

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<sup>1–4</sup>. 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- $\kappa$ 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- $\kappa$ 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- $\kappa$ 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<sup>1–4</sup>; however, it has been difficult to demonstrate these and other possible functions<sup>5</sup> 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 lymphocytes<sup>6</sup> (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 membrane<sup>7</sup>. 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 membrane<sup>8</sup>. 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<sup>9,10</sup>.

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- $\alpha$  (TNF- $\alpha$ )<sup>11,12</sup>. 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<sup>11,12</sup>. We compared the activity of an NF-AT/*lacZ* reporter gene<sup>13</sup> 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<sup>9,14</sup>. 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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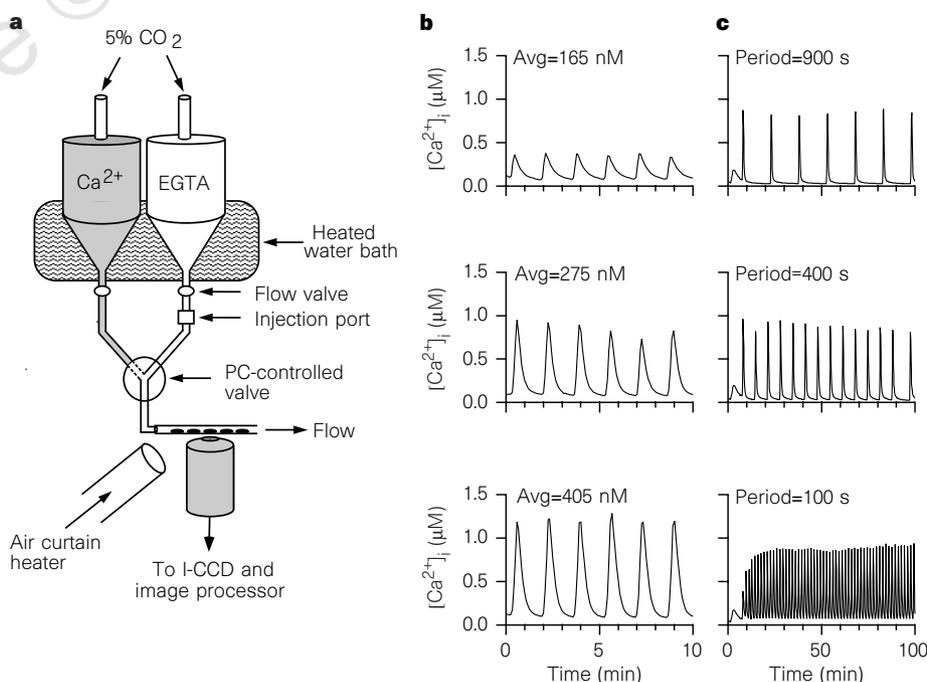
experiment in which the average  $[Ca^{2+}]_i$  was  $\sim 225$  nM; subsequent fluorescence-activated cell sorting (FACS) analysis indicated that 39% of the cells stimulated with  $[Ca^{2+}]_i$  oscillations were *lacZ*<sup>+</sup>, whereas only 19% of the cells with constant  $[Ca^{2+}]_i$  were *lacZ*<sup>+</sup>. This enhancement of NF-AT-dependent transcription was not evident at higher  $[Ca^{2+}]_i$  levels. In cells stimulated to an average of  $\sim 350$  nM  $Ca^{2+}$ , oscillations activated roughly the same amount of *lacZ* expression as constant  $[Ca^{2+}]_i$  elevation (Fig. 2b). The results of 12 paired experiments show that the induction of *lacZ* by constant  $[Ca^{2+}]_i$  elevation falls off sharply below  $\sim 300$  nM  $[Ca^{2+}]_i$ , whereas induction by oscillations is approximately constant down to at least 200 nM (Fig. 2c). The enhancement of NF-AT-dependent gene expression by oscillations thus increases as average  $[Ca^{2+}]_i$  decreases below  $\sim 300$  nM (Fig. 2d). In this way, oscillations appear to increase the ability of small  $Ca^{2+}$  signals to activate NFAT.

A long-standing question in cell signalling is how  $Ca^{2+}$ , with its abundant and varied intracellular targets, is able to achieve specificity and activate only a subset of those targets. One explanation is that variations in oscillation amplitude or frequency may discriminate among different  $Ca^{2+}$ -activated signalling pathways. In T cells, several transcription factors in addition to NF-AT are  $Ca^{2+}$ -sensitive, including NF- $\kappa$ B and Oct/OAP<sup>15,16</sup>. Both factors are involved in the control of IL-2 transcription, and NF- $\kappa$ B also helps to regulate a large number of genes encoding cytokines, growth factors, adhesion molecules and other surface proteins<sup>11,17</sup>. To determine whether the amplitude of the  $Ca^{2+}$  signal can distinguish among transcriptional pathways, we compared the  $Ca^{2+}$  sensitivity of luciferase reporter genes driven by NF-AT, Oct/OAP or NF- $\kappa$ B in Jurkat cells. Phorbol ester (50 nM PDBU) or increased  $[Ca^{2+}]_i$  ( $>600$  nM) alone did not activate NF-AT or Oct/OAP measurably in thapsigargin-treated cells, and only slightly stimulated NF- $\kappa$ B-dependent expression ( $\sim 2.5$ -fold above background). In contrast, the two stimuli acted together in a synergistic fashion to activate NF-AT, Oct/OAP and NF- $\kappa$ B by 150-, 31- and 16-fold, respectively. In each case, the  $Ca^{2+}$ -dependent component of the response was completely suppressed by cyclosporin A, a potent and selective inhibitor of calcineurin,

consistent with a role for this phosphatase in mediating the effects of  $Ca^{2+}$  on these factors<sup>15,18,19</sup>. As shown in Fig. 3a, all three pathways show a similar and highly nonlinear dependence on steady-state  $[Ca^{2+}]_i$ . The pronounced nonlinearity of NF-AT activation is consistent with previous studies<sup>5,20</sup> and is an important determinant of the effects of oscillations on NF-AT signaling efficiency (see below). However, the similar  $Ca^{2+}$  dependencies of the three transcription factors suggests that oscillation amplitude is not likely to contribute significantly to selectivity among these pathways in T cells. In a previous study of B cells<sup>21</sup>, nuclear translocation of NF-AT appeared to be more  $Ca^{2+}$ -sensitive than that of NF- $\kappa$ B; however, the transcriptional activity of these factors was not tested.

As oscillation amplitude is unlikely to discriminate among these three pathways, we tested whether oscillation frequency could. The calcium clamp was used to generate high-amplitude oscillations ( $\sim 1$   $\mu$ M peak) with periods from 100 to 1,800 s. As shown in Fig. 3b, the activation of all three transcription factors shows declines with increasing period. However, NF- $\kappa$ B activation extends to oscillation periods as long as 1,800 s, in contrast to NF-AT and Oct/OAP, whose activity vanishes at periods  $\geq 400$  s. Frequency dependence of NF-AT has also been observed in response to photolytically generated pulses of  $InsP_3$  (ref. 22), indicating that this behaviour is not specific to the use of thapsigargin to deplete stores. The difference in frequency requirements among the three factors is consistent with previous findings that NF- $\kappa$ B persists in the nucleus for  $>16$  min following a brief  $Ca^{2+}$  spike<sup>21</sup>, whereas NF-AT quickly exits following its rapid rephosphorylation<sup>21,23</sup>. Thus, oscillation frequency dictates which combination of transcription factors is active; low frequencies recruit NF- $\kappa$ B alone, whereas high frequencies activate NF-AT, Oct/OAP and NF- $\kappa$ B. These results indicate that  $[Ca^{2+}]_i$  oscillation frequency can discriminate among different transcriptional pathways.

The distinct frequency sensitivities of NF-AT, Oct/OAP and NF- $\kappa$ B suggest that endogenous genes under the control of these factors may also respond differentially to oscillation frequency. We therefore compared the frequency sensitivities of genes encoding IL-2, an



**Figure 1** Generation of synchronized  $[Ca^{2+}]_i$  oscillations in T cell populations. **a**, Diagram of the  $Ca^{2+}$  clamp (see Methods). Oscillations are generated by alternately exposing cells with depleted stores to 0 or 1.5 mM  $Ca^{2+}$ . **b, c**, Average  $[Ca^{2+}]_i$  in 200–300 cells in which the amplitude or frequency of oscillations was

varied. In **b**,  $Ca^{2+}$  was applied for 10 s (top), 15 s (middle), or 30 s (bottom) every 100 s. In **c**,  $Ca^{2+}$  was applied for 30-s periods with a interspike interval of 900 s (top), 400 s (middle), or 100 s (bottom).

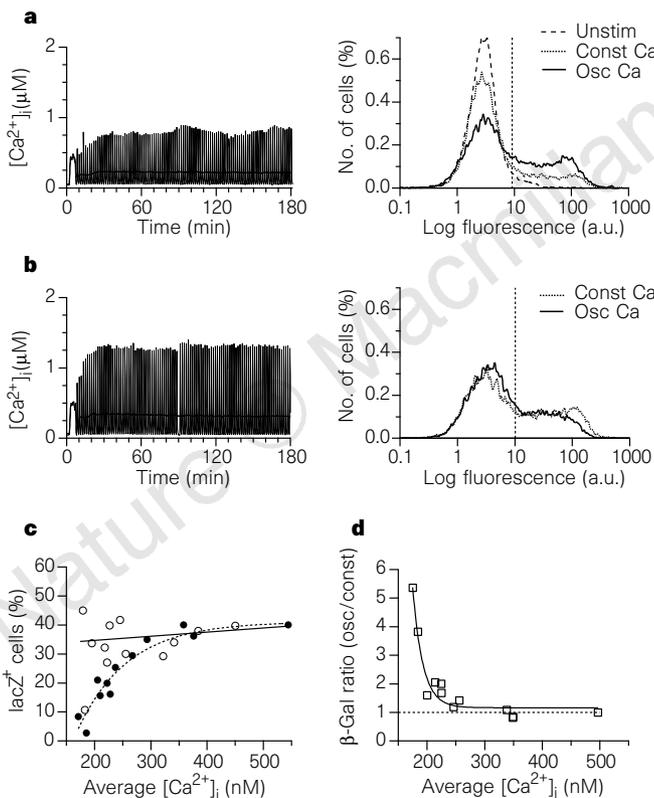
essential growth factor for T cells, and IL-8, a chemokine involved in recruiting immune cells to sites of infection. Both genes can be activated in T cells by  $Ca^{2+}$  ionophore and phorbol ester<sup>24,25</sup>, but their promoters are driven by different combinations of transcription factors: IL-2 expression is highly dependent on NF-AT and Oct/OAP binding<sup>11,26</sup>, whereas IL-8 depends on NF- $\kappa$ B and other factors but not on NF-AT<sup>25,27</sup>. Cells were transfected with luciferase reporter constructs driven by the IL-2 or IL-8 promoters and were stimulated for 3 h with 50 nM PdBu and constant  $[Ca^{2+}]_i$  elevation. Both genes appear to be highly  $Ca^{2+}$ -sensitive, with IL-8 being more sensitive than IL-2 (Fig. 3c). The two genes show different sensitivities to  $[Ca^{2+}]_i$  oscillation frequency (Fig. 3d): IL-2 is not expressed at periods  $\geq 400$  s, whereas IL-8 retains  $\sim 20\%$  of its maximal activity under these conditions. The behaviour of IL-2 resembles that of NF-AT and Oct/OAP, whereas the behaviour of IL-8 is similar to that of NF- $\kappa$ B. These results were supported by measurements of endogenous IL-8 expression in Jurkat cells treated with monensin to prevent secretion<sup>28</sup> (IL-2 expression after 3 h stimulation was too low to measure accurately). FACS analysis of anti-IL-8-stained cells indicated that the frequency dependence of IL-8 expression was similar to that of the reporter gene. Together, these results indicate

that oscillations of different frequencies can lead to the expression of different sets of genes, presumably as a consequence of their effects on the underlying transcription factors.

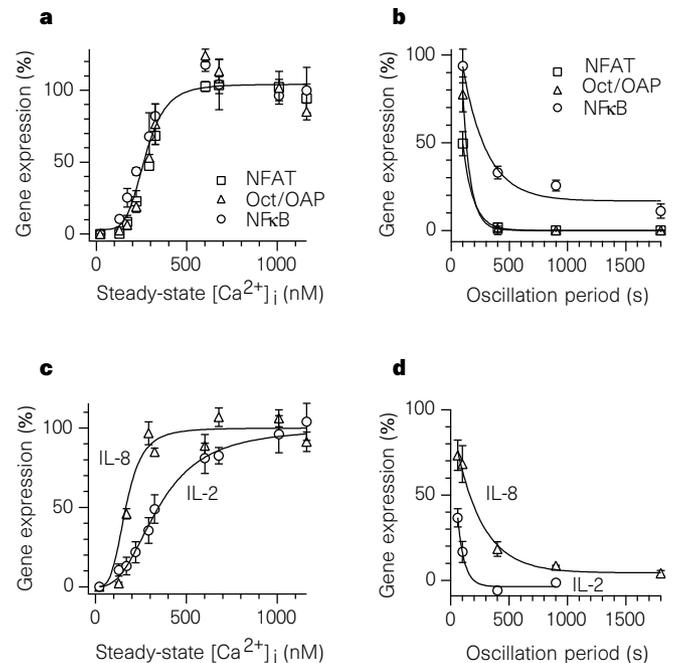
Our results demonstrate two important properties of nuclear signalling by  $[Ca^{2+}]_i$  oscillations. First, oscillations enhance signalling efficiency specifically at low levels of stimulation. This effect arises from the highly nonlinear dependence of transcription on  $[Ca^{2+}]_i$ , so that oscillations periodically exceed the threshold for activation whereas a small constant  $[Ca^{2+}]_i$  rise of the same average magnitude does not. The tendency of  $[Ca^{2+}]_i$  to oscillate at low receptor occupancy in many cells may thus optimize sensitivity to weak external stimuli. Second, oscillations confer specificity on an otherwise highly pleiotropic  $Ca^{2+}$  signal. By differentially controlling the activation of distinct sets of transcription factors and the expression of different genes, oscillation frequency may direct cells along specific developmental pathways<sup>29,30</sup>. Frequency-dependent gene expression is likely to be widespread, as  $Ca^{2+}$ -dependent transcription factors like NF- $\kappa$ B and NF-AT are present in a great variety of cells and oscillations can occur with periods of seconds to hours<sup>1,29</sup>. □

**Methods**

**Cells and solutions.** All experiments were done on the NF-ATZ-DIPA clone of Jurkat T cells, stably transfected with an NF-AT-*lacZ* reporter gene and selected for high *lacZ* inducibility<sup>13</sup>. Experiments were done in RPMI 1640 without



**Figure 2**  $[Ca^{2+}]_i$  oscillations enhance the calcium sensitivity of NF-AT-dependent transcription at low levels of stimulation. **a**, NF-AT-dependent *lacZ* expression in cells stimulated with a low level of  $[Ca^{2+}]_i$ . Left, superimposed  $[Ca^{2+}]_i$  recordings from two cell populations (200–300 cells each) stimulated for 3 h with oscillating  $[Ca^{2+}]_i$  (227 nM average) or constant  $[Ca^{2+}]_i$  (225 nM average). Right, flow cytometry analysis of  $\beta$ -gal activity in the same experiments show that oscillating  $[Ca^{2+}]_i$  activates 39% of the cells while constant  $[Ca^{2+}]_i$  activates only 19%. The vertical gate excludes 97% of the unstimulated cells. **b**, NF-AT-dependent expression at a high level of  $[Ca^{2+}]_i$ . Left, stimulation with oscillating  $[Ca^{2+}]_i$  (340 nM average) or constant  $[Ca^{2+}]_i$  (359 nM average). Right, oscillating and constant  $[Ca^{2+}]_i$  activate 33 and 39% of the cells, respectively. **c**, Percentage of *lacZ*<sup>+</sup> cells induced by various concentrations of oscillating (○) or constant (●)  $[Ca^{2+}]_i$ . **d**, Ratio of the number of  $\beta$ -gal<sup>+</sup> cells activated by oscillating as compared with constant  $[Ca^{2+}]_i$ , as a function of average  $[Ca^{2+}]_i$ , calculated from the data in **c**. All experiments were done in the presence of 50 nM PdBu.



**Figure 3** Effects of  $[Ca^{2+}]_i$  and oscillation frequency on NF-AT-, Oct/OAP-, NF- $\kappa$ B-, IL-2- and IL-8-luciferase reporter genes. **a**, Steady-state  $Ca^{2+}$  sensitivities of NF-AT, Oct/OAP and NF- $\kappa$ B. A plot of the Hill equation is superimposed, with a Hill coefficient ( $n_H$ ) of 4.7 and a  $K_d$  of 270 nM. **b**, Oscillation frequency dependences of NF-AT (□), Oct/OAP (Δ) or NF- $\kappa$ B (○) reporter genes. **c**, Steady-state  $Ca^{2+}$  sensitivities of IL-2 (○) and IL-8 (Δ) reporter genes. Fits to the Hill equation are superimposed (for IL-2,  $n_H = 2.7$  and  $K_d = 346$  nM; for IL-8,  $n_H = 3.6$  and  $K_d = 166$  nM). **d**, Frequency dependences of IL-2 (○) and IL-8 (Δ) reporter genes. Error bars represent s.e.m. of triplicate measurements in 3–5 experiments; where they are not visible they are smaller than the symbols. All experiments were done in the presence of 50 nM PdBu.

phenol red or riboflavins and supplemented with 25 mM HEPES and 0.2% BSA. Solutions were maintained at 4 °C until immediately before each experiment, when they were warmed to 37 °C and supplemented with 50 nM PdBU and either 0.5 mM EGTA (~50 μM free Ca<sup>2+</sup>) or 1 mM Ca<sup>2+</sup> (1.5 mM total Ca<sup>2+</sup>).

**Transfection of the reporter genes.** NF-AT, NF-κB, Oct-1/OAP and IL-2 reporter constructs were provided by J. Goldberg and G. Crabtree and were derived from constructs described previously<sup>16</sup>. Multimeric copies of NFAT (3 copies), NF-κB (4) or Oct/Oap (4) binding sites, linked to a minimal (non-inducible) IL-2 promoter (-74 to +47), were inserted between the *Sma*I and *Hind*III sites in the multiple cloning region of the pGL-3 luciferase reporter vector (Promega). The construct with the intact IL-2 promoter region was made by inserting 371 bp of the IL-2 initiation region (-324 to +47) into the pGL-3 vector. The IL-8 reporter construct was provided by K. P. LeClair<sup>27</sup>.

Transfection of 10<sup>7</sup> cells was by electroporation with 10 μg of the reporter vector, 1 μg of a vector containing large-T antigen and 2 μg of a vector encoding the transmembrane and extracellular domains of CD8. Large-T antigen was used to increase the number of copies of the reporter construct in each cell, and the CD8 construct was used to determine the transfection efficiency. Transfection efficiencies were 30–40%; viability following centrifugation to remove cells killed during electroporation was 85–95%. Experiments were conducted 24–48 h after transfection when the expression of reporter genes was maximal.

**Ca<sup>2+</sup> clamp and Ca<sup>2+</sup> measurements.** Transfected cells were loaded with 2 μM Fura-PE3/AM (Teflabs) for 1 h at 37 °C in loading medium (RPMI 1640, 25 mM HEPES, 2% fetal bovine serum), washed, and incubated for another hour to allow complete de-esterification of the dye. Loaded cells (2–3 × 10<sup>5</sup>) were allowed to adhere to a polylysine-coated laminar flow chamber and were placed on the heated stage of a Zeiss Axiovert 35 inverted microscope. The laminar flow chamber (60 μl volume) was connected to two heated reservoirs containing 1.5 mM and 0 mM Ca<sup>2+</sup> solutions, and pressurized with a mixture of 95% air and 5% CO<sub>2</sub>. At the start of each experiment, cells were treated with 1 μM thapsigargin in 0 mM Ca<sup>2+</sup> solution for 5 min to deplete internal Ca<sup>2+</sup> stores and irreversibly activate CRAC channels. A computer-controlled solenoid valve (General Valve) was used to switch rapidly between the Ca<sup>2+</sup>-containing and Ca<sup>2+</sup>-free solutions flowing into the chamber and over the cells. The solution in the chamber was fully exchanged about once per second and was maintained at 37 °C. Cells were stimulated for 3 h while [Ca<sup>2+</sup>]<sub>i</sub> was measured every 5–10 s by video microscopy as described<sup>10</sup>. Fura-PE3 was calibrated on the microscope in a microcuvette using solutions containing 1 mM EGTA and 10 mM Ca<sup>2+</sup> according to ref. 10.

**Reporter gene assays.** Following stimulation cells were washed from the chamber, lysed by freeze/thawing and subjected to a luciferase assay using standard methods. All measurements were done in triplicate and normalized to the total number of cells determined using a Coulter Counter (Coulter Electronics). β-Galactosidase (β-gal) expression was measured by flow cytometry using the FACS-Gal protocol<sup>13</sup>. Steady-state [Ca<sup>2+</sup>]<sub>i</sub> dependence of luciferase reporter genes (Fig. 3) was determined after 3 h stimulation with 1 μM thapsigargin, 50 nM PdBU and variable extracellular [Ca<sup>2+</sup>]. Luciferase activity was normalized to values between 0 and 100%, given by the responses to TG + PdBU in medium containing 0.5 mM EGTA or 1.5 mM Ca<sup>2+</sup>, respectively.

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## Cell-permeant caged InsP<sub>3</sub> ester shows that Ca<sup>2+</sup> spike frequency can optimize gene expression

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Inositol 1,4,5-trisphosphate (InsP<sub>3</sub>) releases calcium from intracellular stores and triggers complex waves and oscillations in levels of cytosolic free calcium<sup>1–5</sup>. To determine which longer-term responses are controlled by oscillations in InsP<sub>3</sub> and cytosolic free calcium, it would be useful to deliver exogenous InsP<sub>3</sub>, under spatial and temporal control, into populations of unpermeabilized cells. Here we report the 15-step synthesis of a

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