Hippocalcin is a neuronal calcium sensor protein that possesses a Ca\textsuperscript{2+}/myristoyl switch allowing it to translocate to membranes. Translocation of hippocalycin in response to increased cytosolic [Ca\textsuperscript{2+}] was examined in HeLa cells expressing hippocalycin–enhanced yellow fluorescent protein (EYFP) to determine the dynamics and Ca\textsuperscript{2+} affinity of the Ca\textsuperscript{2+}/myristoyl switch in living cells. Ca\textsuperscript{2+}-free hippocalycin was freely diffusible, as shown by photobleaching and use of a photoactivatable GFP construct. The translocation was dependent on binding of Ca\textsuperscript{2+} by EF-hands 2 and 3. Using photolysis of NP-EGTA, the maximal kinetics of translocation was determined (t\textsubscript{1/2} = 0.9 s), and this was consistent with a diffusion driven process. Low intensity photolysis of NP-EGTA produced a slow [Ca\textsuperscript{2+}] ramp and revealed that translocation of hippocalycin–EYFP initiated at around 180 nM and was half maximal at 290 nM. Histamine induced a reversible translocation of hippocalycin–EYFP. The data show that hippocalycin is a sensitive Ca\textsuperscript{2+} sensor capable of responding to increases in intracellular Ca\textsuperscript{2+} concentration over the narrow dynamic range of 200–800 nM free Ca\textsuperscript{2+}.

### Introduction

Calcium regulates numerous cellular processes and Ca\textsuperscript{2+} signals can vary from brief localized release events to a prolonged global rise (Bootman et al., 2001). These diverse Ca\textsuperscript{2+} signals are interpreted by Ca\textsuperscript{2+} sensor proteins (Carafoli, 2002). Little is known about how Ca\textsuperscript{2+} sensors react to differing Ca\textsuperscript{2+} signals, and calmodulin is the only EF-hand Ca\textsuperscript{2+}-binding protein studied in living cells (Craske et al., 1999; Deisseroth et al., 1998; Milikan et al., 2002) and whose in vivo Ca\textsuperscript{2+} affinity has been determined (Persechini and Cronk, 1999). For other EF-hand Ca\textsuperscript{2+}-binding proteins, only in vitro estimates of Ca\textsuperscript{2+} affinity are available. For an understanding of which Ca\textsuperscript{2+}-binding proteins are functionally activated under particular Ca\textsuperscript{2+} signaling conditions, it will be essential to have direct information on their dynamics and Ca\textsuperscript{2+} affinities in living cells. The neuronal calcium sensor (NCS) proteins (Burgoyne and Weiss, 2001) are found mainly in neurons and neuroendocrine cells and are linked with modulation of neurotransmitter release (McFerran et al., 1998; Pongs et al., 1993), regulation of Ca\textsuperscript{2+} (Tsujimoto et al., 2002; Weiss et al., 2000; Weiss and Burgoyne, 2002) and K\textsuperscript{+} channels (An et al., 2000; Guo et al., 2002), regulation of phosphoinositide metabolism (Hendricks et al., 1999; Koizumi et al., 2002), phototransduction (Palczewski et al., 2000), and learning (Gomez et al., 2001). Most of the NCS proteins are NH\textsubscript{2}-terminally myristoylated. Studies on one member of the family, recoverin, showed that the myristoyl group is sequestered in the Ca\textsuperscript{2+}-free form of the protein and following Ca\textsuperscript{2+}-binding a substantial conformational change allows extrusion of the myristoyl group (Ames et al., 1997; Tanaka et al., 1995). This so-called Ca\textsuperscript{2+}/myristoyl switch would allow recovery to associate with membranes in a Ca\textsuperscript{2+}-dependent manner. The Ca\textsuperscript{2+}/myristoyl switch is used by some other NCS proteins (Ivings et al., 2002; O’Callaghan et al., 2002; Spilker et al., 2000, 2002), whereas others such as NCS-1 and guanylyl cyclase-activating protein (GCAP) 2 are membrane associated even at low [Ca\textsuperscript{2+}] (O’Callaghan et al., 2002; Oleshevskaya et al., 1997).

Hippocalcin is an NCS protein most highly expressed in hippocampal neurons (Kobayashi et al., 1992). Its best characterised function is as an inhibitor of neuronal apoptosis through its interaction with neuronal apoptosis inhibitor protein (Lindholm et al., 2002). Hippocalcin has been

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**Abbreviations used in this paper:** EYFP, enhanced yellow fluorescent protein; GCAP, guanylyl cyclase-activating protein; NCS, neuronal calcium sensor; PA, photoactivatable; VILIP, visinin-like protein.

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The online version of this article includes supplemental material.

Address correspondence to Robert D. Burgoyne, Physiological Laboratory, Crown Street, University of Liverpool, Liverpool L69 3BX, England, UK.

Tel.: 44-151-794-5305. Fax: 44-151-794-5337.

email: burgoyne@liverpool.ac.uk

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shown to have a Ca\(^{2+}\)/myristoyl switch mechanism within cells (O’Callaghan et al., 2002), allowing it to be cytosolic at resting [Ca\(^{2+}\)] and translocate to intracellular membranes of the TGN and the plasma membrane in response to an increase in [Ca\(^{2+}\)]. The Ca\(^{2+}\)/myristoyl switch of recoverin has been examined using biochemical and structural approaches, but little work has examined the properties of the switch in NCS proteins in vivo. To address this issue, we have expressed hippocalcin–enhanced yellow fluorescent protein (EYFP) and observed its behavior in cells in response to controlled increases in intracellular [Ca\(^{2+}\)] to characterize the dynamics and the Ca\(^{2+}\) sensitivity of a Ca\(^{2+}\)/myristoyl switch protein in living cells. We demonstrate that hippocalcin is highly sensitive to cytosolic [Ca\(^{2+}\)], but its translocation properties would require Ca\(^{2+}\) elevations prolonged for seconds to tens of seconds rather than very brief Ca\(^{2+}\) transients for a full response. The properties of hippocalcin would mean that it could integrate both temporal and spatial aspects of Ca\(^{2+}\) signals over a tight dynamic range.

Results and discussion

Translocation of hippocalcin in HeLa cells is dependent on Ca\(^{2+}\) binding

HeLa cells, which do not have endogenous hippocalcin, were transfected to express hippocalcin–EYFP, allowing the behavior of hippocalcin to be observed in isolation from neuronal-specific interacting proteins. In HeLa cells, NCS-1 is localized in resting cells to the plasma membrane and TGN—the same as the endogenous protein in neurons (Martone et al., 1999; O’Callaghan et al., 2002). Both hippocalcin–EYFP and untagged neurocalcin \(\delta\) translocate to the same sites following elevation of [Ca\(^{2+}\)], irrespective of time of transfection and expression levels (Ivings et al., 2002; O’Callaghan et al., 2002). The validity of using HeLa cells is supported by the finding that the endogenous closely related protein visinin-like protein \(1\) (VILIP) also translocates to the plasma membrane and TGN in hippocampal neurons (Spilker et al., 2002). Transfected HeLa cells were loaded with the Ca\(^{2+}\) indicator dye Fura red allowing cytosolic [Ca\(^{2+}\)] to be measured in parallel. In an unstimulated HeLa cell, hippocalcin–EYFP was diffusely distributed throughout the cytosol and in the nucleus (Fig. 1). When Ca\(^{2+}\) was elevated, translocation of hippocalcin–EYFP occurred but was slower and not

![Figure 1](http://www.jcb.org/cgi/content/full/jcb.200306042/DC1)
detectable until more than 90 s after Ca\(^{2+}\) elevation (Fig. 1, middle panel). In contrast, the distribution of hippocalcin (E85, E121Q)-EYFP was unaffected by the increase in cytosolic [Ca\(^{2+}\)] (Fig. 1, lower panel), even after 220 s. These data are consistent with a Ca\(^{2+}\)/myristoyl switch requiring Ca\(^{2+}\)-binding to EF-hands 2 and 3 of hippocalcin. The slower kinetics with the EF-hand 3 mutant would be consistent with a reduction in Ca\(^{2+}\) affinity and a need for a higher threshold [Ca\(^{2+}\)] to be reached. Hippocalcin binds 3 Ca\(^{2+}\) ions in vitro (Kobayashi et al., 1993) due to a nonfunctional EF-hand 1 and we do not rule out a requirement for EF-hand 4. Ca\(^{2+}\)-binding to EF-hand 3 may initiate conformational changes in the protein allowing cooperative Ca\(^{2+}\) binding to the other EF-hands as in other NCS proteins (Cox et al., 1994; Permyakov et al., 2000; Senin et al., 2002).

Hippocalcin is freely diffusible in the cytoplasm in resting cells
To determine that hippocalcin in its Ca\(^{2+}\) unbound form is freely diffusible, localized photobleaching was used to probe the movement of hippocalcin–EYFP in transfected HeLa cells (Fig. 2, A and B). A localized area of the cell (Fig. 2 B) was exposed to high-intensity 514-nm light for 30 s. During this period, the whole cytosol rapidly dimmed, indicating that hippocalcin–EYFP was capable of rapid diffusion. Fluorescence was retained in the nucleus, indicating that this is a diffusively isolated region. The retention of nuclear fluorescence and that of surrounding cells indicates the regional specificity of the photobleaching. To examine the speed at which the diffusion of hippocalcin–EYFP occurs, we created a photoactivatable version termed hippocalcin–photoactivatable (PA)–GFP using photoactivatable GFP (Patterson and
This protein was only dimly fluorescent (Fig. 2C) until excited with 430-nm light. The illumination of half of a HeLa cell expressing hippocalcin-PA-GFP allowed the speed of diffusion of the photoactivated hippocalcin-PA-GFP to be measured. In the example shown, fluorescence intensity was monitored at two regions of interest that were 9.37 μm apart. The rate of diffusion of hippocalcin-PA-GFP from the delay between the two regions was 2.9 μm/s. This rate was comparable to that found for PA-GFP (3.2 μm/sec; data not shown). This demonstrates that at resting \([\text{Ca}^{2+}]_{\text{cytosolic}}\) hippocalcin is not membrane-associated but is freely diffusible in the cytosol. The rate of diffusion would be sufficient to account for the translocation process.

Maximal rate of translocation of hippocalcin–EYFP

Ionomycin produces a sustained but slow increase in cytosolic \([\text{Ca}^{2+}]\) in HeLa cells and so the dynamics of hippocalcin–EYFP translocation will be dependent on the rate of this \([\text{Ca}^{2+}]\) rise. Indeed translocation of hippocalcin–EYFP after ionomycin addition occurs only after a lag period of around 2 s and then with a time constant of around 12 s. To assess the true maximum rate of \([\text{Ca}^{2+}]\)-dependent translocation and to rule out any effects on the kinetics of \([\text{Ca}^{2+}]\)-buffering by the expressed proteins, pHippo-EYFP transfected HeLa cells were loaded with the caged \([\text{Ca}^{2+}]\) compound NP-EGTA. Photolysis of the NP-EGTA rapidly increased the cytosolic \([\text{Ca}^{2+}]\) to high levels indicated by the sharp drop in Fura red fluorescence and to an immediate initiation of translocation of hippocalcin (Fig. 3A and B). This translocation could be fitted to first order kinetics with a half time of 0.9 s (mean value from 8 cells) and translocation was complete within <10 s. The initiation of translocation was concomitant with the elevation of \([\text{Ca}^{2+}]\), indicating that the conformational change induced by \([\text{Ca}^{2+}]\) binding is not rate limiting for the translocation.

The trigger \([\text{Ca}^{2+}]\) for translocation of hippocalcin–EYFP

To determine the threshold \([\text{Ca}^{2+}]\) for translocation, transfected HeLa cells loaded with Fura red and NP-EGTA were
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irradiated using low-intensity 360-nm light. This produced a slowly rising ramp in intracellular [Ca$^{2+}$] seen as a decrease in Fura red fluorescence (Fig. 4 A). By calibrating the Fura red fluorescence, the intracellular [Ca$^{2+}$] was calculated. The representative experimental trace (Fig. 4 B) shows the progressive rise in [Ca$^{2+}$] starting at 100 nM. The initiation of translocation was seen when the [Ca$^{2+}$] reached $180$ nM and was half-maximal at around 320 nM. The data were replotted as the percentage of translocation versus [Ca$^{2+}$] and could be fitted by a curve defined by the Hill equation with a $K_{0.5}$ of 324 nM and a Hill coefficient of 3.3 (Fig. 4 C), showing high cooperativity in the response of hippocalcin to [Ca$^{2+}$] elevation. The mean for the trigger concentration was $181 \pm 9$ nM and half maximal translocation was at $293 \pm 20$ nM free Ca$^{2+}$ (11 cells). Translocation was complete as [Ca$^{2+}$] reached $800$ nM, indicating that hippocalcin has a dynamic range of 200–800 nM free Ca$^{2+}$. This is more than tenfold as sensitive to Ca$^{2+}$ than the Kd (5 $\mu$M) for recombinant hippocalcin (Kobayashi et al., 1993), demonstrating the problem with reliance on in vitro analyses. The Ca$^{2+}$ affinity of hippocalcin is higher than that seen for calmodulin, which was around 1 $\mu$M free Ca$^{2+}$ (Persechini and Cronk, 1999).

**Hippocalcin responds to agonist-induced Ca$^{2+}$ elevation and translocation is reversible**

To examine the reversibility of the Ca$^{2+}$/myristoyl switch, hippocalcin–EYFP expressing HeLa cells were exposed to NP-EGTA photolysis to induce translocation (Fig. 5 A, i and ii). This translocation was completely reversed after reduction of cytosolic [Ca$^{2+}$] using 3 $\mu$M ionomycin in a Krebs-Ringer buffer bath solution with 3 mM EGTA present (Fig. 5 A, iii). Translocation was then induced again by the replacement of the bath buffer with Krebs-Ringer buffer containing 3 mM Ca$^{2+}$ (Fig. 5 A, iv). To determine whether hippocalcin would respond to Ca$^{2+}$ transients in-
duced by physiological agonists we used histamine to stimulate
late transfection of HeLa cells. Fig. 5 B shows the initial re-
response in which rapid translocation occurred upon addition
of histamine after a lag of around 2 s. Translocation was
half-maximal at 278 nM free Ca$^{2+}$, similar to that seen with
NG-EGTA. Hippocalcin subsequently dissociated from the
membranes (Fig. 5 C, iii), but hippocalcin could translocate
again to the same sites when ionomycin was added (Fig. 5 C, iv).
This indicates the hippocalcin can respond to agonist-
induced elevation of [Ca$^{2+}$] and also that it acts as a fully re-
versible switch.

Concluding remarks
These experiments have yielded new insights into the behav-
or of hippocalcin in living cells that indicate how it could
integrate both temporal and spatial aspects of Ca$^{2+}$ signals.
The extent of translocation is not just a function of [Ca$^{2+}$] but
also the time that Ca$^{2+}$ remains elevated. Hippocalcin
translocation is more sensitive to increases in intracellular
Ca$^{2+}$ concentration than calmodulin but the full responses
are on the time scale one seconds to tens of seconds. This is
slower than the Ca$^{2+}$ transients seen in neurons due to ac-
tion potentials but longer-lasting Ca$^{2+}$ elevations lasting up
to hundreds of milliseconds are physiologically important in
neurons for the induction of long-term potentiation or long-
term depression (Sabatini et al., 2002; Sjostrom and Nelson,
2002). In a small neuronal process or dendritic spine, hip-
 pacolin could diffuse sufficiently to translocate on such a
time scale. One could imagine how the distinct properties of
Ca$^{2+}$ sensors such as calmodulin and hippocalcin could de-
termine distinct cellular responses to differing Ca$^{2+}$ signals.

Materials and methods
Plasmids and transfection of HeLa cells
EYFP-tagged hippocalcin (pHippo-EYFP) was made as described previ-
ously (O’Callaghan et al., 2002). pHippoE121Q-EYFP and pHippo
(E85,121Q)-EYFP were made using the QuikChange™ site-directed
mutagenesis kit (Stratagene). The photoactivatable hippocalcin construct
pHippo-PA-GFP was made by cutting out the EYFP from pHippo-EYFP
and replacing it with the PA-GFP from pPA-GFP-N1. pPA-GFP-N1 was
generated with the mutations described previously (Patterson and Lip-
pincott-Schwartz, 2002). HeLa cells were grown and transfected as de-
scribed previously (O’Callaghan et al., 2002) and maintained for 8–96 h
before use.

Confocal laser scanning microscopy on living cells
For confocal laser scanning microscopy, live transfected HeLa cells were
examined with either a Zeiss LSM 150 confocal microscope or a Leica
TCS-SP-MP microscope using a 63× water immersion objective with a 1.2
numerical aperture. Cells were loaded with Fura red (Molecular Probes) by
incubation in 5 μM acetoxymethylester and loaded with NP-EGTA (Mol-
ecular Probes) by incubation in 10 μM acetoxymethylester in growth me-
dium for 30 min. The cells were bathed in a Krebs-Ringers solution (145
mM NaCl, 5 mM KCl, 1.3 mM MgCl$_2$, 1.2 mM NaH$_2$PO$_4$, 10 mM glucose,
20 mM HEPES; pH 7.4) with 3 mM CaCl$_2$ and were excited at 488 nm
and light collected at 625–725 nm for Fura red emission and at 525–590 for
hippocalcin–EYFP emission. Ionomycin when used as added to the bath
solution to a final concentration of 3 μM. With phipho-PA-GFP, 488-nm
illumination was used for excitation, and the emission was collected be-
tween 500–550 nm. Photactivation of Hippo-PA-GFP was with 430-nm
laser line illumination at full power of the laser on the Leica TCS-SP-MP
system for 15 s. The photolysis of NP-EGTA was by illumination with 360-
nm laser light at full power for rapid photolysis and at 6% power for gen-
eration of [Ca$^{2+}$]$^*$ complex. Histamine was added to the bath solution at a final
concentration of 100 μM. For calibration of Fura red fluorescence as
[Ca$^{2+}$], the cells were treated with 3 μM ionomycin at the end of the ex-
periment in the presence of 3 mM EGTA to determine Fmim. Due to prob-
lems with photobleaching leading to inaccurate direct determination of
Fmax, a predicted Fmax value at resting [Ca$^{2+}$] (Fmax$r$) was determined
based on the use of a resting [Ca$^{2+}$] of 100 nM that has been well estab-
lished for HeLa cells (Thomas et al., 2000). [Ca$^{2+}$]$^*$ was calculated using a
Kd of 1.40 nM. This calibration method would, if anything, overestimate
rather than underestimate the [Ca$^{2+}$] at which hippocalcin–EYFP translo-
cation was observed. Data for percentage translocation versus [Ca$^{2+}$]$^*$ were
fitted by nonlinear curve fitting using the Hill equation.

Online supplemental material
Figure S1 shows different confocal sections through the same HeLa cell af-
ter treatment with ionomycin. These were selected to show optimal images
of the localization of hippocalcin–EYFP either at the TGN or at the plasma
membrane. Figure S2 shows data averaged from four cells from imaging of
hippocalcin–EYFP and Fura red fluorescence showing that transloca-
tion to the TGN or to the plasma membrane occurs with the same kinet-
ics. Both figures are available at http://www.jcb.org/cgi/content/full/jcb.
200306042/DC1.

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References
An, W.F., M.R. Bowly, M. Bert, J. Cao, H.P. Ling, G. Mendoza, J.W. Hinson,
lation of A-type potassium channels by a family of calcium sensors. Nature.
403:553–556.
Boothe, M.D., P. Lipp, and M.J. Berridge. 2001. The organisation and functions
Burgoyne, R.D., and J.L. Weiss. 2001. The neuronal calcium sensor family of
1994. Cation binding and conformational changes in VILIP and NCS-1,
two neuron-specific calcium-binding proteins. J. Biol. Chem. 269:35807–
32814.
Craske, H., T. Takeo, O. Gerasimenko, C. Vaillant, K. Torok, O.H. Petersen,
and A.W. Tepikin. 1999. Hormone-induced secretory and nuclear transloca-
tion of calmodulin: Oscillations of calmodulin concentration with the nucleus as
to the nucleus supports CREB phosphorylation in hippocampal neurons. Na-
Gomez, M., E. De Castro, E. Guarin, H. Sasakura, A. Kuhara, I. Mori, T. Barfai,
C.I. Bargmann, and P. Nef. 2001. Ca$^{2+}$ signalling via the neuronal calcium
Guo, W., S.A. Malin, D.C. Johns, A. Jeromin, and J.M. Nerbonne. 2002. Modu-
lation of Kv4-encoded K$^+$ currents in the mammalian myocardium by neu-
Hendricks, K.B., B.Q. Wang, E.A. Schnieders, and J. Thorner. 1999. Yeast homo-
dimer of a neuronal frequenin is a regulator of phosphatidylinositol-4-OH ki-
Identification of calcium-dependent binding partners for the neuronal cal-
musor protein neurocalcin & interaction with actin, clathrin and tubu-
ecular cloning of hippocalcin, a novel calcium-binding protein of the recov-
erin family exclusively expressed in hippocampus. Biochem. Biophys. Rs.
Commun. 189:511–517.
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