CT was unable to inhibit ^{125}I-EGF binding to A431 cells. Low concentrations of CT (10 μg/mL) enhanced ^{125}I-EGF binding, thus suggesting a possible cooperativity between EGFr and CT binding to A431 cells. However, this cooperativity was reduced at higher CT concentrations, thus indicating that this effect may have limited significance to the anti-proliferative activity of CT. The results suggest little competitive binding of CT to EGFr. Studies of ^{125}I-EGF binding were also conducted on A431 cells pretreated with CT (or anti-proliferatively inactive preparations of CT) for 30 min at 37°. Under these conditions, CT treatment did not affect ^{125}I-EGF binding (Fig. 7), suggesting that EGFr surface expression was unaltered by treatment.

PLA_2 Activity of CT and Activation of EGFr

To further explore the mechanism of altered cytostatic sensitivity to CT of EGFr-expressing squamous carcinoma cells, the plasma membrane fraction of A431 cells was isolated and incubated with either CT or the inactive MCT and CCT. Membrane protein phosphorylation in treated or untreated preparations was then examined by incubation with [^{32}P]ATP and resolution of the phosphoproteins by SDS–PAGE. A concentration-dependent phosphorylation of EGFr in CT-treated membranes *in vitro* was observed, but no activation with the covalently modified, non-cytotoxic CCT (Fig. 8). The effect of CT was measurable in both control and EGF-treated fractions, thus suggesting cooperativity, not antagonism, between the EGF activation mechanism and CT action.

Studies were also conducted on immunoprecipitated EGFr from CT, or as controls CCT- and MCT-treated A431 cell membranes (30 min, 37°). Following incubation with [^{32}P]ATP, EGFr was immuno-isolated and resolved by SDS-PAGE, and autophosphorylation was monitored as a measure of EGFr activation. As shown in Fig. 9, the phosphorylation of EGFr was stimulated by CT in a concentration-dependent manner but was unaltered by CCT or MCT. Furthermore, CT stimulation of EGFr phosphorylation was still measurable after tyrosine dephosphorylation had been suppressed with sodium-vanadate (2 mM) during the incubation and phosphorylation reaction. However, the magnitude of stimulation in receptor phosphorylation by CT was reduced in vanadate-treated fractions when compared with controls (2- vs 8-fold, respectively).

The modulatory effect of CT on tyrosine phosphorylation was also examined in intact A431 cells. As shown in Fig. 10, incubation of A431 cells with 50 μg/mL CT resulted in stimulation of EGFr tyrosine phosphorylation, which was delayed in onset and maximal only after 30 min of incubation as measured by EGFr tyrosine phosphorylation by immunoblotting of total cell lysates. Although apparent phosphorylation of EGFr itself was reduced after 60 min of incubation, other substrates were also increased measurably in their phosphorylation after 30 min of CT treatment and remained detectably elevated when com-

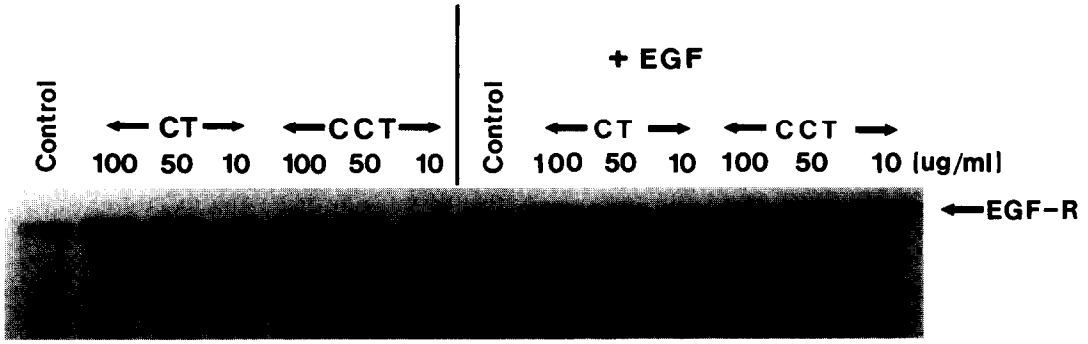

FIG. 8. Effect of CT on protein phosphorylation in plasma membranes derived from A431 cells. A431 cellular membranes were partially purified, and aliquots (50 μg protein in 20 mM HEPES buffer, pH 7.5) were incubated for 30 min at 37° with the indicated final concentration of CT or CCT in the presence (+EGF) or absence of 1 nM EGF. Incubation was continued for 5 min at 4° in the presence of [^{32}P]ATP (10 μCi), 10 mM Mn^{2+} and 0.2 mM Na_3VO_4, and phosphorylation was quenched by heating in SDS-PAGE sample buffer. Phosphoproteins were resolved by SDS–PAGE, and autoradiography was utilized to detect phosphorylation of EGFr on dried gels. Densitometric quantitative analysis of the effect of CT on EGFr phosphorylation demonstrated a 3-, 2.3-, and 0.9-fold increase in receptor phosphorylation over control levels by 100, 50, and 10 μg/mL concentrations of CT, respectively. Similarly, in the presence of EGF, CT stimulated receptor phosphorylation 3.2-, 2.4-, and 1.0-fold over control levels at CT concentrations of 100, 50, and 10 μg/mL, respectively. No more than 20% change in EGFr phosphorylation was detected in any of the CCT-treated samples.

pared with controls for up to 60 min. No detectable change in EGFr phosphorylation was measurable when A431 cells were treated with an equivalent concentration of antiproliferatively inactive CCT or MCT. In addition, CT treatment of ME-180 cells expressing low levels of EGFr (ME-180Par and ME-180Sen) under similar conditions was unable to stimulate EGFr tyrosine phosphorylation (data not shown).

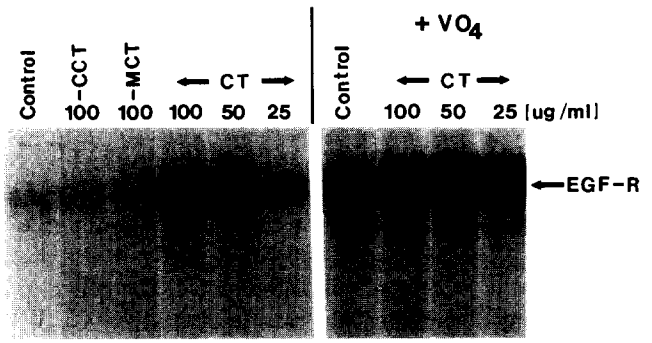

FIG. 9. Effect of CT on EGFr phosphorylation in plasma membranes derived from A431 cells. Aliquots of A431 cellular membranes (50 μg, prepared as described above) were incubated with CT, CCT, or MCT at the concentrations indicated in the presence of 0.2 mM Na_3VO_4 (left) or 2 mM Na_3VO_4 (right) for 30 min at 30°. Phosphorylation was initiated as described in Fig. 7; after 5 min at 4° in the presence of ATP, reactions were terminated with the addition of RIPA buffer (1 ml), and EGFr was immunoprecipitated with A108 monoclonal antibody (2 μg) followed by pansorbin. Immune-complexes were washed three times in RIPA buffer, and immuno-isolated receptor was resolved by SDS–PAGE. EGFr was detected by autoradiography, and the effects of treatment were quantitated by densitometry. CT stimulated 8.5-, 4-, and 1.5-fold increased receptor phosphorylation over control levels at CT concentrations of 100, 50, and 25 μg/mL, respectively. In the presence of higher concentrations of vanadate, CT stimulated 1.8-, 2-, and 1.5-fold increased receptor phosphorylation over control levels at the same respective concentrations of CT as described above.

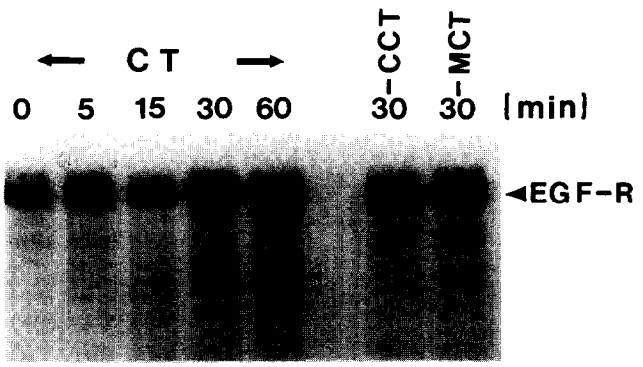

FIG. 10. Effect of CT treatment on EGFr tyrosine phosphorylation in intact A431 cells. Subconfluent A431 cells (~5 × 10^5) were incubated at 37° with 50 μg/mL CT, CCT, or MCT for the time indicated. Monolayers were rinsed and cells released and pelleted by centrifugation. Cell lysates were prepared in RIPA buffer (as described in Materials and Methods) and clarified by centrifugation; equal protein aliquots (50 μg) of the supernatant were resolved by SDS–PAGE, and proteins were transferred to nitrocellulose. After blocking, the nitrocellulose membrane was incubated with anti-phosphotyrosine antibody and antigen detected with ^{125}I-labeled anti-murine Ig. The effects of treatment were detected by autoradiography and quantitated by densitometry. CT stimulated 2.4- and 1.8-fold increased tyrosine phosphorylation of EGFr (and additional substrates of 68–72 kDa as depicted by arrowheads) after 30 and 60 min of incubation, respectively. Earlier time points from CT-treated cells and CCT or MCT treatment of A431 cells for 30 min did not alter EGFr tyrosine phosphorylation by more than 30%.

DISCUSSION

In this report, the antiproliferative activity of CT was examined on squamous carcinoma cells which expressed differences in their EGFr levels. Clonal variants of the ME-180 cell line were established by selection for TNF responsiveness and were characterized subsequently for EGFr expression [14, 15]. Evidence suggests that altered cellular sensitivity to CT was related to intrinsic expression of EGFr and not to the selection procedure utilized to establish variant clones. Primarily, TNF resistance alone does not confer differential sensitivity to CT since both A431 and ME-180R cells are resistant to TNF-induced cytotoxicity but express distinctions in their CT responsiveness and EGFr expression levels [14]. In addition, clonal ME-180 cell variants resistant to TNF in the absence of EGFr overexpression demonstrated no greater sensitivity to CT-induced growth suppression than the ME-180 parental cell population (data not shown). These studies support a correlation between CT-mediated growth suppression and relative EGFr expression in squamous carcinoma cells. However, EGFr dependence is also demonstrable in other cell types as well, supported by the results obtained in studies of colon carcinoma cells that expressed CT sensitivity which partially correlated with EGFr overexpression. Interestingly, the effects of CT on tumor cells do not appear to be correlative with other receptor tyrosine kinases since breast carcinoma cells expressing 50-fold distinctions in their HER2/Neu levels (SKBr-3 or BT-474 and MCF-7 cells, respectively) were equally sensitive to CT (data not shown). Together these data provide evidence for an EGFr expression-specific and -dependent CT sensitivity in both squamous and adenocarcinoma cells, which appear to be independent of the selection process or tumor type.

Experimental evidence suggests that the intrinsic PLA_2 enzymatic activity of CT may play a relevant role in the anti-proliferative effect of toxin since the PLA_2-deficient CT and covalently cross-linked CT were inactive as growth suppressive agents. Moreover, CD, which binds cell surfaces with high affinity but has no detectable PLA_2 activity, exhibited no significant differential activity on cells expressing various levels of EGFr and caused no concentration-dependent stimulation of receptor phosphorylation (data not shown). Therefore, the results suggest that EGFr may be implicated in the anti-proliferative activity of enzymatically active CT.

Binding assays failed to indicate a direct binding of CT to EGFr. Furthermore, CT treatment of A431 cells did not affect significantly subsequent EGF binding to EGF receptors. Pretreatment of A431 cells with EGF for 1–4 hr, which has been shown previously to down-regulate EGFr surface expression [29], prior to incubation with CT did reduce the growth suppressive effects of CT. TNF treatment under identical conditions also had no effect on the CT-mediated growth arrest, thus indicating that specific modulation of CT growth suppressive activity and EGFr surface expression may be linked through a regulatory component downstream of the EGFr signaling pathway. While other EGF-regulated events may play a role in protection of A431 cells from CT-mediated growth suppression (i.e. gene expression, Ca^{2++} mobilization, PLC-γ activation), it is important to note that a 3-fold change in relative expression of EGFr correlates with significant reduction in CT responsiveness (Figs. 1 and 2, compare ME-180S and ME-180R). Therefore, a 2-fold reduction of a surface EGFr expression by EGF-induced internalization may result in significant reduction in CT sensitivity. Initial studies demonstrated an activating effect of CT on EGFr tyrosine phosphorylation which was maximal 30 min after addition of the toxin and required temperatures between 25 and 37° for activation (data not shown). These requirements are consistent with either an enzymatic process or with an induced alteration in membrane fluidity, structure, or composition [30]. However, CT binding alone was unable to induce changes in EGFr function as assessed by studies with the PLA_2-inactive CT preparations. Studies are being conducted to determine the effects of chronic, long-term CT exposure on receptor expression.

CT had no effect on receptor phosphorylation when EGFr was isolated in immune-complexes (A431-derived EGFr bound to A108) and incubated with toxin, which dispels the possibility of a direct binding interaction between CT and EGFr and eliminates the possibility of EGF-like contaminants in the CT preparation (data not shown). Treatment of intact A431 cells with CT activated EGF receptor phosphorylation only 30 min after addition of toxin, thus indicating a delayed onset of the effects on EGFr as previously observed with the membrane preparations. Based upon these results and those derived from EGF competitive binding, the data are consistent with an indirect mechanism of CT activation of EGFr in membrane preparations, which appear to require participation of PLA_2 in the activation process.

The cytotoxic mechanism of PLA_2-containing venomous toxins may provide insight into their mechanism of action and thus give rise to pharmacologically relevant products that mediate PLA_2 action and directly or specifically alter membrane permeability. Previous studies indicate that EGFr function may be altered by changes in non-esterified fatty acids through a protein kinase C-independent process [31]. This effect, also independent of ^{125}I-EGF binding, provides further support for the involvement of phospholipid in the cytotoxic effect of CT observed in the present study which has similar characteristics. An altered cellular membrane integrity may modify the translational mobility of relevant transmembrane proteins which may then enhance their dimerization, a process thought to be important in receptor-activation and subsequent signaling [32, 33]. Analysis of the CT activation process, however, suggests a potential involvement of two different mechanisms of enhanced EGFr tyrosine phosphorylation. First, CT action appears to be additive with respect to EGF binding (Fig. 7) and receptor occupancy and the

activation of EGFr tyrosine phosphorylation. In addition, the fold-stimulation of EGFr phosphorylation by CT in vanadate-treated fractions was reduced when compared with low concentration vanadate control samples, suggesting the involvement of both stimulation of tyrosine kinase activity and inhibition of tyrosine phosphatase activity in the mechanism of CT action on EGFr phosphorylation.

It is interesting to note that both normal growth and cytotoxic signaling result in the liberation of fatty acids and ceramide from membrane phospholipids and sphingoid components, respectively [34–36]. Therefore, the complex signaling pathways involved must be highly regulated and integrated so as to mediate cellular stimulation without toxicity under growth stimulatory conditions or, conversely, to hydrolyze and/or liberate cytotoxic lipids and specific cytokines in anti-tumor pathways [37, 38]. Activation and/or dysregulation of these processes may lead to inappropriate signaling and cellular destruction. The experimental evidence in this report suggests that an enzymatically active CT may modify a selective cellular process normally associated with cell growth and thus also involved in growth suppression. Inappropriate signaling has been shown to result in growth suppression in cells overproducing EGFr [29, 39]. This is mediated by an as yet undefined mechanism, although stimulation of EGFr activation through a PLA_2-dependent process mediated by CT, as is indicated in the present study, may provide a mechanistic example of cellular dysregulation of a growth regulatory pathway. This report provides the first evidence of a role for EGFr overexpression in CT-mediated cell growth suppression which may lead to a better understanding of the mechanism of action of these toxins and their potential clinical implications in the treatment of proliferative disorders. It should be noted, however, that cellular specificity and differential dose-dependent sensitivity to CT and other toxins with PLA_2 activity must be further understood before clinical attempts are envisioned, since direct membrane disruption by high levels of PLA_2 activity may result in indiscriminant cell destruction. In this regard, the potential role of EGFr as a modulator of CT sensitivity will need to be examined in studies of the relative antitumor effect of CT in animals bearing tumors with high or low EGFr expression levels before characteristics affiliated with CT action *in vitro* are clinically relevant.

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