0026-895X/95/010098-07$3.00/0

MOLECULAR PHARMACOLOGY, 48:98–104 (1995).

WIN 17317–3: Novel Nonpeptide Antagonist of Voltage-Activated K^+ Channels in Human T Lymphocytes

ROGER J. HILL,[1] ALISON M. GRANT, WALT VOLBERG, LOTTIE RAPP, CONNIE FALTYNEK, DEBORAH MILLER, KAREN PAGANI, EUGENE BAIZMAN, SU WANG, JOSEPH W. GUILES, and DOUGLAS S. KRAFTE

Departments of Biochemistry (R.J.H., A.M.G., E.B.), Vascular and Biochemical Pharmacology (W.V., D.S.K.), Immunology (L.R., C.F., D.M., K.P., S.W.), and Medicinal Chemistry (J.W.G.), Research Division, Sanofi Winthrop, Inc., Collegeville, Pennsylvania 19426

Received January 12, 1995; Accepted April 17, 1995

SUMMARY

We report the *in vitro* biological characterization of WIN 17317–3 (1-benzyl-7-chloro-4-*n*-propylimino-1,4-dihydroquinoline hydrochloride), a novel inhibitor of voltage-activated (n-type) K^+ channels in human T lymphocytes. WIN 17317–3 inhibits ^{125}I-charybdotoxin binding to n-type K^+ channels with an IC_{50} value of 83 ± 4 nM. WIN 17317–3 demonstrates competitive inhibition of ^{125}I-charybdotoxin binding by increasing its dissociation constant without changing the total number of channels bound and by having no effect on its dissociation rate constant. WIN 17317–3 inhibits whole-cell, n-type K^+ currents with characteristics indicative of open channel block and has an IC_{50} value of 335 nM. The compound is 150-fold selective for n-type K^+ channels, compared with Ca^{2+}-activated, charybdotoxin-sensitive K^+ channels in smooth muscle. In purified $CD4^+$ T lymphocytes activated with either anti-CD3 plus phorbol ester or anti-CD3 plus anti-CD28, WIN 17317–3 decreases interleukin-2 production with EC_{50} values of 0.8 μM and 1 μM, respectively. WIN 17317–3 is a novel, potent, and selective nonpeptide n-type K^+ channel antagonist that inhibits interleukin-2 production in human T lymphocytes.

The most prominent and best characterized ion channel in human T lymphocytes is the voltage-activated n-type K^+ channel (1–3). The channel is selective for K^+, activates at −40 mV, shows cumulative inactivation, and is blocked by both peptide and nonpeptide antagonists (1, 3–5). For example, ChTX, a 37-amino acid peptide purified from the venom of the scorpion *Leiurus quinquestriatus* that was originally described as an inhibitor of Ca^{2+}-activated K^+ channels in muscle (6), is a potent antagonist of n-type K^+ channels. ChTX is active at low nanomolar concentrations (4, 5, 7) and, when radiolabeled, binds to human T lymphocytes with characteristics indicative of binding to n-type K^+ channels (8). Nonpeptide antagonists are significantly less potent (IC_{50} values of 20 μM to 10 mM) and include the nonselective K^+ blockers quinine, 4-aminopyridine, and tetraethylammonium and the Ca^{2+} channel antagonists verapamil and nifedipine (1, 7).

Numerous studies using both peptide and nonpeptide antagonists have indicated that n-type K^+ channels are specifically involved in T lymphocyte activation. For example, antagonists of this K^+ conductance inhibit both mitogen- and antigen-stimulated T lymphocyte proliferation, with similar rank orders of potency (1, 7, 9–11). The effects on proliferation are the result of decreased IL-2 production (7, 10–12) mediated by the inhibition of Ca^{2+}-dependent pathways involved in T lymphocyte activation (5, 11). Effects on proliferation have also been demonstrated with peptide antagonists that are specific for n-type K^+ channels (e.g., margatoxin) (5, 11), indicating that blockade of n-type K^+ channels is sufficient to interfere with T cell activation.

Because the production of activated T lymphocytes is essential for initiating and supporting the immune response, T lymphocyte K^+ channels represent a valid therapeutic target for the discovery of anti-inflammatory drugs. However, the development of T lymphocyte K^+ channel inhibitors as anti-inflammatory agents will require the discovery of potent and selective nonpeptide antagonists. Reference nonpeptide antagonists of n-type K^+ channels, such as classical K^+ and Ca^{2+} channel blockers, are not particularly potent or selective. To identify more potent and selective inhibitors of n-type K^+ channels, we recently initiated high-throughput screening of our compound files, using ^{125}I-ChTX binding to T lymphocytes. These efforts have resulted in the identification of a novel series of n-type K^+ channel antagonists. In this report we present data describing the biochemical, electrophysiological, and immunobiological activity of WIN 17317–3 (1-benzyl-7-chloro-4-*n*-propylimino-1,4-dihy-

This work was undertaken and completed as part of the Sterling Winthrop Pharmaceuticals Research Division Drug Discovery Program.
[1] Present address: Department of Cardiovascular and Metabolic Diseases, Pfizer Central Research, Eastern Point Rd., Groton, CT 06340.

ABBREVIATIONS: ChTX, charybdotoxin; IL-2, interleukin-2; PMA, phorbol-12-myristate-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

droquinoline hydrochloride) (Fig. 1), a representative compound from this series that, to our knowledge, is the most potent and selective nonpeptide antagonist of T lymphocyte K^+ channels reported to date.

Experimental Procedures

Materials

^{125}I-ChTX was obtained from New England Nuclear (Boston, MA). ChTX and iberiotoxin were obtained from Peptides International (Louisville, KY). WIN 17317–3 was synthesized at Sterling Winthrop Pharmaceuticals Research Division (Collegeville, PA). Human Jurkat T lymphocytes were obtained from the American Type Culture Collection (Rockville, MD). RPMI 1640 tissue culture medium was obtained from Gibco-BRL (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). All other reagents, unless noted otherwise, were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Jurkat T lymphocytes were grown in suspension in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.5 mg/ml gentamycin. Cells were routinely seeded at 0.25×10^6 cells/ml in 150-cm^2 Falcon tissue culture flasks and incubated at 37° in an atmosphere of 95% O_2/5% CO_2.

^{125}I-ChTX Binding Assays

^{125}I-ChTX binding to T lymphocytes was based on the methodology of Deutsch *et al.* (8). Jurkat T lymphocytes were resuspended in incubation buffer (5 mM NaCl, 5 mM KCl, 320 mM sucrose, 10 mM HEPES, 6 mM glucose, adjusted to pH 8.4 with Trizma base), to give a cell concentration of 1×10^7 cells/ml. Cells (2×10^6/tube) were incubated in polypropylene, 1-ml, deep-well plates in the presence of 25 pM ^{125}I-ChTX, with or without test agents, for 20 min at 22°. Nonspecific binding was determined in the presence of 10 nM ChTX. At the end of the incubation period, samples were filtered through GF/C glass fiber filters that had been presoaked in 0.6% polyethylenimine, using a Mach II harvester (Tomtec, Orange, CT). Samples were washed with 2×1 ml of ice-cold wash buffer (200 mM NaCl, 20 mM HEPES, adjusted to pH 8.0 with Trizma base). Radioactivity bound to filters was measured in a Betaplate liquid scintillation counter (Wallac, Gaithersburg, MD). Initial experiments showed that the characteristics of ^{125}I-ChTX binding under these conditions are identical to those described by Deutsch *et al.* (8) for isolated peripheral T lymphocytes and are indicative of ^{125}I-ChTX binding to n-type K^+ channels.

^{125}I-ChTX binding to vascular smooth muscle was based on the methodology of Vázquez *et al.* (13). Highly purified sarcolemmal vesicles were prepared from bovine aortic smooth muscle as described by Slaughter *et al.* (14) and were stored at −70°. Membrane vesicles, resuspended in incubation buffer (20 mM NaCl, 20 mM Tris·HCl, pH 7.4), were incubated (2 μg/well) in polypropylene, 1-ml, deep-well plates in the presence of 100 pM ^{125}I-ChTX, with or without test agents, for 30 min at 22°. Nonspecific binding was determined in the presence of 10 nM iberiotoxin, a selective inhibitor of Ca^{2+}-activated K^+ channels. At the end of the incubation period, samples were filtered through GF/C glass fiber filters that had been presoaked in 0.3% polyethylenimine, using a Tomtec Mach II harvester. Samples were washed with 2×1 ml of ice-cold wash buffer (100 mM NaCl, 20 mM HEPES/Tris, pH 7.4). Radioactivity bound to filters was measured as described above. Initial experiments showed that the characteristics of ^{125}I-ChTX binding under these conditions are identical to those described by Vázquez *et al.* (13) for the same preparation and are indicative of ^{125}I-ChTX binding to Ca^{2+}-activated K^+ channels.

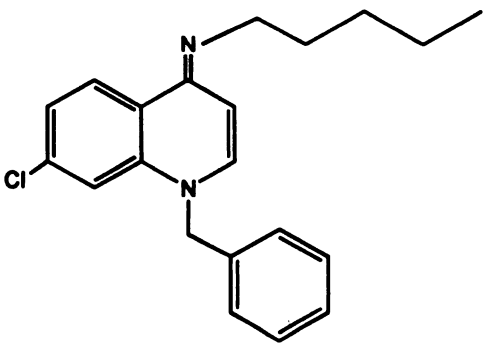

Fig. 1. Structure of WIN 17317–3.

Electrophysiology

Solutions. All recordings were made in extracellular solution of the following composition: 160 mM NaCl, 4.5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4. Patch pipettes (see below) were filled with 154 mM KF, 11 mM EGTA, 1.1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, pH 7.3 (adjusted with KOH). The pipette solution is expected to rapidly equilibrate with the intracellular milieu after establishment of the whole-cell patch-clamp mode (15).

Mechanical recording system. A recording 'chamber' was fabricated by drawing a 1- × 2-cm oval on a glass coverslip, using a wax pencil. The coverslip was then mounted on the stage of a Zeiss inverted microscope placed on a vibration isolation table (Technical Manufacturing Corp., Peabody, MA). Perfusion was allowed by attachment of an inflow line (driven by a MasterFlex pump) and a suction line at opposite ends of the chamber. A drop of Jurkat cells in medium was applied to approximately 300 μl of bath solution on the chamber under no-flow conditions. Cells were allowed to settle to the bottom of the chamber and adhere for approximately 5 min before initiation of flow. Perfusion rates were 4–5 ml/min and experiments were performed at room temperature (21–24°). Mechanical manipulation of pipettes was accomplished using a Narishige hydraulic manipulator (model WR89; Medical Systems Inc., Greenvale, NY).

Patch pipettes. Patch pipettes were pulled from thin-walled glass (TW150–6; World Precision Instruments, Sarasota, FL) on a Flaming-Brown model P-87 micropipette puller (Sutter Instrument Co., San Rafael, CA) and had resistances of 3–5 MΩ when filled with pipette solution. It was not necessary to fire-polish pipettes for use with Jurkat cells.

Electronic recording system. Voltage-clamp recording utilized an Axopatch 1C or 200A amplifier (Axon Instruments, Foster City, CA), and data were digitized with a LabMaster 125-kHz direct memory access board and a Compaq Deskpro 386 computer or ALR 486 computer. All data acquisition and analyses were performed with the pCLAMP software package (Axon Instruments). Current records were digitized at 2 kHz and filtered at 0.5 kHz. Series resistance compensation was used in all experiments.

Experimental protocol. Whole-cell recording modes were established using standard techniques (16). Membrane potential was held at −80 mV and n-type K^+ channel current was measured by applying 150-msec voltage steps to +30 mV every 1 min. Cells were allowed to stabilize for ≥6 min in all cases. Stability was obtained when currents elicited by at least three consecutive voltage steps were superimposable. Pharmacological inhibition was assessed by obtaining peak outward current values during the voltage step described above. Test agent was then continuously perfused at a fixed concentration for 6 min. After this period, current amplitudes were measured every 1 min with the same test pulse as described above. Amplitudes for the first pulse after the 6-min exposure period were used to assess block of closed channels. Amplitudes from the subsequent pulse were used to assess open channel block. Amplitudes in the presence of drug were compared with pre-drug data to determine the percentage of current blocked. A single drug concentration was tested on each cell (i.e., cumulative dose-response curves were not

obtained). Initial experiments showed that the physiological and pharmacological profiles of the currents measured under these conditions are consistent with those previously reported for n-type K^+ channels (1, 4).

Ancillary Pharmacology

The activity of WIN 17317–3 against reference receptors and ion channels was determined in a panel of radioligand binding assays using rat brain membrane receptors. The ^3H-labeled ligands used for each assay were [^3H]prazosin (α_1-adrenergic receptor) (0.1 nM), [^3H]rauwolscine (α_2-adrenergic receptor) (1 nM), [^3H]pyrilamine (histamine H_1 receptor) (1 nM), [^3H]quinuclindinyl benzilate (muscarinic receptor) (0.1 nM), [^3H]nitrendipine (L-type Ca^{2+} channel) (0.1 nM), *N*-[^3H]ethylcarboxamidoadenosine (adenosine receptor) (4 nM), [^3H]dihydroalprenolol (β-adrenergic receptor) (0.1 nM), [^3H]flunitrazepam (benzodiazepine receptor) (1 nM), [^3H]SCH23390 (dopamine D_1 receptor) (0.1 nM), [^3H]raclopride (dopamine D_2 receptor) (1 nM), [^3H]naloxone (opioid receptor) (1 nM), [^3H]lysergic acid diethylamide (serotonin receptor) (1 nM), and [^3H]glibenclamide (ATP-sensitive K^+ channel) (0.1 nM).

Isolation and Activation of $CD4^+$ Lymphocytes

Peripheral blood mononuclear cells were isolated from healthy donors using Ficoll-Paque (Pharmacia Biotech), and monocytes were removed by adherence to Corning tissue culture flasks. $CD4^+$ lymphocytes were isolated by negative selection using antibodies against Mo1 (Coulter), B4 (Coulter), CD16 (Caltag), and CD8 (Caltag) and goat anti-mouse IgG superparamagnetic Microbeads (Miltenyi Biotec, Sunnyvale, CA) in conjunction with a Macs type B2 column. The nonmagnetic unretained fraction was >97% positive for the cell surface marker CD4, as determined by fluorescence-activated cell sorting. $CD4^+$ lymphocytes were resuspended in RPMI 1640 tissue culture medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 0.5 mg/ml gentamycin.

Lymphocytes (1×10^5 cells/well) were activated by two methods, i.e., incubation with immobilized antibodies to CD3 (30 ng/well; AMAC) plus 1 ng/ml PMA or incubation with antibodies to CD3 (300 pg/ml) and CD28 (500 ng/ml; Biodesign International) plus M-450 Dynabeads coated with goat anti-mouse IgG (Dynal A.S). Lymphocytes were incubated for 20–22 hr at 37°, in the presence or absence of stimulants, in an atmosphere of 95% O_2/5% CO_2. Viability after stimulation by either method was >98%, as determined by trypan blue dye exclusion.

Measurement of IL-2 Production

After 20–22 hr of stimulation, culture supernatants were harvested for analysis by sandwich enzyme-linked immunosorbent assay using monoclonal antibody to human IL-2 for capture (Biosource International) and a polyclonal antibody to human IL-2 (Genzyme) for detection of bound IL-2. Recombinant human IL-2 (R & D Systems) was used for the standard curve. Bound antibody was detected using alkaline phosphatase-conjugated goat $F(ab')_2$ fragments to rabbit immunoglobulin (Tago) and alkaline phosphatase substrate (Sigma Diagnostics). Absorbance at 405 nm was determined using a Molecular Devices UV MAX plate reader.

Data Analysis

Analysis of data to determine values for IC_{50}, K_d, B_{max}, and k_{-1} was performed using standard equations and PC- and VAX-based nonlinear least-squares regression analysis programs.

Results

Inhibition of ^{125}I-ChTX binding by WIN 17317–3. WIN 17317–3 inhibited ^{125}I-ChTX binding to n-type K^+ channels in a concentration-dependent manner, with complete inhibition being observed at 10 μM (Fig. 2). WIN 17317–3 had an

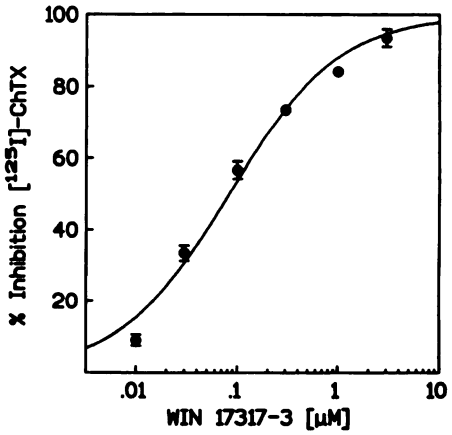

Fig. 2. Inhibition of ^{125}I-ChTX binding to n-type K^+ channels by WIN 17317–3. Jurkat T lymphocytes were incubated for 20 min at 22° with 25 pM ^{125}I-ChTX in the presence of increasing concentrations of WIN 17317–3. Specifically bound ^{125}I-ChTX was determined as described in Experimental Procedures and is plotted as percentage inhibition of ^{125}I-ChTX binding versus test concentration of WIN 17317–3. Data are presented as means ± standard errors (eight experiments). A *smooth curve* was fitted to the data via nonlinear, least-squares, regression analysis, using the equation % inhibition = $A + (B - A)/[1 + (10^C/10^X)^D]$, where X is log [WIN 17317–3], C is log [WIN 17317–3] at the mid-point (IC_{50}), A and B are the bottom and top plateaus of the curve, respectively, and D is the slope factor (Hill coefficient).

IC_{50} value of 83 ± 4 nM and a Hill slope of 0.8 ± 0.1 (mean ± standard error, eight experiments). The nature of inhibition was examined by determining the effect of WIN 17317–3 on concentration-dependent ^{125}I-ChTX binding (Fig. 3). The data were best fit by a competitive model of inhibition where WIN 17317–3 increased the apparent K_d for ChTX without changing the total number (B_{max}) of channels bound. The nature of inhibition was also investigated by determining the effect of WIN 17317–3 on the dissociation rate constant (k_{-1}) for ^{125}I-ChTX dissociation from T lymphocyte K^+ channels (Fig. 4). WIN 17317–3 had no significant effect on the k_{-1} for ^{125}I-ChTX, suggesting that WIN 17317–3 is a competitive inhibitor of ^{125}I-ChTX binding to T lymphocyte K^+ channels.

Inhibition of n-type K^+ currents by WIN 17317–3. Fig. 5, *inset*, illustrates the manner in which current block by WIN 17317–3 develops. The magnitude of closed channel block at 3 μM was 26 ± 2% (mean ± standard error, five cells). During this pulse, however, the rate of current decay was accelerated in a concentration-dependent manner, suggesting that WIN 17317–3 blocks a postactivation state of the n-type K^+ channel. We describe this as open channel block, and we quantitatively assessed the magnitude by applying another depolarizing pulse to +30 mV, 1 min after pulse 2. This 1-min delay was necessary to allow the drug-independent channel inactivation process to recover completely. The dose-response curve for open channel block assessed in this manner is illustrated in Fig. 5. The IC_{50} value of WIN 17317–3 for open channel block was 335 nM.

Ancillary pharmacology of WIN 17317–3. WIN 17317–3 was tested in a panel of radioligand binding assays for activity against standard receptors and other ion channels. WIN 17317–3 bound weakly to α_2-adrenergic, histamine H_1, and muscarinic receptors and L-type Ca^{2+} channels ($1\ \mu M > IC_{50} < 3\ \mu M$) and very weakly or not at all to adenosine A_1, α_1-adrenergic, β-adrenergic, benzodiazepine, dopamine D_1 and D_2, opioid, and serotonin receptors and

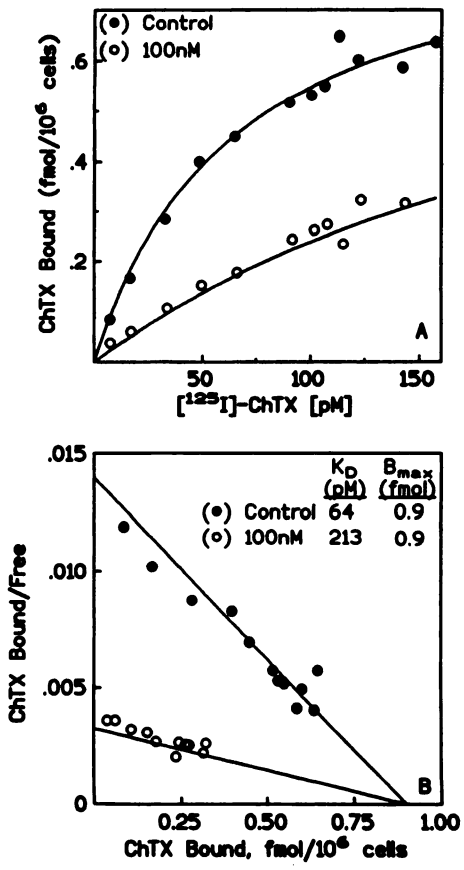

Fig. 3. WIN 17317–3 inhibition of concentration-dependent ^{125}I-ChTX binding to n-type K^+ channels. Jurkat T lymphocytes were incubated for 20 min at 22° with increasing concentrations of ^{125}I-ChTX, with or without 10 nM ChTX, in the absence (●) or presence (○) of 100 nM WIN 17317–3. ^{125}I-ChTX binding was measured as described in Experimental Procedures. A, Data plotted as ^{125}I-ChTX specifically bound versus ^{125}I-ChTX concentration. *Smooth curves* were fit to the data using the equation for competitive inhibition, ^{125}I-ChTX bound = B_{max}·[^{125}I-ChTX]/[[^{125}I-ChTX] + K_d·(1 + [WIN 17317–3]/IC_{50})], where B_{max} is the maximal binding capacity for ^{125}I-ChTX, K_d is the dissociation constant of ^{125}I-ChTX, and IC_{50} is the estimated IC_{50} of WIN 17317–3. The best fit of the data, as determined by nonlinear regression analysis, was obtained with a B_{max} of 0.9 fmol/10^6 cells, a K_d of 64 pM, and an IC_{50} of 0.03 μM. B, Scatchard transformation of the data illustrated in A. *Lines* were fit to the data using the equation (B_{max} − ^{125}I-ChTX bound)/[K_d·(1 + [WIN 17317–3]/IC_{50})], with the estimates for B_{max}, K_d, and IC_{50} determined from nonlinear regression analysis in A. Data are presented as means of triplicate determinations of a representative experiment.

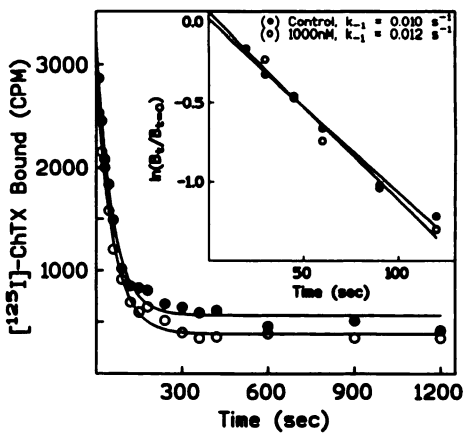

Fig. 4. Effect of WIN 17317–3 on the dissociation kinetics of ^{125}I-ChTX. Jurkat T lymphocytes were incubated for 20 min at 22° with 25 pM ^{125}I-ChTX. At the end of the incubation, dissociation of ^{125}I-ChTX was initiated by dilution (1/20) of samples in incubation buffer, in the absence (●) or presence (○) of 1000 nM WIN 17317–3. Incubation was continued for increasing times after dilution, and ^{125}I-ChTX bound at each time point was measured as described in Experimental Procedures. Data are plotted as ^{125}I-ChTX bound versus time. *Smooth curves* were fitted to the data via nonlinear, least-squares, regression analysis, using the equation ^{125}I-ChTX bound = $A \cdot e^{-BX}$, where X is time (in sec), A is the magnitude of the exponential function, and B is the dissociation rate constant (k_{-1}). *Inset*, semilogarithmic representation of the first-order dissociation reactions, with estimates of the k_{-1} for ^{125}I-ChTX in the absence (●) and presence (○) of 1000 nM WIN 17317–3. Data are presented as the means of six replicate determinations of a representative experiment.

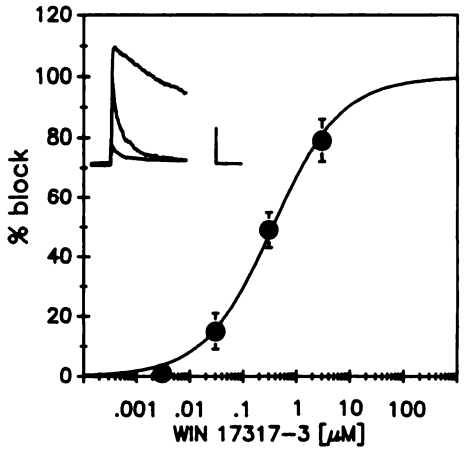

Fig. 5. Dose-response curve for open channel block of n-type K^+ current by WIN 17317–3. Inhibition (%) was measured at each test concentration and plotted as the mean ± standard error. Single concentrations were tested for each cell (five cells/concentration). The *smooth curve* is a fit to the data using the equation % block = [1 + (IC_{50}/[WIN 17317–3])^n]$^{-1}$, where IC_{50} is the concentration for 50% block and n is the Hill coefficient. The best fit to the data was obtained with an IC_{50} value of 335 nM and an n value of 0.7. *Inset*, current records in control (*largest amplitude trace*), after a 6-min exposure to 3 μM WIN 17317–3 with no depolarizing pulse (*intermediate amplitude trace*), and after a subsequent pulse 1 min later (*smallest amplitude trace*). *Calibration bars*, 50 msec and 500 pA.

ATP-sensitive K^+ channels (IC_{50} ≥ 10 μM). WIN 17317–3 was also tested for activity against Ca^{2+}-activated ChTX-sensitive channels in vascular smooth muscle. WIN 17317–3 had an IC_{50} value of 14 μM for inhibition of ^{125}I-ChTX binding to bovine aortic smooth muscle (Fig. 6), compared with its IC_{50} value of 83 nM for inhibition of ^{125}I-ChTX binding to T lymphocytes. This indicates that WIN 17317–3 is >150-fold selective for voltage-activated ChTX-sensitive K^+ channels.

Inhibition of IL-2 production by WIN 17317–3. The effect of WIN 17317–3 on IL-2 production from activated $CD4^+$ lymphocytes is illustrated in Fig. 7. Lymphocytes were activated by anti-CD3 plus PMA (protocol 1) or anti-CD3 plus anti-CD28 (protocol 2). WIN 17317–3 decreased IL-2 production in a concentration-dependent manner, with EC_{50} values of 0.8 μM and 1 μM for protocol 1 (Fig. 7A) and protocol 2 (Fig. 7B), respectively. ChTX, included as a reference inhibitor of n-type K^+ channels, decreased IL-2 production with EC_{50} values of 4 nM and 5 nM for protocols 1 and 2, respectively. To confirm that WIN 17317–3 inhibition of IL-2 production was not due to cytotoxicity, $CD4^+$ lymphocytes were activated (as described above) in the presence of increasing concentrations of WIN 17317–3, and cell viability was assessed by trypan

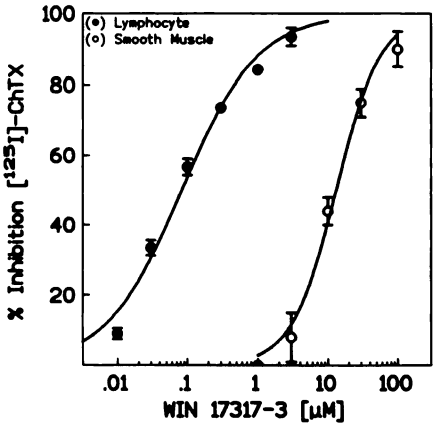

Fig. 6. Comparison of WIN 17317–3 inhibition of ^{125}I-ChTX binding to voltage-activated versus Ca^{2+}-activated K^+ channels. ^{125}I-ChTX binding to Jurkat T lymphocytes and bovine aorta was measured as described in Experimental Procedures. Percentage inhibition of ^{125}I-ChTX binding to T lymphocyte n-type K^+ channels (●) (eight experiments, data from Fig. 2) and smooth muscle Ca^{2+}-activated K^+ channels (○) (two experiments) is plotted versus the test concentration of WIN 17317–3. Data are presented as means ± standard errors. *Smooth curves* were fit as described in the legend to Fig. 2.

blue exclusion. WIN 171317–3 had no effect on $CD4^+$ lymphocyte viability at up to 10 μM, with a 30–37% decrease being observed at 30 μM.

Discussion

The results presented in this report describe the identification and characterization of WIN 17317–3, a nonpeptide antagonist of n-type K^+ channels in human T lymphocytes. Previously reported nonpeptide inhibitors of n-type K^+ channels include quinine, 4-aminopyridine, tetraethylammonium, verapamil, and nifedipine (1, 7). However, as n-type K^+ antagonists, these agents are 100-1000-fold less potent than WIN 17317–3 and are not as selective. Thus, the 4-iminoquinolines represent a new class of n-type K^+ channel antagonists, and WIN 17317–3 is, to our knowledge, the most potent and selective nonpeptide inhibitor of this channel reported to date. Because n-type K^+ channels have been implicated in T lymphocyte activation (1, 7, 9–11), additional optimization of WIN 17317–3 may lead to the development of a novel drug for the treatment of inflammation.

WIN 17317–3 inhibits ^{125}I-ChTX binding to n-type K^+ channels (IC_{50} of 83 nM) by an apparent competitive interaction at the ChTX binding site. The competitive mechanism of inhibition is supported by both equilibrium and kinetic binding data. Under equilibrium conditions, WIN 17317–3 increases the dissociation constant of ^{125}I-ChTX without changing the total number of channels bound, an observation consistent with competitive inhibition. Kinetic data illustrate that WIN 17317–3 has no effect on the rate of dissociation of ^{125}I-ChTX, even at a concentration 10-fold higher than its equilibrium IC_{50}. If the inhibition of ^{125}I-ChTX binding by WIN 17317–3 were allosteric, we would likely have observed a drug-induced increase in the dissociation rate constant, as has been reported for similar drug interactions with other ion channels and receptors (17–19). Thus, taken together, the equilibrium and kinetic data are consistent with a competitive interaction of WIN 17317–3 with the ChTX binding site on n-type K^+ channels.

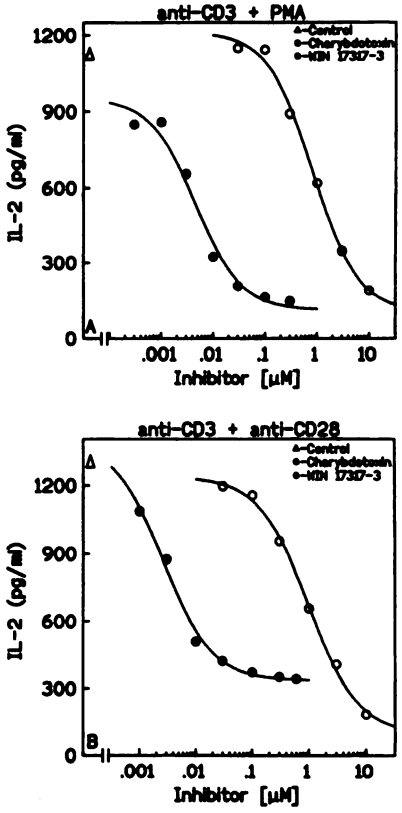

Fig. 7. Inhibition of IL-2 production by WIN 17317–3. $CD4^+$ lymphocytes were isolated from human peripheral blood mononuclear cells as described in Experimental Procedures. Lymphocytes were activated by anti-CD3 plus PMA (protocol 1) (A) or by anti-CD3 plus anti-CD28 (protocol 2) (B), in the presence of increasing concentrations of test agent, and IL-2 production was measured after 22 hr, as described in Experimental Procedures. IL-2 production is plotted versus test concentration of inhibitor. Data for protocols 1 and 2 are presented as means of representative experiments performed in duplicate. *Smooth curves* were fitted to the data via nonlinear, least-squares, regression analysis using the equation IL-2 (pg/ml) = $A + (B - A)/[1 + (10^X/10^C)]$, where X is log [inhibitor], C is log [inhibitor] at the mid-point (EC_{50}), and A and B are the bottom and top plateaus of the curve, respectively.

The interaction of WIN 17317–3 with the ChTX binding site results in functional block of T lymphocyte K^+ channels. The manner in which current block develops was investigated by determining the effect of WIN 17317–3 on both the peak amplitude and the rate of decay of n-type K^+ currents elicited by a single test depolarization. The major effect observed in the presence of WIN 17317–3 is a drug-induced increase in the rate of decay of n-type K^+ currents (Fig. 5, *inset*), suggesting preferential block of open channels by WIN 17317–3. It is possible that WIN 17317–3 increases the rate of channel inactivation, as opposed to blocking a postactivation state of the channel. A concomitant increase in the time necessary for recovery from inactivation would also be necessary to explain our results, because the second test pulse in the presence of drug shows current block, as opposed to recovery of peak amplitudes with enhanced inactivation rates. We did not investigate longer recovery times, and it is possible that current amplitudes would have recovered with longer periods at −80 mV. The simplest interpretation, however, is that the drug blocks open n-type K^+ channels and, in the absence of data to the contrary, we have described blockade as open channel block.

This pattern of block is similar to that reported for blockade of n-type K^+ channels by 4-aminopyridine (20) but contrasts with the manner in which current block develops in the presence of ChTX (4). The major effect observed in the presence of ChTX is a decrease in the peak current amplitude, indicative of closed channel block. It is interesting to note that, although our binding data suggest that WIN 17317–3 and ChTX bind to the same site on n-type K^+ channels (see above), their respective mechanisms of channel blockade appear to be distinct. In keeping with drug-receptor interactions reported for sodium and calcium channels (17, 18, 21), the distinct current-blocking characteristics of WIN 17317–3 and ChTX may reflect state-dependent blockade of n-type K^+ channels. Additional studies, however, will be required to fully test this hypothesis. Also, under the conditions of the binding assay (i.e., low ionic strength), voltage-dependent properties such as channel activation and inactivation would be shifted to the left (22). The end result of this shift would be to drive channels toward postactivation states and it is, therefore, reasonable to assume that WIN 17317–3 binds to the same channel state in both the binding and electrophysiological assays. If WIN 17317–3 binds to the same site or a portion of the binding site for ChTX, then the low ionic strength conditions of the binding assay may also increase the affinity of the drug for its receptor, as previously reported for ChTX (8). This would explain the greater potency of WIN 17317–3 for ^{125}I-ChTX displacement, compared with channel blockade.

WIN 17317–3 is 10–300-fold selective for n-type K^+ channels, compared with reference receptors and ion channels. Of particular note is its very weak activity against ChTX-sensitive, Ca^{2+}-activated K^+ channels in smooth muscle (IC_{50} of 14 μM), suggesting distinct pharmacophores for drug binding to n-type versus Ca^{2+}-activated K^+ channels. This is in keeping with the recently reported selectivity of the peptide inhibitor margatoxin for n-type K^+ channels (23). Margatoxin is a potent inhibitor of ^{125}I-ChTX binding to voltage-activated K^+ channels in lymphocytes but does not inhibit ^{125}I-ChTX binding to Ca^{2+}-activated K^+ channels in smooth muscle. Thus, like margatoxin, WIN 17317–3 is a selective inhibitor of voltage-gated, ChTX-sensitive channels and, as such, represents a novel nonpeptide tool with which to further define the role of n-type K^+ channels in lymphocyte activation.

This report does not address the specificity of WIN 17317–3 for other voltage-gated K^+ channels, such as those found in brain or heart. Clearly this is a very important issue that requires detailed investigation before this class of compounds can be developed as anti-inflammatory agents. In a separate study (24), we provide preliminary data on the selectivity of WIN 17317–3 and its analogues for the delayed-rectifier K^+ channel in heart. Using [^{3}H]dofetilide as a ligand for cardiac K^+ channels, we determined that WIN 17317–3 is 20-fold selective for lymphocyte K^+ channels. Additional studies will determine, for this series, whether this biochemical selectivity correlates with a functional selectivity versus cardiac K^+ channels.

The effect of WIN 17317–3 on lymphocyte activation was investigated by determining its effect on IL-2 production from activated $CD4^+$ T lymphocytes. WIN 17317–3 decreases IL-2 production (EC_{50} of 1 μM) stimulated by either anti-CD3 plus PMA or anti-CD3 plus anti-CD28, without significant effects on cell viability at up to 10 μM. Therefore, it is likely that the significant decrease in IL-2 observed in the presence of 1 μM WIN 17317–3 is due to a specific effect on the lymphocyte activation pathway and is not the result of a nonspecific effect related to cytotoxicity. However, we cannot rule out the possibility that there is some degree of cellular toxicity that does not affect trypan blue exclusion at concentrations of WIN 17317–3 of $\leq$10 μM.

The EC_{50} value of WIN 17317–3 for inhibition of IL-2 production (1 μM) is close to its IC_{50} value of 0.3 μM for inhibition of n-type K^+ currents, suggesting that the drug-induced decrease in IL-2 production is related to inhibition of n-type K^+ channels. One would anticipate that inhibition of IL-2 production would be shifted to the right of channel blockade, because a significant percentage of channels would probably need to be blocked before membrane depolarization and the subsequent inhibition of calcium influx could occur. This correlation compares favorably with several recent reports. For example, the peptide antagonists ChTX, noxiustoxin, and margatoxin inhibit both n-type K^+ currents and T cell receptor-induced IL-2 production at nanomolar concentrations (7, 11, 12). In the present study, ChTX, included as a reference control, decreased IL-2 production with an EC_{50} value of 4 nM, a value similar to those recently reported by Lin *et al.* (11) (0.3 nM) and Freedman *et al.* (12) (1 nM) for inhibition of IL-2 production in anti-CD3- or phytohemagglutinin-stimulated human peripheral T lymphocytes, respectively. A correlation between inhibition of n-type K^+ channels and inhibition of T lymphocyte activation has also been reported for nonpeptide antagonists such as quinine and 4-aminopyridine. These agents inhibit both n-type K^+ currents and mitogen-induced T lymphocyte activation, with IC_{50} values of 30 μM and 3 mM, respectively (1, 7). In the present study, WIN 17317–3 inhibited n-type K^+ channels and T lymphocyte activation at similar concentrations (see above), thus providing additional support for an important role for n-type K^+ channels in the T lymphocyte activation pathway.

In conclusion, we have identified WIN 17317–3, a potent and selective nonpeptide antagonist of voltage-activated K^+ channels in human T lymphocytes. Additional characterization and optimization of this compound may lead to the development of a new drug for the treatment of inflammation.

References

1. DeCoursey, T. E., K. G. Chandy, S. Gupta, and M. Cahalan. Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis? *Nature (Lond.)* **307**:465–468 (1984).
2. Matteson, D. R., and C. Deutsch. K^+ channels in lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature (Lond.)* **307**:468–471 (1984).
3. Gallin, E. K. Ion channels in leukocytes. *Physiol. Rev.* **71**:775–811 (1991).
4. Sands, S. B., R. S. Lewis, and M. D. Cahalan. Charybdotoxin blocks voltage-gated K^+ channels in human and murine T lymphocytes. *J. Gen. Physiol.* **93**:1061–1074 (1989).
5. Leonard, R. J., M. L. Garcia, R. S. Slaughter, and J. P. Reubin. Selective blockers of voltage-gated potassium channels depolarize human T lymphocytes: mechanism of the antiproliferative effect of charybdotoxin. *Proc. Natl. Acad. Sci. USA* **89**:10094–10098 (1992).
6. Miller, C., E. Moczydlowski, R. Latorre, and M. Phillips. Charybdotoxin, a protein inhibitor of single calcium-activated potassium channels from mammalian smooth muscle. *Nature (Lond.)* **313**:316–318 (1985).
7. Price, M., S. C. Lee, and C. Deutsch. Charybdotoxin inhibits proliferation and interleukin 2 production in human peripheral blood lymphocytes. *Proc. Natl. Acad. Sci. USA* **86**:10171–10175 (1989).
8. Deutsch, C., M. Price, S. Lee, V. F. King, and M. L. Garcia. Characterization of high affinity binding sites for charybdotoxin in human T lympho-

cytes: evidence for association with the voltage-gated potassium channel. *J. Biol. Chem.* **266**:3668–3674 (1991).
9. Deutsch, C., D. Krause, and S. C. Lee. Voltage-gated potassium conductance in human T lymphocytes stimulated with phorbol ester. *J. Physiol. (Lond.)* **372**:405–423 (1986).
10. Chandy, K. G., T. E. DeCoursey, M. D. Cahalan, C. McLaughlin, and S. Gupta. Voltage-gated potassium channels are required for human T lymphocyte activation. *J. Exp. Med.* **160**:369–385 (1984).
11. Lin, C. S., R. C. Boltz, J. T. Blake, M. Nguyen, A. Talento, P. A. Fischer, M. S. Springer, N. H. Sigal, R. S. Slaughter, M. L. Garcia, G. J. Kaczorowski, and G. C. Koo. Voltage-gated potassium channels are required for human T lymphocyte activation. *J. Exp. Med.* **177**:637–645 (1993).
12. Freedman, B. D., M. A. Price, and C. Deutsch. Evidence for voltage modulation of IL-2 production in mitogen-stimulated peripheral blood lymphocytes. *J. Immunol.* **149**:3784–3794 (1992).
13. Vázquez, J., P. Feigenbaum, G. Katz, V. F. King, J. P. Reuben, L. Roy-Contancin, R. S. Slaughter, G. J. Kaczorowski, and M. L. Garcia. Characterization of high affinity binding sites for charybdotoxin in sarcolemmal membranes from bovine aortic smooth muscle. *J. Biol. Chem.* **264**:20902–20909 (1989).
14. Slaughter, R. S., J. L. Shevell, J. P. Felix, M. L. Garcia, and G. J. Kaczorowski. High levels of sodium-calcium exchange in vascular smooth muscle sarcolemmal membrane vesicles. *Biochemistry* **28**:3995–4002 (1989).
15. Marty, A., and E. Neher. Tight seal whole-cell recording, in *Single Channel Recording* (B. Sakmann and E. Neher, eds.). Plenum Press, New York, 107–121 (1983).
16. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**:85–100 (1981).
17. Hill, R. J., H. J. Duff, and R. S. Sheldon. Class I antiarrhythmic drug receptor: biochemical evidence for state-dependent interaction with quinidine and lidocaine. *Mol. Pharmacol.* **36**:150–159 (1989).
18. Postma, S. W., and W. A. Catterall. Inhibition of [^{3}H]batrachotoxin A 20α-benzoate to sodium channels by local anesthetics. *Mol. Pharmacol.* **25**:219–227 (1984).
19. Rothman, R. B., and T. C. Westfall. Allosteric coupling between morphine and enkephalin receptors *in vitro*. *Mol. Pharmacol.* **21**:548–557 (1982).
20. Choquet, D., and H. Korn. Mechanism of 4-aminopyridine action on voltage-gated potassium channels in lymphocytes. *J. Gen. Physiol.* **99**:217–240 (1992).
21. Hondeghem, L. M., and B. G. Katzung. Antiarrhythmic agents: the modulated receptor mechanism of action of sodium and calcium-blocking drugs. *Annu. Rev. Pharmacol. Toxicol.* **24**:387–423 (1984).
22. Hille, B. *Ionic Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA (1984).
23. Garcia-Calvo, M., R. J. Leonard, J. Novick, S. P. Stevens, W. Schmalhofer, G. J. Kaczorowski, and M. L. Garcia. Purification, characterization, and biosynthesis of margatoxin, a component of *Centruroides margaritatus* venom that selectively inhibits voltage-dependent potassium channels. *J. Biol. Chem.* **268**:18866–18874 (1993).
24. Michne, W., J. W. Guiles, A. M. Treasurywala, L. A. Castonguay, C. A. Weigelt, B. O'Connor, W. A. Volberg, A. M. Grant, C. Chadwick, D. S. Krafte, and R. J. Hill. Novel inhibitors of potassium ion channels on human T lymphocytes. *J. Med. Chem.*, in press.

Send reprint requests to: Roger J. Hill, Department of Cardiovascular and Metabolic Diseases, Pfizer Central Research, Eastern Point Rd., Groton, CT 06340.