Korean J Physiol Pharmacol
Vol 10: 19−24, February, 2006

Differential Functional Expression of Clotrimazole-sensitive Ca^{2+}-activated K^+ Current in Bal-17 and WEHI-231 Murine B Lymphocytes

Haifeng Zheng, Jae Hong Ko, Joo Hyun Nam, Yung E Earm, and Sung Joon Kim

Department of Physiology and Biophysics, Seoul National University College of Medicine, Seoul 110−799, Korea

The intermediate conductance Ca^{2+}-activated K^+ channels (SK4, IKCa1) are present in lymphocytes, and their membrane expression is upregulated by various immunological stimuli. In this study, the activity of SK4 was compared between Bal-17 and WEHI-231 cell lines which represent mature and immature stages of murine B lymphocytes, respectively. The whole-cell patch clamp with high-Ca^{2+} (0.8 μM) KCl pipette solution revealed a voltage-independent K^+ current that was blocked by clotrimazole (1 mM), an SK4 blocker. The expression of mRNAs for SK4 was confirmed in both Bal-17 and WEHI-231 cells. The density of clotrimazole-sensitive SK4 current was significantly larger in Bal-17 than WEHI-231 cells (−11.4±3.1 Vs. −5.7±1.15 pA/pF). Also, the chronic stimulation of B cell receptors (BCR) by BCR-ligation (anti-IgM Ab, 3 μg/ml, 8∼12 h) significantly upregulated the amplitude of clotrimazole-sensitive current from −11.4±3.1 to −53.1±8.6 pA/pF in Bal-17 cells. In WEHI-231 cells, the effect of BCR-ligation was significantly small (−5.7±1.15 to −9.0±1.00 pA/pF). The differential expression and regulation by BCR-ligation might reflect functional changes in the maturation of B lymphocytes.

Key Words: B lymphocyte, Potassium channel, Ca^{2+}-activated K^+ channel, Clotrimazol, WEHI-231, Bal-17

INTRODUCTION

K^+ channels in immune cells are involved in various cell functions, including the control of membrane potential, differentiation, and cell-volume regulation. The voltage-gated Kv1.3 channel and the Ca^{2+}-activated intermediated-conductance K^+ channels, named SK4 (IKCa1), are predominant K^+ channel families expressed in lymphocytes (George-Chandy et al, 2004). There are four members (SK1-3 and SK4) of Ca^{2+}-activated K^+ channels that form Ca^{2+}-activated, small- to intermediate-conductance K^+ channels in various excitable and non-excitable tissues. SK4, also named as IKCa1 because of their intermediate conductance, are expressed in a variety of epithelial tissues and lymphocytes (Joiner et al, 2001). Differing from SK1-3, SK4 are insensitive to apamin, but can be inhibited by clotrimazole and charybdotoxin (Panyi, 2005). SK1-3 and SK4 are activated by higher than 100 nM $[Ca^{2+}]_i$ and exhibit weak inward rectification in symmetrical potassium solution.

In lymphocytes, Ca^{2+} influx activates SK4, and subsequent membrane hyperpolarization maintains the electrical driving force for the sustained Ca^{2+} entry (Fanger et al, 2001). Antigenic stimuli of T lymphocyte have been reported to upregulate the expression of SK4 channels and the membrane current activity (George-Chandy et al, 2004). Recent studies have also shown that SK4 channels of the murine spenic B cells and human peripheral B lymphocyte are upregulated by antigenic stimuli (Partiseti et al, 1993; Wulff et al, 2004).

Because the responses of lymphocytes to the same antigenic stimuli vary widely depending on their maturation stages, the underlying mechanisms for such variability are of interest to investigate. Although the differential expression of K^+ channels during mouse T cell development has been recognized in the early period of patch clamp studies (McKinnon & Ceredig, 1986), no further investigation has been performed as far as we are aware of.

In the bone marrow, the immature B lymphocytes die via apoptosis processes by cross-linking membrane-bound IgM-type B cell receptors (BCR-ligation). The apoptosis of stimulated immature B lymphocytes is a negative selection that is crucial in avoiding self-reactivity. In contrast, the mature B lymphocytes of BCR ligation with specific antigen undergo proliferation and differentiation into plasma cells and memory B cells (DeFranco, 2000; Kurosaki, 2002). We recently compared ion channels and Ca^{2+} signals between Bal-17 and WEHI-231 cell lines that represent mature and immature stages of murine B lymphocytes, respectively. Briefly summarizing, a chronic stimulation of B cell receptors (BCR-ligation) induced several-fold higher rise of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$[k1]) in WEHI-231 than Bal-17 cells (Nam et al, 2003). WEHI-231 cells expressed

Corresponding to: Sung Joon Kim, Department of Physiology and Biophysics, Seoul National University College of Medicine, 28 Yeongeon-dong, Jongno-gu, Seoul 110-799, Korea. (Tel) 82-2-740-8230, (Fax) 82-2-763-9667, (E-mail) sjoonkim@snu.ac.kr

ABBREVIATIONS: BCR, B cell receptor; SK, small conductance Ca^{2+}-activated K^+ channel; IKCa1, intermediate-conductance Ca^{2+}-activated K^+ current; SOCE, store-operated Ca^{2+}-entry.

higher level of background K^+ channels and lower level of TRPM7 cation channels than Bal-17 cells (Nam et al, 2004; Kim et al, 2005). In spite of the importance of SK4 in lymphocytes, however, the differential functional expression of SK4 between mature and immature B lymphocytes has not been compared. In this study, we compared the expression of the Ca^{2+}-activated K^+ current in Bal-17 and WEHI-231 cells. Also, changes in the functional expression of SK4 after BCR-ligation were analyzed.

METHODS

Cell culture

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 (Invitrogen, Seoul, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 50 μM 2-mercaptoethanol (Sigma Korea, Seoul, Korea), and 1% penicillin/streptomycin (Invitrogen, Seoul, Korea). All cells were incubated at 37°C in 95% O_2 and 5% CO_2.

Electrophysiology

Cultured cells were transferred into a bath mounted on the stage of an inverted microscope (IX-70, Olympus, Osaka, Japan). The bath (0.15 ml) was superfused at 5 ml/min, and voltage clamp experiments were performed at room temperature (22~25°C). Patch pipettes with a free-tip resistance of about 2.5 megohms were connected to the head stage of a patch-clamp amplifier (Axopatch-1D, Axon Instruments). Liquid junction potentials were corrected with an offset circuit before each experiment. Unless mentioned otherwise, a conventional whole-cell clamp was achieved by rupturing the patch membrane after making a giga-seal. A steady-state perforation was usually achieved within 5 min after making a giga-seal. pCLAMP software version 7.0 and Digidata-1322A (both from Axon Instrument) were used for the acquisition of data and the application of command pulses. The resting membrane potential described in this study was measured under the zero-current clamp condition of the whole-cell patch clamp. Voltage and current data were low pass filtered (5 kHz), stored in a Pentium-grade computer and analyzed using pCLAMP software version 9.0 and Origin version 6.1 (Microcal Software Inc., Northampton, MA). 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.

Experimental solutions

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 a pH of 7.4 (titrated with NaOH). High KCl bath solution was used (in mM): 140 KCl, 5 NaCl, 1 $MgCl_2$, 1.3 $CaCl_2$, 5 glucose, and 10 HEPES, at pH 7.4 (titrated with KOH). The Ca^{2+}-free pipette solution contained (in mM) 135 KCl, 6 NaCl, 0.5 $MgCl_2$, 3 MgATP, 10 EGTA and 5 HEPES at pH 7.2 (titrated with KOH). The 0.8 μM free calcium pipette solution (high Ca^{2+}-KCl solution) contained (in mM) 135 KCl, 6 NaCl, 0.5 $MgCl_2$, 3 MgATP, 10 EGTA, 5 HEPES and 8.4 $CaCl_2$ at pH 7.2 (titrated with KOH).

RT-PCR

RNAs were extracted from Bal-17 and WEHI-231 cells by guanidinium acid-phenol-chloroform method. RNA quantification was assessed at 260 nm. Single-strand cDNA was synthesized from 5 μg of total RNA using M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The cDNA was amplified by PCR with specific primers. The sequences of the sense- and antisense primers were 5'-CGGGGCACCT CACAGACACACT-3', 5'-CGCCGCTGACTCCTTCATCTCT-3', respectively (Tamarina et al, 2003). Conditions for PCR reaction were 1x (94°C, 5 min); 30x (94°C, 1 min; 63°C, 1 min and 72°C, 1 min); and 1x (72°C, 1 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

RESULTS

To establish whether SK4 is present in the murine B lymphocyte, the clotrimazole-sensitive outward K^+ current was analyzed by using the conventional whole-cell patch clamp method. To obtain a brief current-voltage relation (I/V curve), ramp-like depolarizing pulses from -100 to 100 mV (0.1 V/s) were applied to Bal-17 and WEHI-231 cells (Fig. 1A, B). The I/V curves obtained with high Ca^{2+}-KCl pipette solution showed significantly larger outward current than those obtained with Ca^{2+}-free KCl in the pipette, indicating the presence of Ca^{2+}-activated K^+ conductance. With high Ca^{2+}-KCl pipette solution, the SK4 channel blocker clotrimazole (1 μM) markedly decreased the outward current from 596.6$\pm$51.17 to 307.7$\pm$29.66 [k2]pA at 80 mV in Bal-17 cells. With Ca^{2+}-free KCl in the pipette, the size of the outward current inhibited by clotrimazole was greatly reduced. However, a partial decrease was consistently observed especially at depolarized clamp voltages (315.0$\pm$44.4 to 249.6$\pm$34.61 [k3]pA, Fig. 1A right panel). Similar results were obtained in WEHI-231 cells which were dialyzed with either high Ca^{2+}- or Ca^{2+}-free KCl pipette solution (Fig. 1B). In general, however, the amplitude of outward current was smaller in WEHI-231 than in Bal-17 cells, as summarized in Fig. 3. As a next step, RT-PCR analysis was performed in Bal-17 and WEHI-231 cells to examine whether the cells contain the molecular candidate of SK4 channels. Both Bal-17 and WEHI-231 cells showed positive RT-PCR product at the expected size (Fig. 1C).

The sensitivity to clotrimazole and RT-PCR analysis indicate the expression of SK4 in the murine B cell lines. To precisely analyze the amplitude of SK4 current, pharmacological blocker specific for SK4 is required. However, the substantial decrease of outward current by clotrimazole even in the Ca^{2+}-free pipette solution was questionable for the usefulness of the clotrimazole-sensitive outward current as an indicator of SK4 current. As shown in Fig. 3, the question of pharmacological specificity is crucial in WEHI-231 cells where the total outward current is generally smaller than Bal-17 cells. For this reason, the experimental protocol was modified hereafter.

In addition to their Ca^{2+}-dependence, SK4 is characterized by the weakly inwardly rectifying property of I/V curves. Such properties imply that the clotrimazole-sensitive current at negative clamp voltage would reflect more specifically the activity of SK4. For the purpose of measuring inward K^+ current, we replaced the extracellular NaCl

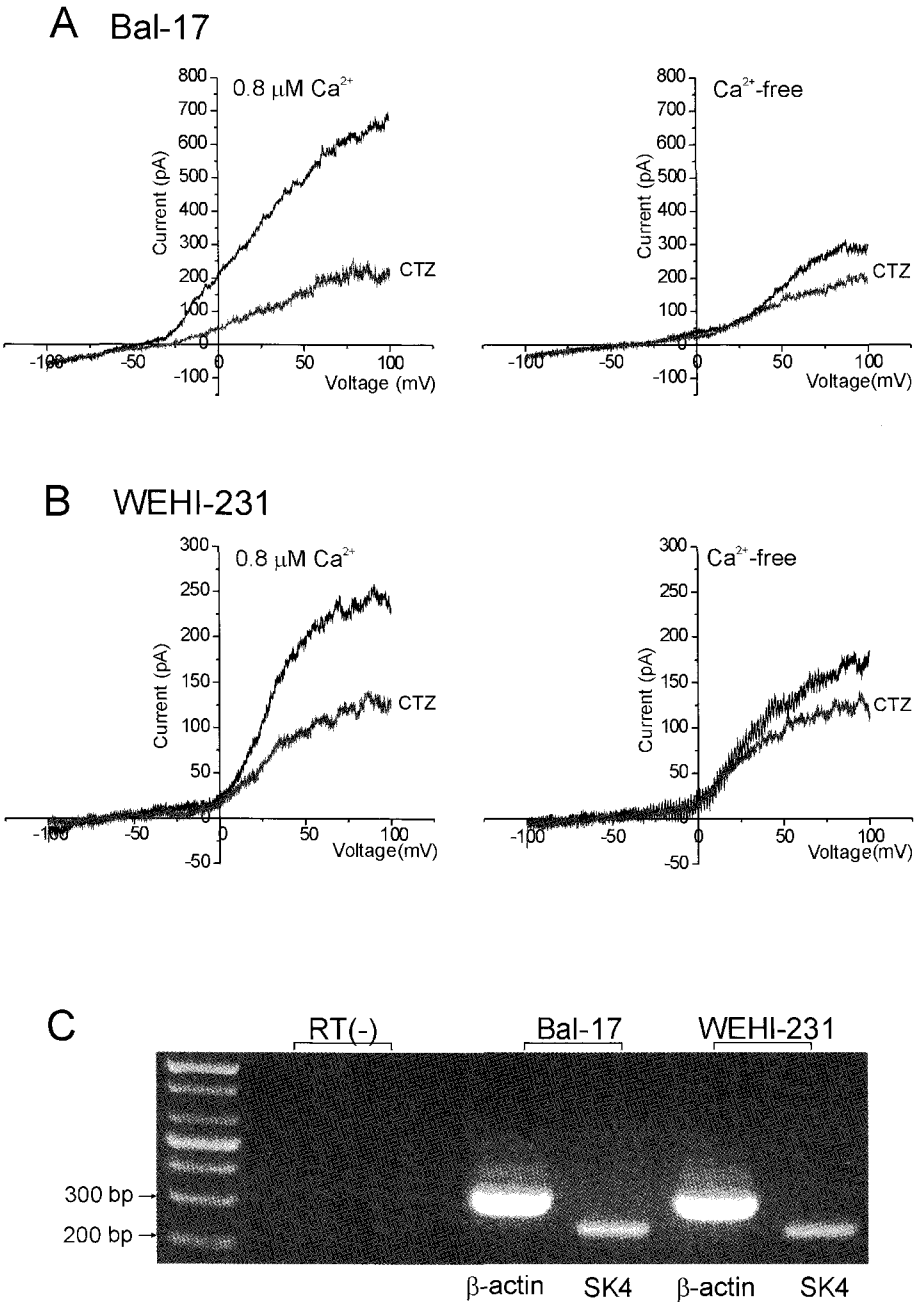

Fig. 1. SK4 channels in murine B lymphocytes. (A) Representative I/V curves of outward current obtained by depolarizing ramp pulses from -100 to 100mV with high-Ca^{2+} and Ca^{2+}-free pipette solution in Bal 17 cells. Clotrimazol (CTZ, 1 μM), a specific blocker of SK4, markedly inhibited the outward currents with high internal Ca^{2+} (left, n=10), whereas only weakly with Ca^{2+}-free pipette solution (right, n=5) in Bal 17 cells. (B) Results of the same experiment done in WEHI-231 cells dialyzed with high Ca^{2+} and Ca^{2+}-free pipette solution (n=10 and 5 respectively). (C) RT-PCR analysis of cDNA for SK4 in Bal 17 and WEHI-231 cells. Control PCR experiments were conducted without reverse transcriptase in the cDNA reaction (RT(−)).

with KCl (KCl bath solution), and I/V curves were obtained by applying a voltage ramp from −100 to 100 mV. As expected, an application of clotrimazole (1 μM) decreased both the inward and outward current with high-Ca^{2+} KCl pipette solution, whereas only the outward current was inhibited with Ca^{2+}-free KCl pipette solution (Fig. 2).

To determine whether the functional expression of SK4 channels could be regulated by BCR-ligation, we pretreated Bal 17 and WEHI-231 cells with anti-IgM antibody (2.5 μg/ml) for 8~12 hr. The I/V curve was obtained with sym-

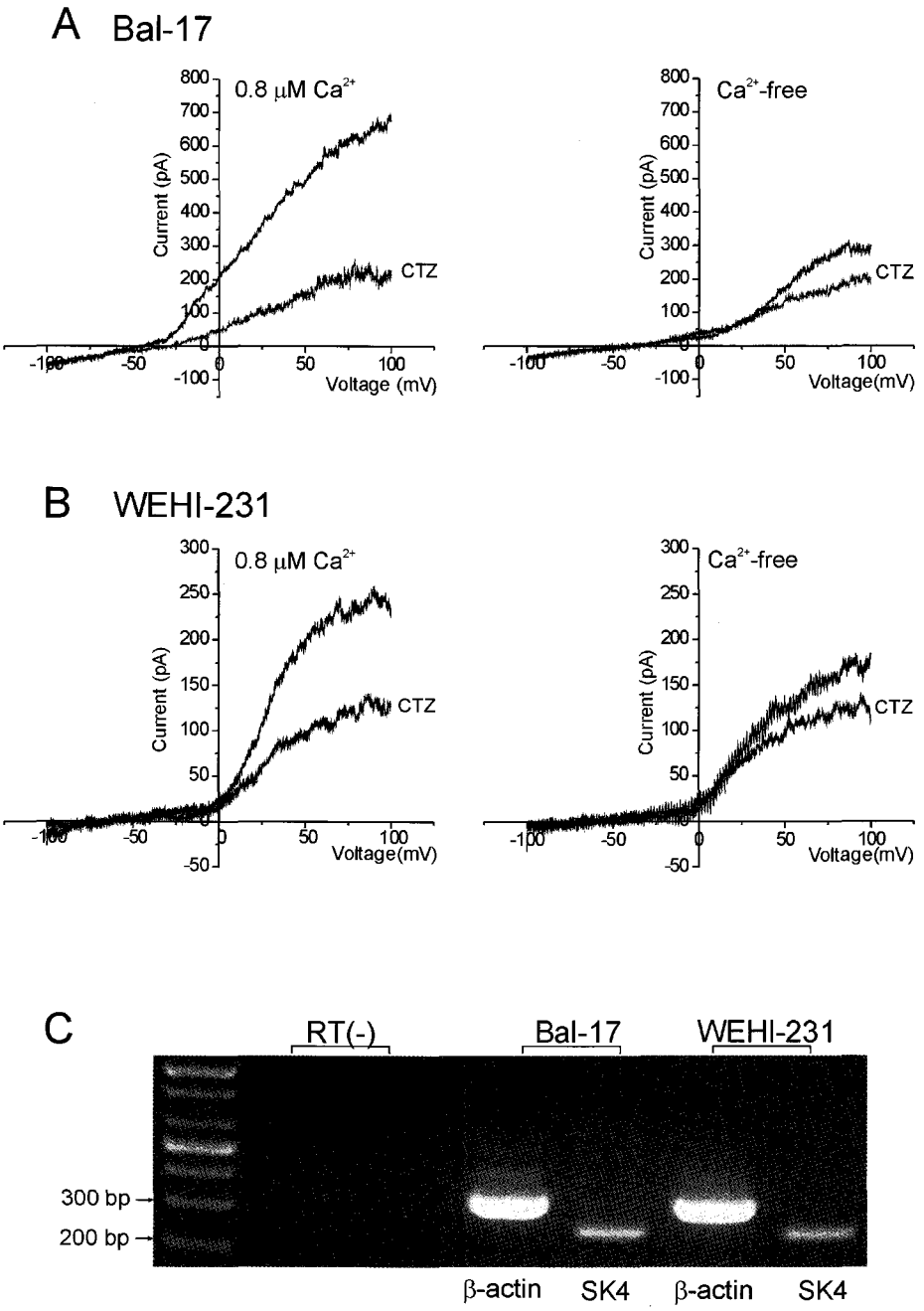

Fig. 2. The activity of SK4 in symmetrical potassium solution. The extracellular NaCl was replaced with KCl, and the I/V curve was obtained by applying a voltage ramp from −100 to 100 mV. CTZ (1 μM) decreased both the inward and outward currents with high-Ca^{2+} pipette solution (left panel), whereas only the outward current was inhibited with Ca^{2+}-free pipette solution (right panel) in Bal 17 (A) and WEHI-231 cells (B).

metrical KCl solution and the current amplitude was normalized to the plasma membrane area by dividing with the membrane capacitance (pA/pF). In summary, the clotrimazole-sensitive current at −80 mV increased from −11.4± 3.10 to −5.7±1.15[k4] pA/pF after BCR ligation in Bal-17 cells. In WEHI-231 cells, the clotrimazole- sensitive current was significantly smaller than Bal-17, and the BCR ligation increased the inward current from −5.7±1.15 to −9.0± 1.00[k5]. Interestingly, not only the clotrimazole-sensitive current but also the clotrimazole-insensitive current was increased by BCR-ligation in Bal-17 and WEHI-231 cells (Fig. 3).

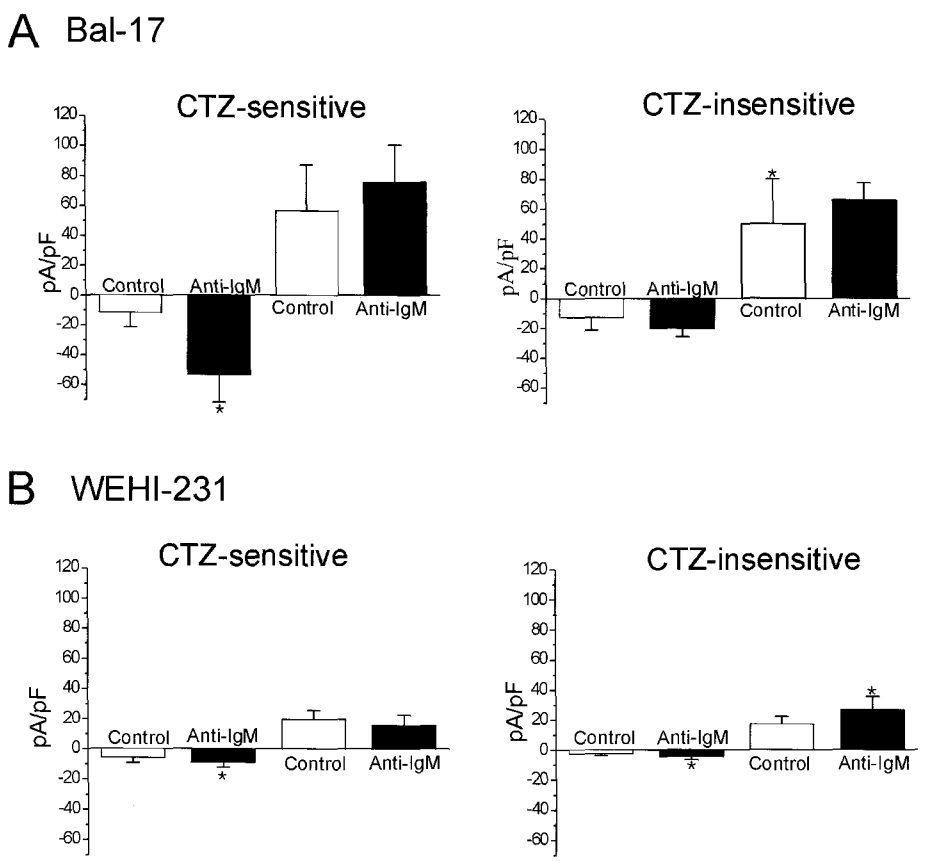

Fig. 3. Effects of BCR ligation on CTZ-sensitive and -insensitive K^+ currents in murine B lymphocyte. For each cell, the I/V curves was obtained with symmetrical KCl solution and the current amplitudes measured at −80 mV and +80 mV were normalized to the membrane capacitance (pA/pF). Bar graphs are summaries of CTZ-sensitive (left panel) and CTZ-insensitive (right panel) current amplitudes. For BCR-ligation, cells were pretreated with Anti-IgM (2.5 μg/ml) for 8~12 hr. (A) and (B) are summarized data obtained from Bal 17 and WEHI-231, respectively (mean±*S.E.M.*, n=10 for each cell line, $*p<0.05$).

DISCUSSION

We found in this study that the amplitude of SK4 current is about two-fold higher in mature B cell line than immature one. Also, the BCR-dependent signaling increased the functional expression of SK4 channels, which was more prominent in Bal-17 than WEHI-231 cells. Not only the clotrimazole-sensitive SK4 but also the clotrimazole-insensitive K^+ current, most probably the Kv current, was generally larger in Bal-17 than WEHI-231 cells. Although the present study was performed in B lymphoma cell lines, the well-known properties of WEHI-231 cells which reflect the immature B cells suggest that the differential expression of SK4 and clotrimazole-insensitive K^+ channels might be valid also in the development of native murine B lymphocytes. The higher expression of SK4 in Bal-17 than WEHI-231 is in contrast with the higher expression of PIP2-sensitive background K^+ channels in WEHI-231 than Bal-17 (Nam et al, 2004). In another study, however, we found that TRPM7 cation channels are more abundant in Bal-17 than WEHI-231 cells (Kim et al, 2005).

The differential expression of SK4 current has also been found in the subsets of Th (T helper) lymphocytes, Th1 and Th2 (Fanger et al, 2000). Th1 and Th2 are divided, due to their synthesis of cytokine interferon (IFN)-γ and interleukin (IL)-4, respectively (Paul and Ahmed, 2003). Measurment of the clotrimazole-sensitive K_{Ca} currents revealed that the amplitudes were higher in Th1 than Th2 cells, which explains the lower level of store-operated Ca^{2+} entry (SOCE) because the amplitudes of Ca^{2+}-release activated Ca^{2+} channel (CRAC) current were same between the two subsets (Fanger et al, 2000). In a similar context, the higher level of K_{Ca} (SK4) in Bal-17 might be reflected as a stronger SOCE. In fact, we recently found that the initial rate of SOCE was faster in Bal-17 than WEHI-231 cells (Nam et al, 2003). Although we did not directly compare the CRAC currents between Bal-17 and WEHI-231 cells in the present study, the differential level of SK4 might at least partially explain the slower rate of SOCE in WEHI-231.

Similar to the upregulation of SK4 after chronic BCR stimulation (>8 h) of Bal-17 cells, Wulff *et al* (2004) recently reported that, in naive and IgD^+CD27^+ memory B cells, that the SK4 current was more than 40 fold increased by 48 hr stimulation of phorbol compound and ionomycin. Thus, the upregulation of SK4 by specific

stimuli seems to be a common property for T and B lymphocytes. In our present study, the stimulation by BCR-ligation was limited to 8~12 hr, since more sustained stimulation induced severe cell death of WEHI-231.

BCR ligation leads to phosphorylation of Bruton type protein tyrosine kinase (Btk) and activation of phospholipase C γ, which induces the release of diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 leads to the release of calcium from intracellular Ca^{2+} stores via the IP3 receptor and triggers SOCE, while DAG activates PKC, which phosphorylates several intracellular substrates, leading to the activation of another transcription factor (*e. g.* NF κB) (Kurosaki, 2002; Panyi, 2005). At present, there is no clue on which of the complex signaling cascades mentioned above is related to up-regulation of SK4 after BCR-ligation.

In summary, both the expression of SK4 and the upregulation of their functional expression are more prominent in mature B cell line (Bal-17) than immature one (WEHI-231). It remains to be investigated whether such differential expression pattern is also present in native B cells at different stages of their development, i.e. bone marrow and spleen B cells.

ACKNOWLEDGEMENT

This work was supported by grant No.R01-2005-000-10231 (Sung Joon Kim) from the Basic Research Program of the Korea Science & Engineering Foundation.

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