Slow and persistent increase of $[Ca^{2+}]_c$ in response to ligation of surface IgM in WEHI-231 cells

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Abstract WEHI-231 and Bal 17 B cell lines are representative models for immature and mature B cells, respectively. Their regulation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) was compared using fura-2 fluorescence ratiometry. The ligation of B cell antigen receptor (BCR) by anti-IgM antibody induced a slow but large increase of $[Ca^{2+}]_c$ in WEHI-231 cells while not in Bal 17 cells. The thapsigargin-induced store-operated Ca^{2+} entry (SOCE) of Bal 17 cells reached a steady state which was blocked by 2-aminoethoxydiphenyl borate (2-APB). On the contrary, the thapsigargin-induced SOCE of WEHI-231 cells increased continuously, which was accelerated by 2-APB. The increase of $[Ca^{2+}]_c$ by BCR ligation was also enhanced by 2-APB in WEHI-231 cells while blocked in Bal 17 cells. The Mn^{2+} quenching study showed that the thapsigargin-, or the BCR ligation-induced Ca^{2+} influx pathway of WEHI-231 was hardly permeable to Mn^{2+}. The intractable increase of $[Ca^{2+}]_c$ may explain the mechanism of BCR-driven apoptosis of WEHI-231 cells, a well-known model of clonal deletion of autoreactive immature B cells.

Key words: B cell; WEHI-231; B cell antigen receptor; Apoptosis; Cytosolic Ca^{2+} concentration

1. Introduction

A multitude of cellular processes are controlled through Ca^{2+} signaling. Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) is increased either by release from Ca^{2+} stores, or by influx across the plasma membrane. In B cells, the regulatory pathways of $[Ca^{2+}]_c$ is believed to control cell proliferation, differentiation and apoptotic processes [1,2]. Cross-linking of B cell antigen receptor (BCR) activates a series of protein tyrosine kinases (PTKs) [3]. Several of these PTKs (e.g. Syk and Btk) are involved in the activation of phospholipase Cγ, producing inositol 1,4,5-trisphosphate (IP_3) which subsequently mobilizes intracellular Ca^{2+} stores via IP_3 receptor. The resulting decrease in the Ca^{2+} content within Ca^{2+} stores is expected to trigger store-operated Ca^{2+} entry (SOCE) and a tonic increase of $[Ca^{2+}]_c$. A low but sustained increase in $[Ca^{2+}]_c$ is believed to be essential for B cell survival and proliferation and activation of transcription factors such as NFAT [4,5].

A distinctive feature of immune system is the balanced fine-tuning between the growth and the death by apoptosis. In bone marrow, the immature B cells with membrane-bound immunoglobulins reactive to autoantigens are arrested in the cell cycle and eliminated through the process of apoptosis. In contrast, the mature B-lymphocytes, once activated by specific antigens, undergo a second round of proliferation and selection in the secondary lymphoid organs to mature into memory cells [6,7].

Bal 17 and WEHI-231 cells are representative murine B lymphoma cell lines that reflect the characteristics of mature and immature B cells, respectively [8–10]. The apoptotic response of WEHI-231 cells to BCR ligation is especially well known, and has been investigated as a model of stimulation-induced apoptosis of B cells at their immature stage [8–10]. Although many early signal transduction events through the BCR have been elucidated, the biochemical events leading to apoptosis of immature B cells are not entirely clear. Previous studies about the underlying mechanism of apoptosis have revealed complex signaling pathways and among them, an unchecked increase in $[Ca^{2+}]_c$ may regulate several key steps in the apoptotic pathways (e.g. caspases and calpains) of various cell types [11,12].

Here we compared the response of $[Ca^{2+}]_c$ to BCR ligation and the SOCE in Bal 17 and WEHI-231 cells. With the comparative study, we could observe differences between two cell lines. An intractable increase in $[Ca^{2+}]_c$ by BCR ligation and by SOCE may suggest a distinctive pathway of Ca^{2+} influx in WEHI-231 cells, a well-known model of immature B cells.

2. Materials and methods

2.1. Cells and agents

WEHI-231 and Bal 17 cells were grown in 25 mM HEPES RPMI 1640 media (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 50 μM 2-Mercaptoethanol (Sigma, St. Louis, MO, USA), and 1% penicillin/streptomycin (Gibco). All cells were incubated at 37°C in 95% O_2/5% CO_2. The chemicals and drugs used in this study were purchased from Sigma, and the rabbit F(ab')2 anti-mouse IgM antibody from Zymed Laboratories Inc (Oxnard, CA, USA). The antibody was applied into the cuvette to make a final concentration of 2.5 μg/ml.

2.2. $[Ca^{2+}]_c$ measurement

The measurement of $[Ca^{2+}]_c$ was done in HEPES-buffered physiological salt solution (PSS) containing 145 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose and pH was titrated to 7.4 with NaOH. Cells were harvested using

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; BCR, B cell antigen receptor; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration; SOCE, store-operated Ca^{2+} entry; IP_3, inositol 1,4,5-trisphosphate

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HEPES-buffered PSS, loaded with fura-2 acetoxymethyl ester (5 μM, 35 min, 37°C), and washed twice with fresh solution. The fluorescence was monitored in a stirred quartz-microcuvette (1 ml) in the thermostated cell holder of fluorescence spectrophotometer (CAF-110, Jasco, Japan) at the wavelengths of 340 and 380 (excitation), and 510 nm (emission). Obtained results were calibrated by adding 10 μM ionomycin with 10 mM $CaCl_2$, which gives the maximum value of fluorescence ratio (340 nm/380 nm, R_{max}), and 35 mM EGTA which gives the minimum value of fluorescence ratio (R_{min}). The $[Ca^{2+}]_c$ was calculated from the equation introduced by Grynkiewicz et al. [13]. The measured basal $[Ca^{2+}]_c$ was between 80 and 120 nM.

2.3. Mn^{2+} quenching experiment

At the isobestic wavelength of 360 nm, the fura-2 fluorescence intensity is not influenced by $[Ca^{2+}]_c$ changes [13]. Mn^{2+} has been shown to quench fura-2 after binding to the dye. The slope of fura-2 fluorescence quenching trace at 360 nm in the presence of Mn^{2+} is therefore regarded as an index of divalent cation influx [14]. Experiments were carried out by adding 200 μM $MnCl_2$ in Ca^{2+}-free medium that excludes Ca^{2+} competition for the divalent cation entry pathway and enhances the observed fluorescence quenching resulting from Mn^{2+} entry. Following measurement of basal Mn^{2+} entry, 10 μM ionomycin was added to reveal maximum quenching, and for the conversion of fluorescence intensity into normalized percent scale.

2.4. Data analysis and statistics

The data are presented as original recordings and bar graphs of mean ± S.E.M. When necessary, Student's *t*-test was applied and $P < 0.05$ was regarded as significant.

3. Results

3.1. Striking increase of $[Ca^{2+}]_c$ upon BCR engagement in WEHI-231 cells

After fura-2 loading, the resting $[Ca^{2+}]_c$ of Bal 17 and of WEHI-231 cells were 89 ± 2.9 nM ($n = 30$) and 83 ± 2.3 nM ($n = 30$), respectively. In Bal 17 cells, the engagement of BCR by anti-IgM Ab (2.5 μg/ml) induced a transient increase of $[Ca^{2+}]_c$ while WEHI-231 cells did not show a significant immediate response (Fig. 1A, $n = 6$ and 5, respectively).

The $[Ca^{2+}]_c$ responses of B cells were also compared after a sustained BCR ligation. After loading the cells with fura-2, anti-IgM Ab was added to 4 ml of cell suspension (about 5×10^5 cells/ml), incubated at 37°C with gentle shaking (1 cycle/s). At 15, 30, 60, and 90 min after stimulation, 1 ml of cell suspension was sampled out and $[Ca^{2+}]_c$ was measured. Experiments were repeated in five different batches of cultured cells and the mean values of $[Ca^{2+}]_c$ were plotted (Fig. 2B). A striking feature was that the sustained BCR ligation (> 30 min) of WEHI-231 cells (closed squares) induced a large increase in $[Ca^{2+}]_c$ while the same stimulation of Bal 17 cells (open circles) showed only a weak rise of $[Ca^{2+}]_c$.

3.2. Different characteristics of thapsigargin-induced SOCE in Bal 17 and WEHI-231 cells

In many kinds of cells including lymphocytes, a depletion of intracellular Ca^{2+} stores activates SOCE pathways [15,16]. Although the BCR ligation in WEHI-231 cells did not evoke an immediate Ca^{2+} response, one of plausible effects of sustained BCR ligation could be a slow emptying of Ca^{2+} stores, and the Ca^{2+} influx induced by the store-depletion might partly explain the increase of $[Ca^{2+}]_c$ in B cells. Therefore, subsequent studies were undertaken to compare the SOCE between Bal 17 and WEHI-231 cells. A general protocol to prove the presence of SOCE is 'Ca^{2+}-add-back procedure': in the absence of extracellular Ca^{2+}, an inhibitor of endoplasmic reticulum (ER) Ca^{2+}-ATPase or Ca^{2+}-releasing agonists are

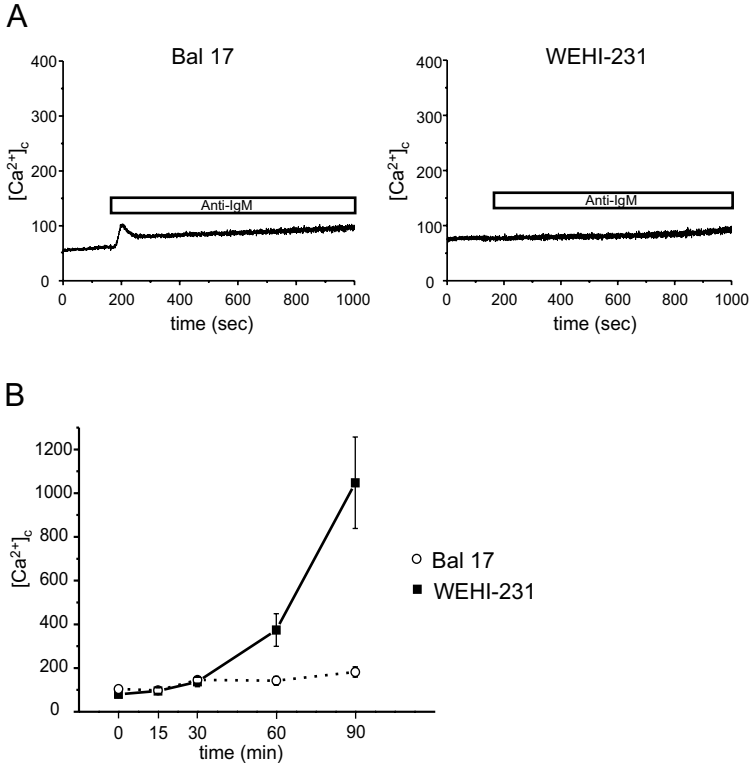

Fig. 1. Effects of BCR ligation on $[Ca^{2+}]_c$ of B cells. A: Representative traces of the $[Ca^{2+}]_c$ of Bal 17 (left panel) and WEHI-231 cells (right panel) and their response to anti-IgM antibody (2.5 μg/ml). B: Summary of the Ca^{2+} response to sustained stimulation with anti-IgM Ab. Means ± S.E. of $[Ca^{2+}]_c$ of Bal 17 (open circles) and WEHI-231 cells (closed squares) are plotted against the duration of stimulation with anti-IgM Ab ($n = 5$, respectively).

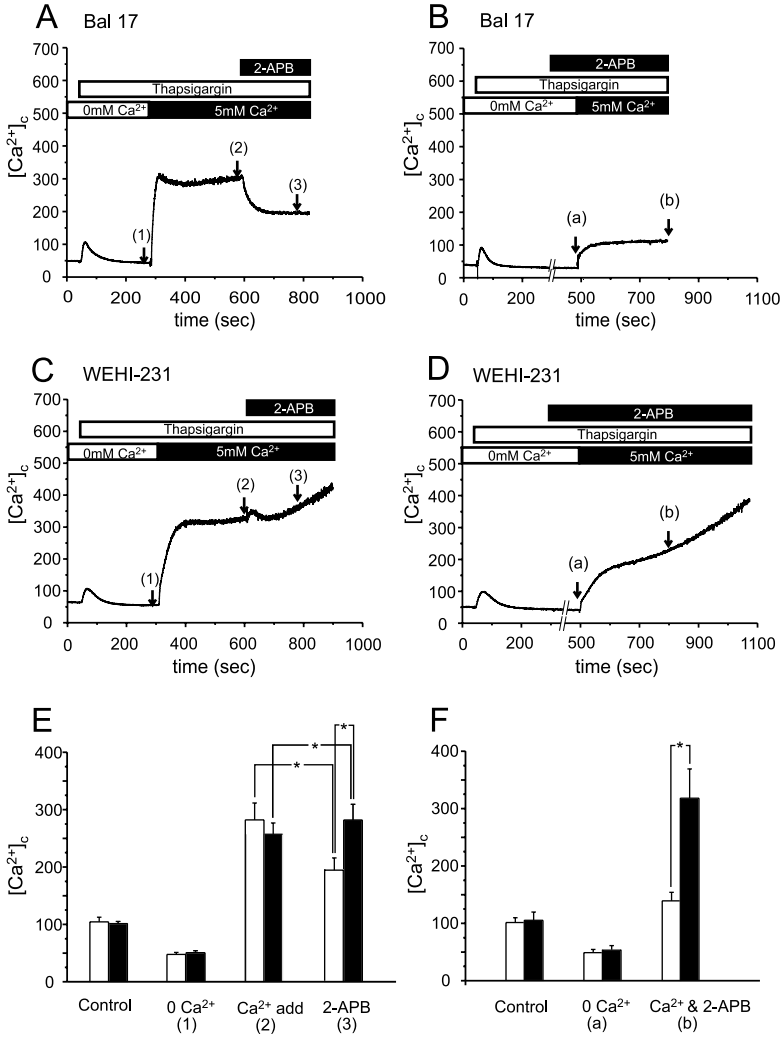

Fig. 2. Thapsigargin-induced SOCE in B cells. The extracellular Ca^{2+} was chelated by adding 1.5 mM of EGTA (0 mM Ca^{2+}). The addition of thapsigargin induced a transient increase of $[Ca^{2+}]_c$ in Bal 17 (A,B) and WEHI-231 cells (C,D). The addition of Ca^{2+} (5 mM $CaCl_2$) induced a rise of $[Ca^{2+}]_c$. in both cell-line cells ($SOCE_{Tg}$). Fifty μM 2-APB was applied either before (B,D) or after the addition of Ca^{2+} (A,C). Note that $SOCE_{Tg}$ was inhibited by 2-APB treatment in Bal 17 cells while enhanced in WEHI-231 cells. Bar graphs present the summary of the Ca^{2+} responses to experimental protocols shown in A and C (graph E), and in B and D (graph F). Open bars, Bal 17 (mean ± S.E., $n = 5$); closed bars, WEHI-231 (mean ± S.E., $n = 5$). In E and F, the mean values of initial $[Ca^{2+}]_c$ before the addition of EGTA are also shown (control). Numbers and small letters in parentheses indicate the timing of $[Ca^{2+}]_c$ measurement (see downward arrows in the original traces above). Asterisks indicate statistically significant difference ($P < 0.05$).

applied, then Ca^{2+} is added to see whether the Ca^{2+} influx was enhanced by prior depletion of Ca^{2+} store [15,16].

In this experiment, firstly, the extracellular Ca^{2+} was removed by chelating with 1.5 mM EGTA and the cells were treated with 1 μM thapsigargin which would induce a passive leak and depletion of Ca^{2+} in ER via inhibiting the Ca^{2+}-ATPase activity of ER. The application of thapsigargin to cells in Ca^{2+}-free condition (chelated by EGTA) showed a transient increase of $[Ca^{2+}]_c$ in both Bal 17 and WEHI-231 cells. On supplying extracellular $CaCl_2$ (5 mM), $[Ca^{2+}]_c$ was increased to above resting concentration in both Bal 17 and WEHI-231 cells (Fig. 2). In Bal 17 cells, the thapsigargin-induced SOCE ($SOCE_{Tg}$) was suppressed by the addition of 50 μM 2-aminoethoxydiphenyl borate (2-APB; Fig. 2A) which has been recently reported as a blocker of SOCE channel in the immune cells [17]. The blocking effect of 2-APB was more prominent when applied before the addition of $CaCl_2$ (Fig. 2B).

Opposite to the aforementioned inhibitory effect of 2-APB, $SOCE_{Tg}$ of WEHI-231 cells was not suppressed but enhanced by 2-APB. Fig. 2C demonstrates a representative result of the $SOCE_{Tg}$ of WEHI-231 cells. On adding 2-APB, with some delay, an additional increase of $[Ca^{2+}]_c$ was observed. The application of 2-APB before adding $CaCl_2$ also facilitated the $SOCE_{Tg}$ in WEHI-231 cells. In Fig. 2D, when pretreated with 2-APB, there appeared an intractable increase of $[Ca^{2+}]_c$ which did not saturate during the time observed after the addition of $CaCl_2$ (> 10 min). Summarized results from five experiments are shown as bar graphs in Fig. 2E,F.

3.3. *Combined effects of BCR ligation and Ca^{2+}-add-back procedure on $[Ca^{2+}]_c$*

In WEHI-231 cells, more than 30 min of BCR ligation was required to show a discernible increase in $[Ca^{2+}]_c$. Interestingly, when the stimulation of BCR ligation was combined with the aforementioned 'Ca^{2+}-add-back procedure', the

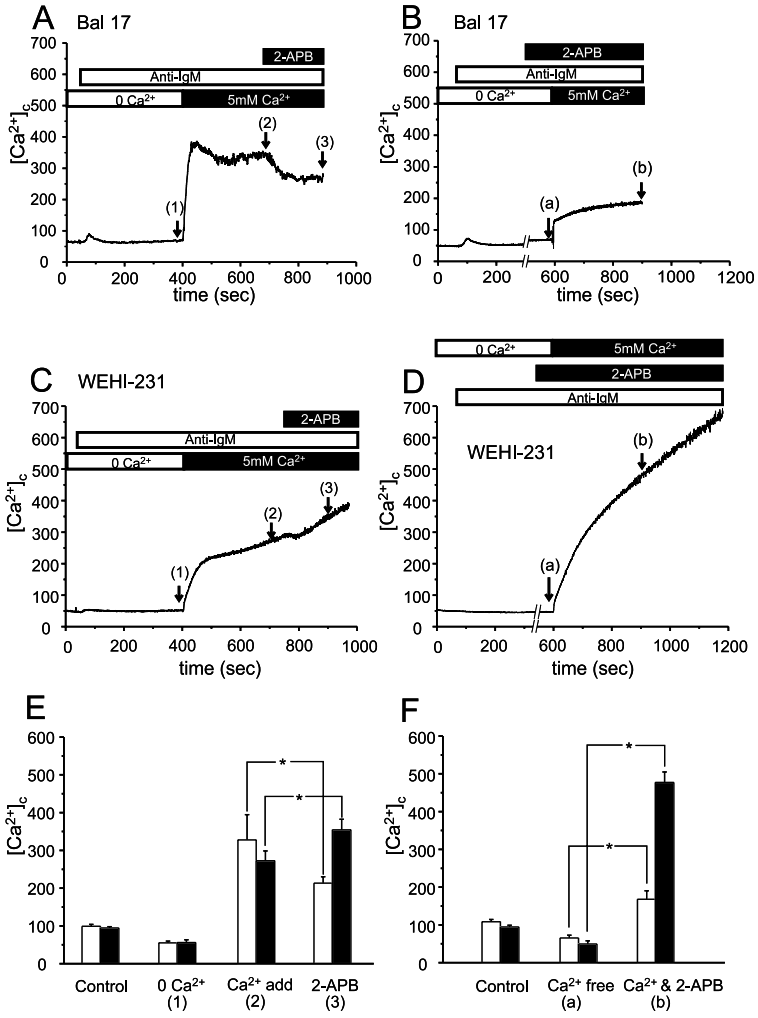

Fig. 3. Combined effects of BCR ligation and Ca^{2+}-add-back procedure in B cells. The extracellular Ca^{2+} was chelated by adding 1.5 mM of EGTA from the initial control. The application of antibody (anti-IgM) induced a transient increase of $[Ca^{2+}]_c$ in Bal 17 (A,B) while not in WEHI-231 (C,D). The addition of Ca^{2+} (5 mM $CaCl_2$) induced a rise of $[Ca^{2+}]_c$ in both cell lines. Fifty μM of 2-APB was applied either before (B,D), or after the re-addition of Ca^{2+} (A,C). Note the 2-APB-induced augmentation of Ca^{2+} response in WEHI-231 (C,D). Bar graphs present the summary of the Ca^{2+} responses to experimental protocols shown in A and C (graph E), and in B and D (graph F). Open bars, Bal 17 (mean ± S.E., $n = 5$); closed bars, WEHI-231 (mean ± S.E., $n = 5$). The timing of each measurement is indicated as downward arrows with numbers or with small letters in the original traces above. Asterisks indicate statistically significant difference ($P < 0.05$).

Ca^{2+} response of WEHI-231 cells was accelerated. For this experiment, after chelating extracellular Ca^{2+} with 1.5 mM EGTA, cells were stimulated with anti-IgM Ab for six min and then 5 mM $CaCl_2$ was added (Fig. 3).

In Bal 17 cells, the application of anti-IgM Ab induced a transient increase of $[Ca^{2+}]_c$, which we interpreted as the release of stored calcium ions (Fig. 4A,B). In this condition, the response of $[Ca^{2+}]_c$ to re-addition of $CaCl_2$ was considered as reflecting the SOCE induced by BCR cross-linking ($SOCE_{BCR}$). The $SOCE_{BCR}$ of Bal 17 cells was inhibited, although not completely, by the application of 2-APB (Fig. 3A). The inhibition of $SOCE_{BCR}$ appeared more effective when 2-APB was applied before the addition of $CaCl_2$ (Fig. 3B).

In WEHI-231 cells, the immediate Ca^{2+} response to BCR ligation was negligible and the initial increase of $[Ca^{2+}]_c$ on adding $CaCl_2$ was smaller than that of Bal 17 cells. However, the combined stimulation of BCR ligation and Ca^{2+}-add-back procedure increased the $[Ca^{2+}]_c$ of WEHI-231 cells continuously, which was not blocked, or even slightly accelerated by the addition of 2-APB (Fig. 3C). The pretreatment with 2-APB unequivocally facilitated the aforementioned combined effects in WEHI-231 cells; a slow, but intractable increase of $[Ca^{2+}]_c$ appeared on adding $CaCl_2$ after the application of 2-APB, which did not saturate during the time of experiment (Fig. 3D). Summarized results are shown as bar graphs in Fig. 3E,F.

In Figs. 2 and 3, the initial rate of Ca^{2+} increase on adding $CaCl_2$ seemed lower in WEHI-231 cells than in Bal 17 cells. To compare this, $[Ca^{2+}]_c$ was measured during the initial 15 s after the addition of $CaCl_2$, then the slope of Ca^{2+} increase was calculated. In Bal 17 cells, the initial rates of Ca^{2+} increase were 12.8 ± 1.83 and 11.1 ± 1.44 nM/s when stimulated with thapsigargin and BCR ligation, respectively ($n = 5$). In contrast, the corresponding rates of WEHI-231 cells were 4.7 ± 0.74 and 2.2 ± 0.26 nM/s, when stimulated with thapsigargin and BCR ligation, respectively ($n = 5$).

3.4. *The low Mn^{2+} permeability of SOCE pathway in WEHI-231 cells*

Mn^{2+} ions reportedly permeate SOCE channels, and the

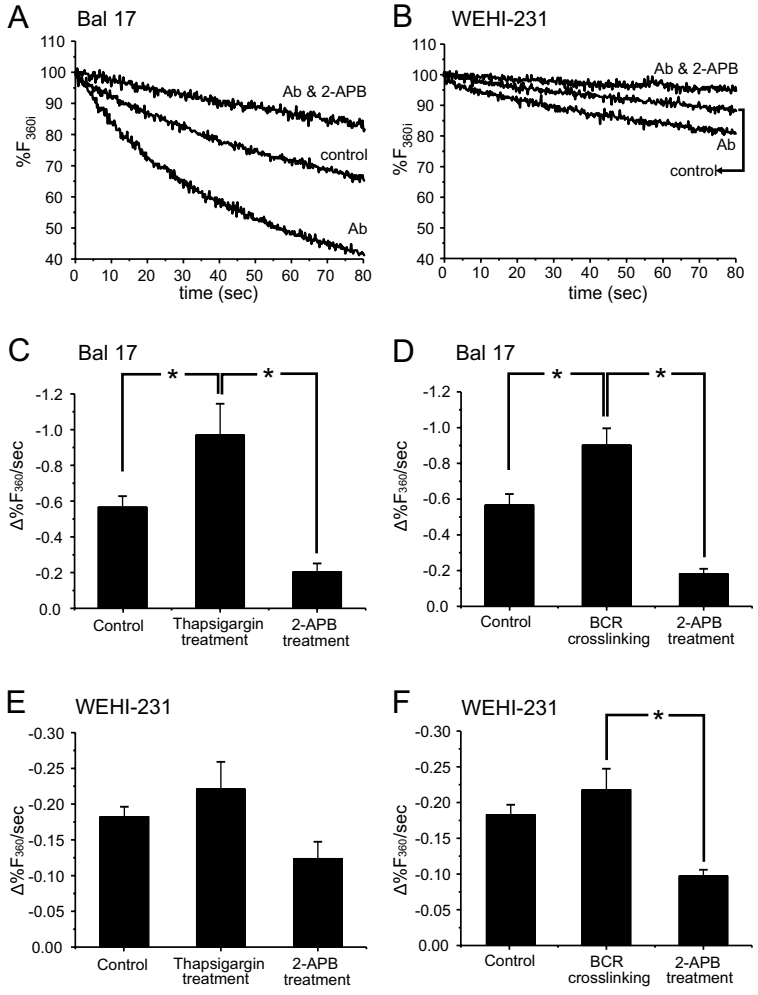

Fig. 4. Mn^{2+} influx in Bal 17 and WEHI-231 cells. A,B: Relative intensity of fura-2 fluorescence excited by isobestic wavelength of light (360 nm). The fluorescence was normalized to its initial intensity at the timing of Mn^{2+} application (0 s) under various conditions (e.g. BCR ligation with or without 2-APB). In each experiment, the decay of fluorescence between 5 and 35 s was fitted to a linear function, and the mean values of the slope were shown as bar graphs below. C–F: $n = 5$. A,C,D: Bal 17; B,E,F: WEHI-231. Asterisks indicate statistically significant difference ($P < 0.05$).

Mn^{2+}-induced decay of fura-2 fluorescence has been regarded as an indicator of SOCE activity [14]. Here the fluorescence intensity of fura-2 excited by the wavelength of 360 nm (F_{360}) was normalized to the initial value measured at the timing of Mn^{2+} application ($\%F_{360}$). Fig. 4A,B presents representative traces of Mn^{2+} quenching study where both cell lines were stimulated by BCR ligation before the addition of $MnCl_2$. The rate of $\%F_{360}$ decay was significantly increased by the application of thapsigargin or by BCR ligation in Bal 17 cells. The pretreatment with 2-APB blocked the effects of both agents on the rate of Mn^{2+} influx (Fig. 4).

Compared with Bal 17 cells, the background influx of Mn^{2+} was weak in WEHI-231 cells (Fig. 4, note different scales of vertical axis in Fig. 4C–F). Moreover, the effects of thapsigargin or BCR ligation on Mn^{2+} influx were statistically insignificant ($P > 0.05$, Fig. 4E,F, $n = 5$). The inhibitory effect of 2-APB was, however, still observed under conditions inducing $SOCE_{BCR}$ (Fig. 4F).

4. Discussion

In this study, we demonstrated that the BCR engagement of WEHI-231 cells induced an intractable increase of $[Ca^{2+}]_c$. Comparing the SOCE of Bal 17 and WEHI-231 cell lines, there were striking differences in their responses to a pharmacological agent (2-APB) which was previously reported as a blocker of SOCE channels [17].

Membrane immunoglobulin BCRs mediate a wide variety of biochemical signals such as the tyrosine phosphorylation, acceleration of phosphoinositol metabolism, increase of $[Ca^{2+}]_c$, and protein kinase C activation [1–3]. B cells demonstrate quite different cellular responses to the same kind of BCR engagement depending on their developmental stage, and the underlying signal transduction pathways between immature and mature B cells have attracted many researchers. Most of those studies have focused on the pattern of protein phosphorylation, involvement of lipid rafts and related signaling proteins whereas the knowledge of differences in the Ca^{2+} response has been limited [6,8,18–20].

The WEHI-231 lymphoma cells have been extensively studied as an example of an immature B cell line that undergoes growth arrest and apoptosis in response to membrane IgM cross-linking [8–10]. Recently it was shown that calpain, a Ca^{2+}-dependent protease, was specifically associated with

BCR-induced apoptosis of WEHI-231 cells [21]. In another report, a variant cell line of WEHI-231 cells that was resistant to the apoptotic BCR stimulation showed a reduced Ca^{2+} response compared with control WEHI-231 cells [10]. Our present data newly demonstrate that the sustained stimulation of BCR (>30 min) elicits prominent increase of $[Ca^{2+}]_c$ in WEHI-231 cells, which is never observed in Bal 17 cells, a model of mature B cells (Fig. 1). However, since the present study was confined to a single kind of cell line representing each stage of development, it should be considered with caution to directly extrapolate our results to the natural B cells in bone marrow.

In WEHI-231 cells, although the immediate Ca^{2+} response to BCR ligation was very small or indistinguishable, the sustained Ca^{2+} response appeared to be accelerated by combined application of 'Ca^{2+}-add-back procedure' (Fig. 3C). At present, it is not clear whether the stimulation of BCRs in WEHI-231 cells could induce Ca^{2+} influx simply by emptying intracellular Ca^{2+} stores or by recruiting other co-operative mechanism(s) as well. In that respect, it was notable that the Ca^{2+} response to BCR ligation was more prominent than $SOCE_{Tg}$ in WEHI-231 cells (compare Figs. 2 and 3). An intriguing interpretation might be that the intracellular event accompanying the SOCE might have augmented the original Ca^{2+} response to BCR engagement, or vice versa.

Another striking difference between WEHI-231 and Bal 17 cells was the opposite effects of 2-APB on the SOCE. 2-APB was originally described as a non-competitive antagonist of the IP_3 receptor, and the inhibition of SOCE by 2-APB has been taken as an evidence for the hypothesis of conformational coupling between store-operated channels and IP_3 receptors [16]. However, more recent works commonly suggest that 2-APB directly blocks the SOCE of various immune cells independent of the IP_3 receptor [17,22]. An opposite sensitivity of SOCE to 2-APB between two cell lines supports the hypothesis that different types of Ca^{2+}-permeable channels are expressed in WEHI-231 and Bal 17 cells.

In addition to the blocking effect of SOCE, 2-APB exerts an inhibitory effect on the Ca^{2+}-ATPase of endoplasmic reticulum (SERCA) [23]. Since the inhibition of SERCA could be different depending on the isoforms of SERCA expressed, one cannot exclude the possibility that the SERCA of WEHI-231 cells might have been more strongly inhibited by 2-APB. However, the facilitating effect of 2-APB was similarly observed under the pretreatment with thapsigargin, a well-known SERCA blocker (Fig. 2), indicating that an action mechanism of 2-APB other than SERCA inhibition was requested to explain the Ca^{2+} responses in WEHI-231 cells. Moreover, the inhibition of SERCA by 2-APB occurs at higher concentrations (IC_{50} > 300 μM) [23] than used in this study (50 μM).

Although 2-APB has facilitatory effects on the SOCE of WEHI-231 cells, it was not successful to demonstrate corresponding results in the Mn^{2+} influx experiment (Fig. 4). Recently, it was reported that physiological stimuli of human neutrophil recruit diverse SOCE pathways with differential sensitivity to 2-APB and with different permeabilities to divalent cations [24]. In a similar context, the major Ca^{2+} influx channels activated by BCR engagement in WEHI-231 cells might be practically impermeable to Mn^{2+}, which impaired the assessment of channel activity with the Mn^{2+}-quenching method. Interestingly, the minute Mn^{2+}-permeable divalent cation pathway of WEHI-231 cells was inhibited by 2-APB (Fig. 4), indicating that multiple Ca^{2+} influx pathways might exist in tested cells.

In summary, by comparing the change of $[Ca^{2+}]_c$ between Bal 17 and WEHI-231 B lymphoma cells, we demonstrated an obvious difference in the responses elicited by BCR ligation. Since the process of apoptosis could be triggered or reinforced by the tonic increase of $[Ca^{2+}]_c$, our findings may provide an explanation about the mechanism for the BCR-mediated apoptosis of WEHI-231 cells.

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