RATIOMETRIC IMAGING OF $[Ca^{2+}]_i$ DYNAMICS BY UV-LASER SCANNING CONFOCAL MICROSCOPY IN INDO-1-LOADED RAT PANCREATIC ACINUS

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ABSTRACT

Ratiometric imaging of $[Ca^{2+}]_i$ at a millisecond-order was enabled by confocal UV-laser scanning microscopy in Indo-1-loaded pancreatic acinar cells. Stimulation with 10 pM CCK-8 induced a $[Ca^{2+}]_i$ rise in the cellular margin of acinus, which occasionally accompanied an increase in the luminal area, showing Ca^{2+} wave propagation from the periphery to the core of a cell in the confocal plane. The increase in the luminal region never preceded that in the basolateral region. The $[Ca^{2+}]_i$ gradient was always from the basolateral to the luminal region. The inward spread of Ca^{2+} can be interpreted as follows; Ca^{2+} entry occurs through the entire surface of the basolateral plasma membrane. As acinar cells are geometrically conical, the luminal region forms three-dimensionally smaller cytoplasmic space, whereas the basolateral region consists of much larger space. This may cause distinct late arrival of Ca^{2+} wave at the core of the basolateral region compared with the early arrival at that of the luminal region.

$[Ca^{2+}]_i$ imaging is now a widely used technique to investigate spatial and temporal dynamics of intracellular Ca^{2+} dynamics. Fluorescent Ca^{2+} indicators such as Fura-2, Fluo-3, Indo-1, Fura-red, and Calcium-green are widely used in these days. Among these dyes, Fura-2, Indo-1, and Fura-red have an advantage of possible conversion of fluorescence ratio to cytoplasmic Ca^{2+} concentration (2). Measurements using other indicators are principally qualitative rather than quantitative. Ratiometry using two different dyes such as Fluo-3 and Fura-red is strongly dependent on a prerequisite that both dyes have to be intracellularly distributed equally (15), but the prerequisite is not always fulfilled. Indo-1 is advantageous for measuring rapid $[Ca^{2+}]_i$ dynamics as ratiometry can be done with two emission ranges produced by irradiation with a single excitation wavelength. Thus, measurements are not restricted by mechanical changes such as

chopper exchange to irradiate two different excitation wavelengths which are required for Fura-2 ratiometry. There are a few reports which utilized ratiometric imaging (1, 6) or line scanning (14, 17, 18) by UV-laser confocal microscopy using Indo-1. However, imaging intervals were in the range of several tens seconds or even hours and full imaging was not able to be obtained by line scanning. To overcome these disadvantages, $[Ca^{2+}]_i$ imaging of thin optical slice of the cells became recently possible by development of a UV-laser video-rate scanning confocal microscope and spatial and temporal analyses could be achieved (13). In the present experiments, we report spatial and temporal dynamics obtained every 8 frames averaged at a rate of 30 frames/s in Indo-1-loaded pancreatic acinar cells and demonstrate new aspects of $[Ca^{2+}]_i$ dynamics which were first detected with this device.

MATERIALS AND METHODS

Cell Isolation

Acini were isolated by collagenase digestion according to a method described originally by Hootman *et al.* (7) with slight modification as reported previously (3). Briefly, after cervical dislocation and exsanguination, the tail portion of the pancreas was excised and 2.5 ml of purified collagenase (Worthington Biochemical, Freehold, NJ, U.S.A.) solution (65 units/ml) containing 0.01% trypsin inhibitor (Type 1-S, Sigma Chemical, St. Louis, MO, U.S.A.) was injected. After 60-min digestion, the tissue was mechanically disrupted with a glass pipette, filtered, rinsed three times, and resuspended in fresh HEPES-buffered Ringer solution (HR). The composition of HR was as follows (mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgCl₂, 1.13; NaH₂PO₄, 1.0; D-glucose, 5.5; HEPES, 10. The solution was supplemented with 2 mg/ml bovine serum albumin (Sigma), Eagle's minimal essential amino acids (Flow Laboratories, Irvine, Scotland), and L-glutamine (2.0 mM). The pH of the solution was adjusted to 7.4 with NaOH and the solution was gassed with 100% O₂.

Confocal Imaging of $[Ca^{2+}]_i$ in Indo-1-Loaded Acinar Cells

Isolated acini were incubated in HR containing 20 μ M Indo-1/AM for 40 min at 37°C. More than 20 μ M of Indo-1/AM was required to be able to detect appropriate signal with a good S/N in this preparation because of reasons mentioned in a previous report (5). Indo-1-loaded acini were rinsed and resuspended in HR. An aliquot (100 μ l) of acinar suspension was then transferred into a hand-made chamber (5 mm diameter $\times$ 5 mm depth) at the bottom to which Cell-Tak (Collaborative Research, Bedford, MA, U.S.A.)-coated cover glass was adhered with dental wax and the chambers were left for several minutes until acini were attached to the cover glass. Those chambers were kept in a humidified box to avoid evaporation of HR.

The chamber was set on a stage of an inverted microscope (TMD-300, Nikon, Tokyo, Japan) as described previously in detail (5). The acini were perfused with HR at a rate of 1 ml/min with a peristaltic minipump. An acinus of interest was photographed and irradiated with an excitation UV beam (351 nm) produced by an Argon ion laser at a scanning frequency of 1/30 s (the video rate) and

emitted fluorescence was guided through $\times 40$ water-immersion objective (NCF Fluor N.A. 1.15) to a pinhole diaphragm, separated at 445 nm with a dichroic mirror and each fluorescence intensity (> 445 nm and < 445 nm) was detected with two photomultipliers (RCM 8000, Nikon, Tokyo, Japan). To obtain images with good spatial resolution, integration of images to be acquired had to be set at 8 in this experiment. Thus the time resolution was 8/30 s. Data obtained were digitized with 8 bits resolution and stored into an optical disc as raw data. Each pixel gives a spatial resolution of approximately 0.3 μ m and 512 $\times$ 484 pixels were shown on the monitor. Changes in $[Ca^{2+}]_i$ estimated by ratiometry were displayed with pseudocolour on the monitor. Background fluorescence correction and shading correction were made daily before the experiments to compensate for uneven irradiation through objective lens, which results in inaccurate rationing (13). Autofluorescence was negligible under the present condition. Cell-free *in vitro* calibration was done using 25 μ M Indo-1. The solutions used were previously reported by Williams and Fay (22). The stimulation of the acini were made by changing HR without CCK-8 to the solution with 10 pM CCK-8 (Peptide Institute, Minoh, Japan).

Accumulation of the AM-ester dyes in various cell organelles is under debate. In pancreatic acinar cells, the great concern was whether most of the dye can be trapped in secretory granules. We adopted digitonin permeabilization method (19) to examine this possibility. It was found that weaker permeabilization with digitonin (10 or 20 μ M) abolished more than 93% of Fura-2 fluorescence and only 6.4–0.6% of the dye was thought to be localized in cell organelles (Fig. 1). It was also demonstrated that compartmentalization of Indo-1 was much weaker when compared with that of Fura-2 (data not shown). Therefore, fluorescence signal detected in the present experiments is considered to be produced mostly from the cytoplasm.

Morphological Examination

Morphological examination of the acinus used for $[Ca^{2+}]_i$ measurement was carried out to later assess structural configuration of the acinus used for the experiments. With a diamond marker set in a revolver of objective lenses, a circular scratch was made to a small area of the bottom of cover glass on which acinus selected for the experiment was located. The specimens thus marked were im-

Fig. 1 Changes in cellular fluorescence after digitonin permeabilization in Fura-2-loaded rat pancreatic acinar cells. Fura-2 fluorescence measurements (excitation 360 nm; emission 510 nm) were carried out microfluorometrically with Nikon P1 system before, during, and after digitonin perfusion (20 µg/ml). See Materials and Methods and a previous paper (9) for detail.

mersed in a fixative containing 12.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (0.167 M) saline for overnight at 4°C, and were processed for electron microscopy. After careful trimming, successive sections (1 µm thickness) of a marked acinus were cut, stained with toluidine blue, and examined under a light microscope.

RESULTS

Ratiometry Using Indo-1

Fig. 2A shows an acinus used for the experiment, fluorescent images, and the ratio image resulting from the division of these images obtained during 10 pM CCK-8 stimulation. In acinar cells, the cytoplasmic area may be morphologically distinguished into two regions (8); the basal half region where rough-surfaced endoplasmic reticulum is abundant (ER region) and the luminal half region where many zymogen granules are accumulated (ZG region) (Fig. 2A, PhC). In an acinus selected for the experiment (Fig. 2A, PhC and MS), fluorescence intensities of longer wavelengths (> 445 nm, Fig. 2, Denom) and shorter wavelengths (< 445 nm, Fig. 2, Numer) were low in the ZG region but higher in the ER region at rest and after stimulation. Estimated [Ca²⁺]_i was therefore low in the ZG region but

higher in the ER region (Fig. 2, B and C).

Spatial and Temporal Dynamics of [Ca²⁺]_i Detected with Indo-1

Stimulation with 10 pM CCK-8 induced an increase in [Ca²⁺]_i in the confocal plane of isolated rat pancreatic acinar cells (Fig. 2A, Ratio). Temporal analysis demonstrated that in most cases [Ca²⁺]_i at rest was approximately 100 nM and, upon stimulation, it was increased to 400–500 nM which is compatible with the values obtained previously with Fura-2 (3, 9). The [Ca²⁺]_i increase started first in the basolateral region and the increase in the luminal region followed (Fig. 2B). The [Ca²⁺]_i gradient was thus always from the basolateral to the luminal region. Fig. 2C shows spatial dynamics of [Ca²⁺]_i obtained at every 8/30 s. The increase in the luminal region never preceded that in the basolateral region before reaching a plateau level.

Novel features of [Ca²⁺]_i dynamics were observed with the current technique. First, when the acinus was continuously stimulated with a low concentration of CCK-8 (10 pM), weak but not full recurrent Ca²⁺ tides were observed in the basolateral region. Although these changes could be visualized as small spotty fluctuations by pseudocolour representation on the colour monitor, they were too faint to be detected by temporal analysis. This was because window size, with which the program averages pixel values of [Ca²⁺]_i, has some limitation to be minimized and this reduction of the size to the limit gave a decrease of S/N, producing noisy fluctuations superimposed on these faint changes. Second, when the acinus was continuously stimulated with 10 pM CCK-8, the [Ca²⁺]_i increase started from the narrow cellular periphery of the confocal plane and propagated towards the cellular core (inward spread), producing a [Ca²⁺]_i increase on the cell border (Fig. 3A, arrows). Occasionally the [Ca²⁺]_i increase in the luminal region followed that in the basolateral periphery after a short time delay. The Ca²⁺ wave therefore spreads from the outer surface of the cell to the inner cytoplasm. When the secretagogue concentration was increased to a pharmacological dose (100 pM), the spatial gradient became less defined (Fig. 3, B and C).

Strong Irradiation Caused a [Ca²⁺]_i Increase

Current experiments also showed that strong irradiation of excitation beam itself induced an increase in [Ca²⁺]_i as an artifact, which is most likely a

Fig. 2

result from photodamage to the plasma membrane. This view was supported in an experiment without any secretagogues but with irradiation of strong beam power. Actual irradiation strength depends on various factors such as optical components. The ratio values of all area of the selected acinus were low just after exposure to excitation beam. The half area of the acinus was then continued to be exposed to the excitation with no addition of secretagogues, while the rest half was masked by a metal shutter. The ratio values in the exposed area gradually or abruptly increased as strong irradiation was continued but those in the masked area remained unchanged when examined by unmasking the area. Thus the photodamage caused an artificial and irregular increase in [Ca²⁺]_i. Laser power which brings about photodamage to the specimen varies from preparation to preparation and, in the case of acinar preparation, such power strength on the specimen was measured and estimated to be more than 1.5–3 mW. Combination of photodamage and unavoidable photobleaching will be problematic for such dyes that fluorescence decreases as [Ca²⁺]_i increases, because the fluorescence decrease due to both photobleaching and [Ca²⁺]_i increase or either of those cannot be distinguishable. Fura-red is one of examples.

DISCUSSION

Previously, Ca²⁺ imaging carried out by conventional microscopy (3, 8–11, 20, 21) and by con-

focal laser microscopy (12, 16) led to conflicting interpretations for the direction of Ca²⁺ wave propagation in pancreatic acinar cell; namely basolateral-to-luminal or luminal-to-basolateral (4). As was clearly shown, the basolateral-to-luminal gradient was reproduced in the present experiments. However, the spatial gradient became unclear when the secretagogue concentration was increased to a pharmacological dose, which indicates that the direction of gradient depends on secretagogue concentration (9).

As pointed out by others using Fura-red (12), fluorescence intensity in the granular region was very low. Possible reasons for this would be: 1) uptake of the dye may be low in this region, 2) diffusion of the dye may be slow or restricted probably because of the presence of abundant zymogen granules, and 3) inner filter effect which may prevent both irradiation of excitation and emission of fluorescence. Whatever the causes are, changes in this area may not be very accurately detected because of low S/N. Thus, faint and noisy fluctuations of fluorescence intensity of numerator against weak fluorescence value of denominator in this area can automatically be misinterpreted as significant changes in [Ca²⁺]_i.

The periphery-to-core propagation of Ca²⁺ in the confocal plane can be interpreted as immediate induction of Ca²⁺ entry through the entire basolateral plasma membrane except for the luminal plasma membrane that is separated with junctional complexes (Fig. 4). The plasma membrane of acinar cell may be distinguished into two functionally and biochemically different parts; the basolateral membrane which faces the interstitial fluid and the luminal membrane where exocytosis occurs. The basolateral membrane has receptors for secretagogues, ion channels, carriers, transporters, and pumps. The luminal membrane is never exposed to the interstitial fluid. If we assume that acinar cells are geometrically conical, the luminal region of each cell may form three-dimensionally smaller cytoplasmic space, whereas the basolateral region consists of much larger space. Due to this geometrical feature, the distance between the basolateral plasma membrane where Ca²⁺ entry occurs and the optically sliced confocal plane is longer in the basolateral region but shorter in the luminal region. This may cause distinct late arrival of Ca²⁺ wave at the core of the basolateral region compared with the early arrival at the core of the luminal region. The cellular periphery of confocal image is undoubtedly the cytoplasm which is adjacent to the

Fig. 2 Spatial and temporal dynamics of [Ca²⁺]_i detected by real-time (8/30 s interval) ratiometry using Indo-1. (A) PhC: A phase contrast image of freshly isolated rat pancreatic acinus selected for the experiment. Granular regions were visible. MS: A light micrograph of section (1 μm) of the same acinus which was stained with toluidine blue. Six cells were detected in this section. Bar, 20 μm. Denom: An image of Indo-1 fluorescence detected at longer wavelengths (> 445 nm). Numer: An image of Indo-1 fluorescence detected at shorter wavelengths (< 445 nm). Ratio: Pseudocolour display of ratio image calculated from *Denom* and *Numer* after shading correction. (B) Temporal changes in [Ca²⁺]_i analysed at two different regions of confocal plane of a cell indicated by squares in PhC (■, basolateral; □, luminal regions). This drawing shows a part (0–66.7 s) of results of the entire experiment (total of 87 s). Arrows indicate the onset of [Ca²⁺]_i rise. (C) Spatial and temporal changes in [Ca²⁺]_i in the confocal plane. These images show changes at an interval of 8/30 s from 56.7 s to 72 s of B.

Fig. 3

Fig. 4 Possible explanation for spatial and temporal dynamics of [Ca²⁺]_i deduced from the present results. This model shows three-dimensional [Ca²⁺]_i dynamics in a pancreatic acinar cell. The change in [Ca²⁺]_i was detected in a confocal plane by optical sectioning as indicated with a blue plane. Upon stimulation, Ca²⁺ entry (arrows) through all the basolateral plasma membrane may cause a [Ca²⁺]_i increase in the peripheral region of the cell. As the acinar cell is solid-geometrically conical, the distance between the plasma membrane and the confocal plane, where [Ca²⁺]_i is measured, is longer in the basal region and shorter in the luminal region, producing inward propagation of Ca²⁺ as noted in the text. JC, junctional complexes

basolateral plasma membrane. Thus the [Ca²⁺]_i increase first occurs in these regions as Ca²⁺ entry occurs through this boarder during stimulus-secretion coupling induced by physiological concentrations of secretagogues.

This work was in part supported by The Pancreatic Disease Research Foundation.

Received 11 October 1995; and accepted 16 November 1995

Fig. 3 Spatial and temporal dynamics of [Ca²⁺]_i in acinar cells. (A) Peripheral-to-core propagation of Ca²⁺ induced by 10 pM CCK-8 selected and enlarged from Fig. 1C. Arrows indicate peripheral increase in [Ca²⁺]_i. (B) Spatial and temporal changes in [Ca²⁺]_i stimulated with 100 pM CCK-8. The basolateral-to-luminal [Ca²⁺]_i gradient became unclear compared with the gradient produced with 10 pM CCK-8 (Fig. 1). (C) Temporal changes in [Ca²⁺]_i detected at two different regions (basolateral and luminal regions) in a cell. Arrows indicate the onset of [Ca²⁺]_i rise.

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