Snake venom cardiotoxin can rapidly induce actin polymerization in intact platelets

Ruey-Fen LIOU,* Wen-Ching CHANG,† Sin-Tak CHU* and Yee-Hsiung CHEN*†‡

*Institute of Biological Chemistry, Academia Sinica, and †Institute of Biochemical Sciences, College of Sciences, National Taiwan University, Taipei, Taiwan, Republic of China

The action of Taiwan cobra (*Naja naja atra*) venom cardiotoxin on rabbit platelets at 37 °C was characterized by observing cytoskeletal alterations and cell lysis. At a concentration of 21.4 μ M the toxin produced no cell lysis within 30 s, and less than 5 % of the total lactate dehydrogenase activity of intact cells was detected in the suspending medium after the interaction had proceeded for 3 min. The extent of cell lysis was proportional

to toxin concentration and interaction time. Before cell lysis, the toxin caused rapid incorporation of actin monomers into cross-linked actin filaments. The actin incorporation could be inhibited by either the presence of cytochalasin B or increased CaCl_2 concentration in the suspending medium. However, addition of indomethacin did not influence the toxin-induced cytoskeletal change.

INTRODUCTION

Snake venom cardiotoxin (CTX) is a very basic polypeptide of 7 kDa [1]. It is non-neurotoxic, but can cause cardiac arrest, muscle contracture, membrane depolarization and cytolysis [2]. Thus CTX has been described as a cytotoxin, a cytolytic factor, and a membrane-active polypeptide [3–5]. From the results of CTX action on cells of different origins, it has been suggested that the primary target of CTX is on the extracellular surface [6,7]. However, the molecular mechanism of CTX action is unclear. Because the CTX-induced biochemical events in different cells are very diverse, it is hard to construct a general model consistent with the action of CTX at a molecular level. CTX has been demonstrated to induce G-actin polymerization *in vitro* [8]. Whether this kind of activity plays a significant role in disturbing cellular activities remains unclear. In the present paper, we demonstrate that CTX could promote rapidly the formation of cross-linked actin filaments in intact platelets before the occurrence of cell lysis.

EXPERIMENTAL

Materials

Indomethacin, cytochalasin B, NADH, Triton X-100, sodium pyruvate, BSA, Hepes, glucose and citric acid were purchased from Sigma (U.S.A.). The crude venom of Taiwan cobra (*Naja naja atra*) was supplied by Chen Hsin Tong Chemical Co., Taipei. The CTX was then purified on a CM-Sephadex C-25 column as described by Lo et al. [9]. All other chemicals were of reagent grade.

Preparation of platelets

Platelets were prepared from rabbit venous blood by the method of Bevers et al. [10]. Blood from the marginal ear vein of rabbits was mixed with one-ninth volume of the anticoagulant ACD (52 mM citric acid/80 mM trisodium citrate/180 mM glucose). After sedimentation at 400 *g* for 10 min, the platelet-rich plasma

was collected and mixed with an equal volume of ACD. The platelets were pelleted by centrifuging at 400 *g* for 10 min. The pellets were then washed twice in Hepes buffer (137 mM NaCl/2.68 mM KCl/1.7 mM MgCl_2 /25 mM glucose/0.05 % BSA/10 mM Hepes, pH 6.6). The final pellets were resuspended in the same Hepes buffer without BSA at pH 7.5. Cell numbers were determined with a TOIC cell counter (Type C-800, U.S.A.). All the experiments were performed with freshly prepared platelet suspensions [(5–7) $\times 10^8$ cells/ml].

Analysis of actin in platelets

Platelets suspended in Hepes buffer were lysed by addition of an equal volume of a lysis buffer (1 % Triton X-100/10 mM EGTA/100 mM Tris/HCl, pH 7.4). After incubation at 4 °C for 5 min, the mixture was centrifuged at 7600 *g* for 5 min [11]. The 0.5 % Triton X-100-insoluble residues were then solubilized and analysed by SDS/PAGE on a 7.5–15 %-acrylamide linear gradient slab gel by the method of Laemmli [12]. The electrophoresis was conducted at 120 V for 8 h. Polypeptides were detected by staining with Coomassie Blue R-250. The amount of monomeric actin in the pellets was determined by densitometric scans of the protein bands on the gel, using a transmittance scanning densitometer (Hoefer GS 300; U.S.A.).

Assay of lactate dehydrogenase (LDH) activity

This was done by the method of Bergmeyer and Bernt [13].

RESULTS AND DISCUSSION

CTX-Induced platelet lysis

Figure 1(a) displays the kinetics of platelet lysis caused by the action of 0–35 μ M CTX. The appearance of LDH in the suspending medium, expressed in terms of the percentage of total enzyme activity of cells, was proportional to the number of cells lysed. The CTX-induced cell lysis rose greatly with increasing CTX concentration or interaction time, which lasted for several hours.

Figure 1 Kinetics of CTX-induced cell lysis

(a) Platelets were incubated with various concentrations of CTX at 37 °C: $\triangle$, 0; $\blacktriangle$, 1.4 μM ; $\circ$, 4.3 μM ; $\bullet$, 8.6 μM ; $\square$, 17.1 μM ; $\blacksquare$, 34.3 μM . (b) Platelets were treated with 21.4 μM CTX alone ($\circ$) or in the presence of 10.4 μM cytochalasin B ($\blacksquare$) or 100 μM indomethacin ($\blacktriangle$) or 5 mM CaCl_2 ($\bullet$) at 37 °C. In both, LDH leakage from cells at the indicated times was examined and expressed as a percentage of the total enzyme activity of cells (see the text for details).

Figure 2 CTX-induced formation of cross-linked actin filaments in platelets

Platelets were incubated with various concentrations of CTX at 37 °C for 3 min. LDH activity in the suspension medium was measured ($\bullet$). The cells were then lysed in 0.5% Triton and the Triton-insoluble residue was analysed for actin content (see the text for details). The percentage of the total actin of cells present in the TICS was plotted versus CTX concentration ($\blacksquare$). The inset displays the electrophoretic pattern: lane 1, half of total lysate of resting cells; lane 2, Triton-insoluble residue of resting cells; lane 3, Triton-insoluble residue of cells after treatment with 34.3 μM CTX.

As the cellular activity of platelets can be modified by cytochalasin B, indomethacin and CaCl_2 [14–16], their effects on the CTX-induced cell lysis were examined. The results are shown in Figure 1(b). Singly, 10.4 μM cytochalasin, 100 μM indomethacin or 5 mM CaCl_2 did not cause cell lysis (results not

shown). Both indomethacin and CaCl_2 greatly suppressed the CTX-induced cell lysis. Cytochalasin B, on the other hand, had no effect on the CTX-induced cell lysis for the time period tested.

CTX-induced cytoskeletal change

In resting platelets, 40–50% of the total actin is in filamental forms, and some of these filaments form structures that can be sedimented from the lysate of cells in a suitable Triton solution at low centrifugal force [11]. The Triton-insoluble cytoskeletal structures (TICS) are considered to be the major structural elements in the contractile components of platelet cytoskeleton. Any effect leading to the enhancement of actin polymerization and/or incorporation of pre-existing actin filaments into the cytoskeleton in platelets results in an increase in the content of TICS, the major protein component of which is actin.

Figure 2 displays the analyses by SDS/PAGE of actin in TICS of platelets which had been treated with 0–34.3 μM CTX for 3 min; the cell lysis at each CTX concentration is also included in the Figure. Less than 7% of cells were lysed at the highest CTX concentration. A main 43 kDa protein band, apparently monomeric actin according to the previous reports [17–19], was seen in all cases. The content of monomeric actin in TICS is presented as a percentage of total actin in platelets. This should be considered as a relative value, because the centrifugal condition for sedimentation of TICS as well as the Triton content in the lysis buffer might affect the result. In resting platelets, around 12–15% of total actin remained in TICS (cf. lanes 1 and 2 in the inset of Figure 2). Similar actin populations have been shown to be retained in TICS of platelets pretreated in a buffer containing 0.1% Triton. In this condition, the cell morphology and the organization of cytoskeletal actin filaments in the resting cells remain unchanged [19]. When cells were incubated with CTX for 3 min, the actin content of TICS increased remarkably (cf. lanes 2 and 3 in the inset of Figure 2). The higher the CTX concentration, the greater the increase (Figure 2). Apparently,

Figure 3 Kinetics of CTX-induced change in the cytoskeleton of platelets

Cells were incubated with $21.4 \mu\text{M}$ CTX at 37°C for various times, and the actin content of the Triton-insoluble residue of the cell lysate was measured (■). For comparison, cell lysis under the corresponding condition is presented (●).

CTX could induce cytoskeletal changes of platelets, as reflected by its being able to promote the formation of TICS.

The kinetics of the cytoskeletal change and cell lysis by $21.4 \mu\text{M}$ CTX were compared (Figure 3). Cell lysis did not occur in the first 30 s, and less than 5% of the total LDH activity of cells was detected in the suspending medium even after the reaction had proceeded for 3 min (Figure 3). Apparently there was a noticeable lag period, within which the cell membrane remained intact. In contrast, the CTX-induced cytoskeletal change was extremely fast. The actin content of TICS rose rapidly and approached a maximum value 10 s after the incubation (Figure 3). Thereafter the actin content fell slightly, and then remained unchanged for the time period tested. The kinetics were similar to those observed when platelets were activated with thrombin or ADP [20]. It appeared that the CTX action on intact platelets caused a rapid change in the cytoskeleton before cell lysis. CTX, however, did not cause an increase in actin content of TICS from 0.1%-Triton-treated platelets. It is likely that the machinery responsible for the CTX action was disintegrated by changes in the membrane resulting from the mild treatment of cells with 0.1% Triton. Together with the kinetic characteristics mentioned above, this suggested that it was the primary action of CTX on intact platelet membrane, rather than any secondary consequence due to membrane disruption, that caused the cytoskeletal changes. Since the results of CTX action on several kinds of cells did not suggest the existence of protein receptors on the plasma membrane [6,7], the machinery responsible for CTX action thus might involve the membrane lipids, and the CTX action might not reorganize the pre-existing actin filaments. In fact, interaction between CTX and membrane lipids of different origins has been reported [21–23]. Since CTX is not able to penetrate into cells [6,7], it was unlikely that CTX was directly involved in the process of incorporating actin into the cytoskeleton, despite the fact that it was shown to be able to induce actin polymerization *in vitro* [8].

Cytochalasin B is able to prevent the addition of actin molecules to the fast-growing end of actin filaments and thereby prevent the actin polymerization [14]. Pre-exposure of platelets to $10.4 \mu\text{M}$ cytochalasin B at 37°C for 10 min completely abolished the CTX-promoting formation of TICS. This indicates that the CTX action on the extracellular membrane might trigger a rapid change in intracellular environments in favour of actin polymerization. The CTX-induced cytoskeletal change, however,

Figure 4 Effects of indomethacin and CaCl_2 on the CTX-induced incorporation of actin into the cytoskeleton of platelets

Cells were incubated with indomethacin (a) or CaCl_2 (b) at 37°C for 3 min in the absence of CTX (●) or in the presence of $21.4 \mu\text{M}$ CTX (■).

might not be a direct cause for the subsequent cell lysis, since cytochalasin B gave no effect on the CTX-induced cell lysis (Figure 1b).

Interaction of platelets with CTX did not cause cell aggregation, though CTX could enhance the action of platelet-aggregation inducers such as ADP, collagen and thrombin, according to the results of Teng et al. [24]. They suggested that in the presence of platelet-aggregation inducers CTX might promote the mobilization of membrane-bound Ca^{2+} and augment Ca^{2+} flux necessary for activation of endogenous phospholipase A_2 . Since the effect of CTX on platelet aggregation could be inhibited by indomethacin and CaCl_2 [24], their effects on the CTX-induced change in the cytoskeleton were tested (Figure 4). Indomethacin or CaCl_2 alone showed no effect on the formation of TICS in resting cells. Preincubation of platelets with indomethacin was ineffective, but increasing CaCl_2 concentration greatly inhibited the CTX-induced formation of TICS (Figure 4b). Since indomethacin blocks the biosynthesis of prostaglandins and thromboxanes derived from arachidonic acid, and inhibits the platelet membrane-bound phospholipase A_2 [15], it is unlikely that metabolites resulting from activation of phospholipase A_2 by CTX, as suggested by Teng et al. [24], were involved in promoting the CTX-induced formations of TICS. With regard to the effect of CaCl_2 , it has been shown that the cytosolic concentration of Ca^{2+} might increase due to Ca^{2+} infusion at high extracellular Ca^{2+} concentration [16], which gave only a slight effect on the binding of CTX to the membrane of both red blood cells and myocardiocytes [6,7]. The suppression of the CTX-promoting formation of TICS by increasing external CaCl_2 concentration might be relevant to the Ca^{2+} -dependent depolymerization of actin filaments [25,26] or its interference with CTX binding. This awaits further study.

REFERENCES

- Chen, Y. H., Pan, B. T. and Lee, C. P. (1982) *Biochim. Biophys. Acta* **702**, 193–196
- Chang, C. C. (1979) *Handb. Exp. Pharmacol.* **52**, 309–376
- Chen, Y. H., Hu, C. T. and Young, J. T. (1984) *Biochem. Int.* **8**, 329–338
- Condrea, C. (1979) *Handb. Exp. Pharmacol.* **52**, 448–472
- Tu, A. (1977) *Venoms: Chemistry and Molecular Biology*, pp. 301–320, John Wiley and Sons, New York
- Chen, Y. H., Liou, R. F., Hu, C. T., Juan, C. C. and Yang, J. T. (1987) *Mol. Cell. Biochem.* **73**, 69–76

- 7 Tzeng, W. F. and Chen, Y. H. (1988) *Biochem. J.* **256**, 89–95
- 8 Chen, Y. H. and Chu, S. T. (1988) *Biochim. Biophys. Acta* **966**, 266–268
- 9 Lo, T. B., Chen, Y. H. and Lee, C. Y. (1966) *J. Chin. Chem. Soc. (Taipei)* **13**, 25–37
- 10 Bevers, E. M., Comfurius, P. and Zwaal, R. F. A. (1983) *Biochim. Biophys. Acta* **736**, 57–66
- 11 Phillips, D. R., Jennings, L. K. and Edwards, H. H. (1980) *J. Cell Biol.* **86**, 77–86
- 12 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 13 Bergmeyer, H. U. and Bernt, E. (1974) *Methods Enzym. Anal.* (2nd Ed.) **2**, 574–579
- 14 Jesse, R. L. and Franson, R. C. (1979) *Biochim. Biophys. Acta* **575**, 467–470
- 15 Casella, J. F., Flanagan, M. D. and Lin, S. (1981) *Nature (London)* **293**, 302–305
- 16 Tsai, W. J., Chen, J. C. and Wang, C. T. (1988) *Biochim. Biophys. Acta* **940**, 105–120
- 17 Jennings, L. K., Fox, J. E. B., Edwards, H. H. and Phillips, D. R. (1981) *J. Biol. Chem.* **256**, 6927–6932
- 18 Fox, J. E. B., Boyles, J. K., Reynolds, C. C. and Phillips, D. R. (1984) *J. Cell Biol.* **98**, 1985–1991
- 19 Fox, J. E. B., Boyles, J. K., Berndt, M. C., Steffen, P. K. and Anderson, L. K. (1988) *J. Cell Biol.* **106**, 1525–1538
- 20 Pribluda, V. and Rotman, A. (1982) *Biochemistry* **21**, 2825–2832
- 21 Vincent, J.-P., Schweitz, H., Chicheportiche, R., Fosset, M., Balerna, M., Lenoir, M.-C. and Lazdunski, M. (1976) *Biochemistry* **15**, 3171–3175
- 22 Chen, Y. H., Lai, M. Z. and Kao, L. S. (1981) *Biochem. Int.* **3**, 385–390
- 23 Batenburg, A. M., Bougis, P. E., Rochat, H., Verkleij, A. J. and de Kruijff, B. (1985) *Biochemistry* **24**, 7101–7110
- 24 Teng, C. M., Jy, W. and Ouyang, C. (1984) *Toxicon* **22**, 463–470
- 25 Carlsson, L. F., Markey, F., Blikstad, I., Persson, T. and Lindberg, U. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 6376–6380
- 26 Markey, F., Persson, T. and Lindberg, U. (1981) *Cell* **23**, 145–153

Received 13 July 1992/19 October 1992; accepted 22 October 1992