Potassium channels mediate killing by human natural killer cells
(tumor-cell lysis/patch-clamp recording/ion-channel blockers/membrane currents/cell-mediated cytolysis)

LYANNE SCHLICHTER*, NEIL SIDELL†, and SUSUMU HAGIWARA*

*Department of Physiology and Jerry Lewis Neuromuscular Research Center, and †Division of Surgical Oncology, University of California, Los Angeles, CA 90024

Contributed by Susumu Hagiwara, September 9, 1985

ABSTRACT Human natural killer (NK) cells in peripheral blood spontaneously recognize and kill a wide variety of target cells. It has been suggested that ion channels are involved in the killing process because there is a Ca-dependent stage and because killing by presensitized cytotoxic T lymphocytes, which in many respects resembles NK killing, is associated with changes in K and Na transport in the target cell. However, no direct evidence exists for ion channels in NK cells or in their target cells. Using the whole-cell variation of the patch-clamp technique, we found a voltage-dependent potassium (K⁺) current in NK cells. The K⁺ current was reduced in a dose-dependent manner by the K-channel blockers 4-aminopyridine and quinidine and by the traditional Ca-channel blockers verapamil and Cd⁺². We tested the effects of ion-channel blockers on killing of two commonly used target cell lines: K562, which is derived from a human myeloid leukemia, and U937, which is derived from a human histiocytic leukemia. Killing of K562 target cells, determined in a standard 51Cr-release assay, was inhibited in a dose-dependent manner by verapamil, quinidine, Cd⁺², and 4-aminopyridine at concentrations comparable to those that blocked the K⁺ current in NK cells. In K562 target cells only a voltage-dependent Na⁺ current was found and it was blocked by concentrations of tetrodotoxin that had no effect on killing. Killing of U937 target cells was also inhibited by the two ion-channel blockers tested, quinidine and verapamil. In this cell line only a small K⁺ current was found that was similar to the one in NK cells. We could not find any evidence of a Ca⁺² current in target cells or in NK cells; therefore, our results cannot explain the Ca dependence of killing. Our findings show that there are K channels in NK cells and that these channels play a necessary role in the killing process. In contrast, the endogenous channel type in the target cell is probably not a factor in determining target cell sensitivity to natural killing.

Natural killer (NK) cells constitute a functionally distinct subset of peripheral blood lymphocytes (PBL) in humans and in many other species (1, 2). They are morphologically associated with large granular lymphocytes (LGL), of which >70% can have NK activity (3). Results of a large number of studies indicate an important role for NK cells in destruction of tumor cells (reviewed in refs. 1 and 4). Killing by NK cells and by cytotoxic T lymphocytes (CTL) shares three readily identified stages (1, 2, 4–6): (i) There is a Mg-dependent recognition–adhesion stage that is not temperature sensitive. (ii) The programming–for–lysis stage is Ca dependent and has a temperature optimum at 37°C. (iii) During the killer cell–independent lysis stage the target cell swell and lyse even after the effector cell (NK or CTL) has detached. The mechanism(s) of killing by either effector cell type is not well understood. In NK cells it appears to involve vesicular exocytosis from the killer cell (5, 7, 8), release of a soluble lytic factor (9), and possibly insertion into the target cell of tubular complexes analogous to those formed by the C9 fragment of complement (10). The Mg and Ca dependence of different stages in the killing process as well as changes in ion fluxes in killer and target cells (11–13) have led to the hypothesis that ion channels are involved in cell-mediated cytolysis. To date, this hypothesis has not been tested directly and no studies of ion channels in NK cells or in their tumor target cells have been reported.

In the present study, we have examined whether ion channels are involved in killing by human NK cells. Using the whole-cell variation of the patch-clamp technique, we found a voltage-dependent K⁺ current in NK cells that was reduced in a dose-dependent manner by quinidine, 4-aminopyridine (4AP), Cd⁺², and verapamil, at concentrations comparable to those that inhibited killing. In contrast, neither the type nor functioning of the channels in target cells appears to be critical for killing to occur.

METHODS

Cell Preparation. NK cells were separated in the LGL fraction of human peripheral blood mononuclear cells as described (3, 14). Following separation by discontinuous Percoll density centrifugation, cells were further depleted of T lymphocytes by sheep erythrocyte–rosette formation, and this highly enriched population (>90% LGL) (9) was used for electrical recording. Target tumor cells were the NK-sensitive human leukemia cell lines K562 and U937.

Cytotoxicity Assays. For assessing natural killing we used the standard 51Cr-release assay (14). Percent specific lysis was defined as the percent specific 51Cr released, or ([experimental release – spontaneous release]/(maximal release – spontaneous release)) × 100. Experimental release (measured as cpm) was determined from the amount of 51Cr released by the targets when incubated with effector cells in the absence or presence of channel blockers. Spontaneous 51Cr release from isolated target cells was determined. Maximal release was measured after the targets were incubated with 5% (vol/vol) Nonidet P-40 detergent.

Electrical Recording. For electrical recording, a patch electrode of 3- to 8-MΩ resistance was pushed lightly against a LGL and after a high-resistance seal (>20 GΩ) formed, a small negative pressure was applied to the pipette interior to establish a whole-cell recording. Linear components of capacitative and leakage currents were analogue-subtracted and the current was low-pass filtered at 3 kHz. Current and voltage traces were stored on floppy disks using a Nicolet storage oscilloscope and were later printed with an X–Y plotter. Recordings were always begun while cells were continuously perfused with a mammalian Ringer’s solution.

Abbreviations: 4AP, 4-aminopyridine; CTL, cytotoxic T lymphocyte(s); LGL, large granular lymphocyte(s); NK, natural killer; PBL, peripheral blood lymphocyte(s).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
(standard solution) containing (in mM) 150 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgCl₂, 10 d-glucose, and 10 Hepes, adjusted to pH 7.4 with NaOH. The pipette contained one of two "internal" solutions (mM): either (i) 20 NaCl, 1 MgCl₂, 100 potassium aspartate, 15 d-glucose, 10 KHepes, 5 K₂EGTA, 4 MgATP, 0.1 cAMP, and 2 theophylline at pH 7.4 or (ii) a KF solution consisting of 20 NaCl, 1 MgCl₂, 100 KF, 15 d-glucose, 10 KHepes, and 5 K₂EGTA at pH 7.4. The KF internal solution increased the lifetime of the cell but did not change the appearance of the currents. Bathing solutions with different K concentrations were made by substituting KCl for equimolar amounts of NaCl. Junction potentials were measured and corrections were made as in previous work (15). All electrical measurements were made at 22±2°C.

For determining the degree of blockage of the K⁺ current by Ca²⁺ or 4AP, the membrane potential was held at −100 mV and then stepped to a range of voltages for 60 ms each and the peak current at each voltage was measured. Then, each test solution was perfused for 5 min, and the maximum current at each voltage was measured. A different protocol for examining channel blockage by quinidine and verapamil was necessary because blockage by these drugs was use dependent—that is, the current decreased rapidly during a pulse as open channels were blocked. Cells were held at −70 mV for 5 min while the blocker was perfused in and then the membrane potential was stepped a single time to −10 mV. The peak current at −10 mV was taken to represent the amount of resting blockage that occurred at −70 mV, which is close to the resting potential measured in T lymphocytes (16).

RESULTS

Effect of Ion-Channel Blockers on Killing. We tested K⁺ channel blockers (4AP, quinidine), Ca⁺⁺ channel blockers (verapamil, Ca²⁺), and a Na⁺ channel blocker (tetrodotoxin). The first four blockers effectively reduced killing of K562 target cells. Percent inhibition, expressed as the mean ± SEM of n separate experiments using blood from different healthy donors, was 95.9% ± 1.4% (n = 7) for 0.1 mM verapamil, 80.6% ± 4.5% (n = 8) for 0.1 mM quinidine, 63.9% ± 2.2% (n = 4) for 1 mM 4AP, and 83.5% ± 7.3% (n = 6) for 2 mM 4AP. Tetrodotoxin at 3 μM did not reduce killing. We also tested the ability of verapamil and quinidine to inhibit killing of U937 target cells. At 0.1 mM, verapamil and quinidine inhibited killing by 92% and 65%, respectively. None of these drugs was toxic at the highest concentrations used: spontaneous 31Cr release from target cells in the absence of effector cells (PBL) was not changed, and trypan blue exclusion by PBL was unaffected over the 4-hr period required for the killing assay. In control experiments, PBL were depleted of monocytes by removing plastic-adherent cells. Because monocytes suppress NK cell activity in vitro (17), it was important to show that channel blockers do not affect killing through their effects on monocytes. There was no difference in the percent reduction of killing in the presence of channel blockers with and without monocyte depletion (data not shown).

Ion Currents in NK Cells and Target Cells. Having found that several ion-channel blockers inhibit killing we wanted to determine whether it is the ion channels in NK cells or in the target cells that are essential. To do this we used the patch-clamp technique to identify the ion channels and then related the inhibition of killing to the blockage of the different ion channels. Highly enriched preparations of human NK cells were obtained by isolating LGL (spherical, 8-9 μm in diameter) as described in Methods. The results described in Fig. 1 are from two representative cells; in all 54 LGL examined, the macroscopic properties of the current were similar. Fig. 1a shows a family of outward potassium (K⁺) currents recorded in the whole-cell configuration. The membrane potential of the cell was held at −100 mV and positive-going voltage pulses were applied in 10-mV increments. Voltages between −90 and −60 mV produced only small time-independent leakage currents corresponding to an input resistance of >15 GΩ. (Membrane resistances of >10 GΩ were usually observed; cells with resistances of <5 GΩ were discarded.) At −50 mV a time-dependent outward current appeared and as the membrane potential became more positive, the current amplitude increased. A clear delay in the activation of outward current and an increase in the sigmoidal rate of rise were observed. During prolonged depolarizing voltage pulses the current reached a peak and then decreased with time because of a voltage-dependent inactivation process. This can be seen in Fig. 1a at +10 to 30 mV. Moreover, cumulative inactivation occurred between pulses (not shown) as it does in T lymphocytes (18–20). Therefore, to permit recovery from inactivation, 10–30 s was allowed between pulses, the longer times corresponding to move positive voltages where there was more inactivation. Several criteria were used to identify the outward current as a K⁺ current: (i) The sigmoid activation kinetics, slow inactivation, and voltage dependence are similar to the delayed rectifier K⁺ current. (ii) The current was blocked by the K⁺-channel blockers 4AP and quinidine. (iii) The maximal potential for the current was close to the K⁺ equilibrium potential over a wide range of external K⁺ concentrations (Fig. 1d). This curve was derived from Fig. 1c, which shows current–voltage relations for the tail currents in 5, 10, and 20 mM external K⁺ and for activated currents in 50 and 100 mM external K⁺. The zero-current (reversal) potential for each curve is plotted as a function of the external K⁺ concentration in Fig. 1d. The value at 5 mM represents the mean ± SEM for 15 cells bathed in the standard solution. The line indicates a slope of 58 mV per decade change in external K⁺, calculated from the actual concentrations of K⁺ in the internal solution (120 mM) and in the bath. These results show that the current was carried by K⁺. By using the value of the reversal potential (Erev) measured in standard solution, the chord conductance was calculated from the peak outward current and plotted against voltage as shown in Fig. 1b. The conductance–voltage curve is sigmoidal as is typical of voltage-dependent channels, and reaches a maximum of 5.8 nS in this cell. The average maximal value was 2.3 ± 0.3 nS (n = 36). For these calculations, Erev was measured in 19 cells and calculated for the remaining cells. A very similar K⁺ reversal in T lymphocytes has been observed, which is consistent with a reversal of 16 pS (19). If the K⁺ current in NK cells is the same, the maximal conductance represents an average of 140 functioning channels per cell.

The Ca⁺⁺ dependence of the programming-for-lysis stage of killing has led to speculation about the presence of Ca⁺⁺ channels in NK cells. We found no evidence of Ca⁺⁺ currents: (i) When essentially all of the K⁺ current was blocked with 4AP or quinidine no inward current was observed above the noise level of 2–3 pA. (ii) When all internal and external K⁺ was replaced with n-methylglucamine, which neither permeates nor blocks K channels, no Ca⁺⁺ current was seen. (iii) When all external Na⁺ was replaced with 50 mM Bs (which permeates Ca channels) and n-methylglucamine (which was also the internal cation), there was no inward current. Moreover, this lack of inward current suggests that Ca²⁺ and Ba²⁺ are not appreciably permeant through the K⁺ channel, as does the fact that the reversal potential was very close to the K⁺ equilibrium potential. Two Ca⁺⁺ channel blockers (verapamil, Ca²⁺) inhibited killing even though NK cells apparently do not have Ca⁺⁺ channels. We show below that these agents reduce the K⁺ currents in NK cells and that this probably accounts for the inhibition of
Fig. 1. Voltage-dependent K⁺ current in human LGL. (a) Membrane currents recorded by using the whole-cell variation of the patch-clamp technique; outward currents elicited by 60-ms-long voltage-clamp pulses applied every 20 s from a holding potential of −100 mV. Shown are 10-mV steps between −60 and +30 mV. Inward tail currents were present at the end of each pulse when the membrane potential was returned to −100 mV. The voltage at which the tail current reversed to outward (reversal potential, \(E_{\text{rev}}\)) was −80 mV in this cell. Bath solution was the standard Ringer's solution; the pipette contained KF solution. (b) Chord conductance–voltage (g–V) relation. The peak current (\(I_{\text{peak}}\)) at each voltage in a was divided by the voltage minus the observed reversal potential of the tail current—i.e., \(g = I_{\text{peak}}/(V - E_{\text{rev}})\). (c) K⁺ dependence of the reversal potential. Different cell from a and b. Instantaneous current–voltage relations for tail currents in 5, 10, and 20 mM external K⁺ and from the steady-state currents in 50 and 100 mM K⁺. Each 0-pA crossing indicates the reversal potential at the corresponding K⁺ concentration that is indicated beside each curve; internal K⁺ = 120 mM. For tail-current measurements, K⁺ currents were activated for 20 ms at +20 mV and then the membrane potential was stepped to a particular test voltage for 40 ms. The tail current at 2 ms after the beginning of the test pulse was plotted to obtain the reversal potential. (d) Reversal potential in each solution versus the logarithm of the external K⁺ concentration. The value at 5 mM (open circle) is the mean ± SEM of 15 cells. The line shows a slope of 58 mV per logarithm of the external K⁺ as predicted by the Nernst equation using the actual internal and external K⁺ concentrations. killing. Ca-channel blockers (verapamil, diltiazem, Ni²⁺) also block a similar K⁺ current in T lymphocytes (19–21).

Next we identified the ion channels in the two common target cell lines for which we had shown inhibition of killing by ion-channel blockers. Fig. 2 shows that K562 cells have only a voltage-dependent Na⁺ current, whereas U937 cells have only a voltage-dependent K⁺ current. When a K562 cell (spherical, about 16 μm in diameter) was depolarized from a holding potential of −100 mV to about −40 mV an inward time-dependent current appeared (Fig. 2a). With further depolarization the amplitude increased and then decreased and reversed to an increasingly outward current. The rates of activation and inactivation increased as the membrane potential was made more positive and voltage-dependent inactivation to a steady-state level was complete in several milliseconds. Similar currents were seen in all 28 cells examined. This was judged to be a Na⁺ current because (i) the voltage dependence and rate of activation and inactivation are appropriate, (ii) the reversal potential (+44 ± 4 mV, \(n = 6\)) was close to the calculated Na⁺ equilibrium potential (+52 mV), and (iii) tetrodotoxin blocked the current, 0.3 μM blocked >90% of the current and 0.6 μM blocked >95%. The current–voltage relation in Fig. 2c shows that activation starts at about −50 mV and that the amplitude of the current reaches a maximum at about −10 mV. As in NK cells there was no evidence of a Ca²⁺ current in K562 cells. When >95% of the current was blocked with tetrodotoxin, the remaining current had the same voltage dependence and time course of activation and inactivation as the tetrodotoxin-sensitive portion and was therefore considered to be residual Na⁺ current. Moreover, for several cells, NaCl and potassium aspartate in the internal solution were replaced with cesium aspartate to eliminate outward K⁺ or Na⁺ currents. Then, in normal bathing solution, inward (but not outward) Na⁺ currents were observed, and when external Na⁺ was replaced with 25 mM Ca²⁺ and n-methylglucamine all inward current disappeared. Therefore, there was no detectable Ca²⁺ current through Ca channels or through the Na channels, within the 2- to 3-pA resolution of our recordings.

U937 target cells are derived from a human histiocyte leukemia that is considered to represent a macrophage-precursor cell (22). The cells are spherical, about 14 μm in diameter. Depolarizing a U937 cell from a holding potential of −100 mV to −30 mV resulted in a time-dependent outward current (Fig. 2b) that increased in amplitude and rate of rise with further depolarization. This current was carried pre-
dominantly by K⁺ since it reversed very close to the K⁺ equilibrium potential and >95% of the current was blocked by 0.1 mM quinidine. It was similar to the K⁺ current in NK cells but much smaller (Fig. 2c). The maximal conductance was 0.2 ± 0.1 nS (n = 7). Interestingly, untransformed mouse peritoneal macrophage cells have no voltage-dependent currents when they are first isolated but a K⁺ conductance appears with time in culture (23), presumably as they mature. When the K⁺ current in U937 cells was blocked with quinidine there was no remaining time-dependent current; therefore, Ca²⁺ currents must be small or absent in this cell line as well. Our finding that the NK-sensitive cells K562 and U937 have different types of channels indicates that endogenous channel type is probably not a factor in determining target-cell sensitivity to natural killing.

**Blockage of the K Channels Corresponds with Inhibition of Killing.** For the remaining experiments in which the dose-dependent effects of channel blockers on killing were assessed, K562 cells were used as the target cells because they lacked a voltage-dependent K⁺ current. Hence, effects of blocking channels in NK cells could be more easily separated from effects on target cells. Fig. 3a shows that several ion-channel blockers inhibited killing of K562 cells in a dose-dependent manner. In all experiments, the order of potency was verapamil > quinidine > Cd²⁺ > 4AP. The concentrations required to block 50% killing in three to five separate experiments were verapamil, 14 μM; quinidine, 40 μM; Cd²⁺, 328 μM; and 4AP, 1890 μM. Dose dependency was always steepest for 4AP and shallowest for Cd²⁺; hence, the two curves cross at high concentrations. Blocking the Na⁺ channels in the K562 target cells with tetrodotoxin had no effect on killing, even at concentrations (3.1 μM) that were five times higher than required to block >95% of the Na⁺ current.

Since the type of channel (Na or K) in target cells did not seem critical and the Na channels themselves did not seem to be necessary for killing, it appeared that the effect of ion-channel blockers was on the NK cell. This view is supported by a comparison of the relative potency of the drugs for inhibiting killing of K562 target cells with their ability to block currents in NK cells. Fig. 3b shows dose-response curves for reducing the K⁺ current in NK cells. For each experiment the peak current at −10 mV measured in standard solution was taken as the control value. Then blocker was added and the peak current at −10 mV was normalized by dividing by the control value. The same order of effectiveness was seen for reduction of K⁺ current and for inhibition of killing. The concentrations required to reduce the current by 50% in three or four cells were verapamil, 7
were Thus, than inhibitor dose-response current. uM; quinidine, reduces the (24). It shows that K-channel blockers reduce the current is not the same (data not shown). Quinidine and verapamil showed a use-dependent blockage that suggests channels are blocked after they open, whereas 4AP did not show use dependence. Cd²⁺ appeared to act by shifting the voltage-dependent activation of K⁺ current to more positive voltages. For example, at the Cd²⁺ concentration (343 μM) that reduced the current by one-half, the conductance–voltage relation was shifted about 20 mV more positive. This effectively reduces the number of open K⁺ channels at any voltage without necessarily blocking the channels.

**DISCUSSION**

Our results demonstrate that human NK cells have K⁺ channels and that these channels are required during the killing process. Determination of which stage of killing requires functioning K⁺ channels, recognition–adhesion or the lethal hit itself, and what is the mechanism of K⁺-channel involvement will require further investigation. Recently, a K⁺ current was found in mouse clonal CTL, which increased slightly after conjugation between a killer cell and a target cell in the presence of Ca²⁺, suggesting a possible involvement in the lethal hit (18). This current was similar to the K⁺ current we have described for human NK cells. Therefore, K⁺ channels might generally mediate lymphocyte cytotoxicity. Our results also show that the type or functioning of ion channels in isolated target cells is not critical. It will be interesting to determine whether new ion channels appear during the lethal hit and whether they are involved in osmotic swelling and lysis of the target cell.

It is interesting that the K⁺ current in NK cells is reduced by the traditional Ca-channel blockers verapamil and Cd²⁺, although the evidence indicates that it is not a Ca-dependent K⁺ current. Evidence against a Ca dependence includes the following. (i) The voltage dependence and time course of activation and inactivation are similar to delayed rectifier K⁺ channels not to the usual Ca-dependent K⁺ channels (25–27). (ii) Omission of all Ca from the internal solution and addition of 120 mM F⁻ did not affect the current. Fluoride effectively chelates Ca and has been shown to abolish calcium currents in some cells (28). (iii) Increasing the external Ca concentration or adding the Ca ionophore A23187 did not increase the K⁺ current. Potassium aspartate internal solution was used in these experiments to avoid chelating internal Ca. A similar K⁺ current in mouse and in human T lymphocytes is reduced by the Ca-channel blockers verapamil (21), quinidine or quinine (18, 19), and Ni²⁺ (20); however, it also does not appear to be Ca dependent (16, 18, 19). The effect of Ni²⁺ might be similar to that of Cd²⁺ in the present study—that is, a shift in the voltage dependence of activation. Similar shifts in K⁺-current activation can be caused by other divalent cations (e.g., see ref. 29).

Ion channels are often characteristic of cell type and as such may be thought of as membrane surface markers. The cellular origin of human NK cells is not known; myelomonocyte, T lymphocyte, and possible new lineages have been proposed (1). T lymphocytes have a very similar K⁺ channel (16, 18, 19) that appears to be involved in cell activation and proliferation (19–21), whereas K⁺ channels have not been found in freshly isolated macrophages (23). Evidence in favor of a T-cell lineage for NK cells includes some shared surface markers (1) and the K⁺ channels might be considered as further evidence of a common origin.

We are indebted to Prof. S. H. Golub for helpful advice and discussions and to E. Famatiga for excellent technical assistance. This work was supported by National Institutes of Health Grants CA30515, CA12582, CA34442, and NS09012, a Muscular Dystrophy Association grant, and National Institutes of Health Training Grant NS07101.