

# Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes

(scorpion venom/ $K^+$  channels/mitogenesis/lymphokines)

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**ABSTRACT** We demonstrate that blockade of the lymphocyte voltage-gated  $K^+$  channel by charybdotoxin (CTX) inhibits lymphocyte mitogenesis. Charybdotoxin blocks conductance with a  $K_i$  of 0.3 nM and inhibits mitogen- and antigen-stimulated proliferation with a  $K_i$  of 0.5 nM. As opposed to the other blockers of the lymphocyte  $K^+$  channel, the inhibition of mitogenesis by CTX can be overcome selectively by exogenous recombinant interleukin 2 (IL-2); endogenous levels of IL-2 in the culture supernatants of stimulated cells are decreased by the presence of CTX. Our results suggest that the voltage-gated  $K^+$  channel is either directly or indirectly involved in IL-2 synthesis and/or secretion.

We have investigated the functional effects of charybdotoxin (CTX) on mitogenesis. CTX, a 37-amino acid peptide isolated from the venom of the scorpion *Leiurus quinquestriatus* (hebraeus) (1, 2), is a highly specific, potent, and impermeant blocker of the lymphocyte voltage-gated  $K^+$  channel (3, 4). The  $K_i$ , as measured by whole-cell patch-clamp techniques, is  $\approx 0.3$  nM for quiescent primary peripheral blood human T cells of both the helper ( $CD4^+$ ) and suppressor ( $CD8^+$ ) phenotype (4). In all cases (*vide infra*) the inhibitory effects are reversible and do not alter cell viability. As such, CTX could be an important pharmacological tool with which to determine the *in situ* role of this channel in lymphocyte function.

Two lines of evidence suggest that T-cell  $K^+$  channels are involved in proliferation and also underlie volume regulation. First, an increased current density precedes DNA synthesis following mitogen stimulation (5-7), and  $K^+$  channel-dependent volume regulation correlates with  $K^+$  conductance (8). Second, the agents that inhibit the  $K^+$  conductance, as determined by patch-clamp measurements, also inhibit volume regulation and stimulated proliferation (5, 6, 8, 9). These agents encompass a wide variety of drugs, including the so-called  $Ca^{2+}$  channel blockers verapamil, diltiazem, and nifedipine as well as the classical  $K^+$  channel blockers quinine, tetraethylammonium ion, and 4-aminopyridine. Each blocker inhibits both stimulated T-cell proliferation and  $K^+$  conductance with similar, though not identical,  $K_i$  values (9). Even the most potent of these drugs, verapamil, requires 6-7  $\mu$ M for a 50% block of the current, levels at which there are significant questions regarding non-channel-mediated effects of these channel blockers. Though we (5, 6) and others (7, 9) have shown that functional  $K^+$  channel expression is correlated with proliferation, it remains to be proven that  $K^+$  channels are directly, causally related to mitogenesis. Inhibition of cell-cycle progression by  $K^+$  channel blockers could be mediated by other sites/processes that are at least as sensitive as  $K^+$  channels to these inhibitors. It has not yet been possible to eliminate this possibility unequivocally.

Because CTX inhibits the T lymphocyte  $K^+$  current (3) at nanomolar concentrations, it is a promising tool for the identification of functions that are coupled to  $K^+$  channels. At least one  $K^+$  channel-dependent lymphocyte function, volume regulation, is sensitive to CTX (10). An example from a nonlymphoid system is the effect of CTX on contraction in isolated vascular smooth muscle (11). We report here a study of the effects of CTX on human T-lymphocyte  $K^+$  conductance and characterize CTX effects on T-cell function, specifically stimulated proliferation and interleukin 2 (IL-2) elaboration.

## MATERIALS AND METHODS

Heparinized human venous blood was collected from healthy donors. Peripheral blood mononuclear cells (PBMC) were separated by step-gradient centrifugation using a modified Ficoll/Hypaque technique (5). Cell number was measured with a Coulter Counter. Viability as assessed by trypan blue exclusion was routinely  $\geq 95\%$ .

**Electrophysiological Measurements.** For electrophysiological recording, T lymphocytes were selectively adhered to plastic dishes by using the monoclonal antibody OKT11 (Ortho Pharmaceutical), which binds to the pan-T-cell marker CD2. Standard patch-clamp techniques were used to study whole-cell currents (5, 12). The pipette solution contained 130 mM KF, 11 mM  $K_2$  EGTA, 2 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 10 mM Hepes, and KOH to bring the pH to 7.2 and a final osmolarity (osM) of about 280 mosM. Bath solution was 300 mosM at pH 7.3. All experiments were done at room temperature.

**Proliferation Assays.** Lymphocytes were cultured in serum-free RPMI 1640 medium supplemented with fatty acid-free bovine serum albumin (1.25 mg/ml; Sigma), 18  $\mu$ M linoleic acid (Sigma), 1 mM sodium pyruvate (GIBCO), 240 nM ferric nitrate (Fisher), 150 nM transferrin (Sigma), 80 nM sodium selenite (GIBCO), 1 mM L-glutamine, and 100 units of penicillin and 100  $\mu$ g of streptomycin per ml. Cultures contained  $10^5$  cells per 0.2 ml in 96-well flat-bottom tissue culture plates maintained at 37°C in humidified air containing 5%  $CO_2$ . Proliferation was initiated with phytohemagglutinin P (PHA-P, 2  $\mu$ g/ml; Difco). DNA synthesis was determined from the incorporation of [ $^3H$ ]thymidine added to cells (0.2  $\mu$ Ci per well; 1 Ci = 37 GBq) 8 hr before harvest. For all culture conditions, viability on day 3 after stimulation was between 95% and 98%, as measured by trypan blue exclusion.

**Purification of Quiescent T Cells.** The T-cell purification procedure entailed rosetting of PBMC with sheep erythrocytes to facilitate removal of B cells, followed by preferential adhesion of monocytes to tissue culture plastic when the

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Abbreviations: CTX, charybdotoxin; PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2; IL-1, interleukin 1; IL-6, interleukin 6; PHA-P, phytohemagglutinin P.

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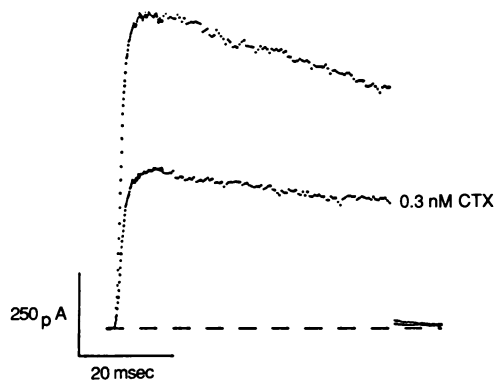


FIG. 1. CTX block of human T-lymphocyte  $K^+$  current. These traces show currents elicited by a 60-msec depolarization from  $-70$  mV to  $+50$  mV. The top current trace was recorded prior to CTX addition to the bathing medium; the bottom trace was recorded 5 min after addition of CTX to  $0.3$  nM final concentration. This concentration of CTX consistently gave  $\approx 50\%$  block of whole-cell current.

rosetted cells were cultured in the presence of serum. The nonadherent cells were treated with monoclonal antibodies against HLA-DR (a surface marker of B cells and activated T cells) and the p55 chain of the IL-2 receptor (found in high concentration on activated T cells) and were eluted from a column of covalently linked goat anti-mouse F(ab')<sub>2</sub> containing Sepharose 6MB (Pharmacia; ref. 13). The resultant population of cells was  $>99\%$  positive for the T-cell surface marker CD2 and  $<1\%$  positive for the monocyte surface marker CD11b (antibody OKM1, Ortho Pharmaceutical), as determined by use of a fluorescence-activated cell sorter to analyze cells that had been labeled with fluorescent antibodies.

**IL-2 Assay.** After 27 hr of stimulation with PHA-P ( $2$   $\mu\text{g}/\text{ml}$ ),  $150$   $\mu\text{l}$  were taken from each well culture and centrifuged at  $2000$  rpm ( $600 \times g$ ), and  $100$ - $\mu\text{l}$  samples were removed for bioassay of IL-2 with an IL-2-dependent murine cell line, CTLL-2 clone (14). DNA synthesis of the CTLL-2 cells is dependent on IL-2 concentration. IL-2 standards for the calibration curve were derived from culture supernatants of an IL-2-producing gibbon ape leukemia cell line (MLA-144). Dilutions of the supernatants from PBMC cultures were added to the CTLL-2 cultures ( $5 \times 10^3$  cells per well). After 24 hr at  $37^\circ\text{C}$  in a  $\text{CO}_2$  incubator, the cells were pulsed with  $0.5$   $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine and harvested 8 hr later. Antibody to the p55 chain of the IL-2 receptor (anti-Tac) was donated by T. Waldmann (National Institutes of Health).

**Reagents.** Purified CTX was donated by C. Miller (Brandeis University). Recombinant IL-2 was obtained from Cetus, and recombinant IL-1 and IL-6 were gifts from J. Elias (University of Pennsylvania), who received them from P.

Lomedico of Hoffmann-La Roche (recombinant human IL-1 $\alpha$ ,  $4 \times 10^7$  units/mg of protein) and P.-K. Sehgal and L. G. May of Rockefeller University (recombinant IL-6). Heat-inactivated human AB serum was obtained from Irvine Scientific.

## RESULTS AND DISCUSSION

**Potency and Properties of CTX in Culture.** To study the effects of CTX on lymphocyte function, we had to establish the functional integrity of the toxin under culture conditions. For this purpose we used whole-cell patch clamp of a T cell as a bioassay (Fig. 1). Incubation of CTX with quiescent or activated lymphocytes for 48 hr in culture did not impair its potency against the lymphocyte  $K^+$  conductance in whole-cell patch clamp. Similarly, the blocking activity of the toxin was unchanged in the presence of  $0.1$ – $1.5\%$  human AB serum. This was true regardless of whether the toxin was preincubated with serum before its presentation to the patch-clamped cell or the toxin was presented to a patch-clamped cell already bathed in serum-containing medium. In contrast, other  $K^+$  channel blockers showed some (but less than  $50\%$ ) increased  $K_i$  for channel blockade in the presence of serum (Table 1). Blocking activity of the toxin was likewise unimpaired in the presence of mitogenic concentrations of PHA-P ( $3$   $\mu\text{g}/\text{ml}$ ) and in the presence of high concentrations of Cetus recombinant IL-2 ( $100$  Cetus units/ml;  $1$  unit/ml is defined as the activity required to produce half-maximal proliferative response in the bioassay). The toxin was also equipotent against the  $K^+$  conductance in 24-hr stimulated lymphocytes [activated with the mitogen phorbol 12-myristate 13-acetate  $\pm$  IL-2 ( $100$  Cetus units/ml)]. In addition, CTX was equally effective at both  $25^\circ\text{C}$  and  $37^\circ\text{C}$ . From these data we conclude that (i) CTX is resistant to functional degradation by lymphocytes under *in vitro* culture conditions, and (ii) interaction with the lymphocyte voltage-gated  $K^+$  channel in whole-cell patch clamp is unaffected by either the simultaneous presence or prior exposure of the cells to mitogen, lymphokine, and/or serum. Also, preincubation of the toxin with mitogen, lymphokine, and/or serum had no effect on its  $K_i$  for channel blockade.

Compared to its better-characterized interaction with  $\text{Ca}^{2+}$ -activated  $K^+$  channels, CTX is a more potent inhibitor of the lymphocyte  $K^+$  channel than it is of the  $\text{Ca}^{2+}$ -activated  $K^+$  channels of rat skeletal muscle ( $K_i \approx 10$  nM in artificial membranes) (16), GH3 anterior pituitary cells ( $K_i \approx 2.1$  nM) (2), or *Aplysia* neurons ( $K_i \approx 30$  nM in sea water) (17). Moreover, cleavage of the two terminal amino acids from the N terminus of the toxin by  $\alpha$ -chymotrypsin did not alter its  $K_i$  against the lymphocyte channel (data not shown) but rendered the toxin inactive against the  $\text{Ca}^{2+}$ -activated  $K^+$  channel of rat skeletal muscle (not shown). Dithiothreitol treat-

Table 1. Effect of serum on the inhibition constants of  $K^+$  channel blockers against the  $K^+$  channel and mitogen-stimulated proliferation

Blocker	Inhibition constants			
	$K^+$ -channel block		Proliferation of PBMC ( <i>n</i> )	
	With serum	No serum	With serum	No serum
CTX	$0.3$ nM	$0.3$ nM	No inhibition	$0.49 \pm 0.13$ nM (4)
Quinine	$30$ $\mu\text{M}$	$22$ $\mu\text{M}$	$82.0 \pm 8.4$ $\mu\text{M}$	$39.5 \pm 11.2$ $\mu\text{M}$ (4)
4-Aminopyridine	$320$ $\mu\text{M}$	$250$ $\mu\text{M}$	$3.8 \pm 0.8$ mM	$0.9 \pm 0.25$ mM (4)
Tetraethylammonium	ND	$8$ mM	$13.5 \pm 1.2$ mM	$8.3 \pm 1.3$ mM (3)

Efficacy of channel block in the presence of serum was assayed electrophysiologically. Cells were placed in whole-cell patch clamp as described in Fig. 1, human AB serum was introduced to the bathing solution, and then drugs were added to the desired concentration. CTX was tested in the presence of up to  $1.5\%$  (vol/vol) serum, and quinine and 4-aminopyridine were tested at  $10\%$  serum. Proliferation was determined as described in Fig. 2. Inhibition constants for quinine, 4-aminopyridine, and tetraethylammonium were determined in the presence of  $10\%$  serum, and all constants are given with standard deviation. The non-CTX data appeared previously in a preliminary report (15).

ment of CTX, which alters the tertiary structure of the protein by breaking the disulfide bonds (16), inactivated the toxin against both channels (not shown).

Similar to the ionic-strength dependence of CTX interaction with the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, we found that in isotonic extracellular medium containing 60 mM NaCl and 176 mM mannitol, the affinity of CTX for the lymphocyte channel increased at least 10-fold compared with its affinity in medium containing salts at physiological ionic strength. These results suggest that electrostatic interaction of CTX with the lymphocyte  $\text{K}^+$  channel may be important, as has been demonstrated for the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel of rat skeletal muscle (18).

**Effect of CTX on Mitogenesis.** Not only  $\text{K}^+$  conductance but also mitogenesis was inhibited by CTX at nanomolar concentrations. CTX inhibited mitogen-driven proliferation of PBMC by  $53\% \pm 15\%$  ( $n = 11$ , seven donors) at concentrations  $> 5$  nM in serum-free cultures (Fig. 2). Higher concentrations of toxin did not significantly increase the effect. No concentration of toxin tested (0.1–100 nM) affected cell viability, which was  $>95\%$  throughout the culture period. Most experiments were done with CTX that was  $\approx 83\%$  pure. It is unlikely that the inhibitory effects of CTX were due to contaminants, as similar results were obtained with highly purified toxin (purity  $> 95\%$ ). The  $K_i$  was  $\approx 0.6$  nM for the example in Fig. 2; the average  $K_i$  was  $0.49 \pm 0.13$  nM ( $n = 4$ ) (Table 1). The decreased peak proliferation on day 3 was not due to slower kinetics of proliferation. The time course of [ $^3\text{H}$ ]thymidine incorporation was not altered in the presence of inhibiting concentrations of CTX. The inhibitory effect of CTX on mitogenesis was observed only if CTX were added within the first few hours after PHA addition, with the maximum effect achieved by preincubation of the cells with toxin for 30 min.

CTX did not inhibit proliferation in cultures that contained human AB serum. Fig. 3 *Left* shows that CTX inhibition of proliferation did not occur when as little as 0.1% human AB serum was present. The mixed human AB serum used in our experiments contained no detectable amounts of IL-2 activity, as measured by a CTLL-2 (mouse T-cell line) proliferation bioassay (see below). Therefore, the uninhibited proliferation in human AB serum suggests that there is a specific interaction of some non-IL-2 serum component with the cells

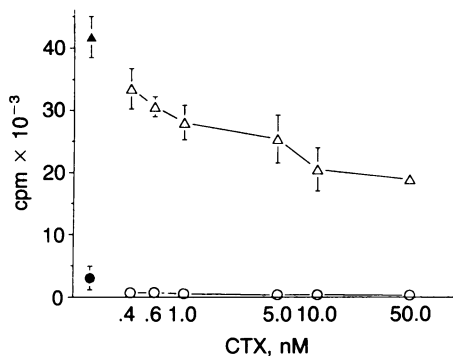


FIG. 2. Dose-response of CTX on stimulated proliferation of peripheral blood lymphocytes. Cells were incubated for 30 min with the indicated concentrations of CTX in serum-free medium and then cultured with CTX still present in the absence (control) (○) or presence (Δ) of PHA-P. PBMC were pulsed with [ $^3\text{H}$ ]thymidine and harvested on day 3 (peak of [ $^3\text{H}$ ]thymidine incorporation). Filled symbols are the corresponding controls without CTX. Each point represents the mean  $\pm$  standard deviation of triplicate samples. The SD of the point at 50 nM CTX was within the drawn symbol. Cell loss in each culture at 72 hr was 8%, 8%, 17%, 7%, 15%, and 14% of control for CTX concentrations of 0.4, 0.6, 5, 10, and 50 nM, respectively. Cell viability of residual cells was  $\geq 95\%$ .

and not degradation of CTX or nonspecific protein binding of the toxin, inasmuch as CTX is equipotent against the  $\text{K}^+$  channel in the presence of serum (see above) and the serum-free medium already contains a significant amount of protein (see *Materials and Methods*). The  $K_i$  values for inhibition of stimulated proliferation by other  $\text{K}^+$  channel blockers are not markedly altered by as much as 10% serum (Table 1).

Therefore, we added several candidate compounds to the serum-free cell cultures to test the ability of each to overcome CTX inhibition. Our initial adjuvants included lymphokines (IL-1, IL-2, and IL-6), added over comparable activity ranges including supra-optimal activities,<sup>†</sup> and hormone (insulin). IL-2 (100 units/ml;  $3.2 \times 10^{-5}$  mg/ml), but not IL-1 (100 units/ml;  $0.3 \times 10^{-5}$  mg/ml) or IL-6 ( $9 \times 10^{-5}$  mg/ml, unit activity undetermined), overcame CTX inhibition of proliferation (Fig. 3 *Center*). Insulin (bovine, Collaborative Research) at 10 pM to 1  $\mu\text{M}$  had no effect on CTX inhibition (data not shown).

**CTX Acts Directly on T Cells.** These results indicate that CTX has a reversible noncytotoxic effect on peripheral blood lymphocyte proliferation. The effect may be directly on T cells or on accessory cells that are also present and necessary for T-cell proliferation with mitogen. The rationale for the latter hypothesis is that (i) accessory cell (i.e., monocyte) function is necessary for T-cell proliferation (19) (accessory cells are responsible both for processing of antigen/mitogen so that it is recognized by the T cell and for production and secretion of peptide hormones that potentiate lymphocyte responses); (ii) macrophage/monocyte-derived cells have  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (20); and (iii)  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels are inhibited by CTX at nanomolar but not subnanomolar concentrations (1, 2, 17).

To determine if the CTX inhibition of proliferation were mediated by accessory cells, we repeated these experiments with purified T-cell preparations. T cells were depleted of accessory cells and stimulated with adhered anti-CD3 (25 ng/ml; Leu-4, Becton Dickinson), a mitogenic monoclonal antibody directed against CD3, a component of the T-cell antigen receptor complex. Proliferation of purified T cells was similarly inhibited by CTX to the same extent as unfractionated PBMC (Fig. 3 *Right*). Inhibition could be overcome with IL-2 (30 units/ml,  $1 \times 10^{-5}$  mg/ml) but not with IL-6 ( $2.7 \times 10^{-5}$  mg/ml). These data suggest that (i) CTX has a direct inhibitory effect on stimulated proliferation of T cells; (ii) the toxin may inhibit IL-2 utilization, synthesis, and/or secretion; and (iii) IL-2 reversal of the CTX-induced inhibition is specific and not a general protein effect.

**CTX Modulates IL-2 Levels.** IL-2 is both produced and utilized by T cells and is a required growth factor for T-cell proliferation (21). Our data suggest a direct effect of CTX on the production and/or utilization of IL-2 by T cells. Since CTX inhibition was overcome by addition of IL-2, it seemed likely that inhibition of proliferation was due to a CTX-induced alteration in the relationship between IL-2 utilization and availability. To test this hypothesis directly, we assayed IL-2 content in cultures grown in the presence (10 nM) and absence of CTX using a CTLL-2 proliferation bioassay. The IL-2 calibration of the bioassay with known standards was identical regardless of whether CTX was present (at 10 nM or equivalent dilution ratios). This indicates that IL-2 content in the presence of CTX is accurately reflected in the bioassays. Moreover, the patch-clamp studies demonstrated that CTX potency was unimpaired by preincubation with 100 units of IL-2 per ml (*vide supra*). Both the PBMC electrophysiology and the CTLL-2 proliferation results indicate that there is no direct interaction between CTX and IL-2. As calculated for

<sup>†</sup>Units/ml are defined as the activity required to produce half-maximal response in the bioassay.

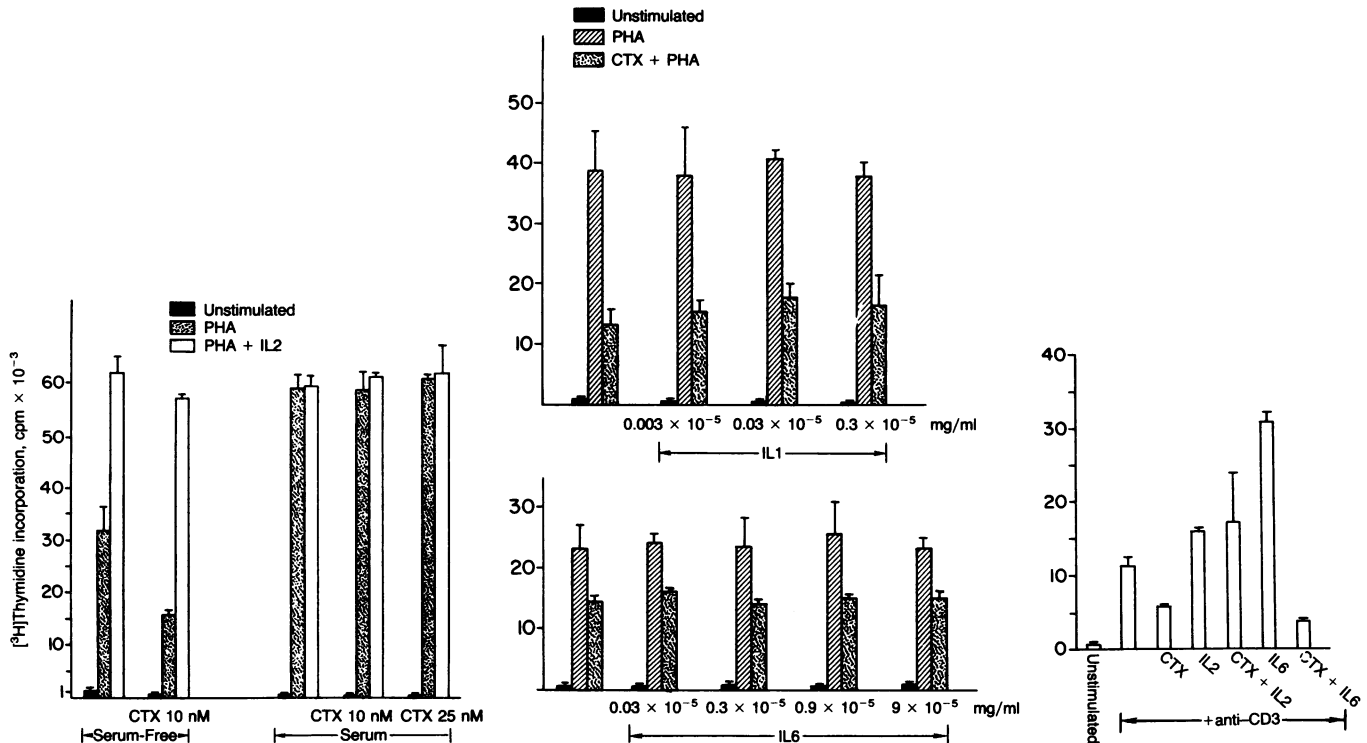


FIG. 3. Effects of lymphokines and serum on CTX-inhibited proliferation. (*Left*) Cells were cultured in the presence of 0, 10, or 25 nM CTX in serum-free or serum-containing (10% human AB serum) medium as described in Fig. 2 except that  $10 \mu\text{g}$  of PHA-P per ml was used in the serum-containing medium. Recombinant IL-2 (100 units/ml;  $3.2 \times 10^{-5}$  mg/ml) was added at the same time as the mitogen. (*Center*) Cells were cultured and stimulated in serum-free medium for 3 days as described for *Left* in the presence of recombinant IL-1 (1–100 units/ml;  $0.003 \times 10^{-5}$  to  $0.3 \times 10^{-5}$  mg/ml) or recombinant IL-6 ( $0.03 \times 10^{-5}$  to  $9 \times 10^{-5}$  mg/ml). (*Right*) Purified T lymphocytes were cultured in serum-free medium in microtiter wells pretreated with goat anti-mouse IgG (Tago) and a murine anti-human monoclonal antibody to CD3 (Leu-4, Becton Dickinson). Cells were exposed to CTX (10 nM) for 30 min prior to exposure to anti-CD3 or anti-CD3 + IL-2 (30 units/ml) or IL-6 ( $2.7 \times 10^{-5}$  mg/ml). Proliferation of cultures to which we added back 1–5% autologous accessory cells was comparable to anti-CD3 + IL-6.

the data shown in Fig. 4A, the IL-2 content in supernatants derived from CTX-containing PHA-P-stimulated cultures was reduced by 41% compared with those derived from cultures containing PHA-P only. A similar decrease (30%, data not shown) in IL-2 content was found for cells cultured with 10 nM CTX in normal minimum essential medium containing 10% human AB serum; however, under these conditions total levels of IL-2 are at least three times that in serum-free conditions (unpublished data). The excess IL-2 in these cultures could account for the lack of effect of CTX on proliferation in serum-containing cultures.

To optimize the levels of IL-2 detected and to assess the effect of CTX on IL-2 utilization, we incubated cells with anti-Tac, a monoclonal antibody to the p55 chain of the IL-2-receptor (22). Because anti-Tac inhibits IL-2 uptake (22), we could prevent binding of endogenously secreted IL-2. In the presence of anti-Tac at  $10^{-2}$  mg/ml, CTX produced a 35% decrease in PHA-P-induced IL-2 content of culture supernatants. Because inhibition of proliferation can be overcome by exogenous IL-2 (*vide supra*), these results suggest that CTX inhibition of mitogenesis is due to modulation of IL-2 synthesis or secretion and not of IL-2 utilization. Synthesis and secretion of lymphokines could involve  $\text{K}^+$  channels, analogous to the involvement of ion channels in secretion of peptide hormones in the neuroendocrine system (23). It is also possible that CTX has some nonchannel-mediated effects, such as modulation of receptor–ligand interactions, which could influence IL-2 elaboration.

There is precedence for inhibition of IL-2 elaboration by another blocker of the lymphocyte voltage-gated  $\text{K}^+$  channel (9). A variety of  $\text{K}^+$  channel antagonists inhibit stimulated proliferation, but this inhibition cannot be overcome by exogenous IL-2 (ref. 9; C.D., unpublished observations).

Only in the case of CTX can exogenous IL-2 restore the full mitogenic response. This suggests that the other blockers have additional sites of interaction in the proliferative pathway, which CTX does not. However, we have demonstrated that tetraethylammonium ion, verapamil, diltiazem, quinine, and nifedipine do not inhibit cell-free translation of RNA from a mouse helper T cell in a rabbit reticulocyte assay (24).

Our results demonstrate a functional consequence of CTX on an immune cell process, namely inhibition of IL-2 elaboration and lymphocyte proliferation. Whether these effects are directly and uniquely mediated by  $\text{K}^+$ -channel blockade remains to be proven. Our results do not preclude the possibility that other sites with high affinity for CTX mediate mitogenesis. However, our preliminary data of  $^{125}\text{I}$ -labeled CTX binding to intact cells (unpublished data obtained in collaboration with Garcia and co-workers at Merck Sharp & Dohme) suggests that the  $\text{K}^+$  channel is the only site of high-affinity interaction.

Regardless of the site of toxin interaction, a mitogenic signal was received by the lymphocytes while in the presence of CTX. Evidence in support of this is (i) the ability of exogenous IL-2 to overcome inhibition of mitogen-stimulated proliferation and (ii) the elaboration of IL-2 by stimulated cells in the presence of CTX. Since CTX inhibits mitogenesis and IL-2 elaboration at nanomolar concentrations, we suggest that the voltage-gated  $\text{K}^+$  channel is either directly or indirectly involved in IL-2 synthesis and/or secretion. To test these possibilities, the effect of CTX on the induction of IL-2 mRNA should be determined for mitogen-stimulated cells.

How might voltage-gated  $\text{K}^+$  channels be coupled to IL-2 production? These channels may set the lymphocyte membrane potential, which is predominantly a  $\text{K}^+$  diffusion potential (25, 26). Alterations in membrane potential could

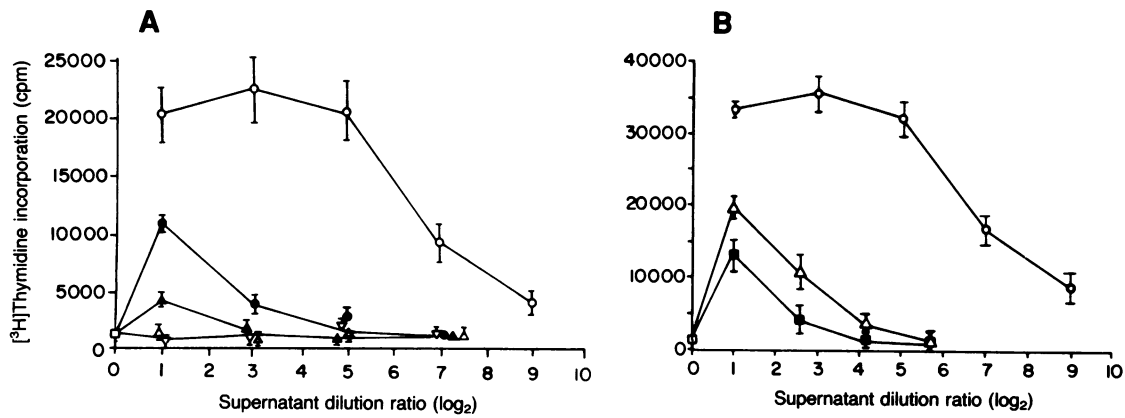


FIG. 4. IL-2 content in CTX-treated cultures. PBMC ( $10^5$  cells in  $200 \mu\text{l}$ ) were stimulated with mitogen and cultured in serum-free medium in the presence and absence of CTX (10 nM). After 27 hr, the supernatants were removed and used to supplement the culture medium of an IL-2-dependent cell line (CTLL-2). The amount of [ $^3\text{H}$ ]thymidine incorporation in the bioassay is proportional to the amount of IL-2 in the supernatant. Each point represents the mean of triplicate cultures. Percentage SEM for each test case was  $<15\%$ . Each curve was used to calculate the dilution at which half-maximal proliferation was obtained. The concentrations in units/ml were derived from the known dilution ratio. IL-2 standards for the calibration curve were derived from culture supernatants of an IL-2-producing cell line (MLA-144) ( $\circ$ ). (A) Results derived from PBMC cultures in the absence of anti-Tac: units of IL-2 per ml were  $<0.1$  for control ( $\nabla$ ) and CTX ( $\Delta$ ) and 5.7 and 3.4 for PHA-P ( $\bullet$ ) and PHA-P plus CTX ( $\blacktriangle$ ), respectively.  $\square$ , No addition to CTLL-2 cultures. (B) Results obtained with cultures that contained anti-Tac ( $10^{-2}$  mg/ml): units of IL-2 per ml were  $<0.1$  for control ( $\square$ ) and 7.1 and 4.6 for PHA-P ( $\Delta$ ) and PHA-P plus CTX ( $\blacksquare$ ), respectively. CTLL-2 proliferation produced by direct addition of IL-2 was not affected by CTX. Similar results were obtained in three independent experiments.

regulate voltage-sensitive  $\text{Ca}^{2+}$  influx. The increase in intracellular free  $\text{Ca}^{2+}$  in T lymphocytes in response to mitogen is sensitive to voltage (27, 28). Cell  $\text{Ca}^{2+}$  may influence IL-2 elaboration (29, 30) and gene expression (J. F. Modiano, personal communication; ref. 31). If CTX blockade of the voltage-gated  $\text{K}^+$  channel results in depolarization of the lymphocyte membrane potential, then this would lead to a smaller increase in free intracellular  $\text{Ca}^{2+}$  in response to mitogen, which would result in decreased production of IL-2.

The observation that no more than 50% of the proliferative response can be inhibited by CTX suggests that there are at least two different components in the mitogenic response, one of which is CTX-sensitive, whereas the other(s) is not. Such components might include two cell populations, two differentially IL-2-dependent pathways, and/or two channel types. For example, a CTX-insensitive contribution to the membrane potential, according to the above-mentioned scenario, could maintain a sufficiently hyperpolarized membrane potential to support limited proliferation.

We thank C. Miller (Brandeis University) for many samples of purified CTX and for assaying the potency of our chymotrypsin- and dithiothreitol-treated toxins against the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel of rat skeletal muscle. We thank D. Krause for the experiments on purified T cells, B. Freedman for the development of the serum-free media, J. Modiano for the IL-2 assays, and T. Waldmann (National Institutes of Health) for anti-Tac. This work was supported by National Institutes of Health Grant GM 41467.

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