Translational Mobility of the Type 3 Inositol 1,4,5-Trisphosphate Receptor Ca$^{2+}$ Release Channel in Endoplasmic Reticulum Membrane*

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The inositol 1,4,5-trisphosphate receptor (InsP$_3$R) is an integral membrane protein in the endoplasmic reticulum (ER) which functions as a ligand-gated Ca$^{2+}$ release channel. InsP$_3$-mediated Ca$^{2+}$ release modulates the cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), providing a ubiquitous intracellular signal with high temporal and spatial specificity. Precise localization of the InsP$_3$R is believed to be important for providing local [Ca$^{2+}$]$_i$ regulation and for ensuring efficient functional coupling between Ca$^{2+}$ release sites by enabling graded recruitment of channels with increasing stimulus strength in the face of the intrinsically unstable regenerative process of Ca$^{2+}$-induced Ca$^{2+}$ release. Highly localized Ca$^{2+}$ release has been attributed to the ability of the InsP$_3$R channels to cluster and to be localized to discrete areas, suggesting that mechanisms may exist to restrict their movement. Here, we examined the lateral mobility of the type 3 isoform of the InsP$_3$R (InsP$_3$R3) in the ER membrane by performing confocal fluorescence recovery after photobleaching of an InsP$_3$R3 with green fluorescent protein fused to its N terminus. In Chinese hamster ovary and COS-7 cells, the diffusion coefficient $D$ was $-4 \times 10^{-10}$ cm$^2$/s at room temperature, a value similar to that determined for other ER-localized integral membrane proteins, with a high fraction ($\sim 75\%$) of channels mobile. $D$ was modestly increased at 37 °C, and it as well as the mobile fraction were reversibly reduced by ATP depletion. Although disruption of the actin cytoskeleton (latrunculin) was without effect, disruption of microtubules (nocodazole) reduced $D$ by half without affecting the mobile fraction. We conclude that the entire ER is continuous in these cells, with the large majority of InsP$_3$R3 channels free to diffuse throughout it, at rates that are comparable with those measured for other polytopic ER integral membrane proteins. The observed InsP$_3$R3 mobility may be higher than its intracellular diffusional mobility because of additional ATP- and microtubule-facilitated motility of the channel.

The inositol trisphosphate signaling pathway is present in nearly all cells. Activation of phospholipase C by G protein-coupled receptors and receptor tyrosine kinases results in the hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate to two products, diacylglycerol and the water-soluble inositol 1,4,5-trisphosphate (InsP$_3$) (1). InsP$_3$ diffuses in the cytoplasm and binds to a receptor, the InsP$_3$R, a polytopic integral membrane protein in the endoplasmic reticulum (ER), activating it as a Ca$^{2+}$ channel to liberate stored Ca$^{2+}$ from the ER lumen into the cytoplasm. This rapid release of Ca$^{2+}$ modulates the cytoplasmic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) providing a ubiquitous intracellular signal with complex features that endow it with high temporal and spatial specificity (1, 2). The InsP$_3$-mediated [Ca$^{2+}$]$_i$ signaling system regulates a diversity of cellular processes, including gene transcription, membrane transport, secretion, contraction, intercellular communication, and synaptic plasticity (1, 3).

The InsP$_3$ signaling pathway is expressed ubiquitously, but it nevertheless provides highly spatially and temporally specific [Ca$^{2+}$]$_i$ signals. This has been attributed to the diversity of InsP$_3$R isoform expression (a family of three InsP$_3$R isoforms exists with different primary sequences derived from different genes with alternatively spliced forms (4, 5)), subcellular distributions of the InsP$_3$R channels (6–10), and complex regulation of the channel by both InsP$_3$ and [Ca$^{2+}$]$_i$ (11, 12). Gating of the InsP$_3$-liganded InsP$_3$R channel is modulated with a biphasic dependence on [Ca$^{2+}$]$_i$ (13–16). Importantly, Ca$^{2+}$ binding to specific sites associated with the channel is necessary for InsP$_3$ to activate it. The requirement for both InsP$_3$ as well as Ca$^{2+}$ binding enables the channel to function as a coincidence detector, which is believed to be important in determining the fidelity of signaling in physiological processes. Furthermore, the requirement for Ca$^{2+}$ binding enables the InsP$_3$R to participate in Ca$^{2+}$-induced Ca$^{2+}$ release, believed to be the fundamental feature that determines the spatial extent and magnitude of InsP$_3$-induced [Ca$^{2+}$]$_i$ signals (2, 17).

[Ca$^{2+}$]$_i$ signals mediated by release from the InsP$_3$R can be highly localized or global throughout the cell. Individual InsP$_3$R-mediated Ca$^{2+}$ release events imaged in Xenopus oocytes and mammalian cells (Ca$^{2+}$ "puffs" and "blips") (18–21) are believed to represent the coordinated gating of channels within clusters that contain variable numbers of InsP$_3$R channels (7, 22, 23). Such highly localized signals can exist in cells because Ca$^{2+}$ diffusion is limited by high concentrations of mobile and immobile Ca$^{2+}$ buffers. Localized [Ca$^{2+}$]$_i$ signals afford the cell the opportunity to regulate physiological processes in discrete environments, with effectors limited to those with appropriate spatial proximity and Ca$^{2+}$ affinity. It has

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† The abbreviations used are: InsP$_3$, inositol 1,4,5-trisphosphate; [Ca$^{2+}$]$_i$, cytoplasmic free Ca$^{2+}$ concentration; CHO, Chinese hamster ovary; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; InsP$_3$R, inositol 1,4,5-trisphosphate receptor; PBS, phosphate-buffered saline.

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been proposed that global signals are produced by the temporally and spatially coordinated recruitment of individual localized release events (17, 18, 24). Increased stimulus strength recruits more release sites more frequently with enhanced Ca\(^{2+}\) release amplitudes (18, 21, 25, 26). Global Ca\(^{2+}\) release manifested as [Ca\(^{2+}\)]\(_i\) waves ensue when the individual release sites become coordinated through the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release.

The ability of localized Ca\(^{2+}\) release events to remain autonomous until cells are maximally stimulated suggests that the spatial separation of Ca\(^{2+}\) release clusters is important to provide stability in the face of the intrinsically unstable regenerative process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Furthermore, the activity of one or a few spatially stable active release sites, which are consistently recruited by repetitive stimulation, determines the initiation sites for [Ca\(^{2+}\)]\(_i\) waves as well as the probability that a global wave will be triggered (19, 25–28). These observations suggest that the spatial organization of Ca\(^{2+}\) signaling molecules is critical in creating the observed diversity of [Ca\(^{2+}\)]\(_i\) signals. The channel permeation and gating properties of the InsP\(_3\)R are of fundamental importance in generating complex Ca\(^{2+}\) release events, but the subcellular organization of the channels, including their localization and density, have also been invoked to account for the characteristics of [Ca\(^{2+}\)]\(_i\) signals in cells (6, 29, 30). A strong correlation between the density of InsP\(_3\)R and the location of local Ca\(^{2+}\) release sites that entrain global [Ca\(^{2+}\)]\(_i\) waves has been observed in exocrine acinar cells (8, 31, 32), whereas no such correlations with channel density were observed in other cells types (19). In addition to local channel density, other factors have also been proposed to account for spatially restricted [Ca\(^{2+}\)]\(_i\) signals, including the presence of mitochondria (21, 33–36), localized differences in InsP\(_3\) sensitivity (4, 5), and functionally discrete Ca\(^{2+}\) stores (6) (29, 37, 38). Unfortunately, immunolocalization of InsP\(_3\)R may provide only limited insights because of isoform nonspecificity of antibodies, weakness or cross-reactivity with other proteins of some of the antibodies used, or low level expression of InsP\(_3\)R and the use of fixed cells. Furthermore, whether the entire ER or only specialized regions are involved in Ca\(^{2+}\) sequestration and/or release is debated (37, 38). Imaging experiments have suggested that the InsP\(_3\)R-sensitive stores are usually continuous (see Ref. 37), but the possibility of functionally discrete stores, created perhaps by restricted intraluminal Ca\(^{2+}\) diffusion, or patchy localization of release and/or uptake sites or Ca\(^{2+}\) binding proteins, is possible. Thus, the relationship between the localization of InsP\(_3\)R and [Ca\(^{2+}\)]\(_i\), signaling properties in cells is an important unresolved issue.

Here we have examined the localization of the InsP\(_3\)R in living cells by imaging GFP fluorescence of a GFP-InsP\(_3\)R fusion protein in which GFP was fused to the N terminus of the rat type 3 InsP\(_3\)R. This methodology ensures absolute specificity of the signal, enabling unambiguous localization of the channel in living cells. In addition, we performed fluorescence recovery after photobleaching (FRAP) experiments of live cells expressing GFP-InsP\(_3\)R. The major questions we addressed are the following. First, is the InsP\(_3\)R mobile in the ER membrane? We hypothesized that mechanisms involved in localizing InsP\(_3\)R would constrain its mobility in the ER membrane. What fraction of the InsP\(_3\)R molecules is immobile? We hypothesized that if the ER was physically discontinuous, a large fraction of the InsP\(_3\)R channels would appear in FRAP to be immobile. Alternately, channel clusters in or protein complexes may not be free to diffuse in the ER membrane. Finally, we asked whether the translational mobility of the InsP\(_3\)R channel changes after disruption of cytoskeletal elements. The InsP\(_3\)R has been proposed to associate with various cytoskeletal elements, particularly molecules linked to the actin cytoskeleton (39–45). Our results indicate that the ER is continuous with most of the GFP-InsP\(_3\)R3 mobile in the ER membrane with a translational diffusion coefficient that, although low, is nevertheless similar to those of other ER-localized polytopic integral membrane proteins of comparable mass. Surprisingly, the relatively low mobility was independent of the actin cytoskeleton, but it was reduced by ATP depletion and disruption of microtubules, suggesting that InsP\(_3\)R mobility in the ER membrane is facilitated by active processes involving the microtubule cytoskeleton.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection—**CHO-K1 (Cricetulus griseus ovary) cells were grown in minimal essential medium without nucleosides (Invitrogen) containing 10% fetal bovine serum and penicillin/streptomycin. COS-7 (Cercopithecus aethiops kidney) cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro) containing 10% fetal bovine serum and penicillin/streptomycin. Both cell types were incubated at 37 °C with 5% CO\(_2\). Cells were plated onto 25-mm diameter round glass coverslips, 24–48 h after transfection using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions, with 3–5 μg of plasmid DNA of either GFP-KDEL (a gift from Dr. Alan Verkman, University of California, San Francisco) or pEGFP-InsP\(_3\)R3. The cDNA encoding pEGFP-InsP\(_3\)R3 was constructed by inserting the rat InsP\(_3\)R3 sequence into BspEII/XhoI and XhoI/EcoRI sites of the pEGFP-C1 vector (Clontech) downstream of EGFP. Three days after transfection, growth medium was replaced with medium containing 250 μg/ml Geneticin (Invitrogen). The medium was then replaced every 2–3 days. Cells were cultured for 1–18 days after transfection.

**Cell Treatments—**Cells on glass coverslips were incubated in a bath solution containing (mM): NaCl (140), KCl (5), MgCl\(_2\) (1), CaCl\(_2\) (2.5), and HEPES (10) during experiments, unless otherwise noted. Photo-bleaching measurements were carried out at both room temperature and 37 °C. In some experiments, cells were preincubated with the metabolic inhibitors 2-deoxy-x-glucose (Sigma) (50 mM) and sodium azide (Sigma) (0.02%) (1 h, 37 °C, for ATP depletion) or nocardazole (Fluka) (100 μM) or Iprunclcin B (Biomol) (2 μM) for 15 min on ice, and then returned to 37 °C bath. For recovery of ATP levels, 10 mM glucose was added to the bath solution, and cells were incubated for 30 min at 37 °C. Transfected cells were also incubated with ER Tracker Blue-White DPX (Molecular Probes) in growth medium (1 μM, 30 min, 37 °C).

**Western Blotting—**CHO and COS-7 cells were rinsed with phosphate-buffered saline (PBS) 48 h after transfection, scrapped from culture flasks, and pelleted. The supernatant was removed, and 1 ml of lysis buffer (10 mM HEPES, 150 mM NaCl, 1% Triton X-100 (w/v), 5 mM EDTA) with protease inhibitor (1 mM benzamidine, 10 μM EDTA, 1 μM leupeptin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, Sigma) was added to the pellet. After 2 min of vortexing, the homog enate was centrifuged at top speed for 20 min (4 °C) on a bench-top centrifuge (Eppendorf). The supernatant was removed, and protein concentration was measured so that 50 μg of total protein was separated on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose, analyzed with an InsP\(_3\)R-specific antibody (Ab-3; Transduction Laboratories) or a GFP-specific antibody (Chemicon International) and, detected by enhanced chemiluminescence (Amersham Biosciences).

**Immunostaining—**CHO and COS-7 cells were seeded onto glass coverslips. The next day they were washed with ice-cold PBS and fixed overnight in −20 °C methanol. The cells were washed in ice-cold PBS-TB (0.3% Triton X-100 and 0.1% bovine serum albumin) and incubated with an InsP\(_3\)R antibody in 1% bovine serum albumin and PBS (1:100) for 2 h at room temperature. After incubation with the primary antibody the cells were washed in ice-cold PBS-TB and incubated for 1 h at room temperature with Alexa Fluor 488 (Molecular Probes) in 1% bovine serum albumin and PBS (1:500). After a final wash in ice-cold PBS-TB, the coverslips were mounted on slides with a drop of Vectashield (Vector Laboratories, Inc.). Cells were visualized with a Zeiss Axiosvert 100M 63×/numerical aperture 1.2 water immersion lens, and images were collected using LSM510 software. For phalloidin staining, CHO cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized in blocking buffer (0.1% saponin, 1% bovine serum albumin in PBS) for 30 min at room temperature, and then incubated with Alexa Fluor 488 phallolidin (Molecular Probes) diluted to 1 unit/200 μl in blocking buffer for 20 min. The cells
were washed three times in PBS and mounted for microscopy in PBS-buffered 50% glycerol.

**Fluorescence Recovery after Photobleaching (FRAP)—** Photobleaching measurements and images of transfected or stained cells were collected on a Zeiss Axiovert 100M confocal microscope using Zeiss LSM510 software. Cells were visualized with a 63× numerical aperture 1.2 apochromat water immersion lens. Cells transfected with a GFP fusion protein were illuminated with an argon ion laser (488 nm) (L absol); ER Tracker Blue-White DPX-stained cells were visualized at 364 nm (Fertigung GmbH Lasermodul UV). Cells chosen for experiments displayed a wide range of fluorescence intensities, which always demonstrated a reticular pattern of fluorescence. Experiments were carried out at room temperature or at 37 °C. A circular region (2–8 μm diameter) in the peripheral part of the cell was photobleached with the 488 nm laser line (25% power, 100% transmission, ~30 s). The prebleach intensity (average of 10 scans) and recovery of fluorescence were monitored by scanning the whole cell at low laser intensity (25% power, 1% transmission, 0.3–2 s) that minimized further photobleaching.

**Analysis of FRAP—** To generate fluorescence recovery curves and determine the time to half-maximum recovery of fluorescence $t_{1/2}$, fluorescence intensities were obtained from three locations: the bleach spot circular region, a region of the same size which was not photobleached (control), on the same cell; and a region off the cell (background). The background intensities were subtracted from the bleach and control intensities, and the results were plotted using the Igor analysis program (WaveMetrics). A first order exponential fit was performed on the bleach curve to determine $t_{1/2}$. $D$ was calculated by $D = \frac{r^2}{4t_1}$ (46) where $r$ was the radius of the bleach spot, $C_{i f}$ was the correction factor for the apparent $D$ calculated assuming two-dimensional diffusion (48, 49). We have therefore not applied a correction factor to account for ER geometry. The mobile fraction ($MF$) was calculated using Equation 1,

$$MF = \left( \frac{F_i - F_0}{C_{i f} / C_{if} - F_i (C_{i f} / C_{if})} \right)$$

(Eq. 1)

where $F_i$ is the fluorescence of the bleach spot before bleaching, $F_0$ is the fluorescence of the bleach spot after bleaching, $C_{i f}$ is the fluorescence of the bleach spot at infinite time, $C_t$ is the fluorescence of the control spot before bleaching, and $C_c$ is the fluorescence of the control spot at infinite time. The loss of fluorescence caused by photobleaching from scanning during the duration of the experiment, the fractional bleach by scanning $C_{i f}$, and $(F_0 - F_i)$ is the loss of fluorescence caused by spot photobleaching. $F_i (C_{i f})$ is the expected intensity of the bleach spot assuming no recovery, $F_t (C_{i f})$ is the expected intensity of the bleach spot assuming full recovery. $MF > 1$ if $F_i > F_t (C_{i f})$.

Statistics were computed using a two-tailed Student $t$ test. Values of $p < 0.05$ were considered significant.

**RESULTS**

GFP was fused to the N terminus of the rat type 3 InsP$_3$R (rGFP-InsP$_3$R3) (Fig. 1A), and the diffusion of the InsP$_3$R in the ER of living cells was determined by confocal imaging of the fusion protein. CHO and COS-7 cells were transiently transfected with either GFP-InsP$_3$R3 or GFP-KDEL, and fluorescence recovery after photobleaching (FRAP) was measured to determine translational diffusion coefficients. GFP-KDEL FRAP provided a measure of small soluble molecule translational diffusional mobility in the ER lumen because the KDEL ER retrieval/retention sequence targets GFP there (48). CHO and COS-7 cells were used because they have been employed in previous studies of ER-localized diffusional mobilities and because InsP$_3$R3 is normally expressed in these cells (50–52). Western blot analysis confirmed the expression of the GFP-InsP$_3$R3 fusion protein in both cell types (Fig. 1B).

Confocal imaging of living transfected CHO (Fig. 2A) and COS-7 (Fig. 2B) cells revealed that the GFP-InsP$_3$R3 was distributed in a reticular pattern throughout the cytoplasm. The protein appeared to be localized to the nuclear envelope as well but was absent from the plasma membrane. These features are reminiscent of an ER morphology. To confirm that the GFP-InsP$_3$R3 localized similarly compared with the endogenous channel, untransfected CHO and COS-7 cells were fixed and

**FIG. 1.** Expression of rat GFP-InsP$_3$R in COS-7 and CHO cells. A, schematic representation of the EGFP-InsP$_3$R fusion protein in the ER membrane. B, Western blot analysis of the expression of GFP-InsP$_3$R3 in COS-7 (left) and CHO (right) cells either transfected or not with GFP-InsP$_3$R3 and probed with InsP$_3$R3 or GFP antibodies. The double arrow indicates GFP-InsP$_3$R3 (upper arrow) and endogenous InsP$_3$R3 (lower arrow). Exposure conditions for the CHO InsP$_3$R3 blot were insufficient to visualize the endogenous InsP$_3$R3 in untransfected cells.

**FIG. 2.** Expressed GFP-InsP$_3$R3 localizes to the ER. A and B, live cell images of GFP-InsP$_3$R3 in CHO (A) and COS-7 (B) cells, demonstrating reticular distribution reminiscent of ER. C and D, immunolocalization of endogenous InsP$_3$R3 in CHO (C) and COS-7 (D) cells. E and F, live cell images of GFP-KDEL in CHO (E) and COS-7 (F) cells. G–I, live CHO cell images of GFP-InsP$_3$R3 (G) stained with the fluorescent ER Tracker dye (H), and the overlay of the two images (I). Bars represent 5 μm.
immunostained for the endogenous InsP$_3$R3 (Fig. 2, C and D, respectively). A similar reticular pattern was observed, suggesting that the N-terminal GFP tag did not influence the normal localization of the InsP$_3$R3 protein. This result is consistent with observations that the determinants of ER localization reside in the C terminus of the InsP$_3$R (53–55) as well as previous observations of a GFP-tagged InsP$_3$R (56). To confirm further that the GFP-InsP$_3$R3 localized to the ER, the distribution of the ER in both CHO and COS-7 cells was determined by visualizing the distribution of transiently expressed GFP-KDEL (Fig. 2, E and F) or ER Tracker Blue-White DPX (Fig. 2, G and H). The distribution of both indicators was highly similar to that of either the endogenous InsP$_3$R3 or the expressed GFP-InsP$_3$R. In single CHO cells, the GFP-InsP$_3$R3 fluorescence was completely colocalized with that of the fluorescent ER Tracker dye (Fig. 2). Taken together, these results demonstrate that transient transfection of rGFP-InsP$_3$R3 results in a normal ER localization of the expressed protein. Thus, FRAP of this construct reports the mobility of the channel in the ER membrane.

Photobleaching of living transiently transfected CHO and COS-7 cells was performed to determine the translational diffusional mobility of the rInsP$_3$R3. Photobleaching was performed using a 63× water immersion objective, which generated bleach spots of 2–8 μm diameter (Fig. 3A). Photobleaching was accomplished by transient removal of attenuating neutral density filter from the laser excitation path. In general, we attempted to bleach the GFP fluorescence in the circular region to ~25% of its starting intensity (Fig. 3B). Fluorescence recovery was then recorded by serial imaging (Fig. 3, C and D). The time course of variation in the fluorescence intensities at both the bleach spot (circle in Fig. 3A) and another location in the cytoplasm (cross-hairs in Fig. 3A) during the experiment depicted in Fig. 3, A–D, were quantitated (Fig. 3E). The fluorescence intensities at both cytoplasmic sites were stable until the bleach. After the bleach, the diminished intensity at the bleach site recovered toward prebleach levels. In the example shown in Fig. 3, recovery was nearly complete. The fluorescence intensity at the remote cytoplasmic site did not change significantly during the course of the experiment. This demonstrates that photobleaching of the GFP was minimal during fluorescence intensity recording subsequent to the bleach and therefore contributed little to the recovery kinetics observed at the bleach site. Nevertheless, data quantification of the recovery kinetics at the bleach site have taken into consideration any observed changes at the remote site in each cell (Equation 1).

The diffusion coefficient ($D$) for InsP$_3$R3 at room temperature in CHO cells was $3.1 \pm 0.2 \times 10^{-10}$ cm$^2$/s ($n = 70$), with a mobile fraction, determined from the asymptote of the recovery curve, of 67 ± 3%. The diffusion coefficient was somewhat higher in COS-7 cells: $D = 4.4 \pm 0.3 \times 10^{-10}$ cm$^2$/s ($n = 60$) with mobile fraction = 77 ± 2% (Table I).

The goal in these studies was to determine the translational diffusional mobility of the InsP$_3$R3 in the ER membrane in living cells. Several control experiments were undertaken to validate the measurement system. First, to confirm that the observed fluorescence recovery was the result of diffusion, we examined the recovery kinetics in fixed cells. The extent of recovery from photobleaching in transiently transfected fixed cells was severely attenuated, but nevertheless finite (Fig. 3F and Table I). As noted, fluorescence recovery in the absence of diffusion defines a reversible recovery process that has been observed previously for both free GFP and GFP fusion proteins (57). The mechanisms responsible for slow reversible recovery of GFP fluorescence are not clear, but they may involve triplet state relaxation or other photophysical phenomena (57). Second, we measured the recovery rates in live cells of other ER-localized probes. ER Tracker Blue-White DPX is a neutral daphoxyl sulfonamide derivative that is expected to partition specifically into the ER membrane and exhibit a higher rate of diffusion (Molecular Probes). The measured $D$ at room temperature was approximately twice that measured for the GFP-InsP$_3$R3, and the mobile fraction was also somewhat higher (~85–90%; Fig. 3G and Table I). The diffusion coefficient for GFP-KDEL was ~15-fold greater than that for GFP-InsP$_3$R3 in both CHO and COS-7 cells, with a mobile fraction ~80–90%
both significantly reduced the mobile fraction by more than half. Depletion was without effect on the diffusion coefficient, but it for that codeplete cellular ATP. In CHO cells, metabolic ties during photobleaching and recovery at bleach (dark points) and control (gray points) in GFP-InsP3R3-expressing cells at 37 °C under control conditions (A), during ATP depletion (B), and after recovery from ATP depletion (C).

(Fig. 3H and Table I). Because technical limitations did not enable bleaching to be achieved in very short times, the diffusion coefficients of highly mobile species such as GFP-KDEL are expected to be underestimated because of consumption of a significant fraction of the total fluorescent molecule pool. Thus, the average D in these two cell types of ~55 × 10^{-10} cm²/s is somewhat smaller than some previously reported values (48, 57, 58). It is however, comparable with that determined for GFP-elastase in the ER lumen (59). For the same reason, the values for the mobile fractions of this construct as well as the GFP-InsP3R3 that we determined probably underestimate somewhat the true mobile fraction. Taken together, these results suggest that the experimental protocols measure the translational diffusional mobility of the InsP3R in the ER membrane.

In both cell types, the measured D for GFP-InsP3R3 was only somewhat sensitive to temperature. In CHO cells, D increased from 3.1 × 10^{-10} cm²/s at room temperature to 3.5 ± 0.5 × 10^{-10} cm²/s (n = 24) at 37 °C, in COS-7 cells, D increased to 5.1 ± 0.7 × 10^{-10} cm²/s (n = 14) from 4.4 × 10^{-10} cm²/s (Fig. 4 and Table II). To investigate the cellular mechanisms that influence the mobility of the InsP3R in the ER membrane, we first examined the effects of ATP depletion. Cells were preincubated with 50 mM 2-deoxy-D-glucose and 0.02% sodium azide for 1 h at 37 °C to deplete cellular ATP. In CHO cells, metabolic depletion was without effect on the diffusion coefficient, but it significantly reduced the mobile fraction by more than half (Table II and Fig. 4). In contrast, metabolic depletion reduced both D as well as mobile fraction in COS-7 cells (Table II and Fig. 4). In each cell type, the effects of ATP depletion were mostly reversed by incubating the cells with 10 mM D-glucose for 30 min at 37 °C. ATP depletion had no obvious effects on the morphology of the ER (not shown), or with previous studies that demonstrated that ATP depletion was without effect on ER structure or continuity in primary fibroblasts (60) or CHO cells measured using a lipid dye (58). Thus, the reversible effects of ATP depletion of the mobility of the InsP3R observed here were not caused by changes in the structure or fragmenting of the ER.

It has been speculated previously that InsP3R interactions with the cytoskeleton may play roles in localizing and retaining the release channels in specific locations in the cytoplasm (45). To determine whether cytoskeletal interactions play a role in restricting InsP3R diffusion mobility, photobleaching experiments were performed on CHO cells that were incubated either in nocodazole (100 μM), which depolymerizes microtubules, or latrunculin B (2 μM), which disrupts actin filaments. Control cells were incubated for equivalent times in the vehicle (1:100 v/v dimethyl sulfoxide) only. The recovery curves from these experiments are shown in Fig. 5, and the results are summarized in Table III. Disruption of the actin cytoskeleton with latrunculin B (Fig. 5D) had no effects (Fig. 5C). In contrast, microtubule disruption with nocodazole significantly reduced by half the diffusion coefficient without affecting the mobile fraction (Fig. 5B).

Again, there were no obvious effects of the inhibitors on the morphology of the ER in the cells analyzed. These results suggest that a microtubule-dependent process facilitates the mobility of the InsP3R in the ER membrane.

### DISCUSSION

The central issue addressed by our studies concerns the extent of translational mobility of the InsP3R in the ER membrane in living cells. Our results demonstrate that the majority (70%) of the expressed GFP-InsP3R3 is freely mobile in the ER membrane, with both the extent and rate of mobility similar to those of other ER-localized integral membrane proteins. Furthermore, the InsP3R3 is freely mobile throughout a single continuous ER because constant photobleaching of a single site in the cell caused the disappearance of GFP fluorescence throughout the rest of the cell (data not shown).

We used FRAP of transiently expressed GFP-tagged InsP3R3 to determine the mobility of the InsP3R in the ER membrane. We assumed, like others, that the relatively polar GFP moiety is unlikely to influence the membrane diffusion of integral membrane proteins to which it is fused (57). We performed colocalization experiments that established the correct ER targeting of the GFP-InsP3R3, which suggests that the GFP moiety is also without effect on the normal localization of the release channel. This conclusion is similar to that reached in a study of a similar construct expressed in human neuroblastoma and salivary gland cells (56) as well as with the expression of other normally ER-localized proteins fused to GFP (57, 58, 60–67). Therefore, we assume that our measurements provide a measure of the diffusional mobility of the InsP3R3 in the ER. Nevertheless, several caveats should be considered when interpreting the results. First, fusion of the GFP to the N terminus of the InsP3R, which is near to the InsP3R binding domain, may perturb normal protein interactions with the channel, including some that might influence its translational mobility. Cytosolic proteins have been discovered which interact with the N-terminal region of the InsP3R (68, 69), which could possibly link the channel to stable cellular structures, although no integral membrane protein or cytoskeleton-related proteins have been described which might possibly be involved in tethering the channel. Second, the FRAP measurements

### Table II

| Cell type       | Experimental conditions | n   | \(D \times 10^{-10}\) cm²/s | MF  |
|-----------------|-------------------------|-----|------------------------------|-----|
| CHO             | Control                 | 24  | 3.5 ± 0.5                   | 0.71 ± 0.04 |
| CHO             | ATP-depleted            | 13  | 3.4 ± 0.7                   | 0.32 ± 0.05 |
| CHO             | ATP depletion recovery  | 6   | 3.0 ± 0.9                   | 0.64 ± 0.10 |
| COS-7           | Control                 | 14  | 5.1 ± 0.7                   | 0.79 ± 0.08 |
| COS-7           | ATP-depleted            | 20  | 3.4 ± 0.5*                 | 0.51 ± 0.05* |
| COS-7           | ATP depletion recovery  | 9   | 5.5 ± 0.8                   | 0.59 ± 0.08 |

* p < 0.05 compared with control.
require expression of a recombinant channel protein at levels that provide sufficient fluorescence signals. If the InsP₃R₃ interacts with other proteins, overexpression of the recombinant channel might saturate those interactions, rendering them insufficient to accommodate all of the expressed InsP₃R₃ protein. Consequently, if these protein interactions normally influence InsP₃R₃ mobility, the mobility we measured would not reflect the normal state. We attempted to control for this possibility in two ways. First, we examined the dimmest cells possible which provided a sufficient fluorescence signal for the measurement because expression was minimal in such cells. Second, we studied cells up to 3 weeks after transfection. By then, the cells generally expressed very low levels of protein, and they had a considerable amount of time to integrate the expressed protein fully into the normal cohort of interactions and locations of the endogenous InsP₃R₃ pool. In each strategy, we found comparable diffusion coefficients compared with cells with brighter signals studied at earlier times, although there was a trend to lower mobile fractions in the 2–3-week expressing cells. It is important to recognize that the mobility and localization of InsP₃R₃ channels will likely be cell type-dependent, whereas we studied only two cell culture lines. Finally, we have examined only the type 3 InsP₃R isoform, and other isoforms may behave differently.

With these caveats in mind, what are the implications of the rates and extent of InsP₃R₃ mobility measured here? The average diffusion coefficient that we determined for CHO and COS-7 cells was \( \sim 4 \times 10^{-10} \text{cm}^2/\text{s} \). This measurement can be compared with similar determinations for other ER-localized proteins. An ER-localized aquaporin-2 water channel had a diffusion coefficient of \( \sim 3 \times 10^{-10} \text{cm}^2/\text{s} \) (57). Like the InsP₃R channel, aquaporin-2 is a tetramer of monomers that each contains 6-membrane helices. Because the intrinsic diffusional mobility of integral membrane proteins is most strongly influenced by the radius of the protein mass that interacts with the lipid membrane (46), the similar diffusion coefficients observed for the InsP₃R and aquaporin-2 may suggest that the mobilities we observed here are either largely governed by the physical interactions between the channel and the ER membrane or that similar processes are involved in the mobility of both proteins. The rate we measured is also similar to that determined for cytochrome P450 in the ER (\( \sim 4.5 \times 10^{-10} \text{cm}^2/\text{s} \)) (60). Although cytochrome P450 is a smaller molecule than the InsP₃R (\( \sim 45 \text{kDa} \) versus \( \sim 310 \text{kDa} \)), it is known to form oligomers as well as complexes with other proteins (64). Lectin receptors on the outer membrane of the nuclear envelope, functionally equivalent to the ER, have \( \sim 4 \times 10^{-10} \text{cm}^2/\text{s} \) (70). The translocon protein translocation complex, which contains 60 transmembrane helices, had \( \sim 4 \times 10^{-10} \text{cm}^2/\text{s} \) (62). Mutant cystic fibrosis transmembrane conductance regulator chloride ion channel (12 transmembrane helices in a monomer) has a diffusion coefficient of \( \sim 10^{-9} \text{cm}^2/\text{s} \) (58), similar to that of another ATP-binding cassette transporter, the transporter for antigen processing (TAP1) (\( D = 12 \times 10^{-10} \text{cm}^2/\text{s} \)) (67). In contrast, ER- or nuclear envelope-localized integral membrane proteins with only one transmembrane helix have much higher rates of diffusion, e.g., emerin (one transmembrane segment, \( 32 \times 10^{-10} \text{cm}^2/\text{s} \) (61); major histocompatibility complex class 1 molecules (one transmembrane segment, \( \sim 40 \times 10^{-10} \text{cm}^2/\text{s} \) (60, 67); vesicular stomatitis virus G protein (one transmembrane segment, \( 40–50 \times 10^{-10} \text{cm}^2/\text{s} \) (66).

The diffusion coefficient is a measure of the surface area randomly sampled by the fluorophore in a given time. With measured \( D \) for the InsP₃R, \( R = 4 \times 10^{-10} \text{cm}^2/\text{s} \), the surface area covered by the channel over the course of 1 min is equivalent to \( \sim 2.5 \mu \text{m}^2 \), with the average distance translated less that 1 \( \mu \)m. Over the course of 15 min, the channel will translate on average \( \sim 3.4 \mu \text{m} \) in any direction. Thus, over short times common in experimental protocols, individual InsP₃R could appear to be relatively stationary in the three-dimensional volume of the ER and cell. Nevertheless, this seems insufficient to account for some published observations of spatially stable active Ca²⁺ release sites (19, 25–28). Mobility of the InsP₃R in the face of release site stability may suggest that the existence of those sites is determined by features associated with the cellular locations rather than by the intrinsic properties or density of InsP₃R at those sites. However, several published observations suggest that cells must have mechanisms to restrict InsP₃R mobility. For example, InsP₃R are concentrated in discrete cell regions in some cell types, for example the apical pole in exocrine cells (9) or at the basolateral pole in other epithelial cells (44). It seems likely that specific retention mechanisms are necessary in these cells to prevent homogenization of the InsP₃R channel density throughout the entire ER. Patch clamp electrophysiology of the outer membrane of the nuclear env-
lope (7) as well as Ca\(^{2+}\) imaging (27, 71) both suggest that InsP\(_3\)Rs likely localize in clusters. These observations also suggest that mechanisms must exist to prevent the channels in clusters from dispersing, although both experimental approaches detect only functional channels, whereas the FRAP measurement reports all channels. It is possible that clusters of functional channels exist in a uniform sea of nonfunctional ones. Our results do not address the mechanisms that restrict InsP\(_3\)R diffusion out of these regions, but they likely include direct or indirect interactions with cytoskeletal proteins. The InsP\(_3\)R has been reported to interact with cytoskeletal components, including ankynin (40, 72, 73), vinculin and talin (74), actin (41) and actin-associated proteins (43, 44), and myosin (39). Nevertheless, there was no effect of disruption of the actin cytoskeleton (41) and actin-associated proteins (43, 44, 72), vinculin and talin (74). The finding was that the measured translational mobility of the InsP\(_3\)R was reduced by pharmacological disruption of microtubules. This observation suggests that the InsP\(_3\)R is associated with microtubule-based motility mechanisms. One intriguing possibility is that such motility depends on microtubule motors, for example kinesin or dynein. Our observation that ATP depletion also reduced the translational mobility of the InsP\(_3\)R is consistent with this model. Microtubule-dependent redistribution of the InsP\(_3\)R1 in the absence of structural changes in the ER has been observed in vascular smooth muscle cells (45). Microtubule disruption has previously been demonstrated to influence Ca\(^{2+}\) release mediated by the InsP\(_3\)R (42, 76, 77). Nocodazole disruption of Ca\(^{2+}\) signals in pancreatic acinar cells was suggested to indicate either that microtubule-mediated organization of the InsP\(_3\)R was important for normal [Ca\(^{2+}\)]i signals or that microtubules directly affected the function of the channel (42). These observations taken together, coupled with our new results, suggest an important role for microtubules in the localization and function of InsP\(_3\)R3 channels in the ER. Further work is necessary to understand the detailed mechanisms by which microtubules interact with the InsP\(_3\)R to influence its mobility and Ca\(^{2+}\) release channel functions.

In summary, we have determined the translational mobility of the InsP\(_3\)R3 channel in the ER membrane in living cells. Our results suggest that the entire ER is continuous, with the large majority of the channel free to diffuse throughout it at rates that are comparable with those measured for other polytropic ER integral membrane proteins. The intrinsic diffusional mobility may be lower than those we measured because of the presence of ATP- and microtubule-facilitated motility of the channel.

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Translational Mobility of the Type 3 Inositol 1,4,5-Trisphosphate Receptor Ca\textsuperscript{2+} Release Channel in Endoplasmic Reticulum Membrane
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