Endogenous expression of TRPV5 and TRPV6 calcium channels in human leukemia K562 cells

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Sustained Ca2+ influx regulates multiple processes in blood cells, including cytokine production, proliferation, and differentiation, which in turn control various immune and inflammatory responses (15, 29). It is generally believed that the major mechanism that mediates Ca2+ influx in blood cells, such as lymphocytes and mast cells, is Ca2+-release-activated Ca2+ (CRAC) channels (14, 36, 28). The role of other Ca2+-permeable channels in blood cells is less clear. The mRNAs for calcium ion channels; single-channel recording; blood cells; leukemia cells

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296: C1098–C1104, 2009. First published March 18, 2009; doi:10.1152/ajpcell.00435.2008.—In blood cells, changes in intracellular Ca2+ concentration ([Ca2+]i) are associated with multiple cellular events, including activation of cellular kinases and phosphatases, degranulation, regulation of cytoskeleton binding proteins, transcriptional control, and modulation of surface receptors. Although there is no doubt as to the significance of Ca2+ signaling in blood cells, there is sparse knowledge about the molecular identities of the plasmalemmal Ca2+ permeable channels that control Ca2+ fluxes across the plasma membrane and mediate changes in [Ca2+]i in blood cells. Using RNA expression analysis, we have shown that human leukemia K562 cells endogenously coexpress transient receptor potential vanilloid channels type 5 (TRPV5) and type 6 (TRPV6) mRNAs. Moreover, we demonstrated that TRPV5 and TRPV6 channel proteins are present in both the total lysates and the crude membrane preparations from leukemia cells. Immunoprecipitation revealed that a physical interaction between TRPV5 and TRPV6 may take place. Single-channel patch-clamp experiments demonstrated the presence of inwardly rectifying monovalent currents that displayed kinetic characteristics of unitary TRPV5 and/or TRPV6 currents and were blocked by extracellular Ca2+ and ruthenium red. Taken together, our data strongly indicate that human myeloid leukemia cells coexpress functional TRPV5 and TRPV6 calcium channels that may interact with each other and contribute into intracellular Ca2+ signaling.

We recently discovered that human myeloid leukemia K562 cells express the endogenous channels that were activated by removing extracellular divalent ions (31). Analysis of the unitary current inward rectification, voltage-dependent gating, and sensitivity to intracellular Mg2+ indicated that they were mediated via TRPV6-like channels. In the present study, we found that TRPV6 and TRPV5 mRNAs are coexpressed in K562 cells. Moreover, the TRPV6 and TRPV5 proteins are coimmunoprecipitated from K562 cell lysates. Electrophysiological studies revealed that monovalent currents recorded in K562 cells differ from the currents via homotetrameric TRPV5 and TRPV6 channels expressed in heterologous systems in their sensitivity to extracellular Ca2+ and ruthenium red. Taken together our data suggest that endogenous TRPV5 and TRPV6 proteins expressed in K562 cells form functional heteromultimeric ion channels with novel properties that are differential peculiarity of Ca2+ channels in human leukemia cells.
MATERIALS AND METHODS

Cell culture. Human myeloid leukemia K562 cell line was obtained from Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia) and cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin in 5% CO2 at 37°C. Cells were plated on glass coverslips (0.4 × 0.4 cm) 1–3 days before the experiment.

Conventional RT-PCR and quantitative RT-PCR (qRT-PCR). Extraction of total RNA from K562 cells, cDNA synthesis, and RT-PCR were done as described previously (31). Primers for TRPV5 and TRPV6 were synthesized according to the published sequences (9). Amplified DNA products for TRPV5 was 312 bp and for TRPV6 was 301 bp. Specificity of amplification was confirmed by sequencing. Quantitative RT-PCR amplification of TRPV5 and TRPV6 cDNA specific fragments was performed with MasterMix (Syntol) and SYBR Green I (Biodye) using ANK-32 Real-time PCR System (Syntol). The primers used were as follows: TRPV5, forward, 5′-TCACAGAGATCGACTCTTG-3′, reverse, 5′-CTCTGATCATGGTCTGTC-3′ (amplified DNA product was 105 bp); TRPV6, forward, 5′-ATGACGCGGATGAGCTGTG-3′, reverse, 5′-ATACCTCCCGTCGGACATG-3′ (amplified DNA product was 110 bp). To avoid false-positive results due to genomic contamination of the samples, the primers spanned an intron at the genomic level. Thermal cycling parameters were 95°C for 5 min and then 45 cycles of denaturation (95°C, 10 s) and annealing/elongation (60°C, 15 s). Samples were subjected to a melting curve analysis and 6% PAAG electrophoresis to confirm the absence of unspecific amplification products and primer-dimers. All reactions were performed in quadruplicate. The abundance of TRPV5/6 transcripts was quantified on the basis of the interpolated cycle number in which fluorescence reached a threshold level (Ct value), using background fluorescence in cycles 7–21 as baseline. The relative abundance of TRPV5 normalized to the TRPV6 expression level, was calculated as 2^(-ΔΔCt), where ΔCt = Ct(TRPV5) − Ct(TRPV6).

Total cell lysate and crude membrane fraction preparations. Cells were incubated in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 1 mM NaF, 1 mM Na2VO3, 50 mM Tris-HCl, pH 7.6, and protease inhibitor cocktail, Sigma) for a 15 min on ice, and then the suspension was gently homogenized. After centrifugation for 10 min at 10,000 g, the supernatant was collected and protein concentration was determined. Obtained cell lysates were used for immunoprecipitation or boiled for 5 min in Laemmli buffer. Solution exchange was performed by bath perfusion and solution concentrations as 2 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protease inhibitor cocktail. Then suspension was centrifuged at 1,000 g for 10 min, and the subsequent supernatant was centrifuged at 200,000 g for 60 min at 4°C (TLA −120.2 rotor, Beckman Coulter Optima TLX Ultracentrifuge). The resulting pellet was solubilized in buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40 and protease inhibitor cocktail) on ice for 1 h. After centrifugation at 14,000 g for 10 min, the supernatant was collected and protein concentration was determined. The sample was then boiled for 5 min in Laemmli buffer. Then 12 μg of protein in sample buffer were separated by SDS-PAGE (8% gel) and analyzed by Western blot.

Immunoprecipitation and Western blotting. For immunoprecipitation, samples of 0.5 μg of total cell protein were diluted to 1 ml with lysis buffer and incubated overnight at 4°C with 2 μg rabbit anti-TRPV5 antibody (Ab; H-99, Santa-Cruz Biotechnology) or 2 μg of rabbit anti-TRPV6 Ab (H-90, Santa-Cruz Biotechnology). Control groups were incubated without antibody in the presence of A/G Agarose (Santa Cruz Biotechnology). Immunocomplexes were precipitated by incubation with 20 μl protein-A/G Agarose for 2 h at 4°C followed by centrifugation. After being washed three times with ice-cold lysis buffer, pellets were diluted with 20 μl of 2× Laemmli buffer and boiled for 5 min. Fifteen microliters of each sample were subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (Hybond-ECL, Amersham). The membranes were blocked for 1 h by 5% nonfat dry milk in T-TBS (0.05% Tween 20 in Tris-buffered saline) and incubated overnight at 4°C with goat anti-TRPV5 antibody (1:200, K-17, Santa Cruz Biotechnology) or rabbit anti-TRPV6 antibody (1:200, Alomone). After washing was completed, blots were incubated with anti-goat (1:3,000, Santa-Cruz Biotechnology) or anti-rabbit (1:3,000, Sigma) horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Detection of horseradish peroxidase-conjugated antibody was done using chemiluminescent substrate (ECL, Amersham) and exposure to X-ray film.

Electrophysiology and data analysis. Single channel currents were recorded using standard outside-out mode of the patch-clamp technique. We used a micropipette puller P-97 (Sutter Instrument, Novato, CA) to manufacture patch pipettes with a resistance of 9–12 MQ when filled with solution. Membrane currents were measured using an Axopatch 200B patch-clamp amplifier (Axon Instruments/Molecular Devices, Eugene, OR) with low-pass filtered at 1 kHz and digitized at 5 kHz with an A/D converter. Data were collected and analyzed with pClamp software. Membrane voltage was calculated as the potential of the intracellular membrane side minus the potential of the extracellular one. The recordings were performed at room temperature (22–23°C) on the stage of an inverted microscope with Nomarsky optics. Data analysis was performed using Microcal Origin 6.2 software (OriginLab). Open channel probability was calculated as NPo = li/l, where i is the mean current, l is the unitary current amplitude, and N is the number of functional channels in the patch. Dose-response curves were fitted using a Hill function of the form \( N_{Po} = \frac{y}{x + IC_{50}^m} \), where X is the concentration of blocker, IC50 is the concentration of blocker that provides 50% inhibition and nH is the Hill coefficient. Data are presented as means ± SE; n = number of the experiments). Error bars are shown where they exceed the symbol size.

Solutions. Patch pipette solution contained (in mM): 140 K-aspartate or Cs-aspartate, 5 NaCl, 10 HEPES-Tris-OH, 10 HEDTA or 10 CaCl2, and 10 d-glucose (pH 7.3). The bath solution in outside-out experiments contained (in mM): 140 Na-methanesulfonate, 5 KCl, 10 HEPES-Tris-OH, 10 N-2-hydroxyethyl-ethylendiamine triacetic acid (HEDTA), and 10 d-glucose (pH 7.3). In some experiments, different concentrations of Ca2+ and EDTA were added into extracellular bath solution to achieve desirable extracellular Ca2+ concentrations as indicated in the results. MaxChelator software (WEBMAXC 2.10) was used to calculate free Ca2+ concentration. In the divalent-chelation-free solution, Ca2+ was omitted and 10 mM HEDTA added to the bath solution. Solution exchange was performed by bath perfusion and was completed within several seconds. All chemicals were from Sigma-Aldridge (St. Louis, MO).

RESULTS

Expression profile of TRPV5 and TRPV6 mRNAs and proteins in K562 cells. Using conventional RT-PCR analysis, we found that 312 bp and 301 bp products that correspond to the TRPV5 and TRPV6 transcripts are expressed in K562 cells (Fig. 1A). The quantitative RT-PCR analysis was performed to determine the relative expression levels of TRPV5 and TRPV6 mRNAs. As shown in Fig. 1, B and C, the expression level of TRPV6 mRNA is 17.74 ± 0.03-fold (n = 4) higher than that of TRPV5 mRNA. Western blot analysis of the protein expression performed with polyclonal anti-TRPV5 Ab and anti-TRPV6 Ab revealed the bands with molecular sizes of ~90 kDa in both total cell lysates and the crude membrane fractions (Fig. 2A). The bands with molecular sizes of 85–100 kDa were
previously identified in lysates of the cells expressing recombinant TRPV5 and or TRPV6 proteins and were attributed to the mature glycosylated TRPV5 or TRPV6 proteins (13). Weak bands were also present at 75 kDa, which correspond to the size of core proteins (13). Because previous studies demonstrated that TRPV5 and TRPV6 channels have a tetrameric stoichiometry and can form heteromultimeric complexes (13), we explored whether endogenous TRPV5 and TRPV6 are physically associated using coimmunoprecipitation approach. Figure 2B shows that bands corresponding to the sizes of glycosylated and nonglycosylated TRPV5 and TRPV6 proteins were detected with anti-TRPV5 Ab or anti TRPV6 Ab in precipitants that were pulled down using anti-TRPV6 Ab and anti-TRPV5 Ab, correspondingly. These data indicate that in K562 cells the endogenously expressed TRPV5 and TRPV6 channel proteins are capable of forming immunopercptible complexes.

**Figure 2.** Expression of TRPV5 and TRPV6 channel proteins in K562 cells. A: Western blots of total cell lysates (lane 1) and crude membrane preparations (lane 2) from K562 cells. Proteins were size-separated by SDS-PAGE and probed with anti-TRPV5 Ab (left) or anti-TRPV6 Ab (right). Molecular masses (kDa) are indicated. B: Immunoblots showing reciprocal coimmunoprecipitation of TRPV5 and TRPV6 proteins from K562 cell lysates. Proteins were immunoprecipitated (IP) with anti-TRPV5 Ab or anti-TRPV6 Ab (indicated with +) or A/G Agarose alone (indicated with –). Western blots (WB) were performed on IP-TRPV5 precipitants using anti-TRPV6 Ab and on IP-TRPV6 precipitants using anti-TRPV5 Ab. Immunoblotting produced bands corresponding to predicted molecular masses of TRPV6 and TRPV5 proteins. Representative blots from four experiments.
higher than that reported previously for recombinant TRPV5 or TRPV6 channels [150 nM-2.9 μM; (9, 33, 35)].

Sensitivity of the endogenous monovalent unitary currents to ruthenium red. From an electrophysiological point of view, TRPV5 and TRPV6 are highly homologous channels. They can be discriminated, however, by their sensitivity to ruthenium red (RR), as TRPV6 has a 100-times lower affinity for RR (IC₅₀ 9 ± 1 μM) than TRPV5 (IC₅₀ 121 ± 13 nM) (9, 25). Therefore, we carried out experiments to test the effects of RR on the activity of the endogenous channels expressed in K562 cells. Figure 5A shows representative single channel recordings in the presence of different concentrations of RR (1–100 μM) in the external solution. The recordings were made in outside-out patches at holding potential −70 mV. Plotting the average NPo values against RR concentration (FIG. 5B) revealed that RR blocks the monovalent currents in K562 cells with an IC₅₀ of 6.5 ± 0.5 μM (n = 6).

DISCUSSION

In the present study we explored the expression and functional properties of TRPV5/6-like channels in human myeloid leukemia K562 cells. We have previously reported the presence of TRPV6 mRNA in K562 cells (31). The present study revealed that in addition to TRPV6 mRNA, the K562 cells also express TRPV5 mRNA, which is consistent with previous report that TRPV5 and TRPV6 mRNAs are coexpress in lymphoid cells (4). Furthermore, we found that anti-TRPV5 and anti-TRPV6 Ab recognize the endogenous proteins with the sizes of 90 and 75 kDa, which correspond to the glycosylated (85–100 kDa) or core (75 kDa) forms of the TRPV5 and TRPV6 proteins, described previously (13). Analysis of the primary structure of TRPV5 and TRPV6 proteins revealed a conserved N-glycosylation sequence in the first extracellular loop (9). Glycosylation is believed to be necessary for protein
stability and assembly of channels in multimeric complex. Previous functional study of heterologously coexpressed TRPV5 and TRPV6 proteins showed that covalent linking of TRPV5 and TRPV6 subunits produces functional channels with novel properties (13). Our finding that endogenously expressed TRPV5 and TRPV6 proteins coimmunoprecipitate indicates that TRPV5 and TRPV6 may physically interact in K562 cells.

It was previously shown that when TRPV5 and TRPV6 proteins were coexpressed in the expression system, they formed heterotetrameric channels that displayed voltage-dependent gating indistinguishable from that of TRPV5 or TRPV6 homotetrameric channels (13). However, heteromultimeric TRPV5/6 channels in expression system differed from homotetrameric TRPV5 or TRPV6 channels in the kinetics of Ca\(^{2+}\)-dependent inactivation and sensitivity to RR block (13). The outside-out single channel recordings presented here and our previous cell-attached and inside-out patch-clamp experi-

Fig. 4. Extracellular Ca\(^{2+}\) block of the monovalent currents. A: single channel activity recorded in outside-out patches at −70 mV and at various extracellular Ca\(^{2+}\) concentrations (0, 10 nM, 10 μM, and 1 mM; indicated above each trace). B: \(N_p\) as a function of extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)). \(N_p\) at each [Ca\(^{2+}\)]\(_o\) was normalized to \(N_p\) at 0 μM [Ca\(^{2+}\)]\(_o\). Each point represents mean ± SE of 3–5 measurements from different patches. Continuous line represents the logistic fit of experimental data.

Fig. 5. Inhibition of channel activity by ruthenium red (RR). A: unitary currents in outside-out patches recorded at −50 mV and at various concentrations of RR (0, 1 μM, 10 μM, and 100 μM; indicated above each trace). B: \(N_p\) as a function of extracellular RR concentration ([RR]\(_o\)). \(N_p\) at each [RR]\(_o\) was normalized to \(N_p\) at 0 μM [RR]\(_o\). Each point represents mean ± SE of 5–6 measurements from different patches. Continuous line represents the logistic fit of experimental data.
ments (31) performed in K562 cells in divalent-free extracellular solution revealed the presence of 30 pS cation channels that displayed several properties typical for homo- and heterotetrameric TRPV5 and TRPV6 channels such as strong inward rectification, voltage-dependent kinetics, block by intracellular Ca$^{2+}$ or Mg$^{2+}$, and voltage-dependent block by extracellular Ca$^{2+}$ (9, 13, 33). However, the IC$_{50}$ value for Ca$^{2+}$ block of endogenous 30 pS cation channels reported here (20 μM) is much higher than that previously reported for homotetrameric TRPV6 channels heterologously expressed in Chinese hamster ovary cells (2.9 μM) (35) or for TRPV5 channels (150 nM) and TRPV6 channels (200 nM) heterologously expressed in HEK-293 (9). Furthermore, we found that RR blocks the endogenous 30 pS channel activity in K562 cells with IC$_{50}$ = 6.5 μM. This value is slightly lower than the IC$_{50}$ reported for RR block of the heterologously expressed TRPV6 channels [9 μM; (9)] and is higher than that of the recombinant TRPV5 channels [121 nM; (25)]. Taken together with our coimmunoprecipitation results, these data indicate that endogenously expressed TRPV5 and TRPV6 proteins may form functional heterotetrameric channels with properties different from those of homotetrameric TRPV5 or TRPV6 channels.

It was previously reported that increase in the TRPV6-to-TRPV5-channel subunits ratio in the heterotetrameric channel affected kinetics of Ca$^{2+}$-dependent inactivation and caused the decrease in the affinity to RR block (13). Given that our quantitative RT-PCR analysis demonstrated that in K562 cells the level of expression of TRPV6 mRNA is significantly higher than that of TRPV5 mRNA and that endogenously expressed channels demonstrate much lower affinity to RR block than recombinant TRPV5 channels, we hypothesize that endogenously coexpressed TRPV5 and TRPV6 proteins form functional heterotetrameric channels that contain more TRPV6 subunits than TRPV5 ones. We speculate that variations in subunit composition of TRPV5/6 channels could provide a mechanism for fine tuning of Ca$^{2+}$ transport kinetics and Ca$^{2+}$-dependent functions, such as proliferation and differentiation, in human myeloid leukemia cells.

The affinity of TRPV5 and TRPV6 as well as other Ca$^{2+}$-selective channels to extracellular Ca$^{2+}$ is related to the existence of high-affinity binding sites within channel pore (7, 2). Mutation within the pore region of TRPV5 channels causes a strongly reduced sensitivity to divalent cations (Cd$^{2+}$) compared with that in wild-type TRPV5 (13). Therefore, the differences in affinity to extracellular Ca$^{2+}$ between recombimantly expressed TRPV5 or TRPV6 channels and the endogenous 30 pS channels in K562 cells, indicate that endogenous channels have a specific pore property, which may be a result of heteromultimeric assembly of TRPV5 and TRPV6 proteins. The pore mutation in leukemia cells cannot be also ruled out.

The expression levels of TRPV5 and TRPV6 channels, which correlate with the alteration of cell proliferation and/or differentiation, are strongly controlled by 1,25-(OH)$_2$D$_3$-dependent manner as shown, for example, in human keratinocytes (18). Therefore, identifying the mechanisms of regulation of TRPV5 and TRPV6 expression and assembly into functional channels of different stoichiometry may enable the development of therapeutic strategies directed to manipulating the expression pattern of these channels and, consequently, the proliferative activity of the leukemia cells.

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