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Changes in the expression of voltage-gated K^+ currents during development of human megakaryocytic cells

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Received 15 October 1996; revised 25 January 1997; accepted 29 January 1997

Abstract

We distinguished four distinct groups of megakaryocytic cells on the basis of their voltage-gated membrane currents. One group of 32 cells (15%), exhibited an inward rectifying current and had a diameter of 12 ± 3.5 μm (mean ± S.D.). A large group of 85 cells (39%) exhibited only a 'leakage-like' current and had a diameter of 15.8 ± 3.7 μm. The other two groups of cells exhibited voltage-gated outward currents. One group consisted of 43 'I-type' cells (19%), with a diameter of 22.3 ± 3.4 μm, for which the maximal outward current occurred for a voltage step from −60 to either 0 or +20 mV. For the last group of 60 'M-type' cells (27%), which had a diameter of 26.7 ± 2.9 μm, the maximal outward current occurred for a voltage step from −60 to +80 mV, the largest voltage step used. The currents recorded in this study, from megakaryocytes having 'leakage-like' currents and 'I-type' currents, were indistinguishable from the voltage-gated currents of the megakaryocytes from myelogenous leukemia patients, in which voltage-gated currents were suppressed (Kapural, L., O'Rourke, F., Feinstein, M.B. and Fein, A. (1995) Blood 86, 1043), suggesting that the megakaryocytes from the myelogenous leukemia patients are a dedifferentiated or less mature form of megakaryocyte.

Keywords: Megakaryocyte; Development; Inward rectifier; Outward rectifier; Myelogenous leukemia; Voltage-gated potassium current

1. Introduction

Recently we reported that human megakaryocytes have a delayed rectifier type of voltage-gated outward K^+ current in their plasma membrane [1] and that this was the only voltage-gated current we could identify in these cells. We also found that this current was either greatly suppressed or absent in megakaryocytes from myelogenous leukemia patients. We suspected, on the basis of findings in other blood cells, that arrested maturation might be responsible for the suppression of the delayed rectifier in the megakaryocytes of these patients. Developmental changes in the expression of voltage-gated outward K^+ currents have been shown to occur in T lymphocytes [2,3]. Also human leukemic cells chemically stimulated to differentiate change their expression of voltage-gated outward K^+ current [4,5]. The main goal of this study was to determine whether there was a developmental explanation for the suppression of the voltage-gated outward current in megakaryocytes of myelogenous leukemia patients [1].

Megakaryocytes are thought to undergo four successive maturational stages as they increase in size and ploidy in the marrow [6,7]. A secondary goal of this study was to determine if these maturational stages are correlated with changes in the expression

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PII S0005-2736(97)00035-7

of the voltage-gated K^+ currents. Studies of voltage-gated K^+ channel expression during megakaryocyte development may have important implications since it has been postulated that these channels may play a role in the differentiation and proliferation of other hematocytes [8–10].

2. Methods

Human megakaryocytes were obtained from the blood laboratory as described previously [1]. None of these clinical samples was obtained for our research experiments. We received the remainder of the sample after all laboratory procedures were complete. This use of human specimens was approved by the institutional review board. The data in this paper were obtained from 220 human megakaryocytes. Of these cells 98 were obtained from bone marrow filters from 11 healthy donors. The remaining megakaryocytes were obtained from bone marrow aspirates from 16 patients with normal marrow. There was no significant difference in the data obtained from megakaryocytes that came from filters or aspirates.

2.1. Identification of megakaryocytic cells

One ml of solution was placed in a recording chamber and left at room temperature for 30 min to allow the cells to settle to the bottom of the chamber. Cells to be recorded from were identified as megakaryocytic by two different methods.

In the first method, using transmitted light microscopy, cells were identified as megakaryocytic if they exhibited a rough, bright surface and attached themselves quickly to the bottom of the recording chamber and spread with an irregular shape. We tested our ability to identify megakaryocytic cells this way by using a fluorescent monoclonal antibody specific for megakaryocytes and platelets to confirm the identification. The bone marrow sample was preincubated with a fluorescent monoclonal antibody specific for the glycoprotein IIb/IIIa receptor (Immunotech, France) for 30 min, and then washed with standard external solution (see below) and placed in the chamber. We used this fluorescent monoclonal antibody because glycoprotein IIb/IIIa is expressed from the early through the late stages of megakaryocyte development and it is also present in platelets [11,12]. Each megakaryocytic cell was first identified using transmitted light and then examined for the presence of fluorescence. Transmitted light (Fig. 1a) and fluorescent (Fig. 1b) images were obtained for

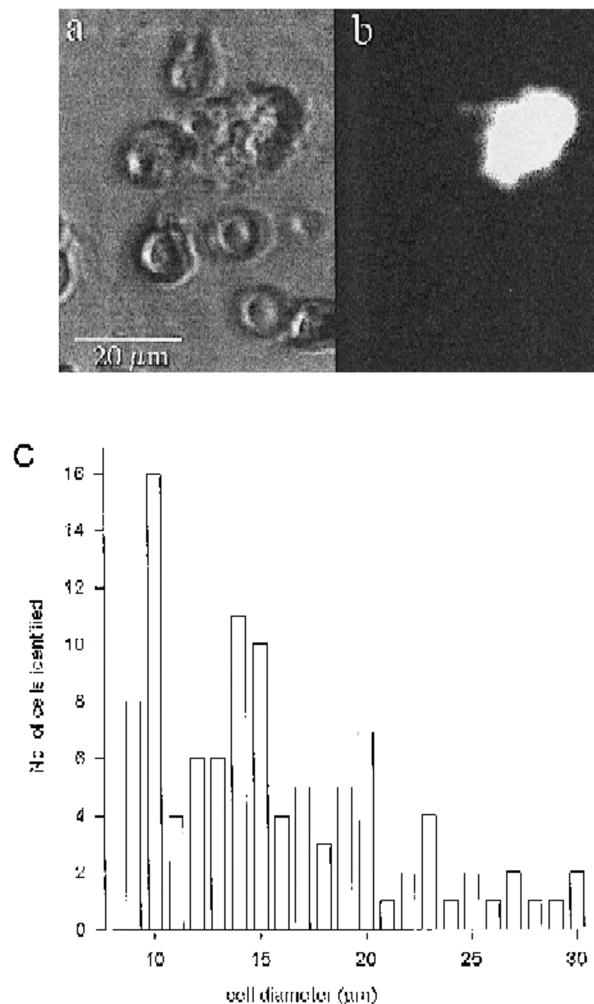


Fig. 1. Identification of megakaryocytic cells. Transmitted light (a) and fluorescent (b) images of human megakaryocytes labeled with fluorescent monoclonal antibody to the glycoprotein IIb/IIIa complex (see Section 2). The transmitted light image (a) shows a single megakaryocytic cell of about 20 μm in diameter, several nearby erythrocytes and a leukocyte. Note that the megakaryocytic cell has adhered and begun to spread on the cover glass that forms the bottom of the chamber. The fluorescent image (b) shows that the megakaryocyte is the only cell labeled by the antibody. (c) Distribution by diameter of cells identified and confirmed as being megakaryocytic cells. We correctly identified 107 cells with diameters between 9 and 30 μm.

each cell using a Bio-Rad MRC-600 laser scanning confocal microscope. Dual channel imaging was used with one channel for fluorescence and the other for transmitted light. The confocal aperture was wide open to collect as much fluorescent light as possible in order to detect all cells that were labeled by the antibody. In this way we correctly identified 107 megakaryocytic cells in the range of 9–30 μm (Fig. 1c). For diameters below about 9 μm we started to make errors in our identification. Therefore we only used cells greater than 9 μm, identified this way, in our patch-clamp experiments. In the second method the bone marrow sample was preincubated with the Gp IIb/IIIa antibody (see above) and we recorded from megakarytocytic cells that were fluorescently labeled by the antibody. We refer to cells identified by the first method as unstained cells and those by the second method as stained cells. The time interval from tissue collection to the acquisition of electrophysiological records was typically between 1 and 6 h.

2.2. Solutions

The standard external solution that bathed the cells was composed of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 10 mM glucose, adjusted to pH 7.4 with 1 M NaOH. For external solution with high potassium chloride, 30 mM NaCl of the external solution was replaced with an equimolar amount of KCl. The standard internal intrapipette solution was composed of 140 mM KCl and 10 mM Hepes and the pH was adjusted to 7.35 with 1 M KOH. We used these external and internal solutions because these were the solutions used in our previous study [1] and the primary goal of this study was to compare the findings reported here to those of the previous study. Also, in our previous study [1] we found that addition of the calcium buffer BAPTA (10 mM) to the internal solution did not change the current–voltage relationship of human megakaryocytes. Where noted, in some experiments 1 mM $MgCl_2$ was added to the intrapipette solution.

2.3. Electrophysiological methods

We employed the whole cell patch clamp technique [13]. Membrane currents were measured at room temperature (21–24°C) using an Axopatch-1D patch clamp amplifier (Axon Instruments, Foster City, CA). Data were acquired and analyzed by a computer (486 or Pentium AT clone) running the PClamp suite of programs (Axon Instruments, Foster City, CA).

Recording pipettes were pulled from 1.5 mm borosilicate glass (# 7052, Garner Glass, Claremont, CA) using a two stage Narishige PB-7 vertical pipette puller and then firepolished on a Narishige MF-9 microforge. Pipette resistances were between 3–10 Mohms. Series resistance compensation was in the range of 45% to 65%, which provided adequate voltage control except for the very largest currents for which there is some inevitable distortion. The Bessel low-pass output filter of the Axopatch amplifier was set at 1 kHz. Voltage steps of 400 ms to 5 s in duration were delivered, unless otherwise noted at intervals of at least 10 s, from a holding potential of −60 mV. The holding potential was set at −60 mV so that the data obtained could be directly compared to our previous measurements in human megakaryocytes [1]. Data analysis was performed using the PClamp programs Clampex and Clampfit (Axon Instruments, Foster City, CA) and Sigma Plot for Windows (Jandel Scientific, San Rafael, CA).

The DAD-6 Superfusion System (Adams and List, New York) was used to locally apply agents to the cell while the bath was superfused with external solution at a rate of 2 ml/min.

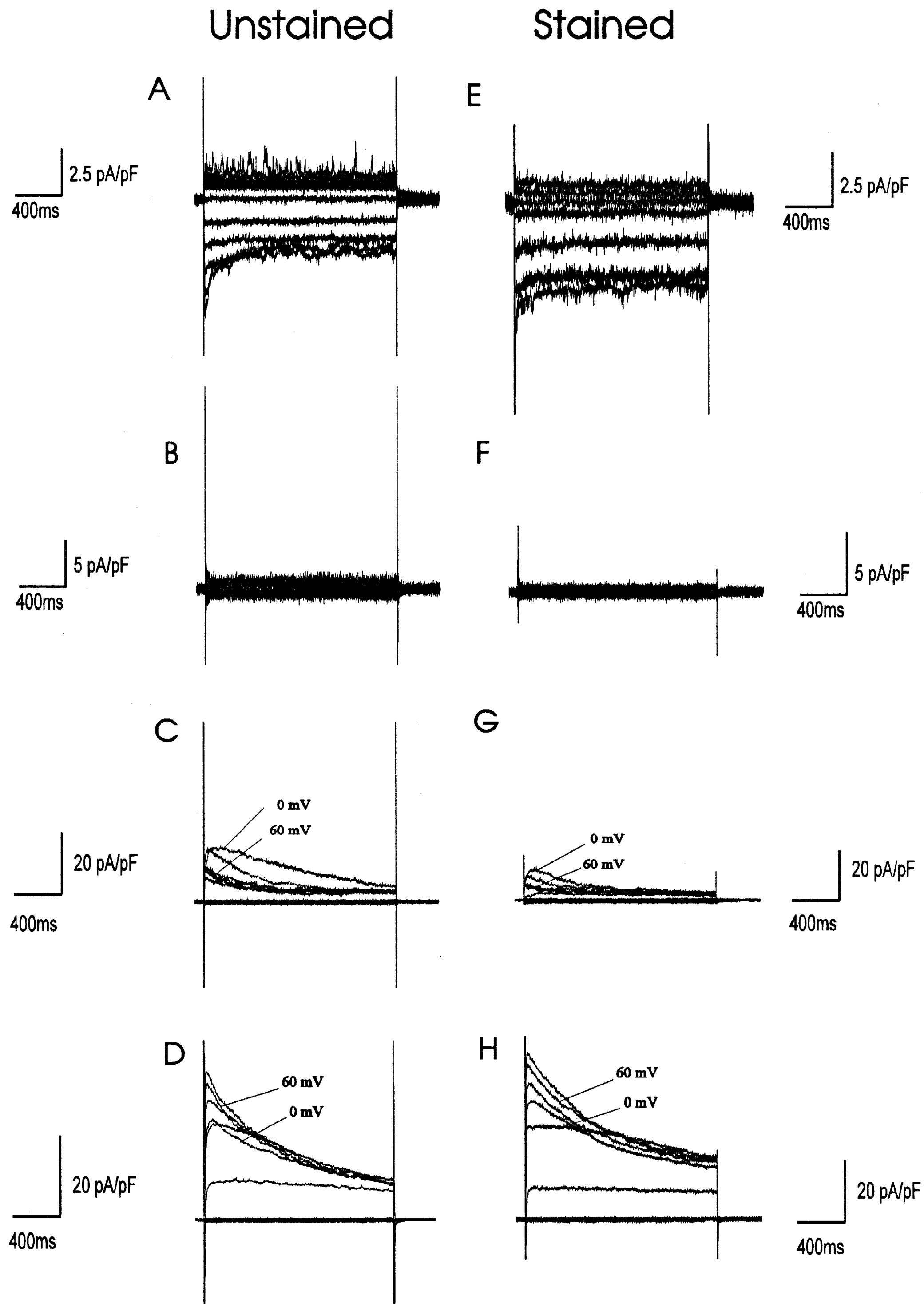
Throughout this paper values are given as the mean ± S.D.

3. Results

3.1. Four groups of cells

We used two different criteria to identify cells, with diameters between 9 and 30 μm, as megakaryocytic (see Section 2). In recordings made from 144 unstained and 76 stained megakaryocytic cells we distinguished the same four distinct groups of cells on the basis of their voltage-gated currents. Examples, from both stained and unstained megakaryocytic cells, of each of the four kinds of voltage-gated currents are given in Fig. 2. In these experiments the cells were held at −60 mV and the voltage was stepped from −160 mV to +80 mV in 20 mV steps

of 1.6 s duration. One group of 32 cells exhibited a voltage-gated inward rectifying current (Fig. 2A and E). Another group of 85 cells exhibited only a 'leakage-like' current without any apparent voltage-gated current (Fig. 2B and F). The other two groups were characterized by the presence of a voltage-gated



outward current (Fig. 2C, D, G and H). In one group of 43 cells the maximal peak voltage-gated outward current occurred for a voltage step to either 0 or +20 mV (Fig. 2C and G) in the other group of 60 cells the maximum current occurred for a voltage step to +80 mV (Fig. 2D and H). We will refer to outward currents of the type in Fig. 2C and G as the I-type of outward current and those in Fig. 2D and H as the M-type of outward current. The only type of voltage-gated currents we previously observed in normal large megakaryocytes, greater than 30 μm in diameter, were similar to the M-type of outward current [1]. We called this type of current M-type because it was found in the large 'mature' megakaryocytes, accordingly we called the other type, I-type for 'immature' since it was found in smaller less 'mature' megakaryocytes.

3.2. Size distribution for each group of cells

As mentioned in the Introduction megakaryocytes are thought to go through four stages of development as they mature in the bone marrow [6,7,14]. In order to compare the four groups of cells we have found (Fig. 2) with these previously described developmental stages we determined the size distribution of each group of cells (see Fig. 3). It can be seen in Fig. 3 that the smallest cells have either a leakage-like current or an inward rectifier and the largest cells have an outward rectifier. These findings are consistent with the idea that megakaryocytes change their expressed ion channels as they mature in the bone marrow.

3.3. Cells with a leakage-like current

A large portion of the cells in this study, 85 of 220, only had a small leakage-like current. For a voltage step to +80 mV the outward current was no more than 40 pA. These cells had a mean diameter of 15.8 ± 3.7 μm (range from 9 to 26 μm) (see Fig. 3). The I–V relationship was unchanged if the holding potential was decreased from −60 mV to −120 mV ($n = 26$) indicating that there is not an underlying voltage-gated current that is simply inactivated at −60 mV. Also, careful examination of the current records at high time resolution, in cells in which the capacitance transients were almost completely compensated, indicated that a rapidly inactivating outward current was not present (data not shown).

We previously found that some of the megakaryocytes greater than 30 μm in diameter, from myelogenous leukemia patients, have leakage-like currents similar to those in Fig. 2B and F, and that treatment, of these cells with forskolin would induce a voltage-gated outward current [1]. Therefore we exposed megakaryocytic cells that had a leakage-like current to forskolin (100 μM) and found that it had no effect on the voltage-gated currents of these cells (data not shown, $n = 8$). We conclude that normal human megakaryocytic cells and 'myelogenous leukemia' megakaryocytes, having leakage-like currents, respond differently to the application of forskolin.

3.4. Cells with I-type of voltage-gated outward current

In trying to better understand the I-type of outward current, we found as shown in Fig. 4A and B, that by changing the holding potential of the I-type of cell from −60 mV to −120 mV the voltage step for which the maximum peak current occurred would change from 0 to +80 mV. This finding indicates that some of the voltage-gated channels in these cells are experiencing significant inactivation at a holding potential of −60 mV.

Fig. 2. Four types of whole cell recordings obtained from human megakaryocytic cells with diameters between 9 and 30 μm. Cells were held at −60 mV and voltage steps 1.6 s in duration were applied from −160 mV to +80 mV in 20 mV increments. (A and E) Megakaryocytic cells exhibiting a voltage-gated slowly inactivating inward current. (B and F) Two of many cells exhibiting a small leakage like current. (C and G) Cells with a small voltage-gated outward current, where the maximum amplitude of the peak currents occurs for a voltage step from −60 mV to 0 mV. (D and H) Cells with a voltage-gated outward current where the maximum amplitude of the peak current corresponds to a voltage step from −60 mV to +80 mV. The cells in A–H were bathed in the standard external solution. The standard internal solution was used in all recordings except A in which an internal solution to which 1 mM $MgCl_2$ was added was used (see Section 2). The terms stained and unstained refers to the method used to identify the cells as being megakaryocytic (see Section 2).

Very slow recovery from inactivation (30–60 s) is characteristic of the *n*-type of voltage-gated outward K^+ current described in T-lymphocytes [2,15]. To examine the possibility that the I-type of current in megakaryocytes is similar to the lymphocyte *n*-type current we applied trains of depolarizing pulses (to +60 mV) of 1000 ms duration from a holding potential of −100 mV every 2 s. We did not observe any cumulative decrease of the current in seven cells (data not shown). Additionally, when *I–V* curves were determined for I-type cells using the same protocol as in Fig. 2, with a pause between measurements of more than 60 s ($n = 12$) there was no change in the *I–V* curve (data not shown). Taken together these findings indicate that the I-type current of megakaryocytes is not similar to the *n*-type current of lymphocytes.

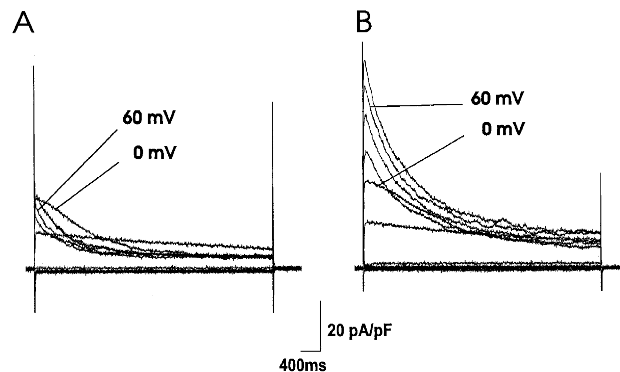


Fig. 4. Two sets of recordings of voltage-gated outward currents from the same megakaryocytic cell with the holding potential for the recording in (A) at −60 mV and that for (B) at −120 mV. Note that the voltage step which elicits the maximum amplitude peak outward current in (A) is to 0 mV and that in (B) is to +80 mV.

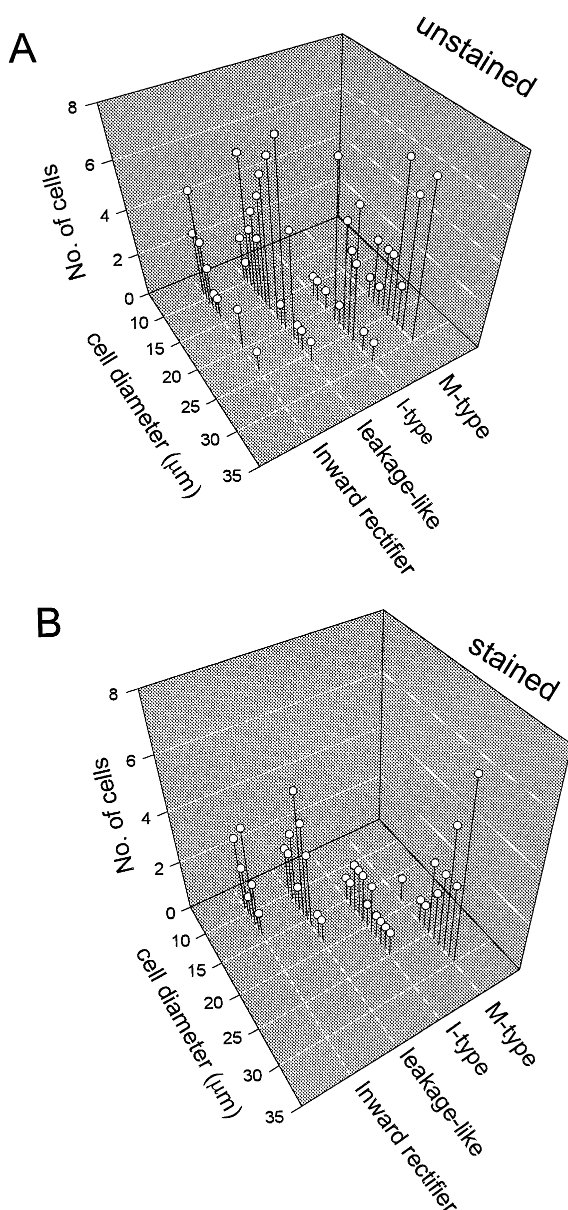


Fig. 3. Size distribution of megakaryocytic cells with the four types of voltage-gated currents described in Fig. 2. The same voltage protocol as in Fig. 2 was used for all the cells. Size distribution of unstained (A) and stained (B) cells. Altogether, 32 cells had an inward rectifying current (see Fig. 2A and E). They ranged in diameter from 9 to 24 μm with a mean and standard deviation of 12 ± 3.5 μm. Eighty five cells only had a small leakage-like current, which was less than 40 pA for a voltage step to +80 mV (see Fig. 2B and F). They ranged in diameter from 9 to 26 μm with a mean and standard deviation of 15.8 ± 3.7 μm. Two groups of cells had an outward rectifying current; either the I-type outward current (Fig. 2C and G) or the M-type outward current (Fig. 2D and H). The former group consisted of 43 cells with diameters in the range from 15 to 30 μm, with a mean and standard deviation of 22.3 ± 3.4 μm. The latter group consisted of 60 cells with diameters from 19 to 30 μm and a mean and standard deviation of 26.7 ± 2.9 μm.

3.5. Zero current potential

Recently we reported that the voltage-gated outward K^+ current is greatly suppressed in megakaryocytes from myelogenous leukemia patients and that the zero current potential of these cells was reduced

about 10 mV when compared to normal human megakaryocytes [1]. These 'myelogenous leukemia' megakaryocytes, with suppressed outward current, exhibited voltage-gated currents similar to the leakage-like or I-type currents. Therefore, we examined the zero current potential of these two cell types. The zero current potential for all the cells with the leakage-like and I-type currents combined was -42.6 ± 9.4 mV ($n = 128$) which is significantly different at the $P = 0.05$ level (unpaired Student's t-test) when

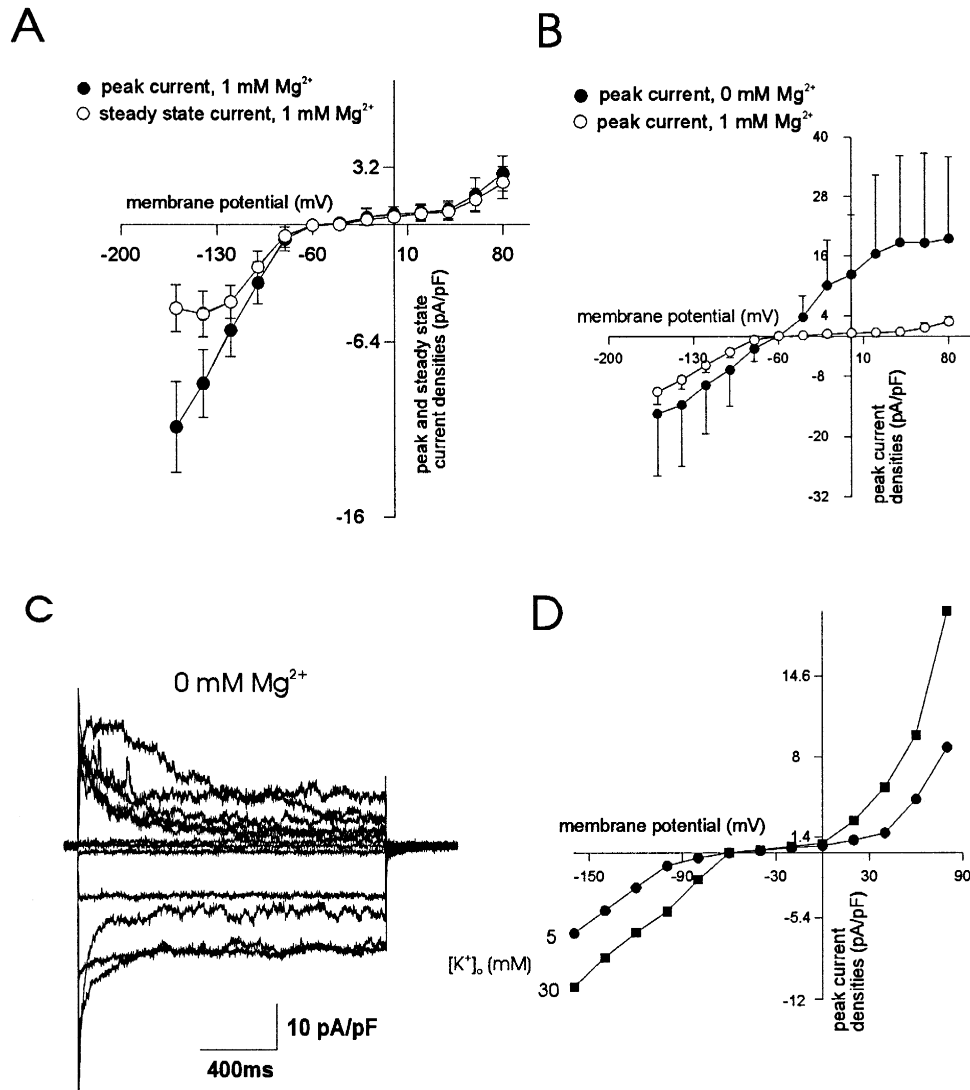


Fig. 5. Voltage-gated inwardly rectifying currents recorded from small megakaryocytic cells with and without Mg^{2+} in the intrapipette solution. (A) Peak and steady state I–V relationship for five megakaryocytic cells with 1 mM Mg^{2+} in the intrapipette solution (see Section 2). (B). Comparison of the peak I–V relationship for five megakaryocytic cells with (open circles) and 11 cells without (filled circles) Mg^{2+} in the intrapipette solution. (C). Example of voltage-gated currents recorded from a small megakaryocytic cell with an inward rectifying current when there was no Mg^{2+} added to the intrapipette solution. The voltage protocol was as in Fig. 2. Note the presence in C of the rapidly inactivating outward current for depolarizing voltage steps, which is not present in Fig. 2A where 1 mM Mg^{2+} was present in the intrapipette solution. (D). I–V relationships of the peak amplitudes of the voltage-gated currents when the cell was bathed with an extracellular solution containing 5 mM KCl (●) or 30 mM KCl (■) and with no Mg^{2+} in the intrapipette solution. The actual current recordings for this cell are given in Fig. 6A.

compared to the cells with the M-type current for which the zero current potential was -51.5 ± 8.9 mV ($n = 60$). It would appear that expression of the M-type outward current, which begins to activate at -40 mV [1], is associated with a decrease in the zero current potential of about 10 mV.

3.6. Cells with an inward rectifier

In Fig. 5A we show the average peak and steady state current–voltage (I–V) relationship for five megakaryocytic cells having a voltage-gated inward rectifying current. An important characteristic of the inward rectifying K^+ current is that intracellular Mg^{2+} acts as an open channel blocker eliminating a fast outward current component, which is present when whole-cell recordings are obtained with Mg^{2+} free intracellular solution [16,17]. Fig. 5C is an example of this outward current component recorded from a megakaryocytic cell, with an inward rectifier, using a Mg^{2+} free intracellular solution. The recording in Fig. 5C should be compared to that in Fig. 2A where Mg^{2+} was present in the intracellular solution. In Fig. 5B the average I–V relationship of the amplitude of the peak current for 11 cells with Mg^{2+} free solution in the recording pipette is compared to the I–V relationship for the five cells in Fig. 5A for which 1 mM Mg^{2+} was present in the recording pipette. In order to confirm that the voltage-gated inward current was indeed a K^+-current we exposed these cells to an external solution with 30 mM KCl (see Section 2). Actual recordings under these conditions are shown in Fig. 6A and the peak I–V relationship in Fig. 5D. As expected the inflection point of the inward current rectification shifted towards more positive voltages and the amplitude of the current at negative voltages increased (Fig. 5D, and for example see [18]). In Fig. 5D the outward current actually increases in the presence of 30 mM KCl although the driving force for a K^+ outward current is actually decreasing, this is the result of a new slowly activating outward current (see Fig. 6 and associated text). Based on the findings in Fig. 5 we conclude that a population of small (mean diameter of 12 μm) megakaryocytic cells have a voltage-gated anomalous inward rectifying K^+ current (IR_K).

The zero current potential for cells with an inwardly rectifying current was -59.9 ± 8.4 mV ($n = 32$) while for the cells with only a leakage-like current it was -39.9 ± 8.6 mV ($n = 85$). Apparently, the inward rectifier serves to maintain the zero current potential hyperpolarized by about 20 mV when compared to the other group of small megakaryocytic cells which have a leakage-like current.

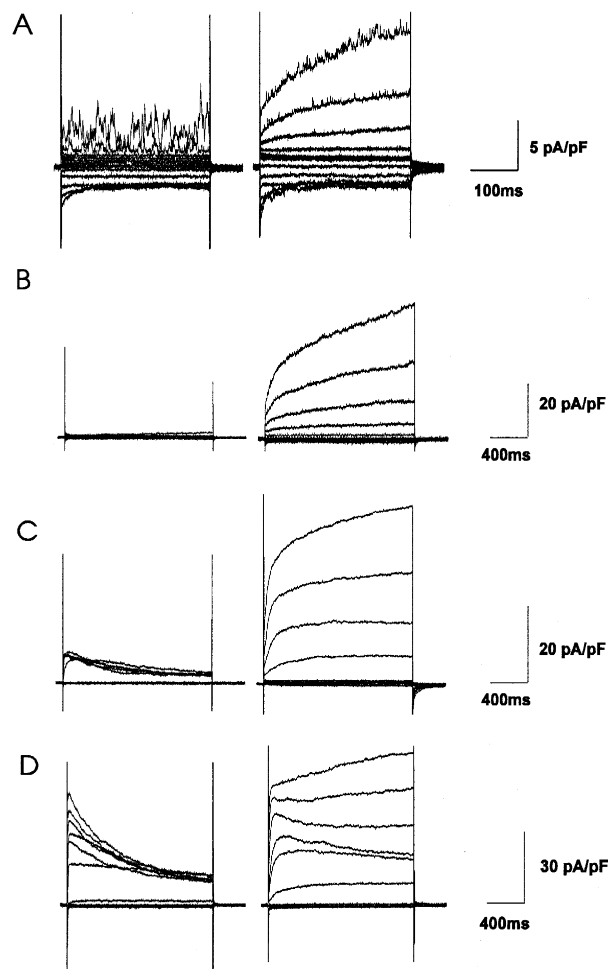


Fig. 6. Induction of a slowly activating voltage-gated outward current upon exposure to a high K^+ (30 mM) external solution. (A–D) On the left are the recordings from each of the four different types of megakaryocytic cells, identified in Fig. 2, in normal external solution. On the right are recordings from the same cells obtained when the external solution was changed to one in which 30 mM of the NaCl was replaced with 30 mM KCl. In each cell a new slowly activating non inactivating voltage-gated outward current is apparent. The voltage protocol was the same as in Fig. 2.

3.7. Slowly activating, non inactivating, voltage-gated outward current in the presence of elevated K^+

In the presence of 30 mM external KCl the cell in Fig. 6A developed a new slowly activating voltage-gated outward current. In order to determine whether this slowly activating outward current was peculiar to cells with an inward rectifier we exposed the other three types of megakaryocytic cells described to 30 mM KCl in the extracellular solution. As can be seen in Fig. 6 all cell types exhibited this new current when exposed to 30 mM KCl. This new voltage-gated current disappeared when the cell was returned to standard external solution. In recordings up to 5 s in duration this current did not inactivate (data not shown). Also the conductance of the membrane increased in proportion to the current (data not shown) indicating that the current was the result of a conductance increase.

Not every cell exhibited this new current. The overall percentage of the megakaryocytic cells examined, with application of elevated external potassium, which exhibited the new current was 45% ($n = 40$). As shown in Fig. 6 the new current was found in all four groups of the megakaryocytic cells identified in Fig. 2. The new current was present in 40% of the cells with an inward rectifier ($n = 10$), 31% of the cells with leakage-like current ($n = 16$), 88% of the cells with the I-type voltage-gated outward current ($n = 8$) and 33% of the cells with the M-type voltage-gated outward current ($n = 6$).

4. Discussion

The data in Fig. 2 demonstrate that in either stained or unstained megakaryocytic cells we can distinguish four classes of cells, on the basis of their voltage-gated currents. We used two independent methods for identifying megakaryocytic cells because each method has its own weakness. First, there are no generally accepted morphological criteria for the identification of living megakaryocytic cells as small as 10 μm in diameter in the light microscope. Although, we tested our ability to identify megakaryocytic cells in the light microscope, see Fig. 1, we expect there will be genuine skepticism concerning our method of identification. On the other hand, although the identification of megakaryocytic cells by the GpIIb/IIIa antibody is reliable, we were concerned that antibody binding might somehow affect the cells voltage-gated channels. Thus on the basis of the two methods taken together, we believe that we have identified four classes of megakarocytic cells having different voltage-gated currents.

Based on the data of Fig. 3 it appears that megakaryocytic cells exhibiting the M-type of voltage-gated current first appear at about 18 μm. This finding correlates well with earlier work which found that both granular and mature megakaryocytes first appear at around 20 μm [6]. Therefore, we suggest that megakaryocytes having the M-type of voltage-gated outward current probably correspond to the previously described mature and granular megakaryocytes. We also suggest that megakaryocytic cells with an inward rectifying conductance correspond to the megakaryoblasts, the least mature form of megakaryocyte [6]. For the remaining two groups of cells (cells with leakage-like current and cells with the I-type of outward current) we suggest that those cells might correspond to promegakaryocytes. These identifications should be considered tentative at best, as they are based primarily on cell size.

We propose that each megakaryocyte goes through a unique sequence of voltage-gated ion channel expression during development. The smallest megakaryocytes express inward rectifying K^+ channels which disappear as the cell grows going through a stage where they exhibit only leakage-like currents. As they continue to grow in size megakaryocytes begin to express voltage-gated outward currents of the I-type and then the M-type. A simple explanation that would account for the I-type and M-type of outward currents is that as the megakaryocyte with the 'leakage-like' current matures it begins to express more than one type of voltage-gated K^+ channel, each with different activation and inactivation characteristics. If the relative number of these channels changes as the cell matures, this might account for I-type and M-type currents. There are only a few studies on the sequence of expression of voltage-gated currents in the development of other hematocytes [2,3]. One similarity between our findings and those studies is that voltage-gated K^+ channel expression is regulated during development of T lymphocytes [3].

When exposed to high potassium in the extracellu-

lar solution, approximately 45% of the cells, exhibited a slowly activating non-inactivating voltage-gated outward current (see Fig. 6). A similar current, termed the IsK current, has been observed in a variety of other cell types including hematocytes [19–21]. It may be that the channels underlying this current are normally present but somehow masked or silenced in developing megakaryocytes. What relationship this IsK-like current has to the voltage-gated currents described in this report is unknown, as is the mechanism by which elevated K^+ causes this current to be revealed.

The currents recorded from megakaryocytes having 'leakage-like' currents and 'I-type' currents, were indistinguishable from the voltage-gated currents of the megakaryocytes from myelogenous leukemia patients, in which voltage-gated currents were suppressed [1], suggesting that the megakaryocytes from the myelogenous leukemia patients are a dedifferentiated or less mature form of megakaryocyte. In these patients, growth of the megakaryocyte appears to occur without the normally associated development of the voltage-gated outward current.

The currents recorded from megakaryocytes having 'leakage-like' currents were similar to the leakage-like currents we found in two megakaryoblastic tumor cell lines (DAMI and CHRF-288-11) and a human erythroleukemia cell line (HEL) [1]. We suggest that all three tumor cell lines are arrested at a relatively early stage of development, at least as far as their voltage-gated currents are concerned.

Acknowledgements

We thank Drs. Edward E. Morse, Peter J. Tutschka, Steven K. Bergstrom and John J. Quinn for supplying us with the human megakaryocytes used in these experiments. We thank Ms. Teresa Murphy and Ms. Judy Gaffney for technical support. This work was supported by grants from the Donaghue Foundation and the University of Connecticut Health Center.

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