

Voltage-gated Potassium Channels Regulate Calcium-dependent Pathways Involved in Human T Lymphocyte Activation

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Summary

The role that potassium channels play in human T lymphocyte activation has been investigated by using specific potassium channel probes. Charybdotoxin (ChTX), a blocker of small conductance Ca^{2+} -activated potassium channels ($P_{K,Ca}$) and voltage-gated potassium channels ($P_{K,V}$) that are present in human T cells, inhibits the activation of these cells. ChTX blocks T cell activation induced by signals (e.g., anti-CD2, anti-CD3, ionomycin) that elicit a rise in intracellular calcium ($[Ca^{2+}]_i$) by preventing the elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. However, ChTX has no effect on the activation pathways (e.g., anti-CD28, interleukin 2 [IL-2]) that are independent of a rise in $[Ca^{2+}]_i$. In the former case, both proliferative response and lymphokine production (IL-2 and interferon γ) are inhibited by ChTX. The inhibitory effect of ChTX can be demonstrated when added simultaneously, or up to 4 h after the addition of the stimulants. Since ChTX inhibits both $P_{K,Ca}$ and $P_{K,V}$, we investigated which channel is responsible for these immunosuppressive effects with the use of two other peptides, noxiustoxin (NxTX) and margatoxin (MgTX), which are specific for $P_{K,V}$. These studies demonstrate that, similar to ChTX, both NxTX and MgTX inhibit lymphokine production and the rise in $[Ca^{2+}]_i$. Taken together, these data provide evidence that blockade of $P_{K,V}$ affects the Ca^{2+} -dependent pathways involved in T lymphocyte proliferation and lymphokine production by diminishing the rise in $[Ca^{2+}]_i$ that occurs upon T cell activation.

The activation of T lymphocytes via antigen receptor is associated with phosphatidylinositol turnover and an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$)¹. Although part of this rise in $[Ca^{2+}]_i$ appears to depend upon Ca^{2+} influx, the precise mechanism responsible for elevation of $[Ca^{2+}]_i$ remains to be determined (1). In addition to Ca^{2+} influx, T cell activation results in enhanced K^+ efflux. The predominant K^+ channel in human T lymphocytes is a voltage-gated channel ($P_{K,V}$) (2, 3) that has properties similar to a delayed rectifier K^+ channel of nerve cells which has been cloned (4–8). Human T cells also possess a variety of small conductance Ca^{2+} -activated K^+ channels ($P_{K,Ca}$) that have recently

been characterized (9). A link between the rise in $[Ca^{2+}]_i$ and the function of these K^+ channels is not completely understood.

The rise in $[Ca^{2+}]_i$ via receptor-operated channels or second messenger pathways is dependent on membrane potential. Lymphocyte membrane depolarization has been shown to result in an inhibition of mitogen-activated Ca^{2+} uptake (10), as well as anti-CD3- and anti-CD2-induced increase in $[Ca^{2+}]_i$ in human T lymphocytes (11, 12) or Jurkat cells (13). $[Ca^{2+}]_i$ elevation induced by thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, is also prevented when lymphocytes are depolarized (10, 14).

Evidence for the involvement of K^+ channels in T cell activation comes from experiments demonstrating that agents known to block K^+ channels inhibit this process (10). PHA-stimulated human T cell activation as measured by IL-2 production, proliferation, and protein synthesis was shown to be inhibited by the nonselective K^+ channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (15). TEA was also reported to inhibit elevation in $[Ca^{2+}]_i$ (16).

¹ Abbreviations used in this paper: α -DdTX, α -dendrotoxin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ChTX, charybdotoxin; 4-AP, 4-aminopyridine; IbTX, iberiotoxin; InsP₃, inositol-1,4,5-triphosphate; MgTX, margatoxin; NxTX, noxiustoxin; $P_{K,Ca}$, Ca^{2+} -activated potassium channel; $P_{K,V}$, voltage-gated potassium channel; TEA, tetraethylammonium.

However, the concentrations of both TEA and 4-AP necessary to cause these effects are very high (mM range), and therefore their effects on T cell activation could be nonspecific (17). Charybdotoxin (ChTX), a 37-amino acid peptide isolated from venom of the scorpion *Leiurus quinquestriatus* var. *hebraeus* (18), blocks with high affinity both $P_{K,Ca}$ in human lymphocytes (9) and $P_{K,V}$ in T lymphocytes from several species (19). The effect of ChTX on human lymphocyte activation has been reported by several groups, but with conflicting results. For example, Price et al. (20) and Bono et al. (21) demonstrated that ChTX inhibited PHA-stimulated T cell proliferation and IL-2 production. Gelfand and Or (22), however, reported that ChTX inhibited cell membrane hyperpolarization in T lymphocytes stimulated by PHA, but had no effect on the proliferative response. Thus, the putative role of K^+ channels in controlling T cell activation remains unclear.

To address this issue, the effects of a variety of peptidyl K^+ channel inhibitors on different activation parameters in human T cells have been investigated. In this study, we demonstrate that ChTX inhibits T cell activation, as measured by either proliferation or lymphokine production, when triggered by pathways associated with a rise in $[Ca^{2+}]_i$. In this respect, the action of ChTX is remarkably similar to that of cyclosporin A and FK-506 (23). Since ChTX inhibits both $P_{K,Ca}$ and $P_{K,V}$, the effects of several selective $P_{K,V}$ blockers, such as noxiustoxin (NxTX) (5, 9) and the recently isolated peptide margatoxin (MgTX) (9, 24), were also investigated on T cell activation to discern which channel is responsible for the profile obtained with ChTX. With the use of these peptidyl inhibitors, we conclude that blockade of $P_{K,V}$ is sufficient to inhibit human T cell activation. This $P_{K,V}$ inhibition appears to cause membrane depolarization (9) which in turn blocks the rise in $[Ca^{2+}]_i$. These results confirm and extend the data of others by identifying $P_{K,V}$ as the relevant ion channel for the in vitro immunosuppressive effects of ChTX.

Materials and Methods

Reagents. ChTX was purified to homogeneity from the scorpion *L. quinquestriatus* var. *hebraeus* obtained from Alomone Laboratories (Jerusalem, Israel) (18). Synthetic NxTX was generously provided by Dr. R. Nutt (Merck Research Laboratories, West Point, PA). Iberiotoxin (IbTX) was isolated from *Buthus tamulus* venom as described (25). α -Dendrotoxin (α -DaTX) was a gift of Dr. M. Blaustein (University of Maryland, Baltimore, MD). MgTX was isolated from venom of the scorpion *Centruroides margaritatus* (24). All peptides were stored lyophilized at -70°C . Before the experiments, the peptides were reconstituted to 10 μM in Tris buffer (20 mM Tris/HCl, 100 mM NaCl, pH 7.4) with 0.1% BSA also present. FK-506 was isolated from *Streptomyces tsukubaensis* by Dr. R. Borris (Merck Research Laboratories). FK-506 was dissolved at 1 mg/ml in ethanol and stored at -70°C . PMA (Sigma Chemical Co., St. Louis, MO) and ionomycin (Calbiochem Corp., San Diego, CA) were dissolved at 10 $\mu\text{g}/\text{ml}$ and 1 mg/ml, respectively, in dimethyl sulfoxide and stored at -70°C . Recombinant human IL-2 was obtained from Amgen Biologicals (Thousand Oaks, CA). OKT3 (anti-CD3) mAb was obtained from Ortho Diagnostic Systems (Raritan, NJ). Affinity-purified rabbit anti-mouse IgG was

obtained from Cappel (Organon Teknica Corp., West Chester, PA). Anti-CD28 (mAbs 9.3) was a gift from Drs. J. A. Ledbetter (Genetic Systems, Seattle, WA) and C. June (Naval Medical Research Institute, Bethesda, MD). Anti-CD2 mAbs T11.2 and T11.3 were gifts from Dr. E. Reinherz (Harvard Medical School, Boston, MA).

Cell Preparations. PBMC were isolated from heparinized blood samples of normal donors using lymphocyte separation medium (LSM; Litton Bionetics, Kensington, MD) density gradient centrifugation followed by lysis of erythrocytes with ACK lysing buffer (Gibco, Grand Island, NY). Highly purified T lymphocytes were further isolated by nylon wool as described previously (23).

Proliferation Assays. Highly purified T lymphocytes were cultured in triplicate samples in flat-bottomed, 96-well microtiter plates (Costar, Cambridge, MA) at 2×10^5 cells per well in complete medium consisting of RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 μM nonessential amino acids, 20 μM 2-ME, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cultures were stimulated with anti-CD2 (T11.2 + T11.3), anti-CD3 (OKT3), anti-CD28 (9.3), or ionomycin, with or without PMA. Either ChTX, NxTX, α -DaTX, IbTX, or FK-506 at indicated concentrations, was added at the beginning of the incubation. The cultures were incubated for 2 d at 37°C , in a 5% CO_2 atmosphere, and T cell proliferation was measured by addition of [^3H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) to each culture (2 μCi per well), 4 h before harvesting. Cultures were harvested on glass fiber filters using a cell harvester (Skatron, Inc., Sterling, VA). Radioactivity was measured using the Betaplate Liquid Scintillation System (model 1205; Pharmacia LKB Nuclear Microtomy Inc., Gaithersburg, MD). All data are shown as the means of triplicate cultures.

Mixed Lymphocyte Reaction. PBMC were cultured in triplicate samples in round-bottomed, 96-well microtiter plates (Costar) at 2.5×10^5 cells per well in complete media as described for the proliferation assay. Equal amounts of irradiated mononuclear cells from a second individual were added to the cultures as stimulator cells. FK-506, ChTX, NxTX, α -DaTX, and IbTX, at indicated concentrations, were added at the beginning of the cultures. The cultures were incubated at 37°C , in a 5% CO_2 atmosphere. T cell proliferation was measured as described for the proliferation assay.

^{125}I -ChTX Binding Assay. To characterize the interaction of NxTX, α -DaTX, and IbTX with the human T cells used in this study, we tested the ability of these peptides to compete for the binding of ^{125}I -ChTX to the preparation as previously described (26). Briefly, monocyte-depleted, E-rosetted lymphocytes (2.5×10^6 cells/ml, 0.4 ml per tube) were incubated in 12×75 -mm polystyrene tubes with 5 pM ^{125}I -ChTX in the absence or presence of increasing concentrations of various toxins. The binding assay is carried out in isotonic sucrose medium containing 10 mM Hepes, 5 mM KCl, 5 mM NaCl, and 6 mM glucose, pH 7.4. The reaction mixtures were incubated for 1 h at room temperature on a rotary shaker. At the end of the incubation period, the samples were diluted with 4 ml of cold quench solution (200 mM NaCl, 20 mM Hepes, titrated to pH 8 with Tris-base) and filtered through glass microfiber filters (GF/C; Whatman Inc., Clifton, NJ) that had been presoaked in 0.6% polyethylenimine. Nonspecific binding was determined in the presence of 10 nM ChTX. Each data point represents the average of triplicate samples.

Measurement of IL-2 and IFN- γ Production. Lymphocytes were cultured in triplicate wells of 96-well microtiter plates and stimulated with OKT3 or ionomycin and PMA. Supernatants were collected 4 or 24 h after the initiation of cultures. The IL-2 level in each supernatant was measured using the IL-2 ELISA assay system

(Collaborative Research, Bedford, MA). IFN- γ was quantitated using a RIA assay system (Centocor, Philadelphia, PA).

Measurement of $[Ca^{2+}]_i$. Most of the flow cytometric $[Ca^{2+}]_i$ determinations were performed as previously described (23). Purified lymphocytes were washed with RPMI 1640 buffered with 10 mM Hepes, pH 7.4. The pellet was resuspended at 2×10^6 cells/ml in RPMI-Hepes containing 1 μ M indo-1 AM (Molecular Probes, Inc., Eugene, OR) and incubated 45 min at 37°C in the dark. The cells were then subjected to centrifugation, resuspended in the original volume of RPMI-Hepes and incubated an additional 45 min at 37°C in the dark. After centrifugation, the cells were resuspended in RPMI-Hepes at 2×10^6 cells/ml and maintained in the dark at room temperature until analysis. A FACS[®] Analyzer/Consort 30 equipped with a 55-megabyte hard disc/tape drive (Becton Dickinson Immunocytometry Systems, San Jose, CA) was adapted to measure intracellular Ca^{2+} using indo-1. An increase in $[Ca^{2+}]_i$ was detected with indo-1 as an increase in the ratio of a lower to a higher emission wavelength (405 nm/485 nm emission) which is proportional to the internal calcium level. Since no standardization curve was determined to correlate intracellular calcium concentration with indo-1 ratio, the data are expressed as relative $[Ca^{2+}]_i$. Indo-loaded lymphocytes were incubated with peptides at 37°C for 15 min. OKT3 (10 ng/ml) was added and the $[Ca^{2+}]_i$ response was monitored for 3–4 min, before rabbit anti-mouse IgG antibody (40 μ g/ml) was added. In some experiments, ionomycin (125 ng/ml) was added and the $[Ca^{2+}]_i$ response was measured. For the membrane depolarization experiments using high potassium buffers, the appropriate buffer was also used as sheath fluid. In more recent experiments, data from the FACS[®] Analyzer sensor head were collected as a continuous file into Consort 40/ μ VAX computer system via a FACS[®] Data Lister (Becton Dickinson). Mean $[Ca^{2+}]_i$ time course data were displayed using KINPRO Software (Becton Dickinson & Co.).

Measurement of Inositol-1,4,5-Trisphosphate ($InsP_3$). Purified T lymphocytes were suspended at 2×10^7 /ml in 0.5 ml of HBSS containing 20 mM Hepes (pH 7.4) and 0.1% FCS in microfuge tubes, and preincubated with OKT3 (1 μ g/ml) at 4°C for 5 min.

All tubes were transferred to a 37°C waterbath and incubated for 2 min. Cells were then activated by adding rabbit anti-mouse IgG antibody (40 μ g/ml). The reaction was terminated at the indicated times by adding 0.2 volumes of ice-cold 100% TCA solution. The concentration of $InsP_3$ in each sample was quantified by using a radioreceptor assay kit (Dupont, Wilmington, DE).

Results

ChTX Only Inhibits T Lymphocyte Proliferation and Lymphokine Production Stimulated by Signal Transduction Pathways Associated with a Rise in $[Ca^{2+}]_i$. Proliferation of T lymphocytes can be induced by antibodies specific for a variety of cell surface molecules. To study the effects of ChTX on these pathways, highly purified T cells were stimulated with either anti-CD2 antibodies (T11.2/T11.3), anti-CD3 + PMA or ionomycin + PMA, all of which trigger a rise in $[Ca^{2+}]_i$. Alternatively, experiments were performed with either anti-CD28 + PMA or IL-2 + PMA, which induce T cells to enter the cell cycle without inducing a measurable rise in $[Ca^{2+}]_i$. FK-506 served as a positive control in these experiments (23). The results in Table 1 suggest that ChTX produces the same pattern of inhibition as FK-506. Thus, ChTX inhibits proliferative responses induced by anti-CD3 + PMA or ionomycin + PMA, but had no effect on IL-2 + PMA-induced proliferation. Therefore, ChTX only inhibits pathways that are associated with a rise in $[Ca^{2+}]_i$.

We next examined the effect of ChTX on IL-2 production using the distinct activation pathways previously described. Lymphocytes, preincubated with ChTX at 37°C for 15 min, were stimulated with anti-CD3 + PMA or anti-CD28 + PMA, and supernatants were collected after 24 h for determination of IL-2. As shown in Fig. 1, ChTX inhibits IL-2 production stimulated by anti-CD3 + PMA in a dose-dependent manner, whereas IL-2 production of lymphocytes stimulated by anti-CD28 + PMA is unaffected.

Table 1. Effect of ChTX on T Lymphocyte Proliferation Stimulated by Various Pathways

Culture	$[^3H]$ Thymidine uptake				
	CD2	CD3/PMA	Ion/PMA	IL-2/PMA	CD28/PMA
	<i>cpm</i>				
Expt. 1					
Control	43,431	47,888	ND	ND	ND
ChTX	18,899	20,058	ND	ND	ND
FK-506	850	2,201	ND	ND	ND
Expt. 2					
Control	ND	ND	87,069	62,325	117,713
ChTX	ND	ND	35,738	62,879	123,581
FK-506	ND	ND	5,788	65,732	135,864

T lymphocytes (10^6 /ml, 0.2 ml/well) were cultured in 96-well plates. ChTX or FK-506 was added at a final concentration of 10 nM. Different stimulants were then added to the cultures as indicated. The final concentration of PMA was 1 ng/ml; anti-CD3, 10 ng/ml; Ionomycin, 250 ng/ml; IL-2, 10 U/ml; anti-CD28, 10 ng/ml; anti-CD2:T11.2, 84 μ g/ml, and T11.3, 56 μ g/ml. The cultures were incubated for 2 d and $[^3H]$ thymidine incorporation determined. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

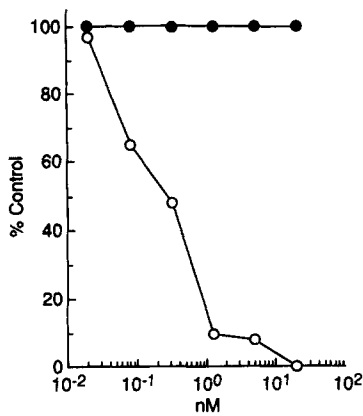


Figure 1. Effect of ChTX on the lymphokine production of activated T cells. Lymphocytes (3×10^6 /ml, 0.2 ml/well) were cultured with appropriate concentration of ChTX in 96-well plates with anti-CD3 (5 ng/ml, ○) or anti-CD28 (200 ng/ml, ●), and PMA (3 ng/ml). Supernatants were collected after 24-h incubation, and the IL-2 level for each supernatant determined. Data are expressed as percent control. Control culture contains 55 U/ml of IL-2 for anti-CD3/PMA and 154 U/ml for anti-CD28/PMA. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

ChTX Inhibits at an Early Stage of T Cell Activation. To investigate the kinetics of the inhibitory effect of ChTX on proliferation, ChTX or FK-506 was added to the lymphocyte cultures at different times after stimulation with anti-CD2 antibodies, T11.2 and T11.3. Data shown in Fig. 2 suggest that ChTX is less effective if added 4 h after activation. Minimal inhibition is observed if ChTX or FK-506 is added 18 h after T cell stimulation. These data imply that ChTX, similar to FK-506, exerts its antiproliferative effect at an early step in T cell activation.

ChTX Inhibits Mitogen-induced Rise In $[Ca^{2+}]_i$. Since ChTX inhibits activation pathways that are associated with a rise in $[Ca^{2+}]_i$, we examined the effect of ChTX on the increase in $[Ca^{2+}]_i$ of anti-CD3-stimulated lymphocytes. Data in Fig. 3 A demonstrate that the inhibitory effect of ChTX on anti-CD3-induced increase in $[Ca^{2+}]_i$ was dose dependent and that ChTX seems to inhibit the early but not

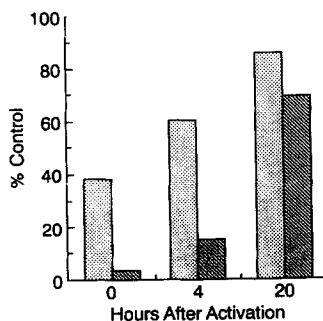


Figure 2. ChTX inhibits at an early stage of T cell activation. T lymphocytes (10^6 /ml, 0.2 ml per well) were cultured in 96-well plates. The cultures were stimulated with anti-CD2 antibodies, T11.2 (84 μ g/ml) and T11.3 (65 μ g/ml), and incubated at 37°C. ChTX (10 nM, ▨) or FK-506 (10 nM, ▩) was added at different times as indicated. Proliferation was determined by $[^3H]$ TdR incorporation 2 d later. Control proliferative response was 127,844 cpm. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

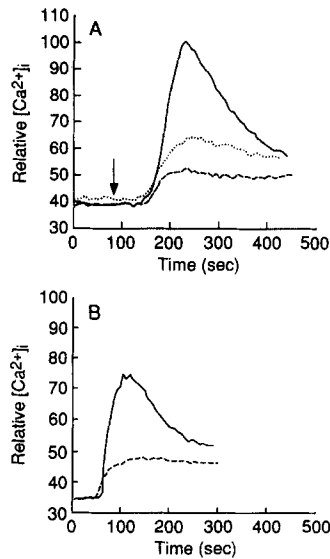


Figure 3. Effect of ChTX on the rise in $[Ca^{2+}]_i$ of activated T cells. Lymphocytes were preloaded with indo-1 and then preincubated with or without different concentrations of ChTX for 15 min at 37°C. OKT3 (5 ng/ml) and rabbit anti-mouse IgG (40 μ g/ml) or ionomycin (125 ng/ml) were added and measurement of relative $[Ca^{2+}]_i$ was performed on the FACS[®] analyzer as described in Materials and Methods. (A) Dose-dependent effect of ChTX on the rise in $[Ca^{2+}]_i$ of OKT3 activated T cells. (Arrow) Point at which rabbit anti-mouse IgG was added. Control (—); ChTX, 10 nM (⋯); 100 nM (---). (B) Effect of ChTX on ionomycin induced rise in $[Ca^{2+}]_i$. Ionomycin was added at time 0. Control (—); ChTX, 100 nM (---).

the late, sustained phase of $[Ca^{2+}]_i$ elevation. Preincubation with ChTX did not induce changes in normal $[Ca^{2+}]_i$ levels. Similarly, ChTX also inhibits ionomycin-induced elevation in $[Ca^{2+}]_i$ (Fig. 3 B). Our results demonstrate that ChTX exerts its effects by blocking the $[Ca^{2+}]_i$ increase associated with T cell activation.

ChTX Has No Effect on Mitogen-induced $InsP_3$ Production. Since ChTX was shown to inhibit the early phase of $[Ca^{2+}]_i$ elevation, which is believed to be associated with the release of Ca from internal stores by $InsP_3$ (1), we next tested whether inhibition of OKT3-induced rise in $[Ca^{2+}]_i$ by ChTX could be due to its inhibitory effect on the production of $InsP_3$. Kinetic studies showed that when T cells were incubated with OKT3 and rabbit anti-mouse IgG, the level of $InsP_3$ induced increased rapidly, and reached the peak around 60 s after activation. Results shown in Table 2 indicate that ChTX preincubation did not inhibit the production of $InsP_3$ in OKT3-stimulated T cells.

$P_{K,V}$ Is the Relevant Target for the Immunosuppressive Effects of ChTX. Since ChTX blocks both $P_{K,Ca}$ and $P_{K,V}$ in human T cells (9), we wished to investigate whether blockade of one or both of these channels was necessary for the immunosuppressive effects observed with the peptide. NxTX and IbTX are peptides purified from other scorpion venoms which share 40 and 68% sequence homology with ChTX, respectively. NxTX inhibits only ChTX-sensitive $P_{K,V}$, but not $P_{K,Ca}$ in human T lymphocytes (9). IbTX consists of a single 4.3-kD polypeptide chain and selectively inhibits the high conductance $P_{K,Ca}$ in smooth muscle. This peptide does not block ChTX-sensitive $P_{K,Ca}$ or $P_{K,V}$ in lymphocytes (9, 26). α -DaTX is a weak blocker of $P_{K,V}$ in lymphocytes (26). As shown in Fig. 4 A, NxTX and α -DaTX inhibit the binding of ^{125}I -ChTX to $P_{K,V}$ in lymphocytes, whereas IbTX has no effect. These results are consistent with previously reported data indicating that ^{125}I -ChTX binding is a monitor of $P_{K,V}$ in human T cells (26).

Table 2. Effect of ChTX on *InsP₃* Production

Expt. No.	Culture	<i>InsP₃</i> , picomole
1	Control	15.05
	ChTX, 100 nM	15.85
2	Control	22.0
	ChTX, 100 nM	18.6

Purified T lymphocytes were suspended at 2×10^7 cells/ml in 0.5 ml of HBSS containing 20 mM Hepes (pH 7.4) and 0.1% FCS in microfuge tubes, and preincubated with OKT3 (1 μ g/ml) at 4°C for 5 min. All tubes were transferred to a 37°C waterbath and incubated for 2 min. Cells were then activated by adding rabbit anti-mouse IgG antibody (40 μ g/ml). The reaction was terminated at 60 s by adding 0.2 volumes of ice-cold 100% TCA solution. The concentration of *InsP₃* in each sample was quantitated by using a radioreceptor assay kit (Dupont, Wilmington, DE). The results are expressed as pmol per 10^7 cells.

The dose-response curves of ChTX, N α TX, α -D α TX, and IbTX on anti-CD3 + PMA-induced proliferation are shown in Fig. 4 B. ChTX and N α TX inhibit this proliferative response with *IC*₅₀ of 3 and 8 nM, respectively, whereas IbTX has no effect. The rank order of potency of these peptides in blocking T cell proliferation (Fig. 4 B) correlates well with their rank order in competing for the binding of ¹²⁵I-ChTX to P_{K,V} in lymphocytes (Fig. 4 A). These results further sup-

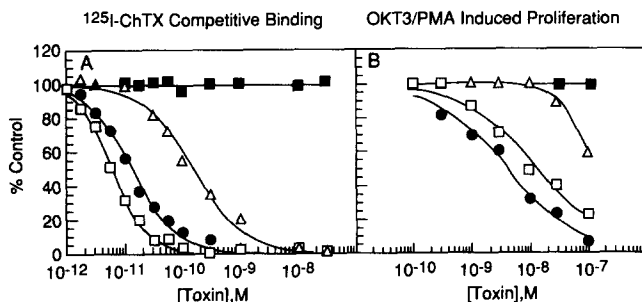


Figure 4. P_{K,V} is the relevant target for the immunosuppressive effects of ChTX. (A) ¹²⁵I-ChTX competition binding assay. Assays were conducted in 12 × 75-mm polystyrene tubes. The assays contained 5 pM ¹²⁵I-ChTX, toxins, at different concentrations as indicated (ChTX, ●; N α TX, □; α -D α TX, △; IbTX, ■) and lymphocytes (2.5×10^6 cells/ml, 0.4 ml per tube) which were suspended in isotonic sucrose medium containing 10 mM Hepes, 5 mM KCl, 5 mM NaCl, and 6 mM glucose, pH 7.4. The mixtures were incubated for 1 h at room temperature and assayed as described. Each data point represents the average of triplicate samples minus nonspecific binding which was determined in the presence of 10 nM ChTX. (B) Dose-dependent effect of K⁺ channel blockers on anti-CD3 and PMA induced proliferation. Highly purified T lymphocytes (2×10^6 /ml, 0.2 ml per well) were cultured in flat-bottomed wells of a 96-well microtiter plate. K⁺ channel blockers were added to the cultures at concentrations as indicated. OKT3 (5 ng/ml) and PMA (1 ng/ml) were added to each well. Cultures were incubated for 2 d and [³H]TdR incorporation for each culture was determined as described in Materials and Methods. The proliferative response for the control was 20,736 cpm. ChTX, ●; N α TX, □; α -D α TX, △; IbTX, ■. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

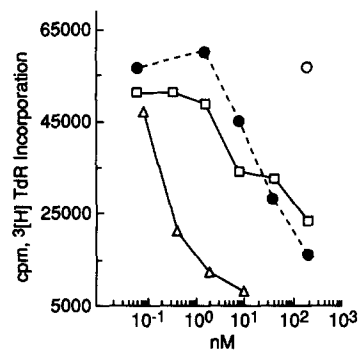


Figure 5. ChTX and N α TX inhibit MLR. T lymphocytes (4×10^6 /ml) were cultured in U-shape bottomed wells (0.1 ml/well) of the 96-well plates. Irradiated mononuclear leukocytes (4×10^6 /ml) from a second individual were added (0.1 ml per well) as stimulator cells. The cultures were incubated with ChTX (●), N α TX (□), or FK-506 (△) at the concentrations indicated. Proliferation was measured by [³H]TdR incorporation 4 d later. The proliferative response for the control (○) was 57,000 cpm. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

port the notion that blockade of P_{K,V} is sufficient to inhibit T cell activation. The difference in the absolute potencies of the peptides in the two assays is most likely due to the fact that binding experiments are carried out in low ionic strength buffer, whereas proliferation effects are monitored in physiological culture media (26).

We next tested the effect of ChTX and N α TX on a MLR in order to better understand if blockade of P_{K,V} can influence T cell function after activation by a more physiologically relevant stimuli. Using this protocol, we found that both ChTX and N α TX inhibit the MLR in a dose-dependent manner (Fig. 5). The *IC*₅₀ for both agents are similar to those found in blocking anti-CD3 + PMA-induced proliferation (Fig. 4 B). These results illustrate that P_{K,V} is the relevant target for the immunosuppressive effect of ChTX.

Blockade of P_{K,V} Inhibits Lymphokine Production. We have previously demonstrated (Fig. 1) that ChTX inhibits IL-2

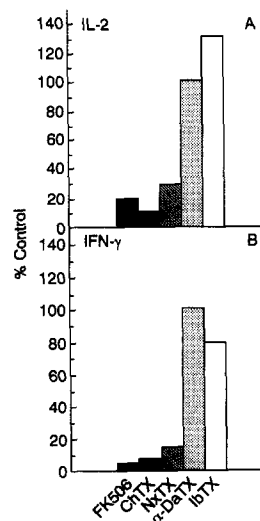


Figure 6. Blockade of P_{K,V} inhibits lymphokine production. T lymphocytes (10^7 /ml, 1 ml/well) were cultured in each well of the 24-well plates. Compounds tested were added at a final concentration of 10 nM. Ionomycin (250 ng/ml) and PMA (10 ng/ml) were then added to the cultures. Supernatants were collected from each culture 4 h later and IL-2 and IFN- γ levels were determined. Data are expressed as percent control. The IL-2 level in control was 450 U/ml. IFN- γ level in the control culture was 50 U/ml. All data are shown as the means of triplicate cultures. SD for each data point is <5%.

production stimulated by anti-CD3 + PMA. To determine whether other pathways that generate a rise in $[Ca^{2+}]_i$ are affected by $P_{K,V}$ inhibition, the effects of toxins on lymphokine production were investigated after T cell stimulation with ionomycin + PMA. For this experiment, larger numbers of cells per culture were used and supernatants were collected 4 h after the addition of the stimulus. ChTX, NxTX, α -DaTX, and IbTX were each tested at 10 nM. ChTX and NxTX, but not α -DaTX or IbTX inhibit IL-2 (Fig. 6 A) and IFN- γ (Fig. 6 B) production by ionomycin + PMA-stimulated lymphocytes. These results are consistent with those found in proliferation and binding assays. Whereas α -DaTX is a weak inhibitor of binding, it had no effect on either proliferation or lymphokine production at a concentration of 10 nM.

The role of $P_{K,V}$ in lymphokine production was further supported by studying the newly isolated peptide MgTX. Electrophysiological data indicate that MgTX is a more potent inhibitor of the human T lymphocyte $P_{K,V}$ than either ChTX or NxTX, and it has no effect on the ChTX-sensitive $P_{K,Ca}$ of lymphocytes (9). The effect of MgTX on IL-2 production of ionomycin + PMA-stimulated T lymphocytes was determined. As shown in Table 3, MgTX inhibits IL-2 production of activated lymphocytes and, as predicted, appears to be more potent than ChTX.

NxTX and MgTX Inhibit Mitogen-induced Rise in $[Ca^{2+}]_i$. To further determine whether NxTX, α -DaTX, IbTX, and MgTX would effect the rise of $[Ca^{2+}]_i$ induced by anti-CD3 as previously demonstrated for ChTX, this parameter of T cell activation was monitored. As shown in Fig. 7 A, 100 nM NxTX blocks the increase in $[Ca^{2+}]_i$, as does ChTX, whereas 100 nM α -DaTX has little or no effect. IbTX at 100 nM, which has no effect on T cell proliferation, also has no effect on the rise in $[Ca^{2+}]_i$ (data not shown). Consistent with its potency in blocking $P_{K,V}$ and lymphokine production, MgTX is the most potent inhibitor of the rise in $[Ca^{2+}]_i$ of all the toxins used in this study (Fig. 7 B). These

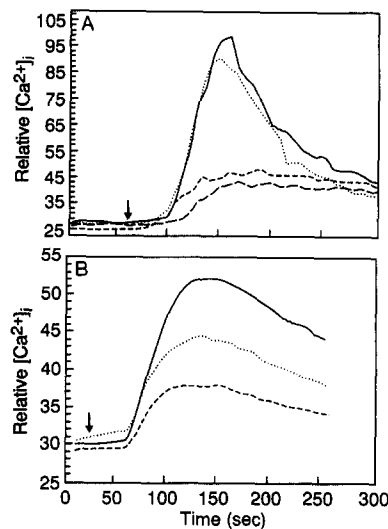


Figure 7. NxTX and MgTX inhibit the rise in $[Ca^{2+}]_i$ of activated T cells. Lymphocytes were preloaded with indo-1 and then preincubated with or without different concentrations of (A) α -DaTX, NxTX; (B) MgTX for 15 min at 37°C. OKT3 (10 ng/ml) and anti-Ig 1:50 dilution were added and measurement of relative $[Ca^{2+}]_i$ was performed on the FACS® analyzer as described in Materials and Methods. (Arrow) Time point at which the second antibody was added. Control (—) (A) α -DaTX, 100 nM (·····); NxTX, 100 nM (---) and ChTX, 100 nM (---); (B) MgTX, 0.3 nM (·····); 3 nM (---).

results suggest that blockade of $P_{K,V}$ prevents the rise in $[Ca^{2+}]_i$ normally associated with activation via the TCR complex.

Lymphocyte Depolarization Blocks the Activation-induced Rise in $[Ca^{2+}]_i$. Since ChTX has been reported to depolarize human T cells (9, 27), we hypothesized that ChTX may inhibit the rise in $[Ca^{2+}]_i$ by its effect on the lymphocyte's resting potential. To further investigate the relationship between membrane potential and $[Ca^{2+}]_i$, we altered the cell's resting potential in a manner that is independent of blockade of $P_{K,V}$. When lymphocytes are suspended in high K^+ so-

Table 3. Effect of MgTX on IL-2 Production

Culture	nM	IL-2
		U/ml
Control		75.0 ± 12.0
ChTX	2 nM	31.2 ± 1.4
	20 nM	2.8 ± 0.2
MgTX	0.5 nM	12.0 ± 0.4
	5 nM	8.2 ± 0.5

Lymphocytes (3×10^6 /ml, 0.2 ml per well) were cultured in 96-well plates with various concentrations of ChTX or MgTX. Ionomycin (250 ng/ml) and PMA (1 ng/ml) were then added to the cultures. After 24-h incubation, supernatants were collected and IL-2 levels determined. All data are shown as the means of triplicate cultures.

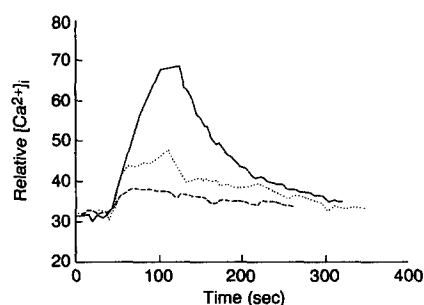


Figure 8. Effect of membrane depolarization on calcium levels of activated lymphocytes. Indo-1-loaded lymphocytes in PBS (—) or 80 mM K^+ solution (---) were stimulated with OKT3 (10 ng/ml) and anti-Ig (1:50 dilution), and measurement of relative $[Ca^{2+}]_i$ was performed as described. In the same experiment, the effect of ChTX (100 nM) was also measured in PBS (·····) or in 80 mM K^+ (data not shown). The zero point represents the point at which second antibody was added.

lution (80 mM), the cell membrane is depolarized (11). Therefore, we examined the effect of changes in membrane potential on the rise in $[Ca^{2+}]_i$ of lymphocytes activated by anti-CD3 in high K^+ medium. Fig. 8 shows that the $[Ca^{2+}]_i$ increase induced by anti-CD3 is inhibited when cells are suspended in high K^+ solutions. Inhibition of both the transient increase and the sustained plateau are similar in magnitude to those observed in the presence of a maximal concentration of ChTX. As expected, addition of ChTX in 80 mM K^+ does not have an additive effect (data not shown).

Discussion

The results presented in this paper demonstrate that ChTX, an inhibitor of $P_{K,V}$ and small conductance $P_{K,Ca}$ in human T cells, blocks the proliferative response and lymphokine production of T cells activated only by pathways that induce a rise in $[Ca^{2+}]_i$, such as those stimulated by anti-CD3, anti-CD2, or ionomycin + PMA. ChTX exerts its immunosuppressive effect in a specific manner since the block in IL-2 production can be bypassed if the cells are activated via the biochemically distinct CD28 pathway. Furthermore, the ability of ChTX to inhibit a MLR implies that a variety of antigen-specific responses may be susceptible to inhibition through this toxin-sensitive pathway. With respect to its selectivity for certain types of lymphocyte activation pathways, the immunosuppressive properties of ChTX are indistinguishable from those of FK-506 and cyclosporine. The sites of action of these immunosuppressants are distinct, however, since FK-506 and cyclosporine interfere with a cytosolic, Ca^{2+} -associated signaling process, and do not have a direct inhibitory effect on the rise in $[Ca^{2+}]_i$ (23).

$P_{K,V}$ has been demonstrated to be the predominant K^+ channel in human T lymphocytes, and its gating properties, conductance, kinetics, and selectivity have been well characterized (2, 3). More recently, human lymphocyte $P_{K,Ca}$ have also been identified and characterized (9). The possible involvement of these K^+ channels in T cell activation has been addressed by different investigators. Early studies suggesting a role for $P_{K,V}$ were not conclusive because the high concentrations of the nonselective K^+ channel blockers TEA and 4-AP required to show inhibition of T cell proliferation may have interfered with cellular processes nonspecifically. Specific K^+ channel toxins have provided an important tool for investigating the role of K^+ channels in T cell activation. In this respect, previous studies have shown that PHA-induced proliferation was inhibited by ChTX, and that the inhibitory effect of ChTX was at the level of IL-2 production (20).

Since ChTX inhibits both $P_{K,Ca}$ and $P_{K,V}$ in human T lymphocytes, blockade of either one or both of these channels may be sufficient to interfere with T cell activation. This question was directly addressed pharmacologically by the use of peptides that are selective for $P_{K,V}$. Electrophysiological data have shown that NxTX and MgTX are selective inhibitors of $P_{K,V}$ in that they do not block $P_{K,Ca}$ in human T lymphocytes (9). α -DaTX is a less potent inhibitor of $P_{K,V}$, whereas IbTX blocks neither $P_{K,V}$ nor $P_{K,Ca}$. The rank order of potency of these peptides is identical in affecting each of

the T cell parameters studied: (a) inhibition of ^{125}I -ChTX binding to human T lymphocytes; (b) proliferation; (c) lymphokine production; and (d) interference with a rise in $[Ca^{2+}]_i$. Therefore, it can be concluded that blockade of $P_{K,V}$ is the relevant target for inhibition of the phenomena investigated in this study.

The role of $P_{K,Ca}$ in human T cells remains unclear. T cell activation by mitogen (27, 28), anti-CD3 (13), or ionomycin (29) is accompanied by membrane hyperpolarization. Hyperpolarization has been suggested to be a consequence of the activation of $P_{K,Ca}$ secondary to the increase in $[Ca^{2+}]_i$, and ChTX was thought to exert its antiproliferative effect by inhibiting $P_{K,Ca}$ (27). Results in this study, however, suggest that $P_{K,V}$, through its ability to set resting potential of T lymphocytes, is the most relevant target for the action of the peptides. It is not known whether a selective inhibitor of $P_{K,Ca}$ would have a similar spectrum of immunosuppressive activities as a $P_{K,V}$ blocker, since such pharmacological probes have not, as yet, been identified.

It is well known that a rise in $[Ca^{2+}]_i$ is a necessary step in the T cell activation cascade. Although it is generally agreed that the early phase of calcium elevation is associated with release of calcium from internal stores, calcium from external sources also contributes to the early phase of calcium elevation. Data obtained by us and others (30) show that in the presence of EGTA (to remove extracellular calcium), the early phase of calcium elevation induced by anti-CD3 was inhibited and subsequently, T cell activation measured by IL-2 RNA transcription (31) or proliferation (32) was also inhibited. These data indicate that a large portion of the early phase of calcium elevation comes from the extracellular source, and that blocking the early phase can lead to the inhibition of T cell activation. In this study we demonstrated that ChTX inhibits the early phase of Ca^{2+} elevation. Whether ChTX inhibits calcium release or calcium influx, or both is not presently known. The current studies also do not allow us to address the role of intracellular Ca^{2+} stores vs. influx in eliciting the rise in $[Ca^{2+}]_i$ directly. Nevertheless, the observation that both the TCR-induced and ionomycin-mediated rise in $[Ca^{2+}]_i$ are blocked by the toxins suggest that $P_{K,V}$ is an important determinant in controlling T cell Ca^{2+} homeostasis.

How does inhibition of $P_{K,V}$ affect the rise in $[Ca^{2+}]_i$? Depolarization of the T cell, whether mediated by blockade of $P_{K,V}$ or by incubation in high K^+ solution, is sufficient to inhibit the rise in $[Ca^{2+}]_i$. Indeed, ChTX, NxTX, and MgTX have recently been shown to depolarize resting lymphocytes (9). The observation that cellular depolarization inhibits the mitogen-induced elevation of $[Ca^{2+}]_i$ has been made previously (10–13), but these earlier studies had not related their finding to blockade of $P_{K,V}$. It is not clear why the effects of ionomycin are also inhibited by ChTX since the ionophore is electroneutral. It is possible that depolarization of the membrane potential by ChTX leads to inhibition of ionomycin-mediated release of intracellular Ca^{2+} . That depolarization inhibits ionomycin-induced $[Ca^{2+}]_i$ was reported earlier (29). Perhaps an internal negative membrane potential drives influx of Ca^{2+} into lymphocytes and de-

polarization limits this process. If Ca^{2+} normally cycles into and out of intracellular stores, limiting Ca^{2+} influx, while allowing cellular Ca^{2+} efflux to occur, will eventually decrease the amount of intracellular Ca^{2+} supplies. In such a model, intracellular Ca^{2+} stores would be in equilibrium with the resting potential, and depolarization would make this Ca^{2+} pool unavailable for release after mitogen stimulation.

In conclusion, this investigation provides a mechanistic basis for the previously observed immunosuppressive effects of ChTX. We have confirmed and extended results indicating

that ChTX inhibits T cell proliferation and lymphokine production when triggered through Ca^{2+} -associated signal transduction pathways. Moreover, similar data have been obtained with $\text{P}_{\text{K,V}}$ selective peptides, suggesting that blockade of $\text{P}_{\text{K,V}}$, because of its role in setting the resting potential in T lymphocytes, leads to inhibition of the activation-induced rise in $[\text{Ca}^{2+}]_i$. The precise mechanism by which changes in membrane potential result in alterations in Ca^{2+} homeostasis remains to be determined. It will also be interesting to determine the relevance of $\text{P}_{\text{K,V}}$ as a target for inhibition of immune responsiveness in vivo.

We would like to thank Drs. C. Cohen and J. Reuben for stimulating discussions throughout this study. We also thank Drs. E. Reinhertz, J. A. Ledbetter, and C. June for generous supply of anti-CD2 and anti-CD28 mAb, respectively.

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Received for publication 6 May 1992 and in revised form 28 October 1992.

References

- Gardner, P. 1989. Calcium and T lymphocyte activation. *Cell* 59:15.
- DeCoursey, T.E., K.G. Chandy, S. Gupta, and M.D. Cahalan. 1984. Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis? *Nature (Lond.)* 307:465.
- Matteson, D.R., and C. Deutsch. 1984. K channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature (Lond.)* 307:468.
- Stuhmer, W., J.P. Ruppersberg, K.H. Schroter, B. Sakmann, M. Stocker, K.P. Giese, A. Perschke, A. Baumann, and O. Pongs. 1989. Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3235.
- Swanson, R., J. Marshall, J.S. Smith, J.B. Williams, M.B. Boyle, K. Folander, C.J. Luneau, J. Antanavage, C. Oliva, S.A. Buhrow, et al. 1990. Cloning and expression of cDNA and genomic clones encoding three delayed rectifier potassium channels in rat brain. *Neuron* 4:929.
- Douglass, J., P.B. Osborne, Y.C. Cai, M. Wilkinson, M.J. Christie, and J.P. Adelman. 1990. Characterization and functional expression of a rat genomic DNA clone encoding a lymphocyte potassium channel. *J. Immunol.* 144:4841.
- Chandy, K.G., C.B. Williams, R.H. Spencer, B.A. Aguilar, S. Ghanshani, B.L. Tempel, and G.A. Gutman. 1990. A family of three mouse potassium channel genes with intronless coding regions. *Science (Wash. DC)* 247:973.
- Grissmer, S., B. Dethlefs, J.J. Wasmuth, A.L. Goldin, G.A. Gutman, M.D. Cahalan, and K.G. Chandy. 1990. Expression and chromosomal localization of a lymphocyte K^+ channel gene. *Proc. Natl. Acad. Sci. USA* 87:9411.
- Leonard, R.J., M.L. Garcia, R.S. Slaughter, and J.P. Reuben. 1992. Selective blockers of voltage gated K^+ channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc. Natl. Acad. Sci. USA* 89:10094.
- Randriamampita, C., G. Bismuth, P. Debre, and A. Trautmann. 1991. Nitrendipine-induced inhibition of calcium influx in a human T-cell clone: role of cell depolarization. *Cell Calcium* 12:313.
- Gelfand, E.W., R.K. Chung, and S. Grinstein. 1984. Role of membrane potential in the regulation of lectin-induced calcium uptake. *J. Cell. Physiol.* 121:533.
- Oettgen, H.C., C. Terhorst, L.C. Cantley, and P.M. Rosoff. 1985. Stimulation of the T3-T cell receptor complex induces a membrane-potential-sensitive calcium influx. *Cell* 40:583.
- Sarkadi, B., A. Tordai, and G. Gardos. 1990. Membrane depolarization selectively inhibits receptor-operated calcium channels in human T (Jurkat) lymphoblasts. *Biochim. Biophys. Acta* 1027:130.
- Gouy, H., D. Cefai, S.B. Christensen, P. Debre, and G. Bismuth. 1990. Ca^{2+} influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular Ca^{2+} stores. A study with the Ca^{2+} endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur. J. Immunol.* 20:2269.
- Chandy, K.G., T.E. DeCoursey, M.D. Cahalan, C. McLaughlin, and S. Gupta. 1984. Voltage-gated potassium channels are required for human T lymphocyte activation. *J. Exp. Med.* 160:369.
- Cahalan, M.D., G. Chandy, T.E. DeCoursey, S. Gupta, R.S. Lewis, and J.B. Sutro. 1987. Ion channels in T lymphocytes. *Adv. Exp. Med. Biol.* 213:85.
- Schell, S.R., D.J. Nelson, H.A. Fozzard, and F.W. Fitch. 1987. The inhibitory effects of K^+ channel-blocking agents on T lymphocyte proliferation and lymphokine production are "nonspecific". *J. Immunol.* 139:3224.
- Gimenez-Gallego, G., M.A. Navia, J.P. Reuben, G.M. Katz, G.J. Kaczorowski, and M.L. Garcia. 1988. Purification, sequence, and model structure of charybdotoxin, a potent selective inhibitor of calcium-activated potassium channels. *Proc. Natl.*

- Acad. Sci. USA.* 85:3329.
19. Sands, S.B., R.S. Lewis, and M.D. Cahalan. 1989. Charybdotoxin blocks voltage-gated K⁺ channels in human and murine T lymphocytes. *J. Gen. Physiol.* 93:1061.
 20. Price, M., S.C. Lee, and C. Deutsch. 1989. Charybdotoxin inhibits proliferation and IL2 production in human peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA.* 86:10171.
 21. Bono, M.R., V. Simon, and M.S. Rosenblatt. 1989. Blocking of human T lymphocyte activation by channel antagonists. *Cell Biochem. and Funct.* 7:219.
 22. Gelfand, E.W., and R. Or. 1991. Charybdotoxin-sensitive, Ca²⁺-dependent membrane potential changes are not involved in human T or B cell activation and proliferation. *J. Immunol.* 147:3452.
 23. Lin, C.S., R.C. Boltz, J.J. Siekierka, and N.H. Sigal. 1991. FK-506 and cyclosporin A inhibit highly similar signal transduction pathways in human T lymphocytes. *Cell. Immunol.* 133:269.
 24. Novick, J., R.J. Leonard, V.F. King, W. Schmalhofer, G.J. Kaczorowski, and M.L. Garcia. 1991. Purification and characterization of two novel peptidyl toxins directed against K⁺ channels from venom of new world scorpions. *Biophys. J.* 59:78a.
 25. Galvez, A., G. Gimenez-Gallego, J.P. Reubin, L. Roy-Contancin, P. Feigenbaum, G.J. Kaczorowski, and M.L. Garcia. 1990. Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion *Buthus tamulus*. *J. Biol. Chem.* 265:11083.
 26. Deutsch, C., M. Price, S. Lee, V.F. King, and M.L. Garcia. 1991. Characterization of high affinity binding sites for charybdotoxin in human T lymphocytes: evidence for association with the voltage-gated K⁺ channel. *J. Biol. Chem.* 266:3668.
 27. Grinstein, S., and J.D. Smith. 1990. Calcium-independent cell volume regulation in human lymphocytes. Inhibition by charybdotoxin. *J. Gen. Physiol.* 95:97.
 28. Tsien, R.Y., T. Pozzan, and T.J. Rink. 1982. T-cell mitogens cause early changes in cytoplasmic free Ca²⁺ and membrane potential in lymphocytes. *Nature (Lond.)* 295:68.
 29. Ishida, Y., and T.M. Chused. 1988. Heterogeneity of lymphocyte calcium metabolism is caused by T cell specific calcium-sensitive potassium channel and sensitivity of the calcium ATPase pump to membrane potential. *J. Exp. Med.* 168:839.
 30. June, C., J.A. Ledbetter, P.S. Rabinovitch, P.J. Martin, P.G. Beatty, and J.A. Hansen. 1986. Distinct patterns of transmembrane calcium flux and intracellular calcium mobilization after differentiation antigen cluster 2 (E rosette receptor) or 3 (T3) stimulation of human lymphocytes. *J. Clin. Invest.* 77:1224.
 31. Gardner, P., A. Alcover, M. Kuno, P. Moingeon, C.M. Weyand, J. Goronzy, and E. Reinherz. 1989. Triggering of T-lymphocytes via either T3-Ti or T11 surface structures opens voltage-insensitive plasma membrane calcium-permeable channels: requirement for interleukin-2 gene function. *J. Biol. Chem.* 264:1068.
 32. Truneh, A., A. Françoise, P. Golstein, and A.-M. Schmitt-Verhulst. 1984. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. *Nature (Lond.)* 313:318.