Supporting Information

Fujita et al. 10.1073/pnas.0900216106

SI Methods

Probes. Recombinant GST fusion proteins containing the mouse PLC-δ1 PH domain (amino acids 1–180) were expressed in *Escherichia coli* and purified by glutathione-agarose resin (Sigma). A PH domain mutant that does not bind to $PI(4,5)P_2$, GST-PH(K30N, K32N) (1), was made by replacing 2 lysine residues at positions 30 and 32 with asparagine by the QuikChange Site-Directed Mutagenesis kit (Stratagene). Rabbit anti-GST antibody was purchased from Bethyl Laboratories. Anti-rabbit IgG antibody and protein A conjugated to colloidal gold were obtained from BioCell and the University Medical Center Utrecht (Utrecht, The Netherlands), respectively.

Cells and Tissues. Human fibroblasts were explanted from the dermis of a healthy adult donor. Mouse embryo fibroblasts were prepared from caveolin-1-null mice and their normal counterparts (Jackson Laboratories). The cells were maintained in DMEM supplemented with 10% FCS, 50 units/mL penicillin, and 0.05 mg/mL streptomycin at 37 °C under 5% CO_2/95% air conditions. For cholesterol depletion, cells were treated with 5 mM methyl-β-cyclodextrin in DMEM for 60 min. Small pieces of vas deferens were excised from adult male ddY mice under anesthesia. The animals were treated according to the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan.

Liposomes. Phosphatidylcholine, phosphatidylinositol (Doosan Serdary Research Laboratory), and phosphoinositides (Avanti, Echelon, and Cell Signaling Technology) were purchased from the respective suppliers. Phosphatidylcholine and either phosphatidylinositol or a phosphoinositide were mixed at defined molar ratios, dried, and vortexed with an aqueous buffer to make liposomes. The liposome samples were either left for 48 h to induce giant liposomes or were pushed through a Miniextruder (Avanti) to make small unilamellar vesicles. The liposomes were pelleted, mixed with a thick sucrose solution to prevent ice crystal formation, and rapidly frozen by the metal sandwich technique (2).

Quick-Freezing and Freeze-Fracture. Cells grown on a small gold foil (≈4 mm^2 in area; 20 μm in thickness) were inverted onto prewarmed 10% gelatin on a gold-plated copper specimen table with the cell side down (2). The cell sandwich was slammed onto the copper block (precooled by liquid nitrogen) by using the rapid-freezing apparatus (3) constructed by John Heuser's laboratory. Mouse tissue pieces were held between 2 aluminum platelets with shallow indentations and were frozen by an HPM010 high-pressure freezing machine (Leica).

For freeze-fracture, the specimens were transferred to a cold stage of a Balzers BAF400 apparatus and fractured between −95 °C and −120 °C and ≈2×10^{-6} millibar. Replicas were made by electron-beam evaporation of Pt/C and C. Three different evaporation protocols were compared in a preliminary experiment: (*i*) Pt/C (2 nm) followed by C (20 nm), (*ii*) C (20 nm) followed by Pt/C (2 nm), and (*iii*) C (2 nm) followed by Pt/C (2 nm) and then by C (20 nm). The 3-step C-Pt/C-C protocol was used for cell specimens.

After thawing, the replicas of liposomes and cultured cells were digested by 2.5% SDS in PBS at 60–70 °C for 1 h to overnight. The replica of mouse vas deferens was autoclaved in the SDS solution, treated in 0.25% trypsin and 0.5 mM EDTA for 60 min at 37 °C, and then digested again by the SDS solution to remove tissue remnants. The replicas were adjusted to 50% glycerol and kept at −20 °C until labeling.

Replica Labeling. Replicas were rinsed with PBS containing 1% Triton X-100 (PBST), blocked with 3% BSA in PBS, and incubated with GST-PH in 1% BSA in PBST at 4 °C overnight. They were subsequently treated with rabbit anti-GST antibody (10 μg/mL) followed by colloidal gold (5-nm or 10-nm)-conjugated protein A (1:70 dilution of the supplied solution), both in 1% BSA in PBST, for 30 min at 37 °C. After each incubation, replicas were rinsed with 0.1% BSA in PBST. Finally, replicas were rinsed with distilled water and picked up on EM grids. The specimens were observed with a JEOL 1400EX electron microscope operated at 100 kV.

Statistical Analysis of Colloidal Gold Labeling. Electron micrographs were digitized with an image scanner (Canon). In some cases, digital images were directly captured by a CCD camera (Hamamatsu Photonics). Areas of 1×1 μm were chosen randomly and analyzed by Ripley's K-function as described previously (4, 5). The result was shown by the normalized L-function. For significance tests, 99% confidence envelopes for complete spatial randomness were generated from 100 Monte Carlo simulations. The labeling density and the nearest neighbor distance were obtained by the Image Processing Tool Kit version 5 plug-in (Reindeer Graphics) for Adobe Photoshop version 6 (Adobe).

Live Imaging. Human fibroblasts were transfected with YFP-PLC-δ1-PH cDNA (kindly provided by Nobukazu Araki) by an Amaxa nucleofector according to the manufacturer's instructions. The fluorescence microscopic images were captured by Axiovert200 equipped with AxioCam (Zeiss), and the fluorescence intensity in the cell surface was analyzed by ImageJ (National Institutes of Health).

1. Stauffer TP, Ahn S, Meyer T (1998) Receptor-induced transient reduction in plasma membrane PtdIns(4,5)P2 concentration monitored in living cells. *Curr Biol* 8:343–346.
2. Fujimoto T, Fujimoto K (1997) Metal sandwich method to quick-freeze monolayer cultured cells for freeze-fracture. *J Histochem Cytochem* 45:595–598.
3. Heuser JE, et al. (1979) Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. *J Cell Biol* 81:275–300.
4. Prior IA, Muncke C, Parton RG, Hancock JF (2003) Direct visualization of Ras proteins in spatially distinct cell surface microdomains. *J Cell Biol* 160:165–170.
5. Fujita A, et al. (2007) Gangliosides GM1 and GM3 in the living cell membrane form clusters susceptible to cholesterol depletion and chilling. *Mol Biol Cell* 18:2112–2122.

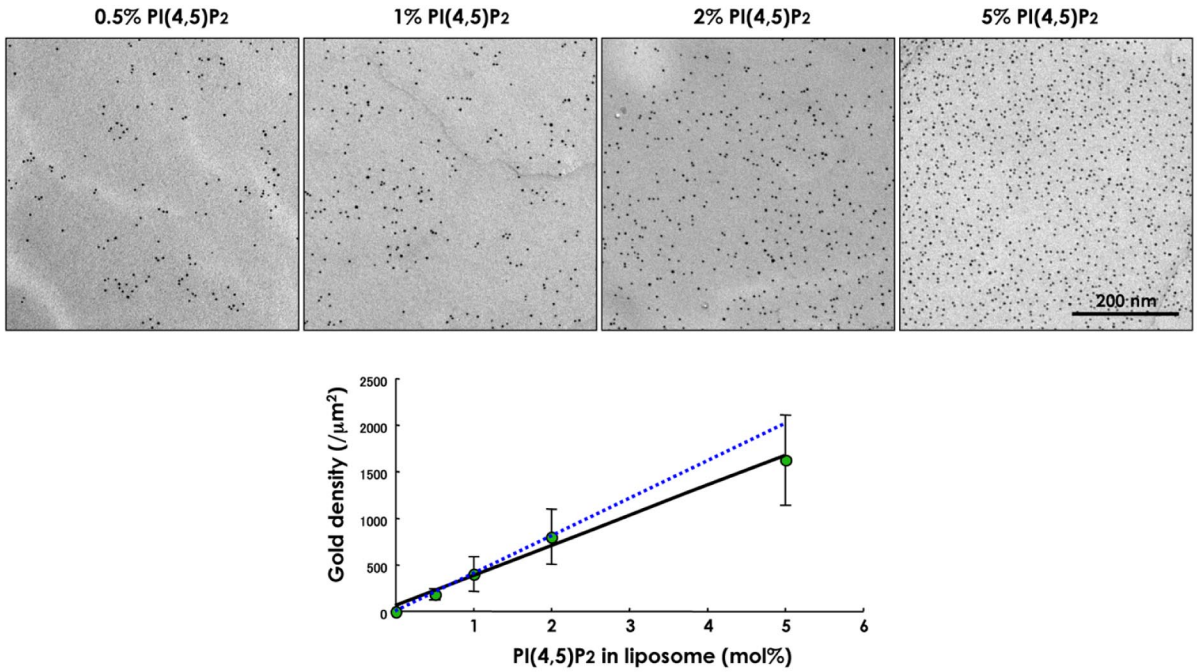

Fig. S1. Labeling of giant unilamellar liposomes made of phosphatidylcholine and 0, 0.5, 1, 2, and 5 mol % of $PI(4,5)P_2$. GST-PH was used at a concentration of 100 ng/mL. The best-fitting lines were drawn by using 4 (0–2 mol %; blue dotted line) or 5 (0–5 mol %; black solid line) points. The labeling occurred largely in proportion to the $PI(4,5)P_2$ content within the whole range, but some saturation was seen at 5 mol %. The labeling in liposomes containing 0.5%, 1%, and 2% $PI(4,5)P_2$ showed weak clustering, probably because chemically synthesized $PI(4,5)P_2$ that has long saturated acyl chains is used.

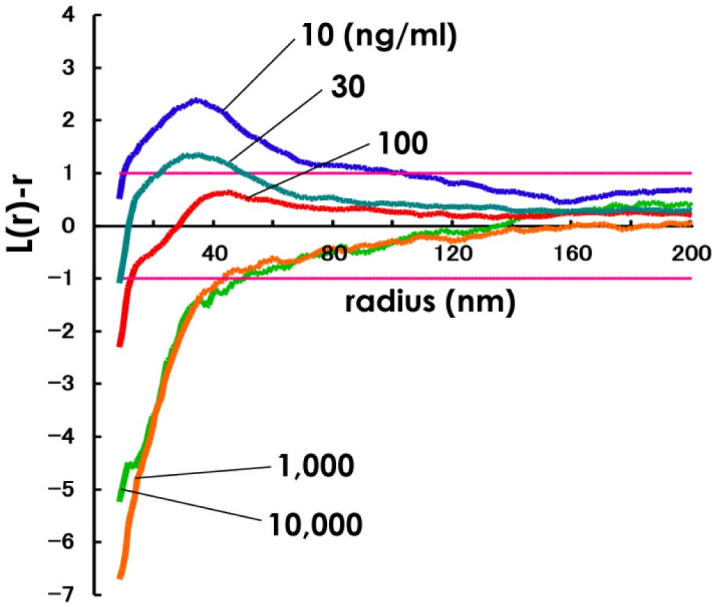

Fig. S2. The effect of the GST-PH concentration. Freeze-fracture replicas of human fibroblasts were probed by 5 different concentrations of GST-PH (10, 30, 100, 1,000, and 10,000 ng/mL) and then incubated with rabbit anti-GST antibody and protein A conjugated to colloidal gold. As the GST-PH concentration was raised, the labeling density increased. The labeling was distributed in a mutually exclusive pattern when GST-PH was used at high concentrations (>1,000 ng/mL), whereas the labeling showed weak clustering when GST-PH was used at low concentrations (<30 ng/mL).

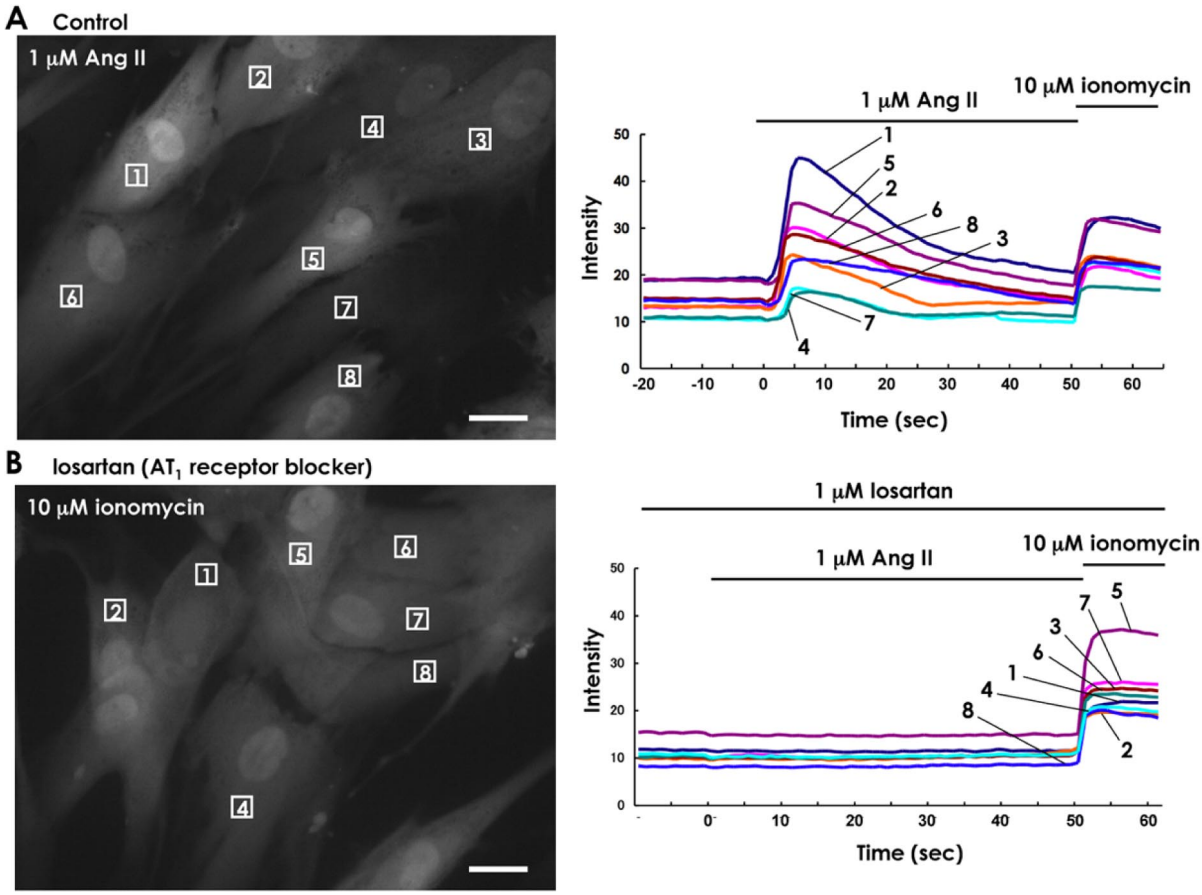

Fig. S3. The $[Ca^{2+}]_i$ change in the human fibroblast monitored by Fluo-4. (*A*) The cells were treated with 1 μM Ang II and then with 10 μM ionomycin at 25 °C. A sharp peak of $[Ca^{2+}]_i$ was observed after the Ang II application, whereas the $[Ca^{2+}]_i$ increase persisted after the ionomycin treatment. The fluorescence intensity measured in the numbered areas of the *Left* is shown in the *Right*. (*B*) The $[Ca^{2+}]_i$ change in the human fibroblast pretreated with 1 μM losartan, a specific inhibitor of the AT1 receptor. The rise of $[Ca^{2+}]_i$ by 1 μM Ang II was completely blocked, whereas the change after the ionomycin treatment was not. (Scale bar, 30 μm.)

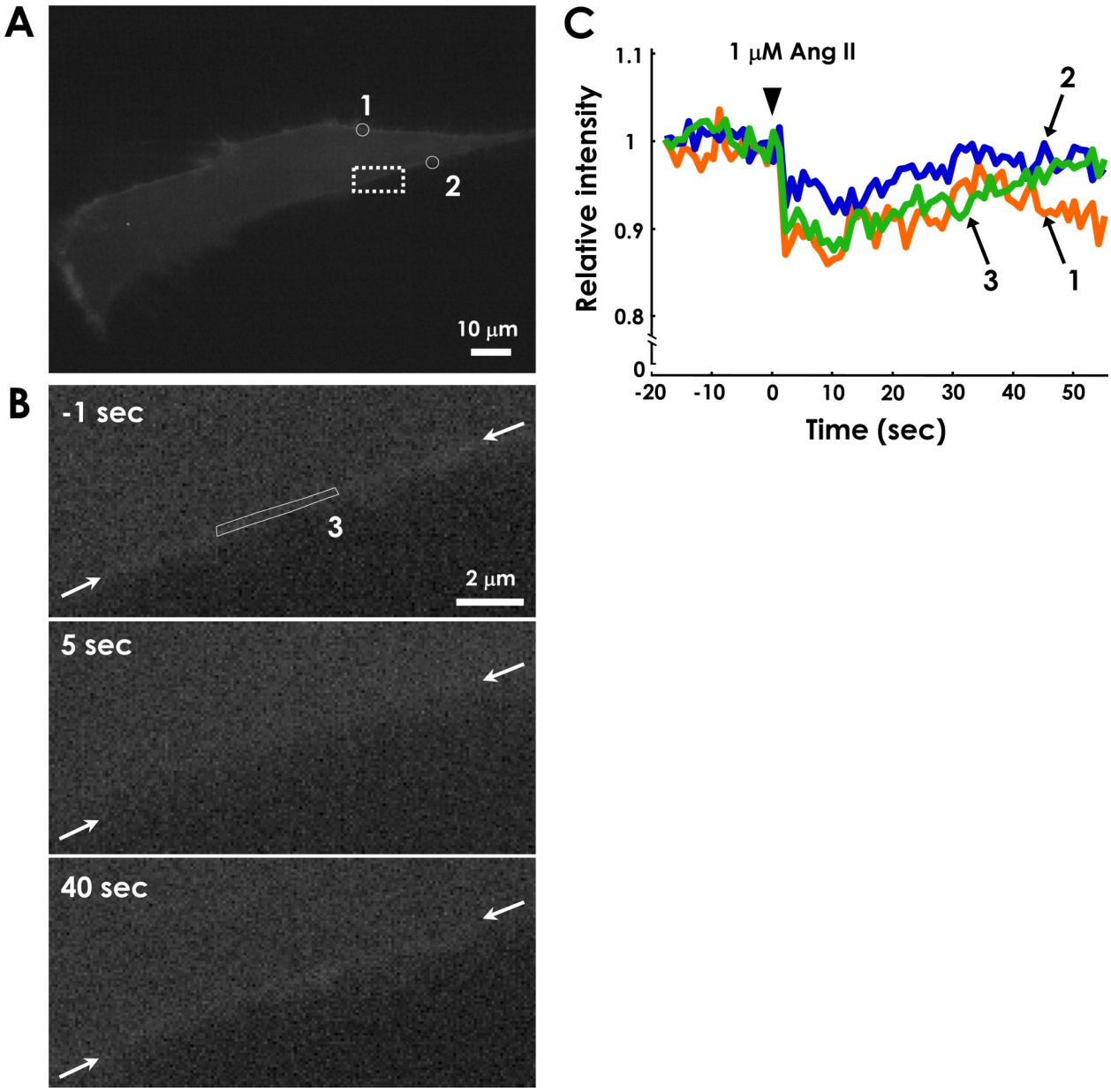

Fig. S4. Live imaging of human fibroblasts expressing YFP-PLC-δ1-PH. (*A*) A low-magnification micrograph of a cell showing the areas where the fluorescence intensities were analyzed (the circled areas 1 and 2). The rectangular area was magnified in *B*. (*B*) A high-magnification view of the cell surface (marked by 2 arrows). The fluorescence intensity of the rectangular area marked as 3 was quantified. (*C*) The time course of the fluorescence intensity change in areas 1, 2, and 3. The intensity decreased to a minimum level 5–10 sec after 1 μM angiotensin II application and gradually recovered.

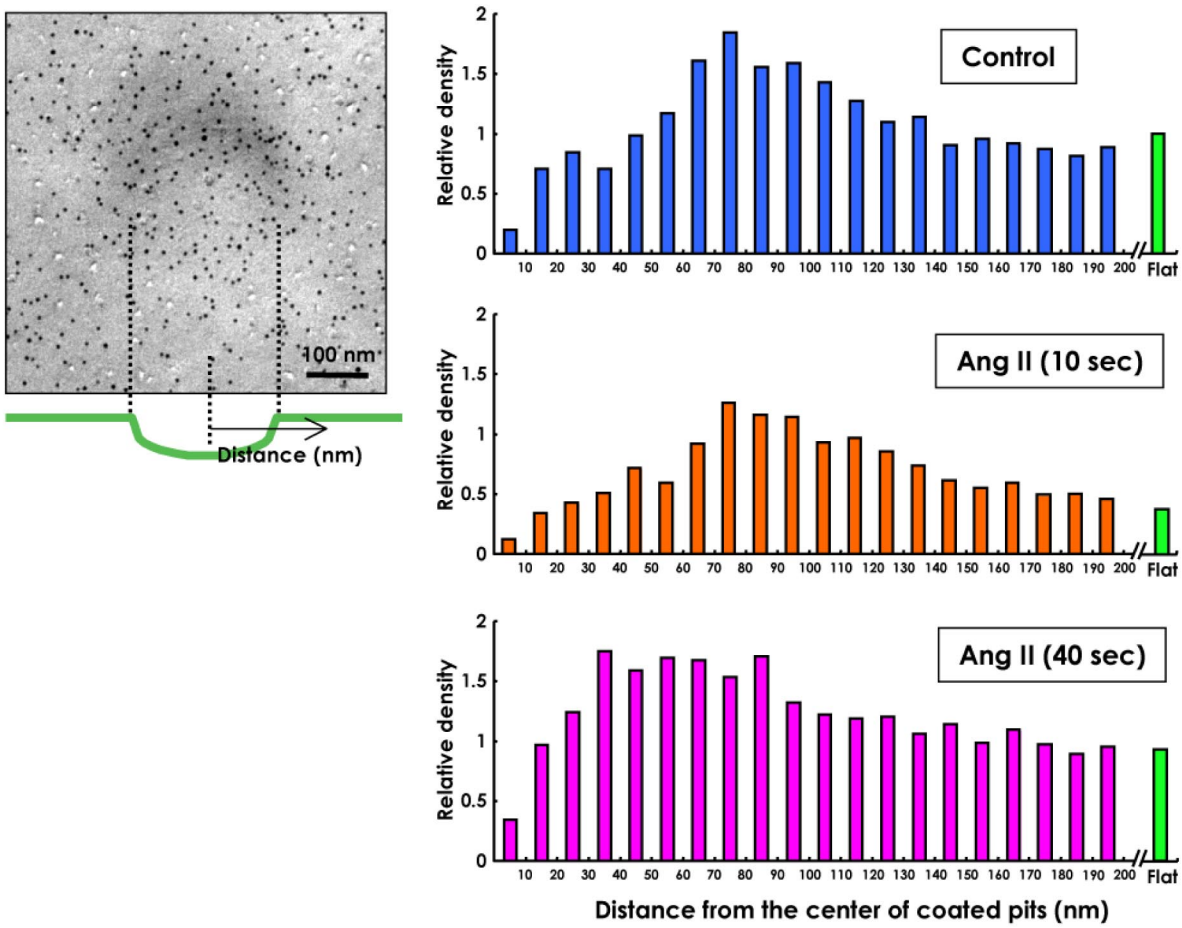

Fig. S5. The labeling of PI(4,5)P_2 in the coated pit. GST-PH was used at a concentration of 100 ng/mL. Distribution of the labeling was measured in relation to the distance from the center of the indentation. The labeling before and at 10 and 40 sec after 1 μM Ang II stimulation was measured for 20 coated pits chosen randomly. The labeling intensity was the highest in the 70- to 100-nm zone from the center. It decreased by about 30% at 10 sec but recovered to the initial level at 40 sec.