Kinesin dependent, rapid, bi-directional transport of ER sub-compartment in dendrites of hippocampal neurons

Hiroko Bannai1,2, Takafumi Inoue2,4,*, Tomohiro Nakayama2,3, Mitsuharu Hattori2 and Katsuhiko Mikoshiba1,2,4

1Laboratory for Developmental Neurobiology, Brain Science Institute, RIKEN, Saitama 351-0198, Japan
2Division of Molecular Neurobiology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
3Department of Pediatrics, Tokyo Women’s Medical University, Tokyo 162-8666, Japan
4Calcium Oscillation Project, ICORP, Japan Science and Technology Corporation (JST), Tokyo 108-0071, Japan

*Author for correspondence (e-mail: tinoue@ims.u-tokyo.ac.jp)

Accepted 4 September 2003
Journal of Cell Science 117, 163-175 Published by The Company of Biologists 2004
doi:10.1242/jcs.00854

Summary

Although spatially restricted Ca2+ release from the endoplasmic reticulum (ER) through intracellular Ca2+ channels plays important roles in various neuronal activities, the accurate distribution and dynamics of ER in the dendrite of living neurons still remain unknown. To elucidate these, we expressed fluorescent protein-tagged ER proteins in cultured mouse hippocampal neurons, and monitored their movements using time-lapse microscopy. We report here that a sub-compartment of ER forms in relatively large vesicles that are capable, similarly to the reticular ER, of taking up and releasing Ca2+. The vesicular sub-compartment of ER moved rapidly along the dendrites in both anterograde and retrograde directions at a velocity of 0.2-0.3 μm/second. Depletion of microtubules, overexpression of dominant-negative kinesin and kinesin depletion by antisense DNA reduced the number and velocity of the moving vesicles, suggesting that kinesin may drive the transport of the vesicular sub-compartment of ER along microtubules in the dendrite. Rapid transport of the Ca2+-releasable sub-compartment of ER might contribute to rapid supply of fresh ER proteins to the distal part of the dendrite, or to the spatial regulation of intracellular Ca2+ signaling.

Supplemental data available online

Key words: Endoplasmic reticulum, Ins(1,4,5)P3R, Neuron, Dendrite, Kinesin, Microtubule

Introduction

Endoplasmic reticulum (ER) is an organelle that is responsible for the storage and release of Ca2+ in eukaryotic cells. In the neuron, Ca2+ regulation by ER plays various roles in neuronal activities, e.g. modification of synaptic vesicle release (Takei et al., 1996), or regulation of nerve growth (Takei et al., 1998). In particular, inositol 1,4,5-trisphosphate [Ins(1,4,5)P3]-induced Ca2+ release (IICR) from intracellular calcium stores through Ins(1,4,5)P3 receptors [Ins(1,4,5)P3Rs] (Berridge, 1993) has been shown to be involved in depotentiation, suppression and input specificity of LTP, and in the induction of LTD in hippocampal neurons (Fujii et al., 2000; Nishiyama et al., 2000). IICR has also been shown to be required for the induction of LTD in the cerebellar Purkinje cells (Inoue et al., 1998), and it is postulated that induction of synaptic plasticity requires this spatially restricted Ca2+ release from ER (Miyata et al., 2000; Wang et al., 2000). Localization of ER membrane and targeting of ER resident Ca2+-release channels, Ca2+ pumps and Ca2+ binding proteins to specific regions in the cell should be an important factor in the spatial regulation of Ca2+ release. Previous morphological and biochemical studies showing heterogeneous distribution of Ca2+ channels on ER and major ER resident proteins in Purkinje cells (Villa et al., 1991; Volpe et al., 1991; Takei et al., 1992) support this idea. However, the distribution of ER membrane in living neurons is little understood.

In addition, ER in the eukaryotic cells is not a static organelle but it is highly dynamic; ER membrane tubules are shown to be highly dynamic in both in neurons and non-neuronal cells (Lee and Chen, 1988; Dailey and Bridgman, 1989; Sanger et al., 1989; Waterman-Storer and Salmon, 1998), and proteins inserted in the ER membrane are also reported to diffuse freely and rapidly on it in COS-7 cells (Nehls et al., 2000). Because neurons are highly polarized cells, transport of newly synthesized proteins and membrane to the distal region should be crucial for the maintenance of
ER functions. In neurons, dynamics of ER in the axon has been well studied. Earlier morphological studies utilizing local cold block showed that smooth ER is transported anterogradely by a distinct mechanism from the fast axonal transport system (Tsukita and Ishikawa, 1980; Ellisman and Lindsey, 1983). Dynamics of ER within the growth cone was also examined and suggested to be microtubule dependent (Dailey and Bridgman, 1989). More recently, we demonstrated that a green fluorescent protein (GFP)-tagged transmembrane region of Ins(1,4,5)P_3R had bi-directional, temperature-sensitive and microtubule-dependent movement that was slower than fast axonal transport in the axon of cultured chick dorsal root ganglion neurons (Aihara et al., 2001). However, the dynamics of ER membrane and of ER resident proteins such as Ca^{2+} channels and Ca^{2+} pumps in the neuron of the central nervous system (CNS) remain to be elucidated especially in the dendrite.

In the present study, we examined the distribution and dynamics of ER labeled with fluorescent proteins in the dendrite of cultured hippocampal neurons. We report here that a sub-compartment of ER exists in a large, vesicular structure that showed rapid, bi-directional movements along the dendrite. This vesicular sub-compartment of ER in living neurons shared an important function of the ER, that is, Ca^{2+} release and uptake. Finally, we show that the movements of the vesicular ER sub-compartment were highly dependent on the integrity of kinesin and microtubules.

**Materials and Methods**

**Construction of fusion proteins**

The construction of GFP-tagged sarcoplasmic/endoplasmic reticulum calcium-ATPase 2a (GFP-SERCA2a) (Aihara et al., 2001), and GFP-tagged Ins(1,4,5)P_3R1 (GFP-Ins(1,4,5)P_3R1) (Zhang et al., 2003) have been described previously. To construct ER luminal marker proteins, GFP-KDEL and RFP-KDEL, we fused the ER targeting sequence that corresponds to the N-terminal 17 amino acids of mouse calreticulin to the N terminus of EGFP or DsRed1 (Clontech, Palo Alto, USA), and inserted an ER retention signal peptide, KDEL, to its C terminus by a two stage PCR strategy (Terasaki et al., 1996; Roderick et al., 1997). The resultant PCR products were subcloned into pcDNA3.1/Zeo+ (Invitrogen, Tokyo, Japan). To construct a dominant negative kinesin fused with GFP (GFP-DNKHC), the tail region (859 aa – end) of the kinesin heavy chain was amplified by PCR using a cDNA clone coding for the human kinesin heavy chain xKHC (KIAA0531) as a template. KIAA0531 was kindly provided by Kazusa DNA Research Institute (Chiba, Japan). The PCR product was subcloned into the EcoRI/BamHI site of pEGFP-C1 (Clontech). GFP-LC3, a marker for autophagosomal (Kabeya et al., 2000), was kindly provided by Drs Mizushima and Ohsumi (National Institute for Basic Biology, Okazaki, Japan). GFP-MST, C-terminal 597 amino acids fused with GFP, was kindly provided by Dr Gelfand (University of Illinois, IL, USA). All the genes were under the control of a CMV promoter.

**Cell culture and transfection**

The primary culture of neurons was prepared from hippocampi of 1-day-old ICR mice, as described previously (Higgins and Banker, 1998). In brief, the hippocampi were treated with 0.5% trypsin for 5 minutes at 37°C, then the cells were dissociated in Hanks’ balanced salt solution (HBSS) containing 0.05% DNase I (Invitrogen) and 12 mM MgSO_4. The dissociated cells were plated on poly-L-lysine (Nacalai Tesque, Kyoto, Japan) or laminin (Koken, Tokyo, Japan)-coated coverslips at a density of 4.6×10^4 to 3×10^5 cells/cm² and cultured in Neurobasal Medium (Invitrogen) supplemented with 2.5 mM L-glutamine (Nacalai Tesque), 2.5% (v/v) B-27 (Invitrogen), 1.25% (v/v) insulin-transferrin-selenium-A (Invitrogen) and antibiotics (250 units/ml penicillin and 250 µg/ml streptomycin). The cultures were transfected with 10 µg of DNAs, usually on days 4-5 in vitro, by a standard calcium phosphate method (Köhmann et al., 1999; Kaether et al., 2000). The transfected cells were used for immunohistochemistry or imaging experiments 2-3 days after the transfection, that is, at days 7 or 8 in vitro.

**Double-labeling experiments and confocal imaging**

For labeling hippocampal neurons with endosomal and lysosomal markers, cells were incubated with 1 mg/ml Texas Red-dextran or fluorescein-dextran (M_f 8×10^4; Molecular Probes, Eugene, USA) overnight (Nakata et al., 1998). To visualize endocytotic vesicles and late endosomes more clearly, other batches of cells were incubated with these tracers for 20-30 minutes, which resulted in no obvious difference from cells treated overnight (data not shown). Mitochondrial tracers, MitoTracker Red CMXRos (Molecular Probes) and MitoTracker Green FM (Molecular Probes) were used at 20 nM and 5 nM for 10 and 30 minutes at 37°C, respectively. An ER tracer, ER-Tracker Blue-White DPX (Molecular Probes) was used at 0.1 µM for 20-30 minutes at 37°C.

For immunostaining, cells were sequentially fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes then with 100% methanol on ice. After permeabilization with 0.1% Triton X-100 in PBS for 10 minutes and blocking with 5% skim milk in PBS, the cells were incubated with anti-BiP (1:400 dilution; Affinity Bioreagents, Golden, USA), anti-calnexin (1:400 dilution; Stressgen Biotechnologies, Victoria, Canada), anti-calreticulin (1:400 dilution; Affinity Bioreagents), or 2.5 mg/ml of 18A10, a monoclonal antibody against Ins(1,4,5)P_3R1 (Maeda et al., 1988). Alexa488 or Alexa594-conjugated IgGs (Molecular Probes) were used as secondary antibodies.

Fluorescence images of neurons were taken under a confocal scanning microscope (FV-300, Olympus, Tokyo, Japan) attached to an inverted microscope (IX70, Olympus) with a 60x objective (NA 1.4 or 1.45, Olympus).

All of the images taken by confocal microscopy were processed and presented after digitally smoothing in order to reduce the noise level. The smoothing filter is implemented using 3x3 spatial convolutions, where the value of each pixel in the selection is replaced with the weighted average of its 3x3 neighborhood. Center pixels are four-fold weighted relative to the surrounding pixels.

**Time-lapse imaging, photobleaching experiment and data analysis**

For the time-lapse imaging experiments, the culture medium was supplemented with 20 mM Hepes (pH 7.3). The cells were visualized under an inverted microscope (IX70, Olympus) and a 60x objective (NA 1.4, Olympus) using standard filter sets and a Hg lamp.

The temperature was maintained at ~37°C by a heating chamber surrounding the microscope stage. Sequential images were acquired with a cooled CCD camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan). Images were taken for 58 mseconds or 117 mseconds every 50 mseconds under continuous illumination.

Data were analyzed using TI Workbench, a custom-made software.
ER luminal Ca\(^{2+}\) imaging

ER luminal Ca\(^{2+}\) imaging was performed as described previously (Hirose et al., 1998; Fujitaya et al., 2001), with some modifications. Cells were loaded with 40 \(\mu\)M mag-fura-2 AM (Molecular Probes) or 20 \(\mu\)M fluo-5N AM (Molecular Probes) for 60 minutes in the culture medium at 37°C. The neurons were then washed in culture medium and incubated for a further 30 minutes to allow complete de-esterification of the intracellular AM esters. After being washed in a physiological salt solution (mM): 150 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 Hepes, 5.6 glucose (pH 7.4), the loaded neurons were permeabilized with 60 \(\mu\)M \(\beta\)-escin (Sigma, St. Louis, USA) for 2 minutes in a Ca\(^{2+}\)-free internal solution (mM): 1 EGTA, 3.3 ATP, 20 Pipes, 20 NaN\(_3\), 112 potassium methanesulfonate (pH 7.0). The cells were subsequently washed with an internal solution containing ~300 nM Ca\(^{2+}\), which was prepared by mixing CaEGTA and EGTA solutions (Hirose et al., 1998).

Ca\(^{2+}\) imaging was carried out at room temperature. Neurons were superfused with the internal solution containing ~300 nM Ca\(^{2+}\), and 1 mM MgATP (Sigma) and 20 \(\mu\)M Ins\((1,4,5)P_3\) (Dojindo Laboratories, Kumamoto, Japan) were added as indicated. Neurons were visualized under an inverted microscope (IX70, Olympus) equipped with a cooled CCD camera (ORCA-ER) and a filter exchanger (OSP-EXA, Olympus). Fluorescence images of neurons were captured every 2 seconds at excitation wavelength of 340 nm and 380 nm (for mag-fura-2) or 480 nm (for fluo-5N), and the ratios (F\(_{340}/F_{380}\) for mag-fura-2 and F/F0 for fluo-5N) of the fluorescence intensities were calculated after subtraction of the background fluorescence and correction for bleaching. The time-course of changes in ratio was further filtered to reduce noise using Igor Pro software (WaveMetrics, Lake Oswego, USA).

Drug preparation

Stock solutions of nocodazole (10 mg/ml, Sigma), cytochalasin D (10 mg/ml, Sigma) and latrunculin A (1 mg/ml, Molecular Probes) were prepared in dimethyl sulphoxide (DMSO), and stored at –20°C. To confirm that the cytoskeleton was disrupted, fixed cells were stained with anti-tubulin antibody (Lab Vision, Fremont, USA) or rhodamine-phalloidin (Molecular Probes). We found no changes in the microtubules or actin structure from control cells exposed to 0.1% DMSO (data not shown).

Antisense treatment

The antisense nucleotide treatment was conducted as described previously (Ferreira et al., 1992; Feiguin et al., 1994; Severt et al., 1999; Kaether et al., 2000). Twenty-four hours after transfection of RFP-KDEL, the neurons were incubated with the oligonucleotides at 5 \(\mu\)M. Twelve hours later, oligonucleotides were again added at 2.5 \(\mu\)M. Treated neurons were used for time-lapse microscopy, 24-28 hours after the first administration of oligonucleotides, that is, 48-52 hours after transfection. We used antisense-oligo DNA corresponding to 5’ flanking –11 to +14 of rat conventional kinesin heavy chain (KHC) (5’-GCCCCGTCCGGCCATCTTTCCTGGCAG-3’) and sense-oligo DNA (5’-CTGGCAGAAAGATGGCGGACCAGGCGCGG-3’) as control. This sequence was reported to suppress the KHC activity in rat hippocampal neurons (Ferreira et al., 1992; Feiguin et al., 1994; Severt et al., 1999; Kaether et al., 2000). Although the mouse KHC has a single displacement of nucleotide within this sequence, this oligonucleotide was also effective in mouse hippocampal neurons (see Results).
Previously, we also demonstrated the movements of vesicles labeled with GFP-tagged transmembrane region of Ins(1,4,5)P_3R in the axons of peripheral neurons (Aihara et al., 2001). Because the direction of vesicle movement observed in that study was solely retrograde, we speculated that these vesicles were transported as a part of the endosomal-lysosomal system probably to be degraded in the cell body. To determine whether the moving vesicles observed in the dendrite were also transported as a part of the endosomal-lysosomal system, we labeled endosomes and lysosomes of neurons with Texas Red-dextran. GFP-KDEL (Fig. 3A), GFP-SERCA2a (Fig. 3B) and GFP-Ins(1,4,5)P_3R1 (Fig. 3C) signals did not overlap with those of Texas Red-dextran both in the cell body and dendrites. We also labeled neurons with MitoTracker Red CMXRs, a mitochondrial marker. The ER marker proteins were located close to mitochondria in hippocampal neurons, as was reported in several cell types (Rizzuto et al., 1998) (for a review, see Rizzuto et al., 2000), but they did not overlap each other (Fig. 3D-F). From these results, we concluded that the vesicular structures labeled by the GFP-tagged ER markers were not endosomes, lysosomes, or mitochondria.

A special marker for vesicular sub-compartment of ER, RFP-KDEL

Interestingly, the RFP-KDEL protein, red fluorescent protein (DsRed1) fused with ER targeting and retention signals, which was designed to be localized inside the ER lumen, showed a patchy staining pattern (Fig. 4A) when expressed in hippocampal neurons, although it showed reticular pattern when expressed in COS-7 cells (data not shown). Co-transfection of the cells with GFP-KDEL and RFP-KDEL revealed that the RFP-KDEL and GFP-KDEL proteins co-localized on vesicles, but only GFP-KDEL was detected on the reticular ER (Fig. 4B). RFP-KDEL was also found on the vesicles labeled with GFP-SERCA2a and GFP-Ins(1,4,5)P_3R1 but not in the reticular structure (Fig. S2, http://jcs.biologists.org/supplemental).

RFP-KDEL-positive vesicles showed rapid bi-directional movements along the dendrites (Fig. 4C; see also Movie 2, http://jcs.biologists.org/supplemental) in the same fashion as the vesicular ER sub-compartment labeled with GFP-tagged ER markers. Vesicles labeled with RFP-KDEL did not disappear and the movement of vesicles did not change even after a brefeldin A treatment (data not shown). Vesicles labeled with RFP-KDEL had a tendency to accumulate at the branch points of dendrites (Fig. 4A, arrowheads), and some of them seemed to form large vacuoles and were less motile than the discrete smaller vesicles. The diameter of the discrete RFP-KDEL-labeled vesicles estimated from DIC images ranged from 0.4 to 1.2 μm (0.7±0.2 μm, mean ± s.d., n=75).

Most of the RFP-KDEL-labeled vesicles were also labeled with an anti-Ins(1,4,5)P_3R1 antibody, 18A10 (Fig. 4D). As was the case with GFP-tagged ER markers, neither the endosomal-lysosomal marker fluorescein-dextran (Fig. 4E), nor the mitochondrial marker MitoTracker Green FM (Fig. 4F) co-localized with the RFP-KDEL protein. A possibility that the RFP-KDEL-labeled vesicles are derived from autophagosome was ruled out by co-expression with an autophagosome-specific marker GFP-LC3 (microtubule-association protein 1 light chain 3; see also Fig. S3, http://jcs.biologists.org/supplemental).

Fluorescence of a portion of a dendrite expressing GFP-SERCA2a (Fig. 2G; see also Movie 1, http://jcs.biologists.org/supplemental) or GFP-Ins(1,4,5)P_3R1 (Fig. 2H; see also Movie 2, http://jcs.biologists.org/supplemental) was photobleached by continuous illumination with laser light. Almost all the fluorescence of the reticulate ER within the illuminated area were photobleached within 20 seconds and never recovered during the illumination. In contrast, vesicles labeled with GFP-SERCA2a or GFP-Ins(1,4,5)P_3R1 rapidly moved into the photobleached region from outside of the photobleached area while still under continuous laser illumination, kept moving for several seconds in the same fashion as those in neurons without such illumination (e.g. Fig. 2B,C), and then disappeared. Because the movements of the fluorescent vesicles were distinct from the other GFP signals on the membrane of the bulk ER, which showed no photo-recovery during the recording period, these vesicles are considered to be specialized compartments of ER that are partitioned from the bulk ER.

The size of the moving vesicles labeled with GFP-KDEL proteins, estimated by differential interference contrast (DIC) images, was relatively large, that is, 0.4-0.8 μm in diameter (0.5±0.1 μm, mean ± s.d., n=44).
We also confirmed that the RFP-KDEL-positive vesicles contained not only Ins(1,4,5)P₃R₁ but also other endogenous ER proteins, such as BiP, calreticulin and calnexin (data not shown). These vesicles were also stained by an ER-specific dye, ER-Tracker Blue-White DPX (data not shown). These results strongly indicate that the RFP-KDEL protein was specifically localized in the vesicular sub-compartment of ER. We therefore used the RFP-KDEL protein as a marker for the vesicular sub-compartment of ER in the following experiments, because its preferential expression in the vesicular sub-compartment enabled more accurate analysis of the properties of the vesicles.

**Ca²⁺ uptake and release from the vesicular sub-compartment of ER**

As mentioned above, some of the proteins contained in the reticular ER were also expressed in the vesicular sub-compartment of ER. This led us to hypothesize that the vesicular sub-compartment has similar functions to those of the reticular ER. Thus, we tested whether the vesicular sub-compartment of ER had the ability to take up and release Ca²⁺. We used a ‘real-time luminal Ca²⁺ monitoring technique’ (Hirose et al., 1998; Fujiwara et al., 2001). Neurons were loaded with low-affinity fluorescent Ca²⁺ indicators, mag-fura-2 AM (Kₐ=≈25 μM) or fluo-5N AM (Kₐ=≈90 μM), and the cell membrane was permeabilized with β-escin to wash out the indicators from the cytosol and to provide direct access for the externally applied drugs to the organelles. Reticular ER takes up Ca²⁺ by activation of a Ca²⁺ pump in the presence of MgATP and releases Ca²⁺ by binding of Ins(1,4,5)P₃ to the Ins(1,4,5)P₃R (Fujiwara et al., 2001).

Fig. 5A,B show the time-course of changes in the luminal Ca²⁺ concentrations monitored with mag-fura-2 and fluo-5N, respectively, in the dendrites of permeabilized neurons. The red traces represent changes in the Ca²⁺ concentration within vesicular sub-compartments of ER labeled with RFP-KDEL, corresponding to the red ellipses (region of interest, ROI) in the images, and traces of other colors represent ROIs in the adjacent areas probably covering the reticular ER. The interval between the time-lapse image frames was 1.5 seconds. (G,H) The photobleaching experiment revealed that the vesicular sub-compartment of ER is separate from the bulk ER. Part of the dendrites of neurons expressing GFP-SERCA2a (G) and GFP-Ins(1,4,5)P₃R₁ (H) were photobleached by continuous laser illumination. The left panels show the images before the photobleaching. All the areas indicated in this image were subjected to photobleaching, and the rectangles indicate the areas shown as time-lapse images. The right panels are time-lapse images taken after the photobleaching reached a steady level during continuous laser illumination, showing that vesicles (arrowheads) moved into photobleached areas. Relative time from the first time-lapse image is indicated below the time-lapse images. In order to visualize the moving vesicles clearly, the images of the first frame (0 seconds) are subtracted from the subsequent images. Scale bars of left images (before photobleaching), 10 μm, and in the time-lapse images, 2 μm. The raw data for G and H without image subtraction are presented as Movies 1 and 2, respectively. http://jcs.biologists.org/supplemental/). All fluorescent images are reversed for a clearer view.
fura-2, 37 vesicles in 17 cells loaded with fluo-5N). These results indicate that at least a certain proportion of the vesicular sub-compartment of ER serves as Ca\(^{2+}\) stores, in addition to the reticular ER. It was not possible to make a clear distinction between the vesicular sub-compartment and reticular ER in permeabilized neurons labeled with other GFP-tagged ER markers (data not shown). For the present, the labeling with RFP-KDEL is the only way to identify the vesicular sub-compartment of ER in permeabilized neurons.

Movement of the vesicular sub-compartment of ER in dendrites

ER in reticular form has been reported to move dynamically in the cytosol (Lee and Chen, 1988; Dailey and Bridgman, 1989; Sanger et al., 1989; Waterman-Storer and Salmon, 1998). The dynamics of reticular ER and the dynamics of Ins\((1,4,5)P_3\)R on the ER membrane have also been observed in the dendritic shafts of hippocampal neurons, and analysis of their movements is currently under way in our laboratory (K. Fukatsu, H.B., S. Zhang, T.I. and K.M., unpublished observations). In this study, we focused on the movements of the vesicular sub-compartment of ER labeled with GFP or RFP-tagged ER marker proteins in the dendrite. As mentioned above, the movements of the vesicular sub-compartment of ER were rapid and bi-directional (Fig. 2, Fig. 4C, and supplemental movies, http://jcs.biologists.org/supplemental/). The bi-directional movements of the vesicles labeled with ER marker proteins were observed in neurons cultured for 4-21 days. Vesicles were never observed inside the spines.

We measured the velocity of moving vesicles labeled with GFP or RFP-tagged ER markers in neurons cultured for 7-8 days (Fig. 6). The average velocity of the vesicle movements was 0.2-0.3 \(\mu\)m/second, as shown in Table 1. The average, range and the distribution pattern of the velocity were similar for the vesicles labeled with the four ER marker proteins. Taken together with the results of the co-localization experiment (Fig. 4B; see also Fig. S2, http://jcs.biologists.org/supplemental) and Ca\(^{2+}\) imaging (Fig. 5), Ins\((1,4,5)P_3\)R1 and SERCA2a are considered to be transported on the same type of vesicles that are labeled with RFP-KDEL and GFP-KDEL.

Kinesin- and microtubule-dependent movement of the vesicular sub-compartment of ER

Finally, we investigated the molecular mechanisms underlying the movement of the vesicular sub-compartment of ER. To determine whether the cytoskeleton, including microtubules and actin filaments, is involved in the vesicle movements, we tested the effects of drugs that disrupt the cytoskeleton on the movement of the vesicular sub-compartment of ER labeled by RFP-KDEL. Nocodazole (10 \(\mu\)g/ml) was used for microtubule disruption, and latrunculin A (1 \(\mu\)g/ml) or cytochalasin D (10 \(\mu\)g/ml) was used for actin disruption, and we confirmed that these treatments were effective in cultured mouse hippocampal neurons by immunocytochemical staining with anti-tubulin antibody (for microtubules) and rhodamine-phalloidin (for actin filaments) (data not shown). Treatment of hippocampal neurons with nocodazole did not change the overall position of the ER, although fragmentation of ER in the reticular structures in small dendritic branches was sometimes observed; latrunculin A and cytochalasin D had no apparent disruptive effect on the structure of reticular ER. The distribution pattern and number of the vesicles labeled with RFP-KDEL were not affected by any of these drugs. In contrast to the minor effects of the drugs on the reticular ER, the movements of the vesicular sub-compartment of ER were severely affected by nocodazole.

![Fig. 3. ER markers did not overlap with markers of endosomes, lysosomes, and mitochondria. (A-C) Double labeling with Texas Red-dextran (red) and GFP-tagged ER markers (green): (A) GFP-KDEL, (B) GFP-SERCA2a and (C) GFP-Ins\((1,4,5)P_3\)R1. (D-F) Double labeling with MitoTracker Red CMX\(_{\text{Ros}}\) (red) and (D) GFP-KDEL, (E) GFP-SERCA2a, and (F) GFP-Ins\((1,4,5)P_3\)R1. The upper images are high power micrographs of the soma and the lower images are those of the dendrites. Note that most of the lysosomal and mitochondrial signals that appear to be outside of the transfected cells arise from surrounding cells. All images were taken with a confocal microscope. Scale bars: 10 \(\mu\)m.](http://jcs.biologists.org/supplemental/)

### Table 1. Velocity of movement of vesicles expressing ER marker proteins

| ER marker proteins | GFP-KDEL | RFP-KDEL | GFP-SERCA2a | GFP-Ins\((1,4,5)P_3\)R1 |
|--------------------|----------|----------|-------------|-------------------------|
| Anterograde (\(\mu\)m/s) | 0.28±0.15 (\(n=97\)) | 0.28±0.19 (\(n=56\)) | 0.33±0.21 (\(n=36\)) | 0.25±0.15 (\(n=43\)) |
| Retrograde (\(\mu\)m/s) | 0.28±0.23 (\(n=139\)) | 0.27±0.18 (\(n=81\)) | 0.26±0.18 (\(n=51\)) | 0.21±0.12 (\(n=63\)) |

Values show mean ± s.d.
Fig. 4. The RFP-KDEL protein was not localized on the reticular ER, but was only seen in the vesicular sub-compartment of ER. 
(A) Expression of RFP-KDEL in a hippocampal neuron observed with a CCD camera. Arrowheads indicate branch points of the dendrites. 
(B) Double-labeling with RFP-KDEL and GFP-KDEL. In contrast to GFP-KDEL that labeled both reticular ER and vesicles, RFP-KDEL fluorescence was found only on vesicles. RFP-KDEL and GFP-KDEL co-localized on the same vesicles (arrowheads). 
(C) Representative movements of vesicles labeled with RFP-KDEL. The net movement of each vesicle (μm) was plotted against time (seconds). 
(D) Double-labeling with RFP-KDEL and endogenous Ins(1,4,5)P₃R1 using monoclonal antibody 18A10. Endogenous Ins(1,4,5)P₃R1 was also found on the RFP-KDEL-labeled vesicles (arrowheads). 
(E,F) Double-labeling with RFP-KDEL (red) and fluorescein-dextran (E; green), or MitoTracker Green FM (F; green). The upper images are high power micrographs of the soma and the lower images are those of the dendrites. The lysosomal and mitochondrial signals that appear to be outside the transfected cells arise from surrounding cells. RFP-KDEL did not co-localize with these markers. B,D-F are confocal micrographs. Scale bars: 10 μm.

Fig. 5. Luminal Ca²⁺ imaging in cultured hippocampal neurons loaded with mag-fura-2 AM (A) and fluo-5N AM (B) and permeabilized with β-escin. (Top left) RFP-KDEL-labeled vesicles, (bottom left) Ca²⁺ indicators. Scale bar, 10 μm. (Top right) Time-course plots of changes in fluorescence intensity of the Ca²⁺ indicators, (right bottom) time-course plots of changes in the ratio (F₃₄₀/F₃₈₀ for mag-fura-2 and F/F₀ for fluo-5N). The ratio plots are indicated after high-cut filtering with a Gaussian filter in order to reduce the noise. The neurons were subjected to Ca²⁺ uptake (1 mM MgATP), washout of MgATP and Ca²⁺ release (20 μM Ins(1,4,5)P₃), as indicated by the horizontal boxes above the traces.
treatment. The population of moving vesicles in the dendrites was significantly decreased by nocodazole treatment ($P<0.02$, Mann-Whitney U-test), but not by latrunculin A treatment (Fig. 7). Nocodazole treatment also decreased the velocity of vesicle movements by ~40% in both anterograde and retrograde directions ($P<0.005$, Mann-Whitney U-test), while latrunculin A did not have any significant effect on the velocity of vesicles ($P>0.2$, Mann-Whitney U-test) (Fig. 8 and Table 2). We also used cytochalasin D to disrupt the actin cytoskeleton, and the result was much the same as that obtained with the latrunculin A treatment. Nocodazole treatment combined with latrunculin A did not result in complete inhibition of the movement as well as treatment with nocodazole alone (Fig. 7), although the velocity of the vesicle was more severely affected by the combination of the drugs than by nocodazole alone (Fig. 8 and Table 2). These results suggest that the movements of the vesicular sub-compartment of ER are highly dependent on the integrity of the microtubules, and the contribution of the actin cytoskeleton to the movement is minor, if any.

Next, we investigated a candidate motor protein that may be responsible for vesicle movements. An ER membrane protein, kinase, was shown to be associated with the microtubule motor protein kinesin (Toyoshima et al., 1992) and to be responsible for the membrane movements in vitro (Kumar et al., 1995), suggesting that kinesin could be involved in the movements of the vesicular sub-compartment of ER. To test this possibility, we examined the effects of overexpression of a dominant-negative kinesin, that is, the tail of the kinesin heavy chain (KHC) fused with GFP (GFP-DNKHC) and treatment with an antisense oligonucleotide against KHC on the movements of the RFP-KDEL-labeled vesicles in the dendrites. Western blot analysis confirmed the decrease in KHC protein levels in antisense-treated cells: the amount of KHC protein in the antisense-treated cells was 40-50% of untreated cells and 50-70% of sense-treated cells (data not shown). Both expression of dominant negative KHC and antisense treatment against KHC did not affect the overall distribution of ER, and the labeling patterns of mitochondrial markers and endosomal/lysosomal markers (data not shown). This result is inconsistent with a previous report (Feiguin et al., 1994) showing that KHC antisense treatment induced loss of ER from the neurites, which may be explained by the difference in the level of reduction of kinesin (>80% of untreated cells in the previous study) and the age of the neurons (DIV1 in the previous study).

In neurons expressing GFP-DNKHC, the number of motile vesicles (Fig. 7) and the velocity of the vesicular movement (Fig. 8 and Table 2) were significantly reduced as compared with those in control neurons expressing GFP instead of GFP-DNKHC (by ~73% for the number of motile vesicles and ~35% for the velocity; $P<0.005$, Mann-Whitney U-test). The antisense oligonucleotide treatment also significantly decreased the number of moving vesicles by ~88% ($P<0.001$, Mann-Whitney U-test) (Fig. 7), and the velocity of vesicles by ~50% ($P<0.005$, Mann-Whitney U-test) compared with those
Dynamics of vesicular ER in neuron in the sense-treated cells (Fig. 8 and Table 2). These data strongly suggest that the movement of vesicular sub-compartment of ER is dependent on the microtubule motor protein, kinesin.

Discussion
Nature of the vesicular sub-compartment of ER
We have examined the distribution and dynamics of ER in dendrites of living hippocampal neuron using fluorescent proteins that are localized on the ER membrane (GFP-SERCA2a and GFP-Ins(1,4,5)P3R1) or inside the ER lumen (GFP-KDEL and RFP-KDEL).

Table 2. Effects of drug treatment, overexpression of dominant-negative kinesin, and antisense oligonucleotide treatment against kinesin on the velocity of vesicle movement

| Condition             | Anterograde (µm/s) | Retrograde (µm/s) |
|-----------------------|--------------------|-------------------|
| DMSO                  | 0.28±0.19 (n=46)   | 0.23±0.17 (n=60)  |
| Latrunculin A         | 0.30±0.18 (n=37)   | 0.24±0.15 (n=51)  |
| Nocodazole            | 0.16±0.11* (n=58)  | 0.15±0.10* (n=48) |
| Nocodazole+Latrunculin A | 0.11±0.07* (n=74) | 0.12±0.09* (n=77) |
| GFP                   | 0.30±0.22 (n=71)   | 0.29±0.26 (n=79)  |
| GFP-DNKHC             | 0.19±0.17* (n=50)  | 0.19±0.15* (n=71) |
| Sense                 | 0.30±0.29 (n=58)   | 0.28±0.21 (n=65)  |
| Antisense             | 0.13±0.10* (n=78)  | 0.15±0.14* (n=77) |

Values show mean ± s.d. *P<0.005 (Mann-Whitney U-test).

First, we showed that a part of the ER exists as vesicular structures that moved dynamically within the dendrites (Figs 1, 2 and 4), in addition to the well-known dense and reticular structure. Because the signal of the ER marker GFP-KDEL, which is expected to diffuse throughout the continuous ER lumen, was found in the vesicular as well as the reticular forms, and because vesicles labeled by GFP-tagged ER marker proteins moved over ~10 µm for at least 20 seconds while being independent of the reticular ER, we consider that at least some proportion of the vesicles are spatially separate from the reticular ER for a considerable period of time. The photobleaching experiment showing that the movements of fluorescent vesicles were quite different from the other GFP signals on the membrane of the bulk ER also supports this idea (Fig. 2G,H; see also Movies 1 and 2, http://jcs.biologists.org/supplemental). Because the fast moving vesicles labeled with ER proteins ran in the photobleached area in dendrites and kept running in it for at least 15 seconds, the lifetime of the vesicular sub-compartment of ER, which is separate from bulk ER, may be at least ~15 seconds.

Our observations strongly suggest that these vesicles are specialized sub-compartments of ER that are separated from the bulk ER. However, it is still unclear whether the vesicular sub-compartment is completely separate from the reticular ER during its movement. Previous studies using electron microscopy clearly showed the existence of vesicles carrying...
endogenous ER proteins such as calsequestrin, SERCA and Ins(1,4,5)P3R, which are entirely separate from the ER cisternae in the neuronal dendrites (Villa et al., 1991; Volpe et al., 1991), and the existence of tubulovesicular smooth ER in the axons (Tsukita and Ishikawa, 1980; Metuzals et al., 1997; Tabb et al., 1998). These observations suggest that there are vesicles carrying ER-specific proteins that are completely separated from the rest of the ER. Morphological studies may be required to clarify whether the vesicular sub-compartment of ER, revealed in this study, corresponds to those previously observed structures or not. Whether the vesicular ER is finally absorbed by the bulk ER by fusion or whether it is totally separate from the bulk ER also remains to be elucidated. We also showed that this vesicular sub-compartment of ER conveyes some of the endogenous ER proteins (Fig. 4), but were distinct from other membranous organelle such as endosomes, lysosomes, mitochondria and autophagosomes (Figs 3, 4; see also Fig. S3, http://jcs.biologists.org/supplemental). An important finding demonstrated in this study is that the vesicular sub-compartment of ER had the ability to function as a Ca2+ store, just like the reticular ER (Fig. 5). Although it is widely accepted idea that the Ca2+ storage site in the neuron is a continuous structure (Berridge, 1998; Petersen et al., 2001), the finding that the vesicular sub-compartment of ER could serve as a Ca2+ storage site suggests the existence of another category of Ca2+ store in the neuron.

The uneven distribution of ER markers, that is, RFP-KDEL resided in the vesicular structure while other GFP-tagged ER markers resided in both the vesicular and reticular structures, may reflect the fact that the ER-resident proteins often distribute unevenly in the ER structures. One of the well known example is the uneven distribution of calsequestrin in ER: calsequestrin is condensed into the vacuolar structure because of its tendency to form oligomers (Wuytack et al., 1987; Villa et al., 1991; Volpe et al., 1991; Villa et al., 1993; Gatti et al., 2001). RFP (DsRed1) also has a strong tendency to form oligomers (Baird et al., 2000). The expression pattern of DsRed2-KDEL (Zhang et al., 2003) that is expected to form less aggregates than RFP (DsRed1)-KDEL was intermediate to those of GFP-KDEL and RFP-KDEL: DsRed2-KDEL labeled both reticular ER and the vesicular sub-compartment, and the latter was labeled much more intensely by DsRed2-KDEL than by GFP-KDEL (unpublished observation). These results suggest that the vesicular structure that RFP-KDEL highlights is not a totally different structure from ER but overlaps with the well-known vesicular structure stained by several ER markers (Villa et al., 1991; Volpe et al., 1991). RFP-KDEL might be sorted exclusively to the vesicular ER fraction by similar mechanisms to those for calsequestrin.

Molecular mechanism underlying the movement of the vesicular sub-compartment of ER

In the present study, we demonstrated for the first time that the vesicular sub-compartment of ER was rapidly transported within dendrites. The movement of the vesicles was bidirectional, with the direction of movement altering occasionally, at an average velocity of 0.2-0.3 μm/second (Fig. 6 and Table 1). The movements were quite similar to those of the ‘large phase-dense organelles (~0.4 μm)’ that were previously observed by video-enhanced phase contrast microscopy (Overly et al., 1996).

The movements of reticular ER have been hypothesized to depend on three mechanisms, namely, (1) sliding movement of ER along microtubules, (2) movement of microtubules to which ER is attached, and (3) movement dependent on the polymerization of the microtubules to which ER is attached (Waterman-Storer and Salmon, 1998). In contrast, the movements of vesicular sub-compartment of ER seemed to depend mainly on a single mechanism, that is, a sliding movement along the microtubules induced by, at least, a plus-end directed motor protein, kinesin (Figs 7, 8 and Table 2). Although we cannot exclude the possibility that other motor proteins such as dynein may also be involved in the movement, the bi-directional movement of the vesicles by kinesin could be explained by the mixed polarity of the microtubules in the dendrites of hippocampal neurons (Baas et al., 1988). It is interesting that the movement of mRNA in the dendrite of neurons, which has been suggested to be kinesin-dependent (Severt et al., 1999), is similar to the movements of the ER sub-compartment in several respects: both movements are bi-directional and the velocity of movement of mRNA granules [~0.1 μm/second on average (Knowles et al., 1996; Rook et al., 2000)] is similar to that of the ER sub-compartment (0.2-0.3 μm/second). In the dendrite, AMPA-type glutamate receptors are also transported by kinesin (Kim and Lisman, 2001; Setou et al., 2002). How can various cargoes such as mRNA, AMPA receptors and ER sub-compartment be targeted to their precise positions in the dendrite by a single class of motor protein, kinesin? The molecular mechanisms underlying the kinesin-dependent transport processes in the dendrite require further studies, such as has been well elucidated for the axonal transport processes (for a review, see Almenar-Queralt and Goldstein, 2001).

In the present study, actin filaments were shown to have little contribution to the transport of vesicular sub-compartment of ER in the dendritic shaft (Figs 7, 8, and Table 2). However, myosin V, one of the actin motors, has been reported to be involved in the movement of vesicular ER in the squid giant axon (Tabb et al., 1998). This discrepancy may have arisen not only from the difference of the cell types but also from the difference of the cellular compartments, i.e., axon or dendrite. The axon and the dendrite have diverse properties. Although the GFP-tagged ER markers labeled vesicular structures both in the axon (Aihara et al., 2001) and the dendrite in this study, the vesicles labeled with GFP-tagged ER markers moved only retrogradely in the axon (Tabb et al., 1998), while the vesicles moved in both directions in the dendrite in this study. Furthermore, the movement of the vesicles was not sensitive to nocodazole in the axon (Aihara et al., 2001) while it was sensitive in the dendrite. Thus, the mechanisms underlying the movement and other properties of the vesicles may be different in the axon and the dendrite. Although the previous work (Aihara et al., 2001) did not show involvement of actin filaments in vesicle movement, it is tempting to speculate that the movement of the vesicular sub-compartment of ER in axons might be largely dependent on actin filaments while those in dendrites are not.

The role of actin filaments and myosin V in the transport of the vesicular sub-compartment of ER in dendrites should be further examined, because actin-dependent movements of ER
vesicles could be crucial in actin-rich domains, such as around the subcortical regions or the dendritic spines (Dekker-Ohno et al., 1996). For the present, our attempt to evaluate the contribution of myosin V to the transport of the vesicular sub-compartment of ER using GFP-tagged dominant negative myosin V (GFP-MST) has not been successful, because mouse hippocampal neurons overexpressing GFP-MST were not viable (unpublished observation), although overexpression experiments of dominant negative myosin V have been reported in many cell lines (Bahadoran et al., 2003; Gross et al., 2002; Hales et al., 2002; Rodriguez and Cheney, 2002; Rudolf et al., 2003). Myosin V might play more crucial roles in cell survival in cultured neurons than in conventional cell lines. Furthermore, experiments using myosin Va knockout mice, where myosin Vb and Vc might compensate for the absence of myosin Va, could be useful to address this issue.

Physiological roles for the transport of the vesicular sub-compartment of ER

Finally, we would like to speculate on the physiological roles of the moving ER vesicles. One possibility is that the vesicular sub-compartment of ER just transports newly synthesized ER proteins from the cell body to distal parts of the dendrites. Vesicles labeled with ER marker proteins traveled at an average velocity of 0.2-0.3 μm/second (Table 1). The speed of the reticular ER movement in newt lung cells was reported to be 0.07 μm/second (Waterman-Storer and Salmon, 1998), and the velocity of the ER membrane proteins [SERCA2α and the transmembrane region of Ins(1,4,5)P3R1] examined in the axons of chick dorsal root ganglion neurons was also 0.07 μm/second (Aihara et al., 2001), implying that ER proteins on the vesicular sub-compartment can be transported 3-4 times faster than those on the reticular ER. Because neurons are highly polarized cells, transport of the vesicular sub-compartment of ER that contains ER resident proteins could greatly contribute to the urgent supply of ER proteins to the distal parts of the dendrites.

Another possibility is that the vesicular sub-compartment is targeted to special regions of the dendrites to function as active Ca2+ stores. The finding that the vesicular sub-compartment of ER takes up and releases Ca2+ supports this idea (Fig. 5). Interestingly, Ins(1,4,5)P3R-mediated Ca2+ waves begin predominantly at branch points on the main apical shaft of the dendrites of rat hippocampal CA1 pyramidal cells (Nakamura et al., 2002) and the ER sub-compartment had a tendency to accumulate at branch points of dendrites in this study, even though the vesicles existed not only at branch points of main shafts but also at those of minor branches (Fig. 4A). Thus, it is tempting to speculate that the transport of the vesicular sub-compartment of ER is involved in the spatial regulation of Ca2+ signaling within the neuron, which is postulated to be important for synaptic plasticity (Finch and Augustine, 1998; Miyata et al., 2000; Wang et al., 2000).

In this study, we examined, for the first time, the dynamics of the ER sub-compartment in the dendrites of mouse hippocampal neurons. We found that the sub-compartment of ER is transported rapidly and bi-directionally in a microtubule- and kinesin-dependent manner. We also demonstrated that this ER sub-compartment could constitute a Ca2+-release pool. The functional dynamics of the vesicles is of interest, as it may be a key to understanding the spatial regulation of neuronal Ca2+ signaling and various other neuronal activities, such as synaptic plasticity.

We are grateful to the Kazusa DNA Research Institute (Chiba, Japan) for providing the human cDNA clone KIAA0531, N. Mizushima and Y. Ohsumi, National Institute for Basic Biology (Okazaki, Japan) for providing GFP-LC3, Dr W. I. Gelfand for providing GFP-MST clone (University of Illinois, IL, USA), Y. Inoue (University of Tokyo, Tokyo, Japan) for giving us advice on analysis of vesicle movement, and Olympus Corporation (Tokyo, Japan) for the assistance with the laser system for the photobleaching experiment. We also thank T. Nakamura (Calcium Oscillation Project, Tokyo, Japan) for valuable discussions, M. Iwai and Y. Tateishi (University of Tokyo, Tokyo, Japan) for assistance with plasmid construction, and N. Ogawa (RIKEN, Saitama, Japan) and K. Fukatsu (University of Tokyo, Tokyo, Japan) for technical assistance. This study was supported by a grant from the Ministry of Education and Science of Japan (to T.I., M.H. and K.M.).

References

Aihara, Y., Inoue, T., Tashiro, T., Okamoto, K., Komiya, Y. and Mikoshiba, K. (2001). Movement of endoplasmic reticulum in the living axon is distinct from other membranous vesicles in its rate, form, and sensitivity to microtubule inhibitors. J. Neurosci. Res. 65, 236-246.

Almenar-Queralt, A. and Goldstein, L. S. (2001). Linkers, packages and pathways: new concepts in axonal transport. Curr. Opin. Neurobiol. 11, 550-557.

Baas, P. W., Deitch, J. S., Black, M. M. and Banker, G. A. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. Proc. Natl. Acad. Sci. USA 85, 8335-8339.

Bahadoran, P., Busca, R., Chiaverini, C., Westbroek, W., Lambert, J., Bille, K., Valony, G., Fukuda, M., Naeyaert, J. M., Ortonne, J. P. et al. (2003). Characterization of the molecular defects in Rab27a, caused by RAB27A missense mutations found in patients with Griscelli syndrome. J. Biol. Chem. 278, 11386-11392.

Baird, G. S., Zacharias, D. A. and Tsien, R. Y. (2000). Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. Proc. Natl. Acad. Sci. USA 97, 11984-11989.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. Nature 361, 315-325.

Berridge, M. J. (1998). Neuronal calcium signaling. Neuron 21, 13-26.

Dalley, M. E. and Bridgman, P. C. (1989). Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons. J. Neurosci. 9, 1897-1909.

Dekker-Ohno, K., Hayasaki, S., Takagishi, Y., Oda, S., Wakasugi, N., Mikoshiba, K., Inouye, M. and Yamamura, H. (1996). Endoplasmic reticulum is missing in dendritic spines of Purkinje cells of the ataxic mutant rat. Brain Res. 714, 226-230.

Ellisman, M. H. and Lindsey, J. D. (1983). The axoplasmic reticulum with myelinated axons is not transported rapidly. J. Neurocytol. 12, 393-411.

Feigun, F., Ferreira, A., Kosik, K. S. and Caceres, A. (1994). Kinesin-mediated organelle translocation revealed by specific cellular manipulations. J. Cell Biol. 127, 1021-1039.

Ferreira, A., Nicol, J., Vale, R. D., Banker, G. and Kosik, K. S. (1992). Suppression of kinesin expression in cultured hippocampal neurons using antisense oligonucleotides. J. Cell Biol. 117, 595-606.

Finch, E. A. and Augustine, G. J. (1998). Local calcium signalling by inositol-1,4,5-trisphosphate in Purkinje cell dendrites. Nature 396, 753-756.

Fujii, S., Matsumoto, M., Igarashi, K., Kato, H. and Mikoshiba, K. (2000). SapIic activity in hippocampal CA1 neurons of mice lacking type 1 inositol-1,4,5-trisphosphate receptors. Learn. Mem. 7, 312-320.

Fujiiwara, A., Hirose, K., Yamazawa, T. and Iino, M. (2001). Reduced IP3 sensitivity of IP3 receptor in Purkinje cells of the ataxic mutant rat. Brain Res. 833-839.

Gatti, G., Trifari, S., Meseali, N., Parker, J. M., Michalak, M. and Meldolesi, J. (2001). Head-to-tail oligomerization of calstecin: a novel mechanism for heterogeneous distribution of endoplasmic reticulum luminal proteins. J. Cell Biol. 154, 525-534.
depression requires release of Ca\(^{2+}\) from separate presynaptic and postsynaptic intracellular stores. *J. Neurosci.* 16, 5951-5960.

Rizzuto, R., Ratto, M. A., Fatton, P., Carru, G., Fay, F. S., Fogarty, K. E., Lifshitz, L. M., Tuft, R. A. and Pozzan, T. (1998). Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca\(^{2+}\) responses. *Science* 280, 1763-1766.

Rizzuto, R., Bernardi, P. and Pozzan, T. (2000). Mitochondria as all-round players of the calcium game. *J. Physiol.* 529, 37-47.

Roderick, H. L., Campbell, A. K. and Llewellyn, D. H. (1997). Nuclear localization of calreticulin in vivo is enhanced by its interaction with glucocorticoid receptors. *FEBS Lett.* 405, 181-185.

Rodriguez, O. C. and Cheney, R. E. (2002). Human myosin-Vc is a novel class V myosin expressed in epithelial cells. *J. Cell Sci.* 115, 991-1004.

Rook, M. S., Lu, M. and Kosik, K. S. (2000). CaMKII \(\alpha\) translocated region-directed mRNA translocation in living neurons: visualization by GFP linkage. *J. Neurosci.* 20, 6385-6393.

Rudolf, R., Kogel, T., Kuznetsov, S. A., Salm, T., Schlicker, O., Hellwig, A., Hammer, J. A., 3rd and Gerdes, H. H. (2003). Myosin Va facilitates the distribution of secretory granules in the F-actin rich cortex of PC12 cells. *J. Cell Sci.* 116, 1339-1348.

Sanger, J. M., Dome, J. S., Mittal, B., Somlyo, A. V. and Sanger, J. W. (1989). Dynamics of the endoplasmic reticulum in living non-muscle and muscle cells. *Cell Motil.* 13, 301-319.

Seto, M., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2002). Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature* 417, 83-87.

Severt, W. L., Biber, T. U., Wu, X., Hecht, N. B., De Lorenzo, R. J. and Jakoi, E. R. (1999). The suppression of testis-brain RNA binding protein and kinesin heavy chain disrupts mRNA sorting in dendrites. *J. Cell Sci.* 112, 3691-3702.

Smith, S. B. and Cunnane, T. C. (1996). Rymodine-sensitive calcium stores involved in neurotransmitter release from sympathetic nerve terminals of the guinea-pig. *J. Physiol.* 497, 657-664.

Spacek, J. and Harris, K. M. (1997). Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. *J. Neurosci.* 17, 190-203.

Taub, J. S., Molyneaux, B. J., Cohen, D. L., Kuznetsov, S. A. and Langford, G. M. (1998). Transport of ER vesicles on actin filaments in neurons by myosin V. *J. Cell Sci.* 111, 3221-3234.

Takehi, H., Eilers, J. and Konnerth, A. (1998). A new class of synaptic response involving calcium release in dendritic spines. *Nature* 396, 757-760.

Takei, K., Stukenbrok, H., Metz, A., Mignery, G. A., Südhof, T. C., Volpe, P. and De Camilli, P. (1992). Ca\(^{2+}\) stores in Purkinje neurons: endoplasmic reticulum subcompartments demonstrated by the heterogeneous distribution of the InsP\(_3\) receptor, Ca\(^{2+}\)-ATPase, and calnexin. *J. Neurosci.* 12, 489-505.

Takei, K., Shin, R. M., Inoue, T., Kato, K. and Mikoshiba, K. (1998). Regulation of nerve growth mediated by inositol 1,4,5-trisphosphate receptors in growth cones of developing PC12 cells. *J. Neurosci.* 18, 282-1705-1708.

Terasaki, M., Slater, N. T., Fein, A., Schmidke, A. and Reese, T. S. (1994). Continuous network of endoplasmic reticulum in cerebellar Purkinje neurons. *Proc. Natl. Acad. Sci. USA* 91, 7510-7514.

Terasaki, M., Jaffe, L. A., Huncutt, G. R. and Hammer, J. A., 3rd (1996). Structural change of the endoplasmic reticulum during fertilization: evidence for loss of membrane continuity using the green fluorescent protein. *Dev. Biol.* 179, 320-328.

Toyoshima, I., Yu, H., Steuer, E. R. and Sheetz, M. P. (1992). Kinectin, a major kinesin-binding protein on ER. *J. Cell Biol.* 118, 1211-1231.

Tsukita, S. and Ishikawa, H. (1980). The movement of membranous organelles in axons. Electron microscopic identification of anterogradely and retrogradely transported organelles. *J. Cell Biol.* 84, 513-530.

Volpe, P., Villa, A., Damiani, E., Sharp, A. H., Podini, P., Snyder, S. H. and Meldolesi, J. (1991). Heterogeneity of microsomal Ca\(^{2+}\) stores in chicken Purkinje neurons. *EMBO J.* 10, 3183-3189.
Wang, S. S., Denk, W. and Häusser, M. (2000). Coincidence detection in single dendritic spines mediated by calcium release. *Nat. Neurosci.* 3, 1266-1273.

Waterman-Storer, C. M. and Salmon, E. D. (1998). Endoplasmic reticulum membrane tubules are distributed by microtubules in living cells using three distinct mechanisms. *Curr. Biol.* 8, 798-806.

Wuytack, F., Raeymaekers, L., Verbist, J., Jones, L. R. and Casteels, R. (1987). Smooth-muscle endoplasmic reticulum contains a cardiac-like form of calsequestrin. *Biochim. Biophys. Acta* 899, 151-158.

Zhang, S., Mizutani, A., Hisatsune, C., Higo, T., Bannai, H., Nakayama, T., Hattori, M. and Mikoshiba, K. (2003). Protein 4.1N is required for translocation of inositol 1,4,5-trisphosphate receptor type 1 to the basolateral membrane domain in polarized Madin-Darby canine kidney cells. *J. Biol. Chem.* 278, 4048-4056.