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Modifying the mechanical property and shear threshold of L-selectin adhesion independently of equilibrium properties

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Interactions between adhesion molecules on two different cells differ from interactions between receptors and soluble ligands in that the adhesion molecule interaction (bond) is often subjected to force. It is widely assumed by cell biologists that the 'strength' of a bond is a simple function of the affinity of one adhesion molecule for the other, whereas biophysicists suggest that bonds have 'mechanical properties' that affect their strength. Mechanical properties are a function of the shape of the energy landscape related to bond formation and dissociation, whereas affinity is related only to the net energy change^{1–5}. Mechanical properties determine the amount by which the kinetics and affinity of bonds are altered by applied force. To date there has been no experimental manipulation of an adhesion molecule that has been shown to affect mechanical properties. L-selectin is an adhesion molecule that mediates lymphocyte binding to, and rolling on, high endothelial venules; these are prerequisites for the emigration of lymphocytes from the bloodstream into lymph nodes. Here we report a selective and reversible chemical modification of a mucin-like ligand that alters the mechanical properties of its bond with L-selectin. The effect of force on the rate of bond dissociation, that is, on a mechanical property, is altered, whereas there is little or no effect of the modification on the rate of bond dissociation in the absence of force. Moreover, the puzzling requirement for hydrodynamic shear flow above a threshold level for L-selectin interactions^{6–9} is dramatically altered.

L-selectin is expressed on leukocytes and binds to a sialyl-Lewis-X-like carbohydrate ligand that is expressed on mucin-like molecules on the endothelium and on other leukocytes^{10,11}. L-selectin is important in initiating rolling interactions between leukocytes in the blood and endothelial cells of the blood-vessel wall. The presence of a small number of L-selectin–ligand interactions at any one time, and rapid bond breakage and formation, allow the zone of adhesive contact to be translated along the vessel wall as cells roll in response to hydrodynamic drag forces^{12–14}. Like all other adhesive interactions, those through selectins can be inhibited from forming, or reversed, by applied force. As shear stress on the vessel wall, a stress which is proportional to the force on a cell, is increased above a certain level, the binding of cells in the bloodstream to the vessel wall can no longer be initiated (Fig. 1a). Cells bound to the vessel wall at moderate wall shear stresses are dislodged at high wall

shear stresses (Fig. 1b). It is puzzling that adhesive interactions through L-selectin do not occur at low shear stresses^{6,8,13}. Rolling adhesions through L-selectin are not initiated at stress levels below about 0.4 dyn per cm² (Fig. 1a), and cells rolling at moderate wall shear stresses are detached when shear is decreased to <0.4 dyn per cm² (Fig. 1b).

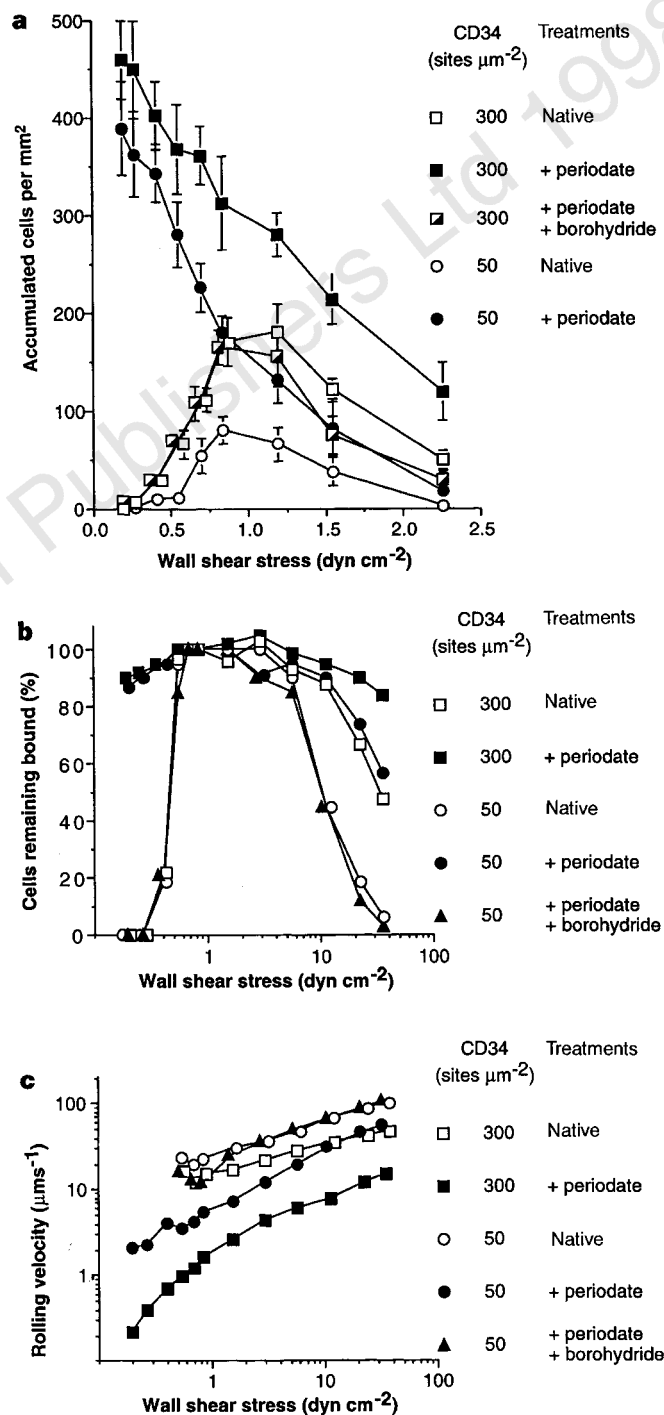


Figure 1 Mild periodate treatment of the L-selectin counter-receptor CD34 abolishes the requirement for a threshold of shear stress for L-selectin-mediated adhesion. This abolition is independent of the strength of rolling adhesions. Substrates bearing the CD34 component of peripheral-node addressin were or were not treated with mild periodate, and were or were not later treated with borohydride. **a**, Accumulation of lymphocytes at different wall shear stresses. **b**, Resistance of lymphocytes accumulated on the substrate at 0.84 dyn per cm² to detachment by subsequent increases or decreases in wall shear stress. **c**, Rolling velocity.

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Chemical modification of enzymes has previously been used to demonstrate that K_M and k_{cat} are separable properties. Mild treatment of L-selectin ligands with periodate increases their binding to L-selectin^{15,16} and we tested whether mechanical properties of the interaction were affected. This chemical modification is highly selective for sialic acid; the exocyclic C8 and C9 carbons are cleaved and the C7 hydroxyl is converted to an aldehyde. We purified CD34, one of the mucin-like ligands for L-selectin^{17,18}, from tonsil. CD34 was then adsorbed to the lower wall of a flow chamber. Leukocyte interactions with the same CD34 substrate were measured before and after treatment of the substrate with mild periodate, and in some cases again after reduction of the sialic acid C7 aldehyde to a hydroxyl by using borohydride. We did not expose leukocytes to any of the reagents and removed them from the flow chamber during chemical treatments of the CD34 substrate. Periodate increased the strength of rolling interactions, as measured by resistance to detachment and slowed rolling velocity, much as did a six- to eightfold increase in CD34 density (Fig. 1b, c)¹⁶. These effects were reversed by reduction of the C7 aldehyde by borohydride. Interactions were specific, because they were completely inhibited by a monoclonal antibody against L-selectin, by EDTA, and by fucoidan (data not shown)¹⁶. The requirement for shear stress at levels above ~ 0.4 dyn per cm^2 for adhesion through L-selectin⁶ was abolished by periodate treatment (Fig. 1a, b). This abolition of the shear threshold was seen even when periodate-treated substrates at 50 sites per μm^2 and native substrates at 300 sites per μm^2 were compared (Fig. 1a); these substrates were similar in terms of the strength of adhesive interactions (Fig. 1b), the rolling velocity (Fig. 1c), and the number of rolling cells that accumulated at 0.8 dyn per cm^2 and above (Fig. 1a). Cells that were accumulated at 0.8 dyn per cm^2 and were rolling on native substrates or substrates treated with periodate and borohydride detached when shear stress was reduced to <0.4 dyn per cm^2 (Fig. 1b). In contrast, cells on

periodate-treated substrates remained rollingly adherent when shear was reduced (Fig. 1b). Therefore, periodate treatment dramatically and reversibly alters adhesive interactions with L-selectin at levels of stress <0.8 dyn per cm^2 , independently of the overall strength of adhesive interactions above 0.8 dyn per cm^2 .

At densities of selectins or their ligands on vessel walls that are too low to support rolling, cells are transiently tethered to vessel walls, that is, they associate and then dissociate^{13,14}. The kinetics of cellular dissociation are first-order and have other properties consistent with dissociation of single bonds^{13,14}, including agreement with kinetics measured for purified molecules¹⁹. The mechanical properties of the tether bond are measurable as the amount by which force on the cell, and hence on the tether bond, increases the dissociation rate constant, k_{off} ^{13,14}. The kinetics of transient tethers to CD34 were measured using automated image analysis. The first 90–99% of the transient tethers to dissociate fit a straight line, showing first-order dissociation kinetics (Fig. 2a, b). The remaining 1–10% of cells dissociated more slowly (not shown), either because of background problems or because of formation of more than one tether. The transient-tether-dissociation rate constant (k_{off}) was independent of CD34 density over the range of 0.5–10 sites per μm^2 on both native and periodate-treated substrates (not shown)¹³.

To quantify the mechanical properties of the receptor–ligand bond, we plotted k_{off} as a function of force on the bond (F_b) (Fig. 3). The relationship between wall shear stress and F_b is linear and has previously been estimated^{13,14}. The increase in k_{off} as a function of force was greater for the native ligand than for the periodate-treated

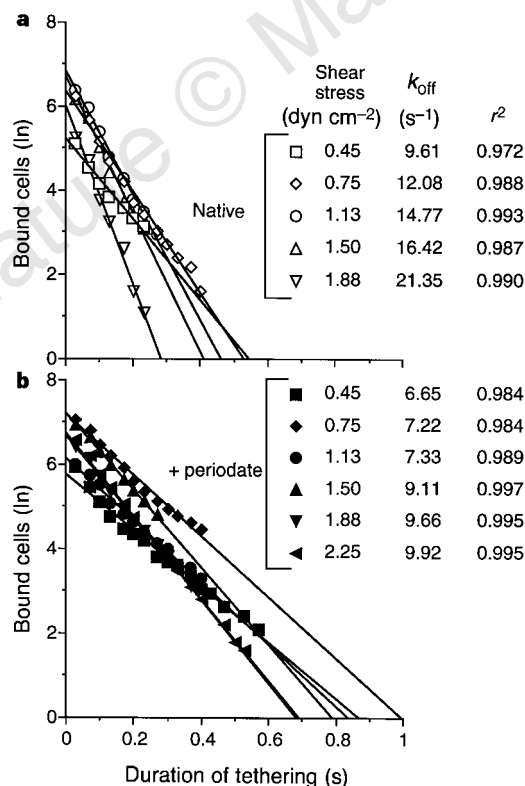


Figure 2 The kinetics of dissociation of transiently tethered neutrophils from native and mild-periodate-treated CD34. Kinetics were measured at different wall shear stresses at 0.5 sites per μm^2 of native CD34 (a) and mild-periodate-treated CD34 (b). r , coefficient of correlation.

	k_{off}^0 (s^{-1})	σ (\AA)	χ^2	d.f.
Native	8.0 ± 0.3	0.18 ± 0.01	3.47	32
+ periodate	5.6 ± 0.2	0.09 ± 0.01	3.56	38
+ periodate + borohydride	5.9 ± 0.6	0.25 ± 0.02	2.79	11

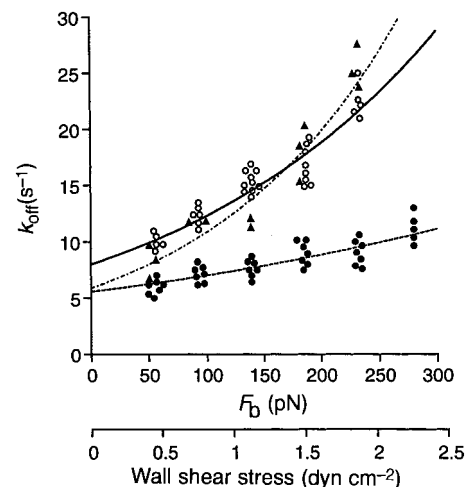


Figure 3 The effect of mild periodate and borohydride treatment on the dissociation kinetics of transient tethers between L-selectin and its ligand and on the mechanical strength of L-selectin–ligand interactions. k_{off} was determined at CD34-site densities from 0.5 to 10 sites per μm^2 . Force on the bond, F_b , was estimated using the length of the lever arm between the tether point on the substrate and the projected centre of the cell on the substrate, as described¹³. Each group of points represents a single wall shear stress; points were separated horizontally so all can be seen. Lines show the fit to Bell's model¹. The values of k_{off} , the bond separation distance (σ), the total χ^2 value, and degrees of freedom (d.f.) are shown. A fit to a Hookean spring model² yielded κ/F_x values (the bond spring constant divided by the fraction of bond stress devoted to dissociation) of 8.6 ± 0.7 , 17.9 ± 2.0 , and 5.9 ± 0.6 N m^{-1} , for native, periodate-, and periodate- and borohydride-modified substrates, respectively, and similar χ^2 values.

ligand (Fig. 3). The mechanical effect of mild periodate was almost completely reversed by reduction with borohydride (Fig. 3). The data were fitted to two theoretical models relating k_{off} to F_b (refs 1, 2). As measured with the exponential constants σ in the Bell model¹ (Fig. 3) or κ/f_k in the spring model² (Fig. 3 legend), the mechanical strength of the tether bond is doubled when an aldehyde is present at the 7-position of sialic acid, compared with either the native structure or the mildly oxidized and reduced structure, which both have a hydroxyl at the 7-position.

The overall mechanical strength of a bond will be a function of the effect of force on both k_{on} and k_{off} . The frequency of transient tethers is related to k_{on} , although not necessarily linearly²⁰. Periodate also seemed to increase the mechanical strength of the tether bond as measured by transient-tether frequency, because, as shear stress was increased from 0.75 to 1.88 dyn per cm², the frequency of tethers to the native substrate fell by 6.5-fold but the frequency of tethers to the periodate-treated substrate fell by only 2.6-fold (Fig. 4).

We have shown that the mechanical properties of a receptor–ligand bond can be altered by chemical modification of the ligand, independently of effects on k_{off} in the absence of force. Thus, the chemical properties that govern mechanical strength and affinity are distinct, analogous to those that govern k_{cat} and K_M for enzymes. Adhesion molecules may therefore differ from one another in both mechanical strength and affinity, and, to understand the function of adhesion molecules in resisting forces *in vivo*, both properties must be measured. The details of how chemistry regulates the mechanical properties of L-selectin ligands are currently unknown. An aldehyde oxygen is more basic than a hydroxyl oxygen, and therefore is a better acceptor for hydrogen bonds²¹. Sialic acid is a key part of the sialyl Lewis-X structure recognized by L-selectin, but it is also present on non-ligand-bearing glycans attached throughout the length of the heavily glycosylated mucin-like region of D34. Mild periodate treatment has less effect on rolling interactions involving sialyl Lewis-X glycolipid than on interactions involving CD34 (ref. 16).

The mechanical properties of a bond can be altered by modifying the elasticity of regions outside the receptor–ligand interface²²; therefore, the C7 aldehyde group on sialic acid might be able to form hydrogen bonds that alter the elastic spring constant of the

mucin-like region of CD34. This might explain the effect on mechanical strength and not on k_{off} in the absence of force. The reversible alteration of the shear-threshold requirement by mild periodate is remarkable, particularly as the same threshold exists over a range of CD34 densities, and as substrates that give equivalent rolling velocities and resistance to detachment at stress levels above 0.8 dyn per cm² nonetheless behave differently from each other at <0.8 dyn per cm². We suggest that the elasticity of the mucin-like region of CD34 may be important in its mechanical properties, and it is interesting that ligands that lack mucin-like regions, fucoidan and sialyl Lewis-X glycolipid do not exhibit a shear threshold⁶. Although the explanation for the shear threshold remains unclear, our data indicate that there may be an important connection between the shear-threshold phenomenon and the mechanical properties of the L-selectin–ligand bond. Furthermore, our results emphasize the importance of mechanical properties for regulating cell adhesion in shear flow. □

Methods

Flow assays. The CD34 component of human peripheral-node addressin was isolated from detergent lysates of human tonsils by successive immunoaffinity chromatography on MECA-79 monoclonal antibody/Sepharose and CD34 monoclonal antibody/Sepharose¹⁸. Drops of 50 μl CD34 (350 to 60 ng ml⁻¹ for rolling experiments and 12 to 0.6 ng ml⁻¹ for transient-tethering experiments, in 0.01 M Tris HCl, 0.15 M NaCl, 0.05% NaN₃, pH 7.5, 0.5% octyl β -D-glucoside) were adsorbed to polystyrene slides overnight at 4 °C. Substrates were washed with PBS and quenched with 2% human serum albumin (HSA). CD34-site density was determined by saturation binding of radiolabelled monoclonal antibody to CD34 (ref. 18). Site densities yielded by input concentrations of ≤ 12 ng ml⁻¹ were extrapolated from higher site-density determinations; as the immobilization procedure and the concentration of the detergent in the adsorption media were constant, site density of adsorbed CD34 was assumed to be proportional to CD34 concentration.

Peripheral blood lymphocytes and neutrophils were purified as described²³. Cells were stored in Ca²⁺- and Mg²⁺-free Hank's balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.5, and 0.25% HSA (H/H medium), and resuspended at 5×10^5 cells per ml in binding medium (HBSS/HEPES containing 2 mM Ca²⁺) before use. CD34 substrates were assembled as the lower wall in the flow chamber and mounted on an inverted phase-contrast microscope¹⁸. Images from a Nikon plan $\times 20$ objective were recorded on videotape. For detachment and rolling-velocity assays²³, cells were perfused through the chamber at 0.84 dyn per cm² until enough had accumulated (~3 min). Non-adherent cells were cleared by perfusion with binding medium at 0.84 dyn per cm². Rolling velocities were determined for 30–50 of the cells observed during detachment assays. Cell displacement was measured over 5–8-s intervals. Velocities were measured only for cells that remained adherent throughout the 10-s period during which a given shear was applied. Cell accumulation was determined by perfusing cells (10^6 ml⁻¹) through the chamber at a range of wall shear stresses for 3 min and counting the rollingly adherent cells.

Treatments. Mild treatment with periodate was carried out after baseline cell-adhesion assays in the flow chamber and washing out of cells. Sodium periodate solution (5 mM) in PBS (pH 7.2) was infused into the flow chamber for 30 min at 4 °C in the dark¹⁶. After further cell-adhesion assays, in many cases substrates were reduced by infusion with 100 mM sodium borohydride (Sigma Chemical) in PBS pH 7.2, at room temperature, for 30 min. After each treatment, the flow chamber was equilibrated with binding medium and adhesion was scored to the same microscopic field of CD34. Cells were detached with 5 mM EDTA in H/H medium before substrate treatments and between tethering and accumulation assays at different shear stresses.

Inhibition with anti-L-selectin or control (IgG) monoclonal antibodies and with fucoidan and EDTA was performed as described¹⁸. All monoclonal antibodies and inhibitors remained during the adhesion assay.

Transient tethers. The duration of transient tethers on CD34 was analysed by a computerized imaging system, consisting of a Pentium computer with MVC 150/40-VL boards (Imaging Technology)²⁴. Cells interacting with the substrate were detected by discriminating their velocity from the hydrodynamic velocity

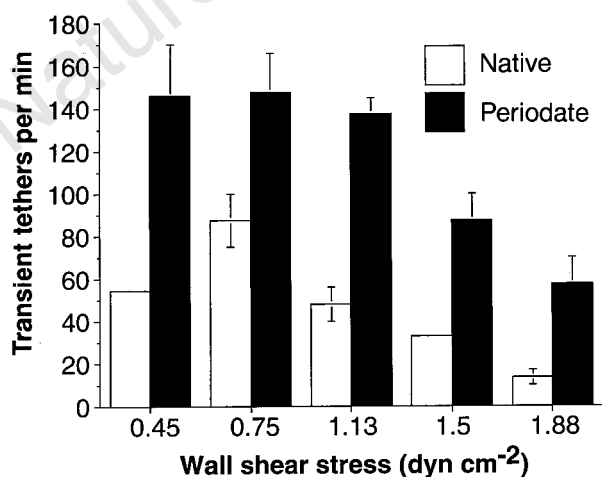


Figure 4 Mild treatment of CD34 with periodate enhances the transient-tethering frequency of neutrophils. We determined the frequency of transient tethers to CD34 at 0.5 sites per μm^2 before and after mild periodate treatment. Neutrophils were at a concentration of 10^6 ml⁻¹. Tethering events were defined as transient when no rolling (<2 μm displacement) occurred while the cell was tethered. After a cell had transiently tethered once, it seemed to have a higher chance of making further transient tethers downstream. Therefore, for cells that made multiple tethers, only the first tether was counted.

distribution of cells free in flow. We analysed enough videotape to obtain 100 to 1,600 tethering events, and plotted the natural log of the number of cells that remained bound as a function of time after initiation of tethering. The most rapidly dissociating 90% or more of tethered cells were used to determine k_{off} .

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Calcium oscillations increase the efficiency and specificity of gene expression

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Cytosolic calcium ($[Ca^{2+}]_i$) oscillations are a nearly universal mode of signalling in excitable and non-excitable cells^{1–4}. Although Ca^{2+} is known to mediate a diverse array of cell functions, it is not known whether oscillations contribute to the efficiency or specificity of signalling or are merely an inevitable

consequence of the feedback control of $[Ca^{2+}]_i$. We have developed a Ca^{2+} clamp technique to investigate the roles of oscillation amplitude and frequency in regulating gene expression driven by the proinflammatory transcription factors NF-AT, Oct/OAP and NF- κ B. Here we report that oscillations reduce the effective Ca^{2+} threshold for activating transcription factors, thereby increasing signal detection at low levels of stimulation. In addition, specificity is encoded by the oscillation frequency: rapid oscillations stimulate all three transcription factors, whereas infrequent oscillations activate only NF- κ B. The genes encoding the cytokines interleukin (IL)-2 and IL-8 are also frequency-sensitive in a way that reflects their degree of dependence on NF-AT versus NF- κ B. Our results provide direct evidence that $[Ca^{2+}]_i$ oscillations increase both the efficacy and the information content of Ca^{2+} signals that lead to gene expression and cell differentiation.

Oscillations in $[Ca^{2+}]_i$ may be advantageous for receptor-mediated signal transduction, for example by increasing the fidelity of low-level signalling, preventing desensitization, or increasing signalling specificity^{1–4}; however, it has been difficult to demonstrate these and other possible functions⁵ for two reasons. First, the amplitude and frequency of $[Ca^{2+}]_i$ oscillations triggered through surface receptors varies among cells and in single cells over time, resulting in a mixture of stimulus waveforms that complicates analysis. Second, surface receptors are often coupled to multiple signalling pathways, making it difficult to ascribe downstream effects to Ca^{2+} alone. We have therefore developed a 'calcium clamp' method for generating homogeneous and synchronous receptor-independent $[Ca^{2+}]_i$ oscillations in large populations of T lymphocytes⁶ (Fig. 1). Ca^{2+} signals leading to T-cell activation are normally generated by a cascade involving antigen binding to the T-cell antigen receptor (TCR), generation of the second messenger inositol 1,4,5-trisphosphate ($InsP_3$), release of Ca^{2+} from internal stores, and Ca^{2+} influx across the plasma membrane⁷. Here we bypass the TCR/ $InsP_3$ pathway by treating Jurkat T cells with thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} -ATPases that depletes internal Ca^{2+} stores and irreversibly activates store-operated Ca^{2+} (CRAC) channels in the plasma membrane⁸. Application of Ca^{2+} to cells treated in this way elevates $[Ca^{2+}]_i$ owing to influx through CRAC channels, whereas removal of extracellular Ca^{2+} allows pumps in the plasma membrane to return $[Ca^{2+}]_i$ to baseline levels. Thus, by rapidly changing the concentration of extracellular Ca^{2+} , it is possible to generate $[Ca^{2+}]_i$ oscillations having a uniform frequency across cells and an amplitude that is relatively constant in each cell over time (s.d., 12.7%) and among cells in the population (s.d., 18.2%; $n = 256$). A further advantage is that this technique probably mimics naturally occurring subcellular gradients of $[Ca^{2+}]_i$, because $[Ca^{2+}]_i$ oscillations triggered through the TCR result from periodic activation of CRAC channels^{9,10}.

We investigated whether oscillations affect the efficiency with which Ca^{2+} signals are detected. NF-AT is a Ca^{2+} -dependent transcription factor expressed in many cells, including T lymphocytes, in which it helps to regulate several immune-response genes including IL-2, IL-4 and tumour-necrosis factor- α (TNF- α)^{11,12}. NF-AT is activated by Ca^{2+} -stimulated dephosphorylation and translocation of a cytoplasmic subunit, which binds to a nuclear subunit induced by protein kinase C or by stimulation of the MAP kinase pathway^{11,12}. We compared the activity of an NF-AT/*lacZ* reporter gene¹³ in Jurkat cells stimulated with an oscillatory or constant elevation of $[Ca^{2+}]_i$ for 3 hours in the presence of 50 nM phorbol-12,13-dibutyrate (PdBu). Oscillations were generated with a period of 100 s, which is similar to the period in intact cells stimulated through the TCR^{9,14}. Oscillation amplitude was adjusted to produce the same average $[Ca^{2+}]_i$ as in the constant- $[Ca^{2+}]_i$ control cells to determine whether the kinetic features of the oscillations confer any signalling advantage relative to a sustained $[Ca^{2+}]_i$ increase, independently of the amount of Ca^{2+} that enters a cell. Figure 2a shows constant and oscillatory $[Ca^{2+}]_i$ stimuli in one

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