Visualizing formation and dynamics of vacuoles in living cells using contrasting dextran-bound indicator: endocytic and nonendocytic vacuoles

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The formation of intracellular vacuoles has been observed by using fixed tissues in a large number of cell types (e.g., hepatocytes, cardiomyocytes, neurons, and different exocrine secretory cell); it reflects wide-ranging pathological conditions and is used as an important indicator of cell damage (5, 10, 23, 24). In particular, the appearance of intracellular vacuoles is hallmark of the morphological changes in pancreatic acinar cells in conditions of acute pancreatitis. These structures were observed in different models of pancreatitis including pancreatic duct obstruction, overstimulation with calcium-releasing secretagogues, and stimulation with bile acids (8, 9, 12, 14–16, 23). In our recent study we have identified aberrant endocytosis as one mechanism for the formation of large intracellular vacuoles in pancreatic acinar cells (18). These vacuoles were visualized by using fluid-phase tracer for endocytosis added to the extracellular solution. The limitation of this technique is that it labels only one specific type of vacuoles. In the present study we decided to utilize contrasting indicator added to the cytosol to visualize, in real time, the formation and dynamics of all large membrane-bounded vacuoles in living cells. We hope that the technique described below will be useful for studies of other cell types in which the formation of intracellular vacuoles reflects important physiological or pathological processes.

MATERIALS AND METHODS

Reagents. Dextran Alexa Fluor 488 10,000 mW, Dextran Texas Red, 3,000 mW, Dextran Cascade Blue 10,000 mW, ER-Tracker Blue-White DPX, MitoTracker Deep Red, and NB-D-C6-ceramide were from Molecular Probes/Invitrogen (Eugene, OR). Collagenase was from Worthington Biochemical (Lakewood, NJ). All other reagents were from Sigma-Aldrich (Gillingham, UK).

Cell isolation and solutions. Mouse pancreatic acinar cells were isolated by collagenase digestion as described previously (22). Pancreata were obtained from adult male mice (CD1) that had been killed by cervical dislocation. In accordance with the Animals (Scientific Procedures) Act of 1986 (UK), training and oversight of procedures were conducted by competent personnel from the University of Liverpool (in compliance with national requirements). The standard extracellular solution used for cell preparation and for perfusion of cells during experiments contained 140 mM NaCl, 4.7 mM KCl, 1.3 mM MgCl2, 1 mM CaCl2, 10 mM d-glucose, 10 mM HEPES (pH 7.4). CCK was added to this solution at concentrations indicated for the specific experiments. Fluorescent probe Dextran Alexa Fluor 488 10,000 mW was added to the extracellular solution to attain a final concentration of 100 µM. The intracellular patch pipette solution contained 120 mM KCl, 10 mM NaCl, 1.5 mM MgCl2, 2 mM MgATP, 100 µM EGTA, and 40 mM HEPES (pH 7.2). NB-D-C6-ceramide was loaded into the cells by incubation with 5 µM of the probe for 15 min at 4°C. The procedure was similar to that described in our previous publication (3). ER Tracker Blue-White DPX was loaded into the cells by incubation with 100 nM of the probe for 20–25 min at 37°C. MitoTracker Deep Red was loaded into the cells by incubation with 100 nM of the probe for 15 min at 37°C. All probes for cellular organelles were removed (by centrifugation) from the extracellular solution prior to the beginning of experiments. Dextran Texas Red 3,000 mW (1 mg/ml) or Dextran Cascade Blue 10,000 mW (1 mg/ml) was added to the patch pipette solution.

Experiments were conducted at room temperature (20–23°C).

Electrophysiological and optical recordings. The whole-cell configuration of the patch-clamp technique was used in this study. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, Kent, UK) and fire polished. The pipettes had a resistance of 3–5 mΩ when filled with the intracellular solution (see Cell isolation and solutions). Further details on patch-clamp protocols used in our laboratory can be found in MATERIALS AND METHODS section of Ref. 2.

Experiments were performed on Zeiss 510 confocal microscope with water immersion objective ×63 numerical aperture 1.2; axial

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resolution was selected at ~1 μm. For excitation of Dextran Texas Red we used a 543-nm laser line; emission was collected in the band 585–615 nm. Dextran Alexa Fluor 488 was excited with a 488-nm laser line and emission collected in the range 505–550 nm. Experiments were conducted in Multi Track configuration (in this configuration the combinations of excitation wavelength and emission filter for specific probes are applied sequentially). NBD-Ce-ceramide was excited with a 488-nm laser line and emission collected between 505 and 552 nm. ER Tracker Blue-White DPX was excited with a 364-nm laser line and emission collected between 475 and 525 nm. MitoTracker Deep Red was excited with a laser line of 633 nm and emission collected with a long-pass filter (650 nm). Dextran Cascade Blue was excited with 357 nm and emission collected between 380 and 430 nm. Four-dimensional imaging of cells was used in some of our experiments to find the region of vacuole formation. In these experiments Z stacks of 10–20 two-dimensional images were collected every 60 s. Two-dimensional images from a specific confocal section were later selected to illustrate the process of vacuole formation.

RESULTS

Visualizing vacuoles using contrasting dextran-bound indicator. The procedure for visualizing the formation of intracellular vacuoles is illustrated in Fig. 1. The vacuole formation is seen as the appearance of a dark area on the background of brightly fluorescent (red color) contrasting indicator (Fig. 1, A and B). The indicator Dextran Texas Red was delivered into the cell via patch pipette (Fig. 1C) in whole cell patch-clamp configuration. Dextran-bound indicator was used in this study to make it more difficult for the probe to cross the membrane of vacuoles. Addition of dextran-bound indicator into the patch pipette does not preclude recording the electrophysiological parameters from the cell; an example of this is the calcium-dependent Cl \(^{-}\) current shown on Fig. 1D. The formation of vacuoles was initiated by supramaximal concentration of CCK (10 or 20 nM). A second indicator, Dextran Alexa Fluor 488, was added together with CCK to the extracellular solution as a probe for fluid endocytosis; note the appearance of fluorescence (green) in the extracellular solution during CCK stimulation (Fig. 1, A and B). Images on Fig. 1A and the profile of fluorescence on Fig. 1B indicate that the large vacuole, formed as a result of CCK stimulation in this specific experiment, does not show fluorescence of Dextran Alexa Fluor 488 and it is therefore of endocytic origin. Figure 1E shows changes of shape of two neighboring nonendocytic vacuoles that could be interpreted as fusion of these structures (Fig. 1E).

Fig. 1. Imaging formation of nonendocytic vacuole. A: images of a cell infused with Dextran Texas Red, before (top row) and after (bottom row) stimulation with 10 nM CCK. Recording started (time 0) ~5 min after establishment of whole cell patch-clamp conditions. Fluorescence of Dextran Texas Red is shown in red. Fluorescence of Dextran Alexa Fluor 488 is shown in green. 3D images of the cell were collected over 60 s. Projection containing large intracellular vacuole, formed following CCK application, is shown. The 360-s image (top right) is the last image collected in this confocal section before CCK application; the 420-s image (bottom left) is the first image collected after CCK application. Profile of fluorescence, plotted in B, was recorded along the white arrow in A (bottom right image). Note the dip in the Dextran Texas Red fluorescence profile (red trace), which corresponds to the vacuole. The profile of fluorescence (here and in other figures) also serves to indicate the size of cells and vacuoles. C: transmitted light image. The patch pipette is visible in the top part of the image. D: calcium-dependent Cl \(^{-}\) current recorded at −30 mV. E: fragment of the cell image (only Dextran Texas Red fluorescence) showing the fusion of a large dark vacuole with a smaller structure.
Combining intracellular and extracellular probes. The extracellular probes were utilized recently by two groups to study exocytosis in living pancreatic acinar cells (11, 20). These investigations observed the formation of large postexocytic \( \Omega \) shapes as a consequence of compound exocytosis. In our previous study we extended this technique to measure endocytosis in the acinar cells and observed the disconnection of postexocytic structures producing large vacuoles (18). Here we combine this method with a new technique for monitoring vacuole formation by using contrasting dextran-bound indicator delivered into the cells via patch pipette.

We found that both nonendocytic and endocytic vacuoles formed in pancreatic acinar cells following supramaximal stimulation with CCK. Combination of intracellular and extracellular fluorescence probes helped us to identify the nature of the vacuoles. For example, in the experiment shown on Fig. 2A a nonendocytic vacuole was formed in the top cell following CCK stimulation, whereas the bottom cell responded by producing an endocytic vacuole of similar size (revealed by internalized fluorescence probe; see also Fig. 2B).

Both nonendocytic and endocytic vacuoles can form in the same cells (Fig. 2C). In this case both types of vacuoles form dark imprints on the brightly fluorescent image of the cell loaded with Dextran Texas Red. The endocytic vacuoles were revealed by the presence of Dextran Alexa Fluor 488. It is interesting to note that the boundary of the image of nonendocytic vacuoles (imprint on Dextran Texas Red fluorescence) was usually sharper than the boundary of endocytic vacuoles.

Observation of vacuole formation following patch-pipette withdrawal. Experiments were also conducted in conditions when the patch pipette was withdrawn following the delivery of the contrasting indicator into the cytosol and the plasma membrane resealed (Fig. 3). In these conditions intracellular pressure changes, which could potentially be induced by changes in the concentration of electrolytes and water, following the stimulation of the cell, cannot be relieved by the patch pipette. The relatively rapid termination of contact between the pipette and the cell interior should also minimize the washout of proteins and other macromolecules from the cytosol. In our experience the probability of successful (without destroying the patched cell) pipette withdrawal increases when the pipette is removed soon (<5 min) after establishing the whole cell configuration. In conditions of our experiments a few minutes

![Image 2](http://ajpgi.physiology.org/)

**Fig. 2.** Visualizing endocytic and nonendocytic vacuoles. **A,** left: transmitted light image of 2 acinar cells (patch pipette is visible in the top part of the image). **A,** middle: nonendocytic vacuole formed in the top cells, visualized with the help of infused Dextran Texas Red (fluorescence of this probe is shown by red color). **A,** right: endocytic vacuole in the bottom cell; green color (here and in C) shows fluorescence of Dextran Alexa Fluor 488, which was added to the extracellular solution with CCK (20 nM). The fluorescence images were recorded following 580 s of CCK stimulation. **B:** overlay and profile of fluorescence. The left image shows overlay of fluorescence of the 2 dextrans (from A). Right: profile of fluorescence recorded along the arrow shown in the image (left). The peak of the fluorescence of Dextran Alexa Fluor 488 (green trace) corresponds to the endocytic vacuole. The dip in the fluorescence of Dextran Texas Red (red trace) corresponds to the nonendocytic vacuole. **C:** 2 types of vacuoles formed in the same cell. Scale bar corresponds to 5 \( \mu \)m. **Left:** transmitted light image of a doublet of pancreatic acinar cells. Top cell was patched and Dextran Texas Red (red color) infused into the cell. Dextran Alexa Fluor 488 (green color) was added into the extracellular solution (together with CCK) and shows endocytic vacuole formed as a result of CCK stimulation (20 nM for 890 s). Overlay of 2 images (right) shows the larger endocytic vacuole and 2 nonendocytic vacuoles above it formed in the same cell.
of pipette-cytosol contact was sufficient to load the dextran-bound indicator. These experiments were conducted on larger clusters. It is known that the morphology of individual pancreatic acinar cells is better maintained in clusters than in isolated cells (13). In these experiments we were also able to observe the formation of intracellular vacuoles (Fig. 3).

Using contrasting indicator for vacuole formation in combination with Golgi, ER, or mitochondrial probes. Nonendocytic vacuoles are frequently formed in parts of the cell that are a few micrometers away from the apical region. One of the organelles present in this region is the Golgi apparatus (3, 19). We found that staining of the NBD-C6-ceramide is compatible with the fluorescence of the contrasting indicator Dextran Texas Red (Fig. 4). We can therefore simultaneously visualize Golgi structures and the formation of vacuoles (note the disruption of Golgi by the growing vacuole; see Fig. 4 and the Supplementary Movie). Previous studies, utilizing electron microscopy, suggested that cytosolic vacuoles form in the vicinity of the Golgi (15, 23); we therefore initially developed a protocol specifically for combined staining of vacuoles and Golgi structures. We later also found combinations of fluorescence-labeled dextran and organelar probes suitable for visualizing vacuoles and the endoplasmic reticulum (ER) (combination of Dextran Texas Red and ER Tracker Blue-White DPX; see Supplementary Fig. 1A) and also vacuoles and mitochondria (combination of Dextran Cascade Blue and MitoTracker Deep Red, Supplementary Fig. 1B).

**DISCUSSION**

Here we have outlined technology for monitoring the formation and dynamics of vacuoles in acinar cells. In the configuration of our experiments, contrasting dextran-bound indicator allowed us to resolve vacuoles that are 0.7 μm in diameter. However, we had difficulty obtaining sufficient contrast for individual secretory granules that are known to be ~0.5 μm (4). The resolution of the new technique, as used in this study, is therefore somewhere between these two values. It is possible that the technique could be further optimized by increasing the concentration of the contrasting indicator and by increasing the intensity of excitation light. Brownian movement of vacuoles/granules (or other organelles) in the cytosol of living cells could be a factor limiting the resolution of this method. Another factor is the nonuniform light-scattering environment of the cytoplasm. For example, the apical part of acinar cells is known to cause strong scattering of visible light (11, 18). Therefore the resolution of the technique could be different in different parts of the cell. Pancreatic acinar cells have well-developed cellular organelles. In the acinar cells of guinea pig pancreas the rough surfaced ER occupies 22% of the acinar cell volume, the mitochondria take up 8.1%, and the Golgi occupies 9.9% of the surface area of cellular membranes (1). It is interesting to note that these organelles do not make a clear imprint on the images of Dextran Texas Red fluorescence. The reason for this is that although the total volume occupied by these organelles is very substantial the individual components are very thin: less than 0.2 μm for ER strands and most of the Golgi cisternae (1, 17). Mitochondria are also less than 0.5 μm in cross section (1, 7). It is possible that small variations of brightness exist over the regions occupied by the organelles but it certainly does not prevent the recordings of vacuole formation (e.g., Fig. 4). Previously, the majority of the studies of vacuole formation in pancreatic acinar cells and other cell types were conducted using fixed tissue. Electron microscopy, visible light microscopy, and fluorescence microscopy were used in these investigations. Although these studies provided remarkable details of the vacuole structure and sug-

Fig. 3. Imaging vacuole formation in a cell following patch pipette withdrawal. A: images of cells before stimulation. The transmitted light image is overlaid with fluorescence image of a cell loaded with Dextran Texas Red. Images were acquired after the patch pipette, used to deliver the indicator into the cell, was withdrawn. B: this image shows the fluorescence of this cell prior to stimulation. C: the same confocal section as in B but following stimulation with CCK (10 nM for 1,480 s). The dark imprint on the red fluorescence image reveals the formation of a nonendocytic vacuole. The fluorescence of Dextran Alexa Fluor 488 (added together with CCK) is shown in green. Note the green area in the right cell, which suggests the formation of an endocytic structure.
gested a plethora of the mechanisms responsible for vacuole formation (9, 12, 15, 23), they lacked the capability to resolve the process of vacuole formation (which can be very fast: \( <60 \) s following stimulation with CCK), the ability to visualize translocation of individual vacuoles, and the dynamics of vacuole interaction with other cellular organelles. The new approach should allow one to visualize transport of individual vacuoles, to study effects of second messengers on the rate of transport and could help to identify molecular motors responsible for this transport. Further refinement of this experimental approach will be necessary to combine all of these with the ability to characterize the origin of vacuoles in live cells. The nonendocytic (dark) vacuoles usually appear in the vicinity of the Golgi apparatus, which could suggest involvement of Golgi components or nearby condensing vacuoles in the formation of these structures. Indeed the notion that large vacuoles, formed as result of caerulein hyperstimulation, look like swollen condensing vacuoles was discussed in a study from Meldolesi’s laboratory (23). The other type of large membrane bounded organelle that drastically increases in number as a result of stimulation with calcium-releasing agonists (6, 23) and is found in the Golgi area (23) is an autophagic vacuole. Both the size and the location of these organelles are similar to that of the dark vacuoles observed in our study. The dark vacuoles could be forming as a result of fusion between different types of organelles; for example autophagic vacuoles have been shown to fuse with late endosomes and lysosomes (21). One cannot also exclude the possibility that the vacuoles are formed by fusion of zymogen granules and lysosomes (15). Combination of optical microscopy, utilizing infused dextran in live cells, followed up by fixation and electron microscopy investigation of the same samples, could be necessary to characterize both the dynamics and the histological nature of the dark vacuoles.

In this study we had to work on a much shorter time scale than in our previous investigation of endocytic vacuoles (18). The reason for this is that cells only rarely survive in patch-clamp experiments beyond 20–30 min. By that time we could indeed observe formation of both nonendocytic vacuoles (which we could not resolve in our previous work because of the absence of a cytosolic marker) and endocytic vacuoles. But the endocytic vacuoles are only in the early stage of their formation and transport and are still clustered in the apical part of the cell. The technology of monitoring the dynamics of both types of vacuoles could be improved by further refinement of the procedure of patch pipette withdrawal; at the moment these experiments are still rather difficult.

In some cells we observed vacuoles immediately following infusion of the Dextran Texas Red (before any stimulation). This prehistory of the vacuole formation revealed by the indicator could identify prior episodes of cell damage that are undetectable by other techniques. The technique there-

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**Fig. 4.** Visualization of a vacuole formation and partitioning of the Golgi apparatus. **A:** top row of images was taken before CCK application, middle row following 50 s of continuous CCK (20 nM) stimulation, and bottom row following 200 s of the CCK stimulation. The vacuole formation is seen as an imprint on the Dextran Texas Red fluorescence (orange). NBD-C₆-ceramide fluorescence (green) highlights Golgi apparatus. Transmitted image of the cells is shown in B. C shows the profiles of Dextran Texas Red fluorescence (orange) and NBD-C₆-ceramide fluorescence (green), recorded along the arrow shown in the image (just above the fluorescence profile).
fore allows one to visualize both already formed vacuoles and de novo generation of vacuoles.

Combination of Dextran Texas Red as a contrasting indicator to visualize vacuoles with NBD-C6-ceramide to label the Golgi apparatus is an example of colabeling of vacuoles and other cellular organelles. We also tested combinations of probes for visualizing vacuoles and ER or vacuoles and mitochondria. The broad range of indicators now available in dextran-conjugated form together with substantial and rapidly increasing range of probes for cellular organelles (including targeted fluorescent proteins) should provide an experimenter with the possibility of visualizing relationships between vacuoles and cellular organelles without major overlap between the spectra of the probes.

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GRANTS

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