Enhancement of Voltage-gated Na⁺ Channel Current Associated with Multidrug Resistance in Human Leukemia Cells

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ABSTRACT

Membrane currents were examined in a drug-sensitive human leukemia cell line (K562) and its multidrug-resistant cell line (K562/ADM) under the whole-cell variation of the patch electrode voltage clamp technique. Most K562 cells showed only the outward current, which was completely suppressed by internal Ca²⁺ ions and 20 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid. In contrast to K562 cells, most K562/ADM cells showed tetrodotoxin-sensitive, voltage-gated Na⁺ channel current in addition to the outward current. Na⁺ current was observed in four of 29 K562 cells examined, while it was observed in 29 of 33 K562/ADM cells. Two revertant cell lines (R1-3, R1-5) did not show Na⁺ current. It was concluded that the amplitude of voltage-gated Na⁺ current increases in association with multidrug resistance in human leukemia cells.

INTRODUCTION

The development of multidrug resistance is a major problem in cancer chemotherapy. One of the proposed mechanisms underlying drug resistance is a change in plasma membrane properties, which is closely related to the expression of M₁, 170,000 glycophorin (P-glycoprotein) on the plasma membrane (1–6), or to the alteration in lipid structural order (7–10). In addition, multidrug-resistant cells possess enhanced energy-dependent drug efflux, common to various antimutator agents (11–15). It has been shown that multidrug resistance can be reversed by Ca²⁺ channel blockers (16–18) and antiarhythmic drugs (19). These findings suggest that ion transport across plasma membrane may be involved in the mechanisms of drug resistance. We have examined the ionic channels in human leukemia cells by electrophysiological methods with the aim of determining what channel develops in association with multidrug resistance.

MATERIALS AND METHODS

Cell Culture. A human leukemia cell line (K562) and its Adriamycin-resistant cell line (K562/ADM), which shows resistance to multidrugs including anthracycline antibiotics and Vinca alkaloids (20), were used in the present experiment. The characteristics of these cells have been described in detail (20, 21). K562 cells were cultured at 37°C under 5% CO₂-humidified air in RPMI 1640 medium containing 10% FCS. K562/ADM cells were continuously cultured in RPMI 1640 medium containing 10% FCS and 300 mg/ml Adriamycin. K562/ADM cells were transferred to the medium without Adriamycin within 2 mo before electrophysiological study. Multidrug resistance of K562/ADM cells is stable for 2 mo in the medium without Adriamycin (20). Electrophysiological analyses were done at 1 to 3 days after subculture.

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3 The abbreviations used are: FCS, fetal calf serum; TTX, tetrodotoxin; HEPES, 1-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid; cAMP, cyclic AMP.
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**Fig. 1.** Membrane current in K562 cells recorded in the standard extracellular medium. In A, the outward current was recorded by using the patch electrode containing standard internal solution (K⁺ ions and 0.5 mM EGTA). Holding potential was −60 mV. The fixed potentials are shown. In B, the outward current was recorded by using the patch electrode containing K⁺ ions and 20 mM EGTA. Holding potential was −60 mV. In C, membrane current was recorded with a patch electrode containing Cs⁺ ions and 20 mM EGTA. Holding potential was −102 mV.

- A: mV
- B: mV
- C: mV

**RESULTS**

Membrane Current in K562 Cells. Fig. 1 shows the representative records of the membrane current in K562 cells. Holding potential, where the membrane potential was steadily kept, was −60 mV. The test pulse was applied every 12 s. The membrane current in most K562 cells was composed of the outward current. There were two types of the outward current. One type (Fig. 1A) showed activation and inactivation processes, whereas another type (Fig. 1B) showed little activation and inactivation. The current-voltage (I-V) relations of Fig. 1, A and B, are plotted as closed circles with a solid line and as closed triangles with a solid line in Fig. 2, respectively. The outward current similar to Fig. 1A was more frequently observed with a patch electrode containing the standard internal solution. By using the patch electrode containing 20 mM EGTA and K⁺ ions, the outward current similar to Fig. 1A was not observed, but the outward current was similar to Fig. 1B. The outward current in Fig. 1A may be Ca²⁺-activated K⁺ current observed in *Helix* neurons (27), because the I-V relation showed an N-shape and it was suppressed by internal 20 mM EGTA.

By using the patch electrode containing 20 mM EGTA and Cs⁺ ions, the outward current was completely suppressed, leaving only leakage current (Fig. 1C). Its I-V relation is plotted as open triangles with a broken line in Fig. 2. The outward current in Fig. 1B may be a steady leakage current. However, we considered this current not to be a leak, because this current showed an outward-going rectification, and it was suppressed by internal Cs⁺ ions in addition to 20 mM EGTA. The outward current without distinctive activation and inactivation has been reported in rat GH3 cells (28). Similar outward currents to Fig. 1, A and B, were also observed in K562/ADM cells. The amplitude of the outward current considerably varied among cells in both cell lines, and a distinctive difference was not recognized between the two cell lines.

Cell Growth and Cellular Uptake of [3H]Vincristine. K562/ADM cells (2 x 10⁴) were cultured at 37°C for 5 h in Falcon culture tubes (no. 2054) containing 2 ml of growth medium (RPMI 1640 medium containing 10% FCS) per tube. Then they were treated with graded concentrations of Adriamycin or vincristine. For examining the effect of TTX, 1 μM TTX was added to the growth medium. The cells were cultured in the presence of the drug(s) and counted with a Coulter Counter 3 days after drug treatment.

Cellular uptake of vincristine was measured in growth medium. K562/ADM cells (2 x 10⁴) in Falcon culture tubes (No. 2054) containing 1 ml of growth medium with 20 mM HEPES buffer were incubated at 37°C with [3H]vincristine (1 μM; specific activity, 4.4 Ci/mM; Amersham), and the amount of intracellular vincristine was measured at 1 h. The above procedures were essentially the same as reported previously (20).

**Fig. 2.** The I-V relation of the outward current in K562 cells. •, record in Fig. 1A; ▲, record in Fig. 1B; Δ, record in Fig. 1C.

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[Note: The image contains graphs and figures that are not transcribed in the text.]

Data were expressed by mean ± SE, and the statistical analysis was done by Student's t test.

Cells used in the present experiment ranged from 10 to 30 μm. The experiments were carried out at room temperature (22–25°C).
Membrane Current in K562/ADM Cells. In contrast to K562 cells, most K562/ADM cells exhibited an inward current (Fig. 3). The outward current was suppressed by using the patch electrode containing Cs+ and 20 mM EGTA. Holding potential was —101 mV. The threshold of the inward current activation was about —40 mV. With greater depolarizing potential steps, the amplitude of the inward current increased and became maximum at about —10 mV. Then the amplitude of the inward current decreased and disappeared at about +50 mV. The I-V relation of the peak inward current (peak current minus leakage current) of this cell is shown in Fig. 4 (squares with a solid line), where I-V relations in the other two cells are shown (triangles with a broken line and circles with a solid line). Although the amplitude of the inward current varied among cells, the I-V relations were similar.

The inactivation time constant of the inward current was examined by plotting the inward current in a semilogarithmic scale. The decaying phase fitted the single exponential, indicating the kinetics followed a first-order process with a single time constant ($\tau_\text{h}$), which is plotted against the membrane potential in Fig. 5 ($n = 4$). The $\tau_\text{h}$ of the inward current in human leukemia cells is thus voltage dependent.

Fig. 6 shows the steady-state inactivation of the inward current recorded in three cells as a function of membrane potential. Holding potential was —100 mV, and the test pulse was fixed to —5 mV. The duration of the prepulse was 100 ms. The steady-state inactivation was measured by normalizing the Na+ current at —5 mV to one in each cell. The amplitude of the inward current decreased as the prepulse was depolarized. The solid line in Fig. 6 was drawn by the formula

$$h_\text{in} = 1 / \left(1 + \exp \frac{V - V_\text{h}}{K_\text{a}}\right)$$

where $V$ is the prepulse potential, and $V_\text{h}$ is the potential at which $h$ becomes 0.5 (~47 mV). The slope parameter $K_\text{a}$ is set to 7.5. The steady-state inactivation approximately fitted this formula. From the above results it was concluded that the inward current was carried through Hodgkin-Huxley type voltage-dependent channels.

Because the kinetics of the inward current was fast, it was considered that the inward current was carried through Na+ channels. For its clarification, the effect of TTX was examined. The application of 100 nM TTX reduced the inward current,
Na* CURRENT IN HUMAN LEUKEMIA CELLS

Fig. 7. A, effect of TTX on the inward current in K562/ADM cell. One μM TTX inhibited the inward current, which was completely recovered after washout of TTX. In the standard extracellular medium, the patch electrode contained Cs* and 20 mM EGTA. Holding potential was −81 mV, and the fixed potential, −11 mV. B, the I-V relation of the inward current during application of 100 nM TTX (V), control. C, inward current in K562/ADM cell recorded in Li* medium. The solution of the patch electrode was the same as in A. Holding potential was −100 mV, and the fixed potential, −13 mV. D, membrane current in K562/ADM cell recorded in 20 mM Ca2+-Na* -free medium. The solution of the patch electrode was the same as in A. Holding potential was −80 mV, and the fixed potential, −6 mV.

Table 1 Comparison of specific Na* current density between K562 and K562/ADM cells

<table>
<thead>
<tr>
<th>No. of examined cells</th>
<th>K562</th>
<th>K562/ADM</th>
<th>R1-3</th>
<th>R1-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells with sodium current</td>
<td>29</td>
<td>33</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Membrane capacity (pF)</td>
<td>29.1 ± 1.80*</td>
<td>29.9 ± 1.83</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Specific sodium current (pA/pF)</td>
<td>0.35 ± 0.21</td>
<td>3.9 ± 0.76*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Significant, P < 0.001.

and after washout of TTX, the amplitude of the inward current recovered (Fig. 7A). The I-V relation during application of TTX was compared to the control in Fig. 7B. The I-V relation did not change by TTX except for the reduction of the inward current. The inhibition was complete with 1 μM TTX. The inward current was also eliminated in Na* -free medium in which Na* ions were isomotically replaced with choline, whereas it was still observed in Li* medium (Fig. 7C). From these results it was concluded that the inward current was carried through voltage-gated Na* channels. One μM verapamil did not suppress Na* current.

Table 1 compares the specific Na* current density between K562 and K562/ADM cells. The peak of maximum inward current was normalized by the membrane capacity which represents the surface area of the cell membrane. Membrane capacity of K562 cells was almost the same as that of K562/ADM cells. Na* current was observed in 4 of 29 K562 cells examined, while it was almost consistently observed in K562/ADM cells (29 of 33). The amplitude of Na* current in K562/ADM cells (3.9 ± 0.76 pA/pF) was significantly greater than that in K562 cells (0.35 ± 0.21 pA/pF) (P < 0.001). For further clarification of this result, membrane current was examined in two revertant cell lines of K562/ADM cells (R1-3, R-15). In these revertant cell lines, Na* current was not observed (n = 10, respectively). Therefore it was concluded that the amplitude of Na* current increased in association with multidrug resistance in human leukemia cells.

We looked for voltage-gated Ca2+ channel current in K562/ADM cells because it has been demonstrated that Ca2+ channel blockers can reverse drug resistance (16–18). However, we could not find macroscopic Ca2+ channel current even when the extracellular Ca2+ ions were raised to 20 mM (n = 10), as shown in Fig. 7D. In the other five cells, membrane current was recorded in 20 mM Ca2+-Na* -free medium using the patch electrode containing 2 mM ATP-Mg, 100 μM cAMP, and 2 mM theophylline. In such a condition we could not again detect Ca2+ current. Similarly Ca2+ channel current was not observed in 80 mM Ba2+-Na* -free medium (n = 5). Ca2+ current was not detected in K562 cells (n = 7).

Effect of TTX on Cell Growth and Drug Uptake in K562/ADM Cells. K562/ADM cells exhibited 630-fold resistance to vincristine compared to K562 cells, but the growth of K562/ADM cells was inhibited by vincristine at over 0.1 μM (Fig. 8A). The addition of 1 μM TTX did further inhibit the growth of K562/ADM cells. In the case of Adriamycin the result was the same (data not shown). Fig. 8B shows the effect of TTX on vincristine uptake in K562/ADM cells. One μM verapamil greatly increased vincristine uptake, while 1 μM TTX did not. These results indicate that voltage-gated Na* channels are not directly involved in the mechanisms of drug resistance.

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**DISCUSSION**

The results in the present experiment revealed the enhancement of voltage-gated Na⁺ channel current associated with multidrug resistance in human leukemia cells. The role of Na⁺ channels on drug resistance was examined by TTX. However, 1 μM TTX, which completely inhibited Na⁺ channels, showed no effect on vincristine uptake in K562/ADM cells. TTX did not potentiate the effect of Adriamycin and vincristine. Therefore, Na⁺ channels appear not to be directly involved in drug resistance, and their functional significance is unclear at present.

Although we could not find voltage-gated Ca²⁺ channel current in K562 and K562/ADM cells, the I-V relation of the outward current exhibited the N-shape, which suggested the existence of Ca²⁺-activated K⁺ channels. Therefore, in human leukemia cells Ca²⁺ ions may enter the cell through other routes than voltage-gated Ca²⁺ channels. The mechanisms of verapamil to reverse drug resistance are still unresolved. These problems remain to be elucidated.

τₙ of Na⁺ current in human leukemia cells was compared to those in rat GH₃ cells (29), bovine chromaffin cells (23), and mouse neuroblastoma cells (30) because the temperature of the experiments and the threshold of Na⁺ channels in these cells are similar to those in human leukemia cells. τₙ in GH₃ cells at a membrane potential of 0 mV was 0.3 ms, in neuroblastoma cells, 0.4 ms, in chromaffin cells, 1.2 ms, and in human leukemia cells, about 2.5 ms. Although τₙ in human leukemia cells is somewhat slower than those in other cells, the values are comparable. The steady-state inactivation parameter, Kₚ, was 7.5 in human leukemia cells. Kₚ in squid giant axons was 7.5 (31), in myelinated frog nerve, 7.0 (32), in mouse neuroblastoma cells, 9.5 (30), and in tunicate egg, 5.8 (33). The values are approximately the same as that of human leukemia cells. Thus Na⁺ channels in human leukemia cells possess similar characteristics to those in excitable tissues.

It has been reported that Na⁺ channels consist of a glycoprotein with three subunits: a major large subunit of molecular weight between 230,000 and 300,000 [α] and two subunits of molecular weight between 30,000 and 39,000 [β₁, β₂] (34, 35). These molecules are different from that of P-glycoprotein, suggesting that another glycoprotein (Na⁺ channel molecule) develops in association with drug resistance.

The enhancement of Na⁺ channel current in K562/ADM cells may be a consequence of an increased expression of normal gene with its amplification (36, 37), because K562/ADM cells actually possess a homogeneously staining region in chromosomes and double minute chromosomes (20). The finding that Na⁺ current could be detected in a few drug-sensitive cells may account for the low expression of the Na⁺ channel gene in these cells similar to the case of P-glycoprotein (4, 6). It has been reported that lipid structural order of the membrane is altered in drug-resistant cells (7–10). Such an alteration of plasma membrane in drug-resistant cells may convert functionally inactive Na⁺ channels to be active.

It may be expected that K562/ADM cells without Na⁺ current (4 of 33 in the present experiment) were revertants. To rule out this possibility the membrane current was examined in 12 K562/ADM cells grown in the medium containing 300 ng/ml Adriamycin just before the electrophysiological study. K562 cells cannot grow in the presence of 300 ng/ml Adriamycin. In 2 of these 12 K562/ADM cells, Na⁺ current could not be detected. Therefore, it was considered that K562/ADM cells without Na⁺ current were not revertants. K562 and K562/ADM cells are the established cells and not the cloned cells. Therefore, the cells may not be entirely homogenous, which might explain the absence of Na⁺ current in a small percentage of K562/ADM cells. This notion may account for the considerable variations in the amplitude of Na⁺ current in K562/ADM cells and the outward current in both cell lines. Another explanation for these variations may be the asynchronous cell cycle, because it is indicated that the amplitude of Ca²⁺ current changes during the cell cycle in the mouse hybridoma cell line secreting immunoglobulins (38).

Recently Schlichter et al. (39) have reported that membrane current of K562 cells is composed of Na⁺ current. In their experiment Na⁺ current is detected in all cells (n = 29), and there is no outward current nor Ca²⁺ current. In our experiment Na⁺ current could be detected in a small portion of K562 cells, and the outward current was consistently observed. Although these discrepancies may be due to the different experimental conditions, we don't have a good explanation at present. Our K562/ADM cells are derived from K562 cells in our laboratory, and Na⁺ current was enhanced in K562/ADM cells. Na⁺ current of their K562 cells may increase if that cell line becomes drug resistant.

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