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Single cell observation of ligand-induced desensitization of B-cell membrane immunoglobulin-mediated calcium signals

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Using a digital imaging fluorescence microscope we have observed the membrane immunoglobulin (mIg)-induced desensitization of calcium signals in individual BAL17 B lymphoma cells which express two kinds of antigen receptors, mIgM and mIgD. The mIgD-mediated desensitization was partly abrogated by pretreating the cells with phorbol 12-myristate 13-acetate (PMA) for 24 h, however, the mIgM-mediated one was not affected by the pretreatment. This supports the idea that at least two mechanisms are operative for mIg-induced desensitization in B cells.

Calcium signal; Fluorescence imaging; B cell; Membrane immunoglobulin; Protein kinase C

1. INTRODUCTION

Resting peripheral B cells express two kinds of antigen receptors, membrane-bound immunoglobulin M (mIgM) and membrane-bound immunoglobulin D (mIgD). Ligand binding to either isotype receptor induces a rapid increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) as well as inositol phospholipid metabolism for cell activation [1–6]. Such kinds of cells are suitable to study the molecular basis for B cell anergy [7,8], which is the inactivation of the signal transducing ability of membrane-bound immunoglobulin receptors.

The mIg-mediated desensitization of calcium signals has been previously observed in B cell populations or sub-populations [9,10]. Here, we have applied a digital imaging fluorescence microscope to observe the desensitization of the calcium signals in individual B cells. Although fluorescence microscopy has been applied to study calcium signals in several different cell types [11–14], it will be shown here that it is a promising method to study ligand-induced desensitization in the single cell. Further, the results suggested that the mIgM-mediated desensitization in BAL17 cells may be different from the mIgD-mediated one.

2. MATERIALS AND METHODS

BAL17 cells were maintained in a culture medium containing RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS (fetal calf serum), streptomycin (100 μg/ml), penicillin G (100 U/ml), L-glutamine (2 mM), and 2-mercaptoethanol (50 μM) [4,6]. Goat anti-mouse IgM H chain-specific antibody (anti-IgM) was prepared as described [15]. Goat anti-mouse IgD (anti-IgD) was a gift from Fred Finkelman (Uniformed Services University of Health Sciences, Bethesda, MD). Fura-2AM was from Dojindo (Kumamoto, Japan) and phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO).

We observed a rapid transient increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$) in an individual BAL17 cell by a digital imaging fluorescence microscope at 37°C. BAL17 cells were harvested from culture dishes to an observation chamber coated with poly-L-lysine (Sigma, St. Louis, MO). Then, the chamber was settled in a CO_2 incubator for 2 h. During the incubation, BAL17 cells became attached to the chamber, after which they were pretreated with medium containing fura-2AM (5 μM) for 40 min at 37°C. Then they were washed with HEPES buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$ at pH 7.2) to remove free fura-2AM in solution. Fura-2-loaded BAL17 cells were stimulated with anti-IgM or anti-IgD.

Fluorescence microscopic images of BAL17 cells were taken under an inverted epifluorescence microscope (Nikon TMD-EFQ) with a silicon-intensified target TV camera (Hamamatsu Photonics 2400-08) and a U-matic video tape recorder (Sony VO-9600). The fluorescence images were analyzed with a digital image processor (Image Sigma II and TVIP-2000, Avionics), which was controlled by a microcomputer (NEC PC-9801VX). The digital frame memory had 512 × 480 pixels, and was installed in the image processor. Each pixel was 8 bit in depth (256 steps for full scale gray level) [12].

Fluorescence measurements of BAL17 cell suspension were done in a 1 cm quartz cuvette using a Shimadzu FR-5000 spectrofluorimeter with an excitation wavelength of 340 nm or 360 nm, and an emission wavelength of 500 nm, at 37°C.

3. RESULTS

Fura-2-loaded BAL17 cells in the observation chamber were stimulated with 8 μg/ml of anti-IgD and the fluorescence microscopic images of the individual BAL17 cells were observed. The ratio of fluorescence excitation images at 340 nm at 360 nm (Ratio F_{340}/F_{360})

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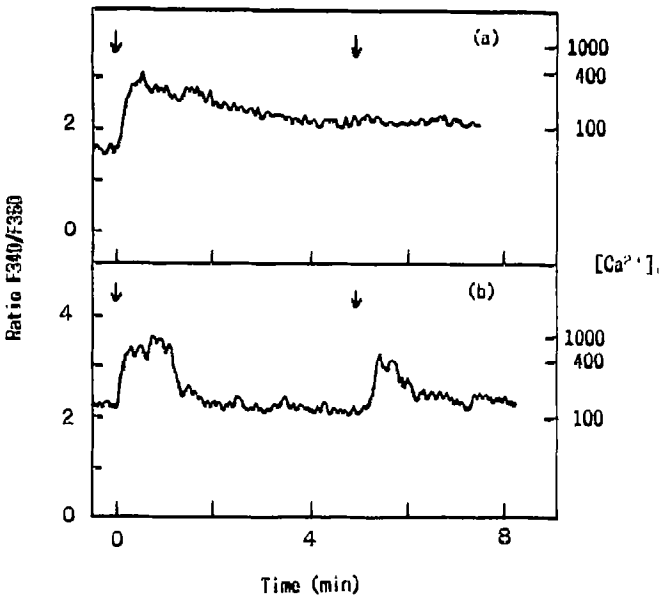

Fig. 1. A typical example of the individual time-courses of $[Ca^{2+}]_i$ in fura-2-loaded BAL17 cells. Here, the cells were first stimulated by 8 μg/ml of anti-IgD and after 5 min the cells were stimulated again by 8 μg/ml of anti-IgM. Arrows indicate the times at which the anti-Ig's were added. (a) A PMA-untreated BAL17 cell; (b) a PMA-treated BAL17 cell.

gave the $[Ca^{2+}]_i$ images [11–13]. Anti-IgD stimulation increased the $[Ca^{2+}]_i$ rapidly in BAL17 cells with a short lag time. Addition of 4–16 μg/ml anti-IgD produced similar increases in the amounts of $[Ca^{2+}]_i$ in B cells. After 5 min the cells were stimulated again, this time with the reciprocal isotype antibody (anti-IgM, 8 μg/ml). A typical example of the individual time-courses of $[Ca^{2+}]_i$ is shown in Fig. 1a. We carried out similar experiments several times and measured more than a hundred individual time-courses of BAL17 cells, however, none of B cells responded to the second antibody stimulation (anti-IgM, 4–16 μg/ml). Conversely, we stimulated BAL17 cells with anti-IgM (8 μg/ml) first and subsequently stimulated them with anti-IgD (8 μg/ml). A typical example of such experiments is shown in Fig. 2a. Although anti-IgM induced $[Ca^{2+}]_i$ of individual B cells, they did not respond to the second antibody stimulation (anti-IgD, 4–16 μg/ml). These results indicated that B cells (BAL17 cells), once activated by anti-IgM or anti-IgD, could no longer be re-activated with the reciprocal isotype antibody binding, which is consistent with the previous findings [9,10]. Then, we confirmed, using a flow cytometer, that the heterologous receptor desensitization did not reflect loss of the receptor from the cell surface (data not shown).

We have previously shown that long-term pretreatment with PMA depletes protein kinase C (PKC) from BAL17 cells [16], so we measured $[Ca^{2+}]_i$ in BAL17 cells pretreated with PMA (100 ng/ml) for 24 h. The $[Ca^{2+}]_i$ in PMA-pretreated BAL17 cells could be increased by the addition of anti-IgD (8 μg/ml), as shown in Fig. 1b. Interestingly, $[Ca^{2+}]_i$ increased again with the addition of the second reciprocal antibody (anti-IgM, 8 μg/ml), showing recovery from the desensitization of calcium signals (see Fig. 1b). One third of PMA-pretreated BAL17 cells, which were able to respond to anti-IgD, were stimulated by the second antibody (anti-IgM), as shown in Fig. 1b, however, the other two thirds were not stimulated. This suggests that the desensitization of calcium signals mediated by IgD receptors was partly induced by PKC activation. On the other hand, this was not true for the desensitization of the calcium signals mediated by IgM receptors. $[Ca^{2+}]_i$ in 24 h PMA-pretreated BAL17 cells never increased with the addition of the second reciprocal antibody (anti-IgG, 4–16 μg/ml), although $[Ca^{2+}]_i$ increased with the first antibody stimulation (anti-IgM, 8 μg/ml) (see Fig. 2b).

4. DISCUSSION

We have shown here, using a confocal fluorescence microscope, that the mIgM-mediated desensitization of mIgD-induced calcium signals was unaffected by the depletion of PKC from BAL17 cells, confirming previous findings that the protein kinase inhibitor, staurosporine, did not affect the anti-Ig-induced heterologous desensitization of mIg receptors in normal mouse B cells [10]. Surprisingly, mIgD-mediated desensitization of mIgM-stimulated $[Ca^{2+}]_i$ was in part abrogated by the PKC depletion, supporting the previous notion that

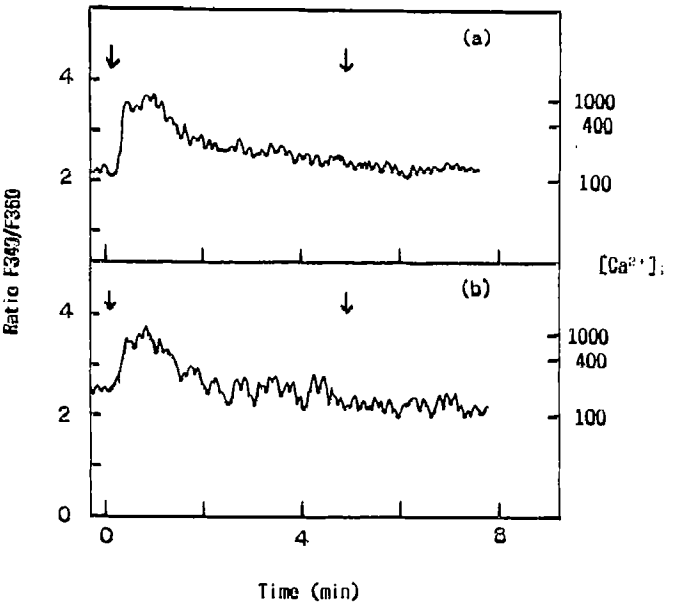

Fig. 2. A typical example of the individual time-courses of $[Ca^{2+}]_i$ in fura-2-loaded BAL17 cells. Here, the cells were first stimulated by 8 μg/ml of anti-IgM and after 5 min the cells were stimulated again by 8 μg/ml of anti-IgD. Arrows indicate the times at which the anti-Ig's were added. (a) A PMA-untreated BAL17 cell; (b) a PMA-treated BAL17 cell.

PKC activation regulates anti-Ig-induced calcium signals [16]. These findings are also consistent with the idea that mIgM-mediated signals are somewhat different from mIgD-mediated ones, as suggested by others [17]. Although at present we cannot explain fully the differences between the findings of Cambier's group and ours, a different sub-population of B cells may reveal a distinct activation pathway, as reported by Liou et al. [18]. The BAL17 cells we used might represent a minor sub-population of normal spleen B cells, which they used for experiments.

Our present findings support the idea that at least two pathways are operative for ligand-induced mIg-desensitization; one is PKC dependent and the other is PKC independent. The method we used in this experiment will be valuable for the analysis of ligand-induced desensitization in various kinds of cells.

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