Observing the Confinement Potential of Bacterial Pore-Forming Toxin Receptors Inside Rafts with Nonblinking Eu³⁺-Doped Oxide Nanoparticles

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ABSTRACT We track single toxin receptors on the apical cell membrane of MDCK cells with Eu-doped oxide nanoparticles coupled to two toxins of the pore-forming toxin family: α-toxin of Clostridium septicum and ε-toxin of Clostridium perfringens. These nonblinking and photostable labels do not perturb the motion of the toxin receptors and yield long uninterrupted trajectories with mean localization precision of 30 nm for acquisition times of 51.3 ms. We were thus able to study the toxin-cell interaction at the single-molecule level. Toxins bind to receptors that are confined within zones of mean area 0.40 ± 0.05 μm². Assuming that the receptors move according to the Langevin equation of motion and using Bayesian inference, we determined mean diffusion coefficients of 0.16 ± 0.01 μm²/s for both toxin receptors. Moreover, application of this approach revealed a force field within the domain generated by a springlike confining potential. Both toxin receptors were found to experience forces characterized by a mean spring constant of 0.30 ± 0.03 pN/μm at 37°C. Furthermore, both toxin receptors showed similar distributions of diffusion coefficient, domain area, and spring constant. Control experiments before and after incubation with cholesterol oxidase and sphingomyelinase show that these two enzymes disrupt the confinement domains and lead to quasi-free motion of the toxin receptors. Our control data showing cholesterol and sphingomyelin dependence as well as independence of actin depolymerization and microtubule disruption lead us to attribute the confinement of both receptors to lipid rafts. These toxins require oligomerization to develop their toxic activity. The confined nature of the toxin receptors leads to a local enhancement of the toxin monomer concentration and may thus explain the virulence of this toxin family.

INTRODUCTION

Single-molecule tracking (SMT) is a powerful approach used to study the complex motion of biomolecules and lipids in living cells (1,2). Labels currently used in fluorescent SMT either suffer from photobleaching, which limits the time a molecule can be tracked, or exhibit blinking, which leads to interrupted trajectories (3) that require complex algorithms to be reconstituted. These problems have led to the synthesis of new generations of fluorophores partially protected from photobleaching (4,5) and quantum dots that do not blink (6,7). In parallel, rare-earth doped nanoparticles (8) and subsequently nano-diamonds (9,10) were introduced. These particles do not blink and are photostable over long periods of time. Recently, up-conversion excitation of rare-earth doped nanoparticles has yielded nonblinking emission under near-infrared excitation (11–13). However, none of these nonblinking fluorescent particles have been used to track single biomolecules. We will show tracking of two toxin receptors in live cells using single nonblinking rare-earth doped nanoparticles. Single-molecule tracking is particularly relevant for toxins because they act at very low concentrations.

We investigate the motion of two bacterial protein toxins, the α-toxin of Clostridium septicum (CSαT) and ε-toxin of Clostridium perfringens (CPεT), both members of the pore-forming toxin family, and their respective receptors in Madin-Darby canine kidney (MDCK) cells. These toxins are secreted by the bacterium as prototoxin monomers, cleaved at their N- and/or C-terminal side to yield activated toxins, bind to receptors on the cell membrane, oligomerize, and form pores that pierce the cell membrane with a β-barrel and cause the death of the cell by uncontrolled ion exchange (14). Through this complicated mechanism, these toxins maintain an extremely high lethality in mice of 10 µg/kg and 100 ng/kg, for CSαT and CPεT, respectively (15,16). The α-toxin binds via glycoporphosphatidylinositol-anchored (GPI-anchored) protein receptors (17,18) to the cell membrane, which have been shown to localize into lipid rafts (19–23) or detergent-resistant membrane domains (DRMs) (24–26), i.e., the fraction of the cell that is not dissolved by a detergent. In contrast to the CSαT, the CPεT receptor has not yet been identified but it has been shown by biochemical methods that CPεT binds to a 37-kDa membrane protein (27), possibly the hepatitis A virus cellular receptor 1 (28), and also acts in DRMs (29,30). To our knowledge, no previous studies on toxin-cell interaction dynamics of pore-forming toxins exist, other than experiments on the binding properties of ensembles of toxin molecules (31).

SMT with nonblinking probes can give long, uninterrupted trajectories that can be used to study the membrane
architecture. The often non-Brownian diffusion of membrane molecules can originate from various mechanisms: lipid rafts (20,21,32–34), cytoskeleton barriers (35–39), tethering to the cytoskeleton (40–42), crowding of molecules (43,44), and intramolecular interactions (45,46). All of these mechanisms can restrict the motion of a molecule so far that it becomes confined. Confined trajectories are often analyzed using the mean-square displacement (MSD) analysis (2,47–49), which gives information about the domain size and diffusion coefficient. The analysis of the density distribution of observed positions gives information about the confining potential (42).

This study investigates the toxin-cell interaction and the motion of the CSaT and the CPeT receptors by SMT with Eu-doped oxide \( \text{Y}_0.6\text{Eu}_{0.4}\text{VO}_4 \) nanoparticles (NPs). First, we demonstrate that these nonblinking nanoparticles are suitable probes for SMT. The obtained trajectories are analyzed by a recently introduced method based on Bayesian inference (51) that extracts most of the information stored in the trajectory, in contrast to the standard MSD analysis, and in particular, the force field felt by the toxin receptor. Biochemical control data show that the toxin-receptor confinement domains we observe require cholesterol and sphingomyelin. Thus, the confinement domains can be attributed to rafts and the potential extracted by the inference to the potential felt by the receptors inside these rafts. These data improve our understanding of how the toxins exploit the cell membrane architecture to achieve high lethality.

**METHODS**

**Single-molecule tracking**

Tracking experiments were performed with a wide-field inverted microscope (Axiovert 100; Zeiss, Oberkochen, Germany) equipped with a 63×, NA = 1.4 oil immersion objective and an electron-multiplying charge-coupled device (QuantEM:512SC; Roper Scientific, Trenton, NJ). NPs were excited with an Ar+ ion laser using the 465.8 nm line. Emission was collected through a 617/8M filter (Chroma Technology, Bellows Falls, VT). Confluent cells on coverslips were incubated with 0.04 nM Na- or Cs-doped CSoT, or CPeT or Clostridium perfringens ε-protoxin (CpeT) for 20 min at room temperature or 37 °C. The concentration is low to avoid oligomerization and observe single NPs (<10 per cell). The sample was then rinsed three times to remove nonbound toxins and nanoparticles. We recorded images at a frame rate of ~20 Hz (exposure time: 50 ms; readout time: 1.3 ms) and an excitation intensity of 0.25 kW/cm² at 21 °C or 37 °C. Similar results were obtained for CpeT.

The toxin receptor position in each frame was determined from a Gaussian fit to the diffraction pattern of the nanoparticles with a homemade algorithm that used MATLAB V8.2 (The MathWorks, Natick, MA).

The synthesis, functionalization of nanoparticles, confinement factor value, cell culture, and MTT test protocols are given in the Supporting Material.

**Determination of domain size \( R_{95} \) and domain area (A)**

Domain sizes were determined by adjusting the length of the radius \( R_{95} \) so that the final circular domain contains 95% of the total number of trajectory points. The area of the confining domain is defined as the area that is enclosed by the circle with radius \( R_{95} \).

**Mean-square displacement analysis**

The mean-square displacement (MSD) analysis was performed according to the literature (47–49) for time-lags \( \tau \) smaller than the total time length of the trajectory divided by 5. The diffusion coefficient \( D_{\text{MSD}} \) was extracted from a linear fit of the points 2–4. Fitting the complete MSD curve with Eq. 1 yields the domain size \( L_{\text{MSD}} \) and the diffusion coefficient \( D_{\text{MSD}} (52,53) \),

\[
\text{MSD}(n\Delta t) = \frac{L_{\text{MSD}}^2}{3} \left( 1 - \exp\left[ -\frac{12D_{\text{MSD}}}{L_{\text{MSD}}^2} n\Delta t \right] \right) .
\]  

**Inference of forces and diffusion coefficient**

We assume that the receptor with mass \( m \) is undergoing molecular diffusion with a diffusion coefficient \( D \) inside an arbitrary potential \( V(\vec{r}) \) in the membrane according to the Langevin equation of motion:

\[
\frac{d\vec{r}}{dt} = -\nabla V(\vec{r}) + \sqrt{2D(\vec{r})}\xi(t),
\]  

The receptor is subject to thermal noise \( \xi(t) \) giving rise to Brownian motion with friction coefficient \( \gamma(\vec{r}) \), which is related to \( D(\vec{r}) \) via the Einstein-Stokes equation \( D(\vec{r}) = \kappa T/\gamma(\vec{r}) \). This relation is a crude approximation of the relation of \( D(\vec{r}) \) and \( \gamma(\vec{r}) \) in the cell membrane. Only the absolute potential depth would be affected, but the shape of the inferred potential will be correct, creating a good starting point for biomolecules of unknown size.

Solving the associated Fokker-Planck equation in Smoluchowski’s overdamped approximation yields the probability transition function (Eq. 3), i.e., the probability of observing a certain displacement from one space-time point \( (r_i,t_i) \) to the next \( (r_j,t_j) \), supposing a value for the diffusion coefficient \( D \) and force \( F \), which are constant at the point of evaluation:

\[
P(\vec{r}_2,t_2|\vec{r}_1,t_1) = \frac{\exp\left(-\frac{\left(\vec{r}_2 - \vec{r}_1 - \vec{F}(t_2-t_1)/\gamma\right)^2}{4D(t_2-t_1)}\right)}{4\pi D(t_2-t_1)}. \]  

The overall probability of observing a certain trajectory for a given set of variables is then computed by multiplying all the displacement probabilities between all individual points in the dataset, assuming that the motion of the molecule is a Markov process. With Bayes’ theorem and the probability transition function in Eq. 3, we can determine the a posteriori probability, i.e., the probability of the parameters taking on certain values, given the observed trajectory.

The diffusion coefficient \( D_{\text{Bay}} \) was evaluated in a global fashion, while the forces were determined for each rectangle of an 8 × 8 mesh of subdomains, assuming that the potential gradient is a constant vector in each subdomain (129 parameters in total, optimized in groups of three variables). Alternatively, it is possible to describe the forces with a polynomial model potential, whose coefficients can be optimized.

The search for the maximum of the a posteriori probability was performed first with a quasi-Newtonian optimization of all parameters simultaneously using the Broyden-Fletcher-Goldfarb-Shannon algorithm with line searches (54). A second optimization using Monte Carlo around the previously determined optimum yields the final values (maximum of the likelihood distribution) for the forces and the diffusion coefficient or for the potential coefficients along with their standard deviations. As explained in the accompanying article (55), the final values were corrected for bias using numerical simulations.
Pharmacological treatments of cells

Where mentioned, we incubated cells with either 500 nM latrunculin B (Calbiochem; EMD Biosciences, San Diego, CA), 20 U/mL cholesterol oxidase (Calbiochem), or 10 U/mL sphingomyelinase (Calbiochem) in HBSS + 10 mM HEPES for 30 min. Incubation with nocodazole (Sigma, St. Louis, MO) was performed at a concentration of 100 nM in HBSS + 10 mM HEPES for 45 min. We rinsed the cells before experiments, except in the case of latrunculin B and nocodazole, where the products were left in solution during tracking.

Successful actin depolymerization was easily confirmed by observing the cell morphology, and a cholesterol quantification kit (Invitrogen, Carlsbad, CA) was used to determine successful cholesterol oxidation (see Fig. S6 in the Supporting Material).

RESULTS

Toxin receptor tracking

We use Y0.6Eu0.4VO4 NPs as probes to study the motion of two protein toxins and their receptors in the membrane of MDCK cells: the α-toxin of Clostridium septicum (CSαT) and the ε-prototoxin of Clostridium perfringens (CPEpT). The properties of these nanoparticles are described in Fig. S1. The principle of the tracking experiment is visualized in Fig. 1 A. Typical trajectories of CSαT and CPEpT receptors are shown in Fig. 1, B and C, respectively. The CPEpT targets the same receptor as the CPεT, so we will refer to the receptor as the CPεT receptor. The mean localization precision achieved in one dimension for our probes in our experimental conditions is 30 nm, the best precision achieved being 17 nm (see Fig. S2). Due to the absence of blinking, uninterrupted trajectories for up to 4600 points, or 240 s, can be collected. Tracking over longer periods is not limited by the NP photostability but by mechanical instabilities of the microscope and cell motion.

All observed trajectories are confined, low-frequency jumps that take place to adjacent confinement zones. In all cases, we observe long-term correlated drifting of the domains, which we attribute to cell motion or mechanical instabilities during acquisition. We analyze only short-term behavior before drift becomes visible. Both toxins under study are known to act on the MDCK cell membrane without entering the cells (27). Concurrent with this, we do not observe receptor internalization. The MSD plot of the trajectories of Fig. 1, B and C, are shown in Fig. 1, D and E, respectively. The good fit by Eq. 1 (red) confirms that the toxin receptors are undergoing confined motion in the cell membrane.

Tracking during the initial incubation with NP-labeled toxins shows that the toxin receptors are confined as soon as they are labeled by the binding of the toxin and do not undergo capture into domains (see Movie S1 in the Supporting Material). This agrees with biochemical data showing that toxin binding, oligomerization, and pore formation all happen within DRM s (29).

To test the specificity of the functionalized NPs, we use a polyclonal antibody against the toxin that inhibits receptor binding. Fig. 2 A shows that the number of bound NPs to the cell membrane decreases drastically to 16% of the initial value if the NP-toxin conjugates are incubated with the antibody before addition to the cells.

Using an MTT assay, the viability of control cells was compared to that of cells incubated with 1), NPs functionalized with APTES; and 2), NPs functionalized with APTES and CPEpT at typical conditions for SMT experiments. Neither the labels nor the labeled toxins have a negative impact on cell viability (Fig. 2 B).

The motion of a CPεT receptor in a live MDCK cell (black) was compared to the motion of a receptor in a fixed cell (red) and to the motion of a NP fixed on the glass coverslip (blue) in Fig. 2 C. Immobilized NPs give an experimental measure of the lowest accessible values for diffusion coefficients and domain sizes: 0.008 ± 0.002 μm²/s and 0.02 ± 0.01 μm², respectively. Fixing the cell greatly reduces the motion of the receptor but does not freeze it out completely, in agreement with Tanaka et al. (56).

The NP polydispersity allows for the investigation of a relationship between measured diffusion coefficients (D_MSD) of labeled receptors and the diameter of the label (Fig. 2 D). No general trend is observed, which implies an absence of a significant drag force on the receptor due to the NP label.
As a final control, we substituted NP labels by small organic fluorophores (Cy3) with a coupling ratio of ~3 fluorophores:1 toxin. The coupling ratio was determined by stepwise photobleaching analysis (57). In both cases, the CPeT receptor motion is confined to small domains in the cell membrane of average length \( L_{MSD} = 350 \pm 230 \text{ nm} \) (mean \( \pm \sigma \)). The obtained average diffusion coefficients are \( \langle D_{MSD}^{\text{NP}} \rangle = 0.06 \pm 0.09 \mu \text{m}^2/\text{s} \) and \( \langle D_{MSD}^{\text{FF}} \rangle = 0.05 \pm 0.06 \mu \text{m}^2/\text{s} \) for NPs and fluorophore (FF) labels, respectively. Furthermore, the overlay of the cumulative distributions in Fig. 2, E and F, for diffusion coefficients \( D_{MSD} \) and domain sizes \( L_{MSD} \) shows no significant difference between NP and organic fluorophore labels.

**Inference reveals forces acting on the receptor within the confining domain**

To analyze the receptor trajectories, we use Bayesian inference introduced in Masson et al. (51), which uses most of the available information that is stored in a trajectory. This offers new possibilities to quantify the motion of biomolecules, particularly by extracting the force map and potential acting on the biomolecule without any assumption on the potential type (55,58).

A model is required to describe the motion of the tracked molecule. We use the general form of the Langevin equation of motion (Eq. 2), where a molecule is undergoing Brownian motion with spatially varying diffusivity in an arbitrary potential. Because local variations of the diffusion coefficient are found to be small (55), we consider one global diffusion \( D_{inf} \). Then we calculate the a posteriori probability for \( D_{inf} \) and forces using Eq. 3 and Bayes’ theorem. The value of a parameter that gives the highest a posteriori probability is the inferred value of that parameter and the width of the a posteriori probability gives a direct measure of the uncertainty of this inferred value (51,55). The method infers the correct values from numerical trajectories calculated for parameters in the range of our experimental conditions and converges quickly (51,55). Furthermore, the program does not find forces within the domain if only reflective walls confine the numerical trajectory (55). We also extracted from the single-particle motion of a bead in a laser trap the forces and potential acting on the trapped bead and found values in agreement with the standard trap characterization techniques.

Using inference, we extract the force map (Fig. 3 B) from the CSeT receptor trajectory shown in Fig. 3 A and find a diffusion coefficient \( D_{inf} = 0.131 \pm 0.006 \mu \text{m}^2/\text{s} \). The receptor is subjected to forces within the domain that force it back toward the center of the confining domain. The a posteriori distributions (Fig. 3 C) are narrow and well peaked, which indicates good convergence to the inferred values. Note that, in contrast, the Cy3 trajectories are too short and contain too little information to yield exploitable results.

Fig. 3 D shows the inferred potential that confines the toxin receptor. In this implementation of the inference method, only the parameters of the potential are optimized along with the global diffusion coefficient, leading to a reduction in computation time with respect to the force map method (51). We start with a fourth-order polynomial, reducing the order and testing if the model still fits the data well. Fig. S3 A shows the inferred potential using a second-order and fourth-order polynomial for a single receptor trajectory. The reduction from fourth- to second-order changes the coefficients of the \( x^2 \) and \( y^2 \) terms by only 10% and the potential values for an ensemble of 37 CSeT receptor and 40 CPeT receptor trajectories differ on average by 10%. Furthermore, the linear contributions are negligible at \(<1\%\) compared to the contributions from the quadratic terms. The potential is often circular as shown by the average ratio of the diagonalized quadratic term coefficients \( k_x \) to \( k_y \) of 1.2 \pm 0.7. We therefore characterize the potential as springlike with a radial spring constant \( k_r \), defined as \( k_r = (k_x^2 + k_y^2)^{1/2} \). The \( k_r \) of the potential in Fig. 3 D is 0.24 \pm 0.01 pN/\mu m.

**FIGURE 2** Control experiments. (A) Normalized number of bound toxin-NP conjugates to MDCK cells during an experiment without \((N = 175)\) and with \((N = 28)\) an antibody against the coupled CPeT. (B) MTT assay results. (C) Trajectories of toxin receptors on live (black) and fixed cells (red). An immobilized nanoparticle on the glass surface (blue). (D) Diffusion coefficients \( D_{MSD} \) as a function of the bound nanoparticle size. (E and F) Cumulative distributions for \( D_{MSD} \) and \( L_{MSD} \), respectively, obtained from the MSD analysis of CPeT receptor trajectories, showing fluorophore labeling (blue dashed line) and NP labels (red).
We also analyzed our results using the radial density distribution of single toxin receptor trajectories. Although inference outperforms Boltzmann statistics techniques (55), it is important, to verify that we obtain similar results. Following Jin et al. (42), the normalized radial density distribution was fitted assuming Boltzmann statistics, using three candidate potentials (see Fig. S3 A). The spring potential best describes the observed trajectory, confirming that the confining potential is springlike.

Comparing the motion of CSαT and CPeT receptors

We chose the diffusion coefficient $D_{\text{inf}}$ and the spring constant $k_r$ of the springlike potential as well as the confinement domain area $A$ to quantify and compare the trajectory characteristics of CSαT and CPeT receptors (Table 1, and see Fig. S4, A–C). All CSαT and CPeT receptors were observed to be mobile and confined, except for 2% of CPeT receptors that were immobile.

Both toxin receptors have the same mean $D_{\text{inf}}$ and $A$ and Kolmogorov-Smirnov (KS) analysis confirms that the distributions are the same. Inferred $k_r$ somewhat differ in their mean values ($0.30 \pm 0.02 \text{ pN/µm}$ and $0.60 \times 0.08 \text{ pN/µm}$ for CSαT and CPeT receptors, respectively) and in their distributions. More CPeT receptors are confined by a stiffer potential.

Temperature (in)dependence of the CSαT and CPeT receptor motion

Toxin receptor trajectories were also recorded at 37°C. Both toxin receptors remain confined. The cumulative distribution for $D_{\text{inf}}$, $k_r$, and $A$ are given in Fig. S4, D–I, and the mean values are summarized in Table 1 for comparison. KS analysis shows that, for both toxin receptors, the change in temperature has no effect, except for an absence of highly confined receptors at 37°C for the CPeT receptors (see Fig. S4 H). At 37°C, all three observed parameter distributions are the same for CSαT and CPeT receptors.

**TABLE 1** Quantification of toxin receptor trajectories on MDCK cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Temperature</th>
<th>$D_{\text{inf}}$ (µm²/s)</th>
<th>$k_r$ (pN/µm)</th>
<th>$A$ (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSαT (N = 72)</td>
<td>21°C</td>
<td>0.16 ± 0.01 (± 0.09)</td>
<td>0.30 ± 0.02 (± 0.2)</td>
<td>0.40 ± 0.05 (± 0.4)</td>
</tr>
<tr>
<td>CPeT (N = 101)</td>
<td>21°C</td>
<td>0.16 ± 0.01 (± 0.14)</td>
<td>0.60 ± 0.08 (± 0.8)</td>
<td>0.40 ± 0.05 (± 0.4)</td>
</tr>
<tr>
<td>CSαT (N = 46)</td>
<td>37°C</td>
<td>0.18 ± 0.01 (± 0.07)</td>
<td>0.30 ± 0.03 (± 0.2)</td>
<td>0.40 ± 0.04 (± 0.3)</td>
</tr>
<tr>
<td>CPeT (N = 43)</td>
<td>37°C</td>
<td>0.13 ± 0.01 (± 0.05)</td>
<td>0.40 ± 0.03 (± 0.2)</td>
<td>0.40 ± 0.05 (± 0.3)</td>
</tr>
</tbody>
</table>
Confinement of the receptors is cholesterol- and sphingomyelin-dependent

Lipid rafts are rich in certain types of more densely packed lipids like sphingomyelin, a feature facilitated by cholesterol. When the membrane is depleted of cholesterol or sphingomyelin, rafts have been shown to disassociate (59).

To pinpoint the nature of these confinement domains, we investigate the role of two molecules that are connected to lipid rafts: sphingomyelin and cholesterol. We first showed that prior cell incubation with cholesterol oxidase reduces the toxicity of both CSaT and CPεT (see Fig. S5), in accordance with Miyata et al. (29). This measurement yields an indication of the behavior of many toxin receptors. However, tracking the same single CPεT receptor (Fig. 4) before and after incubation is much more informative and reveals that oxidizing the cholesterol in the membrane leads to an increase in $A$ and a decrease in $k_r$; $D_{inf}$ increased for this receptor from $0.189 \pm 0.006 \, \mu m^2/s$ to $0.256 \pm 0.009 \, \mu m^2/s$ after 10 min of incubation. Tracking the same receptor during incubation is possible due to the photostability of the NPs. The ensemble of the trajectories shows a reduction of confinement due to cholesterol oxidation (decrease in $k_r$) and an increase in confinement domain area (Fig. 5, $A$–$C$ (see Fig. S7, $A$–$C$), Fig. 6, $A$–$C$ (see Fig. S8, $A$–$C$), and Table 2). A quantity of 18 out of 72 CSaT and CPεT trajectories now yields domain areas larger than $1.2 \, \mu m^2$.

Incubation with sphingomyelinase, which modifies sphingomyelin to ceramide, also induces a decrease in confinement through a larger $A$ and smaller $k_r$ after incubation. $D_{inf}$ increased significantly (see Fig. S9 and Fig. S10).

Confinement of the receptors is independent of the amount of polymerized actin and microtubules

We investigated the effects of actin cytoskeleton depolymerization by latrunculin B and microtubule disruption by nocodazole on the mode of motion of the toxin receptors to determine whether the cytoskeleton plays a role in confinement. MTT assays show no change in toxin potency due to treatment with latrunculin B (see Fig. S5). Moreover, at the single-molecule level, we observe no significant difference for any of the parameters for both toxin receptors (Fig. 5, $D$–$F$ (see Fig. S7, $D$–$F$) and Fig. 6, $D$–$F$ (see Fig. S8, $D$–$F$).
Confinement Potential of Toxin Receptors

**DISCUSSION**

Both pore-forming toxins studied here use receptors confined in small membrane domains. Moreover, the confinement is observed from the very moment the toxin binds to the receptor (see Movie S1), indicating previous recruitment of the receptor to the domain. These confined receptors thus provide a way for the toxins to concentrate their bound monomers, which subsequently increases the probability of monomer oligomerization and finally pore formation. As was suggested previously, this mechanism may explain the high toxicity of these peptidic bacterial toxins.

Concerning the nature of the confining domains, we can put forward the following arguments: GPI-anchored proteins have been shown to localize into confinement zones described as lipid rafts (19–23). The CSaT receptor has been demonstrated to be a GPI-anchored protein (17,18). Furthermore, we have shown that the toxicity of both CPεT and CSaT toxins is decreased by cholesterol oxidation, which is known to destabilize lipid rafts (21,59) in the cell membrane (29). On the single receptor level, experiments

![Figure 5](image1.png)  
**FIGURE 5** Cumulative distribution plots for CSaT receptors. (A–C) Distribution for $D_{Inf}$, $k_r$, and $A$ before (blue line) and after (red dashed line) treatment with cholesterol oxidase, respectively. We oxidized 27 ± 2% of the total cell cholesterol accessible (see Fig. S6). (D–F) Distribution before (blue line) and after (red dashed line) treatment with latrunculin B. (Black line) Position of the greatest difference, which gives the $D$ value used in the KS analysis. The associated $P$-value is compared to the $P_{threshold}$ value obtained from simulations.

![Figure 6](image2.png)  
**FIGURE 6** Cumulative distribution plots for CPεT receptors. (A–C) Distribution for $D_{Inf}$, $k_r$, and $A$ before (blue line) and after (red dashed line) treatment with cholesterol oxidase, respectively. (D–F) Distribution before (blue line) and after (red dashed line) treatment with latrunculin B.

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**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$P_{threshold}$</th>
<th>$P$</th>
<th>$D$-value</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol oxidase</td>
<td>0.05</td>
<td>0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>Latrunculin B</td>
<td>0.05</td>
<td>&gt;0.20</td>
<td>No Difference</td>
</tr>
<tr>
<td>Cholesterol oxidase, Latrunculin B</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>0.61</td>
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$D$-value indicates the greatest difference position, $P$-value *obtained from simulations.**
with cholesterol oxidase, sphingomyelinase, latrunculin B, and nocodazole show that a decrease of cholesterol or sphingomyelin disrupts the confinement domains whereas cytoskeleton modifications do not affect the confinement characteristics. Moreover, we have observed in real-time the transition of single toxin receptors (both for CSαT and for CPεT receptors) from a confined to a quasi-free motion state as a result of cholesterol depletion by cholesterol oxidase. All these data indicate that both receptors are confined in lipid rafts. Although we cannot presently exclude a possible role of protein-protein interactions in the formation of these confinement domains, their dependence on cholesterol and sphingomyelin is a characteristic raft property. Based on the size of these confinement domains and the sizes reported for rafts in the literature in the 10-nm range (34), we attribute our confinement domains to lipid platforms formed by the coalescence of smaller lipid rafts (32,33,60).

Implementing an inference approach that only assumes that the tracked receptor is moving according to the Langevin equation of motion in an arbitrary potential, we observed forces within the confining domain and extracted a corresponding springlike potential with a radial spring constant \( k_r \). It thus appears that forces are present inside these lipid platforms.

The obvious question is: What is the origin of the springlike potential inside these rafts?

It should be noted that springlike confining potentials have been observed for membrane receptors that are directly tethered to the cytoskeleton. Tethering acts as a spring and induces receptor confinement. Spring constants determined for tethered receptors are typically in the range of 1–10 pN/μm, which is an order of magnitude larger than the spring constants determined here. In addition, considering that the GPI-anchored receptor of the CSαT is inserted into the outer leaflet of the membrane, it cannot be tethered to the cytoskeleton. Thus, the springlike potential cannot be explained by direct tethering to actin filaments. However, tethering via intermediate molecules might yield lower spring constants. Nevertheless, the absence of influence of actin depolymerization and microtubule disruption on the raft characteristics speaks against such a hypothesis. It should be noted, though, that we cannot exclude the influence of small compartments fenced off by actin and actin-tethered proteins on short timescales (61). It is, however, highly unlikely that cytoskeleton pickets and fences create the forces observed here because such forces were found to be larger by almost one order of magnitude (62).

We therefore propose that the confining potential is created by molecular interactions within the confining lipid raft. A possible hypothesis is that the hydrophobic mismatch between the protein receptors and their surrounding lipids is weakest at the center of the confinement domain and largest at its borders. The observed forces and potentials could thus be related to line tension effects. Given the presence of numerous lipid species and proteins in cell membranes, the change in solubilization energy of the protein receptors in the lipid membrane environment may be gradual.

**CONCLUSION**

We demonstrated single-molecule tracking experiments using Eu-doped oxide NPs as nonblinking, photostable fluorescent labels allowing efficient discrimination against background and cell fluorescence. We attained a mean

### Table 2: Measured mean values of all CSαT and CPεT receptor trajectories for treatment with cholesterol oxidase and without

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<thead>
<tr>
<th></th>
<th>CSαT</th>
<th>CPεT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± error on the mean (±σ))</td>
<td>(Mean ± error on the mean (±σ))</td>
</tr>
<tr>
<td>Control Trajectories</td>
<td>( N = 34 )</td>
<td>( N = 15 )</td>
</tr>
<tr>
<td>( D_{\text{inf}} )</td>
<td>0.15 ± 0.02 (± 0.1) μm²/s</td>
<td>0.29 ± 0.08 (± 0.3) μm²/s</td>
</tr>
<tr>
<td>( k_r )</td>
<td>0.41 ± 0.05 (± 0.3) pN/μm</td>
<td>0.32 ± 0.05 (± 0.2) pN/μm</td>
</tr>
<tr>
<td>( A )</td>
<td>0.45 ± 0.09 (± 0.5) μm²</td>
<td>0.4 ± 0.1 (± 0.5) μm²</td>
</tr>
<tr>
<td>Cholesterol oxidase Trajectories</td>
<td>( N = 42 )</td>
<td>( N = 30 )</td>
</tr>
<tr>
<td>( D_{\text{inf}} )</td>
<td>0.16 ± 0.01 (± 0.08) μm²/s</td>
<td>0.38 ± 0.07 (± 0.4) μm²/s</td>
</tr>
<tr>
<td>( k_r )</td>
<td>0.26 ± 0.03 (± 0.2) pN/μm</td>
<td>0.11 ± 0.01 (± 0.07) pN/μm</td>
</tr>
<tr>
<td>( A )</td>
<td>0.7 ± 0.1 (± 0.9) μm²</td>
<td>2.8 ± 0.7 (± 4) μm²</td>
</tr>
</tbody>
</table>

### Table 3: Measured mean values of all CSαT and CPεT receptor trajectories for treatment with latrunculin B and without

<table>
<thead>
<tr>
<th></th>
<th>CSαT</th>
<th>CPεT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Mean ± error on the mean (±σ))</td>
<td>(Mean ± error on the mean (±σ))</td>
</tr>
<tr>
<td>Control Trajectories</td>
<td>( N = 38 )</td>
<td>( N = 42 )</td>
</tr>
<tr>
<td>( D_{\text{inf}} )</td>
<td>0.18 ± 0.01(± 0.06) μm²/s</td>
<td>0.18 ± 0.01 (± 0.08) μm²/s</td>
</tr>
<tr>
<td>( k_r )</td>
<td>0.31 ± 0.03 (± 0.2) pN/μm</td>
<td>0.34 ± 0.03 (± 0.2) pN/μm</td>
</tr>
<tr>
<td>( A )</td>
<td>0.44 ± 0.07 (± 0.4) μm²</td>
<td>0.37 ± 0.05 (± 0.3) μm²</td>
</tr>
<tr>
<td>Latrunculin B Trajectories</td>
<td>( N = 58 )</td>
<td>( N = 65 )</td>
</tr>
<tr>
<td>( D_{\text{inf}} )</td>
<td>0.171 ± 0.009 (± 0.07) μm²/s</td>
<td>0.158 ± 0.009 (± 0.07) μm²/s</td>
</tr>
<tr>
<td>( k_r )</td>
<td>0.36 ± 0.04 (± 0.3) pN/μm</td>
<td>0.38 ± 0.04 (± 0.3) pN/μm</td>
</tr>
<tr>
<td>( A )</td>
<td>0.34 ± 0.03 (± 0.2) μm²</td>
<td>0.38 ± 0.04 (± 0.3) μm²</td>
</tr>
</tbody>
</table>
localization precision of 30 nm with an acquisition time of 51.3 ms during several minutes and used this NP labeling to perform the first toxin-cell interaction experiments at the single-molecule level.

Coupling of these NP labels to two members of the pore-forming toxin family leads to the following observations:

1. The toxin receptors of both the CPεT and the CSaT appear to be recruited to confinement domains before toxin binding.
2. Both toxin receptors are confined to domains with the same characteristics, diffusion coefficient of 0.16 ± 0.01 μm²/s and domain areas of 0.40 ± 0.05 μm².
3. The newly introduced inference approach demonstrated the existence of a springlike potential inside these domains with spring constants of 0.30 ± 0.03 pN/μm for both receptors.
4. The confinement domains are cholesterol and sphingomyelin-dependent and cytoskeleton-independent.
5. No significant modifications of the domain characteristics were observed for a temperature change of 21–37°C.
6. These confinement domains are stable over long periods of time (minutes).

Biochemical modifications of the cell membrane and the temperature independence lead us to attribute the confinement zones to actively maintained lipid raft platforms. We attribute the spring-like potential felt by the toxin receptors to molecular interactions within the lipid raft.

The force map and potential determined here are important for the understanding of toxin activity. Indeed, from a physiological point of view, targeting of confined receptors might represent an evolutionary advantage to increase toxicity. They have, moreover, far-reaching implications for cell function in general, because such platforms, believed to be formed by the coalescence of small lipid rafts (32) disassociating and forming on the timescale of seconds (59), are thought to be actively maintained by the cell to perform signaling and trafficking operations (33). The study of toxin-cell interactions thus appears as a valuable tool for the investigation of cell membrane organization.

SUPPORTING MATERIAL

One movie, one table, and 11 figures are available at http://www.biophysj.org/biophysj supplemental/S0006-3495(12)00417-1.

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REFERENCES


49. Reference deleted in proof.


