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Calcium-Modulating Cyclophilin Ligand Desensitizes Hormone-Evoked Calcium Release

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The Ca^{2+}-modulating cyclophilin ligand (CAML) protein causes stimulation of transcription factors via activation of a store-operated Ca^{2+} entry pathway. Since CAML is widely expressed in mammalian tissues, it may be an important regulator of Ca^{2+} store function. In the present study, we investigated the consequence of CAML overexpression on Ca^{2+} signaling using rapid confocal imaging of Fluo3-loaded NIH3T3 fibroblasts. Control and CAML-expressing cells gave concentration-dependent responses to the Ca^{2+} mobilizing agonist ATP. CAML expression reduced the sensitivity of the cells so that higher concentrations of ATP were needed to achieve global Ca^{2+} waves. The amplitudes of Ca^{2+} waves were significantly reduced in CAML expressing cells, consistent with earlier suggestions that CAML causes depletion of internal Ca^{2+} stores. With low ATP concentrations, only local Ca^{2+} release events were observed. CAML did not affect the characteristics of these local Ca^{2+} signals, suggesting that it does not directly affect Ca^{2+} release channels.

The Ca^{2+}-modulating cyclophilin ligand (CAML) protein has been shown to participate in the activation of transcription factors in T cells (1, 2). CAML is implicated in the signal transduction cascade leading from a member of the tumor necrosis receptor family (TACI) to activation of NF-AT, AP-1 and NF-κB transcription factors (1). In addition, when expressed by itself in lymphocytes, CAML stimulates calcineurin thus causing the subsequent activation of NF-AT (2). The stimulation of calcineurin arises due to the elevated cytosolic Ca^{2+} levels observed in CAML expressing cells. The way in which CAML increases cytosolic Ca^{2+} is not entirely clear, but it seems to be by activating the "capacitative" Ca^{2+} entry (CCE) pathway that is normally triggered after hormone-induced release of endoplasmic reticulum (ER) Ca^{2+} stores (3). Consistent with this notion, CAML-induced Ca^{2+} increases are acutely dependent on extracellular Ca^{2+} (2).

CAML is widely expressed in mammalian tissues, with particular abundance in the testis and brain (2). It is a protein of 296 amino acids with three predicted transmembrane sequences in the C-terminal region (2). CAML has been shown to be an integral membrane protein with the N-terminus projecting into the cytoplasm. Only the C-terminal 46 amino acids, which includes two of the three transmembrane sequences, appears to be necessary for elevation of cytosolic Ca^{2+} (4). The bulk of the N-terminus interacts with the TACI receptor on the plasma membrane by a direct protein–protein interaction (1).

CAML colocalizes substantially with SERCA2 Ca^{2+}ATPases and to a lesser extent with calreticulin (5), suggesting that it is expressed in ER Ca^{2+} stores. However, it has no apparent sequence homology to known Ca^{2+} channels (4), and it is therefore unlikely that CAML itself releases Ca^{2+}. Furthermore, in sucrose density gradients it did not cosediment with inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$), indicating that it has no direct effect on these intracellular Ca^{2+} release channels (5). Expression of CAML increases the sensitivity of cells to the Ca^{2+}ATPase inhibitor thapsigargin (4). This observation, along with the apparent constitutive activation of CCE in CAML-expressing cells (2) led to the suggestion that it causes a leak of Ca^{2+} from the ER, perhaps by interaction with Ca^{2+}ATPases (5).

MATERIALS AND METHODS

CAML transfection and cell culture. Low passage NIH3T3 cells (a generous gift from Dr. Martine Roussel, Memphis TN) were grown in DMEM plus 10% v/v fetal calf serum. Cells were transfected by electroporation with plasmid pGFC1, which contains the complete open reading frame of the human CAML cDNA inserted (as a *Xho*I–*Bam*HI fragment) into the multiple cloning site of pEGFP-C1 (Clontech). This plasmid directs expression of the enhanced green fluorescent protein fused at its C-terminus to the CAML protein. Cells were grown in the presence of G418 (1 mg/ml) to select for stable trans-

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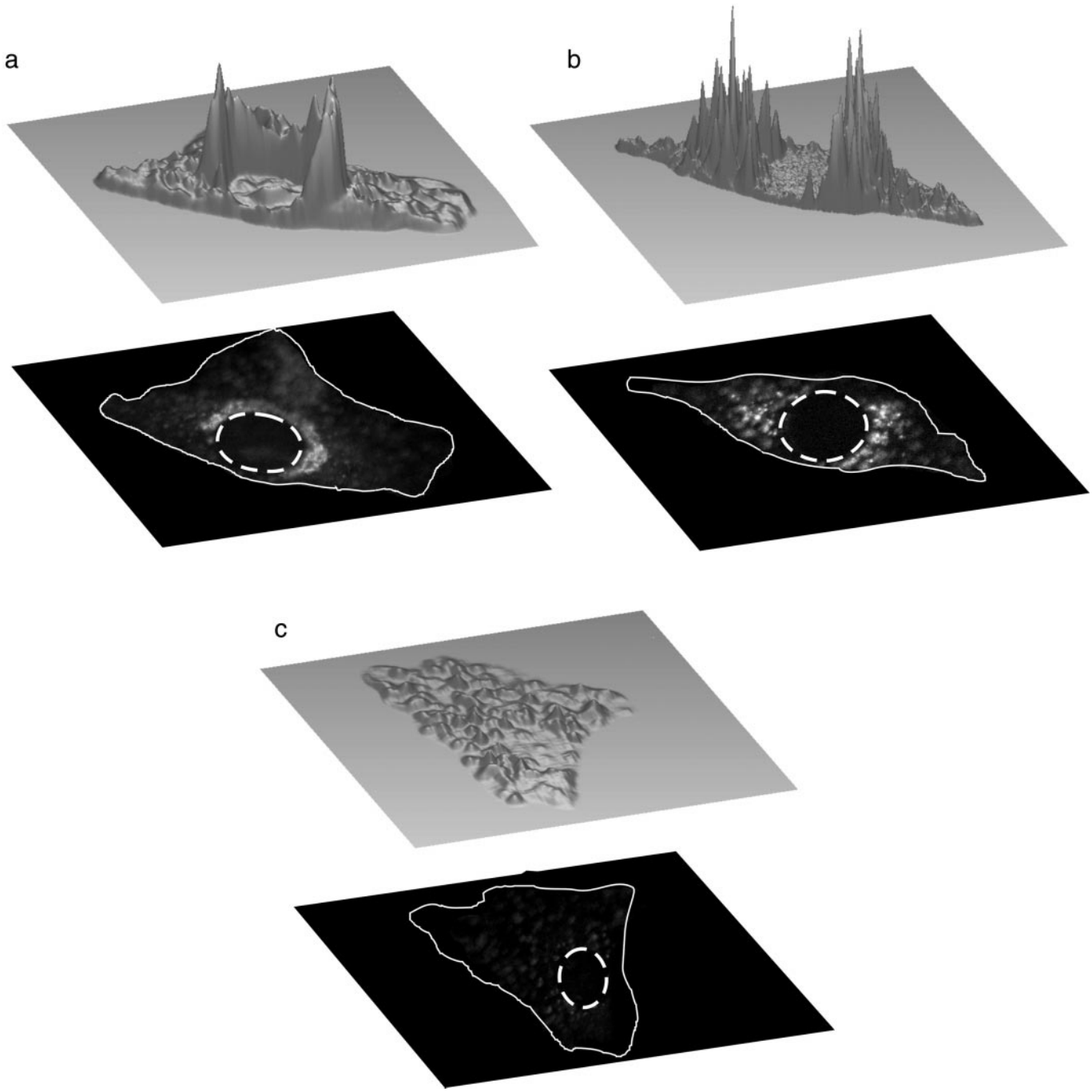

FIG. 1. Spatial localisation of GFP-CAML expressed in NIH3T3 fibroblasts. Cells expressing either GFP-CAML (a and b) or GFP alone (c) were imaged as described under Materials and Methods. The intensity of fluorescence staining is encoded by the height of the surfaces in the 3-D plots (ai–ci). The perinuclear pattern of GFP-CAML expression is apparent from the 2-D images (aii–cii). The position of the nucleus within each cell is marked by the dashed line. The boundary of the cells is indicated by the continuous line.

formants. After 3 weeks culture, cells were sorted by flow cytometry to obtain GFP-bright (i.e., CAML overexpressing) as well as nonfluorescent (control) cell populations.

Confocal imaging. The culture medium was replaced with an extracellular medium containing (mM): NaCl, 121; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; $NaHCO_3$, 6; glucose, 5.5; Hepes, 25; pH 7.3. Cells were loaded with Fluo-3 by incubation with 2 μM Fluo-3 acetoxymethyl ester (Molecular Probes Inc.) for 30 min, followed by a 30-min deesterification period. All incubations and experiments were carried out at room temperature (20–22°C). Confocal cell imaging was performed as described elsewhere (6). Briefly, a single glass coverslip was mounted on the stage of an inverted microscope attached to a Noran *Oz* laser-scanning confocal microscope, equipped with a standard argon-ion laser for illumination. Fluo-3 was excited using the 488-nm laser line, and the emitted fluorescence was collected at wavelengths >505 nm. Images were acquired using the confocal

microscope in image mode at a frequency of 7.5 Hz. Absolute values for Ca^{2+} concentrations were calculated according to a self-ratio method described elsewhere (6). The K_d of Fluo-3 was taken as 810 nM (7).

RESULTS AND DISCUSSION

NIH3T3 cells were stably transfected with GFP-tagged CAML (see Materials and Methods). In agreement with previous observations (5), the subcellular distribution of CAML appeared to be mainly perinuclear (Figs. 1a and 1b). In typical confocal sections, CAML-GFP was located around the nucleus spreading ~20 μm into the cytoplasm from the nuclear envelope (average cell diameter ~150 μm). No CAML-GFP signal was detected in the nucleus itself, and only smaller amounts in the cytoplasm nearer to the plasma membrane. The CAML-GFP signal in the perinuclear region appeared either as a ring of fluorescence (Fig. 1a), or with a punctate distribution (Fig. 1b). No localized fluorescence was detected in control NIH 3T3 cells expressing GFP alone (Fig. 1c).

Superfusion with the $InsP_3$-generating agonist ATP evoked concentration-dependent Ca^{2+} increases in both control and CAML-expressing cells. Although cell populations responded over the same range of ATP concentrations, CAML decreased the sensitivity of the cells to ATP so that fewer cells responded at each ATP concentration. Furthermore, at low ATP concentrations (0.5 and 1 μM) a higher proportion of CAML-expressing cells only showed trains of localized "elementary" Ca^{2+} signals (i.e., Ca^{2+} puffs; for detailed description see Ref. 6) that did not eventually lead to regenerative Ca^{2+} waves (Fig. 2a). In contrast, more of the control cells responded with global Ca^{2+} waves (Figs. 2b and 2c).

The amplitudes (63 ± 6 nM; mean ± SEM; $n = 422$) and range of spatial spreading (2–6 μm) of Ca^{2+} puffs was similar in control and CAML-expressing cells. In many cell types, the Ca^{2+} puffs that are triggered by low agonist concentrations occur around the nucleus (8). Such Ca^{2+} puff sites are denoted as "pacemaker" sites, since they are responsible for elevating cytosolic Ca^{2+} to the point where regenerative Ca^{2+} waves ensue (6). Similarly, NIH3T3 fibroblasts also displayed perinuclear pacemaker Ca^{2+} puffs. In the CAML-expressing cells, the pacemaker sites usually occurred within the region where CAML expression was highest. However, the average distance from the nuclear envelope to the pacemaker Ca^{2+} puff sites (9.5 ± 0.6 μm; mean ± SEM; $n = 173$) was the same in control and CAML-expressing cells, suggesting that CAML did not affect the position of the pacemaker sites. The amplitudes of the global Ca^{2+} waves evoked by ATP differed significantly between control and CAML-expressing cells. In control cells, the amplitudes of Ca^{2+} waves were 1150 ± 64 nM (mean ± SEM; $n = 20$), and in CAML expressing cells the amplitudes of Ca^{2+} waves was decreased to 794 ± 74 nM (mean ± SEM; $n = 15$).

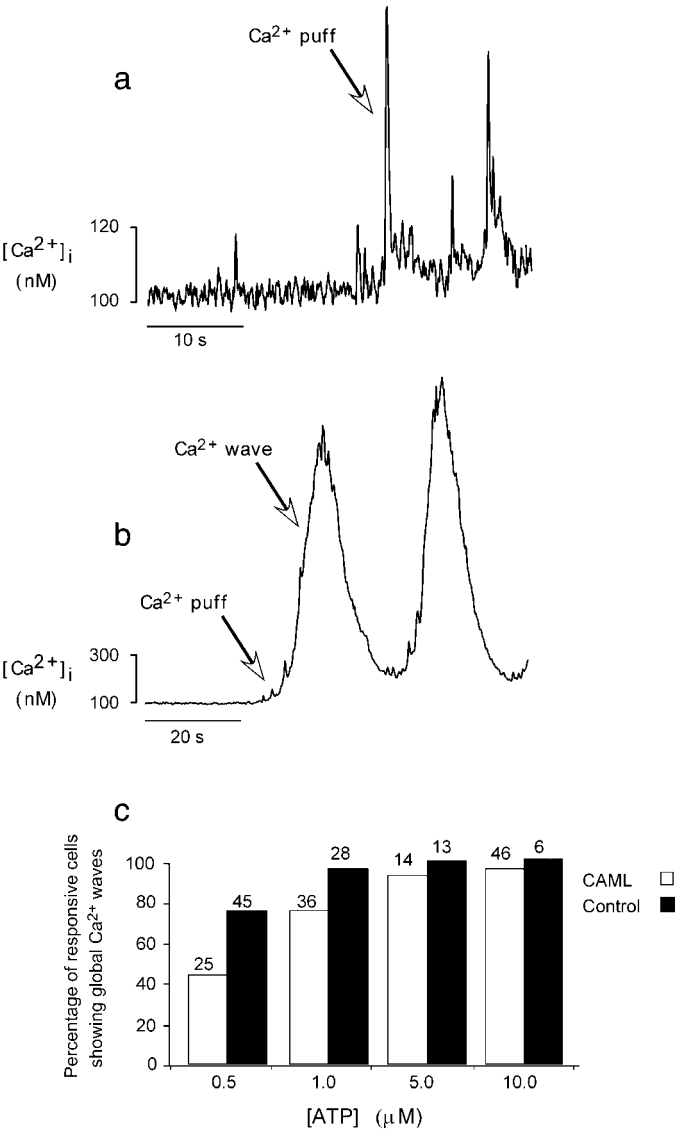

FIG. 2. CAML expression decreases the proportions of cells showing regenerative Ca^{2+} waves. The traces in a and b illustrate typical responses from CAML expressing (a) and control (b) cells. The cells were stimulated from the start of the traces with 500 nM ATP. The control cell displayed global regenerative Ca^{2+} waves, whereas the CAML expressing cell gave only a few sporadic Ca^{2+} puffs that did not lead to a regenerative response. c depicts the concentration response of CAML and control cells to various ATP concentrations. The number of cells monitored is shown at the top of the bars. The data were pooled from at least three independent experiments.

The functions of CAML in mammalian cells are only just emerging. It has been shown to participate in activation of transcription factors either by interaction with TACI, or by solely causing an increase in cytoplasmic Ca^{2+} levels. In the present study, we did not observe higher resting Ca^{2+} levels in the CAML expressing cells. However, the observations that CAML-

expressing cells displayed lower amplitude Ca^{2+} waves and required higher agonist concentrations to produce regenerative Ca^{2+} signals are consistent with CAML causing a modest depletion of internal Ca^{2+} stores. It is possible that CAML might cause different effects depending upon the cell type in which it is expressed.

Although CAML is localized to the cellular region where pacemaker Ca^{2+} puffs occur, it does not appear to modify the activity of $InsP_3Rs$. The characteristics of Ca^{2+} puffs were indistinguishable in control and CAML-expressing cells. Since Ca^{2+} puffs reflect the gating of a cluster of $InsP_3Rs$ on the surface of the ER, changes in channel behavior should be obvious as an alteration of Ca^{2+} puff properties. CAML and $InsP_3Rs$ may therefore be positioned in close proximity, but we would suggest that they do not interact. Therefore, how the ubiquitous CAML protein participates in Ca^{2+} signal transduction remains unclear.

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