Cobra Venom Cardiotoxin (Cytotoxin) Isoforms and Neurotoxin: Comparative Potency of Protein Kinase C Inhibition and Cancer Cell Cytotoxicity and Modes of Enzyme Inhibition†

Shyh-Horng Chiou,‡ Robert L. Raynor,§ Bin Zheng,§ Timothy C. Chambers,§ and J. F. Kuo*,§

Institute of Biochemical Sciences, National Taiwan University, and Institute of Biological Chemistry, P.O. Box 23-106, Academia Sinica, Taipei, Taiwan, and Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Received August 7, 1992; Revised Manuscript Received November 10, 1992

ABSTRACT: Effects of cobra cardiotoxin (cytotoxin) and its isoforms, and neurotoxin, on protein kinase C (PKC) and cancer cells were investigated. A positive correlation existed between hydrophobicities and activities of the toxins to inhibit PKC activity (assayed using phosphatidylserine vesicle, arachidonate monomer, and Triton/phosphatidylserine mixed micelle systems), phorbol ester binding to PKC, proliferation of several cancer cell lines, and phorbol ester-induced HL60 cell differentiation. Their relative hydrophobicities and activities, in a decreasing order, were cardiotoxin-1 ≈ cardiotoxin-3 > cardiotoxin (a mixture of isoforms) > cardiotoxin-4 ≫ neurotoxin (inactive). Under the mixed micelle assay system (containing 0.3% Triton X-100, 8 mol % phosphatidylserine, 2 mol % diolein, and 200 μM $CaCl_2$), cardiotoxin inhibited PKC competitively with respect to phosphatidylserine (apparent K_i of about 0.06 mol % or 2.5 μM), and in a mixed-type manner with respect to both diolein (apparent K_i of about 0.04 mol % or 1.7 μM) and Ca^{2+} (apparent K_i of about 2.9 μM). On the basis of findings that IC_{50} (~0.3 μM) of cardiotoxin for inhibition of HL60 cell proliferation and differentiation was lower than its IC_{50} (9 μM) for PKC inhibition in vitro in the phosphatidylserine vesicle system and that PKC inhibition was the only known molecular mechanism of cardiotoxin, it was suggested that cardiotoxin might be highly membrane interacting and that the observed cellular effects of cardiotoxin might be mediated, in part, via PKC inhibition.

The venoms of the cobra snakes (Elapidae) have been studied extensively both chemically and pharmacologically during the past four decades (Lee, 1979; Karlsson, 1979). Snake toxins isolated from this elapid family may be the most studied group among all snake toxins. The components isolated from the crude venoms, in addition to some miscellaneous proteins/peptides and enzymes, include several major classes of biologically active proteins, such as phospholipase A_2, neurotoxins, and cardiotoxins (also called cytotoxins). The toxins have been used as tools in the studies of various biological phenomena. Especially noteworthy is the use of α-bungarotoxin in the labeling of acetylcholine receptors (Changeux, 1981), leading to the isolation and characterization of the membrane receptors.

Cardiotoxins are a group of very basic polypeptides, present in some snake venoms, which are especially abundant in cobras including Formosan cobra (Lee, 1979). They constitute up to about 40% of total protein in crude venom. Their biological functions are more diverse and their modes of action are less understood compared to neurotoxins. The primary sequences of several closely-related cardiotoxins from the Formosan cobra have been established (Hayashi et al., 1975, 1976; Kaneda et al., 1976a,b; Chiou et al., 1989) and shown to be related to those of neurotoxins (Yang et al., 1969). There is in general no specific noninvasive physical method to distinguish between neurotoxins and cardiotoxins, although these two classes of toxins have discrete pharmacological effects. We reported previously that cobra cardiotoxin (Kuo et al., 1983), like other naturally occurring bioactive amphiphilic polypeptides such as marine worm cytotoxin (Kuo et al., 1983), mastoparan (Raynor et al., 1991), melittin (Katoh et al., 1982) and polymyxin B (Mazzei et al., 1982), is a PKC[1] inhibitor; cobra and marine neurotoxins, in contrast, have no effect on PKC (Kuo et al., 1983). In the present study we employed three cardiotoxin isoforms purified from Formosan cobra venom to investigate a structure–activity relationship regarding their effects on PKC inhibition and cancer cell cytotoxicity and used a cardiotoxin preparation (a mixture of isoforms) to study modes of PKC inhibition.

EXPERIMENTAL PROCEDURES

Materials. Lyophilized crude venom powder from Formosan cobra (*Naja naja atra*), cardiotoxin from Formosan cobra or Thailand cobra (*Naja naja kaouthia*), neurotoxin from Central Asian cobra (*Naja naja oxiana*), PS, arachidonic acid, diolein, tamoxifen, and carboxypeptidase A were purchased from Sigma; ET-18-OCH_3 was from Calbiochem; TPA was from LC Services; [^{3}H]thymidine was from ICN Radiochemicals; [^{3}H]PDBu was from Du Pont–New England Nuclear; PKC inhibitor peptide was from Gibco; K562 and HL60 cells were from American Type Culture Collection; HL60/ADR, originally developed by Bhalla et al. (1985), was a gift of Dr. Robert I. Glazer; and KB-3 and KB-V1 cells (Shen et al., 1986) were a gift of Dr. Michael M. Gottesman.

† This work was supported by Academia Sinica and National Science Council of Republic of China (S.-H.C.), and U.S. Public Health Science Research Grants CA-36777 and HL-15696 and American Cancer Society Research Grant CH-513 (J.F.K.).

‡ National Taiwan University.

§ Emory University School of Medicine.

[1] Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; IC_{50} and EC_{50}, concentrations causing 50% inhibition or activation, respectively.

0006-2960/93/0432-2062$04.00/0

Isolation of Cardiotoxin Isoforms and Neurotoxin. The crude venom powder (495 mg) from Formosan cobra, dissolved in 10 mL of 0.05 M ammonium acetate (pH 5.9), was applied to a TSK CM-650 (total 10 mL) open column and then eluted with a linear gradient of 0.1–0.5 M and 0.7–1.0 M ammonium acetate followed by 1.0 M ammonium acetate. A reversed-phase HPLC column (4.0 × 300 mm, SynChropak RP-C_{18}, 6.5-μm bead) was used to remove the contaminant cardiotoxin-2 from the major cardiotoxin-3 fraction from the above ion-exchange chromatography (Chiou et al., 1992). The purities of the isolated toxins were checked by SDS–polyacrylamide slab gel electrophoresis (5% stacking/14% resolving gel) as described (Laemmli, 1970) with some modifications (i.e., 5% cross-linking N,N'-methylenebis-[acrylamide] in the gel solution). The amino acid compositions were determined with a Beckman 6300 amino acid analyzer using a single-column system based on a conventional ion-exchange chromatography system. The N-terminal sequences of the isolated toxin fractions from the ion-exchange column or fractions rechromatographed from reversed-phase HPLC columns were carried out by automated Edman degradation with a pulse liquid-phase protein sequencer (Model 477A, Applied Biosystems). Briefly, the samples, each containing about 1–5 nmol of protein, were dissolved in 200 μL of 0.1% trifluoroacetic acid and 10 μL of each was used for sequence determinations. For the identification of phenylthiohydantoin derivative of cysteine, toxins were reduced and alkylated with iodoacetamide before the sample was transferred to the sequencer. The electroblotting of protein bands from SDS–polyacrylamide gels to Immobilon-P transfer membranes (Millipore Corp.) for microsequencing of toxin was used to confirm the sequence determined by direct sequencing of the toxin peaks from cation-exchange chromatography. The C-terminal sequences of the HPLC-purified toxins were determined by carboxypeptidase A digestion at 37 °C for 30, 60, and 90 min in 0.1 M ammonium bicarbonate buffer, pH 7.8, and analysis of the released amino acids.

PKC Preparation and Assay and [^{3}H]PDBu Binding. PKC was purified from pig brain extracts as described (Girard et al., 1986); the enzyme preparation was devoid of other contaminating protein kinase activities. Type I (or γ), type II (or β), and type III (or α) isoforms of PKC were prepared by hydroxyapatite chromatography as described by Huang et al. (1986). PKC was assayed under the standard conditions using the PS vesicle system (Girard et al., 1986). Briefly, the reaction mixtures (0.2 mL) contained 5 μmol of Tris/HCl (pH 7.2), 2 μmol of $MgCl_2$, 2 μg of sonicated PS, 40 μg of histone H1, either 0.8 μmol of EGTA or 0.06 μmol of $CaCl_2$, 1 nmol of [γ-^{32}P]ATP (containing about 1 × 10^6 cpm), and the appropriate amount of PKC. The reaction, started with the radioactive ATP, was carried out for 5 min at 30 °C. PKC was also assayed under modified conditions using the arachidonate monomer system, in which 125 μM arachidonate replaced PS (Raynor et al., 1985), or using the Triton X-100/PS mixed micelle system described by Hannun et al. (1985). Binding of [^{3}H]PDBu to PKC was performed as reported elsewhere (Zhou et al., 1988; Raynor et al., 1991).

Cellular Studies. [^{3}H]Thymidine incorporation into various cancer cell lines was performed as described previously for K562 cells (Zheng et al., 1991). TPA-induced differentiation of HL60 cells was determined by cell attachment, and the cell viability was estimated by trypan blue dye exclusion, as reported (Shoji et al., 1988).

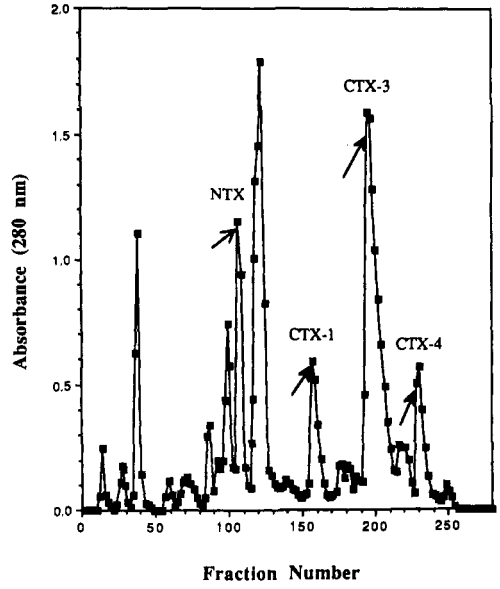

FIGURE 1: Isolation of cardiotoxin isoforms and neurotoxin. Lyophilized crude venom (495 mg) from Formosan cobra, dissolved in 10 mL of 0.05 M ammonium acetate (pH 5.9), was applied to a TSK CM-650 column (10 mL). Elution was carried out in four steps: (A) 0.05 M ammonium acetate (fractions 1–40), (B) a linear gradient of 0.1–0.5 M ammonium acetate (fractions 41–140), (C) a linear gradient of 0.7–1.0 M ammonium acetate (fractions 141–240), and (D) 1.0 M ammonium acetate (fractions 241–280). The fractions were monitored for absorbance at 280 nm. The peak fractions were pooled, desalted, and lyophilized. Arrows indicate one neurotoxin (NTX) and three cardiotoxin (CTX) isoforms (CTX-1, CTX-3, and CTX-4) used in the present study. The nomenclature and identification of toxin isoforms are based on the amino acid and sequence analysis.

RESULTS

Cardiotoxin isoforms 1, 3, and 4 and neurotoxin were purified from crude venom of Formosan cobra (Figure 1) and their primary amino acid sequences are compared (Figure 2). Their amino acid compositions, net charges, pI and M_r values, and hydrophobicities (Hϕ) calculated according to Kyte and Doolittle (1982) without correction of free N-terminal groups, are summarized (Table I). It might be noted that cardiotoxin isoforms, 1, 3, and 4 and neurotoxin have net charges (at neutral pH) of +7, +9, +9, and +3, respectively, and that their hydrophobicities, in decreasing order, are cardiotoxin-3 (0.14) ≈ cardiotoxin-1 (0.12) > cardiotoxin-4 (–0.03) ≫ neurotoxin (–1.27).

Because PKC isozymes α,β and γ were inhibited with similar potencies by cardiotoxin and its isoforms (data not shown), as by mastoparan and other agents (Raynor et al., 1991, 1992), the present PKC preparation, which had not been resolved into the individual isozymes, was used in all studies reported herein. Relative potency of the toxins in inhibiting PKC was compared. Under the standard assay conditions where PKC was activated by PS/Ca^{2+}, their IC_{50} values, in increasing order, were cardiotoxin-3 (0.8 μM) ≈ cardiotoxin-1 (1 μM) < cardiotoxin-4 (2 μM) ≪ neurotoxin (>100 μM) (Figure 3A). The unfractionated cardiotoxins (presumably a mixture of all isoforms) from Formosan cobra (Figure 3A) and Thailand cobra (data not shown) both had a potency (IC_{50} of 1.3 μM) approximating the average of the three isoforms, in agreement with their relative amounts present in the venom (Figure 1). Consistent with inhibition of PKC activity specifically stimulated by TPA when the enzyme was assayed in the presence of suboptimal concentrations of PS/Ca^{2+} (data not shown), binding of [^{3}H]PDBu to PKC was also inhibited

CTX-1: LKCNKLIPIASKTCPAGKNLCYKMFMMSDLTIPVKRGCIDVCPKSNLLVKYVCCNTDRCN

CTX-3: LKCNKLVPLFYKTCPAGKNLCYKMFMVATPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN

CTX-4: RKCNKLVPLFYKTCPAGKNLCYKMFMVSNLTVPVKRGCIDVCPKNSALVKYVCCNTDRCN

NTX: LECHNQQSSQTPTTTGCSGGETNCYKKRWRDHRGYRTERGCGCPSVKNGIEINCCTTDRCNN

FIGURE 2: Comparison of primary amino acid sequence of cardiotoxin (CTX) isoforms 1, 2, and 3 and neurotoxin (NTX) from Formosan cobra venom. The sequences shown were taken from the present and previous studies (Hayashi et al., 1975, 1976; Kaneda et al., 1976a,b; Chiou et al., 1989; Yang et al., 1969). The amino acid residues (denoted by one-letter symbols) in toxins that are different from those in cardiotoxin isoform 1 sequence are underlined.

Table I: Amino Acid Compositions, Net Charges, M_r and p*I* Values, and Hydrophobicities of Cardiotoxin Isoforms and Neurotoxin from Formosan Cobra[a]

parameter	CTX-1	CTX-3	CTX-4	NTX
no. of amino acids/molecule				
nonpolar residues				
Ala	2	2	2	0
Val	4	7	7	1
Leu	6	6	5	1
Ile	4	1	1	2
Pro	4	5	4	2
Met	3	2	2	0
Phe	1	2	2	0
Trp	0	0	0	1
polar residues				
Gly	2	2	2	7
Ser	3	2	2	4
Thr	3	3	3	8
Cys	8	8	8	8
Tyr	2	3	3	2
Asn	5	4	6	6
Gln	0	0	0	3
acidic residues				
Asp	3	2	2	2
Glu	0	0	0	4
basic residues				
Lys	8	9	8	3
Arg	2	2	3	6
His	0	0	0	2
total residues	60	60	60	62
net charge	+7	+9	+9	+3
p*I*	10.04	10.18	10.31	9.74
M_r	6,700	6,747	6,793	6,956
Hϕ	0.12	0.14	−0.03	−1.27

[a] Amino acid compositions and other properties of cardiotoxin isoforms were from Hayashi et al. (1975, 1976), Kaneda et al. (1976a,b), and Chiou et al. (1989), and those of neurotoxin were from Yang et al. (1969). Hydrophobicities (Hϕ) of the toxins were calculated using the hydropathy indices of amino acids (Kyte & Doolittle, 1982) without correction of free N-terminal groups.

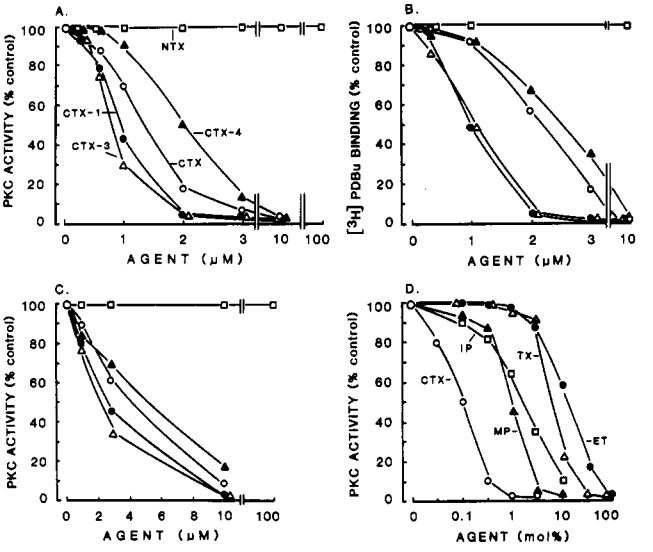

FIGURE 3: Inhibition of PKC activity and [^{3}H]PDBu binding by cardiotoxin and its isoforms, neurotoxin, and other agents. (A) The enzyme was assayed under the standard (PS vesicle) conditions in the presence of 200 μM $CaCl_2$, PS (2 μg/0.2 mL), and varying concentrations of toxins (agents), as indicated. The control activity in the absence of toxins (25.9 pmol/min) was taken as 100%. CTX, cardiotoxin; NTX, neurotoxin. (B) Inhibition of [^{3}H]PDBu binding to PKC by toxins (indicated by the same symbols) used in (A). The enzyme was incubated for 30 min in the presence of PS (1.6 μg/0.2 mL), 10 μM $CaCl_2$, and 32 nM [^{3}H]PDBu (20 000 cpm), with or without 200 μM nonradioactive PDBu, and varying concentrations of toxins (agents), as indicated. The nonspecific binding was <10% of the total binding. The specific binding (6.4 pmol) in the absence of toxins was taken as 100%. (C) The enzyme was assayed under the modified conditions in the presence of 200 μM $CaCl_2$, 125 μM arachidonate (replacing PS), and varying concentrations of toxins (indicated by the same symbols) used in (A). The control activity in the absence of toxins (20.8 pmol/min) was taken as 100%. (D) The enzyme was assayed using the mixed micelle system containing 0.3% Triton X-100, 8 mol % PS, 2 mol % diolein, 200 μM $CaCl_2$, and varying concentrations of cardiotoxin (CTX), mastoparan (MP), ET-18-OCH_3 (ET), tamoxifen (TX), or PKC inhibitor peptide (IP), as indicated. The control activity in the absence of agents (32 pmol/min) was taken as 100%. The unfractionated cardiotoxin used in (A), (B), and (C) was from Formosan cobra, whereas that in (D) was from Thailand cobra; these two preparations had the same inhibitory activity in all parameters studied. In all experiments, the mean values of triplicate assays were presented; standard errors of the means were <±5% (not shown). The findings were confirmed in two or three other experiments.

by the toxins, with IC_{50} values, in increasing order, of cardiotoxin-1 (1 μM) = cardiotoxin-3 (1 μM) < unfractionated cardiotoxin (2.1 μM) < cardiotoxin-4 (2.5 μM) (Figure 3B). Neurotoxin was without effect at 10 μM, the highest concentration tested. Interestingly, similar relative potency of the toxins was also noted when PKC was assayed under the modified conditions where the enzyme was activated by arachidonate/Ca^{2+} (Figure 3C). Because the relative potency of cardiotoxin isoforms in inhibiting phorbol ester binding was similar to that for their inhibition of PKC activity shown above, it is suggested that they acted by inhibiting the enzyme activation process.

The mixed micelle system has been developed and used by Hannun et al. (1985) to study stoichiometry and specificity of PKC activation by lipids. Here, we employed this physically defined system (as opposed to PS vesicles in the standard assay) to investigate PKC inhibition by cardiotoxin and other agents. Under the mixed micelle system (containing 0.3% Triton X-100, 8 mol % PS, 2 mol % diolein, and 200 μM $CaCl_2$), we found that cardiotoxin from Thailand cobra (Figure 3D) or Formosan cobra (data not shown) was far more active than other PKC inhibitors. Their IC_{50} values, in increasing order, were cardiotoxin (0.1 mol % or 4.2 μM) < mastoparan (1 mol % or 42 μM) < PKC inhibitor peptide (2 mol % or 84 μM) < tamoxifen (8 mol % or 336 μM) < ET-18-OCH_3 (13

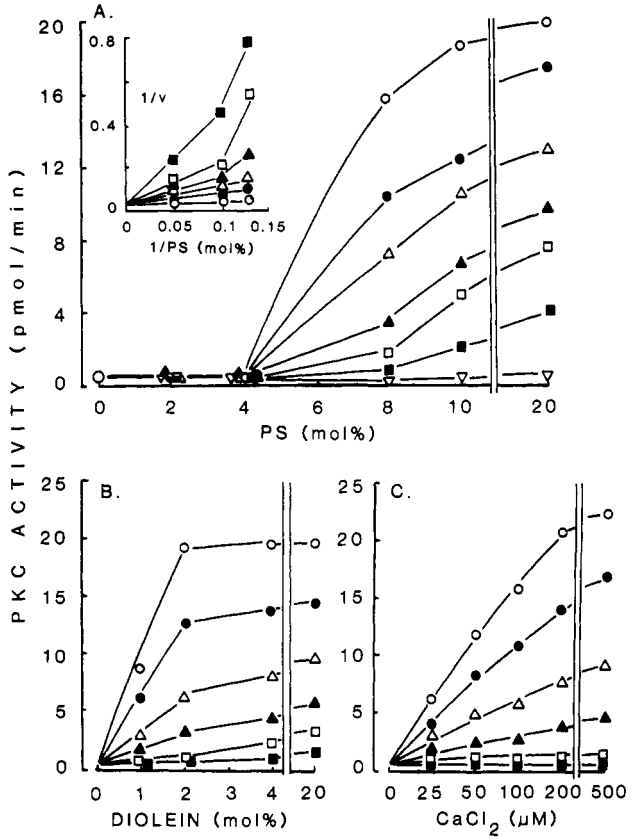

FIGURE 4: Kinetics of PKC inhibition by cardiotoxin (unfractionated, from Thailand cobra). The enzyme was assayed, with varying concentrations of cardiotoxin, using the mixed micelle system in the presence of (A) 2 mol % diolein, 200 μM $CaCl_2$, and varying concentrations (mol %) of PS, (B) 8 mol % PS, 200 μM $CaCl_2$, and varying concentrations (mol %) of diolein, and (C) 8 mol % PS, 2 mol % diolen, and varying concentrations (μM) of $CaCl_2$, as indicated. Symbols used for cardiotoxin concentrations (mol %) were ○ (0), ● (0.05), △ (0.1), ▲ (0.15), □ (0.2), ■ (0.3), and ▽ (1.0). The double-reciprocal plots of the data from (A) are also shown (inset). The data presented in all cases were the mean values of triplicate assays; standard errors of the means were <±6% (not shown). The findings were confirmed in another set of experiments.

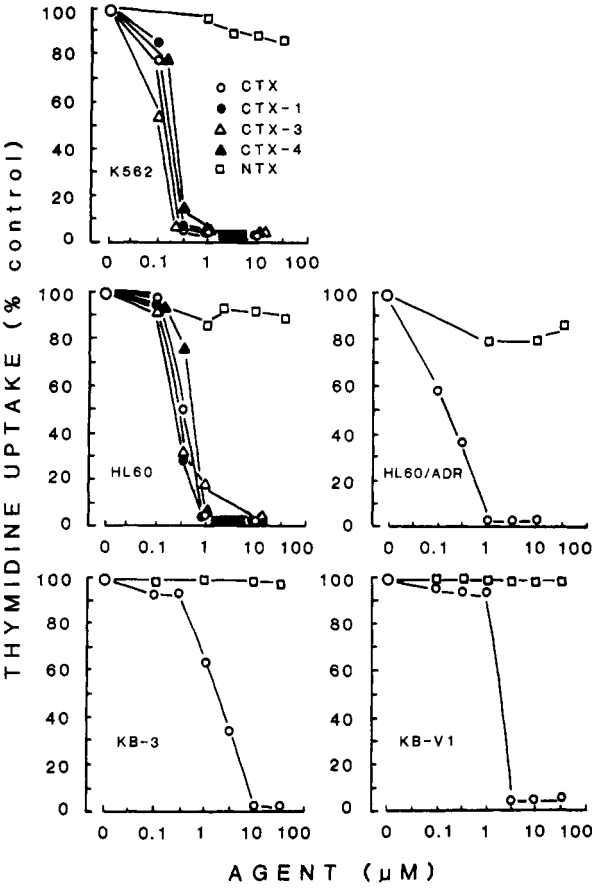

FIGURE 5: Effects of cardiotoxin (CTX, unfractionated), cardiotoxin isoforms, and neurotoxin (NTX), all from Formosan cobra, on proliferation of K562, HL60, HL60/ADR, KB-3, and KB-V1 cells. The cells (10^5 mL^{-1} $well^{-1}$) were incubated for 48 h with various concentrations of toxins as indicated. Incorporation of [^{3}H]thymidine was carried out for 1 h at the final hour of the incubation period.

mol % or 546 μM). These values, while underscoring the specificity and stoichiometry of interactions between PKC/PS/diolein/Ca^{2+} complex and its inhibitors in the mixed micelle assay system, were quite different from the IC_{50} values reported for the same agents on inhibition of PKC assayed using the standard PS vesicle system, i.e., cardiotoxin, 1 μM (Kuo et al., 1983; Raynor et al., 1991); mastoparan, 8 μM (Raynor et al., 1991); PKC inhibitor peptide, 8 μM (this study); tamoxifen, 28 μM (Su et al., 1985); and ET-18-OCH_3, 9 μM (Helfman et al., 1983; Raynor et al., 1991).

Inhibition of PKC by varying cardiotoxin concentrations, assayed using the mixed micelle system, as a function of PS (Figure 4A), diolein (Figure 4B), or $CaCl_2$ (Figure 4C) was examined. Analysis of the kinetic data from Figure 4 and similar experiments by double-reciprocal plots (such as shown in Figure 4A) indicated that cardiotoxin inhibited the enzyme competitively with respect to PS (average apparent K_i of about 0.06 mol % or 2.5 μM), and in a mixed-type manner with respect to both diolein (average apparent K_i of about 0.04 mol % or 1.7 μM) and $CaCl_2$ (average apparent K_i of 2.9 μM). The apparent K_i value of cardiotoxin was similar to the IC_{50} value (0.1 mol % or 4.2 μM) obtained in Figure 3D. The kinetic findings thus suggested that interaction of PS and cardiotoxin with PKC was mutually exclusive and that interactions of diolein or Ca^{2+} with the PKC/PS complex did not exclude interaction of cardiotoxin but the affinity of the activators for the complex was affected by the toxin. The types of PKC inhibition by cardiotoxin with respect to PS, diolein, and $CaCl_2$ in the present study using the mixed micelle system were the same as we reported recently for inhibition by mastoparan (an active factor, tetradecapeptide, from wasp venom) when the enzyme was assayed under the standard PS vesicle system (Raynor et al., 1991). Additional experiments concerning cardiotoxin inhibition with respect to PKC itself were also performed using the mixed micelle system. It was found that the inhibition was not overcome by increasing or even saturating concentrations of the enzyme and that the double-reciprocal plots of the data indicated a complex, undefined inhibition kinetics (data not shown). The findings, taken together, suggested that cardiotoxin might not bind directly to PKC but rather interact with a site (or sites) on PS also shared by PKC.

Studies of effects of cobra toxins were extended to include their cytotoxicity on several cancer cell lines (Figure 5). It appeared that the cardiotoxin isoforms and neurotoxin inhibited [^{3}H]thymidine uptake (cell proliferation) of K562 and HL60 cells with a relative potency similar to that observed for inhibition of PKC activity (Figure 3A,C) or phorbol ester binding to PKC (Figure 3B), i.e., cardiotoxin isoforms 1 and 3 were more active than isoform 4, whereas neurotoxin was practically inactive. It appeared that leukemia cell lines (K562, HL60, and HL/ADR) were more sensitive to cardiotoxin cytotoxicity than epithelial carcinoma cell lines (KB-3 and KB-V1), as indicated by their IC_{50} values of about 0.2

Table II: Comparative Effects of Cardiotoxin (from Formosan or Thailand Cobra) and Neurotoxin on TPA-Induced HL60 Cell Differentiation[a]

treatment	cell attachment (% of total cells)	viability (%) attached cells	viability (%) unattached cells
none (control)	<2		93
TPA (10 nM)	45 ± 2	90	85
cardiotoxin (0.03 μM)	<3		92
cardiotoxin (0.1 μM)	<3		84
cardiotoxin (0.3 μM)	<3		28
TPA (10 nM) + cardiotoxin (0.03 μM)	26 ± 2[b]	86	85
TPA (10 nM) + cardiotoxin (0.1 μM)	17 ± 2[b]	92	77
TPA (10 nM) + cardiotoxin (0.3 μM)	3 ● 1[b]	98	11
neurotoxin (30 μM)	<2		90
TPA (10 nM) + neurotoxin (30 μM)	23 ± 3[b]	94	91

[a] Cells were incubated with toxins for 3 h, followed by incubation with TPA for an additional 21 h. The differentiating cells were indicated by attachment to culture dishes. [b] Significantly different from TPA alone ($p < 0.05$).

and 2 μM, respectively (Figure 5). It might be noted that drug-resistant variants HL60/ADR (Bhalla et al., 1985) and KB-V1 (Shen et al., 1986) were not more resistant to cardiotoxin cytotoxicity than their respective drug-sensitive parental HL60 and KB-3 cells, suggesting that the variants did not exhibit an increased ability to export the toxin out of cells or to reduce binding of the toxin to membranes.

Finally, we examined effects of cardiotoxin (PKC inhibitor) on a cellular response linked to PKC activation, i.e., TPA-induced HL60 cell differentiation. While without effect when present alone, cardiotoxin (0.03, 0.1, and 0.3 μM) potently inhibited TPA-induced cell attachment without affecting cell viability at 0.03 and 0.1 μM (Table II). It is unclear whether cardiotoxin also acted by blocking TPA-induced translocation and down-regulation of PKC, as did another PKC inhibitor ET-18-OCH_3 (Shoji et al., 1988). Neurotoxin, on the other hand, inhibited differentiation only at a very high concentration of 30 μM.

DISCUSSION

Hydrophobicity has been considered a minimal, essential characteristic of substances (ranging from simple organic compounds to complex proteins such as cobra toxins) capable of activating or inhibiting PKC. These substances presumably act by binding to the hydrophobic, regulatory domain of the enzyme and hence modulating its activation by phospholipid membranes, Ca^{2+}, and/or diacylglycerol (or TPA). In the present study, we observed a close correlation between hydrophobicities (Table I) of cardiotoxin isoforms and neurotoxin and their abilities to inhibit PKC activity or phorbol ester binding to PKC (Figure 3). In the intact cells, a positive relationship was also noted for cardiotoxin (a mixture of isoforms, Hϕ estimated to be 0.11) and neurotoxin between their hydrophobicities and abilities to inhibit cell proliferation (Figure 5) and TPA-induced cell differentiation (Table II), i.e., cardiotoxin ≫ neurotoxin. The net positive charges of cardiotoxin isoforms, although contributory toward overall hydropathy of the toxin molecules, did not appear to be crucial because, for example, cardiotoxin-4 (net charge of +9) was less active than cardiotoxin-1 (net charge of +7) (Table I). In previous studies such a positive correlation, however, was not observed between the hydrophobicities of mastoparan analogues and their abilities to stimulate GTPase of G proteins (Higashijima et al., 1990) or to inhibit PKC (Raynor et al., 1992). One possible reason might be that mastoparan, a small tetradecapeptide (M_r of about 1500) from wasp venom, compared to cobra cardiotoxin, having 60 amino acids (M_r of about 6700), lacks certain higher-order structures essential for well-defined functional interactions with the hydrophobic, regulatory domain of PKC.

EC_{50} of diolein for PKC activation in the mixed micelle system was about 1 mol % (Figure 4B), consistent with the data of Hannun et al. (1985) that about 1 molecule of diolein in each micelle is sufficient to activate PKC. Accordingly, IC_{50} of 0.1 mol % for cardiotoxin (Figure 3D) might be translated that $^1/_{10}$ of the toxin molecule in each micelle, or 1 molecule for every 10 micelles, was able to inhibit PKC activated by diolein. IC_{50} values for mastoparn and PKC inhibitor peptide were 1 and 1.5 mol %, respectively (Figure 3D), implying that about 1 molecule of either peptide per micelle was sufficient to counteract the stimulatory effect of diolein. The apparent high efficacy of cardiotoxin might be due to its large size and surface (potentially containing several functional hydrophobic loci), and hence it could function as multiple inhibitor molecules. Other PKC inhibitors tamoxifen and ET-18-OCH_3 had IC_{50} values of 7.5 and 13 mol %, respectively (Figure 3D); they were rather inefficacious stoichiometrically, perhaps reflecting their nonspecific interactions with PS in the micelle. The IC_{50} values for the inhibitors in the mixed micelle system, mentioned above, were quite different from those obtained using the standard PS vesicle system (see Results section for the values). In both systems, however, cardiotoxin was the most potent inhibitor. It is a matter of conjecture as to which assay system could yield information that is more physiologically relevant. Although the PS vesicle system resembles the phospholipid bilayer in biomembranes, the mixed micelle system can provide insights into stoichiometry and specificity of interactions between PKC and its regulators. One potential disadvantage of the mixed micelle system is that it requires large amounts (or high concentrations) of PKC regulators. For example, EC_{50} of 1 mol % of diolein (Figure 4B) equals 42 μM, compared to that of 1 μM in the PS vesicle system (Raynor et al., 1991); IC_{50} of 13 mol % for ET-18-OCH_3 (Figure 3D) equals 546 μM, compared to that of 9 μM in the PS vesicle system (Raynor et al., 1991). Accordingly, the requirement of high concentrations of the regulators might preclude application of the mixed micelle system for certain agents because of their insolubility or scarcity. Because of limited supplies of the isoforms, a cardiotoxin preparation (a mixture of isoforms) was used in kinetic studies in the mixed micelle system (Figure 4). It is plausible that a more pronounced difference in inhibitory potency of the isoforms might be revealed in the mixed micelle than in the PS vesicle system shown in Figure 3.

Because of its amphiphilicity, actions of cardiotoxin could be attributed to its potential generalized, nonspecific interactions with membranes. This possibility might be largely eliminated by following three lines of evidence: (a) cardiotoxin potently inhibited PKC activity assayed using synaptosomal membrane as phospholipid cofactor (IC_{50} of 1 μM) without affecting Na,K-ATPase activity in the same membrane preparation (Raynor et al., 1991), (b) its isoforms inhibited proliferation of various cancer cell lines with an order of potency (Figure 5) similar to their inhibition of PKC and phorbol ester binding (Figure 3), and (c) it inhibited proliferation (Figure 5) and TPA-induced differentiation

(Table II) of HL60 cells, IC_{50} being about 0.1 μM, without causing cell lysis. It might be noted that the IC_{50} of cardiotoxin for the above cellular effects was lower than for PKC inhibition in vitro. It is unclear at present whether cardiotoxin is highly membrane-bound or even permeable to cells and/or exerts its cellular effects, in part, through PKC inhibition. It is entirely possible that cardiotoxin exerts its cytotoxicity (hence also called cytotoxin) and other biological effects via additional mechanisms that are independent of PKC. Other molecular mechanisms of cardiotoxin actions, in addition to PKC inhibition, however, are yet to be discovered.

ACKNOWLEDGMENT

We thank Stephanie Sanders for expert preparation of the manscript.

REFERENCES

Bhalla, K., Hindenberg, A., Taub, R. N., & Grant, S. (1985) *Cancer Res. 45*, 3657–3662.

Changeux, J. P. (1981) *Harvey Lect. 75*, 85–254.

Chiou, S.-H., Lin, W.-W., & Chong, W.-P. (1989) *Int. J. Pept. Protein Res. 34*, 148–1520.

Chiou, S.-H., Lu, B.-S., & Yu, N.-T. (1992) *Biochem. Int. 26*, 747–758.

Girard, P. R., Mazzei, G. J., & Kuo, J. F. (1986) *J. Biol. Chem. 261*, 370–375.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039–10043.

Hayashi, K., Takechi, M., Kaneda, N., & Lee, C. Y. (1975) *Biochem. Biophys. Res. Commun. 64*, 360–366.

Hayashi, K., Takechi, M., Kaneda, N., & Sasaki, T. (1976) *FEBS Lett. 66*, 210–214.

Helfman, D. M., Barnes, K. C., Kinkade, J. M., Jr., Vogler, W. R., Shoji, M., & Kuo, J. F. (1985) *Cancer Res. 43*, 2955–2961.

Higashijima, T., Burnier, J., & Ross, E. M. (1990) *J. Biol. Chem. 265*, 14176–14186.

Huang, K.-P., Nakabayashi, H., & Huang, F. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8535–8539.

Kaneda, N., Sasaki, T., & Hayashi, K. (1976a) *Biochem. Biophys. Res. Commun. 72*, 1450–1455.

Kaneda, N., Sasaki, T., & Hayashi, K. (1976b) *FEBS Lett. 70*, 217–222.

Karlsson, E. (1979) Snake Venoms, in *Handbook of Experimental Pharmacology* (Lee, C. Y., Ed.) Vol. 52, pp 159–212, Springer, Berlin.

Katoh, N., Raynor, R. L., Wise, B. C., Schatzman, R. C., Turner, R. S., Helfman, D. M., Fain, J. N., & Kuo, J. F. (1982) *Biochem. J. 202*, 217–224.

Kuo, J. F., Kem, W. R., Raynor, R. L., Mazzei, G. J., Schatzman, R. C., & Turner, R. S. (1983) *FEBS Lett. 153*, 183–186.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lee, C. Y. (1979) in *Advances in Cytopharmacology* (Ceccarelli, B., & Clementi, F., Eds.), Vol. 3, pp 1–16, Raven Press, New York.

Mazzei, G. J., Katoh, N., & Kuo, J. F. (1982) *Biochem. Biophys. Res. Commun. 109*, 1129–1133.

Raynor, R. L., Zheng, B., & Kuo, J. F. (1991) *J. Biol. Chem. 266*, 2753–2758.

Raynor, R. L., Kim, Y. S., Zheng, B., Vogler, W. R., & Kuo, J. F. (1992) *FEBS Lett. 307*, 275–279.

Shen, D., Cardarelli, C., Hwang, J., Cornwell, M. M., Richert, N., Ishii, S., Pastan, I., & Gottesman, M. M. (1986) *J. Biol. Chem. 261*, 7762–7770.

Shoji, M., Raynor, R. L., Berdel, W. E., Vogler, W. R., & Kuo, J. F. (1988) *Cancer Res. 48*, 6669–6673.

Su, H.-D., Mazzei, G. J., Vogler, W. R., & Kuo, J. F. (1985) *Biochem. Pharmacol. 34*, 3649–3653.

Yang, C.-C., Yang, H.-J., & Huang, J.-S. (1969) *Biochim. Biophys. Acta 188*, 65–77.

Zheng, B., Woo, C. F., & Kuo, J. F. (1991) *J. Biol. Chem. 266*, 10031–10034.

Zhou, Q., Raynor, R. L., Wood, M. G., Jr., Menger, F. M., & Kuo, J. F. (1988) *Biochemistry 27*, 7361–7365.