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VOLTAGE-GATED POTASSIUM CHANNELS ARE REQUIRED FOR HUMAN T LYMPHOCYTE ACTIVATION

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The involvement of ion channels in T lymphocyte activation is suggested by reports of membrane depolarization (1–3), hyperpolarization (2, 4), increased ion fluxes across the membrane (5–9), and increased intracellular free calcium (4, 10) after mitogenic stimulation. Furthermore, various stages of T lymphocyte activation are inhibited by substances known to block ion channels in other tissues (11–15). Using the gigaohm seal recording technique (16), we and others (15, 17) recently demonstrated the existence in human T lymphocytes of a voltage-gated potassium (K) channel that resembles delayed rectifier K channels of nerve and muscle. There are ~200–300 channels in each T lymphocyte (15, 18). The K channels open when the membrane is depolarized, but do not appear to be sensitive to levels of internal calcium (15, 18) known to activate the calcium-activated "maxi" K channel (19). Furthermore, calcium channels have not been detected (15, 17, 18); thus, the voltage-gated K channel appears to be the main channel in human T lymphocytes.

Phytohemagglutinin (PHA)[1] is a potent mitogen of human T lymphocytes (20). In voltage-clamped human T lymphocytes, PHA at mitogenic concentrations causes K channels to open upon depolarization more rapidly and at more negative potentials (15). [^{3}H]Thymidine ([^{3}H]TdR) incorporation by T lymphocytes after PHA stimulation is inhibited by the classic K channel blockers, tetraethylammonium (TEA) and 4-aminopyridine (4-AP), and by quinine, a blocker of calcium-activated K channels in other tissues (19), at doses that block K currents in voltage-clamped lymphocytes, suggesting that voltage-gated K channels may play a role in T lymphocyte activation (14, 15).

In this paper, we describe in-depth analysis of the effects of K channel blockers on T lymphocyte activation. In particular, the effects of K channel blockers on [^{3}H]TdR incorporation, protein synthesis, interleukin 2 (IL-2) production, and the expression of the IL-2 receptor (Tac antigen), are reported. These experi-

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[1] *Abbreviations used in this paper:* allo-MLR, allogeneic mixed lymphocyte response; 4-AP, 4-aminopyridine; FH, Ficoll-Hypaque; HBSS, Hanks' balanced salt solution; [^{3}H]TdR, tritiated thymidine; IL-2, interleukin 2; MNC, mononuclear cells; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; SRBC, sheep erythrocytes; TEA, tetraethylammonium.

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ments further characterize the role of the K channel in events leading to PHA- or alloantigen-induced activation of human T lymphocytes.

Materials and Methods

Isolation of T Lymphocytes. Heparinized (20 U/ml) peripheral venous blood was obtained from healthy volunteers. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque (FH) density gradient, washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) supplemented with 25 mM Hepes and resuspended in HBSS at 4×10^6 cells/ml. T lymphocytes were purified by rosetting MNC with 2-aminoethyl isothiouronium bromide hydrobromide–treated sheep erythrocytes (SRBC) and separating rosetted T lymphocytes from nonrosetted non-T cells on FH gradient. SRBC attached to T lymphocytes were lysed by 17 mM Tris buffer, pH 7.2, containing 134 mM ammonium chloride. T lymphocytes were washed three times with HBSS and resuspended in RPMI 1640 containing 25 mM Hepes, 10% pooled, heat-inactivated human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), hereafter referred to as medium. The cells were >95% viable determined by trypan blue dye exclusion and contained >90% T lymphocytes as assessed by Leu-1 (Pan-T; Becton, Dickinson & Co., Mountainside, CA), monoclonal antibody binding, and fluorescence-activated cell sorter analysis (B-D FACS Systems, Sunnyvale, CA).

K Channel Blockers. 4-AP (Sigma Chemical Co., St. Louis, MO) and TEA (Eastman Kodak Co., Rochester, NY) were recrystallized before use in experiments. Quinine was obtained from Sigma Chemical Co. Verapamil and diltiazem were gifts of Dr. Richard Tsien, Yale University, New Haven, CT.

Voltage Clamp Experiments. K currents were studied in the whole cell conformation using the gigaohm seal recording technique (16), under conditions described elsewhere (15). Blocking of K currents in voltage-clamped T lymphocytes by verapamil and diltiazem added to the bathing solution was estimated from the reduction of the peak current elicited by a large depolarizing voltage step, 30–80 ms in duration, repeated every 30 s, from a holding potential of −80 mV. This pulsing procedure results in each K channel being open ~0.1% of the time, an estimate of the average expected in intact T lymphocytes.

Effect of K Channel Blockers on PHA-stimulated [^{3}H]TdR Incorporation. 10^5 T cells in medium were plated in 96-well, round-bottomed microtiter plates (Nunc, Denmark) and cultured at 37°C in humidified 5% CO_2 in the presence of PHA-P (Difco Laboratories, Detroit, MI), 8 μg/ml, with or without K channel blockers for 57 h. [^{3}H]TdR (sp act, 4 Ci/mM; ICN Pharmaceuticals, Irvine, CA) was added (1 μCi/well) for the final 9 h of culture. The cells were harvested onto a glass filter strip by automated multiple sample harvester (Flow Laboratories, Inc., McLean, VA). [^{3}H]TdR incorporation was determined by a liquid scintillation counter and normalized to that in PHA-treated cells in the absence of blocker. All experiments were done in triplicate. Background counts were <200 cpm.

Effect of K Channel Blockers on Allogeneic Mixed Lymphocyte Response. MNC (1×10^6/ml) irradiated with a cesium source (2,500 rad) were used as stimulator cells. Responder MNC (1×10^5 cells/well) were mixed with an equal number of allogeneic stimulator cells and incubated with or without K channel blockers for 5 d at 37°C in 5% CO_2 atmosphere. [^{3}H]TdR was added (1 μCi/well) for the last 8 h of culture. Viability was assessed by trypan blue dye exclusion on day 5.

Effect of 4-AP on CCRF-HSB2. CCRF-HSB2 is a human T lymphoblastoid cell line derived from an acute lymphoblastic leukemia (21). Using recording conditions described previously (15) we examined the identity of the ion channels in four cells of this cell line. In addition we examined the effect of 8.6 mM 4-AP on the spontaneous [^{3}H]TdR incorporation of this continuously dividing cell line. Briefly, 1×10^5 cells in medium were distributed in 96-well microtiter plates and incubated with or without 4-AP for 16 h at 37°C in 5% CO_2 atmosphere. The cultures were pulsed with [^{3}H]TdR (1 μCi/well) at the onset of culture.

[^{3}H]Leucine Incorporation. 10^5 T lymphocytes per well were cultured with PHA, 8

μg/ml, with or without quinine, 4-AP, or TEA, as described above, for 44 h. Resting cells were cultured in medium for this period. [^{3}H]Leucine (3 μCi/well; sp act, 50 μCi/mM; ICN Pharmaceuticals) was added to the cells for the final 4 h of culture as previously described (22). Cells were harvested onto a glass fiber filter strip and washed with 5% trichloroacetic acid and 95% ethanol. The [^{3}H]leucine incorporation into the acid-insoluble fractions were determined by liquid scintillation counting and normalized as described earlier. All experiments were performed in triplicate.

Tac Expression on Activated T Lymphocytes. The monoclonal antibody against the IL-2 receptor, Tac, was a gift of Dr. Thomas A. Waldmann, National Institutes of Health. The development and characterization of this monoclonal antibody has been described (23). T lymphocytes were cultured in 15 × 75-mm test tubes (2×10^6 cells per tube) with PHA at a final concentration of 100 μg/ml, with or without 4.6 mM 4-AP, 20 mM TEA, or 200 μM quinine for 40 h. These concentrations of blockers have been shown to completely inhibit [^{3}H]TdR incorporation in human T lymphocytes (15). Resting T lymphocytes were suspended in medium for the same duration. The cells were then washed with phosphate-buffered saline (PBS), pH 7.2, incubated on ice with an appropriate dilution of Tac monoclonal antibody for 30 min, washed three times with PBS, incubated on ice with fluoresceinated goat anti-mouse IgG Fc $F(ab)_2$ fragment (Cappel Laboratories, Cochraneville, PA) for 30 min, washed three times with PBS, and resuspended in 0.5 ml PBS. The proportion of positive cells was determined by a fluorescence-activated cell sorter analyzer (B-D FACS Systems, Sunnyvale, CA).

Since we used high concentrations of PHA (final concentration, 100 μg/ml) for our studies of the expression of Tac antigen and IL-2 production (see below) we examined the effects of concentrations of PHA from 8 to 166 μg/ml on 4-AP-induced inhibition of [^{3}H]TdR incorporation.

IL-2 Production. MNC were cultured (2×10^6 cells per tube) in the presence of PHA (final concentration, 100 μg/ml) with or without 7.8 mM 4-AP for 48 h. Resting MNC were suspended in medium alone for the same duration. After centrifugation, the IL-2-containing supernatants were collected, filtered, and stored at −20°C until used.

IL-2 Microassay. Supernatants were tested for IL-2 activity by their ability to sustain proliferation of murine cytotoxic T lymphocytes harvested from long-term IL-2-dependent CTLL cultures (24). The CTLL2 cell line was a gift from Dr. Steven Gillis, Immunex Corporation, Seattle, WA. 5×10^3 CTLL cells were cultured for 20 h in 200 μl of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (10%), 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine with or without a 1:5 dilution of the putative IL-2-containing supernatants or varying dilutions of a purified human IL-2 standard (gift of Dr. Seven Gillis). The characterization of this IL-2 preparation has been described (25). The final concentration of 4-AP in the supernatants diluted 1:5 was 1.56 mM. As a control, 1.56 mM 4-AP was added along with purified IL-2 (final concentration, 750 U/ml) to CTLL cells to determine whether this concentration of 4-AP would nonspecifically inhibit [^{3}H]TdR incorporation by CTLL cells. [^{3}H]TdR (1 μCi/well) was added for the final 4 h of incubation and the [^{3}H]TdR incorporation determined. The IL-2 activity was expressed as a percentage of a purified IL-2 standard equivalent to 750 U/ml.

Effect of Exogenous IL-2 on 4-AP Inhibition of Mitogenesis. 10^5 T lymphocytes per well were cultured with PHA (8 μg/ml) with or without 4-AP or purified IL-2 (final concentration, 330 U/ml) for 64 h. [^{3}H]TdR was added (1 μCi/well) for the final 16 h. [^{3}H]TdR incorporation was normalized to that in PHA-treated cells in the absence of 4-AP.

Two-dimensional Gel Electrophoresis. T lymphocytes (2×10^6/ml) in medium were cultured for 2, 6, 12, and 24 h in sterile 12 × 75-mm tubes in the presence of 10 μg/ml PHA with or without 8.6 mM 4-AP. Control T lymphocytes were suspended in medium alone for the same duration. Cells were then centrifuged, suspended in 200 μl methionine-free medium, labeled with [^{35}S]methionine (sp act, 1,220 Ci/mM; 22.5 μCi/culture) for 2 h, washed with HBSS, and the cell pellets stored at −70°C until used. The incorporation of [^{35}S]methionine into protein in lymphocytes has been shown to be linear for at least 2 h (26). Two-dimensional gel electrophoresis was performed by the O'Farrell technique

with modifications (27). A mixture of molecular weight markers (Sigma Chemical Co.), carbonic anhydrase (29×10^3 mol wt), egg albumin (45×10^3), bovine albumin (66×10^3), phosphorylase *b* (97×10^3), and β-galactosidase (116×10^3), was used for molecular weight calibration. Calibration of the first dimension isoelectric focusing gel was performed using a combination of a flat membrane electrode (MI-104; Microelectrodes, Inc, Londonderry, NH) and a micro-reference electrode (MI-401; Microelectrodes, Inc). Autoradiographs were developed for 60 h.

Results

Effect of Verapamil and Diltiazem on PHA-stimulated Mitogenesis

4-AP, TEA, and quinine inhibit PHA-induced mitogenesis in a dose-dependent manner with the same potency sequence as for channel block (14, 15, and Table I). We have extended these studies on pharmacological agents that both block T lymphocyte K currents and inhibit mitogenesis. We recently reported (14) the block of K currents in human T lymphocytes by several organic calcium channel antagonists (14), which have been reported to block channels other than calcium channels in other cells (28). Block of K currents in human T lymphocytes by these drugs was complex. K channel block was relieved by hyperpolarization (voltage dependence) and was enhanced by repeated depolarizing pulses (use dependence) (14). Verapamil and diltiazem, two organic calcium channel blockers (28, 29), inhibited PHA-induced mitogenesis (Fig. 1 and Table I) at concentrations similar to those required to block the T lymphocyte K channel. The dotted curves in Fig. 1 show dose-response curves for K current block by the two drugs, estimated as described in Materials and Methods, assuming that one drug molecule binds to each K channel.

Effect of K Channel Blockers on the Allogeneic Mixed Lymphocyte Response

In the allogeneic mixed lymphocyte response (allo-MLR), T lymphocytes are stimulated to proliferate by the major histocompatibility antigens on allogeneic stimulator cells (30). We extended our studies on T lymphocyte activation by examining the effect of K channel blockers on the allo-MLR. Quinine, 4-AP, and TEA inhibited the allo-MLR in a dose-dependent manner (Fig. 2). The allo-MLR was more sensitive to the K channel blockers than PHA-induced mitogenesis (15). If allogeneic stimulator cells do not enhance the probability of K channel opening in responder T lymphocytes to the same extent as does PHA (15), a lower concentration of K channel blocker might be required to inhibit K currents. These experiments show that functional K channels are required for the proliferation of allogeneic cell–stimulated T lymphocytes, as was found for PHA-induced mitogenesis (15).

Time Course of 4-AP Block of PHA-stimulated [^{3}H]Thymidine Incorporation

Mitogens must remain bound to the T lymphocyte membrane for at least 20 h to induce mitogenesis (31). The time course of K channel block of mitogenesis was examined, revealing a similar temporal requirement for functional K channels early during mitogenesis. 4-AP (4.6 mM) was added to PHA-treated cells at various times after the PHA addition and was left in culture until the end of the experiment when [^{3}H]TdR incorporation was measured. Consistent with our previous results (15), 4.6 mM 4-AP added concomitantly with PHA completely

TABLE I

Potency of K Channel Blockers on K Channel Currents and [^{3}H]Thymidine and [^{3}H]Leucine Incorporation

50% block of:	Verapamil	Quinine	Diltiazem	4-AP	TEA
K currents	6	14	60	190	8,000
PHA-induced mitogenesis	24	33	114	2,100	13,000
PHA-induced protein synthesis	ND	76	ND	1,500	29,000

Values are micromolar concentrations required to produce 50% block. ND, not done. The values for block of K currents and inhibition of mitogenesis by TEA, 4-AP, and quinine were taken from reference 16. Block of K currents by verapamil and diltiazem in voltage-clamped T lymphocytes was determined as described in Materials and Methods. Diltiazem was studied in one cell, verapamil in four cells. PHA-induced mitogenesis and protein synthesis were assayed as described in Materials and Methods. Mitogenesis results are the mean of three to four experiments, each done in triplicate on T lymphocytes from a different donor. Protein synthesis data are the mean of eight experiments each performed in triplicate on T lymphocytes from different individuals.

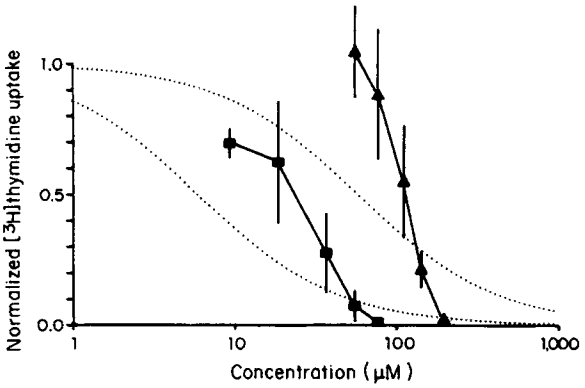


FIGURE 1. Effect of verapamil and diltiazem on PHA-stimulated T lymphocyte mitogenesis. Verapamil (■) and diltiazem (▲) were added at time zero to T lymphocytes (1×10^5 cells/well) cultured in the presence of 8 μg/ml PHA. The data points indicate the mean ± SD of five experiments, each experiment done in triplicate and with a different donor. The data are normalized to the [^{3}H]TdR incorporation in T lymphocytes treated with PHA alone. The dotted curves are dose-response curves for K current inhibition, assuming one drug molecule–one channel stoichiometry, with one-half inhibition at 6 μM for verapamil and 60 μM for diltiazem.

inhibited mitogenesis (Fig. 3). Addition of 4-AP at later times revealed an escape from 4-AP inhibition that commenced at ~24 h and was complete by 48 h. 4-AP added at the same time as [^{3}H]TdR did not inhibit PHA-induced [^{3}H]TdR incorporation.

Effect of 4-AP on the [^{3}H]TdR Incorporation of the HSB2 Cell Line

In four voltage-clamped cells of a human T lymphoblastoid cell line, CCRF-HSB2 (obtained from Dr. G. Granger, University of California, Irvine), we did not detect K channels using the recording conditions described in our earlier report (15), nor was [^{3}H]TdR incorporation by these continuously dividing cells significantly altered by 8.6 mM 4-AP (n = 3, mean ± SD, 8,367 ± 2,506 cpm without 4-AP, 5,731 ± 1,729 cpm with 4-AP), a concentration that completely abolishes PHA-induced [^{3}H]TdR incorporation in human T lymphocytes (15).

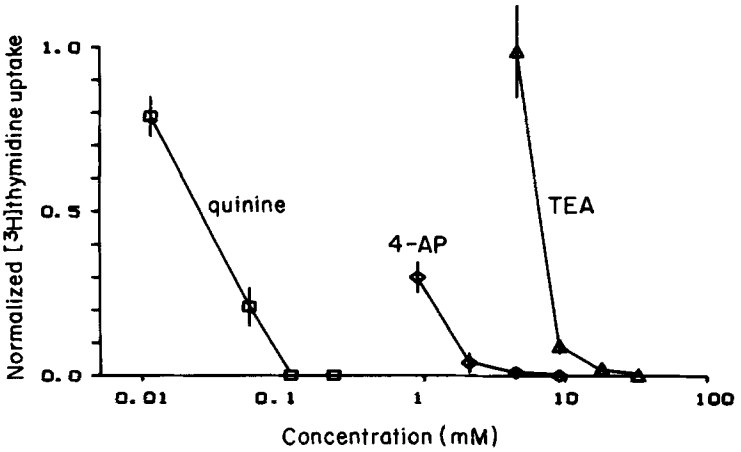


FIGURE 2. Effect of K channel blockers on allogeneic MLR. 4-AP, TEA, or quinine were added at time zero to cultures consisting of 1×10^5 irradiated stimulator MNC and 1×10^5 allogeneic responder MNC and incubated for 5 d. Each data point is the mean ± SEM of six experiments, each done in triplicate on a different stimulator-responder combination. Responder MNC were left in either 4.5 mM 4-AP, 18.1 mM TEA, or 113 μM quinine for the same length of time and viability was assessed by trypan blue dye exclusion. The number of viable cells per well was determined in three experiments, each experiment being done in triplicate on a different donor. 4-AP and TEA did not decrease the viability significantly, whereas quinine did (MNC in medium alone, $1.01 \times 10^5 \pm 0.31 \times 10^5$ cells/well; MNC with 4-AP, $0.80 \times 10^5 \pm 0.24 \times 10^5$ cells/well; MNC with TEA, $0.81 \times 10^5 \pm 0.18 \times 10^5$ cells/well; MNC with quinine, $0.54 \times 10^5 \pm 0.24 \times 10^5$ cells/well; mean ± SD).

Effects of K Channel Blockers on Protein Synthesis in Human T Lymphocytes

Protein synthesis appears to be necessary for the commitment of T lymphocytes to DNA synthesis (32–33). Furthermore, proteins such as IL-2 and the IL-2 receptor (Tac) play a major role in T lymphocyte mitogenesis (23, 34). We therefore studied the effects of K channel blockers on various aspects of protein synthesis to determine whether these substances, at concentrations that inhibit [^{3}H]TdR incorporation, also reduce protein synthesis. Total protein synthesis was measured by [^{3}H]leucine incorporation into the acid-insoluble fractions in the cell. Two-dimensional gel electrophoresis was used to study the synthesis of individual proteins in the presence of the K channel blocker, 4-AP. The effect of K channel blockers on Tac antigen expression and IL-2 production was also examined.

K channel blockers inhibit [^{3}H]leucine incorporation. Fig. 4 shows that quinine, 4-AP, and TEA inhibited the PHA-inducible increase in [^{3}H]leucine incorporation, measured at 44 h, with the same potency sequence (Table I) as for the block of K channel currents and inhibition of mitogenesis. PHA-induced mitogenesis was more sensitive to inhibition by TEA and quinine than was PHA-stimulated protein synthesis (Table I). For example, 33 μM quinine produced roughly 50% inhibition of [^{3}H]TdR incorporation, whereas 76 μM quinine was required to inhibit [^{3}H]leucine incorporation to the same extent.

Two-dimensional gel electrophoresis. PHA caused a marked elevation in the rate of synthesis of most proteins within 6 h compared with unstimulated T lymphocytes (Fig. 5 *B* and *A*, respectively). 4-AP reduced PHA-induced protein

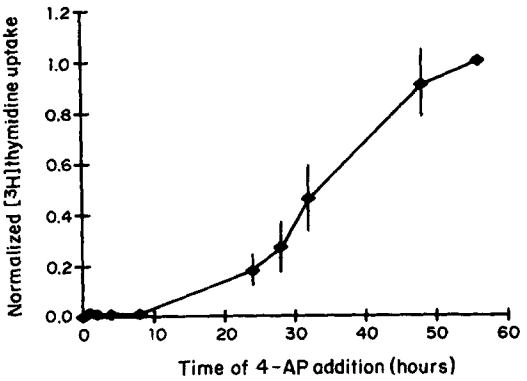


FIGURE 3. Temporal sensitivity to 4-AP inhibition of mitogenesis. Each point is the mean ± SD of three to four experiments, each done in triplicate on T lymphocytes from a different donor. PHA was added at time zero, and 4-AP was added at the times shown on the abscissa. The data are normalized to the [^{3}H]TdR incorporation in PHA-activated T lymphocytes in the absence of 4-AP.

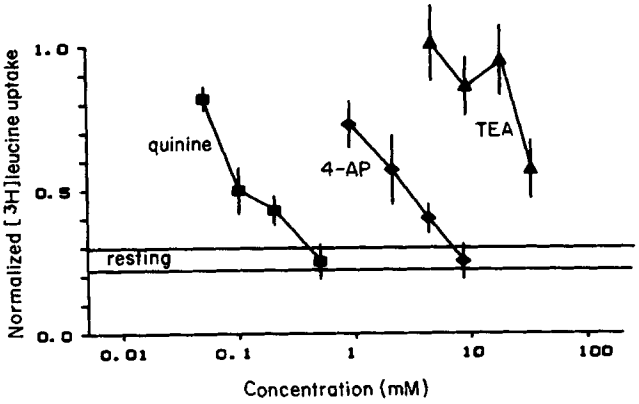
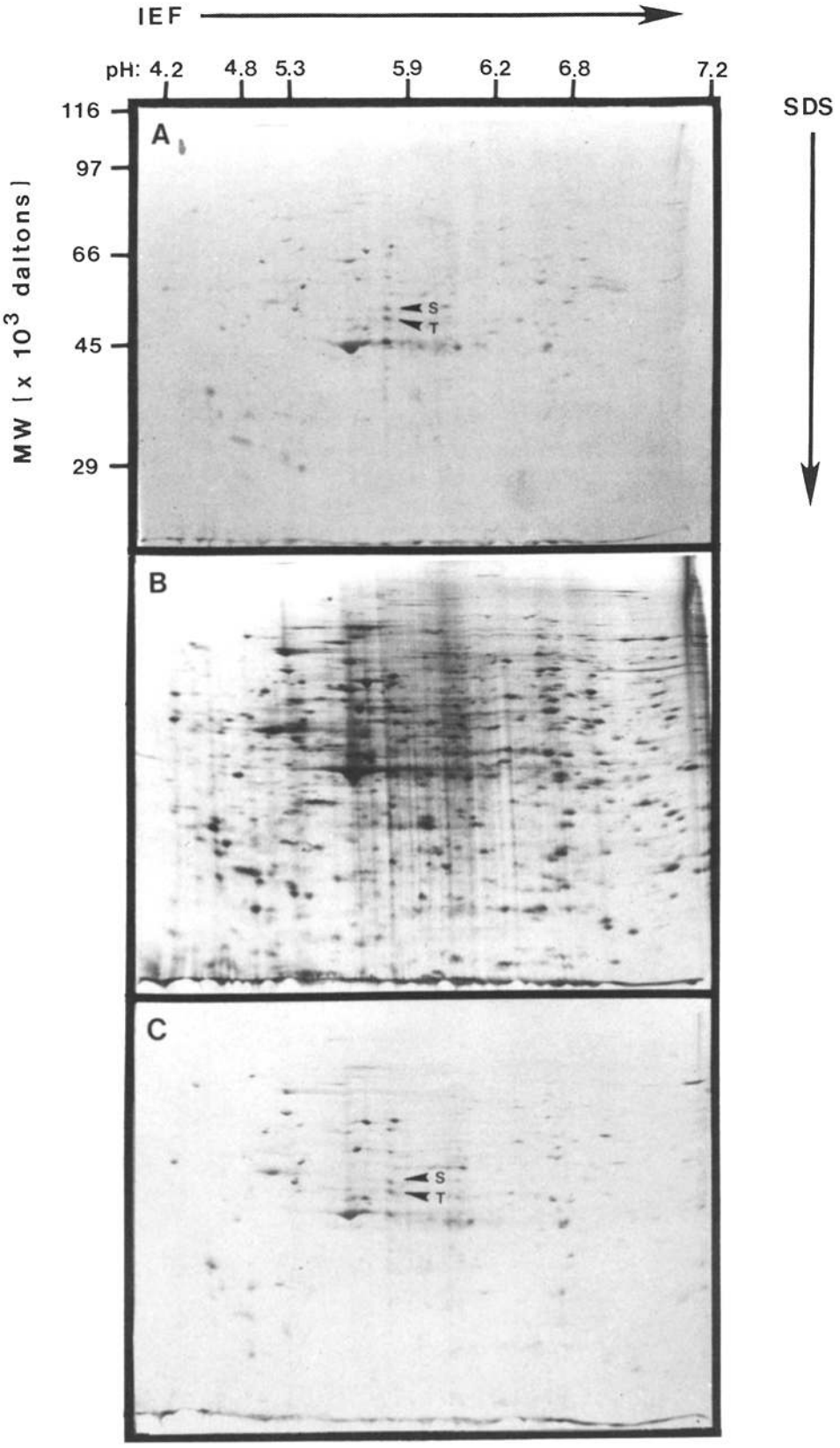


FIGURE 4. Inhibition of [^{3}H]leucine incorporation by quinine (■), 4-AP (♦), and TEA (▲), all normalized to the value obtained in PHA-activated T lymphocytes in the absence of blockers. [^{3}H]Leucine incorporation into the acid-insoluble fraction was measured 44 h after PHA addition. Each point shows the mean ± SEM of eight experiments, each done in triplicate on T lymphocytes from a different donor. The two horizontal lines show the range (±1 SEM) of [^{3}H]leucine incorporation by resting lymphocytes from the same eight experiments.

synthesis to the level in unstimulated T lymphocytes, in gels at 6, 12, and 24 h after the addition of PHA, although some proteins appeared to be synthesized at an enhanced rate compared with resting cells (visual assessment, Fig. 5*C*). These data corroborate the [^{3}H]leucine data (Fig. 4) and, in addition, show that protein synthesis is not inhibited in a nonselective manner by 4-AP at concentrations that suppress [^{3}H]TdR incorporation.

Of interest are two proteins, S and T, visible as two dense spots in gels of unstimulated T lymphocytes. These proteins appear to be reduced in gels of T lymphocytes examined 24 h after PHA activation (visual assessment), in contrast to the increase in intensity of nearly all the remaining visible proteins in the gel (Fig. 5). Preliminary results indicate that this apparent reduction occurs at least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4.8
5.3
5.9
6.2
6.8
7.2
SDS
MW [x 10^3 daltons]
116
97
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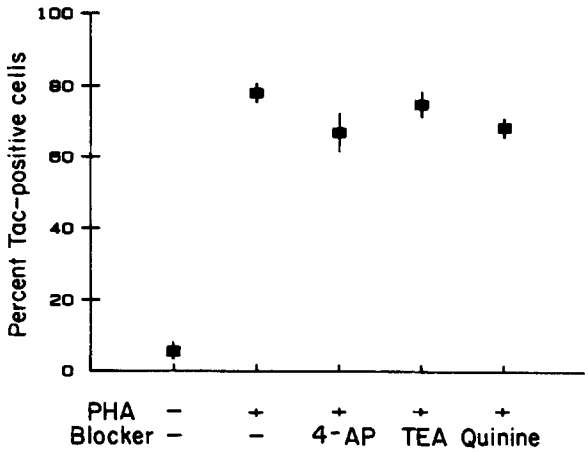

FIGURE 6. Effect of K channel blockers on the fraction of T lymphocytes expressing Tac antigen 48 h after PHA addition. Each point is the mean ± SEM of four experiments. The first data point starting from the left shows the low level in unstimulated T lymphocytes. The other points show Tac expression in T lymphocytes stimulated with PHA in the absence or presence of three different K channel blockers.

as early as 6 h after addition of PHA. In the presence of 4-AP and PHA, proteins S and T were present at approximately the same density as in resting T lymphocytes. Protein S has a molecular weight of ~54 × 10^3 and a pI of ~5.8. Protein T has the same pI and a molecular weight of ~52 × 10^3.

K channel blockers do not affect the expression of the IL-2 receptor (Tac antigen). Fewer than 5% of unstimulated T lymphocytes expressed Tac antigen. The majority of PHA-activated T lymphocytes expressed the Tac antigen on their surface (Fig. 6). The expression of Tac was not altered by K channel blockers at concentrations that inhibit mitogenesis (Fig. 6). The lack of inhibition by the blockers is not due to the high concentration of PHA (100 μg/ml) used for these experiments, since lower PHA concentrations had the same effect (data not shown) and increasing the PHA concentration from 8 to 166 μg/ml did not override the 4-AP block of mitogenesis (Table II). Furthermore, IL-2 production (see below) induced by 100 μg/ml PHA was reduced by 4-AP.

4-AP reduces IL-2 production. PHA-activated MNC synthesized substantially more IL-2 than unstimulated MNC (Fig. 7). Supernatants from MNC treated simultaneously with 4-AP (7.8 mM) and PHA had significantly less IL-2 activity than supernatants from PHA-stimulated MNC. This result could be due to reduced IL-2 production by 4-AP-treated MNC or to a direct inhibitory effect of residual 4-AP in the supernatants on the CTLL cell line. We addressed the latter possibility by adding the same final concentration of 4-AP along with pure IL-2 to CTLL cells and found that this concentration of blocker did not nonspecifically inhibit [^{3}H]TdR incorporation by CTLL cells (Fig. 7).

FIGURE 5. Two-dimensional gels. (*A*) Resting T lymphocytes. (*B*) T lymphocytes 24 h after addition of PHA. (*C*) T lymphocytes 24 h after addition of PHA together with 4-AP. Isoelectric focusing (IEF) is shown from right to left, and molecular weight separation from top to bottom. Arrows show the position of proteins S and T. 2 × 10^6 T cells were used in each experiment.

TABLE II

Effect of PHA on 4-AP Inhibition of Mitogenesis

PHA concentration	Normalized [^{3}H]TdR incorporation			
	4-AP concentration (mM)			
	0.9	2.1	4.3	8.6
μg/ml				
8	0.8	0.05	0.003	0.003
16	0.82	0.05	0.02	0.02
33	0.5	0.05	0.006	0.002
166	0.3	0.03	0.008	0.009

Each value is the mean (cpm) of triplicate cultures normalized to the [^{3}H]TdR incorporation by T lymphocytes treated with the corresponding concentration of PHA in the absence of blocker.

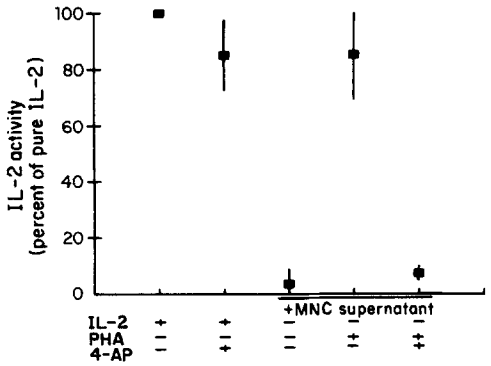

FIGURE 7. Effect of 4-AP on IL-2 production. IL-2 activity was measured using the IL-2-dependent CTLL2 cell line. The vertical axis represents the IL-2 activity in the supernatants expressed as a percentage of purified IL-2 (final concentration 750 U/ml) which was arbitrarily assigned a value of 100% (first data point starting from the left). The direct effect, if any, of 4-AP on [^{3}H]TdR incorporation by the CTLL2 cell line was examined by adding a mixture of purified IL-2 (final concentration, 750 U/ml) and 4-AP (final concentration, 1.56 mM) and is displayed in the graph as the second data point from the left. The IL-2 activity in the supernatants are shown as data points 3, 4, and 5. Each data point is the mean ± SEM of two or three experiments, each performed in triplicate on a different individual.

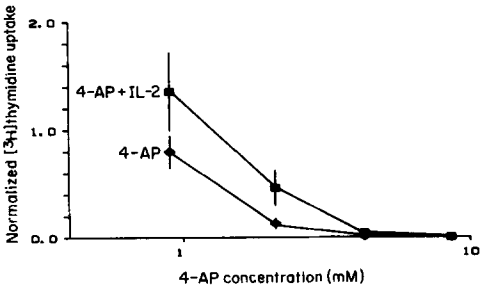

FIGURE 8. Effect of IL-2 on 4-AP inhibition of [^{3}H]TdR incorporation. Each point is the mean ± SEM of four experiments done in triplicate on T lymphocytes from different donors. The diamonds show the inhibitory effect of 4-AP alone, comparable to that found earlier (15). The squares show the increase in [^{3}H]TdR incorporation in experiments in which IL-2 (final concentration, 330 U/ml) was added together with 4-AP and PHA. All data are normalized to the [^{3}H]TdR incorporation measured in PHA-activated T lymphocytes in the absence of 4-AP and IL-2.

Effect of IL-2 on 4-AP block of [^{3}H]TdR incorporation. Since IL-2 production was reduced by 4-AP, we added purified IL-2 to T lymphocytes treated with 4-AP and PHA to determine whether restoring IL-2 concentration would relieve the inhibition of mitogenesis by 4-AP (Fig. 8). IL-2 partially alleviated the inhibitory effect on mitogenesis of low concentrations of 4-AP, but not of higher 4-AP concentrations that completely inhibit mitogenesis.

Discussion

Alteration of the gating characteristics of the human T lymphocyte voltage-gated K channel, by mitogenic concentrations of PHA (15), and the reversible inhibition of PHA- and allogeneic MNC-stimulated [^{3}H]TdR incorporation by K channel blockers (14, 15, and present study), suggest a requirement for functional voltage-gated K channels in human T lymphocyte activation. Of the five compounds studied thus far in detail, verapamil is the most potent inhibitor of both mitogenesis and T lymphocyte voltage-gated K currents (Table I). The block of mitogenesis exhibits a steeper dependence on blocker concentration than does the channel block itself (Fig. 1, reference 15), indicative of a nonlinear relationship between the number of conducting channels and the biochemical events culminating in cell division. The data in Table I suggest that mitogenesis is inhibited when a substantial fraction (perhaps 50%) of the K channels are blocked (14, 15).

In intact T lymphocytes, the potencies of the blockers may be somewhat different than those measured in voltage-clamped cells, since the degree of K channel block by 4-AP (34, 35), verapamil, and diltiazem (14) depends upon membrane potential and also on the frequency of channel opening. If each K channel were open on the average less than about 0.1% of the time, then, based upon their dependence on the frequency of channel opening, the half-blocking concentrations of verapamil and diltiazem would be underestimated. On the other hand, if, as is likely (1, 2, 4), the resting potential of intact lymphocytes is more positive than −80 mV, the holding potential used when measuring block, then the half-blocking concentrations listed might be too low for 4-AP and too high for diltiazem and verapamil, based upon their voltage dependence. Although these considerations preclude making precise quantitative comparisons between the agents, the parallel potency sequence for channel block and the inhibition of mitogenesis, indicates that voltage-gated K channels are necessary for mitogen-stimulated human T cell activation.

Several lines of evidence indicate that inhibition of mitogenesis by K channel blockers is not due to nonspecific toxicity. T lymphocytes treated with K channel blockers are viable as assessed by trypan blue dye exclusion (13, 15, and Fig. 2 legend), by the ability to respond normally to PHA after wash-out of blocker (13, 15), by the ability to continue synthesizing proteins at the same rate as unstimulated T lymphocytes (Fig. 4), and by the 'normal' expression of the Tac antigen (Fig. 6). Tetramethylammonium (TMA), an analog of TEA, neither blocks the channel nor inhibits mitogenesis (15). 4-AP added at the same time as [^{3}H]TdR did not inhibit PHA-stimulated mitogenesis, indicating that 4-AP does not nonspecifically inhibit [^{3}H]TdR uptake and incorporation (Fig. 3). The human T lymphoblastoid cell line CCRF-HSB2 does not appear to express K

channels (see Results). [^{3}H]TdR incorporation by these continuously dividing cells was not altered by 8.6 mM 4-AP, a concentration that completely inhibits mitogenesis in human T lymphocytes. These data argue against a direct toxic effect of K channel blockers on T lymphocytes.

In the present report we have demonstrated that 4-AP completely inhibits mitogenesis if added at any time during the first 20–30 h after PHA stimulation, but not if added later. Verapamil, a calcium channel blocker (28, 29) that also blocks the T lymphocyte K channel (14), inhibits mitogenesis with a similar temporal dependence (13). Mitogens must remain in contact with T lymphocytes during the same period to induce proliferation (31). Taken together, the data suggest that continued activation of K channels for 20–30 h by mitogen may enable a sequence of events which culminate in [^{3}H]TdR incorporation.

Proteins play an important part in T lymphocyte activation (23, 32–33, 36). Potassium channel blockers reduce PHA-induced protein synthesis in human T lymphocytes to the level in unstimulated T lymphocyte with a potency sequence parallel to that for block of K currents and inhibition of mitogenesis. [^{3}H]TdR incorporation was completely abolished by concentrations of K channel blockers that only partially inhibited [^{3}H]leucine incorporation. One interpretation of these data is that protein synthesis at the transcriptional or translational levels or at both is dependent on functional K channels. Alternatively, events triggered by PHA leading to enhanced protein synthesis may require functional K channels. However, it is also conceivable that the K channel blockers might have a direct effect on protein synthesis that is independent of, but with a dose dependence and pharmacological sensitivity similar to, their K channel blocking action. The last possibility seems unlikely since preliminary studies indicate that 4-AP, at concentrations that inhibit protein synthesis in T cells, does not suppress [^{35}S]-methionine incorporation in a cell-free translation system (unpublished data). In summary, the data are consistent with the interpretation that the inhibition of mitogenesis by K channel blockers is due to inhibition either of protein synthesis or of an event leading up to it.

At 40 h, the PHA-induced expression of Tac antigen on activated T lymphocytes was not altered by K channel blockers at concentrations that completely inhibit [^{3}H]leucine incorporation, suggesting that the expression of this protein during T lymphocyte activation may be a posttranslational event. This interpretation is consistent with the proposal that Tac expression involves exposure of preformed cryptic molecules by membrane rearrangement (37). Cyclosporin A also suppresses mitogen-induced [^{3}H]TdR incorporation without altering Tac expression (38) and may act at a similar stage as K channel blockers or may directly block the K channel. It may be possible to stage T lymphocytes in a quiescent state with Tac expressed by using K channel blockers or cyclosporin A. This would help in examining the intracellular events after the binding of IL-2 to its receptor sites on activated T cells.

IL-2, a protein synthesized by a subpopulation of T lymphocytes, plays a major role in T lymphocyte mitogenesis (36). We assessed the effect of 4-AP on IL-2 production to determine whether functioning K channels are necessary for this process. IL-2 production was reduced by 4-AP to resting levels (Fig. 7), suggesting a dependence on functional K channels. This diminished production may be

related to an effect of 4-AP on T lymphocyte or macrophage K channels. Interleukin 1, a product of monocytes/macrophages, is necessary for IL-2 production (39). A K channel in mouse peritoneal macrophages resembling the K channel in human T lymphocytes has recently been described (40). It is conceivable that inhibition of this K channel in monocytes/macrophages might decrease interleukin 1 synthesis, contributing to the reduced IL-2 production by T lymphocytes.

The suppression of IL-2 production may contribute to the inhibition of mitogenesis by K channel blockers. Purified IL-2 was added to T lymphocyte cultures treated with PHA and 4-AP to determine whether the inhibitory effect of 4-AP could be overcome by reconstituting the IL-2 activity in the culture. Exogenous IL-2 partially relieved 4-AP inhibition of mitogenesis, at low, but not at high concentrations of 4-AP, demonstrating that reconstitution of IL-2 activity in the culture is not in itself sufficient to restore T lymphocyte proliferation. Other factors such as the early increase in intracellular free calcium, the expression of the transferrin receptor, and the events following binding of IL-2 to the IL-2 receptor, are also necessary for T lymphocyte activation (4, 8–10, 41–46) and might be dependent on functional K channels. Studies are in progress to determine whether these events can be inhibited by K channel blockers.

Two-dimensional gel electrophoresis provided a visual corroboration of our quantitative [^{3}H]leucine data. Resting T lymphocytes had a low rate of protein synthesis. Consistent with the report by Lester et al. (47), an increase in the synthesis of several proteins was evident 6 h after activation with PHA. 4-AP added together with PHA reduced PHA-stimulated synthesis of the majority of proteins to the level in unstimulated T lymphocytes, although some proteins continued to be synthesized at an increased rate. This result demonstrates that 4-AP, at the concentrations used to inhibit [^{3}H]TdR incorporation, does not nonselectively suppress protein synthesis. Of interest are two proteins, S and T, with the same pI (~5.8) but different molecular weights (approximately 54×10^3 and 52×10^3, respectively), suggesting a precursor-product relationship. Proteins S and T are visible as two dense spots in gels of resting T lymphocytes but appear to be less prominent or absent in gels of T lymphocytes 6 h after PHA stimulation, in contrast to the increase in intensity of all the other proteins. This result could reflect reduced synthesis of the two proteins. Alternatively, the two proteins could be modified and occupy different positions on the gel. 4-AP, at concentrations that inhibit protein synthesis, appears to prevent the reduction or disappearance of these proteins. The S and T proteins may be involved in maintaining T lymphocytes in a resting state. Cytoplasmic- and ribosomal-associated factors that inhibit cell-free protein translation are present at a significantly higher specific activity in unstimulated T cells than in T lymphocytes treated with PHA (48–50). These regulatory factors, which have not been characterized, have been suggested to maintain lymphocytes in a quiescent state. The relationship, if any, between these factors and proteins S and T remains to be determined.

Earlier reports demonstrated a rapid, mitogen-induced, dose-dependent increase in cell-associated calcium in lymphocytes (8–10, 42, 43), which was suggested to constitute a mitogenic signal (9, 43). Stimulation of T lymphocyte

[^{3}H]TdR incorporation by the calcium ionophores A23187 and ionomycin (44, 45), the dependence of lectin-induced mitogenesis on extracellular calcium (7, 9, 10), and the recent demonstration of a mitogen-induced increase in intracellular free calcium in mouse thymocytes (4), support this hypothesis. Proponents of the calcium hypothesis have postulated the existence of calcium (46) and calcium-activated potassium channels (2, 4) to account for the mitogen-induced rise in intracellular calcium and the membrane hyperpolarization in T lymphocytes. Calcium channels (15, 17, 18) or calcium-activated K channels (15, 18) have not been detected in human T lymphocytes using a variety of recording conditions. We and others have shown that the K channel blockers 4-AP and TEA, the calcium channel blockers verapamil and diltiazem (28, 29), and quinine, which blocks calcium-activated K channels in other tissues (19), inhibit PHA-stimulated T lymphocyte activation (13–15) and/or mitogen-stimulated membrane hyperpolarization (2). For all five agents, these effects occur at concentrations that block K currents in voltage-clamped human T lymphocytes (14, 15, Fig. 1, Table 1), suggesting that inhibition of T cell activation by these agents is related to their ability to block voltage-gated K channels in T cells.

Voltage-gated K channels in squid axons are reported to be measurably permeable to calcium (51). It is conceivable that the mitogen-stimulated increase in intracellular free calcium in T lymphocytes is due to the passage of calcium through voltage-gated K channels. This notion could account for the parallelism between the elevation of intracellular free calcium for 24 h (10) and the temporal sensitivity of lectin-stimulated mitogenesis to K channel block (Fig. 3). Alternatively, entry of calcium into T lymphocytes could be electrically silent.

In conclusion, functioning voltage-gated K channels appear to be required for T lymphocyte activation. These K channels may be involved in the mitogenic signal, or may play a permissive role enabling a sequence of events culminating in [^{3}H]TdR incorporation. Functional voltage-gated K channels appear to be required during or prior to mitogen-induced protein synthesis, but are not required during late stages immediately preceding [^{3}H]TdR incorporation. The combined use of immunological, biochemical, and electrophysiological techniques offers great potential in understanding the changes that take place when eukaryotic cells are transformed from a resting to the activated and proliferating stage.

Summary

The calcium channel blockers, verapamil and diltiazem, inhibit phytohemagglutinin (PHA)-induced mitogenesis at concentrations that block the T lymphocyte K channel currents. K channel blockers also inhibit the allogeneic mixed lymphocyte response in a dose-dependent manner with the same potency sequence as for block of K currents. K channel blockers inhibit PHA-stimulated mitogenesis only if added during the first 20–30 h after PHA addition, but not later, indicating a requirement for functional K channels during this period. We investigated the effect of K channel blockers on various aspects of protein synthesis for two reasons: first, protein synthesis appears to be necessary for the events leading to DNA synthesis, and second, the increase in the protein synthetic rate commences during the first 24–48 h after PHA addition. PHA-induced

total protein synthesis was reduced to the level in unstimulated T lymphocytes by K channel blockers in a dose-dependent manner with the same potency sequence as for the block of K currents and inhibition of [^{3}H]thymidine incorporation. Two-dimensional gel electrophoresis demonstrated that although the synthesis of the majority of proteins was reduced by K channel blockers to the level in unstimulated T cells, some proteins continued to be synthesized at an enhanced rate compared with resting cells. Two proteins, S and T, detected by two-dimensional gel electrophoresis in unstimulated T lymphocytes, appeared to be reduced in intensity in gels of PHA-treated T lymphocytes, in contrast to the increased synthesis of the remaining proteins. 4-Aminopyridine (4-AP), at concentrations that inhibit protein synthesis, prevented the apparent PHA-induced reduction of proteins S and T. These proteins may play a role in maintaining the T lymphocyte in a resting state and may be related to the translation inhibitory factors reported to be present at a higher specific activity in quiescent T lymphocytes than in PHA-activated T cells. The expression of the IL-2 receptor (Tac) during T lymphocyte activation was not altered by K channel blockers, whereas the production of interleukin 2 (IL-2) was reduced to the level in unstimulated T lymphocytes. Exogenous IL-2 partially relieved the inhibition of mitogenesis by low, but not by high, concentrations of 4-AP. These experiments clarify the role of K channels in T lymphocyte activation and suggest that functional K channels are required either for protein synthesis or for events leading to protein synthesis.

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