Receptor-mediated calcium signal playing a nuclear third messenger in the activation of antigen-specific B cells

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ABSTRACT We have studied receptor-mediated calcium signals in antigen-specific B cells (trinitrophenol-specific B cell clone, TP67.21) using a confocal fluorescence microscope with an argon ion laser (488 nm) and a He-Cd laser (325 nm). Confocal fluorescence images of fluo-3 loaded B cells, excited by an argon ion laser, became much brighter and more nonhomogeneous than those before antigen stimulation. Time-dependent fluorescence changes in intensities were abrupt and quite similar to the patterns of the intracellular calcium ion concentration $[Ca^{2+}]_i$ observed by a conventional fluorescence microscope using fura-2. From the morphological patterns of the calcium images, the parts of the bright fluorescence seemed to belong to the nucleus in B cells. To confirm the above events we measured the confocal fluorescence images of the nucleus. From the fluorescence images of co-loaded Hoechst 33342 (a DNA-specific fluorescent probe), which excited by a He-Cd laser, the brighter parts of the fluo-3 fluorescence intensities were identified to the nucleus in B cells. This suggested the possibility that the increased intranuclear calcium ions may play a nuclear third messenger in B cells.

INTRODUCTION

In virtually all system in which ligand binding to receptor induces increased polyphosphoinositide hydrolysis, ligand binding also induces a rapid increase in intracellular free calcium levels (1-3). However, inositol trisphosphate receptors are suggested to play a key role in the coupling between cytoplasmic second messenger and nuclear third messenger triggered events (4-8). This suggests the possibility that the receptor-mediated calcium signals may transfer not only to the cytoplasm but also to the nucleus (3, 7).

The intracellular free calcium concentrations have been measured by fluorescent calcium ion indicators. In addition, recently, a digital imaging fluorescence microscope together with use of the fluorescent calcium indicators has enabled us to measure the intracellular free calcium concentration in an individual cell (9-12). However, a conventional fluorescence microscope is not suited to study the heterogeneity of the intracellular calcium distribution because of its poor spatial resolution (13, 14). Compared to the conventional light microscopy, confocal scanning microscopy has a more superior capability for directly visualizing the three-dimensional structure inside cells. Thus, we have tried here a single cell to study the calcium signals in antigen specific B cell clones using a confocal fluorescence microscope with an argon ion laser (488 nm) and a He-Cd laser (325 nm).

Then, membrane forms of immunoglobulin (mIg) serve as antigen receptors on B cells. Antigen binding to the mIg or cross-linkage of the mIg on B cells has been shown to induce an increase in intracellular free calcium ion concentration together with an increase in inositol phospholipid metabolism (15-18). In this paper, we suggested the possibility that antigen binding to B cell receptors transferred calcium signals not only to the cytoplasm but also to the nucleus.

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MATERIALS AND METHODS

Materials

Fluo-3-AM, fura-2-AM and Hepes were obtained from Dojindo (Kumamoto, Japan). Hoechst 33342 (bisbenzimide H 33342) was obtained from Calbiochem (La Jolla, CA). TMA-DPH (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene) was obtained from Molecular Probes (Eugene, OR). RPMI 1640 was from Gibco (Grand Island, NY). Preparation of trinitrophenol-conjugated ovalbumin was followed in our previous paper (11). In our present experiments, an average of 21 trinitrophenyl groups was conjugated with ovalbumin.

Cell line

Antigen-specific B cell clones (TP67.21) directed against 2,4,6-trinitrophenyl (TNP) hapten were established in our previous paper (19). They were maintained in a culture medium containing RPMI 1640 supplemented with 5% FCS (fetal calf serum), penicillin G (100 U/ml), streptomycin (100 μg/ml), and 2-mercaptoethanol (50 μM).

Instrumentation

Confocal fluorescence microscopic images of B cells were taken under a confocal scanning fluorescence microscope system (MRC-600; Bio-Rad, Cambridge, MA) with an inverted epifluorescence microscope (Nikon TMD-EFQ; Tokyo) (7). A pinhole aperture in the detection path of the confocal microscope was 3 mm and we used a glycerine-immersed 1.4 NA (numerical aperture) 60× Nikon Planapo and a 0.85 NA 40× Nikon Fluor objectives. The temperature of the observation chamber was controlled at 37°C. A conventional digital imaging fluorescence microscope was used in combination with an inverted epifluorescence microscope (Nikon TMD-EFQ) with a silicon intensified target TV camera (Hamamatsu Photonics 2400-08; Hamamatsu) and a U-matic video tape recorder (Sony, Tokyo). The fluorescence images were analyzed with a digital image processor (Image Sigma II and TVIP-2000; Avionics, Tokyo), which was controlled by a microcomputer (NEC PC-9801VX) (11).

RESULTS

Confocal fluorescence images of calcium signals

First fluo-3 loaded B cells (TP67.21) were stimulated with antigen (trinitrophenol-conjugated ovalbumin, final 2 μg/ml). The confocal fluorescence images of individual B cells were observed using an argon ion laser (488 nm). The fluorescence intensity of individual B cells increased after short lag times. Many of the cells (TP67.21) responded to trinitrophenol-conjugated ovalbumin and increased their fluo-3 fluorescence intensities as shown in Fig. 1. Addition of 0.2–4 μg/ml of trinitrophenol-conjugated ovalbumin induced similar fluorescence intensity changes in B cells. In a control experiment, we added ovalbumin (without trinitrophenol groups) to B cells; no significant fluorescence intensity increase was observed as shown in Fig. 1 *e*. A typical example of the time-dependent confocal fluorescence images of fluo-3 loaded B cells is shown in Fig. 2 *a*. Here, the sequential fluorescence images in an antigen-stimulated B cell are shown (*top row,* left to right). Fluo-3 fluorescence images were collected by 8-s intervals. These sequential fluorescence images corresponded to the cell whose time course was shown in Fig. 1 *a*. Fluorescence pseudo-images of B cells showed that the fluorescence intensities before antigen stimulation were dim and inhomogenious ones. After the stimulation, the fluorescence intensities became much greater and more nonhomogeneous, suggesting the increases of intracellular calcium ion concentration in B cells (see Fig. 2 *a*).

At the present time it is difficult to determine directly the intracellular free calcium ion concentration $[Ca^{2+}]_i$ from the fluo-3 fluorescence intensities by confocal fluorescence microscopy. However, the time-dependent profiles shown in Fig. 1 resembled closely the profiles of the fura-2 fluorescence ratio images (excitation at 340 nm or 360 nm, emission at 500 nm) for the $[Ca^{2+}]_i$ which were measured in the same condition by a conventional fluorescence microscope. These results supported the idea that the fluo-3 fluorescence images in B cells reflected mostly the images of $[Ca^{2+}]_i$ in B cells. From the fura-2 images by a conventional fluorescence microscope it was estimated that the $[Ca^{2+}]_i$ in B cells increased from 80–120 nM at the resting levels to 200–300 nM at the activated levels.

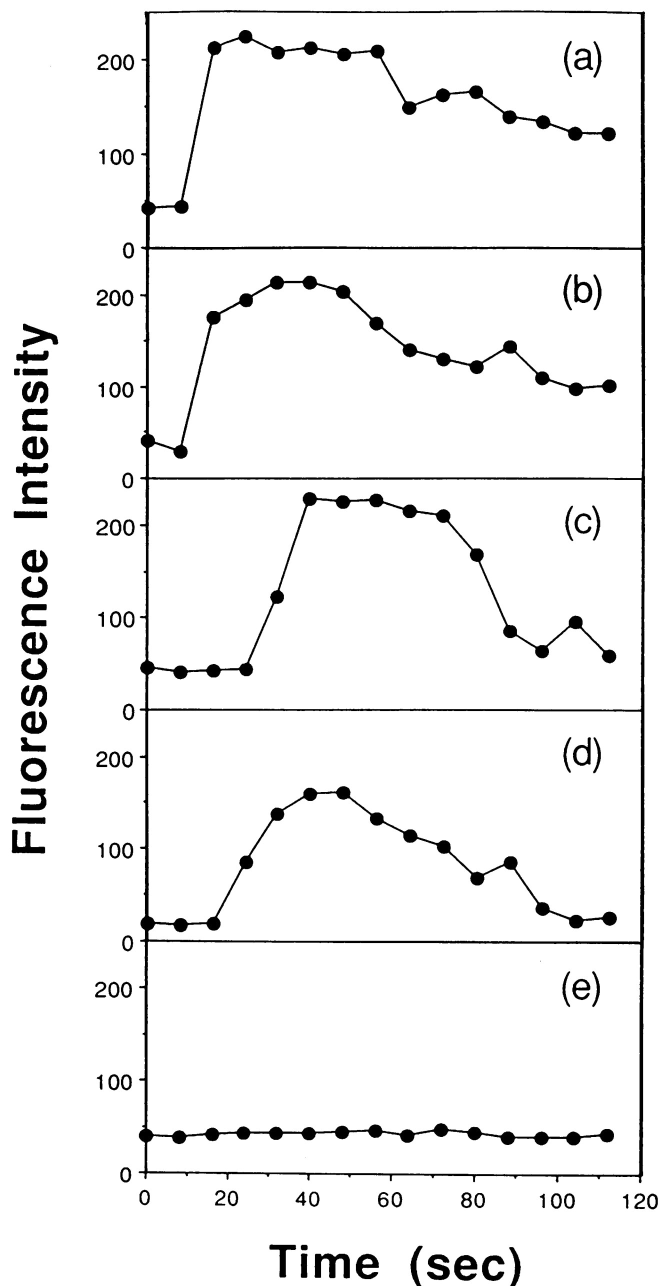
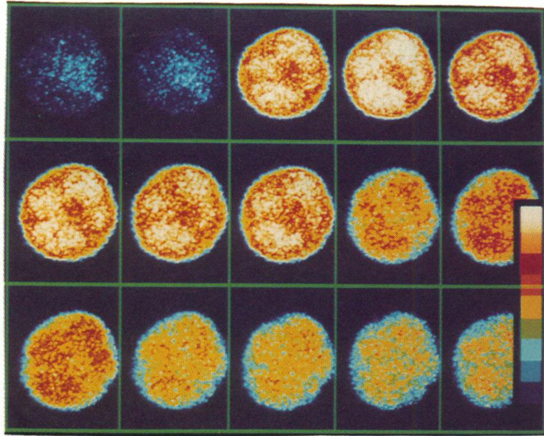


FIGURE 1 Time-courses of the fluorescence intensity changes of fluo-3 loaded individual B cells (TP67.21) after the addition of antigen at 37°C. The vertical axes were relative fluorescence intensities which subtracted the background fluorescence intensities. (*a–d*) Addition of trinitrophenol-conjugated ovalbumin (2 μg/ml). Time-courses of *a* and *d* corresponded to the cell-1 and cell-3 shown in Fig. 2 *b*, respectively. (*e*) Addition of ovalbumin without trinitrophenol (a control experiment).

Confocal fluorescence images of the nucleus

From the morphological patterns of the calcium images in Fig. 2 *a*, the parts of the bright fluorescence seemed to be the nucleus in B cells. To confirm this we tried to measure the confocal fluorescence images of the nucleus in B cells. Here, we used Hoechst 33342 (a DNA-specific fluorescent dye) for the fluorescence images of the nucleus (20). Before the confocal fluorescence imaging we measured fluorescence spectra of Hoechst 33342 and fluo-3 co-loaded B cells as shown in Fig. 3. Fig. 3 *a* shows the fluorescence spectra of the B cells excited at 325 nm. These fluorescence spectra were quite similar to those of the B cells which were loaded with Hoechst 33342 alone and were independent of the antigen stimulation (see Fig. 3 *a*). Then, Fig. 3 *b* shows the fluorescence spectra of

(a)



(b)

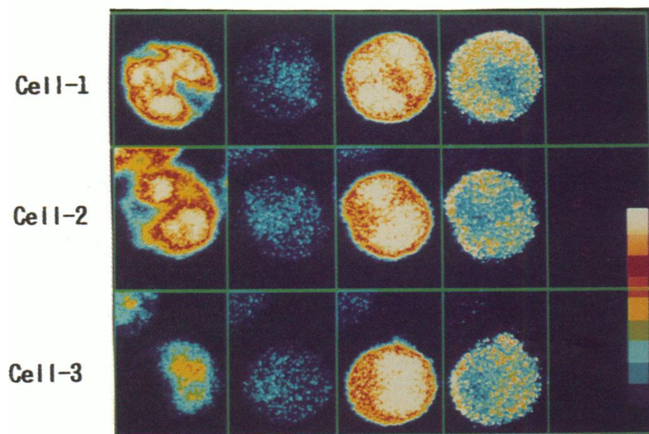


FIGURE 2 (*a*) Confocal fluorescence pseudo-images of fluo-3 loaded B cells (TP67.21) after the addition of trinitrophenol-conjugated ovalbumin (2 μg/ml). Sequential fluorescence images of antigen-stimulated cells are shown from top row (left to right). Fluorescence images were collected by 8-s intervals at 37°C. Trinitrophenol-conjugated ovalbumin was added between the first frame and the second frame. (*b*) Confocal fluorescence pseudo-images of the Hoechst 33342 and fluo-3 coloaded B cells (TP67.21) at 37°C. Three typical examples are shown (cell-1, 2, and 3). Cell-1 is the same cell shown in Fig. 2 *a*. In each example, the first column represents: confocal fluorescence images from a DNA-specific fluorescent probe (Hoechst 33342) by the excitation of a He-Cd laser (325 nm). The second and the third columns: confocal fluorescence images from a calcium ion-specific probe (fluo-3) by the excitation of an argon ion laser (488 nm) before and after antigen stimulation, respectively. The fourth column: ratios of fluo-3 fluorescence images after antigen stimulation. Here, rationing was done by using the fluorescence images of the second and the third columns.

the B cells excited at 488 nm. They were dependent on antigen stimulation and were similar to the spectra of B cells which were loaded with fluo-3 alone. These results suggested that Hoechst 33342 neither affect the fluo-3 fluorescence images in B cells nor fluo-3 affect the Hoechst 33342 fluorescence images.

Then, we measured the confocal fluorescence images of B cells co-loaded with fluo-3 and Hoechst 33342 using a He-Cd laser (excitation at 325 nm and emission above 390 nm). For example, we showed three typical fluorescence images of the nucleus together with the calcium images in B cells (see Fig. 2 *b*). To determine the location of the nucleus exactly, we added membrane-specific fluorescent-probe TMA-DPH in the observation chamber just before the measurements. TMA-DPH showed outlines of B cells. The fluorescence images of the nucleus in B cells (the first column in Fig. 2 *b*) gave the similar fluorescence patterns such as the brighter parts of the calcium images (the third column in Fig. 2 *b*). As the depth of the optical section was 0.7 μm in our present experimental conditions, it was concluded that the optical slice was thin enough to contain only the nucleus or cytosol.

Further, to correct inhomogeneities in dye intensity and distribution inside B cells, we measured ratios of fluo-3 fluorescence images after the antigen stimulation to those before the stimulation as shown in Fig. 2 *b* (the *fourth column*). While true ratioing was not possible with fluo-3, the ratios using fluorescence images prior to stimulation (the *second column* in Fig. 2 *b*) showed the existence of non-homogeneous fluorescence images in the fourth column of Fig. 2 *b*. That is, the parts whose fluo-3 fluorescence intensities were dim before antigen stimulation (the second column in Fig. 2 *b*), became much brighter after the stimulation (the third column in Fig. 2 *b*). These brighter parts in the fluo-3 fluorescence images were consistent with the brighter parts in the ratio images (the *fourth column* in Fig. 2 *b*). These kinds of phenomena were observed in a large number of B cells (TP67.21) which were measured in this experiment. This indicated that the inhomogeneities of the fluo-3 fluorescence images after antigen stimulation shown in the third column of Fig. 2 *b* were mostly derived from the inhomogeneities of $[Ca^{2+}]_i$ distribution in B cells, not merely from the inhomogeneities of dye distribution. Percentages of the increments in fluo-3 fluorescence ratio intensities were usually much higher in the nucleus than in the cytoplasm as shown in Fig. 4. These results suggested that the receptor-mediated calcium signals were transferred not only to the cytoplasm but also to the nucleus.

DISCUSSION

At the present time, we can not determine exactly that the fluo-3 fluorescence intensity observed by the confocal fluorescence microscope is solely attributable to the

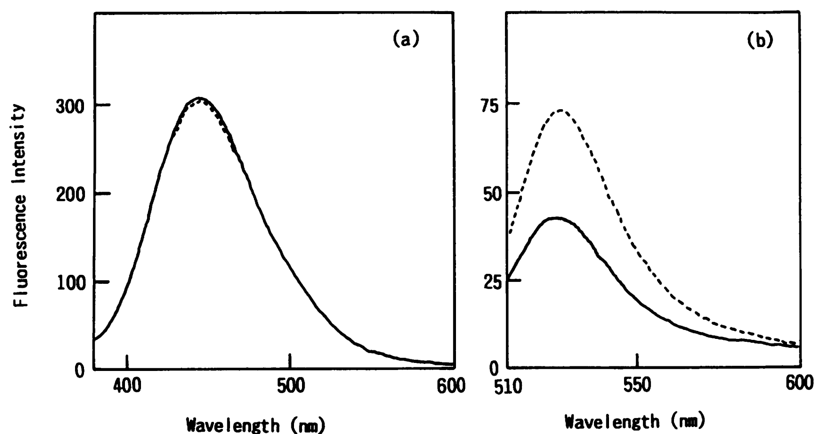


FIGURE 3 Fluorescence spectra of Hoechst 33342 and fluo-3 co-loaded B cells (TP67.21) at 37°C. (*a*) Fluorescence spectra of B cells excited at 325 nm. A continuous line shows the spectrum before antigen stimulation. A dotted line shows the spectrum at 30 s later after antigen stimulation. (*b*) Fluorescence spectra of B cells excited at 488 nm. A continuous line shows the spectrum before antigen stimulation. A dotted line shows the spectrum at 30 s later after antigen stimulation.

$[Ca^{2+}]_i$ in B cells. However, the patterns of the fluorescence intensity changes after the antigen-stimulation were quite similar to the patterns of the time-courses of the $[Ca^{2+}]_i$ observed by the conventional fluorescence microscope using fura-2 fluorescence. Thus, the present results indicated that the fluorescence intensity of the fluo-3-loaded B cells reflected mostly the concentration of calcium ions within the cells. From the confocal fluorescence images of fluo-3 and Hoechst 33342 co-loaded B cells in Fig. 2 *b*, it was suggested that the calcium signals transferred not only to the cytoplasm but also to the nucleus (7). Similar kinds of events have been observed in T cell clones after antigen stimulation (unpublished results). These results suggested the possibility that the increased intranuclear calcium ions after antigen stimulation may play a nuclear third messenger in B cells. As the calcium ion concentration gradients have been shown to exist between the nucleus and the cytoplasm (6, 7, 10), it was suggested that there are mechanisms which modulate the transport of calcium ions across the nuclear membrane and regulate intranuclear calcium ion concentration (1).

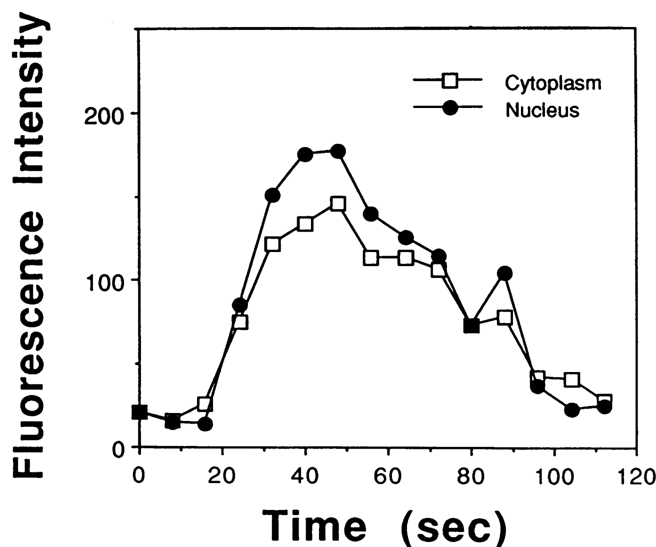


FIGURE 4 Time-courses of the fluorescence intensity changes of the nucleus and cytosol in a B cell. The vertical axes were relative fluorescence intensities which subtracted the back ground fluorescence intensities. This example was obtained from the fluorescence images shown in Fig. 2 *b* (cell-3).

Received for publication 17 August and in final form 11 November 1992.

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