Confocal fluorescence microscopy for studying thapsigargin-induced bivalent-cation entry into B cells

Yukiko OKAMOTO,* Tadahide FURUNO,* Teruaki HAMANO† and Mamoru NAKANISHI*‡

*Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabe-dori, Mizuho-ku, Nagoya 467,

and †The Second Department of Internal Medicine, Hyogo College of Medicine, Nishinomiya, Hyogo 663, Japan

We studied thapsigargin-induced bivalent-cation entry into antigen-specific B cells (TP67.21) with a confocal fluorescence microscope. Confocal fluorescence images of fluo-3-loaded B cells showed that thapsigargin-stimulated Ca^{2+} signals were transferred not only to the cytoplasm but also to the nucleus. In the absence of external Ca^{2+} ions, the free Ca^{2+} concentrations both in the cytosol and in the nucleus declined to basal levels by 5 min after addition of thapsigargin. However, subsequent addition of Ca^{2+} in the external medium made the fluo-3 (fura-2)

fluorescence intensity rise, reflecting the fact that Ca^{2+} accumulated again in the nucleus as well as in the cytoplasm. Then, we added Ba^{2+} and Mn^{2+} instead of Ca^{2+} , because Ba^{2+} and Mn^{2+} are known to enter via Ca^{2+} channels. The addition of Ba^{2+} and Mn^{2+} in the external medium quenched the fluo-3 fluorescence both in the nucleus and in the cytoplasm of B cells. This suggested the possibility that the increase in intranuclear Ca^{2+} after thapsigargin stimulation may come from the cytoplasm, not from the nuclear stores.

INTRODUCTION

It is known that ligand binding to receptors induces a rapid increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), together with increased polyphosphoinositide hydrolysis [1–3]. In addition, it is reported that inositol trisphosphate receptors play a key role in the coupling between cytoplasmic second-messenger and nuclear third-messenger-triggered events [4–8]. These results suggest the possibility that the receptor-mediated Ca^{2+} signals may transfer not only to the cytoplasm but also to the nucleus [3,8].

By confocal fluorescence microscopy we have shown recently that antigen-stimulated Ca^{2+} signals were transferred not only to the cytoplasm, but also to the nucleus, in mouse B cells (TP67.21 and BAL17) and rat basophilic-leukaemia cells (RBL-2H3) [8–10]. These types of Ca^{2+} signal in the nucleus were also observed after fertilization of sea-urchin eggs [11]. In addition, it was reported that isolated nuclei display an ATP-dependent uptake of Ca^{2+} ions, of which about 20% was subsequently released by inositol trisphosphate [1]. Thus it seems that Ca^{2+} ions stored within the space between the nuclear membranes may be released to the cytosol when inositol trisphosphates bind to receptors on the nuclear outer membrane, because the nucleus contains an autonomous phosphoinositide signalling system just as complex as that present at the plasma membrane [12–14]. But such a mechanism would not explain the increase in nuclear Ca^{2+} ions that occurs after activation of lymphocytes or sea-urchin eggs as described above [8,10,11], because there is no evidence for inositol trisphosphate receptors on the nuclear inner membranes [12].

In this paper we have studied the thapsigargin-induced bivalent-cation entry into B cells by confocal fluorescence microscopy. We found that thapsigargin increased cytosolic and nuclear Ca^{2+} by depleting Ca^{2+} from intracellular Ca^{2+} stores, and that the depletion of the intracellular Ca^{2+} stores induced the

Ca^{2+} -influx pathway. Further, we found that Ba^{2+} and Mn^{2+} entered B cells through Ca^{2+} channels and quenched the fluorescence of intracellular Ca^{2+} indicators (fluo-3 and fura-2). From these results we have discussed the mechanism of the intranuclear Ca^{2+} signalling pathway in B cells.

MATERIALS AND METHODS

Fluo-3 AM, fura-2 AM (AM, acetoxymethyl ester) and Hepes were obtained from Dojindo (Kumamoto, Japan). Hoechst 33342 (bisbenzimidazole H 33342) was obtained from Calbiochem (La Jolla, CA, U.S.A.). TMA-DPH [1-(4-trimethylammoniumphenyl)-6-phenylhexa-1,3,5-triene] was obtained from Molecular Probes (Eugene, OR, U.S.A.). RPMI 1640 was from Gibco (Grand Island, NY, U.S.A.). Trinitrophenol-conjugated ovalbumin was prepared as described in our previous paper [15,16]. In our present experiments, an average of 21 trinitrophenyl groups was conjugated to ovalbumin. Thapsigargin and ionomycin were obtained from Sigma (St. Louis, MO, U.S.A.).

Antigen-specific B-cell clones (TP67.21) directed against 2,4,6-trinitrophenyl (TNP) hapten were established previously [17]. They were maintained in a culture medium containing RPMI 1640 supplemented with 5% fetal-calf serum, penicillin G (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and 2-mercaptoethanol (50 μM).

Confocal-fluorescence-microscopic images of B-cell clones (TP67.21) were taken under a confocal-scanning-fluorescence microscope system (MRC-600, Bio-Rad) with an inverted epifluorescence microscope (Nikon TMD-EFQ) [9,10]. A pinhole aperture in the detection path of the confocal microscope was 3 mm, and we used a glycerol-immersed 1.4 NA (numerical aperture) 60 $\times$ Nikon Planapo and a 0.85 NA 40 $\times$ Nikon Fluor objective. Fluo-3-loaded B cells (TP67.21) were excited at 488 nm with an argon-ion laser, and the fluorescence emission was observed above 515 nm. The temperature of the observation

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenylhexa-1,3,5-triene.

‡ To whom correspondence should be addressed.

chamber was controlled at 37 °C. Confocal fluorescence images of nuclei in B cells were measured by using a He–Cd laser (325 nm) together with a DNA-specific fluorescence probe, Hoechst 33342 [18].

Fluorescence measurements of B-cell suspensions were done in a 1 cm quartz cuvette with a Shimadzu FR-5000 spectrofluorometer at 37 °C [16].

RESULTS

Thapsigargin has been shown to increase cytosolic Ca^{2+} in various cells by causing Ca^{2+} to be released from intracellular Ca^{2+} stores [19–21]. Here, we first stimulated fluo-3-loaded B cells (TP67.21) with thapsigargin (0.2–40 nM), and observed time-dependent fluorescence images of individual B cells, using a confocal fluorescence microscope with an argon-ion laser (488 nm) and a He–Cd laser. Confocal fluorescence images of B cells before and after addition of thapsigargin (20 nM) in the absence of external Ca^{2+} ions are shown in Figure 1. Fluo-3 fluorescence images of B cells before thapsigargin stimulation were dim and non-homogeneous, but became much brighter, and the lack of homogeneity became more enhanced, after stimulation, as shown in Figure 1. Compared with the confocal fluorescence images of Hoechst-33342 co-loaded cells, the regions of bright fluorescence in fluo-3-loaded cells were assigned to the nucleus in B cells [10]. A typical example of time-courses of the fluo-3 fluorescence intensities after addition of thapsigargin (20 nM) is shown in Figure 2. Figure 2 indicated that thapsigargin induced rises in the free Ca^{2+} concentrations in the nucleus as well as in the cytoplasm of B cells, in the absence or presence of external Ca^{2+} ions. In the absence of external Ca^{2+} , the free Ca^{2+} concentrations both in the nucleus and in the cytosol declined to the basal level by 5 min after addition of thapsigargin. However, in the presence of external Ca^{2+} (1 mM), the free Ca^{2+} concentrations remained at a much higher level at 5 min after thapsigargin stimulation, as shown in Figure 2(b). This was due to Ca^{2+} entry from the external medium. The mechanism for regulation of the Ca^{2+} entry is not yet defined; however, it has been suggested that thapsigargin-induced Ca^{2+} entry is mediated by a capacitative Ca^{2+} -entry pathway that is activated in response to Ca^{2+} -pool depletion [22–24]. Thapsigargin-induced nuclear and cytosolic Ca^{2+} signals observed here were quite similar to the antigen-induced Ca^{2+} signals of B cells (TP67.21) in our previous paper [10].

At present, it is difficult to determine directly $[\text{Ca}^{2+}]_i$ from the fluo-3 fluorescence intensity by confocal fluorescence microscopy. Therefore, we estimated $[\text{Ca}^{2+}]_i$ from the fluo-3 fluorescence intensity values by using the following equation [25]:

$$[\text{Ca}^{2+}]_i = [(F - F_{\min.}) / (F_{\max.} - F)] \times K_d \quad (1)$$

where K_d is the dissociation constant for Ca^{2+} binding to fluo-3 (400 nM at 37 °C, pH 7.3 [26]), F is the fluorescence intensity of the sample, and $F_{\min.}$ and $F_{\max.}$ are the fluorescence intensities of the sample in 1 mM EGTA and 2 mM CaCl_2 respectively. The $[\text{Ca}^{2+}]_i$ values calculated from eqn. (1) are also shown in Figure 2. These values are consistent with the $[\text{Ca}^{2+}]_i$ values obtained from fura-2 fluorescence-ratio intensities of the B-cell suspensions, as shown in Figure 3. In addition, the time-dependent profiles shown in Figure 2 resembled closely the $[\text{Ca}^{2+}]_i$ profiles of fura-2-loaded B-cell suspensions which were observed by a fluorescence spectrophotometer (Figure 3). These results supported the idea that fluo-3 fluorescence images in B cells reflected mostly the images of $[\text{Ca}^{2+}]_i$ in B cells. From the fura-2 fluorescence-ratio intensities (F_{335}/F_{358}) shown in Figure 3, it was

Figure 1 Confocal fluorescence images of fluo-3-loaded B cells (TP67.21) before and after addition of thapsigargin (20 nM) in the absence of external free Ca^{2+} ions (with 1 mM EGTA)

Here, a typical example is shown: a fluorescence image of a fluo-3-loaded B cell before thapsigargin stimulation (left); a fluorescence image of the fluo-3-loaded B cell after thapsigargin stimulation (after 80 s) (middle); a DNA image of the Hoechst 33342 co-loaded B cell which was excited by a He–Cd laser (325 nm). To determine the location of the nucleus exactly, we added the membrane-specific fluorescent probe TMA-DPH into the observation chamber. TMA-DPH indicated an outline of the B cell (right).

Figure 2 Typical example of time courses of the fluorescence-intensity changes of fluo-3-loaded individual B cells (TP67.21) after addition of thapsigargin at 37 °C

○, Fluo-3 fluorescence intensities in the nucleus; ●, those in the cytoplasm. The $[\text{Ca}^{2+}]_i$ values were estimated by using eqn. (1). For calculating $F_{\min.}$ and $F_{\max.}$, ionomycin (2 μM) was added to the cells [25]. (a) Addition of thapsigargin (20 nM) in the absence of external free Ca^{2+} (with 1 mM EGTA). (b) Addition of thapsigargin (20 nM) in the presence of external free Ca^{2+} (1 mM).

estimated that $[\text{Ca}^{2+}]_i$ in B cells increased from around 50 nM at the resting levels to 300–500 nM at the activated levels.

Then, in the absence of external Ca^{2+} , the free Ca^{2+} concentrations in B cells declined to basal levels by 5 min after addition of thapsigargin (see Figure 2a). In this case, subsequent addition of external Ca^{2+} (1 mM) made the fluo-3 fluorescence intensity rise, reflecting accumulation again of Ca^{2+} both in the nucleus and in the cytoplasm. The amounts of Ca^{2+} entry were dependent on the initial concentrations of thapsigargin (0.2–40 nM), and 20 nM thapsigargin induced sufficient Ca^{2+} entry, as shown in Figure 4(a). The results were consistent with the findings that the Ca^{2+} -influx pathway was induced by the depletion of the in-

Figure 3 Time courses of $[Ca^{2+}]_i$ in fura-2-loaded B-cell suspensions (TP67.21) after addition of thapsigargin (20 nM), observed by a fluorescence spectrophotometer at 37 °C

(a) In the absence of external free Ca^{2+} (with 1 mM EGTA); (b) in the presence of external free Ca^{2+} (1 mM).

tracellular Ca^{2+} stores with thapsigargin [19–21]. In all these experiments, the fluo-3 fluorescence intensities increased more in the nucleus than in the cytoplasm (see Figure 4a).

To determine whether the increased intranuclear Ca^{2+} ions are transferred from the cytoplasm, or whether they are released from the nuclear stores, we used Ba^{2+} and Mn^{2+} instead of Ca^{2+} . Ba^{2+} and Mn^{2+} are known to enter via Ca^{2+} channels and are sensed by fura-2 and fluo-3 [27,28]. We first compared the fluorescence spectra of fluo-3 in the presence of saturating concentrations of the bivalent cations. Emission spectra of fluo-3 complexes of Ca^{2+} , Ba^{2+} or Mn^{2+} between 500 and 550 nm are shown in Figure 5. The quantum yields of fluo-3 complexes of Ba^{2+} and Mn^{2+} were much lower than that of Ca^{2+} , although they were higher than that of free fluo-3 (with EGTA). In addition, when we added equimolar Ba^{2+} or Mn^{2+} to the Ca^{2+} solution, the quantum yields of the mixture decreased drastically, as shown in Figure 5 (broken lines). The quantum yield of an equimolar mixture of Ca^{2+} and Ba^{2+} was between the quantum yield of Ca^{2+} solution and that of Ba^{2+} solution. However, the quantum yield of an equimolar mixture of Ca^{2+} and Mn^{2+} was greatly decreased, and it was close to the quantum yield of the solution of Mn^{2+} alone. It must be due to the reason that Mn^{2+} bound to fluo-3 about 70-fold as strongly as Ca^{2+} did [28]. This characteristic made it possible to examine channel activities in the plasma and nuclear membranes of B cells after stimulation with thapsigargin. In Figure 6 we stimulated B cells (TP67.21) with thapsigargin (20 nM) in the absence of external Ca^{2+} ions, and after 5 min we added Ba^{2+} (or Mn^{2+}) in the external medium instead of Ca^{2+} . The addition of Ba^{2+} (Mn^{2+}) in the external medium increased the fluo-3 fluorescence intensities in B cells. Although the quantum yields of fluo-3 complexes of Ba^{2+} and Mn^{2+} are much smaller than that of Ca^{2+} , it was suggested that Ba^{2+} (Mn^{2+}) permeated Ca^{2+} channels and increased the fluo-3 fluorescence intensities both in the nucleus and in the cytoplasm.

Figure 4 Thapsigargin-induced entry of Ca^{2+} in B cells, and the effects of Ba^{2+} and Mn^{2+} on the entry

B cells (TP67.21), in the Ca^{2+} -free medium, were first depleted of intracellular Ca^{2+} with thapsigargin (TG, 20 nM), and the Ca^{2+} entry was then initiated by addition of 1 mM Ca^{2+} in the external medium. Fluo-3 fluorescence intensities in individual B cells were observed by a confocal fluorescence microscope with an argon ion laser at 37 °C. ○, Fluo-3 fluorescence intensities in the nucleus; ●, those in cytoplasm. (a) Addition of 1 mM Ca^{2+} at 5 min after thapsigargin stimulation; (b) 1 mM Ba^{2+} was added just after addition of 1 mM Ca^{2+} ; (c) 1 mM Mn^{2+} was added just after addition of 1 mM Ca^{2+} .

To examine channel activities of Ba^{2+} and Mn^{2+} further, we added Ba^{2+} or Mn^{2+} (1 mM) in the external medium just after Ca^{2+} entry upon re-introduction of Ca^{2+} (Figures 4b and 4c). Fluo-3 fluorescence intensities were decreased much more abruptly by addition of Ba^{2+} and Mn^{2+} (Figure 4). These results indicated that Ba^{2+} and Mn^{2+} were transferred across both the plasma membranes and the nuclear membranes to quench the fluo-3 fluorescence in the nucleus as well as in the cytoplasm. These results suggested the possibility that the increased intranuclear Ca^{2+} ions in B cells (TP67.21) may come from the cytoplasm, not from the nuclear Ca^{2+} stores.

DISCUSSION

We have studied here by confocal fluorescence microscopy the effects of bivalent cations on the thapsigargin-induced Ca^{2+} signals in B cells. Confocal fluorescence images showed that thapsigargin-induced Ca^{2+} signals were transferred not only to the cytoplasm but also to the nucleus in B cells (TP67.21). In the absence of external Ca^{2+} ions, the free Ca^{2+} concentrations in B

Figure 5 Fluorescence spectra of fluo-3 complexed with bivalent cations (Ca^{2+} , Ba^{2+} and Mn^{2+})

Spectra were obtained in 10 mM Hepes buffer (pH 7.3) containing $1 \mu\text{M}$ fluo-3 free acid and 1.3 mM cations indicated. Fluorescence excitation was measured at 488 nm. The free fluo-3 ('EGTA') spectrum was obtained in the presence of 1 mM EGTA and in the absence of metal cations.

Figure 6 Thapsigargin-induced entry of Ba^{2+} and Mn^{2+} into B cells

B cells (TP67.21), in the Ca^{2+} -free medium, were first depleted of intracellular Ca^{2+} with thapsigargin (TG; 20 nM), and Ba^{2+} and Mn^{2+} entry was then initiated by addition of 1 mM Ba^{2+} (or Mn^{2+}) in the external medium. O, Fluo-3 fluorescence intensities in the nucleus; ●, those in the cytoplasm. (a) Addition of 1 mM Ba^{2+} at 5 min after thapsigargin stimulation; (b) addition of 1 mM Mn^{2+} at 5 min after thapsigargin stimulation.

cells declined to basal level by 5 min after addition of thapsigargin; however, subsequent addition of external Ca^{2+} ions showed that Ca^{2+} accumulated again both in the nucleus and in the cytoplasm. These results were consistent with the

findings that the Ca^{2+} -influx pathway was induced by depletion of the intracellular Ca^{2+} stores with thapsigargin [19–21]. This characteristic made it possible to examine channel activities in the plasma and the nuclear membranes of B cells after addition of thapsigargin.

In our present experiments, Ba^{2+} and Mn^{2+} , which are known to enter via Ca^{2+} channels, were transferred from the external medium to the nucleus as well as to the cytoplasm of B cells after addition of thapsigargin (Figure 6). In addition, when we added Ba^{2+} or Mn^{2+} in the external medium just after Ca^{2+} entry upon re-introduction of Ca^{2+} , fluo-3 fluorescence intensities were abruptly quenched (Figures 4b and 4c). This suggested that Ba^{2+} and Mn^{2+} transferred from the external medium to the nucleus as well as to the cytoplasm and quenched the fluo-3 fluorescence in B cells. These types of fluorescence quenching were also observed when Ba^{2+} and Mn^{2+} were added just after addition of Ca^{2+} into the external medium of the antigen (trinitrophenyl-conjugated ovalbumin)-stimulated B cells (TP67.21) (results not shown).

These results suggested the possibility that the increased intranuclear Ca^{2+} ions in B cells (TP67.21) after thapsigargin (or antigen) stimulation may come from the cytoplasm, not from the nuclear stores. It should be mentioned here, however, that the coincident time course of nuclear and cytosolic Ca^{2+} signals are indeed consistent with the latter driving the former, but that the rather slow time course for each of these does not explain fully why the cytoplasmic Ca^{2+} signals occur before the nuclear Ca^{2+} signals. Thus, the present results do not preclude a nuclear store of mobilizable Ca^{2+} .

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