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Higher Expression of TRPM7 Channels in Murine Mature B Lymphocytes than Immature Cells

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TRPM7, a cation channel protein permeable to various metal ions such as Mg^{2+}, is ubiquitously expressed in variety of cells including lymphocytes. The activity of TRPM7 is tightly regulated by intracellular Mg^{2+}, thus named Mg^{2+}-inhibited cation (MIC) current, and its expression is known to be critical for the viability and proliferation of B lymphocytes. In this study, the level of MIC current was compared between immature (WEHI-231) and mature (Bal-17) B lymphocytes. In both cell types, an intracellular dialysis with Mg^{2+}-free solution (140 mM CsCl) induced an outwardly-rectifying MIC current. The peak amplitude of MIC current and the permeability to divalent cation (Mn^{2+}) were several fold higher in Bal-17 than WEHI-231. Also, the level of mRNAs for TRPM7, a molecular correspondence of the MIC channel, was significantly higher in Bal-17 cells. The amplitude of MIC was further increased, and the relation between current and voltage became linear under divalent cation-free conditions, demonstrating typical properties of the TRPM7. The stimulation of B cell receptors (BCR) by ligation with antibodies did not change the amplitude of MIC current. Also, increase of extracellular $[Mg^{2+}]_c$ to enhance the Mg^{2+} influx did not affect the BCR ligation-induced death of WEHI-231 cells. Although the level of TRPM7 was not directly related with the cell death of immature B cells, the remarkable difference of TRPM7 might indicate a fundamental change in the permeability to divalent cations during the development of B cells.

Key Words: B lymphocyte, TRPM7, Nonselective cation channel, Cell death, Bal-17, WEHI-231

INTRODUCTION

Bal 17 and WEHI-231 cells are representative murine B lymphoma cell lines that represent the characteristics of mature and immature B cells, respectively. The apoptotic response of WEHI-231 cells to the stimulation of cross-linking B cell receptors (BCR-ligation) is especially well known, and has been investigated as a model of stimulation-induced apoptosis of B cells at their immature stage (Igarashi et al, 1994; Wu et al, 1995; Tasker & Marshall-Clarke, 2000). Compared with the biochemical signals and their differences between these two cell lines, however, their properties of ion channels related with the Ca^{2+} signal and membrane potential are poorly understood.

We have recently reported that the WEHI-231 cells showed intractable increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in response to BCR ligation, which was not observed in their counterpart of mature B cell line, Bal-17 (Nam et al, 2003). In addition, the investigation of ion channels in WEHI-231 cells by using patch clamp techniques found that novel types of background K^+ channels with big conductance (340 pS, BK_{bg}) were expressed, which was very rarely expressed in the mature B cell model, Bal-17. The BK_{bg} channels in WEHI-231 are under the tonic inhibitory control of MgATP, and this inhibitory effect of MgATP is mediated by the phosphoinositide (PI) phosphorylation mechanism (Nam et al, 2004).

Recently, a nonselective cation channel called TRPM7 (abbreviation of transient receptor potential membrane melastatin 7 ion channel) was found in various cells, including DT-40 B cells. The TRPM7 activity is inhibited by intracellular Mg^{2+} ion (MIC, Magnesium Inhibited Cation current) or MgATP (I_{MagNuM}, Magnesium Nucleotide-inhibited Metal ion current) (Nadler et al, 2001; Runnels et al, 2001; Hermosura et al, 2002). The MIC channels (TRPM7) show outwardly rectifying voltage-dependence in physiological solution. Although the inward current through TRPM7 is small under physiological conditions, TRPM7 is permeable to various divalent cations, including Ca^{2+} and Mg^{2+}. Due to their permeability to Mg^{2+} and sensitivity to intracellular Mg^{2+}, TRPM7 channels have been sug-

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ABBREVIATIONS: TRPM7, melastatin 7 type of transient receptor potential channel; MIC, magneium inhibited cation current; I_{MagNuM}, magnesium nucleotide-inhibited meta lion current; 2-APB, 2-aminoethoxydiphenyl borate; BCR, B cell antigen receptor; $[Ca^{2+}]_c$, cytosolic Ca^{2+} concentration.

gested to play a central role in cellular Mg^{2+} homeostasis and viability of cells (Nadler et al, 2001; Schmitz et al, 2003). In addition to the Mg^{2+} homeostasis and the potential Ca^{2+} influx pathway, the permeability of TRPM7 to various divalent cations make it the best candidates for cellular entry of trace metal ions like Zn^{2+} (Monteilh-Zoller et al, 2003). In addition to TRPM7, other members of TRPM family proteins (TRPM2, TRPM4, TRPM5) are expressed in various cell lines of lymphocytes and have been suggested to play intriguing roles in the modulation of immune responses (Perraud et al, 2004).

As mentioned above, not only the targeted deletion of TRPM7 in a chicken B-cell line induced growth arrest and cell death, but also the over-expression killed HEK cells within 24 h (Nadler et al, 2001; Schmitz et al, 2003). Thus, it is highly likely that the endogenous TRPM7 activity is tightly regulated. The vital role of TRPM7 played in the Mg^{2+} homeostasis lead us to speculate that there might be a significant difference in the levels of TRPM7 during the developmental stage of B cells. In this study, therefore, the amplitude of MIC (I_{MagNuM}) and the level of transcripts (mRNA of TRPM7) were compared between WEHI-231 and Bal-17 cells.

METHODS

Cells and agents

Mouse B lymphocytes with properties of immature B cells (WEHI-231) and mature B cells (Bal 17) were grown in 25 mM HEPES RPMI 1640 media (Gibco, Grand Island, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, USA), 50 μM 2-mercaptoethanol (Sigma, St. Louis, USA), and 1% penicillin/streptomycin (Gibco). All cells were incubated at 37°C in 95% O_2/ 5% CO_2. The pipette solution for whole-cell patch clamp contained (in mM) 145 Cs-methansulfonate, 8 NaCl, 10 HEPES, 3.6 $CaCl_2$ and 10 EGTA with pH 7.2 (titrated with CsOH), and the calculated free Ca^{2+} activity was about 80 nM/L. The normal bath solution for the whole-cell patch clamp contained (in mM) 145 NaCl, 3.6 KCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 5 glucose, and 10 HEPES with pH 7.4 (titrated with NaOH). For each cell, the size of membrane capacitance was obtained for later standardization of current amplitudes. 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, USA).

Electrophysiology

The whole-cell patch clamp (Axopatch-1D, Axon Instruments, Forster City, USA) was performed at room temperature (22~25°C) in a bath mounted on the stage of an inverted microscope (IX-70, Olympus, Osaka, Japan). pCLAMP software v.7.0 and Digidata-1200A (both from Axon Instrument) were used for the acquisition of data and the application of command pulses. Membrane capacitance was measured in each cell by using the capacitance cancellation circuit of Axopatch-1D amplifier. Data were represented as mean$\pm$*S.E.M.* Student's t test was used to test for significance at the level of 0.05.

Mn^{2+}-quench experiment

At the isobestic wavelength of 360 nm, the fura-2 fluorescence intensity is not influenced by $[Ca^{2+}]_c$, but quenched by Mn^{2+}. The slope of fluorescence quenching trace is regarded as an index of divalent cation influx (Merrit et al, 1989). 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 the measurement of Mn^{2+} entry, background fluorescence was confirmed by adding 5 μM ionomycin to convert fluorescence intensity into normalized percent scale.

RT-PCR

Total RNA was isolated using the RNA STAT-60 (TEL-TEST Inc., Friendswood, TX). First-strand cDNA was synthesized from 1 μg of total RNA and oligo d(T) primed reaction by SuperScript™ reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's instructions. PCR (PTC-0150 MiniCycler™; MJ Research Inc., Waltham, MA) reaction was performed using 2.5 units of Taq polymerase (Takara Bio Inc., Shiga, Japan) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM each of dATP, dTTP, dGTP and dCTP, 10 μM each of sense and antisense primers, and DNA template. Temperature cycling proceeded as follows: 1 cycle at 95°C for 5 min and 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min. For semi-quantitative analysis of RT-PCR products, β-actin, a housekeeping gene, was used as an internal standard control. PCR products were then subjected to gel electrophoresis on a 1.5% agarose gel containing ethidium bromide. Finally, images of DNA gel were digitally captured by a gel documentation system (Gel Doc 2000, Bio-Rad LSD, USA), and the intensities of each band were quantified by an image analyzing software (Quantity One, Bio-Rad LSD).

The primers used were as follows: mTRPM 7 sense 5'-TCT GTG AGT ACC CCA TCC-3' ; mTRPM7 antisense 5'-TCT GTG AGT ACC CCA TCC-3' ; β-actin sense 5'-AAG ATC CTG ACC GAG CGT GG-3'; β-actin antisense 5'-AAG ATC CTG ACC GAG CGT GG-3', and their specificity was verified by restriction enzyme digestion of the PCR products. The amplified fragment of TRPM 7 was digested with *EcoRV* (Roche Molecular Biochemicals) to confirm the digested result with expected size.

Cell death assay

WEHI-231 cells were seeded in 6-well plates at 2.5×10^5 cells/mL in complete medium and grown for 24 h. The cells were treated with 2.5 μg/mL of the rabbit $F(ab')_2$ anti-mouse IgM antibody (Zymed Laboratories Inc.) and different concentrations of $MgCl_2$. At the end of the incubation, Trypan Blue dye was added to cells on a hematocytometer slide at a ratio of 1:1, and preparations were viewed under a standard light microscope. The percentage of dead cells was determined by the following formula; percent cell death=100$\times$(number of dead cells)/(total number of cells).

RESULTS

After making a whole-cell configuration with Bal-17 or WEHI-231 cells, depolarizing ramp pulses were applied repetitively at every 5 s. The current to voltage relation (I/V curves) of Bal-17 cells thus obtained clearly indicated spontaneous development of outwardly rectifying current that was reversed at 0 mV, an expected property of nonselective cation channels under this particular experimental condition (Fig. 1A). The steady-state development of outwardly rectifying current typically took four to six minutes, and the maximum amplitudes at 100 mV ranged from 800 to 2000 pA (202±12.9 pA/pF, n=34) in Bal-17 cells. In WEHI-231 cells, basically similar pattern of outwardly rectifying current was induced by the dialysis with Mg^{2+}/ATP-free pipette solution (Fig. 1B). However, the amplitude of current developed was much smaller than that of Bal-17 (33±4.4 pA/pF at 100 mV, n=35, Fig. 1C).

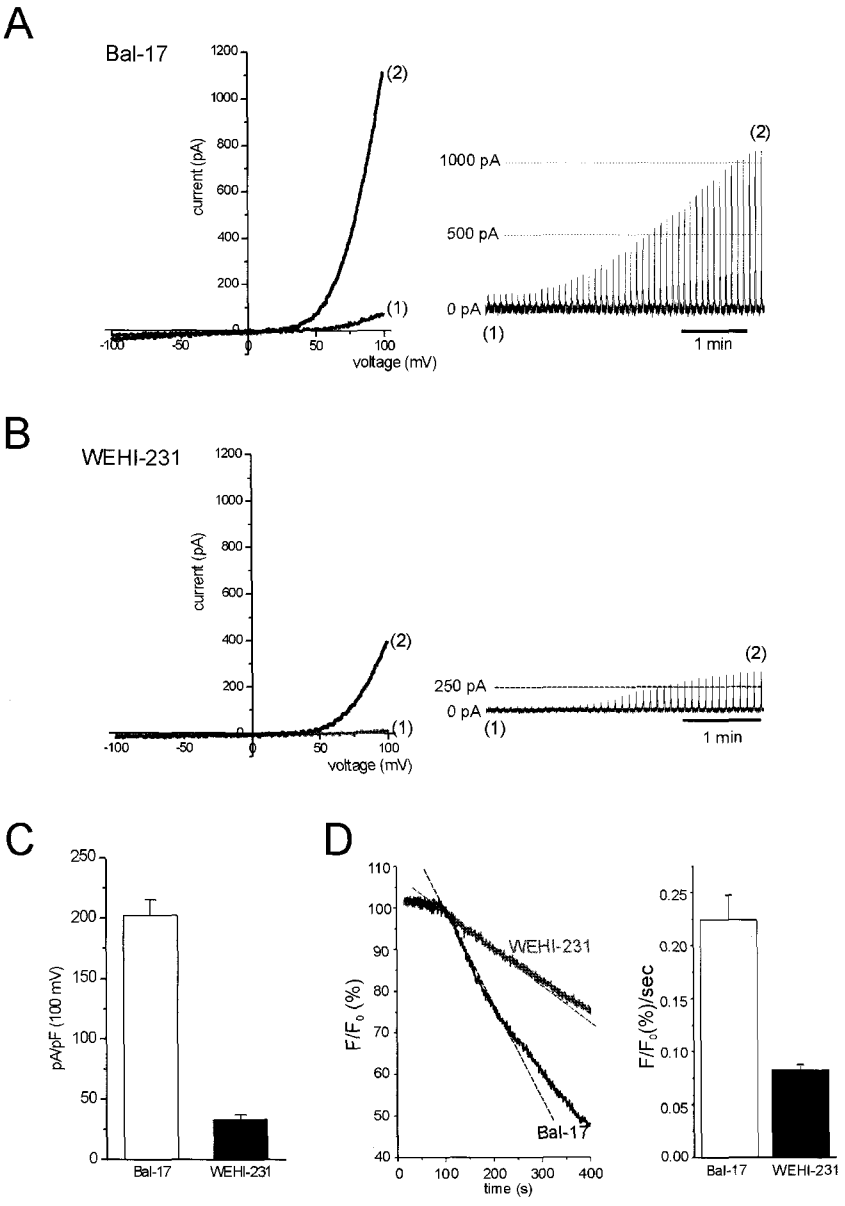

Fig. 1. MIC current and divalent cation influx in B lymphocytes. (A, B) Ramp depolarizing pulses (from −100 to 100 mV, 0.4 V/s) were repetitively applied at every 5 s. The left panels are the current/voltage relations (I/V curve) of the initial (1) and steady-state (2). The original chart traces demonstrates the spontaneous development of outwardly rectifying currents (right panels). (C) Summary of the peak amplitudes (at 100 mV, normalized to the membrane capacitance) of steady-state MIC current. (D) Mn^{2+} quenching study. Left column, trace of fura-2 fluorescence (360 nm) normalized to the control level (F/F_0 (%)). Right column, summary of fluorescence decay rate (dotted lines of the left column) measured in Bal-17 and WEHI-231 cells.

In both Bal-17 and WEHI-231 cells, an addition of 2 mM $MgCl_2$ to the pipette solution blocked almost completely the development of the outwardly rectifying current (n = 5, data not shown), consistent with the property of MIC current (Nadler et al, 2001; Runnels et al, 2001; Kozak & Kahalan, 2003).

Because the MIC channels are permeable to various divalent cations, the membrane permeability to Mn^{2+} was compared. In both Bal-17 and WEHI-231 cells loaded with fura-2, the addition of Mn^{2+} (0.2 mM) to the divalent-free bath solution continuously decreased fura-2 fluorescence (Mn^{2+}-quenching technique, see Methods). The slope of decay indicated the background permeability to divalent cations, which was about three fold higher in Bal-17 cells than WEHI-231 cells (Fig. 1D).

A well-known electrophysiological property of MIC current is that the outwardly rectifying I/V curve becomes linear by removing the extracellular divalent cations (diva-

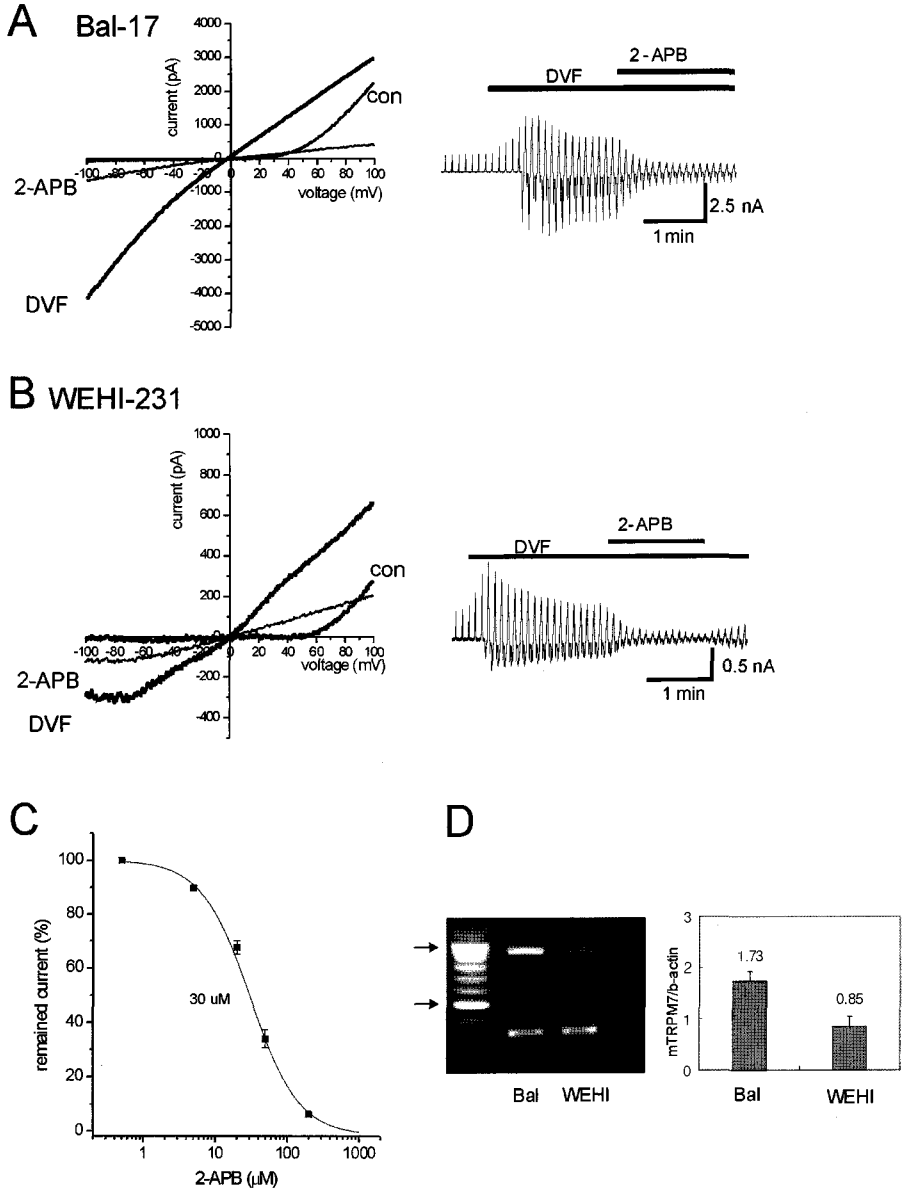

Fig. 2. Effects of DVF and 2-APB consistent with the presence of TRPM7 transcripts in B lymphocytes. (A, B) Steady-state I/V curves of MIC current obtained in normal bath solution (con), divalent-free (DVF) condition, and application of 50 μM 2-APB. Original chart traces of membrane currents reflect the overall effects of DVF and 2-APB (right columns). (C) Concentration-dependent inhibition of MIC current by 2-APB. The remaining current at 100 mV was normalized and means ± S.E. were fitted by the function $\{I/I_{con} \times 100\ (\%) = 100/(1 + (\text{tested concentration}/IC_{50})^n)\}$. The IC_{50} is 30 μM. (D) RT-PCR analysis for TRPM7 and β-actin. Higher level of TRPM7 signal was observed in Bal-17 than WEHI-231 cells. The intensity of TRPM7 was normalized to the β-actin signal and the summarized results are shown (n=7, right panel).

lent-free, DVF) (Hermosura et al, 2002; Monteilh-Zoller et al, 2003). In Bal-17 cells, after confirming the steady-state MIC in normal bath solution, the bath perfusate was changed to DVF solution. The DVF condition initially increased the outward current and then induced inward current. At the steady-state in DVF solution, the slope of I/V curve showed a linear shape or slight inward rectification in Bal-17 cells (Fig. 2A). The MIC of WEHI-231 cells was also increased and the I/V curve became linear in DVF solution, although the amplitude of current was much smaller than Bal-17 (Fig. 2B). 2-aminoethoxydiphenylborate (2-APB), a blocker of the $InsP_3$-receptors and various cation channels, has also recently been shown to block the MIC (Hermosura et al, 2002; Prakriya & Lewis, 2002). As seen in Fig. 2C, the MIC of Bal-17 was also blocked by 2-APB in a concentration-dependent manner (Fig. 2C). Similar decrease of the current amplitude by 50 μM 2-APB was confirmed in WEHI-231 cells (n=3, data not shown).

Among the cloned TRP cation channels in vertebrate cells, TRPM7 and hetero-oligomer of TRPM6 and TRPM7 show intracellular Mg^{2+}-sensitive outwardly rectifying cationic currents (Nadler et al, 2001; Chubanov et al, 2004). The semi-quantitative RT-PCR analysis demonstrated that both Bal-17 and WEHI-231 expressed TRPM7, and that the normalized level of TRPM7 in the Bal-17 was about two fold higher than WEHI-231 (Fig. 2D). However, neither of them were found to express TRPM6 in six trials or RT-PCR with three different sets of primers (data not shown).

Since the stimulation of B cell receptors by cross-linking the membrane IgM-like receptors (BCR-ligation) evokes various intracellular signals, a question arises whether the BCR-ligation changes the MIC currents in Bal-17 and WEHI-231 cells. Thus both Bal-17 and WEHI-231 cells were stimulated with anti-IgM antibody (1.5 μg/ml) for 15-20 hours, then the peak amplitude of MIC currents were measured. Although a number of Bal-17 cells stimulated by the BCR-ligation showed large MIC currents (right panel of Fig. 3A as an example), the difference was not statistically significant (Fig. 3B). In WEHI-231 cells after 15~20 hours of BCR-ligation, we also measured the amplitudes of MIC current, and found no difference from the control level (Fig. 3B).

As mentioned in *Introduction*, it has recently been proposed that the TRPM7 channels fulfill a central ubiquitous role in mediation of Mg^{2+} uptake in vertebrate cells and cell viability (Nadler et al, 2001; Schmitz et al, 2003; Montell, 2003). Thus, we tested whether higher driving force for Mg^{2+} influx might rescue the apoptosis of WEHI-231 cells. The number of dead WEHI-231 cells were counted by trypan blue exclusion assay at 4, 6, 14, and 20 hours after the application of anti-IgM antibody

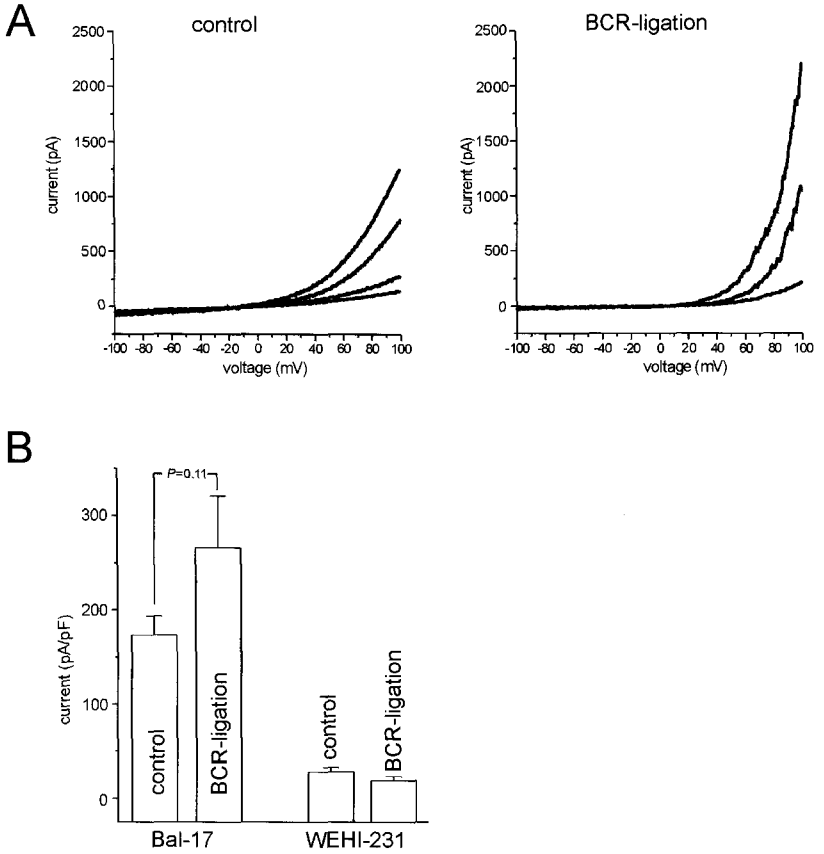

Fig. 3. Test of BCR-ligation on the MIC currents. (A) Representative I/V curves reflecting the development of MIC current in control (left) and BCR-ligated (right) Bal-17 cells. For BCR-ligation, cells were treated with anti-IgM Ab (2.5 μg/ml) for 15 20 hours. (B) Summary of the effects of BCR-ligation on MIC currents in Bal-17 and WEHI-231 cells.

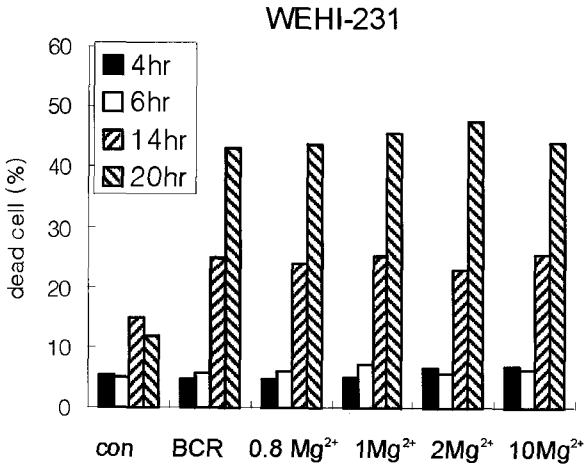

Fig. 4. No effects of increasing $[Mg^{2+}]_{ext}$ on the BCR-ligation-induced death of WEHI-231 cells. The number of dead cells were counted at 4, 6, 14 and 20 hours after the BCR-ligation (anti-IgM Ab, 2.5 μg/ml) in each culture dish and displayed as a bar graph. The same procedure was repeated after the addition of $MgCl_2$, as indicated in the bar graph.

(BCR-ligation) to the cell culture with different concentrations of $MgCl_2$. The proportion of dead cells after the BCR-ligation, however, was not changed by the increase of $MgCl_2$ up to 10 mM (Fig. 4).

DISCUSSION

In this study, both the whole-cell patch clamp and the RT-PCR studies indicate that MIC (TRPM7) channels are several folds higher in Bal-17 than WEHI-231 cells. The higher permeability of Bal-17 cells to the divalent cation is also evident, based on the Mn^{2+} quenching experiment (Fig. 1D). Compared with previous reports, the level of native MIC current in Bal-17 (202 ± 12.9 pA/pF at 100 mV) seems to be significantly larger than other types of cells which are not over-transfected with TRPM7. In fact, the size of MIC current in WEHI-231 (30 pA/pF at +100 mV) is comparable with those obtained in microglia (Jiang et al, 2003), HEK-293, RBL-2H3, and Jurkat-T cells (Nadler et al, 2001).

Among the Ca^{2+} permeable channels characterized in the immune cells, the Ca^{2+}-release-activated Ca^{2+} (CRAC) channel is the most widely known, and it is typically observed under depletion of intracellular Ca^{2+} stores. However, it has recently been found that many of the previous studies on CRAC currents were 'contaminated' by MIC channels since they were performed in divalent-free condition and notably omission of Mg^{2+}/MgATP from the pipette solution (Bakowski & Parekh, 2002; Hermosura et al, 2002; Prakriya & Lewis, 2002). In our present study, for the selective recording of MIC current, the $[Ca^{2+}]_c$ was clamped at 80 nM to prevent the store depletion and the activation of CRAC channels (Hermosura et al, 2002; Prakriya & Lewis, 2002).

Since the expression of TRPM7 is very critical for the survival and Mg^{2+} homeostasis of cells (Nadler et al, 2001; Montell, 2003; Schmitz et al, 2003), we initially thought that the lower expression of TRPM7 in WEHI-231 immature B lymphocytes might be relevant with the distinctive apoptotic responses to the BCR-ligation. However, a simple addition of $MgCl_2$ to the extracellur medium did not change the apoptotic tendency of WEHI-231 cells. Since the free Mg^{2+} in the cytosol is already maintained at millimolar or submillimolar concentrations, close to the extracellular $[Mg^{2+}]$, some fluctuation of Mg^{2+} influx might result in negligible changes of the intracellular $[Mg^{2+}]$.

The TRP family with four sub-families (TRPC, TRPV, TRPM and TRPP) comprises 25 types of channels with six trans-membrane domains (Clapham, 2003). Among the eight types of TRPM channels, the TRPM6 and TRPM7 are unique because they also contain functional kinase domains, whose function is a matter of controversy (Runnels et al, 2001; Montell, 2003; Dorovkov & Ryazanov, 2004; Perraud et al, 2004). In addition to the Mg^{2+} homeostasis and the potential Ca^{2+} influx pathway, the permeability of TRPM7 to various divalent cations make it the best candidate for cellular entry of trace metal ions like Zn^{2+} (Monteilh-Zoller, 2003).

In human, the mutation of TRPM6 causes hypomagnesemia (Schlingmann et al, 2002) due to failure of hetero-oligomerization with TRPM7 and membrane trafficking (Chubanov et al, 2004). Since the mRNAs for TRPM6 were not found in this study (data not shown), only the homomers of TRPM7 seem to be responsible for the MIC current in B lymphocytes. The absence of TRPM6 is consistent with the reported expression pattern; TRPM6 is specifically expressed in kidney and small intestine, two organs known to play a key role in controlling Mg^{2+} reabsorption (Schlingmann et al, 2002; Perraud et al, 2004).

It has been reported that TRPM7 channels are under the regulation of membrane phosphoinositides (PIP_2) (Runnels et al, 2003). Since the stimulation of B cells increases PIP_2 synthesis (Saito et al, 2003), we speculated that the persistent BCR-ligation might increase the amplitudes of MIC. In Bal-17 cells, although the amplitudes of MIC currents showed an increasing tendency upon the BCR-ligation, the change was not statistically significant (Fig. 3A and B). Similar study was also performed in the microglial cells where the MIC (TRPM7) current density was not affected by culturing in a medium containing microglia activating agents (Jiang et al, 2003).

In summary, we found TRPM7 channel current in Bal-17 and WEHI-231 B cell lines that represent mature and immature stages of development, respectively. Although the TRPM7 current was not sensitively regulated by extracellular signals, a large difference in the level of TRPM7 expression might suggest a role in the development of B lymphocytes. Further investigation about the role of TRPM7 proteins in the development of native B lymphocytes would be of an intriguing theme.

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