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EFFECTS OF Ca^{2+} AND MEMBRANE SURFACE CHARGE ON THE DIRECT LYTIC ACTIVITY OF COBRA CARDIOTOXIN - A Membrane Spin Assay

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SUMMARY

Direct membrane lytic activity of cobra (Naja Naja atra) cardiotoxin was studied by measuring the release of trapped spin label (2,2,6,6-tetramethyl-piperidinyl-oxyl)-choline chloride from liposomes. Results suggest that (a) presence of membrane negative charge and Ca^{2+} are required for optimal direct lytic activity of cardiotoxin, (b) direct Ca^{2+} binding to cardiotoxin may affect the equilibriu between its membrane active and inactive form.

In addition to neurotoxin, cardiotoxin is the second membrane active constituents of cobra venom (1). It is known that neurotoxins block the neuromuscular transmission by curare-like action, but mechanism of action of CTX* still remains to be elucidated. CTX from cobra is composed of 60 residues in a single polypeptide chain cross-linked by four disulfide bridges (1,2). CTX affects various kinds of cells both excitable and non-excitable, causing depolarization of the membrane and consequently impairing both the function and structure of the cell (3,4,5). It has been reported that relatively high Ca^{2+} concentration (10 mM) antagonized a number of pharmacological effects of CTX (6,7,8). Moreover direct measurements with labeled CTX demonstrate Ca^{2+} in high concentrations interferes with binding of the toxin to anxons (9) and to skeletal muscle (8) which support the view that CTX acts primarily on membrane Ca^{2+} binding site.

The unique basic nature of CTX has been postulated to function as direct lytic factor by being able to penetrate the lipid bilayer and combine with acidic phospholipids thus causing membrane lysis (10,11).

* CTX = Cardiotoxin.

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A membrane spin assay (12) has been used in the present study to evaluate this hypothesis.

In the present communication we provide evidence to show that (a) negatively charged amphiphiles promote, but are not required for the lytic activity of CTX and (b) Ca^{2+} is required for optimal lytic activity of CTX.

MATERIALS AND METHODS

Cardiotoxin was isolated from the venom of Naja naja atra (4), the trace of phospholipase A_2 present in the CTX fraction was removed by repeated rechromatography on CM-Cellulose column, using gradients of ammonium acetate buffer (0.15 - 0.9M). (2,2,6,6-tetramethyl-piperidinyl-oxyl)-choline chloride was prepared as described (13). Egg phosphatidyl choline, sphingomyelin, dicetylphosphate and cholesterol were purchased from Sigma Chem. Co., St. Louis, Mo. and stearylamine was obtained from Grand Island Biological Co., Grand Island, N.Y. Other chemicals and solvents used were reagent grade. Egg phosphatidyl choline was purified by preparative thin layer chromatography before use.

Preparation of liposomes and direct lytic studies : 50 µl of the prepared stock solution (which consists of 20 mM egg phosphatidyl choline, 15 mM cholesterol with or without 2.2 mM dicetylphosphate or stearylamine) is placed into a small test tube and the solvent is removed under reduced pressure and finally dried in dessicator under vacuum for 1 hour. The liposomes were formed by swelling the dried lipids with 50 µl of 0.10 M TEMPO-choline chloride** in 0.15 M NaCl using a vortex mixer and few glass beads. The resulting milky liposomes were transferred into dialyzing bags and dialyzed against three changes of 0.15 M NaCl (1 litre) to remove the untrapped TEMPO-choline chloride. The liposomes were diluted 20 times prior to each assay.

The stock solutions of CTX and Ca^{2+} were also prepared in 0.15 M NaCl.

Appropriate amount of stock solutions of CTX and Ca^{2+} were mixed in a small culture tube which had been coated with sphingomyelin§ and then made up to a volume of 25 µl with 0.15 M NaCl. 25 µl of the already diluted (20x) liposomes was then added into each tube for a total volume of 50 µl (final CTX concentration: 16 µM; Ca^{2+} concentration: 0, 0.5 mM, 4.0 mM and 20 mM). Each mixture was immediately mixed with a vortex mixer and transferred into 25 µl sampling pipet (coated with sphingomyelin) and monitor the high-field peak of the released TEMPO-choline chloride spin label as a function of time was recorded on a Varian E-6 X band electron spin resonance (ESR) spectrometer at 20 ± 1°C. Total lysis is induced by rapidly freezing and thawing the mixture in liquid nitrogen.

RESULTS AND DISCUSSION

Figure 1 shows the release of trapped TEMPO-choline

** TEMPO-choline chloride = (2,2,6,6-tetramethyl-piperidinyl-oxyl)-choline chloride

§ Coating the culture tube with sphingomyelin decrease its surface reactivity and stabilizes the liposome from spontaneous lysis.

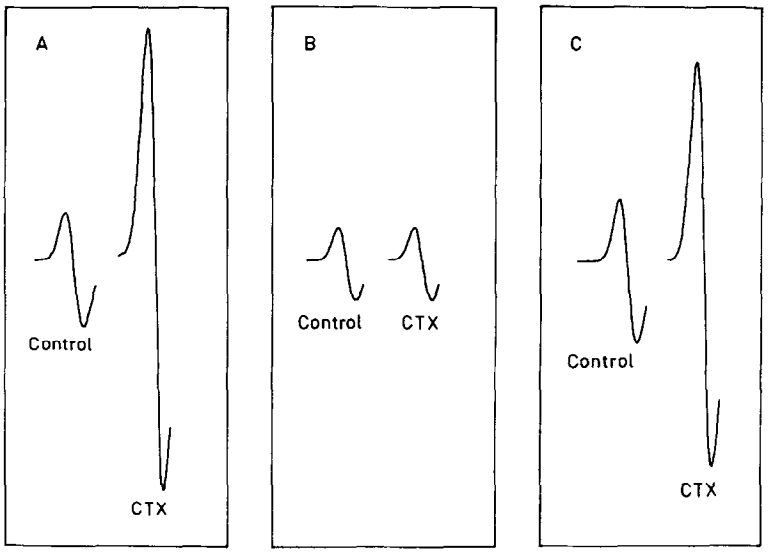

Figure 1. The high-field peak intensity of released TEMPO-choline chloride spin label from (A) Zwitter ionic liposome (egg phosphatidyl choline: cholestero in molar ratio of 2.0:1.5), (B) Positively charged liposome (stearylamine: egg phosphatidyl choline: cholesterol in molar ratio of 0.22:2.0:1.5), (C) Negatively charged liposome (dicetylphosphate: egg phosphatidyl choline: cholesterol in molar ratio of 0.22:2.0:1.5). After 24 hours incubation of liposomes with or without CTX (16 μM) at 20 ± 1°C. The instrument gains used in (A), (B), and (C) are 10 x 10^2; 5 x 10^2 and 6.3 x 10^2 respectively.

chloride from neutral, positively cherged and negatively charged liposomes ‡. As can be seen, both neutral and negatively charged liposomes (Figure 1A and 1C) are susceptible to the direct lytic action of CTX. However, positively charged liposome (Figure 1B and Table I) is completely resistant. These results suggest that although a negative charge at the membrane surface may promote the binding of CTX, it is not required for the lytic activity of CTX as Zwitter ionic liposomes are also effectively lysed by CTX. The resistance of positively charged liposomes to CTX is not an absolute one, as in the presence of Ca^{2+} lytic activity was detected (see Figure 2 and Table I). Therefore, the decreased affinity of CTX to positively charged liposomes may be responsible for results observed in Figure 1B. Concentration dependent Ca^{2+} stimulation and inhibition of CTX activity were shown in Figure 2. As can be seen Ca^{2+} at 0.5 to 4.0 mM range stimulates while at 20 mM inhibits CTX activity towards liposomes irrespective of membrane surface charge. It is possible that direct Ca^{2+} binding

‡ Mutilamellar liposomes used in the present study preclude a more quantitative analysis of the direct lytic activity of CTX. However, in future studies, we hope to use unilamellar vesciles.

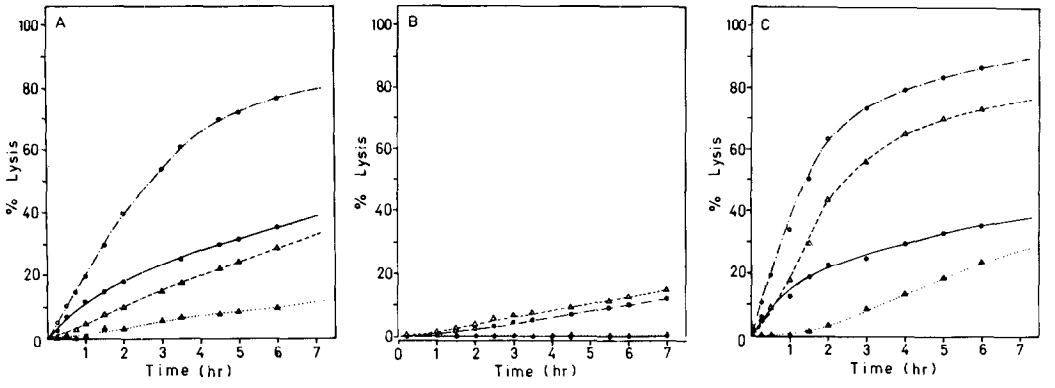

Figure 2. Effect of Ca^{2+} concentration on the rate of TEMPO-choline chloride spin label released from (A) Zwitter ionic liposome (egg phosphatidyl choline: cholesterol in molar ratio of 2.0:1.5), (B) Positively charged liposome (stearylamine: egg phosphatidyl choline: cholesterol in molar ratio of 0.22:2.0:1.5) and (C) Negatively charged liposome (dicetylphosphate: egg phosphatidyl choline: cholesterol in molar ratio of 0.22:2.0:1.5) in 0.15M NaCl at 20 ± 1°C by CTX (16 μM) (—●—●—); CTX (16 μM) + Ca^{2+} (0.5 mM) (-·-○-·-○-·-); CTX (16 μM) + Ca^{2+} (4.0 mM) (--△--△--) and CTX (16 μM) + Ca^{2+} (20.0 mM) (···▲···▲···). Final egg phosphatidyl choline concentration was 500 μM. Percent lysis was obtained by monitoring the normalized time dependence increase in the high-field peak intensity as shown in Figure 1.

Table I

Direct Lytic Activity Of CTX Measured At 24 Hours

Sample	% Lysis *		
	Stearylamine liposome (Positively Charged)	Neutral liposome (Zwitter Ionic)	Dicetylphosphate liposome (Negatively Charged)
CTX (16 μM)	0	90	70
CTX (16 μM) + Ca^{2+} (0.5 mM)	50	100	95
CTX (16 μM) + Ca^{2+} (4.0 mM)	50	85	90
CTX (16 μM) + Ca^{2+} (20.0 mM)	20	60	60

* Percent lysis obtained was normalized against the buffer and Ca^{2+} concentration as indicated.

at low concentrations to CTX convert it to a membrane active form and Ca^{2+} binding at higher concentrations (20 mM) convert to an inactive form presumably through a Ca^{2+} induced conformation change.

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