

A component of innate immunity prevents bacterial biofilm development

Pradeep K. Singh^{*}, Matthew R. Parsek[†], E. Peter Greenberg^{‡§} & Michael J. Welsh^{*§||}

^{*} Department of Internal Medicine, [‡] Department of Microbiology, and ^{||} Department of Physiology and Biophysics and Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, Iowa 52242, USA [†] Department of Civil Engineering, Northwestern University, Evanston, Illinois 60208, USA [§] W. M. Keck Foundation Microbial Communities and Cell Signaling Laboratory, Iowa City, Iowa 52242, USA

Antimicrobial factors form one arm of the innate immune system, which protects mucosal surfaces from bacterial infection^{1–3}. These factors can rapidly kill bacteria deposited on mucosal surfaces and prevent acute invasive infections^{1–4}. In many chronic infections, however, bacteria live in biofilms, which are distinct, matrix-encased communities specialized for surface persistence^{5–7}. The transition from a free-living, independent existence to a biofilm lifestyle can be devastating, because biofilms notoriously resist killing by host defence mechanisms and antibiotics^{5,8}. We hypothesized that the innate immune system possesses specific activity to protect against biofilm infections. Here we show that lactoferrin, a ubiquitous and abundant constituent of human external secretions, blocks biofilm development by the opportunistic pathogen *Pseudomonas aeruginosa*. This occurs at lactoferrin concentrations below those that kill or prevent growth. By chelating iron, lactoferrin stimulates twitching, a specialized form of surface motility, causing the bacteria to wander across the surface instead of forming cell clusters and biofilms. These findings reveal a specific anti-biofilm defence mechanism acting at a critical juncture in biofilm development, the time bacteria stop roaming as individuals and aggregate into durable communities.

Their occurrence in chronic infections brands bacterial biofilms a major medical problem^{5,6,8}. The airway infection by *P. aeruginosa* that afflicts people with cystic fibrosis is a prime example of a biofilm infection^{6,7}. Once this infection develops, *P. aeruginosa* colonize the airways for life, causing lung destruction and eventually death⁹. Normal mucosal surfaces resist biofilm infections despite

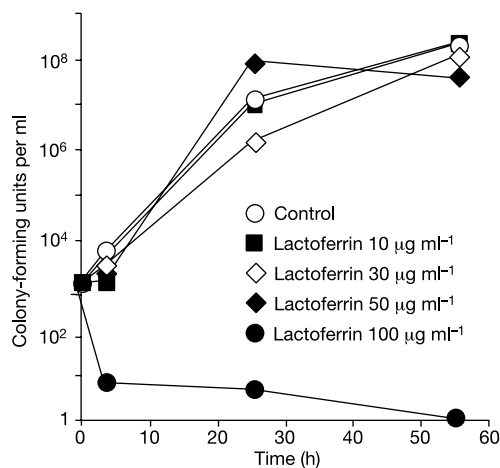


Figure 1 Growth of *P. aeruginosa* in the presence of lactoferrin. Results are representative of three experiments.

continual exposure to pathogenic bacteria. Rapid killing of deposited organisms probably accounts for some of this resistance¹. At times, however, bacteria remain on mucosal surfaces for prolonged periods, for example, during infections such as bronchitis, conjunctivitis, or infections associated with foreign bodies. This led us to hypothesize that mucosal surfaces might possess an anti-biofilm defence, perhaps using antimicrobial factors, a phylogenetically conserved limb of the innate immune system¹⁰.

To test this hypothesis, we asked whether human lactoferrin could inhibit biofilm development in *P. aeruginosa*. We investigated the effect of lactoferrin because it is ubiquitous, and it is among the most abundant proteins present in surface secretions. Substantial concentrations of iron-unsaturated lactoferrin¹¹ are found in tears (1–4 mg ml⁻¹)¹² and airway secretions (~0.4–1.0 mg ml⁻¹)^{13,14}. In addition, breast milk delivers large amounts (~3–7 mg ml⁻¹)¹⁵ to the undeveloped digestive systems of babies. At high concentrations, lactoferrin is known to limit bacterial growth by sequestering iron. In this regard it acts like other nutrient-depriving host defence molecules, such as transcobalamins (which bind vitamin B12)¹⁶ and calprotectin (which binds zinc)¹⁷. Lactoferrin can also be bactericidal by binding lipopolysaccharide and disrupting bacterial membranes, and it can enhance killing by other antibiotics^{18,19}. To determine whether lactoferrin has anti-biofilm activity that is distinct from these known properties, we examined the effect of a subinhibitory concentration of lactoferrin (20 µg ml⁻¹) on biofilm

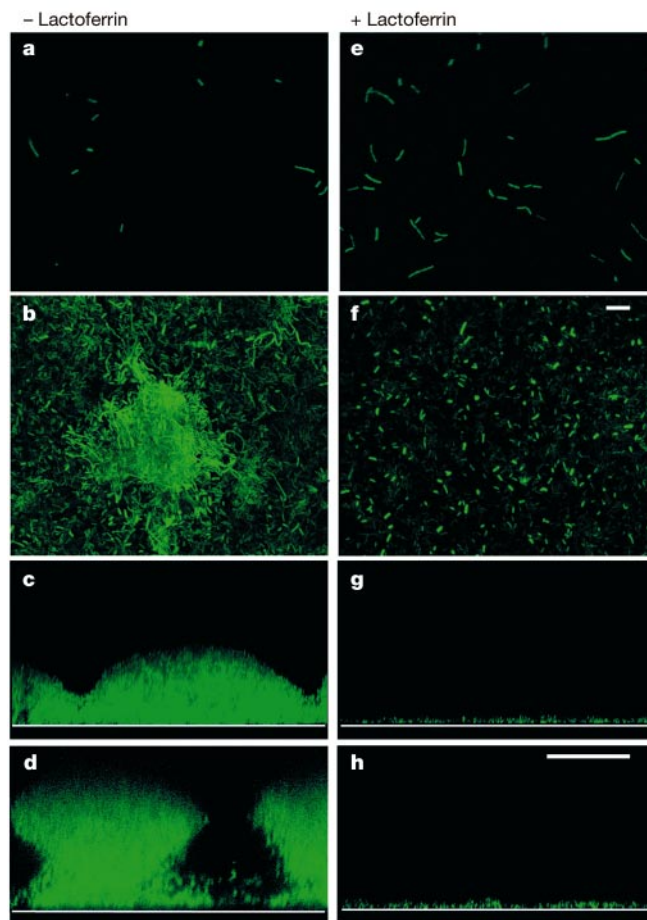


Figure 2 Confocal microscopic images of GFP-labelled *P. aeruginosa* in biofilm flow cells perfused with lactoferrin-free (a–d) and lactoferrin-containing (20 µg ml⁻¹) (e–h) media. Images were obtained 4 h (a, e), 24 h (b, f), 3 days (c, g) and 7 days (d, h) after inoculating the flow cells. Images a, b, e and f are top views (x–y plane); scale bar, 10 µm. Images c, d, g and h are side views (x–z plane); scale bar, 50 µm. Results are representative of six experiments.

development. This concentration of lactoferrin did not affect the growth rate of free-swimming *P. aeruginosa* strain PAO1 (Fig. 1).

To evaluate the effect of lactoferrin on biofilm formation, we grew *P. aeruginosa* expressing green fluorescent protein (GFP) in continuous-culture-flow cells and followed biofilm development over time. Flow cell chambers were continuously perfused with biofilm medium with or without lactoferrin. In medium without lactoferrin (Fig. 2a–d), we observed the typical stages of biofilm development^{20,21}. Initially, bacteria attached to the surface (Fig. 2a). Microcolonies were evident after 24 h (Fig. 2b). After 3 days, the microcolonies had enlarged (Fig. 2c). By day 7, towering pillar and mushroom-shaped biofilms had developed (Fig. 2d). Lactoferrin disrupted this pattern of development (Fig. 2e–h). Attached bacteria (Fig. 2e) multiplied, but they failed to form microcolonies (Fig. 2f). Even after prolonged incubation, the bacteria did not assemble into differentiated biofilm structures; in the presence of lactoferrin they remained in a thin layer (Fig. 2g, h). In contrast, exposing mature, 5-day-old biofilms to lactoferrin-containing medium for 48 h failed to alter their structure (not shown). Thus, once they had developed, biofilms were resistant to lactoferrin.

Because lactoferrin prevented biofilm development, we performed additional studies to confirm that the low concentration of lactoferrin did not prevent growth. To show that lactoferrin-containing medium in flow cells could support growth of *P. aeruginosa*, we cultured bacteria in the effluent from a biofilm chamber. *P. aeruginosa* doubled six times in 22 h in this conditioned medium (not shown), verifying that even spent lactoferrin-treated medium did not limit growth. Second, we measured the dividing times of attached bacteria in flow cells using time-lapse video microscopy. Lactoferrin increased the dividing time of attached

cells by 27% (93 min without lactoferrin compared with 127 min with 20 $\mu\text{g ml}^{-1}$ lactoferrin). Although this reduced growth rate could decrease the size of microcolonies and biofilms, it could not account for the complete absence of biofilm structure induced by lactoferrin.

Although the time-lapse microscopy showed only small differences in dividing times, it revealed that lactoferrin markedly altered bacterial movement. These differences are represented in Fig. 3a and b, tracing the movement of representative bacteria over the surface of a flow cell. In the absence of lactoferrin (Fig. 3a), the parent bacterium moved across the field of view. When the parent cell divided, the two daughter cells remained near the point of parent cell division. When a daughter cell divided, its progeny also remained near the point of the original cell division. Thus, a microcolony began to form. In the presence of lactoferrin (Fig. 3b), the parental cell also moved across the field of view, and divided into two daughter cells. With lactoferrin, however, the daughter cells moved away from the point of cell division. When one of the daughter cells divided, its progeny also left the site of cell division (time-lapse microscopy images are available as Supplementary Information).

To analyse the changes quantitatively, we defined three behaviours and classified the actions of 40 parental cells and their offspring over three generations. Bacteria that remained stationary from the time they were created by cell division to the time they themselves divided were called squatters. Bacteria that moved away from the division site were called ramblers, and cells that detached from the surface and were swept away by the flow of medium were called flyers. The relative proportions of bacteria engaged in these different behaviours are shown in Fig. 3c and d. In the absence of lactoferrin, most cells were squatters, fewer cells were flyers, and rambling was rare. In the presence of lactoferrin, a significantly larger proportion of cells exhibited rambling behaviour and fewer were squatters. In both cases, the predominant behaviour (squatting without lactoferrin, and rambling with lactoferrin) became more

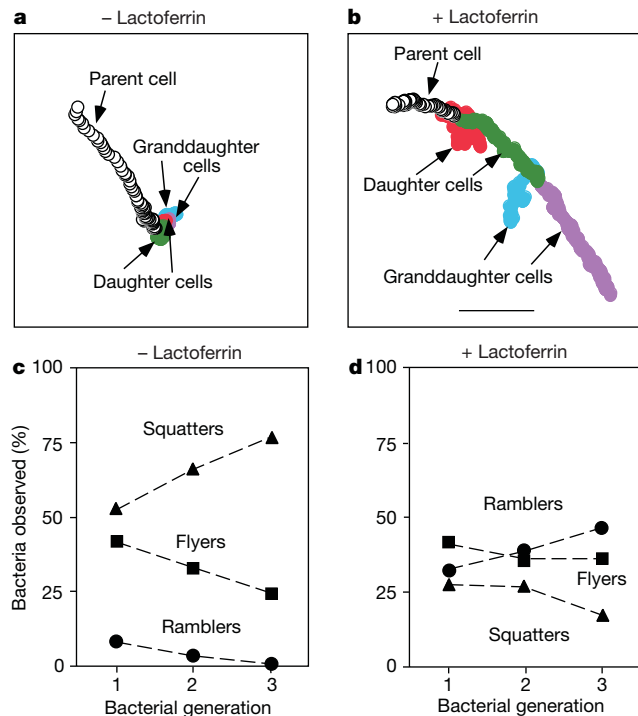


Figure 3 Representations of bacterial behaviours. **a, b**, Movement of representative cells without **(a)** and with **(b)** lactoferrin. Each point represents bacterial position at 1-min intervals. Scale bar, 20 μm . **c, d**, Effect of lactoferrin on bacterial behaviour without **(c)** and with **(d)** 20 $\mu\text{g ml}^{-1}$ lactoferrin. Three behaviours were defined: squatting, flying and rambling, and the proportion of bacteria engaged in each behaviour was assessed over three generations. Data were collected from six different movies and represent the behaviour of a total of 462 bacteria. Proportions of squatters and ramblers were different in control and lactoferrin media ($P < 0.001$, binomial distribution test).

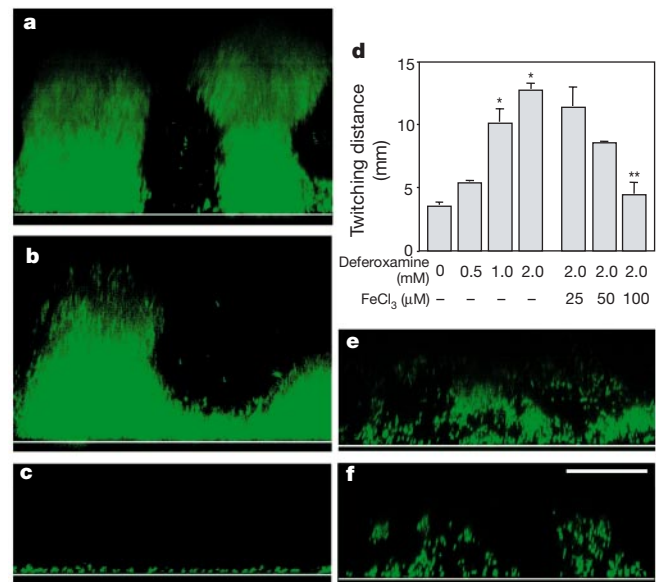


Figure 4 Role of iron in twitching motility and biofilm development. **a–c**, Effect of iron-saturated lactoferrin on biofilm formation in wild-type *P. aeruginosa*. **a**, No lactoferrin; **b**, Fe-saturated lactoferrin; **c**, Fe-unsaturated lactoferrin. **d**, Effect of iron chelation by deferoxamine on *P. aeruginosa* twitching motility. Asterisk, $P < 0.001$, one-way ANOVA, as compared with no deferoxamine. Double asterisk, $P < 0.005$, one-way ANOVA, as compared with 2 mM deferoxamine without FeCl_3 . **e, f**, Effect of control **(e)** and lactoferrin-containing **(f)** medium on biofilm formation by a non-twitching mutant. Scale bar, 50 μm . Similar results were obtained in three other experiments.

prevalent in subsequent bacterial generations.

We next investigated the mechanism of lactoferrin's action on biofilm development. To examine the role of iron, we compared the activity of iron-saturated lactoferrin to iron-unsaturated lactoferrin (Fig. 4a–c). Unlike iron-unsaturated lactoferrin, iron-saturated lactoferrin did not prevent biofilm formation by *P. aeruginosa*. Conalbumin, a lactoferrin-like host defence protein from chicken eggs, functioned similarly; it prevented biofilm formation in the iron-unsaturated state, but not when saturated with iron (not shown). We also studied deferoxamine, an iron chelator unrelated to lactoferrin that has a lower iron-binding affinity than lactoferrin^{22,23}. At subinhibitory concentrations, it prevented biofilm formation, and time-lapse studies showed that deferoxamine also stimulated bacterial surface motility (not shown). However, with deferoxamine, microcolonies did occasionally form, probably owing to its lower iron-binding affinity. Together, these results suggest that lactoferrin blocks biofilm formation in *P. aeruginosa* by sequestering free iron.

We hypothesized that the increased surface motility induced by iron chelation was due to twitching, a specialized form of surface locomotion mediated by type 4 pili²⁴. To test this, we performed twitching motility assays in which *P. aeruginosa* was inoculated at a point on the bottom of agar plates, and the rate at which bacteria spread over the agar–plastic interface was measured. Deferoxamine stimulated twitching motility in a dose-dependent manner, and this response was blocked by adding iron (Fig. 4d). Thus, as free iron levels decreased, twitching motility increased.

To test further whether lactoferrin prevented biofilm development by stimulating twitching motility, we examined its effect on a *P. aeruginosa* twitching mutant. We reasoned that the mutant would form biofilms in the presence of lactoferrin. The mutant formed microcolonies and irregularly shaped biofilms in both the absence and presence of lactoferrin (Fig. 4e, f), although the biofilms formed in the presence of lactoferrin were somewhat smaller. This stands in contrast to the twitching wild-type strain, where differentiated biofilm formation was completely blocked by lactoferrin (Fig. 4a, c). Taken together, these results indicate that lactoferrin prevents biofilm formation by stimulating bacterial twitching motility. Furthermore, the concentration of lactoferrin that had this effect did not limit the growth of free-swimming bacteria and only slightly reduced the growth of attached cells. Once bacteria were living in an established biofilm, they lost sensitivity to lactoferrin.

Biofilm bacteria are extraordinarily resistant to killing by antimicrobial agents^{5,6,8}. We hypothesized that the surface-attached bacterial layers that formed in the presence of lactoferrin would be less resistant than differentiated biofilms that formed in the absence of lactoferrin. To test this, we grew bacteria in a biofilm reactor on small removable discs. This allowed the antimicrobial susceptibility of the bacterial community to be tested with its

multicellular structure intact. Bacteria were grown with or without conalbumin. Conalbumin was used in these studies because the cost of lactoferrin was prohibitive for the large volume of medium required, and, as described above, lactoferrin and conalbumin affected biofilm formation similarly. After 48 h, the discs were removed from the reactor (and from the conalbumin), and two agents were tested: H₂O₂, which neutrophils use in the oxidative killing of bacteria, and tobramycin, an antibiotic used clinically to treat *P. aeruginosa* infections. Control biofilms were resistant to both agents: 1,000 µg ml⁻¹ tobramycin and 500 mM H₂O₂ had minimal effects on viability after 4 h of treatment (Fig. 5). In contrast, growth in conalbumin decreased resistance to both agents in a dose-dependent manner. Thus, in addition to inhibiting structural differentiation, iron chelation limited the development of an important functional consequence of biofilm formation, antimicrobial resistance.

These findings can be viewed from two perspectives. For the host, the development of a biofilm infection on a normally sterile mucosal surface can have disastrous consequences. Our data suggest that lactoferrin has a previously unrecognized role in host defence. In addition to its well-known bactericidal and bacteriostatic actions, it blocks the formation of *P. aeruginosa* biofilms at a low concentration, keeping the bacteria more vulnerable to killing. This function may serve as a failsafe mechanism to prevent bacteria that survive initial defences from assuming the intractable biofilm state. Secondary immune responses may then be better able to combat the infecting organisms. As noted above, biofilms form in the airways of cystic fibrosis patients and on other compromised mucosal surfaces^{5–7}. Although our data do not address how this occurs in the presence of lactoferrin, there is evidence that lactoferrin may be inactivated by proteolytic cleavage in the lungs of cystic fibrosis patients²⁵. Other reports have shown that the levels of free iron are increased in airway secretions of cystic fibrosis patients^{26,27}. However, these observations were made in patients with established airway infections, and thus their relevance to the pathogenesis of *Pseudomonas* biofilm infection is unclear.

From the bacterial point of view, biofilms are a growth mode specialized for long-term colonization of surfaces. Our data indicate that a higher level of iron is required for biofilm formation than is needed for growth. If the iron level is acceptable, *P. aeruginosa* is cued to stop moving, form microcolonies, and eventually develop into biofilms. If iron levels are not sufficient, the *P. aeruginosa* cells keep moving. This response may protect the bacteria from constructing complex, durable biofilm structures in locations where iron, a critical nutrient, is in short supply. □

Methods

Bacterial strains, plasmids, and growth conditions

Pseudomonas aeruginosa strain PAO1 containing the GFP plasmid pMRP9-1 (ref. 28) was used for most studies. Where indicated, an isogenic twitching motility mutant (a PAO1 pilHIIJK deletion mutant from J. Kato) containing pMRP9-1 was used. Biofilm medium consisted of 1% Trypticase Soy Broth (Difco). In the growth experiments, about 10³ bacteria from an overnight culture were added to 5 ml of biofilm medium containing various concentrations of lactoferrin or deferoxamine (Sigma). Cultures were incubated in acid-washed tubes at 37 °C with shaking. Colony-forming units were determined by plate counting. The concentration of lactoferrin that inhibited bacterial growth varied somewhat for different lots of lactoferrin but was never less than 30 µg ml⁻¹. The concentration of deferoxamine that slowed growth was never less than 5 mM. *P. aeruginosa* with pMRP9-1 was also grown in lactoferrin-containing effluent medium from flow cells. After 1 day of bacterial growth, effluent was collected on ice, filter sterilized, and growth was assessed as above.

Biofilm experiments

For studies of biofilm formation, wild-type *P. aeruginosa* PAO1 and the twitching motility mutant were grown in flow cells similar to those described previously²⁸; the size of the flow channel was 5 × 35 × 1 mm. An overnight culture diluted to 10⁷ cells per ml in fresh biofilm medium was used as the inoculum and flow was arrested for 45 min. Flow of biofilm medium with and without 20 µg ml⁻¹ of Fe-unsaturated or Fe-saturated lactoferrin, conalbumin, or 3–5 mM deferoxamine (Sigma) was then initiated at a rate of 170 µl min⁻¹. Images were obtained using a Bio-Rad scanning confocal microscope. Bacterial movement was assessed by using time-lapse images acquired at 1 min intervals.

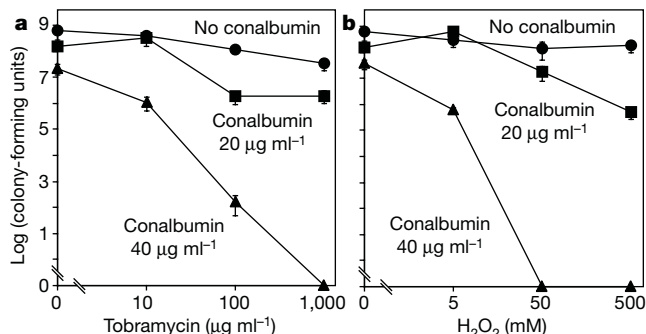


Figure 5 Effect of conalbumin on the antimicrobial susceptibility of *P. aeruginosa* biofilms to tobramycin (a) and H₂O₂ (b). Data are mean ± s.e.m., n = 6 from three different experiments.

Motion of bacterial cells was traced visually by following individual cells. VOXblast software (VayTek) was used to obtain the *x-y* coordinates of bacterial cells. Bacterial behaviors were classified as follows. Squatters remained within a 15 μm circle drawn around the point of parental cell division until the time of its cell division. Ramblers remained attached to the growth surface, but moved outside the circle. Flyers detached and were carried away by media flow. Dividing times of attached bacteria were measured by counting the number of frames between cell divisions; 60 bacterial divisions were observed and the dividing times were averaged.

Pseudomonas aeruginosa biofilms for susceptibility tests were grown in a rotating disc reactor²⁹. Fe-unsaturated conalbumin (Sigma) was added to standard biofilm medium at indicated concentrations for the duration of biofilm growth. Discs and attached bacteria were then washed three times in distilled water, and treated for 4 h in 1 ml of H₂O₂ (Fisher Scientific) or tobramycin (Eli Lilly) at indicated concentrations. The treated discs were washed three times, and bacteria were removed and dispersed in 2 ml sterile PBS by homogenization (Brinkman Homogenizer). Viable cell numbers were enumerated by plate counting.

Twitching motility assays

Plates for twitching motility assays consisted of biofilm medium plus 1% Noble agar (Difco). Indicated concentrations of deferroxamine and FeCl₃ (Sigma) were added to molten agar. Plates were dried overnight at room temperature, and *P. aeruginosa* with pMRP9-1 was point inoculated at the bottom of the Petri plate³⁰. After 3 days, the twitching distance along the plastic-agar interface (at the bottom of the agar plate) was measured.

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Supplementary Information accompanies the paper on Nature's website (<http://www.nature.com>).

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Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to P.K.S. (e-mail: pradeep-singh@uiowa.edu).

Single and multiple vesicle fusion induce different rates of endocytosis at a central synapse

Jian-Yuan Sun*, Xin-Sheng Wu* & Ling-Gang Wu

Departments of Anesthesiology, Anatomy and Neurobiology, Washington University School of Medicine, St Louis, Missouri 63110, USA

* These authors contributed equally to this work

During synaptic transmission, neurotransmitter-laden vesicles fuse with the presynaptic membrane and discharge their contents into the synaptic cleft. After fusion, the vesicular membrane is retrieved by endocytosis for reuse. This recycling mechanism¹ ensures a constant supply of releasable vesicles at the nerve terminal¹. The kinetics of endocytosis have been measured mostly after intense or non-physiological stimulation^{2–13}. Here we use capacitance measurements to resolve the fusion and retrieval of single and multiple vesicles following mild physiological stimulation at a mammalian central synapse. The time constant of endocytosis after single vesicle fusion was 56 ms; after a single action potential or trains at ≤ 2 Hz it was about 115 ms, but increased gradually to tens of seconds as the frequency and the number of action potentials increased. These results indicate that an increase in the rate of exocytosis at the active zone induces a decrease in the rate of endocytosis. Existing models^{5,10}, including inhibition of endocytosis by Ca²⁺, could not account for these results—our results suggest that an accumulation of unretrieved vesicles at the plasma membrane slows endocytosis. These findings may resolve the debate about the dependence of endocytosis kinetics on the stimulation frequency^{2,3}, and suggest a potential role of regulation of endocytosis in short-term synaptic depression.

To detect single vesicle endocytosis, both the miniature excitatory postsynaptic current (mEPSC) and the membrane capacitance (C_m) at the calyx of Held (nerve terminal) were simultaneously recorded (Fig. 1a). We increased the mEPSC rate to 24 ± 2 Hz (*n* = 46) by including 10 mM EGTA and 7.5 mM Ca²⁺ in the