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MECHANISM OF ACTIVATION BY Ca^{2+} OF HEMOLYSIS INDUCED BY LYTIC POLYPEPTIDE EQUINATOXIN II

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Equinatoxin II is a cytolytic polypeptide from the sea anemone *Actinia equina* L. which forms pores in natural and artificial membranes. In the present study, we show that the mechanism by which Ca^{2+} activates toxin-induced hemolysis of human red blood cells consists of the ability of Ca^{2+} which enters the cell presumably through preformed toxin-induced pores and acts inside the cell to increase the rate of toxin binding to the membrane. As a result, a larger amount of pores is produced thus increasing the rate of hemolysis. In addition, the data reveal that equinatoxin II induces hemolysis of RBC interacting with a limited number of toxin-binding sites (receptors) with an upper estimate of $(180\pm35)\cdot10^3$ sites per one cell. The total number of toxin-binding sites does not depend on the presence of Ca^{2+}. These data strongly suggest that specific receptor must exist on the erythrocyte membrane to mediate the hemolytic action of this toxin.

KEY WORDS: erythrocyte, hemolysis, divalent cations, equinatoxin II.

Ca^{2+} МЕХАНІЗМ АКТИВАЦІЇ ГЕМОЛІЗУ, ВИКЛИКАНОГО ЛІТИЧНИМ ПОЛІПЕПТИДОМ ЕКВІНАТОКСИНОМ ІІ

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Еквінатоксін ІІ – цитолітичний поліпептид морської анемони *Actinia equina* L., який утворює пори в природних і штучних мембранах. У даній роботі ми показуємо, що механізм, за яким Ca^{2+} активує токсин-індукований гемоліз еритроцитів людини, полягає у здатності Ca^{2+} проникати в клітку ймовірно через заздалегідь утворені токсином пори і викликати усередині клітки збільшення швидкості зв'язування токсину з мембраною. В результаті утворюється більша кількість пор, що і збільшує швидкість гемолізу. Крім того дані показують, що еквінатоксин ІІ викликає гемоліз еритроцитів, взаємодіючи з обмеженою кількістю місць зв'язування токсину (рецепторів) з щонайбільш $(180\pm35)\cdot10^3$ місць на одну клітину. Загальна кількість місць звязування токсину не залежить від присутності Ca^{2+}. Ці дані переконливо свідчать, що на еритроцитарній мембрані повинні існувати специфічні рецептори, які опосередковують гемолітичну дію цього токсину.

КЛЮЧОВІ СЛОВА: еритроцит, гемоліз, двовалентні катіони, еквінатоксин ІІ.

Ca^{2+} МЕХАНИЗМ АКТИВАЦИИ ГЕМОЛИЗА, ВЫЗВАННОГО ЛИТИЧЕСКИМ ПОЛИПЕПТИДОМ ЭКВИНАТОКСИНОМ ІІ

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Эквинатоксин ІІ – цитолитический полипептид морского анемона *Actinia equina* L., который образует поры в естественных и искусственных мембранах. В данной работе мы показываем, что механизм, по которому Ca^{2+} активизирует токсический гемолиз эритроцитов человека, состоит в способности Ca^{2+} проникать в клетку, вероятно через предварительно образованные токсином поры, и вызывать внутри клетки увеличение скорости связывания токсина с мембраной. В результате получается большее количество пор и увеличивается скорость гемолиза. Кроме того данные показывают, что эквинатоксин ІІ вызывает гемолиз эритроцитов, взаимодействуя с ограниченным количеством мест связывания токсина (рецепторов), максимум $(180\pm35)\cdot10^3$ мест на

одну клетку. Общее количество мест связывания токсина не зависит от присутствия Ca^{2+}. Приведенные данные доказывают, что специфические рецепторы должны существовать на эритроцитарной мембране для того, чтобы опосредовать гемолитическое действие этого токсина.
КЛЮЧЕВЫЕ СЛОВА: эритроцит, гемолиз, двухвалентные катионы, эквинатоксин II.

INTRODUCTION

Equinatoxin II from *Actinia equina* L. is a lethal and cytolytic toxin [1] belonging to the family of pore-forming, basic polypeptides with molecular mass of 16-20 kDa, produced by sea anemones. It is hemolytic [2, 3], cytotoxic [4] cardiotoxic [5] and causes platelet aggregation [6] and lung damage [7]. It is able to lyse lipid vesicles [2, 8] and form ion channels in planar lipid bilayers [2]. Although molecular mechanism of equinatoxin-induced RBC lysis is still incompletely understood the later observations suggest that the formation of cation-selective pores comprising three or four toxin molecules and permeable also to non-electrolytes [2, 8, 9] is responsible for this effect. The effective hydrodynamic diameters of such pores have been estimated to be around 2 nm [2, 3]. Despite the fact that equinatoxin II causes erythrocyte and platelet lysis at very low concentrations (nanomoles) and are generally inhibited by sphingomyelin [2, 3] there is no agreement whether or not a specific receptor is required to promote the action of this toxin. In common with the lytic action of other toxins [10-12], the hemolytic ability of equinatoxin II depends on the presence of divalent cations [3, 9, 13], particularly Ca^{2+}. It was reported that the effect of Ca^{2+} depends on the concentration of cations and chemical modification of the structure of toxin molecule [3]. However, the nature of the influence of cations on toxin-induced hemolysis is still not well understood. In this paper we considered primarily the mechanism of action of Ca^{2+} on hemolysis of RBC induced by equinatoxin II.

MATERIALS AND METHODS

In the present experiments only fresh blood was used. Some blood drops from donor's finger were mixed with 10 ml of isotonic Tris buffered saline (TBS) (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and washed twice by centrifugation (2000×g, 3 min). RBC pellet (30 μl) was suspended into 0.5 ml of TBS and used over period of hours as stock-suspension. Chlorpromazine/HCl, A23187 and trehalose from were from Sigma, PEG 4000 from Ferak, raffinose from Pfanstiehl Carbohydrates (Waukegan, IL). Equinatoxin II was a gift from Dr. P.Macek (University of Ljubljana, Slovenia) and Dr. G. Menestrina (Povo Trento, Italy). Other reagents were the highest grade available.

Hemolysis assay. The dynamics of erythrocyte hemolysis and alteration in their shape during interaction with toxin were measured spectrophotometrically [2, 3, 14, 15]. RBC suspensions were constantly stirred in TBS and their apparent absorbance at 720 nm was recorded continuously. Stock RBC suspension (6-7 μl) was placed into a spectophotometer cuvette (2 ml), so that the initial absorbance was 0.12-0.13. This value corresponds to a concentration in the cuvette of ~$0.8 \cdot 10^6$ cells/ml, as detected by a Coulter-Counter. Aliquots of toxin from concentrated stock solution prepared in TBS (40 μg/ml) or other substances (1 M for Ca^{2+}, Zn^{2+}, Cd^{2+} and EDTA, 1.4 M for sucrose, 2 M for trehalose, 0.5 M for raffinose, 3.3 mg/ml for chlorpromazine) were added directly to a cuvette with or without of erythrocyte suspension. Time of mixing was approximately 2 sec. Because absorbance is proportional to cells concentration, the measured rate of absorbance changes is proportional to the rate of hemolysis [2, 15]. In all cases the rate of hemolysis was expressed in arbitrary units (a.u.) calculated from kinetic curves as the tangent of α (tgα), where α is an angle between linear part of the absorbance curve and time axis. One arbitrary unit corresponds to the rate of

changing in absorbance as $8.3 \cdot 10^{-4}$ absorbance units per one second. All experiments were carried out at room temperature (20-22°C).

Treatment of RBC with ionophore A23187.RBC suspension (100 μl) with concentration of $3 \cdot 10^8$ cells/ml was incubated in TBS at room temperature for 1 h in the presence of 5 μM A23187 and indicated amount of Ca^{2+} or EDTA. After incubation, an aliquot of RBC suspension (8-10 μl) was introduced into the cuvette containing 2 nM toxin with or without of 1 mM CaC_2 and kinetic of hemolysis was followed by hemolysis assay.

Incorporation of the toxin into the RBC membrane under protection of PEG 4000. RBC suspensions (100 μl) with concentration of $3 \cdot 10^8$ cells/ml were first mixed with 100 μl of 60 mM solutions of PEG 4000 prepared in TBS. If needed appropriate $CaCl_2$ was added to obtain a final concentration of 1 mM. After that, aliquots of concentrated toxin solutions were added to each sample to obtain desired number of toxin molecules per one cell. During subsequent incubation (up to 3 h at room temperature) aliquots of RBC were transferred into a cuvette with or without of 1 mM $CaCl_2$ but free of toxin and processed as described above. There were no hemolysis in any samples during incubation up to 6 h.

RESULTS

Fig. 1 shows that Ca^{2+} leads to activation of toxin-induced hemolysis decreasing the lag-time and increasing the hemolysis rate. In addition, Ca^{2+} at higher concentrations (20 mM) initiates shape transformation of cell toward spherical form before hemolysis to occur, as judged from the increase in absorbance and disappearance of absorbance noise [14, 15].

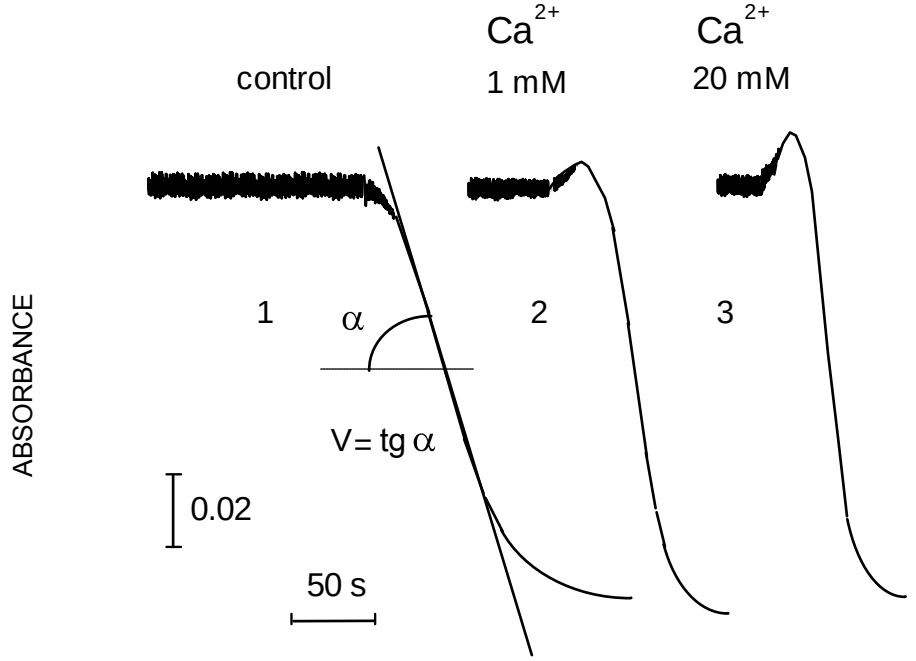

Fig. 1. Typical changes in absorbance during toxin-induced hemolysis at final concentration of toxin of 2 nM in the absence and presence of Ca^{2+}. Cells at final concentration ~$0.8\ 10^6$ cells/ml were introduced into the medium containing toxin and cations.

Stimulating effect of Ca^{2+} depends on the concentration of cations, showing saturation behavior with the maximal rate at the concentrations above 10-20 mM. So, the presence of Ca^{2+} leads to two three-fold increase in the total rate of hemolysis induced by the same amount of toxin. This effect depends also on the concentration of toxin being more pronounced at low concentration. Similar but two-fold lower in magnitude effect was

demonstrated by Mg^{2+}. In contrast, Zn^{2+} ions inhibited toxin-induced hemolysis in concentration dependent manner and fully prevented it at concentration of 1 mM and higher (data not shown). It turned out that the stimulation of hemolysis by Ca^{2+} under experimental conditions depicted in Fig. 1 is not a maximal one that Ca^{2+} ions are able to produce. In addition, it was found that Ca^{2+} effect was dependent on time point of addition of cations to the medium containing cells and toxin. The maximal stimulation was attained adding cations appr. at 2/3 of whole control lag-period. In this case Ca^{2+} increased additionally by 20-30% the rate of hemolysis and also activated the rate of shape transformation. These data show that Ca^{2+} can participate in time-dependent process of pore initialization and formation. In principle, Ca^{2+} effects may be due to influence of Ca^{2+} on toxin-induced pore (for example on its dimension), on specific Ca^{2+}-dependent toxin receptors (sites) distinct from those which normally bind toxin, or both. To compare dimension of pores formed by toxin in the presence and absence of Ca^{2+}, inhibition of toxin-induced pore by variety of inhibitors was performed. Data from Table 1 show that divalent cations Cd^{2+} and Zn^{2+} as well as trehalose completely inhibit both types of hemolysis. Effect of cations was reversible, because EDTA added later was able to release hemolysis (not shown). This implies that cations electrostatically interact with toxin-induced pore and close its. Osmotic protectants sucrose and raffinose inhibited hemolysis by some extent. However, inhibition of Ca^{2+}-activated hemolysis was larger compared with hemolysis induced by toxin in the absence of Ca^{2+}. This clearly indicates that Ca^{2+} does not increase the dimension of pores.

RBC at final concentration of ~10^6 cells/ml were introduced into TBS containing 2 nM of equinatoxin II in the presence or absence of 1 mM $CaCl_2$ and time course of hemolysis was recorded as in Fig.1. After the onset of hemolysis, when 90% of initial absorbance was reached aliquots of inhibitors from concentrated stock solutions (2-120 μl) were added to obtain indicated final concentration. Inhibition of both types of hemolysis was assessed as a ratio V_{inh}/V_C where V_{inh} -the rate of hemolysis in the presence of inhibitors; V_C - the corresponding rate of control hemolysis in the absence of inhibitors.

Table 1. Inhibition of toxin-induced hemolysis.

Hemolytic medium	control	sucrose (30 mM)	raffinose (30 mM)	trehalose (30 mM)	Cd^{2+} (1 mM)	Zn^{2+} (1 mM)
Toxin	1	0.6±0.1	0.6±0.1	0	0	0
toxin+Ca^{2+}	1	0.6±0.1	0.3±0.1	0	0	0

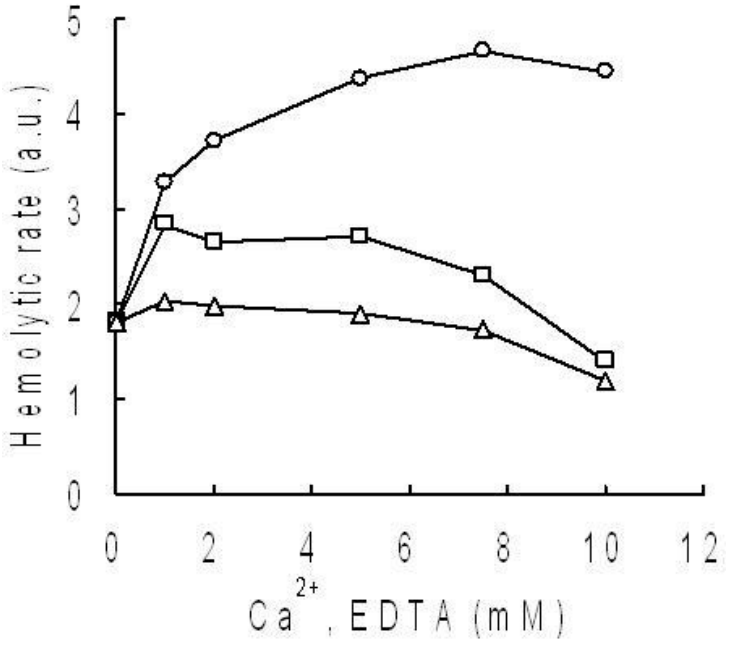

Fig. 2. Effect of Ca^{2+} (○), EDTA (Δ) and equimolar mixture of Ca^{2+} and EDTA (□) on hemolysis induced by 2 nM of toxin. Experimental conditions are the same as in Fig. 1.

Therefore, stimulating effect of this cation may be due to the increased pore number with similar properties. In some extent this is confirmed by equivalent action of other protectants. Interestingly, the trehalose being a smaller molecule than raffinose, completely inhibited hemolysis suggesting a specific interaction (not only as an osmotic protectant) with constituents of toxin-induced pore. To investigate further a possible role of Ca^{2+}-binding sites in the mechanism of activation, Ca^{2+} effects were studied in the presence of equimolar EDTA. EDTA alone exhibited slightly inhibitory effect only at relatively high concentration (Fig. 2). Equimolar mixture of Ca^{2+} and EDTA at low concentration activated hemolysis with extent lower than that produced by the corresponding concentration of Ca^{2+}, although finally inhibited hemolysis in a manner close to EDTA as a concentration is increased. This result suggests a competition between Ca^{2+}-binding site, Ca^{2+} and EDTA with compared affinity. At low concentration of cations, Ca^{2+}-binding site binds preferably Ca^{2+} ions and activates hemolysis, whereas at high concentration EDTA became more potent helating Ca^{2+} from Ca^{2+}-binding site thus inhibiting hemolysis. Additional experiments revealed that the time course of hemolysis in the presence of the mixture of Ca^{2+} and EDTA with proportion 2:3 (1 mM Ca^{2+} and 1.5 mM EDTA) is very close to control one. These experimental conditions were chosen to investigate kinetic effects of helating Ca^{2+} in the case of Ca^{2+}-activated hemolysis.

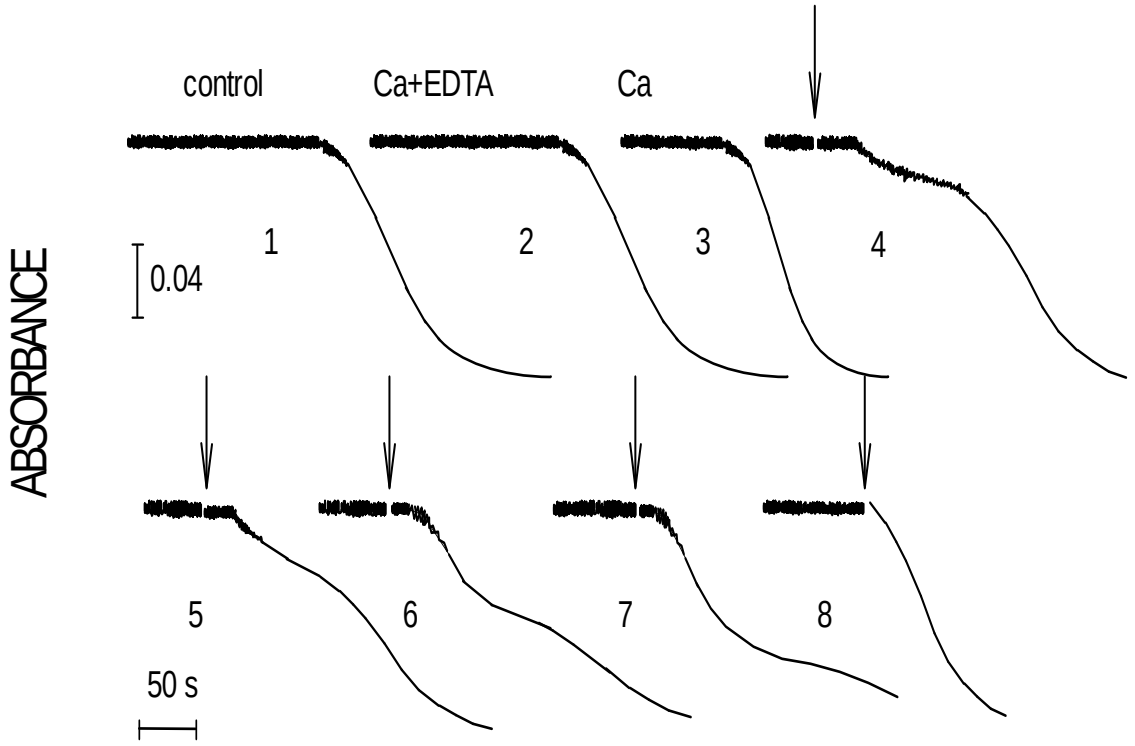

Fig. 3. Typical spectrophotometer traces obtained during activation by Ca^{2+} (1 mM) and inhibition by EDTA (1.5 mM) of hemolysis induced by equinatoxin II (2 nM). Arrows indicate addition of a portion of EDTA into the cuvette. Labels without arrows denote that Ca^{2+} or EDTA were added to the medium containing toxin prior to the cells.

Data presented in Fig. 3 show that effect of EDTA on time-course of hemolysis is complex and depends on the time of addition of EDTA during lag-period preceding hemolysis. When EDTA was added immediately prior the onset of hemolysis its only slightly (within statistical experimental error) reduced the rate of hemolysis compared with the corresponding control (traces 2 and 8). In fact, EDTA is practically ineffective at the stage of cell lysis. Acting at the initial stages of the process EDTA cancels activating effect of Ca^{2+} (traces 1, 2, 3). Adding later, EDTA initiates unusual biphasic response when separate lysis of cell sub-populations is occurred. These data may be adequately interpreted on the basis of time-dependent chelating by EDTA of Ca^{2+}, previously bound to Ca^{2+}-specific site. Indeed, in analogy with Ca^{2+} when some time is required to produce an activating effect, (existence of lag-time in hemolysis) EDTA is also required some time to remove Ca^{2+} from membrane binding site. This is why the cells undergoing hemolysis are yet not sensitive to the action of EDTA. However, if EDTA had sufficient time to helate Ca^{2+}, it is able to stop Ca^{2+}-activated component of lysis, and cells will finally lyse by Ca^{2+}-independent component. This is confirmed by the observation that the total hemolytic time, in fact, is the same in all cases irrespective of Ca^{2+} and EDTA, while exact time-courses of hemolysis may be significantly different. In this respect the action of EDTA is different from its corresponding action on releasing of hemolysis induced by melittin and inhibited previously by cations Rudenko, Nipot 1996) when effect of EDTA was recognized immediately after addition to the cells. This strongly suggests that Ca^{2+}-dependent site for toxin is buried into the membrane and initially is poor accessible for Ca^{2+} as well as EDTA. This also agrees with the interpretation

that the lag-time reflects the time required for toxin molecules to reach corresponding binding site through putative potential barrier. In any case, lag-time in toxin action significantly differs this type of RBC lysis from lysis of lipid vesicles, which is occurred without any visible delay [2, 3]. The later data suggest that the site responsible for the action of Ca^{2+} has intracellular location. The results presented in Table 2 confirm this proposal because loading cells by Ca^{2+} in the presence of ionophore A23187 makes them more prone to lysis as compared with control.

RBC at concentration $3 \cdot 10^8$ cells/ml were incubated in TBS at room temperature for 1 h in the presence of 5 μM A23187 and indicated amount of Ca^{2+} or EDTA. After incubation aliquots of RBC (5-7 μl) were transferred into spectrophotometer cuvette to detect hemolysis induced by 2 nM of toxin as described in Materials and Methods. V - the rate of hemolysis; V_c - the rate of hemolysis of control non-treated cells in the medium free of Ca^{2+}.

Table 2. Effect of RBC treatment by A23187 on toxin-induced hemolysis

parameter	non-treated control	A23187 +1 μM Ca^{2+}	A23187 +10 μM Ca^{2+}	A23187 +100 μM Ca^{2+}	A23187 +100 μM Ca^{2+} +1 mM EDTA
V/V_c	1	1.9±0.1	1.6±0.1	1.4±0.1	0.9±0.1
V/V_c 1 mM Ca^{2+}	2.7±0.9	1.7±0.1	1.5±0.1	1.5±0.1	2.5±0.9

As seen, even 1 μM of Ca^{2+} is sufficient to obtain a maximal activation suggesting that relatively low intracellular concentration of Ca^{2+} is required to produce an effect. It is worth noting that in contrast to native cells, ionophore-treated cells have lost their ability to be activated by extracellular Ca^{2+} in hemolytic media. We conclude, therefore, that Ca^{2+} activates hemolysis entering the cell presumably through toxin-induced pores and acts from the cell interior.

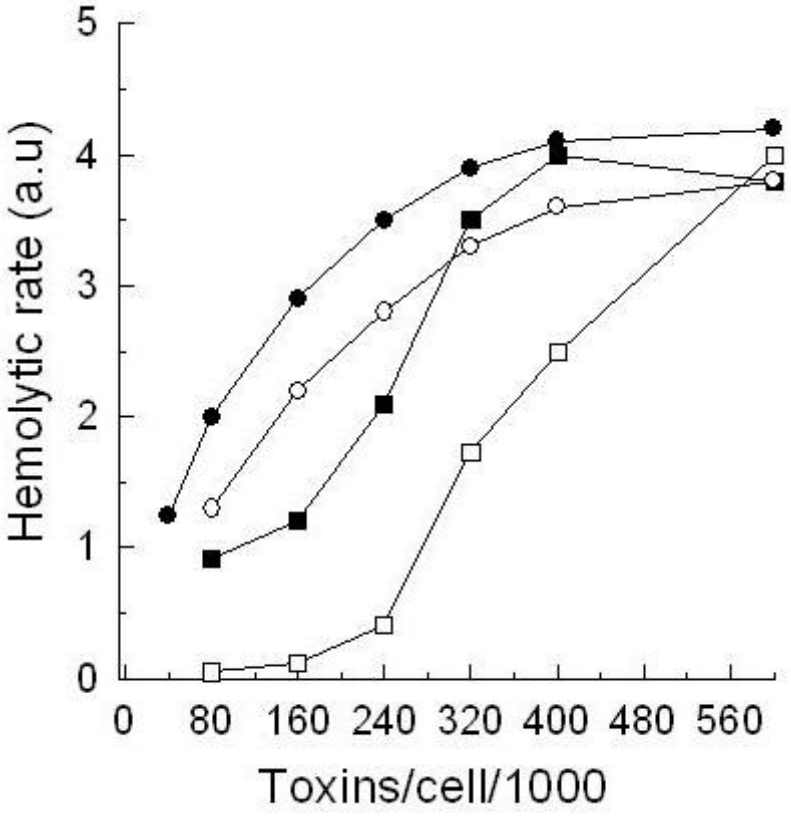

Fig. 4. Dependence of the rate of hemolysis on the amount of toxin molecules bound to RBC membrane. Cells were incubated with the toxin for 5 min (□, ■) or 2 h (○, ●) in the presence (closed symbols) or absence (open symbols) of 1 mM $CaCl_2$. Other conditions are described in Materials and Methods. Representative results from four independent experiments.

To resolve the problem whether Ca^{2+} binds to a special class of sites thus opening additional amount of pores (Ca^{2+}-specific pores) or all toxin-induced pores are identical and under regulatory control of Ca^{2+} (Ca^{2+}-dependent pores) we have compared some properties of steady-state pores formed in the presence or absence of Ca^{2+}. These experiments also give possibility to estimate a number of toxin binding sites (receptors) per one cell. RBC were first incubated in the media containing 30 mM PEG 4000. This concentration of PEG fully protected the cells against hemolysis during prolonged period of incubation up to 6 h even in the presence of maximal concentration of toxin (600,000 per one cell). During incubation, toxin molecules bind to the RBC membrane and form a steady-state pores. This is followed by the fact that the rates of hemolysis after transferring the cells into TBS free of PEG and toxin were, in fact, the same after incubation with toxin for 1, 2 and 3 hs (not shown). Dose-response curves shown in Fig. 4 demonstrate saturation behavior where maximal rates of hemolysis do depend on the presence or absence of Ca^{2+} for cells incubated with toxin for 2 h. Treating these dependencies as Hill plots one can obtain an upper estimate of toxin-binding sites which lies within a range of (180 ±35) 10^3 (mean ±S.D., n=4) toxin molecules per cell and is not Ca^{2+}-dependent. The role of Ca^{2+} became clear comparing corresponding dependencies measured after 5 min of incubation at the same conditions. In this case Ca^{2+} significantly increased rate of hemolysis at all toxin concentrations. Because in these type of the experiments the cells were finally exposed and lysed in the medium free of toxin, it is conceivable that the rate of hemolysis should be directly proportional to the concentration of

toxin in the membrane. This leads to the conclusion that Ca^{2+} significantly (about 10-fold) increases the rate of toxin binding to RBC membrane. Interestingly, that after incubation of the cells under PEG protection for 2 h in the presence of toxin, Ca^{2+} in hemolytic medium was unable to stimulate hemolysis further regardless of toxin concentration (not shown).

RBC at concentration $3 \cdot 10^8$ cells/ml were incubated in TBS under PEG 4000 protection (30 mM) for 2 h and at toxin concentration to give 80,000 toxin molecules per cell. Inhibition was measured as in Table 1.

Table 3. Inhibition of hemolysis caused by steady-state toxin-induced pores

Incubation medium	control	sucrose (30 mM)	raffinose (30 mM)	trehalose (30 mM)	Cd^{2+} (1 mM)	Zn^{2+} (1 mM)
toxin	1	0.4±0.1	0.16±0.06	0	0	0
toxin+Ca^{2+} (1 mM)	1	0.4±0.1	0.15±0.06	0	0	0

Data in Table 3 comparing inhibitory properties of rank of inhibitors relative to steady-state pores formed in the presence and absence of Ca^{2+} reveal, in fact, no differences between them in contrast to dynamic pores formed during hemolysis (Table 1).

DISCUSSION

A number of bacterial toxins that affect target cells either from the exterior surface or by entering and altering some intracellular process have been shown require Ca^{2+} for their activity. For instance, E.coli hemolysin or Adenylate cyclase toxin (AC) are both Ca^{2+}-binding proteins, and interaction of the toxin molecules with Ca^{2+} results in a major conformational changes [10, 11, 16]. This Ca^{2+}-induced conformational change [16] is probably necessary but not sufficient for insertion and toxin delivery, however both delivery of catalytic domain and the process that results in the hemolytic event are Ca^{2+}-dependent. In the case of equinatoxin II, Ca^{2+} effect on toxin activity is different from above mentioned at least in one important aspect, namely that direct toxin-Ca^{2+} interaction is not involved in toxin activity. This is followed by the fact that RBC pre-treated by the ionophore A23187 in the presence of Ca^{2+} became almost as sensitive to toxin action as the cells treated by toxin in the presence of Ca^{2+} (Table 2). In addition, ionophore-treated cells have completely lost their ability to be activated by extracellular Ca^{2+}.

The present data reveal that the mechanism by which Ca^{2+} activates toxin-induced hemolysis is relatively simple. The major role of Ca^{2+} is to promote toxin binding to the membrane. This conclusion is based on the following observations: 1) appearance of a visible toxin-induced shape transformation (Fig. 1) at high Ca^{2+} concentrations as an indicator of binding [14, 15], 2) data from Fig. 4 (lines for 5 min) indicate about 10-fold increase in the rate of toxin binding to RBC membrane, 3) inability of Ca^{2+} to increase hemolysis caused by preformed steady-state toxin-induced pores irrespective of whether or not these pores have been formed in the presence or absence of Ca^{2+} , 4) identical features of steady-state pores with respect to the action of inhibitors. The role of Ca^{2+} as an agent that participates mainly in the process of pore initialization is confirmed further by the differences between the steady-state and dynamic pores. In general the dynamic pore which is formed under toxin gradient is larger than a steady-state pore as indicated by the reduced ability of sucrose and raffinose to inhibit hemolysis induced by dynamic pores (Tables 1 and 3). It is obvious that the dimension

of pores increases rising toxin gradient, because potent inhibitors of steady-state pores raffinose, Cd^{2+} and trehalose were all ineffective in preventing hemolysis at high toxin concentrations (20-150 nM) used to introduce a desired number of toxin molecules into the membrane (not shown). Only PEG 4000 entirely blocked hemolysis at those circumstances. Interestingly, when toxin incorporation and pore formation have been completed, it results in steady-state pores which are already blocked by inhibitors. This points to highly conductive transitory pore state which arises during pore growth. In fact, this result contradicts to the model of oligomerization of toxin molecules in the process of pore formation (the more oligomers, the more is the membrane permeability) [2, 3] and must await more detailed examination. Taking this in mind, it is possible that diameter of equinatoxin-induced pore determined in some works [2, 3] was overestimated insofar as experimental conditions allowing to obtain a dynamic pore were used.

Approach used here permits to make an upper estimate of a number of toxin-binding sites bearing by the one cell. The value obtained lies within the range of $(180\pm35)\cdot10^3$ sites per one cell and should be considered as an upper estimate. This ensues from the suggestion of complete binding of all toxin molecules to the cells and an absence of non-specific binding, when toxin molecule binds to the membrane but does not participate in pore formation. An existence of such non-lytic sites was demonstrated in the case of melittin [15] and can not be a *priori* excluded for equinatoxin II. Furthermore, we noticed that diluted toxin samples are able to lose spontaneously their activity by several fold during course of the experiment. This is an additional reason that the number of sites may be overestimated. Altogether the data presented here leads to the following model of toxin-induced hemolysis and the mechanism of action of Ca^{2+}. They demonstrate for the first time that equinatoxin II induces hemolysis interacting with only limited number of membrane sites. This strongly suggests that a specific receptor must exist on erythrocyte membrane to mediate the action of this toxin. The observed mode of action of Ca^{2+} is also compatible with this view. Indeed, Ca^{2+} applies inside the cell resulting in about 10-fold increase in the rate of toxin binding to RBC membrane although, it seems it does not increase a total number of toxin-binding sites. The increased binding might be probably due to the conformational change of receptor but not of toxin and exposure of additional functional groups that enhances dramatically the affinity of receptor to the toxin. As follows from the kinetic experiments (Fig. 3), such conformational change is reversible since EDTA could eliminate activating effect inflicted by Ca^{2+} in a time-dependent manner. Calmodulin seems to be hardly directly involved in Ca^{2+} activity because known inhibitor of calmodulin chlorpromazine acted in the same direction activating toxin action both in the presence and absence of Ca^{2+} (data not shown). This is in contrast to the action of some calmodulin inhibitors relative to hemolysis induced by viruses, Staphylococcus aureus α-toxin or complement where they act in opposite direction as compared with the action of cations [10].

Thus, the model presented here is compatible with experimental data and gives a new insight in our understanding of the mechanism of action of equinatoxin II which, in some respect, is unique among the other toxins and pore-forming substances.

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