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

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 cytolysin, a cytolysis, a direct lytic 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 MgCl2/25 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) x 10^6 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 μM 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.

Abbreviations used: CTX, cardiotoxin; LDH, lactate dehydrogenase; TICS, Triton-insoluble cytoskeletal structures.
‡ To whom correspondence should be addressed.
Platelets were incubated with various concentrations of CTX at 37 °C: △, 0; △, 1.4 μM; ○, 4.3 μM; □, 8.6 μM; □, 17.1 μM; ■, 34.3 μM. (b) Platelets were treated with 21.4 μM CTX alone (○) or in the presence of 10.4 μM cytochalasin B (■) or 100 μM indomethacin (△) or 5 mM CaCl₂ (●) 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).

As the cellular activity of platelets can be modified by cytochalasin B, indomethacin and CaCl₂ [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 CaCl₂ did not cause cell lysis (results not shown). Both indomethacin and CaCl₂ 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 filamentous 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,
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 μM 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 μM 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, 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++ and augment Ca++ flux necessary for activation of endogenous phospholipase A2. Since the effect of CTX on platelet aggregation could be inhibited by indomethacin and CaCl2 [24], their effects on the CTX-induced change in the cytoskeleton were tested (Figure 4). Indomethacin or CaCl2 alone showed no effect on the formation of TICS in resting cells. Preincubation of platelets with indomethacin was ineffective, but increasing CaCl2 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 A2 [15], it is unlikely that metabolites resulting from activation of phospholipase A2 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 CaCl2, it has been shown that the cytosolic concentration of Ca++ might increase due to Ca++ infusion at high extracellular Ca++ concentration [16], which gave only a slight effect on the binding of CTX to the membrane of both red blood cells and myocardicocytes [6,7]. The suppression of the CTX-promoting formation of TICS by increasing external CaCl2 concentration might be relevant to the Ca++-dependent depolymerization of actin filaments [25,26] or its interference with CTX binding. This awaits further study.

REFERENCES
2 Chang, C. C. (1979) Handbook of Pharmacology 82, 309–376
Received 13 July 1992/19 October 1992; accepted 22 October 1992