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RESEARCH PAPER

F199E substitution reduced toxicity of *Clostridium perfringens* epsilon toxin by depriving the receptor binding capability

Jingjing Kang[a], Jie Gao[a], Wenwu Yao[b], Lin Kang[b], Shan Gao[b], Hao Yang[b], Bin Ji[b], Ping Li[b], Jing Liu[b], Jiahao Yao[b], Wenwen Xin[b], Baohua Zhao[a], and Jinglin Wang[b]

[a]Life Science Institute of Hebei Normal University, Shijiazhuang, Hebei Province, PR China; [b]State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Beijing, PR China

ABSTRACT

Epsilon toxin (ETX), a potent toxin, is produced by types B and D strains of *Clostridium perfringens*, which could cause severe diseases in humans and domestic animals. Mutant rETX^{F199E} was previously demonstrated to be a good vaccine candidate. However, the mechanism concerned remains unknown. To clarify how F199E substitution reduced ETX toxicity, we performed a series of experiments. The results showed that the cell-binding and pore-forming ability of rETX^{F199E} was almost abolished. We speculated that F199E substitution reduced toxicity by depriving the receptor binding capability of ETX, which contributed to the hypothesis that domain I of ETX is responsible for cell binding. In addition, our data suggested that ETX could cause Ca^{2+} release from intracellular Ca^{2+} stores, which may underlie an alternate pathway leading to cell death. Furthermore, ETX induced crenation of the MDCK cells was observed, with sags and crests first appearing on the surface of condensed MDCK cells, according to scanning electron microscopy. The data also demonstrated the safety and potentiality of rETX^{F199E} as a vaccine candidate for humans. In summary, findings of this work potentially contribute to a better understanding of the pathogenic mechanism of ETX and the development of vaccine against diseases caused by ETX, using mutant proteins.

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Introduction

Epsilon toxin (ETX) is produced by types B and D strains of *Clostridium perfringens* and causes rapidly fatal enterotoxaemia in livestock, mainly sheep, and other sensitive animals including goats and cattle,[1] resulting in heavy economic losses every year.[2,3] ETX is secreted in an inactive prototoxin and is converted to a mature active protein when the N- and C-terminal peptides are removed by proteases, such as trypsin, α-chymotrypsin and λ-protease.[4,5] The toxicity of mature active protein increases by approximately 1000-fold in contrast to the minimally toxic prototoxin.[4] Its lethal activity is slightly below that of the botulinum neurotoxins and the lethal dose by intraperitoneal injection in mice is 65–110 ng/kg[4,6]. The toxin accumulates mainly in the kidneys and brain when injected intravenously into rats, resulting in injury of cerebral blood vessels and neuronal cells.[7] *Clostridium perfringens* is the third most common cause of foodborne illness in the United States.[8,9] Moreover, human cell lines such as the Caucasian renal leiomyoblastoma (G-402) cell line and human ACHN cells are sensitive to ETX.[10,11] Potentially active in humans, ETX is considered to be a potential biological weapon, classified as a category B biological agent by the U.S. Centers for Disease Control and Prevention (CDC).[11]

Circular dichroism (CD) spectroscopy declared that ETX is rich in β-strands.[12] Crystal structure of ETX showed that there are 3 structural domains. Domain I contains a group of key amino acid residues (Tyr196, Phe199, Tyr29, Tyr30 and Tyr36), suggesting a relationship of binding to receptor.[13,14] Domain II is a β-sandwich including a 2-stranded sheet and a 5-stranded sheet, and is likely to be involved in oligomerization.[15] Domain III is also a β-sandwich with one 3-stranded and one 4-stranded sheet, playing a role in mediating ETX insertion into membranes.[16]

ETX toxicity has been tested on a few sensitive cell lines including the Madin Darby Canine Kidney (MDCK), the Caucasian renal leiomyoblastoma (G-402), ACHN, and murine renal cortical collecting duct principal (mpkCCDcl4) cell lines.[17,18] The MDCK cell was commonly used to study the effects of the epsilon toxin.[1,19,20] It is reported that epsilon toxin formed ~2 nm-wide pores in MDCK cells membrane and led to membrane permeabilization which contains a rapid decrease

CONTACT Wenwen Xin xinww@hotmail.com No. 20, Dongda Street, Fengtai District, Beijing, 100071, China; Baohua Zhao zhaobaohua@hebtu.edu.cn No. 20 Road East. 2nd Ring South, Yuhua District, Shijiazhuang, Hebei, 050024, China; Jinglin Wang wjlwjl0801@sina.com No. 20, Dongda Street, Fengtai District, Beijing, 100071, China.

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in intracellular K^+, and an increase in Na^+ and Cl^-, and a delayed increase in Ca^{2+}[21].

Vaccines have been used extensively over the past decades to prevent disease in domesticated livestock, which was demonstrated to be an efficient way.[11,18,22,23] Our laboratory previously constructed mutant protein rETX^{F199E}, which is of low-toxicity but retains their immunogenicity and was selected as a promising candidate vaccine against animal enterotoxemia and human disease caused by *Clostridium perfringens*. The mutant rETX^{F199E} could protect the immunized mice against a challenge of a 100×LD_{50} dose of recombinant wild-type ETX.[6] However, the specific mechanism of the attenuated rETX^{F199E} remains unknown. To investigate whether the 199th amino acid residue is involved in maintaining ETX biological activity, we performed a series of experiments including testing the cytotoxicity of recombinant toxins toward MDCK cells, binding of toxins to MDCK cells, assessing the ability of recombinant toxins to form pores, and analysis of the structure change of the mutant.

Results

Expression and purification

The expressed recombinant toxins have a 6×His-tag at the C-terminal, therefore the recombinant proteins were purified using a Ni^{2+} chelating affinity chromatography resin column.[6] Both rETX^{F199E} and rETX exhibited a high-level soluble expression. We also expressed rETX without His-tag at the C-terminal, and there were no statistical differences between the toxicities of rETX with and without His-tag (Fig. S1). However, in the same scale of expression, the level of soluble expression of rETX with His-tag was higher than that without a His-tag at the C-terminal (Fig. S2). Therefore, rETX with His-tag was used in subsequent assays. Purified rETX^{F199E} and rETX have similar high purity, for only one band was observed in Coomassie brilliant blue stained gel (Fig. S3). Equal amounts of protein were used in the following assays.

Cytotoxicity assay

To detect the activity of recombinant mutant protein, equal amounts of epsilon toxin and the mutant epsilon toxin proteins were added to MDCK cells. Then the viability of MDCK cells was determined using an MTS assay. The CT_{50} (50% lethal dose of cells) was calculated. The result showed that MDCK cells were effectively killed by the wild-type epsilon toxin. In contrast, the mutant protein including a substitution at F199 exhibited an attenuated cytotoxicity (Fig. S4). In addition, cytotoxicity of rETX^{F199E} (CT_{50} was 99,581 ng/mL) on MDCK cells was approximately 553-fold less than that of rETX (CT_{50} was 180 ng/mL). These findings indicated that the amino acid residue F199E substitution had a significant influence on ETX activity ($p < 0.05$).

Thermal stability of rETX^{F199E} mutant protein

A thermal stability assay was performed to analyze the structural difference between rETX and rETX^{F199E}. T_m is a characteristic of protein stability. When the temperature is changed, the structure of protein changed, with exposure of hydrophobic regions. The dye could bind to exposed hydrophobic regions of the unfolded proteins and the fluorescent signal has a significant increase. Therefore, the stability of a protein can be determined by monitoring the changes in fluorescence representing changes in temperature. As shown in Table 1 and Fig. 1, the T_m of the rETX^{F199E} and rETX are 50.00 ± 0.88°C and 60.95 ± 0.33°C respectively. The melting temperature of rETX^{F199E} was determined to be markedly different from that of wild-type epsilon toxin, indicating that some alterations of the structures of rETX^{F199E} had probably occurred, when compared with the wild-type ETX.

Circular dichroism

To find out whether the amino acid residue F199 mutation affects the ETX structure, circular dichroism (CD) spectroscopy was performed. Figure 2 showed the CD spectra and secondary structures proportion of the 2 proteins. The CD spectra of the 2 proteins showed a similar degree of ultraviolet absorption between 200 nm and 260 nm (Fig. 2C), which is expected for proteins rich in β-sheet. A small deviation (between 185 and 200 nm) was observed in the spectrum of mutant proteins rETX^{F199E} compared with the wild-type toxin (Fig. 2C). To further evaluate the CD spectra, the spectra were analyzed using the CDNN software (Gerald,. B. CD spectroscopy Deconvolution, version 2.1, 1997). The wild-type and mutant proteins were predicted to have similar compositions of α-helix, β-strand, β-turns, and unordered structure (Fig. 2A and B). No obvious differences were observed between the secondary structures proportion of the 2 proteins (Fig. 2D). This result indicated that rETX^{F199E} and rETX possess similar structures.

Binding to MDCK cells

Results from the cytotoxicity suggested that the cytotoxicity of the rETX^{F199E} mutant protein had a significant decrease ($p < 0.05$). It is reported that clustered and surface-accessible aromatic amino acids can mediate binding of the toxin to cells and its receptor.[15] To confirm the interaction between the rETX^{F199E} and MDCK cells, On-Cell Western assay was performed. The MDCK cells were incubated with mutant protein (4 μg/mL or 40 μg/mL) for 30 minutes at 37°C. As shown in Fig. 3, rETX was more likely to bind to MDCK cells. In contrast, the mutant protein rETX^{F199E} was defective in binding to MDCK cells. The binding ability of rETX^{F199E} was almost abolished, indicating that the amino acid residue F199E substitution had substantial influence on ETX binding ($p < 0.05$). To further study the ability of the mutant protein to bind to MDCK cells, the confocal microscopy assay was performed and the

Table 1. The Tm (°C) of recombinant toxins.

Groups	Tm ± SD(n = 8)
rETX	60.95 ± 0.33°C
rETX^{F199E}	50.00 ± 0.88°C
PBS	—
Water	—

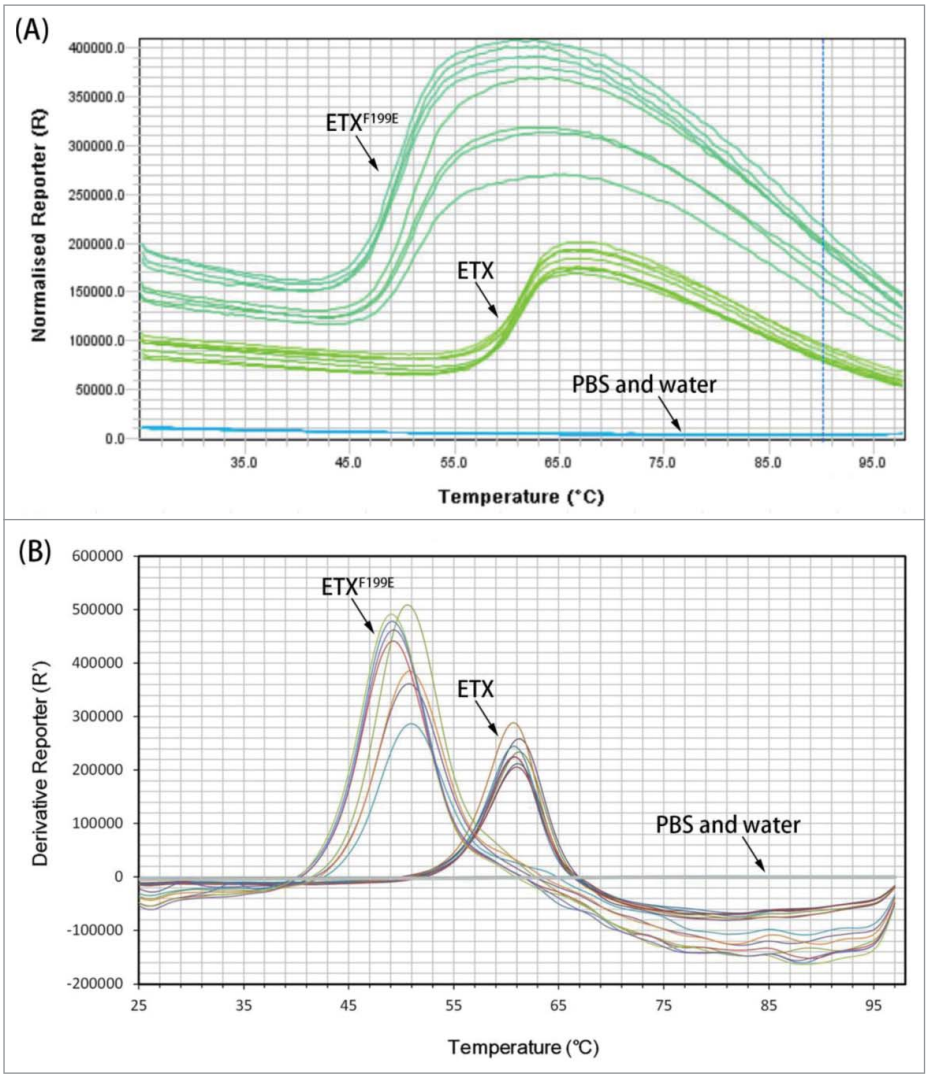

Figure 1. Melt curve of recombinant toxins. (A) Fluorescence signal versus temperature. (B) Derivative value of fluorescent signal. In this assay, 8 replicates were used for each sample.

result showed that the binding ability of rETX^{F199E} to MDCK cells was substantially reduced than that of rETX (Fig. 4). The result of confocal microscopy assay is in close agreement with that of the On-Cell Western assay.

Heptamer formation

Both the On-Cell Western assay and confocal images of MDCK cells against toxins showed that rETX^{F199E} was not able to bind to MDCK cells. It has been previously reported that wild-type epsilon toxin could bind to cells and formed a heat-and SDS-resistant oligomeric complex in MDCK cell membranes.[14,24] Thus, a western blot assay was performed to test whether the amino acid residue F199E substitution could prevent the toxin from heptamer formation. As expected, wild-type epsilon toxin could bind to cells and formed heptamer formation, approximately 200 kDa (Fig. 5). However, rETX^{F199E} was defective in heptamer formation. These data probably suggest that the amino acid residue F199E substitution affected the heptamer formation of epsilon toxin.

Determination of intracellular calcium concentration

In a previous study, epsilon toxin was shown to be a pore-forming toxin, assembling into oligomeric complexes in the plasma membrane of sensitive cells and leading to dysregulated ion homeostasis including a decrease in intracellular K^+ and an increase in intracellular Ca^{2+}, which could cause cell death.[7,21] To investigate whether rETX^{F199E} could alter the intracellular Ca^{2+} concentration, we examined the degree of fluctuations of intracellular Ca^{2+} concentration in MDCK cells. Figure 6A showed that rETX induced a significant increase in the intracellular Ca^{2+} concentration in MDCK cells when the extracellular Ca^{2+} was available ($p < 0.05$). The intracellular Ca^{2+} concentration reached the peak after 10–20 min. However, the rETX^{F199E} mutant protein could not lead to an increase in intracellular Ca^{2+} concentration. The same result was achieved when the toxin concentration was 10-fold higher (Fig. 6B). The 2 control groups were examined using buffer without Ca^{2+}. Surprisingly, rETX was still able to induce a significant increase in the intracellular Ca^{2+} concentration in MDCK cells when the extracellular Ca^{2+} was absent, although the spike of Ca^{2+} was delayed. This result indicated that intracellular Ca^{2+} stores were also involved in ETX induced cytotoxicity.

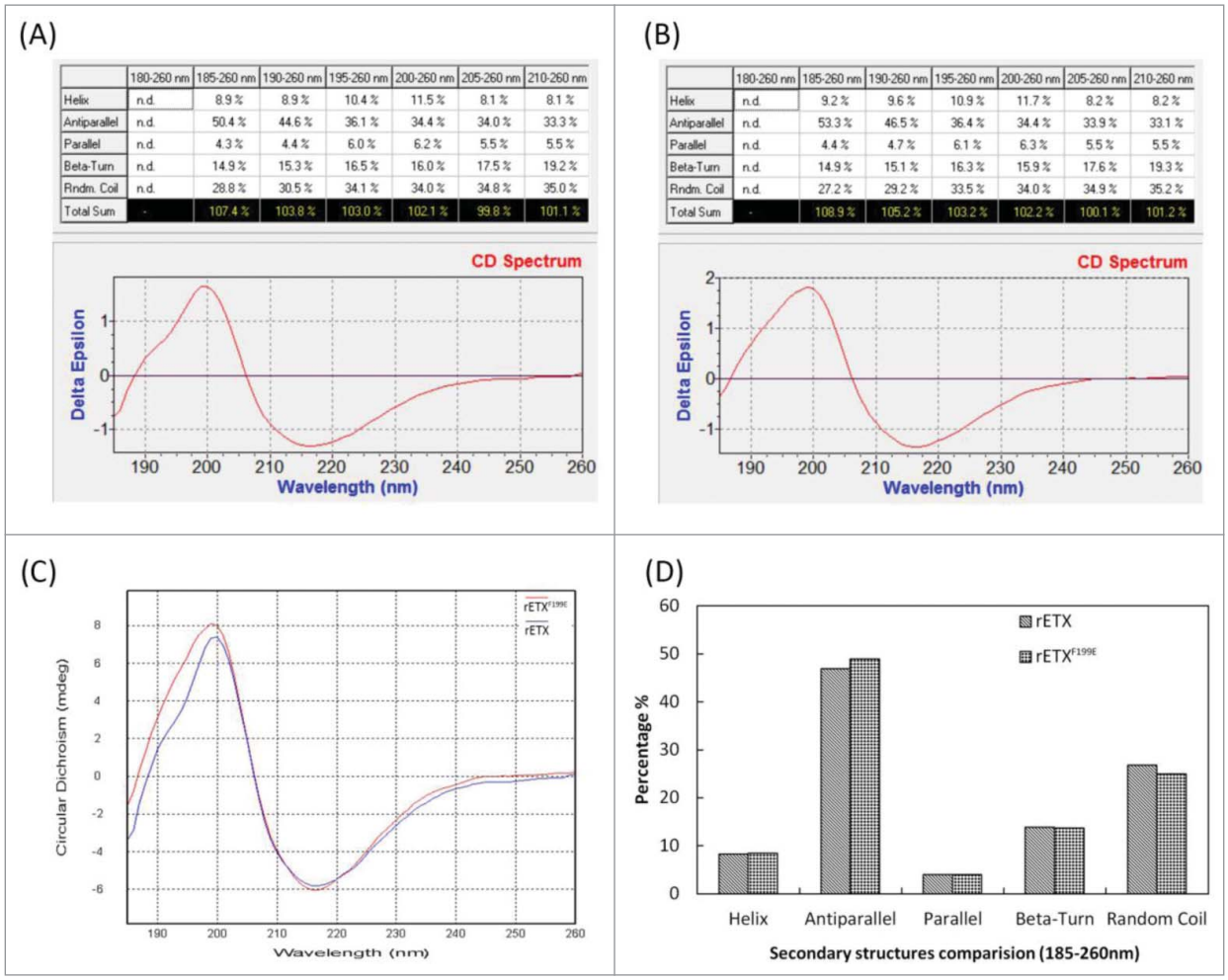

Figure 2. CD spectra and secondary structures proportion of wild-type and mutant epsilon toxin. (A) The CD spectra and secondary structures proportion of rETX. (B) The CD spectra and secondary structures proportion of rETX^{F199E}. (C) Comparison between the CD spectra of the 2 proteins. (D) Relative ratio of different types of secondary structures of the 2 proteins.

Scanning electron microscopy

It was reported that the diameter of the membrane pore formed by epsilon-toxin in MDCK cells was at least 2 nm.[21,25] To further survey pore-forming function in the plasma membrane of MDCK cells, scanning electron microscopy was performed. The result showed that there are no obvious differences between MDCK cells treated with PBS (Fig. 7A and D) and those treated with rETX^{F199E} (Fig. 7B and E). In contrast, sags and crests were observed on the surface of MDCK cells when MDCK cells were treated with wild-type epsilon toxin (Fig. 7C and F), which was rarely seen on the surface of MDCK cells treated with rETX^{F199E}. In addition, cells treated with rETX were condensed (Fig. 7C). It is likely that the amino acid residue F199E substitution causes this difference.

Discussion

Our previous work showed that rETX^{F199E} is potentially a good vaccine candidate, possessing extremely low cytotoxicity and strong immunogenicity and providing a powerful protection against ETX challenge in mice. Substitution of the 199th amino acid dramatically abolished the toxicity of ETX. However, the specific mechanism remains unknown. To clarify how F199E substitution reduced ETX toxicity, we performed a series of experiments.

The expressed recombinant ETX (rETX) with His-tag (without 13 N-terminal and 23 C-terminal residues) was used as the

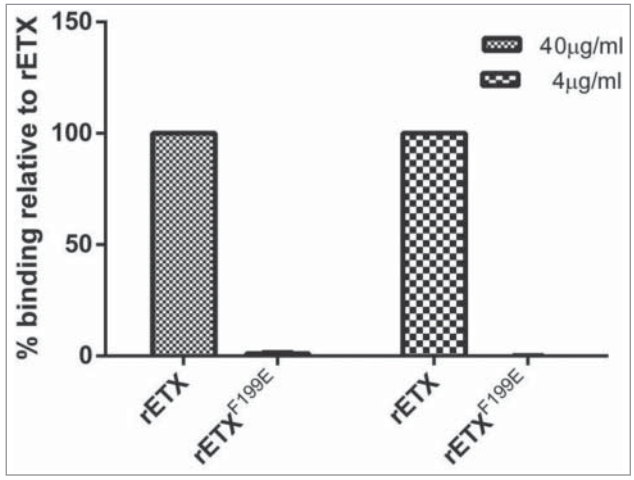

Figure 3. Binding of the recombinant toxins to MDCK cells. MDCK cells were mixed with 100 μl of toxins (4 μg/mL or 40 μg/mL). Mouse anti-His monoclonal antibody (1:500) and FITC conjugated goat anti-mouse IgG (1:200) was used to detect the bound protein. Values are mean ± SD, n = 3.

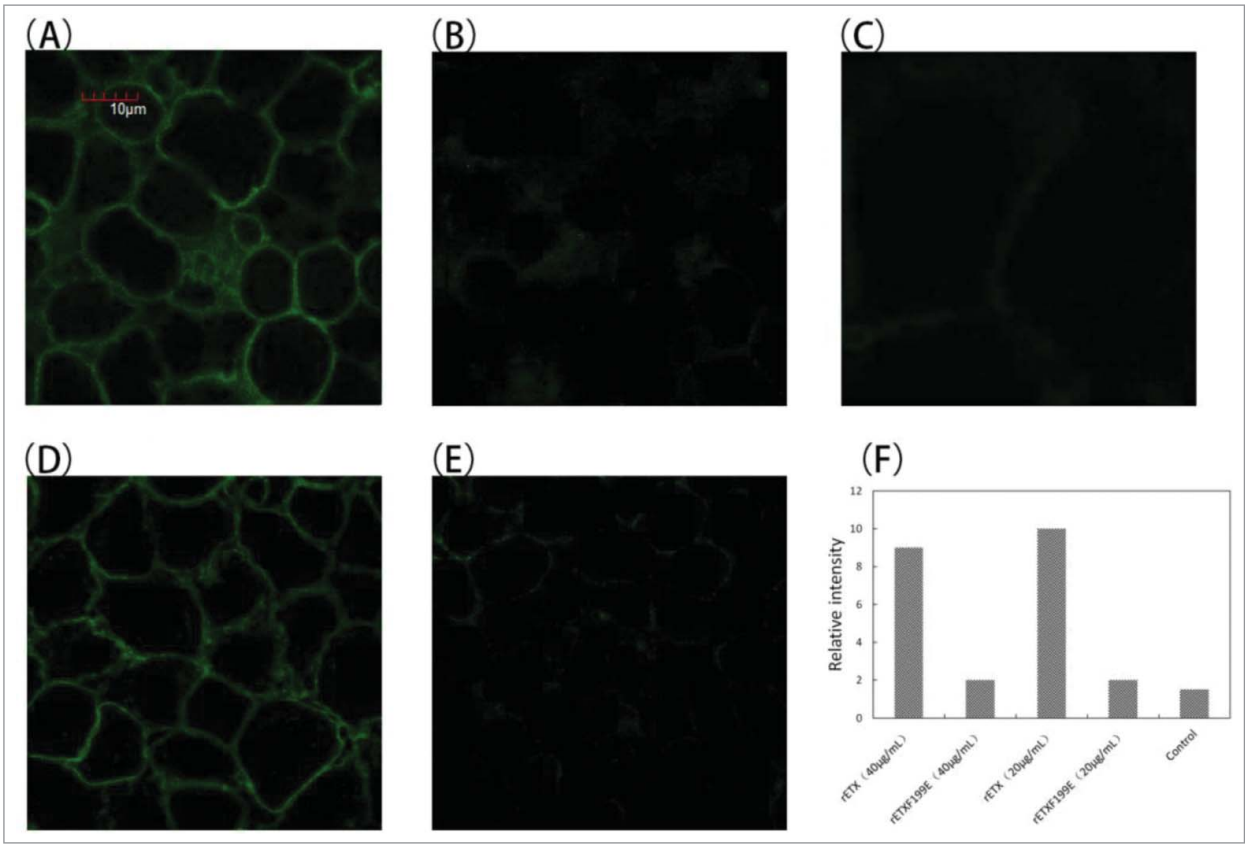

Figure 4. Binding of toxins on the surface of MDCK cells. Confocal microscopy assay was performed as described in experimental procedures to test the binding proteins. (A) Cells were treated with rETX(20 μg/ml), (B) cells were treated with rETX^{F199E}(20 μg/ml), (C) cells were treated with PBS, (D) cells were treated with rETX(40 μg/ml), (E) cells were treated with rETX^{F199E}(40 μg/ml), (F) Relative fluorescent intensity of photos of the 5 groups. Cells were fixed and stained with anti-His monoclonal antibody and goat anti-mouse IgG (H + L).

substitution of the natural ETX. We demonstrated that 6×His-tag did not reduce the activity of rETX.

Amino acid F199 was previously demonstrated to be located within a possible cell-binding motif.[15] Previous studies also concluded that rETX^{F199E} may be misfolded based on thermal stability assay and the deviation observed in the CD spectrum without further study.[14] However, the 3-dimensional structure of rETX^{F199E} was previously modeled based on homology-modeling, indicating that there was no obvious change in the structure of rETX^{F199E} compared with wild-type ETX. The

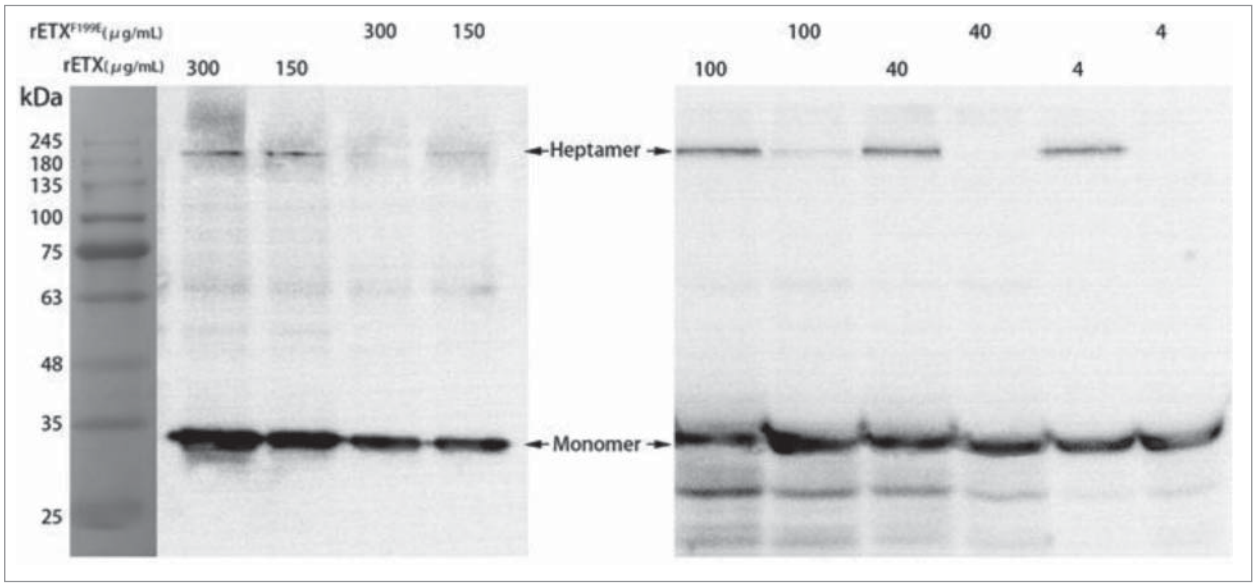

Figure 5. Analysis of heptamer formation by rETX and rETX^{F199E}. MDCK cells were treated with 200 μl of toxins (4 μg/mL to 300 μg/mL) for 30 min at 37°C. Samples were solubilized, and analyzed by SDS-PAGE, and then immunoblotted with an anti-His monoclonal antibody and a HRP-coupled goat anti-mouse IgG antibody (1:50,000). The result was photographed using an AE-1000 cool CCD image analyzer.

melting temperatures of rETX^{F199E} and rETX were different by as much as 10°C, which is in close agreement with the previous study.[14] This phenomenon indicated that the structures of rETX^{F199E} probably possessed some changes when compared with wild-type ETX. However, the CD spectrum in this study indicated that rETX^{F199E} and rETX possessed similar structures, and the difference between the 2 proteins was not as obvious as described previously.[14] Moreover, rETX^{F199E} was found to exhibit similar immunogenicity and antigenicity with rETX.[26] Also, rETX^{F199E} was highly expressed in a soluble form.[26] Therefore, we speculated that there was a possibility that rETX^{F199E} may not be misfolded, and how F199E substitution reduced ETX toxicity was further studied in this work. Our study showed that the F199E substitution could significantly reduce the ability of the toxins to bind to MDCK cells. The binding capability of rETX^{F199E} to MDCK cells was largely abolished, suggesting that F199 is implicated in receptor binding. Previous research showed that F199 was involved in a possible cell-binding motif consisting of a set of amino acids (Y29, Y30, Y36, Y196 and F199).[15,27,28] Phenylalanine is a hydrophobic residue in the cluster of aromatic residues. Likewise, the hydrophobic tryptophan (W190) in domain I has also been previously implicated in receptor binding.[15,27] The results contributed to the hypothesis that domain I of ETX is responsible for cell binding. However, the actual receptor of ETX has not been determined yet and consequently the exact region of ETX that interacted with the receptor remains unknown. Some proteins have been suggested as potential receptors for ETX such as hepatitis A virus receptor 1 (HAVCR1)[17] and the myelin and lymphocyte protein (MAL).[29] However, there is no sufficient evidence to confirm the identity of the receptor of ETX. Bokori-Brown suggested that domain III containing a β-octyL-glucoside-binding region is probably functioned in receptor binding.[13] Our previous study also showed that C-terminal peptide reduced toxicity of ETX probably *via* covering receptor-binding region in domain III.[30] Therefore, there is still a possibility that the receptor binding region is located in domain III.

The inconsistency between the thermal stability and CD analysis of rETX and rETX^{F199E} actually occurred. This may be because CD analysis mainly reflects the secondary structure, while thermal stability reveals the conformation of the protein. Therefore, we speculated that the polypeptide chain of rETX^{F199E} was correctly folded into secondary structures, and some changes occurred when the secondary elements were folded into a 3-dimensional structure. Conformation of a protein is flexible and is more likely to change with the substitution of an amino acid.

Faint heptamer signal of rETX^{F199E} appeared when high concentrations (no less than 100 μg/mL) of mutant toxins were used in immunoblotting assay, indicating very low cytotoxicity of rETX^{F199E}. Actually, rETX^{F199E} exhibit toxicity on MDCK cells at high concentrations and the CT_{50} of rETX^{F199E} was approximately 100 μg/mL[26].

Many studies have shown that ETX affects its targets by forming pores. However, an increasing number of studies suggested that an alternate pathway leading to cell death was also involved. Co-existence of pore-forming and non-pore-forming actions has been proposed for ETX on renal cells.[31] Wioland et al reported that ETX induced an increase in extracellular glutamate, and produced oscillations of intracellular Ca^{2+} concentration in oligodendrocytes and these effects occurred without any change in the transmembrane resistance of

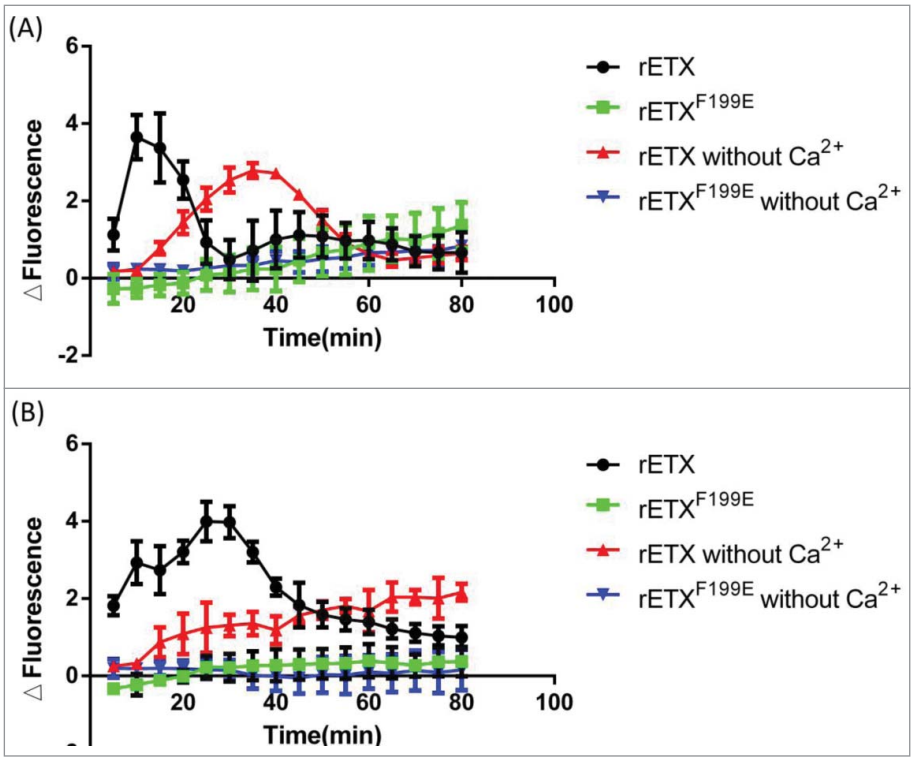

Figure 6. Ability of recombinant toxins to trigger an increase in intracellular Ca^{2+}. Changes in intracellular calcium were monitored using Fluo-8®AM as described in Experimental Procedures. MDCK cells were incubated with 4 μg/ml toxins (A) or 40 μg/ml toxins (B) and fluorescence was measured at 5 minute intervals in 80 min. Data points represent mean values ± SD, n = 3.

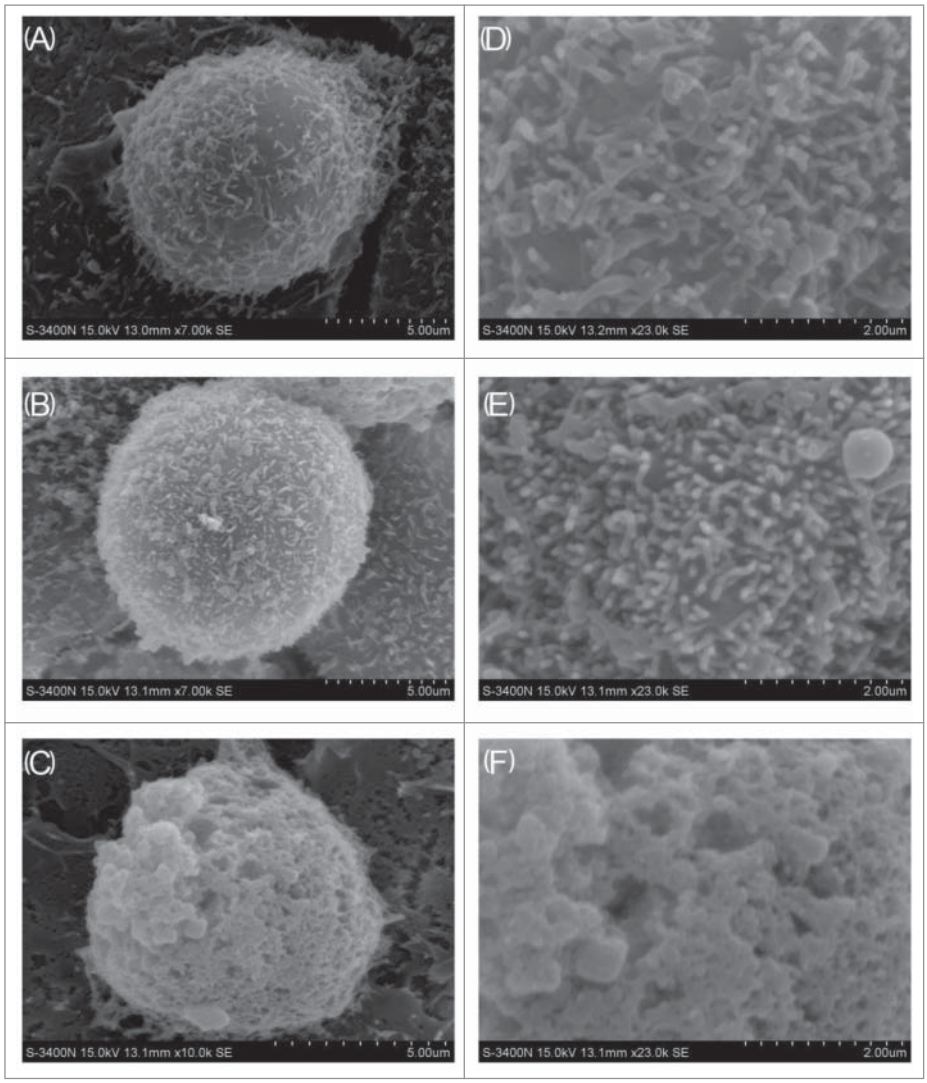

Figure 7. Morphological effect of toxins. Scanning electron microscopy photos of MDCK cells treated with PBS (A), rETX^{F199E} (B), and wild-type epsilon toxin (C). D, E, and F are partial enlarged views of A, B, and C, respectively.

oligodendrocytes, inferring that ETX acts through a pore-independent mechanism.[32] Moreover, demyelination occurs in very low ETX concentrations (< 0.01nM)[33] at which it has not been established that ETX can form heptamers. We also found that extracellular Ca^{2+} and intracellular Ca^{2+} stores were both involved in ETX induced increase in intracellular Ca^{2+} concentration. In MDCK cells Ca^{2+} was released from intracellular stores by ETX when the extracellular Ca^{2+} was absent. This suggested that ETX not only induced Ca^{2+} inflow by forming pores, but also could lead to an increase in intracellular Ca^{2+} by an alternate pathway.

Previous studies demonstrated that the increase in intracellular Ca^{2+} correlated with the formation of large pores in MDCK cells.[21,34] The increase in intracellular Ca^{2+} was consequently considered as a sign of pore forming.[14] However, Wioland et al reported ETX produces oscillations of intracellular Ca^{2+} concentration when ETX acts using non pore-forming mechanisms.[32] We also found ETX could lead to the release of intracellular Ca^{2+} stores, which are probably not dependent on pore forming. In addition, there is still a possibility that epsilon-toxin modified a specific ion channel which could increase the cell membrane permeability.[7] Therefore, the fluctuation of intracellular Ca^{2+} was considered as an index of cellular effect of ETX on MDCK cells rather than an index of pore-formation.

Pore-forming toxins like *Staphylococcus aureus* α-toxin and Streptolysin O commonly bind to receptors on cell membrane as monomers, followed by formation of polymers, with the formation of polymers requiring the toxins to first bind to the receptors on the target cell membrane.[35,36] We also found that the ability of rETX^{F199E} to form a complex was reduced or even abolished, in an extent similar to that of reduction in cell binding, indicating that receptor binding is required to form heptamers or pores. Therefore, receptor binding is believed to be the first step by which ETX functions as a cytotoxin.

It was previously reported that *Escherichia coli* hemolysin A (Hly A) specifically binds to receptors on the cell membrane at low concentrations while unspecifically binds to the cell membrane at high concentrations.[37,38] This result showed that the binding capability of rETX^{F199E} was abolished at both low and high concentrations and toxicity of rETX^{F199E} was eliminated as well. Therefore, the cell binding of ETX is different from Hly A and receptor-binding is required consistently at varied concentrations of toxins. In addition, the binding capability of wild type ETX was saturable, suggesting that the binding of ETX is

receptor dependent. This finding is consistent with those of previous studies, which have shown that there is a putative protein receptor located at detergent resistant membrane (DRMs) in the apical cell membrane.[7,24,29,39] However, previous studies also showed that epsilon toxin was capable of forming channels in lipid bilayers without the need of a receptor, although with less efficiency.[21,25] We also found that rETX could occasionally form slight heptamer in PBS without cells. This is not contradicted to receptor-mediated binding of cells, as many cytolytic toxins such as α-toxin from *S. aureus* and aerolysin from *Aeromonas sobria* form pores in lipid bilayers in the absence of receptors, whereas they all need a receptor for biological activity.[21]

Epsilon toxin could form pores in the membrane of MDCK cells, however, few studies observed the changes of the membrane surface. It was reported that the morphological effects of epsilon toxin on cells commonly included a condensation of the nucleus and a progressive swelling of the cells.[1] We found that within the first 60 minutes, ETX induced crenation of the MDCK cells as a result of cell shrinkage, followed by a gradual volume increase and finally lysis of the cells (Fig. S5). This is probably because ETX initiates a series of events including the early intracellular decrease in the monovalent ions K^+ and Cl^-, slightly delayed for Na^+ increase, and the slower increase in Ca^{2+} [21], which causes a decrease in intracellular osmolality first and an increase later. Also, sags and crests were observed on the surface of the condensed MDCK cells in scanning electron microscopy. This indicated that the membrane was extremely disrupted after the shrinkage and swelling. In addition, all the cells observed in scanning electron microscopy appeared in a round shape and almost detached. This may be because MDCK cells exhibited weak adhesion to glass coverslip used in this assay. Most of the cells dropped off after rinsing and dehydrating. The rest of the cells appeared round and were almost detached.

The mutant protein $rETX^{F199E}$ exhibited reduced cytotoxicity, binding, and pore-forming activity on MDCK cells. Previous studies have shown that mutant ETX proteins with amino acid substitutions near F199 are defective in binding MDCK cells, but are not defective in binding human ACHN cells.[13] Binding assay of toxins on human ACHN cells was also performed, and we found rETX was more likely to bind to ACHN cells. In contrast, $rETX^{F199E}$ was defective in binding to ACHN cells (Fig. S6). The cytotoxicity assay of toxins in ACHN cells was also performed, and the CT_{50} (50% lethal dose of cells) of ETX in ACHN cells was 157.08 μg/mL. However, $rETX^{F199E}$ did not show any cytotoxicity in ACHN cells (Fig. S6). Actually, it is not clear if $rETX^{F199E}$ is defective in binding other target cells such as the cerebellar granule cells, because it is not known whether ETX exploits a single receptor shared by all possible targets. For instance, MAL is a likely receptor candidate expressed by MDCK and brain oligodendrocytes[29] but not expressed by neurons. However, some of neurons such as the cerebellar granule cells are ETX targets.[40]

Some species of *Clostridium perfringens* are able to cause severe diseases in humans.[11] Toxoid vaccines against enterotoxemia are widely used in animals.[18] However, there is no vaccine for humans against disease caused by ETX currently. Moreover, variable immune responses and inflammatory responses following vaccination of toxoid were reported.[18,41] Therefore, toxoid vaccines could hardly be used in humans. Amino acid substitution of a protein toxin is a promising way to develop a mutant vaccine for human. The notoxicity of $rETX^{F199E}$ in human ACHN cells indicates that $rETX^{F199E}$ could be potentially used in humans as a vaccine candidate.

Conclusions

In this study, the mechanism of attenuated toxicity of $rETX^{F199E}$ was clarified. The data demonstrated that F199E substitution reduced toxicity by abolishing the receptor binding capability of ETX on MDCK cells and ACHN cells. Whether $rETX^{F199E}$ is defective in binding other target cells such as the cerebellar granule cells is not clear. We found that ETX could cause the Ca^{2+} release from intracellular Ca^{2+} stores. It is demonstrated for the first time that the intracellular Ca^{2+} stores contribute to the increase in Ca^{2+} level in ETX treated MDCK cells, which may be an alternate pathway leading to cell death. This finding provides new insight into the cytotoxic mechanism of ETX. The data also demonstrated the safety of $rETX^{F199E}$, which contributes to our previous work that $rETX^{F199E}$ is potentially a good vaccine candidate for animals and humans. More studies will be required to explore $rETX^{F199E}$ as a vaccine for humans. With the inefficiency of receptor binding of $rETX^{F199E}$, this mutant protein could also be considered as a platform for receptor binding studies. In summary, these findings potentially contribute to understanding the pathogenic mechanism of ETX and the development of vaccine against diseases caused by ETX, using mutant proteins. Unfortunately, the actual receptor of ETX has not been determined yet, although many studies are attempting to find the answer. Consequently, many questions remain unanswered such as the exact region of ETX that interacts with the receptor. Receptor studies will answer many of the questions and provide targets to develop antidotes against ETX intoxication or enterotoxemia, which should be further investigated in the future.

Materials and methods

Expression and purification of $rETX^{F199E}$ and rETX

Our laboratory constructed the recombinant plasmids pTIG-His-*etx* and pET-His-*etx*^{F199E}, which expressed rETX (without the 13 N-terminal and 23 C-terminal sequences) and $rETX^{F199E}$ (rETX containing an F199E mutation) respectively as described previously.[6,42] The $rETX^{F199E}$ and rETX were expressed in soluble forms in the *E. coli* BL21 (DE3) strain induced by 0.5 mM IPTG overnight at 16°C. The recombinant epsilon toxins were purified using an Ni^{2+} affinity chromatography column (GE Healthcare, United States) as described previously.[6,42] The purified proteins were analyzed using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. The gel was stained by Coomassie brilliant blue.

Cytotoxicity

MDCK cells were bought from The Chinese Academy of Sciences. A sensitive clone was achieved and authenticated to ensure

the consistency of MDCK cells. MDCK cells (3∼4 × 10^4 cells/well) were grown in 96-well plates with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and cultured in a 5% CO_2 incubator for 24 h at 37°C. The toxin protein used in this assay was freshly purified. The cells were incubated with serial dilutions of toxin protein in DMEM (100-μl final volume in each well) for 24 h at 37°C in a 5% CO_2 incubator. An equal volume of DMEM culture was added to 96-well plates and used as a blank. After washing with phosphate-buffered saline (PBS) 3 times, each well (20 μl) was treated with 100 μl of culture medium containing 20 μl of MTS (3-(4, 5-dimethylthiazol-2-yl) -5(3-carboxymethoxyphenyl) -2-(4-sulfopheny)-2H-tetrazolium, inner salt) (Promega, United States) at 37°C for 3∼4 h. The absorbance value at 490 nm was then measured using a SPECTRA MAX plus plate reader (Molecular Devices, United States). Percentage of cell viability was confirmed as follows: the mean absorbance value of an experimental group/that of a control.[43] The CT_{50} of the toxins was calculated using the improved Karber's method according to the formula as described previously.[26]

Binding of mutant toxin to MDCK cells

MDCK cells (3∼4 × 10^4 cells/mL) were seeded in a 96-well plate with DMEM containing 10% FBS for 24 h at 37°C. Monolayers were mixed with 100 μl of toxins (4 μg/mL or 40 μg/mL). Mouse anti-His monoclonal antibody (EarthOx, United States) (1:500) and FITC conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, United States) (1:200) was used to detect the bound protein. The fluorescence signal was detected using the Varioskan Flash Multiplate Reader (Thermo Scientific, United States).

Confocal microscopy assay

MDCK cells (1 × 10^5 cells/well) were cultured in cell culture dishes (NEST, shanghai, China) for 24 h and treated with 2 different concentrations of toxin respectively. The cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then washed 3 times with 200 μl PBS. BSA (5%) was used to block the dish for 2 h. Monolayers were incubated for 60 min at room temperature with the anti-His monoclonal antibody after being washed 3 times with PBS and then were washed another 3 times. The cells were incubated with FITC conjugated goat anti-mouse IgG (H + L) for 45 min at room temperature. After being washed 3 times again with PBS, the cell culture dishes were observed using IX81 confocal microscopy (Olympus, Japan). Confocal images were produced with exposure time of 19.481s, HV of 800, C.A. of 135, and offset of 6%.

Heptamer formation

MDCK cells were plated at 1∼2.5 × 10^5 cells per well in a 24-well plate for 24 h at 37°C, and the cells were treated with 200 μl of mutant proteins (4 μg/mL to 300 μg/mL) for 30 min. Toxin was removed and the cells were subsequently washed with PBS. Cells in each well were lysed in 300 μl of lysis buffer (1% SDS, 1% TritonX-100, 50 mM Tris-HCl, pH 7.4). Lysates were heat denatured at 100°C for 5 min and electrophoresed on a 12% SDS polyacrylamide gel, and were analyzed by immunoblotting using an anti-His monoclonal antibody followed by a HRP-coupled goat anti-mouse IgG antibody (Beijing TransGen Biotech Co, China). The result was analyzed and photographed using the AE-1000 cool CCD image analyzer.

Intracellular calcium determination

Cell monolayers in 96-well plates were incubated with 0.1 ml HBSS (5 mM KCl, 6 mM glucose, 12 mM $MgCl_2$, 125 mM NaCl, 25 mM HEPES, pH 7.5) containing 4 mM Fluo-8®AM (AAT Bioquest, United States) in a 5% CO_2 incubator for 1 h at 37°C. The cells were washed 3 times with 0.2 mL of HBSS, and then the mutant protein was diluted with HBSS containing or without calcium chloride and added to the well. Fluorescence signals associated with Ca^{2+} concentrations were determined using Varioskan Flash Multiplate Reader (Thermo Scientific, United States).

Thermal stability

Thermal stability assay of the recombinant toxins $rETX^{F199E}$ and rETX was performed using the Protein Thermal Shift Dye Kit (Applied Biosystems, United States) according to the manufacturer's instructions. 12.5 μl of toxins (0.1 to 1 mg/mL stock), 5 μl of Protein Thermal Shift Buffer and 2.5 μl of Diluted Protein Thermal Shift Dye (8×) were added to strips of 8 tubes and were pipetted up 10 times to mix well. The melting temperature (Tm) was determined using the StepOnePlus Real-Time PCR system (Applied Biosystem, United States) with a 1% thermal gradient from 25°C to 99°C. The measured channel was set as ROX with an excitation filter of 580 ± 10 nm and an emission filter of 623 ± 14 nm.

Circular dichroism

Circular dichroism (CD) spectroscopy was performed in Tsinghua University. CD spectra in the far UV region (185–260 nm) were obtained with a Chirascan plus spectropolarimeter (Applied Photophysics, United Kingdom) using a 0.1 cm path length cuvette. Protein was buffer exchanged into 15 mM phosphate buffer (pH 7.4). Spectra were obtained using the average of 3 scans with a data pitch of 1 nm, time-per-point of 0.5 s, and bandwidth of 1 nm. The temperature was controlled at 37°C throughout data acquisition.

Scanning electron microscopy

For scanning electron microscopy study, MDCK cells (1 × 10^5 cells/well) were grown to confluence in a round glass coverslip (12 mm diameter, Electron Microscopy Sciences). The coverslips were placed in a 24-well plate in a 5% CO_2 incubator for 24 h at 37°C and then incubated with 300 μl toxins (40 μg/ml or 20 μg/ml) for 20 min. The samples were immediately fixed in 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for 4 h. Then the samples were rinsed, dehydrated in graded alcohol and acetone, and the samples were baked, plated with gold. Finally, such cultures and untreated control were

analyzed and photographed by S-3400N scanning electron microscopy (HITACHI, Japan).

Statistical analysis

Cell binding assay data and Pore-forming assay data were analyzed using analysis of variance (ANOVA) and student's paired t-test. $^{*}P < 0.05$ represents statistical significance between the 2 groups.

Abbreviations

CD	Circular dichroism
CDC	Centers for Disease Control and Prevention
ETX	*Clostridium perfringens* epsilon toxin
FBS	fetal bovine serum
MDCK	the Madin Darby Canine Kidney cell line
PBS	phosphate-buffered saline

Disclosure of potential conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Author contributions

Wenwen Xin, Baohua Zhao and Jinglin Wang conceived and designed the experiments; Jingjing Kang performed the experiments; Jie Gao and Wenwu Yao analyzed the data; Lin Kang, Shan Gao, Hao Yang, Bin Ji, Ping Li, Jing Liu and Jiahao Yao contributed reagents/materials/analysis tools; Jingjing Kang and Wenwen Xin wrote the paper.

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