Perforin Binding and Rearrangements Assessed by QCM-D

Analysis of Perforin Assembly by Quartz Crystal Microbalance Reveals a Role for Cholesterol and Calcium Independent Membrane Binding*

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ABSTRACT

Perforin is an essential component in the cytotoxic lymphocyte mediated cell death pathway. The traditional view holds that perforin monomers assemble into pores in the target cell membrane via a calcium–dependent process, and facilitate translocation of cytotoxic proteases into the cytoplasm to induce apoptosis. While many studies have examined the structure and role of perforin, the mechanics of pore assembly and granzyme delivery remain unclear. Here we have employed quartz crystal microbalance with dissipation monitoring (QCM-D) to investigate binding and assembly of perforin on lipid membranes, and show that perforin monomers bind to the membrane in a cooperative manner. We also found that cholesterol influences perforin binding and activity on intact cells and model membranes. Finally, contrary to current thinking, perforin efficiently binds membranes in the absence of calcium. When calcium is added to perforin already on the membrane, the QCM-D response changes significantly, indicating that perforin becomes membranolytic only after calcium binding.

Cytotoxic lymphocytes have the capacity to eliminate virally infected or otherwise compromised cells by releasing stored cytotoxins from granules and/or by engaging death receptors. One stored cytotoxin is the pore-forming protein, perforin, which facilitates entry of other cytotoxins such as serine proteases (granzymes) into the target cell (1). Granule-mediated target cell death is absolutely dependent on perforin. Mice deficient in perforin are unable to control viral infection (2,3) and patients with inactivating mutations in the perforin gene present with the disease familial hemophagocytic lymphohistocytosis (3). At higher concentrations perforin is cytolytic in its own right. At lower doses, perforin synergizes with granzymes to induce apoptosis, however the mechanism by which perforin facilitates granzyme entry into the target cell remains a topic of debate. Perforin-induced membrane damage is essential for granzyme delivery (4) and it seems likely that both complete perforin pores and...
incomplete pores may contribute to the passage of granzymes (5).

Perforin is a 60 kDa member of the Membrane Attack Complex/Perforin (MACPF) family of proteins. It comprises a MACPF domain, an epidermal growth factor-like domain and a C2 calcium-binding domain (6,7). Perforin monomers oligomerize to form 16-20 nm diameter pores in the plasma membrane of target cell (8,9). Pore formation and subsequent cell lysis is calcium-dependent, as demonstrated in assays using red blood cells (RBCs) and nucleated mammalian cells (10). Calcium binding is important for the molecular stabilization of perforin, inducing conformational changes that expose several aromatic residues (involved in membrane binding) in the C2 domain (11).

Perforin has structural similarities to the bacterial cholesterol-dependent cytolysins (CDCs). CDCs have been extensively studied and a robust model describing their pore formation exists (12). CDC monomers bind to the membrane and oligomerize into a circular pre-pore on the membrane surface. Conformational changes then take place within each subunit. This involves the unfurling of two key α-helices to form β-strands, together with vertical collapse of the pre-pore towards the membrane surface. In the case of the well-studied CDC, Streptolysin O (SLO), a large ~30 nm pore forms in the plasma membrane of target cell (8,9). Pore formation and subsequent cell lysis is calcium-dependent, as demonstrated in assays using red blood cells (RBCs) and nucleated mammalian cells (10). Calcium binding is important for the molecular stabilization of perforin, inducing conformational changes that expose several aromatic residues (involved in membrane binding) in the C2 domain (11).

In contrast to CDCs, perforin pores show no indication of vertical collapse, suggesting that there are significant differences in their pore-forming mechanisms (9). Both perforin arcs and full pores are commonly observed in vitro, however, currently it is unclear whether perforin forms a pre-pore complex prior to insertion into the membrane, or if there is a threshold of oligomer size before insertion occurs (4,8,23-27).

In this study we have used QCM-D to investigate the binding and rearrangement of perforin on membranes. We find that perforin exhibits a complex binding mechanism, in which cooperation between monomers occurs during the binding process. Perforin binding and activity is enhanced if cholesterol is present in the membrane. Strikingly, we find that perforin binds membranes in the absence of calcium, and that the introduction of calcium to membrane-bound perforin results in perforin rearrangement in the membrane.

**EXPERIMENTAL PROCEDURES**

**Reagents** - 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (cat. 850345P, Avanti polar lipids), Cholesterol (Chol) (cat. C8667, Sigma-Aldrich), 3-mercaptopropionic acid (MPA) (cat. M5801, Sigma-Aldrich), 28% ammonium hydroxide solution (cat. CAS 1336-21-6, Ajax Finechem), 30% hydrogen peroxide solution (cat. 108597, Merck Millipore), CaCl2 (cat. 10035-04-8, Ajax Finechem), HEPES (cat. 7365-45-9, Amresco), Ethylenediaminetetraacetic acid (EDTA) (cat. ED, Sigma-Aldrich), L-α-Phosphatidylcholine from egg yolk (eggPC) (cat. P3556, Sigma-Aldrich), Methyl-β-cyclodextrin (MβCD) (Sigma-Aldrich), NaCl (cat. 7647-14-5, Amresco), Nystatin (Sigma-Aldrich).

**Buffers** - HEPES buffered saline (HBS): 20 mM HEPES, pH 7.5, 150 mM NaCl; HEPES buffered saline with calcium (HBS-Ca2+) as above with 2 mM CaCl2; Low salt buffer (LS): 20 mM HEPES, pH 7.4, 30 mM NaCl; Low salt buffer with calcium (LS-Ca2+) as per LS plus 2 mM calcium; HBS with EDTA: 20 mM HEPES, pH 7.5, 150 mM NaCl and 2 mM EDTA. All buffers were made up in double distilled water (MilliQ water) and then filtered through a 0.22 µm filter and degassed. HBSS: 10 mM HEPES, 0.2% (w/v) BSA in Hank’s balanced salts solution, pH 7.2; HE: 10 mM HEPES, pH 7.2, 0.2% (w/v) BSA, 150 mM NaCl, 5 mM CaCl2.

**Proteins** - Recombinant mouse perforin was produced as previously described (28). Recombinant mouse perforin mutants, were generously provided by Dr. Voskoboinik, purified as previously described (10,29). The SLO expression plasmid, a gift from Prof. Bhakdi, was produced and purified as previously described (18).

**Liposome preparation** - Liposomes were prepared as described (21,30). Briefly, DMPC, eggPC and cholesterol powder was dissolved to 5 mM stock solution in ethanol-free chloroform.
Lipids were mixed, at ratios of 40:60 (v/v) DMPC:Chol or 50:50 (v/v) eggPC:Chol. Chloroform was evaporated under a gentle stream of nitrogen gas and remaining solvent removed by vacuum desiccator for 2 hours. Lipids were then stored at -20°C. On the day of use lipids were rehydrated in 1 ml HBS or HBS-Ca\(^{2+}\) to a final concentration of 0.5 mM at 37°C for 1 hour, vortexed for 2-5 minutes and sonicated for 7 min 1-3 times at ~50°C. Liposome suspensions were stored at 4°C for up to 7 days.

**QCM chip cleaning and surface modification** - Gold coated sensor chips were cleaned by incubating in a solution of ammonium hydroxide:hydrogen peroxide:water (1:1:3 v/v) at 70°C for 15-20 min as described previously (30). The gold surface was then washed and modified with 1 mM MPA solution made up in propan-2-ol for at least 1 hour at room temperature.

**QCM experiments** - QCM-D measurements were performed using an E4 system with flow cells (Q-Sense, Sweden) as described previously (21,30). Briefly, changes to the resonance frequency (Δf) and energy dissipation (ΔD) were measured simultaneously. All plots presented here are of the 7th harmonic unless stated. All experiments were conducted at 22°C. A lipid bilayer was achieved by deposition of liposomes in HBS or HBS-Ca\(^{2+}\) until a change in frequency of 25-30 Hz (31). Lipid layers were washed with a low salt buffer (LS or LS-Ca\(^{2+}\)) to encourage osmotic stress to burst any intact liposomes. After a baseline was established in the buffer, protein was added to the system at a rate of 50 µl/min. Once all the protein was used the flow was stopped and an equilibration period, with no flow, was monitored before a wash step was introduced to remove any unbound material from the surface with HBS or HBS-Ca\(^{2+}\) at 300-500 µl/min.

**sRBC lysis assays** - Sheep erythrocytes were washed in 0.9% saline solution to remove any contaminating haemoglobin in the buffer. The erythrocytes were then counted and resuspended at 2x10\(^8\) cells/ml. Perforin was serially diluted in HBS±Ca\(^{2+}\) and 2x10\(^7\) cells were added to each dilution in a final volume of 200 µl. This was then incubated at 37°C or 22°C for 20 mins and supernatant collected and its absorbance measured at 405 nm to detect the release of haemoglobin.

**Mammalian cell lysis assay** - Jurkat cells were washed twice with HBSS then resuspended at 2x10\(^6\) cells/ml in the same buffer. Cells were pre-treated with 5 mM MβCD or nystatin for 30 min at 37°C then added to an equal volume of perforin diluted in HE with 5 mM MβCD or nystatin. Cell viability was determined by Trypan Blue dye exclusion following a further 30 min incubation at 37°C.

**Theoretical model** - Change in mass (Δm) on the surface of the chip can be calculated according to the Sauerbrey equation (32):

\[
\Delta m = -C(\Delta f/N)
\]

Where C is the mass sensitivity constant (in this case 17.7 ng/cm\(^2\)) and N is the harmonic/overtone number (the 7th was used).

The kinetic form of the Langmuir adsorption model provides the simplest descriptor for the dynamics of adsorption at a solid-solution interface (33):

\[
\Gamma_{\max} = k_{\text{ads}}(1-\theta) \left[ M \cdot C_b + k_{\text{des}} \theta / k_{\text{ads}} (1-\theta) + M \right] - k_{\text{des}} \theta
\]

where \(\Gamma_{\max}\) is the maximum adsorbed material in mol/m\(^2\) (determined using the Sauerbrey equation for a saturating concentration), \(M\) is a mass transport factor that represents how quickly molecules can get to the surface in ms\(^{-1}\), \(k_{\text{ads}}\) is the rate of adsorption in ms\(^{-1}\), \(k_{\text{des}}\) is the rate of desorption in mol m\(^{-2}\)s\(^{-1}\) and \(C_b\) is the bulk concentration mol m\(^{-3}\). The fractional surface coverage, \(\theta\), is calculated by dividing the adsorbed amount at a given time and \(\Gamma(t)\) by the maximum adsorbed amount (\(\Gamma_{\max}\)). At low surface coverage, the adsorption process is assumed to be broadly controlled by diffusion of molecules to the surface, and so the initial slope of \(\theta\) vs \(t\), i.e. d\(\theta\)/dt is used to calibrate \(M\) (34).

**RESULTS**

**Perforin binding and assembly measured by QCM-D** - To assess the binding and assembly of perforin, gold-coated sensors were modified with MPA and then treated with DMPC-Chol liposomes to form a lipid bilayer on each of the four sensors (31). A different concentration of perforin was introduced to each chamber and changes in dissipation and frequency were monitored in real time. After the flow was stopped, changes in the dissipation and frequency were monitored for a further 20 min. A HBS-Ca\(^{2+}\) solution was introduced to remove non-specifically bound material. In this system, a
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decrease in frequency over time indicates that mass is accumulating on the surface of the lipid bilayer, and the change in frequency ($\Delta f$) is proportional to the accrued mass on the sensor according to the Sauerbrey equation (32). Perforin showed a two-phase, concentration-dependent binding to the lipid layer (Fig 1A). Interestingly, at higher concentrations (100 and 200 nM), $\Delta f$ continued to increase after the flow of perforin-containing solution ceased. This was reproducible and may be due to a small amount of perforin binding during the equilibration period (as the chamber solution contains perforin). The perforin-lipid interaction appears to be very stable as there was no loss of mass evident during the subsequent wash step (Fig 1A).

Changes in dissipation ($\Delta D$) in real time were also measured. Dissipation is a measure of the energy lost (dissipated) from the sensor: if the fluidity of the surface increases it will be more energy dissipating and thus $\Delta D$ increases. By contrast, if the layer becomes more rigid it will not absorb as much of the dissipating energy and $\Delta D$ will remain constant or decrease. This allows us to assess how perforin changes the viscoelastic properties of the lipid layer, presumably as monomers oligomerize and insert. We observed a small initial increase in $\Delta D$, followed by a decrease, then finally a large and sustained increase, indicating that perforin binding and activity changes the fluidity of the lipid layer in a multi-phase manner (Fig 1A). Like $\Delta f$, $\Delta D$ also increased during the equilibration period, which indicates that protein already in the chamber continues to change the lipid layer. Perforin data plotted as $\Delta f$ versus $\Delta D$ ($\Delta f$-$\Delta D$ or signature trace) shows several transitions consistent with a multi-step process (Fig 1B). The overall trend, is upward and toward the right and is indicative of protein-protein and/or protein-lipid organization (35,36). The $\Delta f$-$\Delta D$ profile is complex and suggest that there are several processes occurring. Attributing specific steps to events or pore formation is difficult. However, along with protein-protein associations, there are regions where the $\Delta f$-$\Delta D$ trace reflects a decrease (or no change) in $\Delta D$ during an increase in $\Delta f$. These features indicate that the membrane is more rigid, possibly be due to protein insertion into the membrane and/or conformational changes in the protein structure.

To further analyse changes in the frequency and dissipation data, the first derivative was analysed for the concentrations of perforin shown in Fig 1A. The first derivative emphasizes changes in the gradient of the frequency and dissipation. These changes reflect specific kinetic events during protein binding to the membrane, conformational changes to the protein on the membrane, or in some cases other effects such as densification or solvation. The first derivative of the frequency data ($\Delta f'$) shows two minima, indicating that two events occur as perforin interacts with membrane. The first minimum appears to become saturated at 100 nM perforin as there is no further increase in the rate with 200 nM perforin. The second minimum is strongly concentration-dependent and does not reach saturation (Fig 1C). Similarly, the first derivative of $\Delta D$ ($\Delta D'$) also shows two maxima and a minimum, which indicates the viscoelasticity of the composite layer is changing due to perforin (Fig 1C). A simple interpretation of the above results is that the first event represents initial binding of perforin monomers to the bilayer until a critical concentration is reached, which leads to the second, more rapid, event reflecting reorganization / assembly of monomers into pores on the surface. It is conceivable that the second event accelerates as the forming oligomers offer high(er) affinity binding sites for incoming monomers.

Changes in frequency and dissipation are measured using several different harmonics, which are integer multiples of the fundamental frequency of the quartz sensor. Harmonics enable measurements at various depths from sensor’s surface, at higher harmonics the magnitude of oscillation is less and therefore these only probe material close to the sensor surface. Analysis of the harmonics, for a single perforin concentration (100 nM), shows a very small but reproducible difference in $\Delta f$ over the four harmonics ($3^{rd}$ – $9^{th}$). This difference is a spreading effect post binding, where the highest $\Delta f$ value was in the $3^{rd}$ harmonic which decreases to through to the $9^{th}$ harmonic. This indicates that there was more mass detected in the $3^{rd}$ (further away from the sensor surface), potentially on top of the surface of the membrane, compared to the $9^{th}$ (closer to the sensor surface), which reflects perforin in the membrane (Fig 1D). Comparison of the harmonic data for $\Delta D$ shows a more defined yet even increase over a small $\Delta D$ range ($1.5x10^{-6}$), evident after the initial binding period. This suggests post binding protein rearrangement on the membrane surface.

One possible explanation for the variation in harmonics is that during perforin oligomerization, monomers lose coupled water
molecules and become less energy dissipating on the surface of the membrane compared to free monomers. Upon perforin insertion, the majority of the protein is still on the surface with a smaller proportion in the membrane, closer to the sensor surface. Thus the larger portion of the oligomer on the surface appears heavier and more energy dissipating. Hence the spread in the harmonics is attributed to the proportions of protein on the surface versus protein inserted in the membrane.

When analysed as ∆f-∆D, all the harmonics follow the same trend, as expected, and spread out after initial binding, where the 3rd harmonic displays the largest shift to the right (Fig 1E). An increase in mass and dissipation in the ∆f-∆D plot is indicative of protein-protein association (35,36). As all ∆f and ∆D harmonics followed the same trend, only the 7th harmonic for is displayed for comparison in subsequent experiments.

To assess the kinetics of perforin binding to the lipid membrane, the data were compared with the Langmuir isotherm model of adsorption for a similar overall rate. This comparison clearly indicates that perforin adsorption does not obey simple first-order kinetics, providing additional evidence for a more complex, cooperative process. Specifically, over the first 12 min (during the initial binding period) perforin does not follow the Langmuir isotherm model, and it is evident there is more than one rate of binding or more than one process occurring (Fig 1F). This may indicate an element of cooperation in perforin binding, or a surface rearrangement step. It is possible that as monomers bind, they provide higher affinity binding sites for incoming monomers, thus increasing the rate of binding once a critical surface coverage is achieved. More complex kinetic modelling could not be performed due to insufficient knowledge of perforin oligomerization and surface interactions.

**Perforin activity is enhanced in the presence of cholesterol** - Composition of the membrane plays a significant role in binding and/or activity of pore forming proteins (e.g., the CDCs (37), pleurotolysin (38), and *Vibrio cholerae* cytolsin (39)), however, the membrane requirements for perforin pore formation remain largely unstudied. It has been suggested that cholesterol may play a role in dictating the type of pore formed by perforin, where full barrel stave pores predominate in high cholesterol membranes and proteo-lipidic hemi-pores are more common when the concentration of cholesterol is lower (27,40).

To examine the requirement for cholesterol in perforin-mediated lysis of nucleated mammalian cells, we used methyl-β-cyclodextrin (MβCD) to remove cholesterol from the plasma membrane (41,42). Perforin-mediated lysis of MβCD treated and untreated cells was assessed by Trypan blue exclusion, and compared to lysis by the CDC, SLO. As expected, SLO was unable to lyse mammalian cells pre-treated with MβCD, confirming effective extraction of cholesterol from the plasma membrane (data not shown). In comparison, perforin lysed cells pre-treated with MβCD but, compared to untreated cells, a five-fold higher concentration was required to lyse 50% of the target cell population (Fig 2A). This indicates that cholesterol plays an important role in perforin pore formation on mammalian cells. MβCD has been used with perforin previously, but in the context of nucleated cells where it inhibits endocytosis (4). These results were concordant with ours in showing that perforin activity is suppressed by MβCD. Furthermore, this study showed that MβCD reduces perforin binding to the plasma membrane (4). We also pre-treated mammalian cells with nystatin, which disrupts cholesterol-rich plasma membrane microdomains but does not remove cholesterol from the bilayer (42). Interestingly, nystatin had no effect on perforin’s lytic ability (Fig 2A); which suggests that cholesterol must be present but not necessarily specifically organized, in the membrane.

Next we used QCM-D to compare perforin binding to DMPC or DMPC:Chol. This showed perforin can bind to both model membranes, however less perforin bound when cholesterol was absent (Fig 2B). Significantly, the trace for ∆D was substantially different with and without cholesterol. There were more pronounced fluctuations in ∆D when cholesterol was present, indicating a larger impact on the structure and fluidity of the membrane (Fig 2B). This was also clearly evident in the ∆f-∆D signature trace (Fig 2C).

Analysis of first derivative of the frequency data indicates that the two-phase binding pattern remains evident when cholesterol is absent, although the rates are lower (Fig 2D). Changes in the first derivative of the dissipation data show a similar trend, which are reduced in amplitude (Fig 2D). This is consistent with the observation that lysis of intact cells by perforin is reduced but not abolished when cholesterol is removed from the membrane. Overall, these results suggest that although perforin does not
require cholesterol to bind membranes such as DMPC, post-binding events such as assembly or pore formation require lipid configurations including free (non-sequestered) cholesterol.

**Perforin binds to membranes in the absence of calcium -** Lysis by perforin is calcium-dependent. It is currently thought that calcium is required for initial binding of perforin to the plasma membrane through the C2 domain. We investigated the requirement of calcium for binding and activity of perforin on DMPC:Chol membranes by QCM-D. Surprisingly, we observed that perforin efficiently binds membranes in the absence of calcium (with 2 mM EDTA added to chelate any trace calcium) (Fig 3A). Binding in the absence of calcium appeared to be as stable as when calcium was present, because little or no material was removed from the membrane at a wash buffer flow rate of 300 µl/min (Fig 3A). By contrast, the changes in dissipation were very different when calcium was absent. In the presence of EDTA the dissipation steadily decreased, indicating that the protein had little or no effect on the viscoelastic properties of the membrane (Fig 3A). This was also evident in the δf-δD signature traces (Fig 3B). Equivalent results were obtained for perforin in HBS lacking both calcium and EDTA, confirming that this effect was due to the absence of calcium (data not shown). This suggests that in the absence of calcium, perforin can associate with the membrane but cannot form pores. To ensure that perforin used in this experiment was competent to form pores, we tested flow-through solutions from the QCM-D cells for activity by red blood cell lysis assays. As expected, solutions containing calcium lysed the red blood cells whereas those lacking calcium (and containing EDTA) did not (Fig 3C). When excess calcium (5 mM) was added to the EDTA-containing solution, lytic activity was restored (Fig 3C).

To further investigate binding of perforin to DMPC:Chol membranes in the absence of calcium, we used an inactive perforin mutant (D429A), which has one calcium binding residue mutated. This mutant reportedly does not bind to red blood cells and hence is non-lytic (10), which was confirmed by red blood cell lysis assay (data not shown). Using QCM-D, in the presence of calcium, D429A bound to the membrane, although much less efficiently than wild type perforin (wt Pfn, Fig 3D). Strikingly, dissipation data showed absolutely no impact on membrane properties (Fig 3D). To further compare the D429A binding and viscoelastic changes to the membrane, we analysed the first derivatives of the frequency and dissipation data (Fig 3E). The δf trace for D429A appeared somewhat similar to wt Pfn but with slower and a less efficient association with the membrane (Fig 3E). The δD trace for D429A shows little change over time (Fig 3E). This is in stark contrast to wt Pfn and indicates that although D429A can (weakly) bind to the membrane, post-binding events are inhibited.

In the absence of calcium, D429A bound DMPC:Chol similarly to wt Pfn, confirming that perforin has calcium-independent membrane binding capacity (Fig 3F). δD traces were identical, indicating absence of post-binding events (Fig 3F). Removal of unbound perforin, with a wash step, and subsequent introduction of calcium into the system resulted in a large change in dissipation (and smaller change in frequency) with wt Pfn but not D429A (Fig 3F). The first derivatives for these data are very similar for wt Pfn and D429A until calcium is introduced producing the dramatic change in both δf and δD for wt Pfn but not D429A (Fig 3G and H). Thus these results indicate that (i) perforin associates with phospholipids independently of calcium, (ii) membrane-bound perforin is able to bind calcium, and (iii) that calcium is critical for post-binding events in pore formation.

**Decreasing the oligomerization rate changes the QCM-D trace for perforin membrane interactions -** To identify the QCM-D signatures of perforin assembly and pore formation we used the point mutant R213E, which forms pores at a slower rate than wt Pfn (29). The slower rate of R213E lysis was confirmed using an RBC lysis assay at 22°C (QCM-D working temperature) for 40 min, (data not shown). Wt Pfn and R213E were then analysed by QCM-D. The δf data for wt Pfn and R213E were comparable (Fig 4A), indicating that the mode of membrane binding of this mutant is unaffected. However there was a small but reproducible increase in the rate of binding of R213E (Fig 4A). The shape of the δD trace for R213E was also comparable to wt Pfn, and again the changes produced by the mutant occurred slightly faster (Fig 4A). A small but notable difference in both δf and δD was evident during the equilibration period (when the flow was stopped), in that the wt Pfn traces continued to increase over time but the R213E traces did not (Fig 4A). These differences were further highlighted in the δf-δD signature trace where the R213E curve followed a similar path as the wt Pfn curve, being only slightly temporally
displaced (events occurring slightly earlier). In addition, there was no final extension in the trace of the R213E mutant as seen for wt Pfn (Fig 4B). The first derivatives of the frequency and dissipation for R213E show that the second process for both Δf' and ΔD' shifted to the left and increased in rate compared to wt Pfn (Fig 4C). This suggests that the second process (or at least part of the second process) reports perforin oligomerization/insertion in the membrane. When the data was plotted against the Langmuir isotherm model, it was evident that the R213E mutant resembles wt Pfn until approximately 8 min when it diverges (Fig 4D). To analyse these differences further, increasing concentrations of R213E mutant were tested (Fig 4E). These data show that at the highest concentration (200 nM) the R213E mutant does not induce an increase in ΔD once the flow has ceased. This contrasts with wt Pfn, where changes in ΔD during this period were quite pronounced (Fig 4E and Fig 1A). Once again, when plotted as Δf-ΔD, clearly the Δf-ΔD signatures are missing a final “extension” at the end of the trace (Fig 4F), similar to 50 nM wt Pfn (Fig 1B). Finally, the first derivatives for Δf and ΔD data once again indicated that R213E behaves similarly, but not identically, to wt Pfn. A notable difference is the decreased magnitude of the ΔD' response during the incubation period (Fig 4G) compared with 200 and 100 nM wt Pfn (Fig 1C). Together, these results suggest that the rate of oligomerization is reflected by the QCM-D signature of perforin, and that post-binding events can be investigated using this approach.

Perforin and SLO differ in their membrane binding and pore forming characteristics - Previously we characterized SLO binding and pore formation using QCM-D (21). The data obtained for SLO can be compared with the results for perforin as they were obtained under similar conditions, on Au-MPA with DMPC:Chol (50:50). However, it is important to note the considerable size difference between recombinant SLO and perforin. Perforin is expressed as a hexa-histidine tagged monomer of 60 kDa, whereas SLO is a MBP fusion protein that is 97 kDa. Therefore, it is expected that the same concentration of SLO would produce a higher Δf maxima than perforin due to its larger hydrated mass per molecule. This was indeed the case, despite obvious differences in binding rates: at the same concentration SLO has a larger Δf maxima than perforin (Fig 5A). Additionally, ΔD appears to be substantially different, again SLO has a significantly larger ΔD compared with perforin (Fig 5A). This implies that the larger SLO molecule is more energy dissipating than perforin, as expected. This is also true for the oligomeric structures formed by SLO vs perforin. The large SLO pores would cause a higher ΔD in comparison to the smaller oligomers that are formed by perforin (approximately half the size).

Comparison of the signature QCM-D Δf-ΔD plots revealed obvious differences between SLO and perforin, not only in magnitude but also in their overall membrane binding trends (Fig 5B). In particular, SLO binds in an energy dissipating manner in 3 stages, whereas perforin shows complex multi-staged processes, two of which are consistent with a stiffer, less energy dissipating surface. This comparison suggests that the mechanism of pore formation differs between SLO and perforin, and that the membrane responds differently as a consequence.

Comparison of the first derivative of SLO and perforin functions reveal both traces display two processes or events (maxima) for Δf at similar time points (Fig 5C). Analysis of Δf' for SLO showed that the second process was related to oligomerization (21). This may also be the case for perforin given the similarities, however, this remains speculative. While the Δf' traces appear similar, there are important discrepancies such as the difference in the magnitude of the two processes (minima). SLO displays two similar events of similar magnitude which both increase with increasing concentration, whereas the first process for perforin is concentration independent and only the second increases with concentration (Fig 5C). The ΔD' traces show that initially perforin differs from SLO, with a decrease in ΔD' below zero that is never seen for SLO, however, this is followed by a subsequent process (positive), which is a common feature at approximately the same time point (Fig 5C).

Finally, when plotted against the Langmuir isotherm model for single order binding, it is obvious that only SLO follows a first order kinetic model, suggesting there is an element of cooperation or surface rearrangement in perforin binding that is very different to SLO (Fig 5D). These data highlight the complexity of pore formation and suggest that while there are similarities between SLO and perforin, the mechanism of pore formation is different.

Comparing individual SLO and perforin traces shows that there are also differences in the response of the harmonics in both Δf and ΔD (Fig 5E and F). In the Δf trace for 100 nM SLO there is a spread in harmonic data, with the 9th being
smaller and increasing to the 3rd (Fig 5E). In contrast, perforin shows less spread in \( \Delta f \) (Fig 5F). Comparing \( \Delta D \) harmonic data for SLO they essentially overlay, whereas, perforin shows a large spread with the 9th being the most energy dissipating. These differences indicate that SLO and perforin are interacting with the membrane in subtle but distinctly different ways. The spread in harmonics in \( \Delta f \) indicates that there is mass through the lipid layer, whereas, a spread in \( \Delta D \) indicates that the layer is not equally as viscoelastic throughout the layer. The spread in \( \Delta f \) may be larger for SLO due to the increased mass of the individual molecules and oligomers, compared to perforin, which shows a less prominent spreading (but it is still evident). Both traces indicate that there is likely to be protein insertion into the membrane, however, differences in the behaviour of the harmonics in \( \Delta D \) indicates that the effect (or organization/activity) on the membrane differs.

**DISCUSSION**

We have used QCM-D to investigate the association and interaction of perforin with membranes. We find that perforin binds to membranes in a multi-step process: binding (but not subsequent events) is possible in the absence of calcium, and cholesterol is important for activity. These results help to further dissect the mechanism of pore formation by perforin.

**Role of cholesterol and calcium in perforin–membrane interactions** - Our results indicate that cholesterol affects the lytic activity of perforin, although unlike CDCs it is not essential for binding. There are mixed reports on the importance of cholesterol for perforin function. Early work suggested that the absence of cholesterol in liposomes makes no difference to perforin pore formation (43,44). Recently, however, it has been suggested that cholesterol dictates the type of pore formed by perforin. In cholesterol rich membranes, fully formed (barrel stave) pores are the dominant species, whereas incomplete proteo-lipidic pores occur more often in membranes that have less cholesterol (27,40). We suggest that cholesterol contributes to bilayer structure or fluidity to provide an optimal binding and assembly stratum for perforin.

Perforin requires calcium for lytic and granzyme delivery function. Our experiments show perforin binding to a phospholipid bilayer can occur in the absence of calcium, suggesting that calcium-independent association of individual perforin monomers with membrane may contribute to perforin’s function, in addition to calcium-mediated monomer-monomer binding and/or rearrangement on the membrane. Recent structural studies suggest perforin has three calcium-chelating sites in its C2 domain, two high and one low affinity site (9,11). The two high affinity sites are likely occupied at cytoplasmic calcium concentrations, whereas the low affinity site is occupied only at higher calcium concentrations (likely >0.2 mM). D429 is an important residue in the low affinity site responsible for a conformational change which allows repositioning of several residues, thus potentiating binding of aromatic side chains at the tip of the C2 domain to the membrane (11). In our studies, stable calcium-independent binding is evident for both wt Pfn and D429A, however, it is not associated with changes to the viscoelasticity of the lipid bilayer. When calcium is introduced, wt Pfn produces a large change in energy dissipation that is not seen with D429A. This increase in the dissipation is probably due to rearrangement of perforin into oligomers and/or insertion into the lipid layer.

**Oligomerization** - Perforin oligomerization is proposed to be facilitated by electrostatic interaction between subunits (9,29). Several residues are known to play a role in this interaction, including R213. The QCM response suggests the slowly oligomerizing mutant R213E exhibits a subtle but faster change to the second stage of perforin binding, as highlighted by the first derivative data (\( \Delta f' \)). Unlike wt Pfn, there is no steady increase in the dissipation (or frequency) once the mutant binds to the membrane, which is possibly due to a delay in oligomerization and insertion into the membrane. If binding is cooperative, delayed insertion may stabilize or increase binding opportunities for free monomers, explaining the faster second stage.

**Model for pore formation** - Models for perforin pore formation have been guided by those built to explain CDC pores. The current model for CDC pore formation invokes the assembly of monomers into a stable circular intermediate (the pre-pore), which then collapses and inserts into membrane. This model dictates that insertion into the membrane only occurs after complete ring formation is achieved. However, to date a perforin pre-pore has not been described, and by cryo-EM inserted pores show no signs of vertical collapse (9).

Our results further highlight differences between perforin and SLO (a CDC). This is
demonstrated by the differences in both frequency and dissipation when each toxin interacts with a lipid layer. Furthermore, these differences are clearest when the frequency and dissipation are plotted with respect to each other ($\Delta f - \Delta D$), showing that they do not overlay nor do they follow the same trend. In addition, it seems that the order of binding differs between SLO and perforin, where SLO displays first order kinetics and perforin does not. Taken together this suggests that their mechanisms of binding and pore formation are diverse.

The mechanism of perforin pore formation is still elusive and many questions remain. Based on our work, any model must now incorporate a requirement for a specific phospholipid configuration including cholesterol, and accommodate cooperative monomer binding (Fig 5G). In addition, a more complex role for calcium should be considered as it appears to function in both binding and post-binding events. (Fig 5G). Whether perforin forms complete prepore intermediates and then inserts into the membrane remains unclear: it is possible that monomers are able to insert into the membrane as they join the growing assembly, and this creates transient proteo-lipidic pores that may become complete pores or remain incomplete (Fig 5G). It has recently been reported that AFM images of perforin indeed include a mixture of arcs and fully formed rings (45). The full rings have a mean diameter of 20 nm which is similar to previous reports using EM (8,9,23-27,45).

CONCLUSION

This study reveals that the kinetics of perforin monomer binding to membranes is a complex cooperative process that involves calcium and cholesterol for optimal activity. QCM-D shows that perforin does not exhibit first order binding, suggesting cooperation between monomers during the binding process. Perforin binding and activity is enhanced when cholesterol is present in the membrane. Strikingly, perforin binds membranes in the absence of calcium, and the introduction of calcium to membrane-bound perforin results in perforin chelating calcium and subsequent rearrangement of the membrane. QCM-D offers a new approach to understanding the dynamics of pore formation, and evaluating therapeutic candidates that potentiate or inhibit perforin binding and activity.

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AUTHOR CONTRIBUTIONS: PIB and SES conceived and coordinated the study with involvement of LLM and MED. SES performed the QCM-D experiments. The paper was written by SES and PIB with contributions from LLM, JCW, MED and RFT. SP and RFT provided expertise in QCM-D and theoretical analysis, respectively. CHB performed and analysed experiments shown in Figure 2A. JAT provided recombinant wt perforin and expert advice. All authors reviewed the results and approved the final version of the manuscript.
REFERENCES


FIGURE LEGENDS

FIGURE 1. Perforin binding and activity monitored by QCM-D. (A) QCM-D trace for 25 nM (yellow), 50 nM (green), 100 nM (blue) and 200 nM (red) wt Pfn. \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. Time points where the flow rate was stopped and/or the sensors were washed are indicated. Data shown are representative of three or more experiments for each concentration. (B) \( \Delta f-\Delta D \) trace for 25 nM, 50 nM, 100 nM and 200 nM wt Pfn, coloured as in (A). Changes in direction of the trace are highlighted by small arrows and inflection points are indicated by \( \Delta f \) (left axis) plotted as dotted lines. Time points where the flow rate was stopped and/or the sensors were plotted on the right axis

FIGURE 2. Cholesterol is important for perforin activity. (A) Perforin mediated lysis of mammalian cells is decreased in the absence of cholesterol. Cells pre-treated with MβCD (black), nystatin (green) or untreated (red) were incubated with increasing concentrations of wt Pfn. Specific wt Pfn induced lysis is plotted as % cell lysis. Representative curves of four or more experiments for each treatment are shown. (B) QCM-D trace for 100 nM wt Pfn on DMPC alone (black) or with cholesterol (red). \( \Delta f \) (right axis) is plotted as solid lines and \( \Delta D \) (left axis) is plotted as dotted lines. As per (A), time points where the flow was stopped and restarted for the wash are indicated, and the data shown are representative of three or more experiments. (E) \( \Delta f-\Delta D \) trace for 100 nM wt Pfn showing the spread over the 3rd, 5th, 7th and 9th harmonics (lightest to darkest respectively). \( \Delta f \) (right axis) is plotted as solid lines and \( \Delta D \) (left axis) is plotted as dotted lines. As per (A), time points where the flow was stopped and restarted for the wash are indicated, and the data shown are representative of three or more experiments. (F) The wt Pfn binding rate does not overlay the Langmuir isotherm adsorption model for a simple first order process. 200 nM wt Pfn, data from panel (A), is plotted as theta (θ) against the theoretical Langmuir isotherm adsorption model over time. Experimental data (wt Pfn) is plotted as a solid line and the model data is plotted as a broken line.

FIGURE 3. Perforin binds membranes in the absence of calcium. (A) QCM-D trace for 100 nM wt Pfn in the presence of HBS-Ca\(^{2+}\) (black) or HBS containing 2 mM EDTA (grey). \( \Delta f \) (right axis) is plotted as solid lines and \( \Delta D \) (left axis) is plotted as dotted lines and are representative of three separate experiments. (B) \( \Delta f-\Delta D \) trace for wt Pfn data shown in (A). (C) Lysis assay using the flow through from the flow cells in (A): sRBC were treated with 100 µl of each flow through with or without the addition of excess calcium (5 mM). Graph displayed as % specific lysis ((Abs595 – Background) / Max. Abs595 – Background)x100. (D) QCM-D trace of 100 nM wt Pfn (black) and D429A (green). \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. Data are representative of three or more experiments. (E) The first derivative of the frequency and dissipation functions shown in panel (A) for wt Pfn (black) and D429A (green). \( \Delta f \) (Hz)/dt (min) (\( \Delta f' \)) is plotted on the left axis in darker colours and \( \Delta D \) (10\(^{16}\))/dt (min) (\( \Delta D' \)) is plotted on the right axis in lighter colours. (F) QCM-D trace of 100 nM wt Pfn (black) and D429A (green) in HBS containing 2 mM EDTA. At the time indicated, buffer was exchanged for HBS-Ca\(^{2+}\) post binding. \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. (C) The first derivative of the frequency function shown in panel (F) for wt Pfn (black) and D429A (green). The time points where the flow rate was stopped and 2 mM calcium was introduced into the system are indicated. (H) The first derivative of the dissipation function shown in panel (F) for wt Pfn (black) and D429A (green). Time points indicated as in (F) and (G).

FIGURE 4. Rate of oligomerization impacts the QCM-D profile of perforin. (A) QCM-D trace for 100 nM wt Pfn (black) and R213E (pink). \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. Data are representative of three experiments. (B) \( \Delta f-\Delta D \) trace for wt Pfn (black) and R213E (pink), data from panel (A). (C) The first derivative of the frequency and dissipation functions shown
in panel (A) for wt Pfn (black) and R213E (pink). \( df \) (Hz)/dt (min) plotted on the left axis in darker colours and \( dD \) (10^-6)/dt (min) is plotted on the right axis in lighter colours. (D) Neither wt Pfn (black) or R213E (pink) fit the Langmuir Isotherm model, instead they largely overlay each other. (E) QCM-D trace for 25 nM (yellow), 50 nM (green), 100 nM (blue) and 200 nM (red) R213E perforin. \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. Data are representative of three or more experiments. (F) \( \Delta f - \Delta D \) trace of data from panel (E). (G) The first derivative of the dissipation function shown in panel (E) for 25 nM (yellow), 50 nM (green), 100 nM (blue) and 200 nM (red) R213E perforin. \( df \) (Hz)/dt (min) plotted on the left axis in darker colours and \( dD \) (10^-6)/dt (min) is plotted on the right axis in lighter colours.

FIGURE 5. QCM-D comparison of SLO and Perforin. (A) Representative QCM-D traces of 100 nM SLO (black) and Pfn (red) on Au-MPA containing DMPC:Chol bilayers. Only the binding (0- ~20 min) and rest period are shown, no wash. \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines. (B) \( \Delta f - \Delta D \) trace for 100 nM SLO (black) and Pfn (red) data from (A). (C) The first derivative of the frequency and dissipation functions shown in panel (A) for 100 nM SLO (black) and Pfn (red), data from (A). \( df \) (Hz)/dt (min) plotted on the left axis (darker solid lines) and \( dD \) (10^-6)/dt (min) is plotted on the right axis (lighter thin line). (D) Langmuir isotherm adsorption model (grey broken line) plotted against experimental data for SLO (black) and Pfn (red). (E) QCM-D traces of 100 nM SLO on Au-MPA containing DMPC:Chol bilayers. Only the binding (0- ~20 min) and rest period shown, no wash. \( \Delta f \) (right axis) plotted as solid lines and \( \Delta D \) (left axis) plotted as dotted lines, 3rd to 9th harmonics are coloured from lightest to darkest as indicated. (F) QCM-D traces of 100 nM Pfn on Au-MPA containing DMPC:Chol bilayers. Graph plotted as in (E). (G) Perforin (made up of a C2 domain (green), EGF-like domain (black line), MACPF domain (yellow) containing two bundles of helices (red)) is able to bind to the membrane in a calcium (small orange circles) independent conformation, that is unknown, and through the calcium dependent C2 domain (green). Once bound in the correct orientation perforin then provides a higher affinity binding site for other monomers to bind, thus cooperation is seen when binding to the membrane. Oligomerization occurs and either perforin is capable of insertion whilst the ring of monomers grows, or a rate limiting number of monomers must be bound (similar to a pre-pore) before insertion. Finally, either a complete or incomplete pore is formed to perforate the membrane.
FIGURES

FIGURE 1
FIGURE 2

A

B

C

D

Perforin Binding and Rearrangements Assessed by QCM-D
FIGURE 3

A

B

C

D

E

F

G

H

Perforin Binding and Rearrangements Assessed by QCM-D
FIGURE 4

Perforin Binding and Rearrangements Assessed by QCM-D
Perforin Binding and Rearrangements Assessed by QCM-D

FIGURE 5

A

B

C

D

E

F

G

Monomer  Intermediate  Pore

1. Binding  2. Oligomerisation  3. Insertion
Analysis of Perforin Assembly by Quartz Crystal Microbalance Reveals a Role for Cholesterol and Calcium Independent Membrane Binding
Sarah E. Stewart, Catherina H. Bird, Rico F. Tabor, Michael E. D'Angelo, Stefania Piantavigna, James C. Whisstock, Joseph A. Trapani, Lisandra L. Martin and Phillip I. Bird

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