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Factors in snake venoms that increase capillary permeability

ROGER A. MILLER, ANTHONY T. TU, *Department of Biochemistry, Colorado State University, Fort Collins, CO 80523, USA*

Abstract—Capillary permeability increasing (CPI) activity is a phenomenon of the microvasculature caused by many agents such as snake venoms, histamine, 5-hydroxytryptamine (5-HT), prostaglandins and leukotrienes. Since no systematic study has been done to determine what components of snake venom cause CPI activity, a CPI factor from *Naja naja atra* (Taiwan cobra) venom was isolated using intravenous injections of Evan's blue dye as the indicator of increased permeability and the factor's properties were extensively studied. Cardiotoxin from *Naja naja kaouthia* (Thailand cobra) and Mojave toxin from *Crotalus scutulatus scutulatus* (Mojave rattlesnake) venoms demonstrated CPI activity. Postsynaptic neurotoxins from an elapid and a hydrophid and myotoxin *a* from *Crotalus viridis viridis* (prairie rattlesnake) showed no CPI activity at the dose studied. The purified CPI active component from *Naja naja atra* venom was found to have cardiotoxic activity. Therefore, Elapidae cardiotoxins are CPI active factors. However, CPI activity is not due to cardiotoxins alone as the presynaptic neurotoxin, Mojave toxin, also showed CPI activity. Selective inhibitors were used to indicate possible mechanisms of action on the capillaries by *Naja naja atra* venom and *Crotalus scutulatus scutulatus* venom. The histamine H_1-receptor blockers diphenhydramine, promethazine, and cyproheptadine were effective against both venoms in preventing increased capillary permeability. These results suggested that histamine release activity is the most likely mechanism resulting in CPI activity from these venoms.

Capillary permeability increasing (CPI) activity has been observed with many different vasoactive compounds such as bradykinin (Elliott et al 1960), histamine, 5-hydroxytryptamine (5-HT) (Spector & Willoughby 1964), prostaglandins (Feigen 1981), and snake venoms (Ohtani & Takahashi 1988).

CPI activity is normally tested using albumin bound with synthetic dyes such as Evan's blue. In normal conditions, the albumin-dye complex does not permeate through the capillary membrane. However, when the capillary is challenged by some agents such as snake venoms, its permeability increases and the plasma proteins are suddenly allowed to penetrate the membrane.

Little study has been done on CPI-components from snake venoms. The main question is whether the CPI-causing component is identical in all snake venoms or whether there are several totally different components responsible for the same phenomenon.

Materials and methods

Venoms and toxins. Naja naja atra venom was collected in Taiwan. Mojave toxin was isolated according to Bieber et al (1975) and Cäte & Bieber (1978). *Crotalus scutulatus scutulatus* venom was purchased from Miami Serpentarium. Cardiotoxin from *Naja naja kaouthia* venom, neurotoxin I and neurotoxin II from *Naja naja oxiana* venom were purchased from Sigma. Purified *Lapemis hardwickii* neurotoxin was isolated by the method of Tu & Hong (1971) and myotoxin *a* from *Crotalus viridis viridis* venom, was isolated using the method of Cameron & Tu (1977).

Capillary permeability increasing assay. The Evan's blue dye assay method is based on the method of Miles & Wilhelm (1955).

Correspondence to: A. T. Tu, Department of Biochemistry, Colorado State University, Fort Collins, CO 80523, USA.

White Swiss Webster mice (18–25 g) were injected intravenously (i.v.) with a solution of 1·0 (% w/v) Evan's blue dye in phosphate buffered saline (PBS, pH 7·4). The 1·0 μg dose of the test fractions was injected intradermally in the mid-dorsal skin. After 15 min the mice were killed and the skins removed. A blue spot where the dye-complexed albumin had penetrated beyond the capillaries indicated increased capillary permeability. Two cross-sectional diameters were measured to the nearest mm for each spot and the area estimated by using the formula for determining the area of an ellipse ($A = \pi ab$).

For the CPI inhibitor study, the inhibitors were mixed with the *Naja naja atra* venom or *Crotalus scutulatus scutulatus* venom to give a concentration of 333 μg for the inhibitor and 20 μg mL^{-1} for the venom before intradermal injection. The length of time from injection to killing was 1 h.

Fractionation of a Naja naja atra *CPI Factor.* The absorbance at 280·0 nm was determined for each test-tube fraction after each step and plotted. After each step, fractions were checked for purity using sodium dodecylsulphate polyacrylamide gel electrophoresis. Spectropor dialysis tubing with a molecular weight cut off of 1000 or 2000 was used for dialysis.

Step one: Naja naja atra venom (3·0 g) was applied to a column of Sephadex G50-50 (2·5 cm × 82 cm) pre-equilibrated with 0·01 M sodium tetraborate, pH 9·0, elution buffer. Fractions of 5·0 mL were collected. Each peak fraction was tested for CPI activity. The peak fractions with the greatest CPI activity were pooled, dialysed, and lyophilized.

Step two: The CPI active peak fraction from step one was applied to a column of carboxymethylcellulose (2·5 cm × 35 cm) pre-equilibrated with phosphate buffered saline (PBS) consisting of 0·145 M NaCl and 0·005 M sodium phosphate, pH 7·4. Fractions of 4·0 mL were collected. A 500 mL linear salt gradient from 0·145 M NaCl to 2·0 M NaCl was applied at fraction 55. The peak fractions were pooled, dialysed, lyophilized and assayed for CPI activity.

Step three: Sephadex G50-50 (superfine) (2·5 cm × 82 cm) column chromatography was the final step in the isolation of the CPI factor. The active peak from step two was applied to the pre-equilibrated column and eluted with PBS, pH 7·4. Fractions of 5·0 mL were collected. The single peak fraction was pooled, dialysed, lyophilized, assayed for CPI activity and purity checked using SDS-PAGE.

Biochemical characterization. The molecular weight was determined using 2-mercaptoethanol and SDS-PAGE. Isoelectric focusing slab gel was used to estimate the pI of the *Naja naja atra* CPI factor. The amino acid composition was determined for the *Naja naja atra* CPI factor using high performance liquid chromatography (HPLC) (Beckman model 334T) with a C_{18} reverse-phase column (4·6 mm × 25 cm) based on the method of Heinrikson & Meredith (1984). The phospholipase A_2 activity of the *Naja naja atra* CPI factor was checked using the titration method of Wells & Hanahan (1969). The hyaluronidase activity of the *Naja naja atra* CPI factor was checked using the turbidimetric method of DiFerrante (1956). The 50% lethal dosage (LD50) (i.v. mice) for the *Naja naja atra* CPI factor was estimated using six doses and five mice for each dose and determined from the curve at 50% survival (Litchfield & Wilcoxon 1949). The cardiotoxicity of the *Naja naja atra* CPI

factor was determined using the method of Walker (1977). Rat isolated hearts were perfused with Kreb-Henseleit solution with injections of test samples of 0·1 $\mu g\,mL^{-1}$, 1·0 $\mu g\,mL^{-1}$, and 10 $\mu g\,mL^{-1}$ and the reactions of ventricular pressure, heart beats and time were monitored.

Results and discussion

Isolation of the CPI factor from the venom of the Taiwan cobra is shown in Fig. 1. Each fraction was assayed for CPI activity. Only two fractions showed CPI activity in addition to the most active fraction, (peak III; shaded fraction Fig. 1A) In the second step, peak III (515 mg) was applied to a column of carboxymethyl cellulose which gave the chromatographic pattern shown in Fig. 1B. Two fractions, IIIA and IIIB were obtained; the IIIB fraction had CPI activity (0·85 cm^2), while the IIIA fraction had none. Fraction IIIB (108 mg) was then applied to the third and final step of the Sephadex G50-50 column and gave the chromatographic pattern shown in Fig. 1C. The single fraction (44–51) IIIB1 was CPI active (0·94 cm^2) and showed one band of approximately 7000 mol. wt on SDS-PAGE gels. CPI activity of the purified CPI factor from *Naja naja atra* venom was compared with that of *Naja naja kaouthia* cardiotoxin, Mojave presynaptic neurotoxin (Mojave toxin), Lapemis postsynaptic neurotoxin, *Naja naja oxiana* short chain (neurotoxin II) and long chain (neurotoxin I) postsynaptic neurotoxins and myotoxin *a*. Phosphate buffered saline solution was used as a negative control. Those that resulted in positive CPI activity were the purified CPI factor from *Naja naja atra* venom (0·94 cm^2), *Naja naja kaouthia* cardiotoxin (0·95 cm^2) and Mojave toxin (3·46 cm^2). All of the postsynaptic neurotoxins and the myotoxin *a* had no CPI activity at the dose tested.

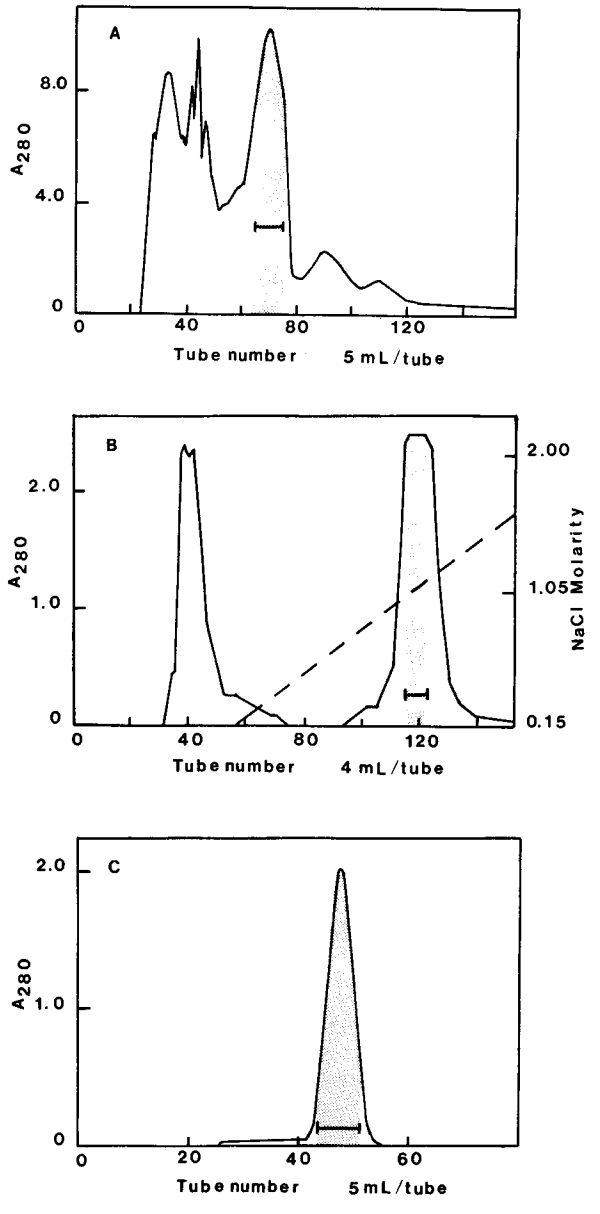

FIG. 1. Isolation of *Naja naja atra* capillary permeability increasing (CPI) Factor. For details see the material and methods section. (A) Step one: Sephadex G50-50 column chromatography (B) Step two: CM-cellulose ion-exchange chromatography (C) Step three: Sephadex G50-50 column chromatography.

In order to speculate on the mechanism of CPI activity, several inhibitors were used at the selected doses to look for possible inhibition of CPI activity of the *Naja naja atra* and *Crotalus scutulatus scutulatus* venoms. The histamine H_1-receptor blocker diphenhydramine and the histamine H_1-receptor blockers with anti-5-HT activity (promethazine, cyproheptadine) totally inhibited both venoms. Heparin sulphate inhibited only the cobra venom. Hydrocortisone, an inhibitor of phospholipase A_2, did not inhibit the CPI activity of *Naja naja atra* venom but did partially inhibit the *Crotalus scutulatus scutulatus* venom from a control area of 1·53 cm^2 to an inhibited area of 0·79 cm^2. Acetylsalicylic acid, cimetidine and aprotinin had no inhibitory effects on the two venoms.

Biochemical properties. The Molecular weight of *Naja naja atra* CPI factor was approximately 7000 by SDS-PAGE and 7464 as calculated from amino acid composition. Both the CPI factor and cardiotoxin had the same mobility indicating they have the same apparent molecular weight. The amino acid analysis of the *Naja naja atra* CPI factor gave the results shown in Table 1. Note that there are no Glx (glutamine and glutamic acid) or His (histidine) residues, which is also the case for the cardiotoxins of *Naja naja atra* (Narita & Lee 1970) and *Naja naja kaouthia* (Joubert & Taljaard 1980). The Ile (isoleucine) residue was used as the minimum value residue and this gave the total residues per molecule of 65 for the CPI factor. The LD50 for the *Naja naja atra* CPI factor was determined to be approximately 1·1 $mg\,kg^{-1}$ body weight (i.v., mice) which is similar to that for cardiotoxins. The *Naja naja atra* CPI factor and the *Naja naja kaouthia* cardiotoxin both formed a single band at the cathode edge of the isoelectric focusing slab gel indicating a pI greater than pH 9·5. The *Naja naja atra* CPI factor was cardiotoxic to a rat isolated heart by stopping it in a contracted state in 7–9 min when it was perfused with a solution of 1·0 $\mu g\,mL^{-1}$ of the factor. The heart stopped in 3 min when perfused with a solution of 10 $\mu g\,mL^{-1}$ of the factor. The *Naja naja atra* CPI factor had no phospholipase A_2 activity, nor hyaluronidase activity.

The present investigation indicates that different toxins (Mojave toxin, cobra cardiotoxin, and *Naja naja atra* CPI factor) show capillary-permeability-increasing activity. This clearly indicated that the CPI activity is expressed by several venom compounds which are not identical. As to the mechanism of CPI activity, the conclusion that may be drawn is that both *Naja naja atra* venom and *Crotalus scutulatus scutulatus* venom seem to cause histamine release, as supported by the fact that the histamine H_1-receptor blockers inhibited the increased capillary permeability of these venoms. Usually, a cobra venom contains several cardiotoxins (isotoxins) (Narita & Lee 1970; Joubert & Taljaard 1980; Kaneda & Hayashi 1983) so it may be the CPI factor we isolated is a cardiotoxin.

Table 1. Amino acid composition of the capillary permeability increasing factor from *Naja naja atra* venom.

Amino acid	24 h	48 h	72 h	Corrected value	Normalized (Ile)	Residues/ molecule	Minimum mol. wt.
Asx	1·70	1·84	1·55	1·70	5·48	5	630·21
Glx	0·00	0·00	0·00	0·00	0·00	0	0·00
Ser	0·62	0·67	0·64	0·64	2·08	2	181·07
Gly	0·57	0·66	0·68	0·64	2·06	2	117·48
His	0·00	0·00	0·00	0·00	0·00	0	0·00
Thr	0·86	0·72	0·76	0·78	2·51	3	254·15
Ala	0·69	0·56	0·59	0·61	1·98	2	140·93
Pro	1·75	1·45	1·00	1·40	4·53	5	440·01
Arg	0·86	0·64	0·70	0·73	2·36	2	368·49
Tyr	1·02	0·94	1·01	0·99	3·19	3	520·71
Val	2·48	1·99	2·15	2·21	7·13	7	706·69
Met	0·57	0·44	0·57	0·53	1·70	2	222·92
1/2 Cys	2·96	2·92	2·50	2·79	9·02	9	921·64
Ile	0·40	0·22	0·31	0·31	1·00	1	113·18
Leu	2·12	1·85	1·98	1·99	6·41	6	725·52
Phe	0·78	0·66	0·71	0·72	2·32	2	340·90
Lys	4·36	4·03	4·51	4·30	13·89	14	1780·42
Trp*							
Total residues/molecule						65	
Molecular weight							7464·29

* Trp not determined.

The present investigation also established that the CPI assay can be used successfully as a direct isolation assay for CPI factors. This assay ability can facilitate the study of purified toxins and the effects of CPI agents on the microvasculature.

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