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Selective blockers of voltage-gated K^+ channels depolarize human T lymphocytes: Mechanism of the antiproliferative effect of charybdotoxin

(electrophysiology/calcium-activated potassium channels/scorpion toxins)

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ABSTRACT **Charybdotoxin (ChTX), a K^+ channel blocker, depolarizes human peripheral T lymphocytes and renders them insensitive to activation by mitogen. We observed four types of K^+ channels in human T cells: one voltage-activated, and three Ca^{2+}-activated. To discern the mechanism by which ChTX depolarizes T cells, we examined the sensitivity of both the voltage-activated and Ca^{2+}-activated K^+ channels to ChTX and other peptide channel blockers. All four types were blocked by ChTX, whereas noxiustoxin and margatoxin blocked only the voltage-activated channels. All three toxins, however, produced equivalent depolarization in human T cells. We conclude that the membrane potential of resting T cells is set by voltage-activated channels and that blockade of these channels is sufficient to depolarize resting human T cells and prevent activation.**

Charybdotoxin (ChTX), a peptide purified (1, 2) from the venom of *Leiurus quinquestriatus* (var. hebraeus), has been shown to block mitogen-induced proliferation of human peripheral T lymphocytes (3–5). This inactivation can be reversed by addition of interleukin 2 (IL-2) (3, 4, 6), and it affects only Ca^{2+}-dependent activation pathways (6). Pretreatment of resting T cells with ChTX prevents mitogen-stimulated increases in cytosolic [Ca^{2+}] required for the production of IL-2 (3–6).

A plausible explanation that has been advanced for the action of ChTX on T-cell activation is that it blocks channels maintaining the cell's resting potential. Depolarization alone appears sufficient to inactivate resting T cells, since simply raising the extracellular K^+ concentration prevents mitogen-induced changes in cytosolic Ca^{2+} concentration, in synthesis and release of IL-2, and in proliferation (7, 8). Depolarization of unstimulated human peripheral blood mononuclear cells by ChTX has been reported (ref. 5; for review, see refs. 9–11). The depolarization produced by ChTX is to the same potential (−35 to −25 mV) as that produced by a variety of other, less potent, K^+ channel blockers (12), though the lack of specificity of most K^+ channel blockers makes it difficult to precisely account for their antiproliferative effects (13). Despite some reports that ChTX failed to inhibit activation of T cells (14–16), the link between depolarization by ChTX and its action as an antiproliferative agent is strong; only under conditions where ChTX depolarizes T cells does it block proliferation.

The action of ChTX as a K^+ channel blocker has been well characterized, both electrophysiologically and biochemically. ChTX was originally described as a selective blocker of Ca^{2+}-activated K^+ (K_{Ca}) channels (17–21). However, in T cells, ChTX has been shown to block voltage-gated K^+ (K_v) channels (22). Binding experiments using ^{125}I-labeled ChTX confirmed the existence of high-affinity sites associated with K_v channels in human T cells (23). Since its initial description in human T cells (24, 25) the K_v, or "*n*-type" (26), current has been implicated in the control of T-cell proliferation. This current accounts for most of the K^+ conductance in resting, peripheral human T cells. Genomic and cDNA clones encoding a K^+ channel ($K_{V1.3}$) with the properties of the *n* channel have been isolated from a variety of tissues, including human T cells (16). Whether the K_v channels in T cells consist exclusively of $K_{V1.3}$ subunits is unproved, but their sensitivity to ChTX is almost certainly determined by the presence of this gene product (27).

In addition to the prominent voltage-gated conductance, both rodent (28) and human (29) T cells also express K_{Ca} channels. In the present study, we identify three separate (based on conductance) ChTX-sensitive K_{Ca} channels in resting human T cells. Since both the K_v and K_{Ca} channels are ChTX-sensitive, the central question concerning the action of ChTX as an antiproliferative agent in T cells is the relative contribution of the K_v vs. K_{Ca} channels toward setting the resting potential. To address this question, we took advantage of two other channel-blocking peptides. Noxiustoxin (NxTX) and margatoxin (MgTX), a peptide isolated from the venom of the scorpion *Centruroides margaritatus*, have been shown to block cloned rat $K_{V1.3}$ channels (27, 30). We found that MgTX, like NxTX, is also a potent inhibitor of the K_v current in human T cells. Furthermore, MgTX and NxTX do not block any of the other ChTX-sensitive channels, yet they, like ChTX, depolarize resting T cells. A preliminary report of some of these findings has been presented in abstract form (31).

MATERIALS AND METHODS

Reagents. ChTX was synthesized by a solid-phase method (32). MgTX was purified from venom of the scorpion *C. margaritatus* as described (30). Synthetic NxTX was provided by R. Nutt (Merck Sharp & Dohme). Iberiotoxin (IbTX) was purified (33) from *Buthus tamulus* venom (Sigma). [^{3}H]Tetraphenylphosphonium (Ph_4P^+) was obtained from NEN/DuPont.

Human Peripheral Blood T Lymphocytes. Purified T cells were prepared by a procedure based on the "E-rosetting" method (34). The purified T cells were washed in RPMI 1640 and maintained in that medium for use within 24 hr.

Current Measurements. Standard whole-cell and single-channel patch-clamp techniques were employed (35). Electrodes were fire-polished (2–10 MΩ) and, for single channel recordings, the tips were coated with beeswax. Seals ranged

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Abbreviations: Ph_4P^+, tetraphenylphosphonium; IL-2, interleukin 2; ChTX, charybdotoxin; MgTX, margatoxin; NxTX, noxiustoxin; IbTX, iberiotoxin; K_{Ca}, Ca^{2+}-activated K^+; K_v, voltage-gated K^+.
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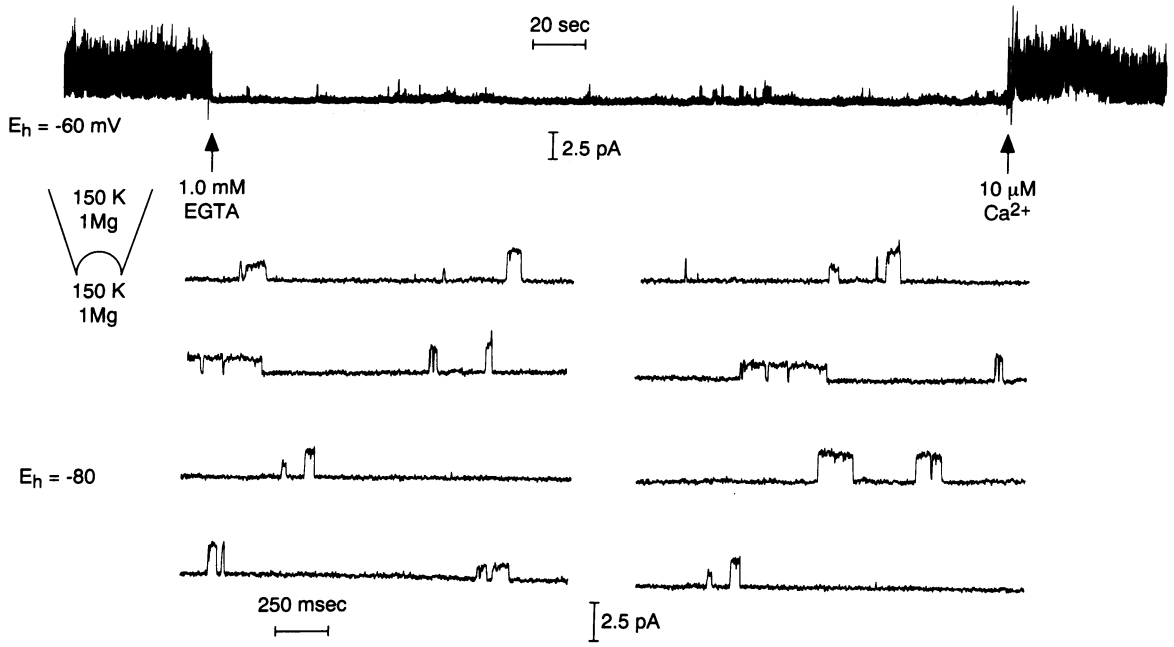

FIG. 1. Ca^{2+}-activated channels in excised inside-out patches. Patches were pulled from human T cells into a solution containing 150 mM KCl and 1 mM $MgCl_2$ (high-K saline) with no Ca^{2+} buffering (contaminating Ca^{2+} at ≈ 2 μM). The same solution was used for pipet filling. Top trace: EGTA was added to the chamber at the time indicated by the arrow to produce an initial concentration of ≈ 1 mM. The patch was superfused with EGTA-free saline for 5 min, followed by addition of 10 μM $CaCl_2$ to the chamber. Lower traces: individual channel openings from another patch (holding potential, -80 mV) with an expanded time scale. E_h, holding potential.

from 10 to 100 GΩ. No series resistance or leakage compensation was employed. Recordings were obtained at room temperature in a 1.0-ml perfusion chamber with flow rates of ≈ 2 ml/min. Electrode and bathing solutions are described in the text and figure legends.

Membrane Potential Measurements. Procedures for measuring uptake of $[^3H]Ph_4P^+$ and the conversion to membrane potential were as described (36). Measurements were taken in saline (135 mM NaCl/5 mM KCl/1.8 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes·NaOH, pH 7.4/10 mM glucose). The internal volume of the lymphocyte was estimated as 113 fl.

RESULTS

Ca^{2+}-Activated Channels in Excised Patches. Fig. 1 shows recordings of Ca^{2+}-activated channels from excised patches of human T lymphocytes. In the top recording, channel activity is seen to decrease abruptly upon injection of EGTA into a perfusion chamber containing an inside-out patch held at -60 mV. After 5 min of washing with EGTA-free saline, addition of Ca^{2+} caused an immediate return of channel activity. The lower traces show K_{Ca} currents in another inside-out patch containing fewer channels and with an expanded time base. Two classes of channel openings, based on amplitude, were detected. These channels were also readily apparent immediately following formation of on-cell patches, but their activity usually declined after a few minutes, suggesting that the process of seal formation may have transiently increased the cytosolic Ca^{2+} concentration. Infrequently (1/19 patches), we observed a third type of K_{Ca} channel with a larger unitary amplitude. The low probability of observing the third channel type in patches was intriguing, since, as discussed below, all three types were always seen in whole-cell recordings. Fig. 2 shows unitary current vs. membrane potential plots for the two predominant K_{Ca} channels under two different conditions. In Fig. 2*A*, the data were obtained from channels in an inside-out patch bathed in symmetrical K^+ saline (150 mM). The linear plots have slopes of 13 pS and 21 pS. In six additional experiments, conductances ranged from 11 to 14 pS for the smaller channel and from 21 to 23 pS for the larger one. The infrequently observed third channel type had a unitary conductance of 35 pS in symmetrical KCl. All of the K_{Ca} channels could be distinguished from the K_v channels, based on unitary conductance (30–31 pS measured under our conditions) and by the fact that the K_v channels were silent at holding potentials more negative

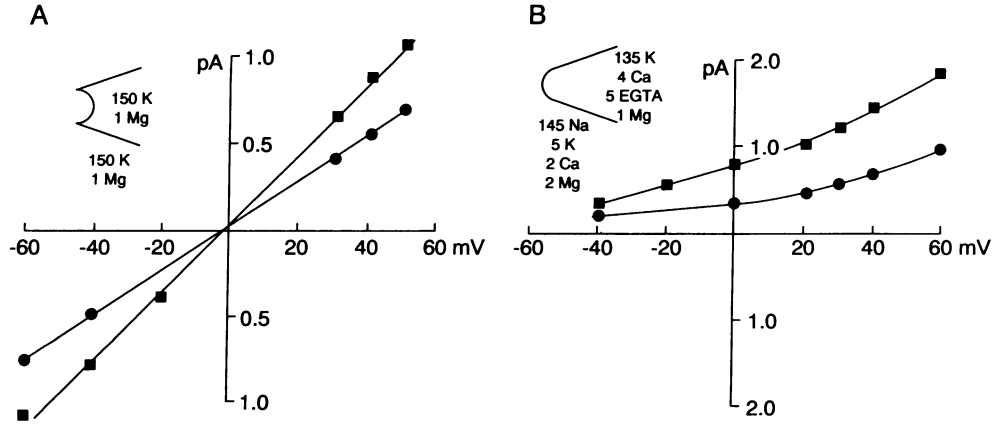

FIG. 2. Conductance and ion selectivity of Ca^{2+}-activated channels in excised patches. Data were obtained from inside-out (*A*) or outside-out (*B*) patches. Mean amplitudes of single-channel currents are plotted against membrane potential under symmetrical K^+ (*A*) or bi-ionic (*B*) conditions. Compositions (mM) of pipet and bath solutions are shown (*Insets*). Theoretical reversal potential for K^+ selective conductance: 0 mV in *A*; -83 mV in *B*.

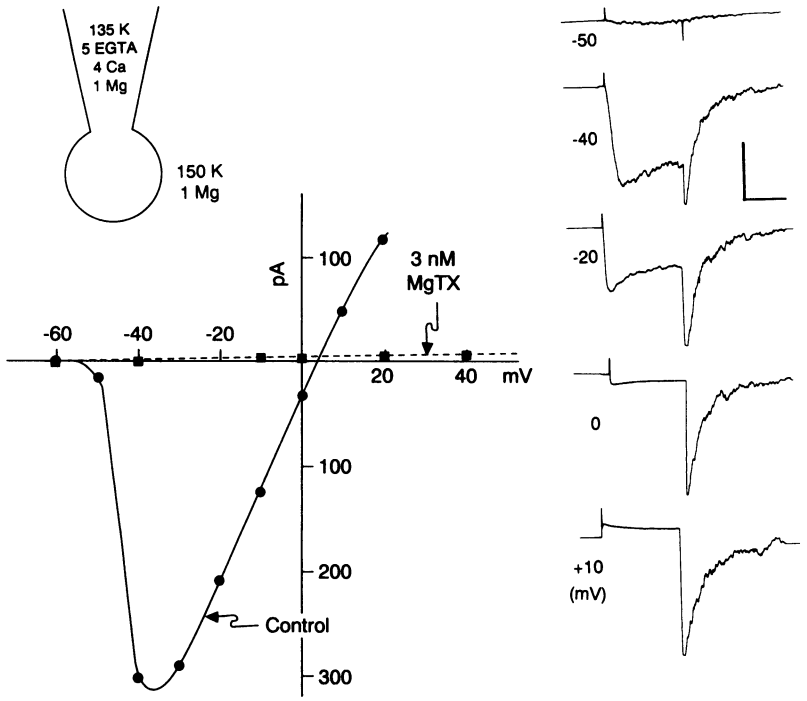

FIG. 3. MgTX blocks the voltage-activated channels. A lymphocyte was voltage-clamped in the whole-cell mode. A series of depolarizations lasting 250 msec were delivered from a holding potential of −70 mV. Sample current responses at right (calibration bars: vertical, 200 pA; horizontal, 125 msec). The peak current recorded from each pulse is plotted against the test potential. Data are shown from the same cell before (●) and after (■) addition of 3 nM MgTX to the bath. Pulses were delivered at 30-sec intervals. Compositions (mM) of pipet and bath solutions are shown (*Inset*). Predicted reversal potential for K^+: +3 mV.

than −70 mV. The data in Fig. 2*B* were obtained from channels in an outside-out patch with asymmetric K^+ and Na^+. Under this condition, the extrapolated reversal potential for the currents is close to the K^+ equilibrium potential, demonstrating the selectivity of both channels for K^+.

K^+ Channel Recordings Under Whole-Cell Clamp. Conditions were selected for whole-cell voltage clamp that permitted measurements of both macroscopic K_v currents and unitary currents from K_{Ca} channels in the same cell. Cells were bathed in 150 mM K^+ saline (symmetrical K^+), the membrane potential was held at −80 mV, and EGTA was used to buffer the intracellular free Ca^{2+} to 0.5 or 2 μM. Voltage pulses to membrane potentials more positive than −55 mV elicited macroscopic K_v currents that reversed near 0 mV, followed by large tail currents due to the slow deactivation of the channels (Fig. 3). The characteristics of

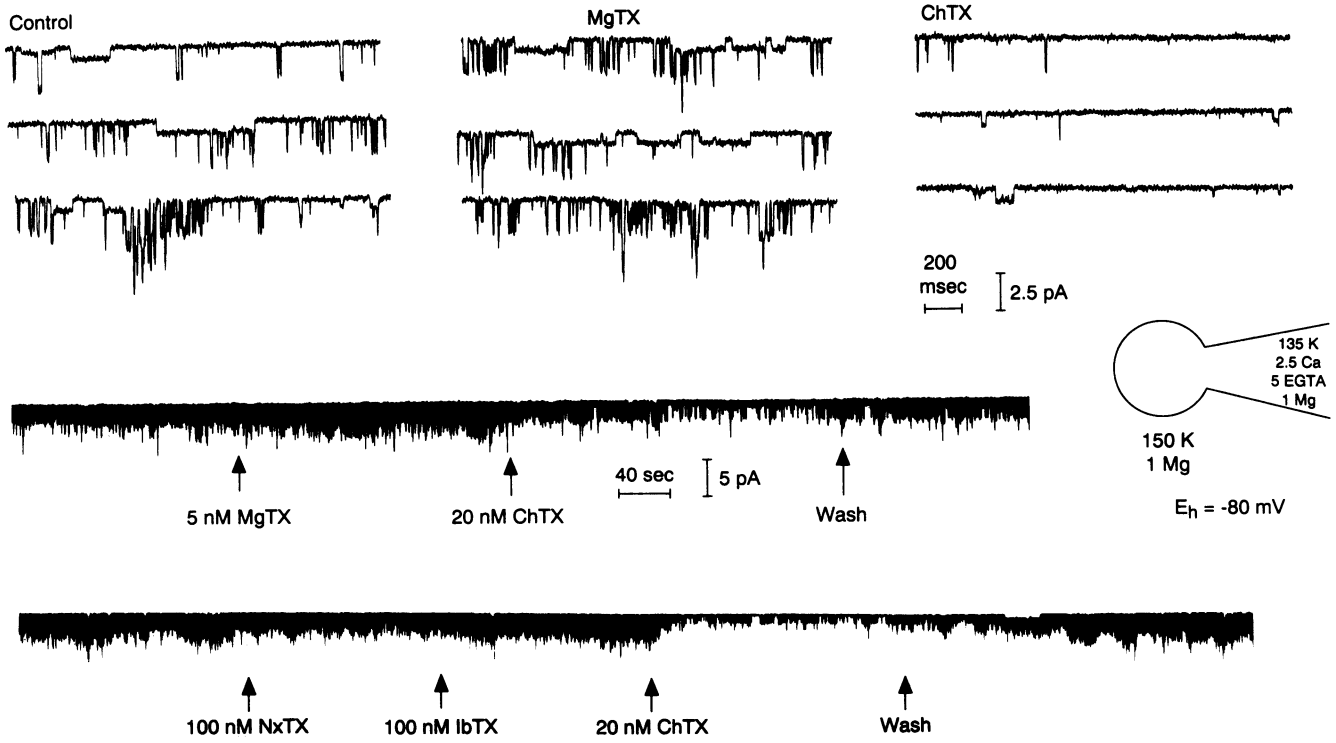

FIG. 4. Effect of peptide K^+ channel blockers on Ca^{2+}-activated channels. Data from two separate experiments are shown. Lymphocytes were voltage-clamped in the whole-cell mode to −80 mV. Ca^{2+} was buffered to ≈0.5 μM with EGTA. The time-compressed recordings show the effects of ChTX vs. MgTX (middle trace) or ChTX vs. IbTX and NxTX (bottom trace) on channel activity. Arrows indicate the times at which toxins were added at the concentrations indicated. The upper traces, showing individual channel openings, were excerpted from the continuous record shown by the middle trace. Three classes of openings were observed (conductances: 13, 22, and 35 pS). Compositions (mM) of pipet and bath solutions are shown (*Inset*).

activation of the macroscopic currents were similar to those recorded in normal Na^+ saline, but the currents inactivated more slowly in the high-K^+ saline. It was often possible to resolve individual openings of the K_v channels during the decay of the tail currents. The single-channel conductance of the K_v channels in symmetrical K^+ was 31 pS. Fig. 3 also shows that 3 nM MgTX completely blocked the voltage-gated currents. Under these conditions, the IC_{50} for MgTX is well below 1 nM. NxTX has been shown to block the *n*-type K^+ current in Jurkat cells (human T-cell leukemia line) with an IC_{50} below 1 nM (37). We found that NxTX also blocked the T-cell K_v currents in the high-K^+ solution, but unlike MgTX, its potency was greatly reduced: The currents were inhibited by about 80% in 10 nM NxTX, and 50 nM NxTX inhibited them completely.

With Ca^{2+} present at 0.5 or 2 μM in the pipet, the combined activity of many channels was readily apparent at −80 mV. At low Ca^{2+} (0.5 μM), individual openings were resolved and the conductances matched those of the three classes of K_{Ca} channels observed in excised patches. The 35-pS channel displayed isolated openings, in contrast to the 13- and 21-pS channels, which opened in bursts. Fig. 4 shows an example of the effects of four K^+ channel-blocking peptides on the K_{Ca} channels measured under whole-cell conditions in high external K^+. The activity of all three classes of K_{Ca} channels was reduced significantly and reversibly by ChTX. None of the channels was affected by MgTX at 5 nM or NxTX at 100 nM, concentrations that completely blocked the voltage-gated channels. Some inhibition of the K_{Ca} channels was observed with NxTX at 1 μM. MgTX had no effect on the K_{Ca} channels up to 100 nM, the highest concentration tested. As an independent test of the selectivity of MgTX, we measured the effects of ChTX and MgTX on the linear membrane conductance between −120 and −60 mV, where the contribution of current through K_v channels should be negligible. The experiments were carried out in normal saline, with whole-cell patch pipets containing 2 μM Ca^{2+} (EGTA-buffered) or 0 Ca^{2+} (EGTA alone). The whole-cell slope conductance with pipets containing 2 μM Ca^{2+} was 61 ± 0.3 pS (n = 7), compared to 18 ± 0.2 pS (n = 7) with the Ca^{2+}-free pipets. The additional conductance elicited by high internal Ca^{2+} was blocked by ChTX (20 nM), but not by MgTX (4 nM). IbTX is a selective and potent (K_d of 1.5 nM) blocker of the "Maxi-K" ChTX-sensitive K_{Ca} channel found in many cell types (33, 38). IbTX had no effect on any of the lymphocyte channels. Likewise, apamin (up to 200 nM), which reportedly (39) blocks most K_{Ca} channels in Jurkat cells, had no effect (data not shown). To date, we have not identified a selective inhibitor of the K_{Ca} channels in human T cells.

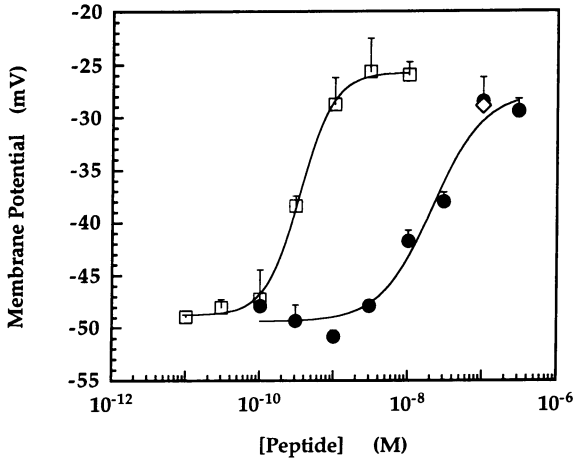

FIG. 5. ChTX, NxTX, and MgTX depolarize resting T cells. The average membrane potential of populations of resting T cells was measured by using $[^3H]Ph_4P^+$. The dose-dependent depolarizations caused by MgTX (□) and ChTX (●) are plotted. The depolarization produced by a saturating concentration (100 nM) of NxTX (◇) is included for comparison. All values are means of triplicate determinations; standard errors are shown where they exceed the size of the symbol.

Effects of Channel-Blocking Toxins on Membrane Potential. Measurements of membrane potential (Fig. 5) were obtained by monitoring the uptake of $[^3H]Ph_4P^+$ in T lymphocytes. Ph_4P^+ permeates cell membranes and partitions according to the transmembrane field (36). Using this technique, we calculated the membrane potential of resting human T cells to be approximately −50 mV. Addition of MgTX or ChTX produced a concentration-dependent depolarization (MgTX ED_{50}, 350 pM; ChTX ED_{50}, 30 nM) of these cells. The maximum depolarization induced by NxTX was similar in magnitude to that brought about by ChTX or MgTX. The cells depolarized to between −30 and −25 mV. The greater efficacy of MgTX in causing depolarization correlates with its higher potency as a channel blocker and argues strongly against a significant contribution of K_{Ca} channels in setting the resting potential of unstimulated T cells. Comparing the dose–response relationship for depolarization vs. block of K_v, we estimate that maximal depolarization requires inhibition of >95% of the voltage-gated channels.

DISCUSSION

We report here the existence of two types of K_{Ca} channels not previously described in human T cells. In cell-attached and excised patches, two low-conductance (13 and 21 pS) channels were consistently observed. A third type of channel, with a conductance of 35 pS, was observed only infrequently in patches. All three channels, however, were routinely identified under whole-cell clamp with Ca^{2+} buffered at 0.5 μM or above. The 13- and 21-pS channels may correspond to the two low-conductance K_{Ca} channels recently described in rat thymic cells (40). A 35-pS K_{Ca} channel was previously reported in human T cells (29). All of the K_{Ca} channels we observed were sensitive to ChTX, but none were affected by 100 nM NxTX or MgTX. IbTX, a selective inhibitor of large-conductance "Maxi-K" K_{Ca} channels, had no effect on any channels in human T cells.

Since MgTX and NxTX, selective blockers of the K_v channels, can depolarize resting human T cells, it appears that the activity of those channels is sufficient to maintain the membrane potential of nonactivated cells. It is noteworthy that the resting potential of unstimulated T cells lies at the threshold of activation of the K_v channels; perhaps the sharp voltage dependence of activation of these channels serves to "clamp" the resting potential within a narrow range. Despite the pronounced inactivation of the K_v currents exhibited during prolonged voltage-clamp pulses, two pieces of evidence support the idea that sufficient numbers of those channels are open to maintain the resting potential. First, under physiological conditions, the separation between the voltage midpoints of activation and inactivation results in a significant population of channels available to open near the resting potential (41, 42). Second, inactivation of the K_v current is incomplete at all voltages, and this maintained component of the current can be blocked by MgTX (31). The role of the K_{Ca} channels in resting T cells remains unclear. It is possible that cytoplasmic Ca^{2+} levels in resting cells rarely rise to the point where the K_{Ca} channels can open. Following activation, however, with the attendant rise in cytoplasmic Ca^{2+} concentration, the K_{Ca} channels may control the membrane potential, as evidenced by the ability of ChTX to block the hyperpolarization that follows stimulation by mitogens or Ca^{2+} ionophores (5, 12, 14, 15, 40, 43).

The mechanism by which depolarization prevents activation of T cells apparently involves the attenuation of the increase in cytosolic Ca^{2+} that normally occurs following receptor–ligand coupling (44). This rise in Ca^{2+} is necessary for mitogen-induced activation, although activation by exogenous IL-2 can occur downstream from the Ca^{2+}-dependent processes (45). The relationship between membrane potential and Ca^{2+} during T-cell activation is a subject of considerable debate, with conflicting views of the relative importance of release from intracellular pools vs. entry through plasmalemmal channels (46, 47). Nonetheless, it is clear that depolarization diminishes the rise in intracellular Ca^{2+} normally produced by mitogen activation. Both ChTX and NxTX have been shown to inhibit this rise in intracellular Ca^{2+}, prevent IL-2 release, and block proliferation in an IL-2-reversible manner (4, 6). It seems reasonable to conclude that the stasis produced by the K^+ channel toxins is a simple consequence of their ability to block the K_v channels.

In summary, the present findings support the hypothesis that the K_v channel determines membrane potential in nonactivated human T cells and that block of this channel is sufficient to cause depolarization and prevent activation by mitogen. The discovery of potent, selective blockers of the ChTX-sensitive K_v channel in human T cells may ultimately lead to additional methods of immunosuppression.

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