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Fluorescence digital image analysis of the inositol trisphosphate-mediated calcium transient in single permeabilized parietal cells

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The *myo*-inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} mobilization in single saponin-permeabilized and fura-2-loaded parietal cells was analysed by a fluorescence digital image processor. When the cells were incubated with ATP, free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) increased in some restricted cytoplasmic regions showing discontinuous plateau and in the peripheral cytoplasm showing continuous $[Ca^{2+}]_i$ gradient towards the plasma membranes. When treated with IP_3, the high plateau enlarged to the entire cytoplasm and (a) new higher plateau(s) appeared and enlarged again in a transient manner. The IP_3-induced Ca^{2+} transient was also observed by fluorescence microphotometry of the single cells or by fluorescence spectrophotometry and $^{45}Ca^{2+}$ uptake experiment of the cell population.

Fluorescence digital imaging; Ca^{2+} transient; Inositol trisphosphate; Permeabilized cell; (Parietal cell, Guinea pig)

1. INTRODUCTION

myo-Inositol 1,4,5-trisphosphate (IP_3) is known to be an essential cellular second messenger in many cells [1] and triggers Ca^{2+} release from intracellular Ca^{2+} pool, perhaps located in non-mitochondrial compartments such as endoplasmic reticulum and sarcoplasmic reticulum [1–3]. In the gastric parietal cells, an ATP-dependent and IP_3-sensitive Ca^{2+} pool is presumably located in smooth surfaced vesicles [4,5].

The IP_3-mediated Ca^{2+} release has been studied by the permeabilized cell population by fluorescence spectrophotometry or radioactive Ca^{2+}. In an effort to understand the effect of IP_3 on the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) in single gastric parietal cells, fluorescence digital image of the cells previously loaded with Ca^{2+}-sensitive dye fura-2 was analysed to measure the spatial distribution and temporal changes of $[Ca^{2+}]_i$.

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Abbreviations: fura-2, 1,(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylpheoxy)ethane-*N,N,N′,N′*-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid

2. MATERIALS AND METHODS

2.1. *Preparation of gastric glands and parietal cells*

The gastric glands were prepared from guinea pig (Hartley, male, 250 g) as described [6,7]. The mucosal cells were dissociated from glands and fractionated by Percoll density gradient ultracentrifugation (30000 × *g* for 15 min at 4°C) [8,9]. A fraction enriched in parietal cells was recovered at a density region from 1.043 to 1.050 g/ml. The proportion of parietal cells in this fraction was 87 ± 2% (six determinations).

2.2. *Fura-2 loading*

Parietal cells (10^9) were suspended in 10 ml of a tissue culture medium Medium-199 (Nissui, Japan) containing 10 mM Mops and 25 mM $NaHCO_3$ (pH 7.4) and were loaded with fura-2 by incubating the cells with 20 μl of fura-2 acetoxymethyl ester (1 mM stock solution, Dojindo, Japan) and 10 μl of 25% (w/w) Pluronic F-127 (BASF, Wyandotte, USA). After constant gassing with 95% O_2/5% CO_2 for 15 min at 37°C [9,10], the cell suspension was rinsed twice, resuspended and kept in the fresh Medium-199 at 4°C.

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Just before use, parietal cells (10^7) were resuspended in 10 ml of the 'cytosol buffer' with the following composition: 20 mM NaCl, 150 mM KCl, 5 mM $MgSO_4$, 0.2 mM KH_2PO_4, 0.8 mM K_2HPO_4, 0.49 mM $CaCl_2$, 1 mM EGTA, 25 mM $NaHCO_3$ and 5 mM glucose in 10 mM Mops-Tris buffer at pH 7.2. The Ca^{2+} concentration was fixed at 180 nM [11]. The culture medium also contained 1% bovine serum albumin, 10 μg/ml oligomycin, and ATP-regenerating system (5 mM creatine phosphate and 50 μg/ml creatine phosphokinase). To this cell suspension saponin was added at a final concentration of 75 μg/ml and incubated for 20 min at 37°C. The cells were rinsed and resuspended in the cytosol buffer in the absence of saponin [5]. For digital image analysis, parietal cells (10^5 cells/100 μl) in the cytosol buffer were layered on a cover-glass slip (thickness <0.12 mm) previously coated with Cell-Tak (Cosmo-Bio, Japan), an adhesive protein of the marine mussel, *Mytilus edulis* [12], and mounted in a Rose chamber (Ikemoto, Japan) and refilled with 2.4 ml of the cytosol buffer containing 1 mM ATP. Saponin and some reagents were added by a microforge manipulator.

2.3. *Digital image analysis*

Digital image processing was carried out as described [18–20] with some modifications. The fura-2 loaded parietal cells were layered in the chamber and put on the microscope stage previously warmed up to 37°C by a thermostatted heater stage. The microscopic system consisted of an Olympus IMT-2-RFL (Olympus, Japan) with DAPO 100× UV (NA 1.3) objective. Cells were excited by ultraviolet light at 340 nm and 380 nm through narrow bandpass filters (band width 10 nm), 25% transmission neutral density filters, and a 455 nm dichroic mirror. The sequential image was collected through a single broad band pass filter (510 nm, band width 30 nm). A DC-stabilized high pressure mercury lamp (HBO 100W/2) was fitted with a computer-associated excitation filter changer. Video images were acquired by a silicon-intensified target camera (SIT camera, CTC-9000, Ikegami, Japan). The high voltage and gain of the camera were kept under manual control. The analogue output was fed either directly into an image display or indirectly into a high vision VTR (S-VHS video, Toshiba, Japan) equipped with a video timer. The output was digitized to a resolution of 512 × 480 pixels (× 8 bit) by a color image analyzer CIA-102 (Olympus) with 2 M byte frame memory (A/D convert). Images were integrated (16 bits) to improve S/N ratio and calculated to 340–380 nm ratio image on the image analyzer using a host computer HP-310 (Hewlett Packard, USA), and were reconverted to the analogue signal on the image display with black and white or pseudo color. The p*Ca* of the cells was converted from 340/380 nm fluorescence ratio using a software-associated fluorescence microphotometer (IMT-2-OSP, Olympus) interfaced to photometry control units (OSP-CBI and OSP-OPU, Olympus). These images were constructed as a three dimensional plot of the 45° angular polygons by the 7475 A graphics plotter (Hewlett Packard) according to our software system. Background images, presumably due to leak of cell-associated fura-2 after saponin-permeabilization, were subtracted from the cell images. Autofluorescence of the parietal cells was negligible. The $[Ca^{2+}]_i$ calculated from the ratio measurement was independent of cell thickness, microscopic optics and illuminations [14].

2.4. *Ca^{2+} measurement by fluorescence spectrophotometry*

Isolated and saponin-permeabilized parietal cells (10^6) were resuspended in 2 ml of the cytosol buffer in a cuvette. The fluorescence was recorded by a Hitachi 650-60 fluorescence spectrophotometer (Hitachi, Japan) as described [9,10] according to the methods of Tsien et al. [13] and of Grynkiewicz et al. [14].

2.5. *Ca^{2+} measurement by isotope $^{45}Ca^{2+}$*

The permeabilized cells were incubated for 20 min at 37°C with 1.0 μCi of $^{45}Ca^{2+}$ (25 mCi/mg, New England Nuclear, USA) in the presence of 1.0 mM ATP, then IP_3 (5 μM) or Ca^{2+} ionophore ionomycin (1 μM) was added. The incubation was stopped by adding 2 ml of the same cytosol buffer containing 2 mM EGTA but no $^{45}Ca^{2+}$. The solutions were filtered through Millipore filters under mild suction [4,5]. The β-ray radioactivity remaining on the filter pads was counted as described [4,5].

2.6. *Materials*

The sources of some of the reagents mentioned above have been given [4–10]. Instruments equipped with a digital image processing system have been described in section 2.3.

3. RESULTS

Fig.1 shows three dimensional mapping of the fura-2-loaded single gastric parietal cells intensified by a digital image processing microscope. Vertical heights at each pixel in these images are proportional to the p*Ca* of the cells which was calculated from the 340/380 nm fluorescence ratio of fura-2 acid by fluorescence microphotometry in vitro in Mops-Tris buffer (160 mM KCl, 20 mM NaCl, 5 mM $MgSO_4$, 0.2 mM KH_2PO_4, 0.8 mM K_2HPO_4, 1 mM ATP in 10 mM Mops-Tris buffer, pH 7.2). This buffer also contains various concentrations of Ca^{2+} (p*Ca*; from 5.5 to 8.0) which was prepared by Ca^{2+}/EGTA buffer at 24°C according to the method of Fabiato and Fabiato [11] as modified by Oiki and Okada [20]. Before permeabilization of the cells with saponin, $[Ca^{2+}]_i$ was almost homogeneous except for the peripheral cytoplasm just beneath the plasma membranes (fig.1A).

When the cells were treated with saponin for 10 min in the presence of ATP and ATP-regenerating system, $[Ca^{2+}]_i$ increased uniformly in some restricted cytoplasmic area(s), thus creating (a) discontinuous $[Ca^{2+}]_i$ plateau(s) (p*Ca* 6.9) (fig.1B). This discontinuous pattern is unlikely to be due to an artifact produced by digital image processing because continuous $[Ca^{2+}]_i$ gradients still exist at the peripheral cytoplasm just beneath

the plasma membranes. We could not, however, identify the precise location of the plateau area(s) in the cell.

When the cells were stimulated with IP_3, the above $[Ca^{2+}]_i$ plateau(s) enlarged to the almost entire cytoplasm and a new higher $[Ca^{2+}]_i$ plateau(s) (p*Ca* 6.6) was created within 10 s as shown in fig.1C, which again rapidly enlarged towards the plasma membranes (fig.1D–F).

Individual parietal cells, when treated with IP_3, responded with maximally 1.75-fold or 5.03-fold increase in $[Ca^{2+}]_i$ in the average and in the restricted region(s), respectively. These results were obtained by both a software-associated fluorescence microphotometer and a pixel for pixel map interfaced to the computer. At 40 s after IP_3 stimulation, the lowest levels of $[Ca^{2+}]_i$ was 50 nM (p*Ca* 7.3), the first, second and third plateau levels were 126 nM (p*Ca* 6.9), 251 nm (p*Ca* 6.6) and 316 nM (p*Ca* 6.5), respectively.

The IP_3-induced $[Ca^{2+}]_i$ increase was brief, lasting approximately 60 s and the high $[Ca^{2+}]_i$ plateau levels returned to the original level throughout the cytoplasm, leaving the first $[Ca^{2+}]_i$ plateau(s) in some restricted regions of the cytoplasm. This process may reflect the Ca^{2+} re-uptake into the store(s).

Fig.2A–C shows the ATP-dependent Ca^{2+} uptake into the store(s) and release of accumulated Ca^{2+} by IP_3 or Ca^{2+} ionophore ionomycin treatment as measured by the microphotometer in single cells (A), by the fluorescence spectrophotometer in the cell population (B), and by radioactivity measurement in the cell population (C), respectively. In fig.2A–C, there was a rapid uptake of Ca^{2+} by permeabilized cells following addition of ATP. This Ca^{2+} uptake into the cells may reflect Ca^{2+} removal by non-mitochondrial system(s) such as smooth surfaced membrane vesicles, because this process was insensitive to mitochondrial uncouplers such as antimycin and oligomycin and was quantitatively similar to that taken up by sub-microsomal fractions [4,5]. Addition of IP_3 resulted in a rapid release of Ca^{2+} into the cytoplasm followed by a re-uptake of Ca^{2+} into the ATP-dependent pool(s) in the presence of ATP-regenerating system, resulting in a transient increase in fluorescent signal of fura-2 or in a transient decrease of cell-associated $^{45}Ca^{2+}$. The Ca^{2+} ionophore ionomycin also caused a release of Ca^{2+} from the store(s). In the latter case, however, re-uptake of Ca^{2+} was not observed because of marked increase in permeability of the membranes to Ca^{2+} by the ionomycin treatment [4].

4. DISCUSSION

The Ca^{2+} cycling in the gastric parietal cells appears to proceed in the following way [4–6,15,16]. Step 1: the IP_3-mediated Ca^{2+} release from the store(s), presumably smooth surfaced vesicles in the apical cytoplasm. Step 2: the Ca^{2+} efflux from the cell by a calmodulin-regulated pump or Na^+-Ca^{2+} antiporter. Step 3: local but prolonged Ca^{2+} entry from the extracellular space and the following Ca^{2+} efflux from the cell (local Ca^{2+} cycling). Step 4: the ATP-dependent Ca^{2+} re-uptake into the deleted intracellular pool(s) by the Ca^{2+} entering from outside of the cells. This process may be enhanced by hormone dissociation from the receptor [15,16].

The present experiments using the permeabilized cells eliminate steps 2 and 3 and simplify the analysis of the IP_3-induced Ca^{2+} release from an ATP-dependent Ca^{2+} re-uptake to the Ca^{2+} store(s). Furthermore, the digital image processing can provide the information on the IP_3-induced $[Ca^{2+}]_i$ change in the single cells.

When treated with IP_3, about 40% of the parietal cells in the gastric glands responded as described in section 3. In marked contrast, all of these cells responded to Ca^{2+} ionophore ionomycin. This result suggests that the responsibility of each parietal cell to IP_3 shows marked heterogeneity.

We found that the distribution of $[Ca^{2+}]_i$ in the cytoplasm of the saponin permeabilized and ATP-treated parietal cells reveals marked heterogeneity and discontinuity, showing plateaus with high $[Ca^{2+}]_i$ in the restricted region(s) of the cytoplasm. When the single permeabilized cells were treated with IP_3, the $[Ca^{2+}]_i$ plateau region(s) enlarged towards the plasma membranes and new higher plateau regions successively appeared and enlarged. This discontinuous distribution pattern is in marked contrast to the continuous increase in $[Ca^{2+}]_i$ in the peripheral cytoplasm towards the plasma membrane. At present it is not yet clear why and how such discontinuous $[Ca^{2+}]_i$ distribu-

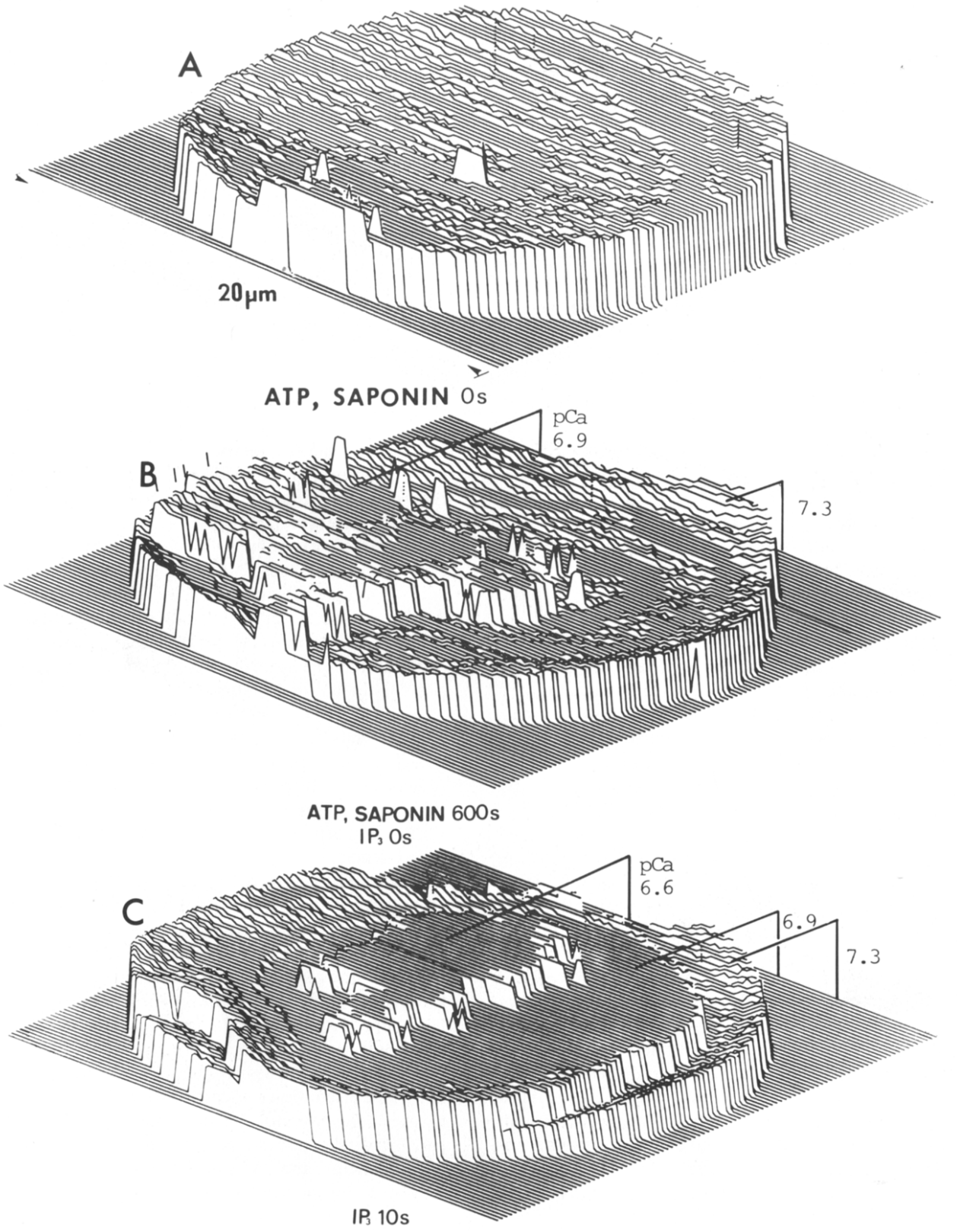

Fig.1. For legend see p. 34.

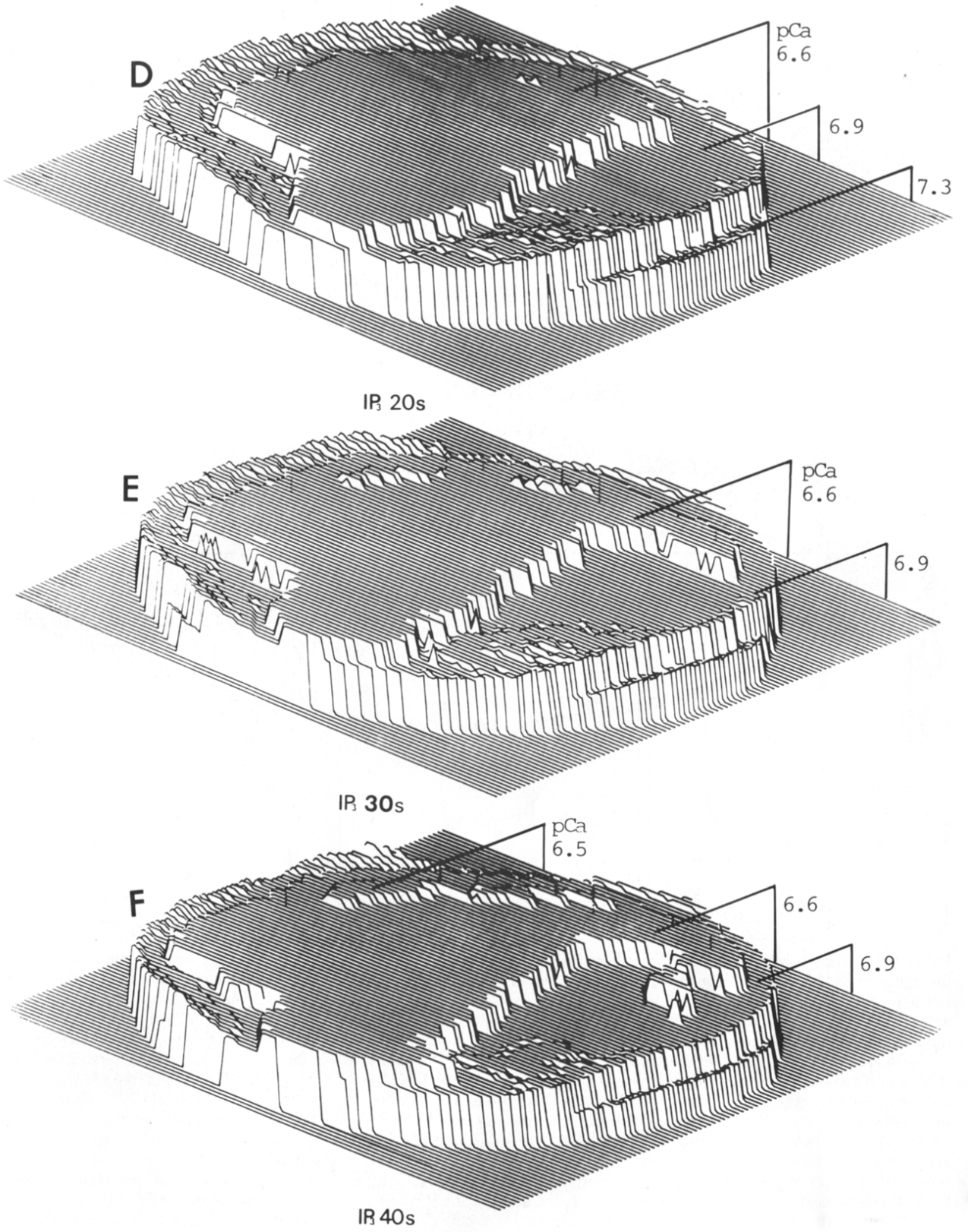

Fig.1. For legend see p. 34.

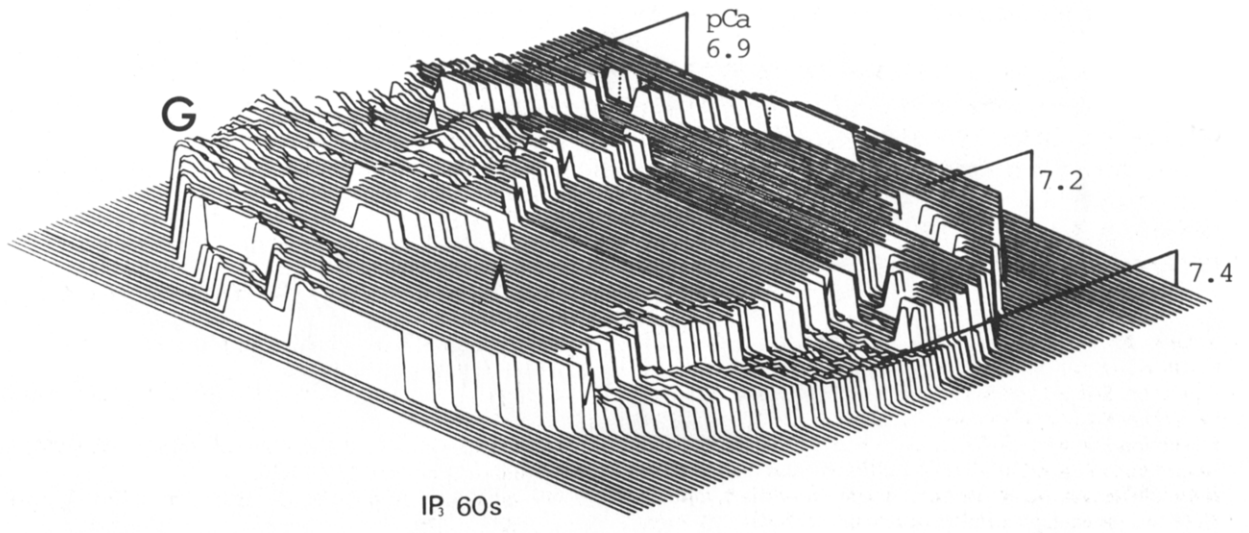

Fig.1. Computer graphics of the spatial and temporal changes in $[Ca^{2+}]_i$ induced by IP_3 in saponin-permeabilized and ATP-supplied single parietal cells. The concentrations of the reagents used in this study were similar to those in fig.2. The data are representative of 3 independent experiments.

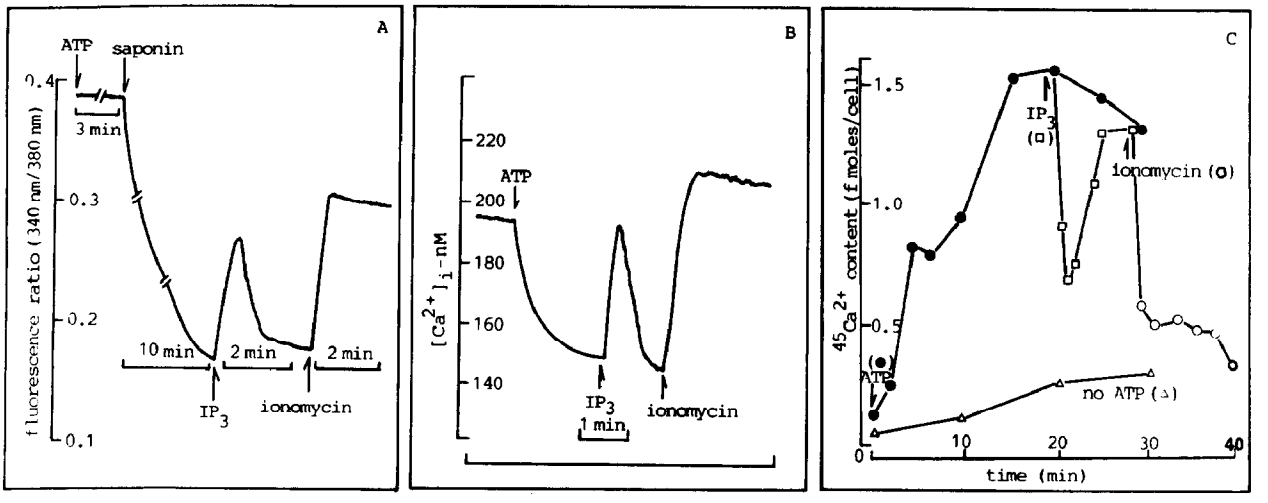

Fig.2. Ca^{2+} released by IP_3 or Ca^{2+} ionophore ionomycin in saponin-permeabilized parietal cells measured by a fluorescence microphotometer (fura-2) in single cells (A), by a fluorescence spectrometer (fura-2) in the cell population (B), or by $^{45}Ca^{2+}$ in the cell population (C). (A) IP_3 transiently increased $[Ca^{2+}]_i$. In marked contrast, ionomycin irreversibly led to a 1.94-fold increase in average $[Ca^{2+}]_i$ and all of the $[Ca^{2+}]_i$ gradients within the cells dissipated. The fluorescence ratio (340/380 nm) corresponds to pCa in the following way: ratio $R = 0.2$, p$Ca = 7.5$; $R = 0.4$, p$Ca = 7.2$; $R = 0.6$, $p_{Ca} = 6.9$; $R = 0.8$, p$Ca = 6.6$; $R = 1.0$, p$Ca = 6.5$. The data are average $[Ca^{2+}]_i$ of three associating cells. Only one cell responded to IP_3 in this case, as shown in fig.1. (B) The resting $[Ca^{2+}]_i$ in the saponin-permeabilized cell population (10^6 cells) was 203.7 ± 32.7 nM ($n = 3$) in the presence of 180 nM Ca^{2+} in the outer environment. ATP caused a substantial decrease in $[Ca^{2+}]_i$ that reached 145 nM. IP_3 led to an increase in $[Ca^{2+}]_i$ up to 200 nM in a transient manner. In contrast, ionomycin led to an irreversible increase in $[Ca^{2+}]_i$ up to 215 nM. (C) The quantity of $^{45}Ca^{2+}$ taken up by ATP treatment into the saponin-permeabilized cells in cell population (10^6 cells) was 1.58 ± 0.42 fmol/cell ($n = 4$), which corresponds to 0.2 nmol $^{45}Ca^{2+}$/mg protein. IP_3 led to 53% loss of cellular $^{45}Ca^{2+}$ within 1 min in a transient manner and to a re-uptake of $^{45}Ca^{2+}$ released from the store(s). Ionomycin caused a substantial release of ATP-accumulated $^{45}Ca^{2+}$ reaching to the level of ATP-independent $^{45}Ca^{2+}$ uptake. In figs 1 and 2, the concentrations of reagents used were as follows: ATP, 1.0 mM; saponin, 75 μg/ml; IP_3, 5 μM; ionomycin, 1 μM. Each datum (A, B and C) is a mean of at least three independent experiments.

tion is created, maintained, and enlarged in the cytoplasm.

The IP_3-induced Ca^{2+} transient was also obtained by fluorescence microphotometry of the single cells or by fluorecence spectrophotometry and $^{45}Ca^{2+}$ uptake experiment of the cell population. This study will provide valuable information on IP_3-mediated spatial and temporal $[Ca^{2+}]_i$ changes in parietal cells of the gastric gland.

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