Selective Blocking of Voltage-Gated $K^+$ Channels Improves Experimental Autoimmune Encephalomyelitis and Inhibits T Cell Activation

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Selective Blocking of Voltage-Gated K\(^+\) Channels Improves Experimental Autoimmune Encephalomyelitis and Inhibits T Cell Activation\(^1\)

Christine Beeton,* Jocelyne Barbaria,* Pierre Giraud,† Jerome Devaux,‡ Anne-Marie Benoliel,§ Maurice Gola,† Jean Marc Sabatier,¶ Dominique Bernard,* Marcel Crest,§ and Evelyne Béraud\(^2\)*

Kaliotoxin (KTX), a blocker of voltage-gated potassium channels (Kv), is highly selective for Kv1.1 and Kv1.3. First, Kv1.3 is expressed by T lymphocytes. Blockers of Kv1.3 inhibit T lymphocyte activation. Second, Kv1.1 is found in paranodal regions of axons in the central nervous system. Kv blockers improve the impaired neuronal conduction of demyelinated axons in vitro and potentiate the synaptic transmission. Therefore, we investigated the therapeutic properties of KTX via its immunosuppressive and symptomatic neurological effects, using experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. The T line cells used to induce adoptive EAE were myelin basic protein (MBP)-specific, constitutively contained mRNA for Kv1.3, and expressed Kv1.3. These channels were shown to be blocked by KTX. Activation is a crucial step for MBP T cells to become encephalitogenic. The addition of KTX during Ag-T cell activation led to a great reduction in the MBP T cell proliferative response, in the production of IL-2 and TNF, and in Ca\(^{2+}\) influx. Furthermore, the addition of KTX during T cell activation in vitro led a decreased encephalitogenicity of MBP T cells. Moreover, KTX injected into Lewis rats impaired T cell function such as the delayed-type hypersensitivity. Lastly, the administration of this blocker of neuronal and lymphocyte channels to Lewis rats improved the symptoms of EAE. We conclude that KTX is a potent immunosuppressive agent with beneficial effects on the neurological symptoms of EAE. The Journal of Immunology, 2001, 166: 936–944.

Voltage-gated potassium channels (Kv)\(^3\) serve an important function in regulating neuronal excitability (1). Kv1.1-Kv1.6 are present in the CNS (2). Kv1.1 and Kv1.2 are found in particular in paranodal regions of axons (3, 4). Kv blockers such as aminopyridines (APs) restore the conduction of demyelinated axons in vitro and potentiate synaptic transmission (4–8). Therefore, Kv blockers have received much attention as a potential mean of symptomatic therapy for neuropathies such as multiple sclerosis (MS). Although 4-AP appears efficacious in some patients, its therapeutic index is so narrow that there are often unpleasant and potentially toxic side effects (9, 10), limiting the dose to be used. These side effects may result from AP blocking a vast array of Kv such as Kv1.1-Kv1.5, Kv3.1, Kv3.3, and Kv3.4, and Kv4.1-Kv4.3. Therefore, we hypothesized that compounds displaying more selectivity for Kv may possess a better therapeutic index than does 4-AP. We selected a highly selective blocker of Kv1.1 and Kv1.3, kaliotoxin (KTX) (11), as a potential candidate drug for MS therapy.

In contrast, the predominant type of Kv expressed by human, mouse, and rat T cells is the n type channel, composed of four Kv1.3 α subunits (12). Kv1.3 is involved in T cell activation and proliferation (13). High affinity blockers have been used to demonstrate that Kv1.3 blockade depolarizes the T cell membrane and attenuates the Ca\(^{2+}\) signaling pathway that is crucial for lymphocyte activation (14). Because T lymphocytes must be activated to initiate and support the immune response, T lymphocyte Kv1.3 represents a valid therapeutic target for the discovery of anti-inflammatory drugs, as supported by the suppression of T cell activation by selective peptide and nonpeptide inhibitors of this channel (toxins, Ref. 15–17; WIN-17317-3, Ref. 18; CP339818, Ref. 19; UK78282, Ref. 20; correolide, Ref. 21; and alkoxypsoralsen, Ref. 22). This idea has also been documented by an in vivo mini pig model for delayed-type hypersensitivity (DTH) (21, 23).

On the basis of these arguments, designing molecules such as KTX that act on neuronal Kv1.1 and T cell Kv1.3 would make it possible to combine the symptomatic treatment of neurological deficits with immunosuppressive effects. To date, none of the selective Kv blockers have been tested for neurological T cell-mediated disease treatment.

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory and demyelinating autoimmune disease of the CNS, a model widely used to study MS. EAE is mediated by MHC class II-restricted, myelin Ag-specific CD4\(^+\) T cells (24). These cells are of the Th1 type, which produce IL-2, IFN-γ, and TNF. EAE can be

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**Abbreviations used in this paper: Kv, voltage-gated potassium channel(s); EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; MBP, myelin basic protein; KTX, kaliotoxin; CTX, charybotoxin; MgTX, margatoxin; α-DTX, α-dendrotoxin; DTH, delayed-type hypersensitivity; AP, aminopyridine; SN, supernatant; RT, reverse transcriptase.
induced in Lewis rats by adoptive transfer of myelin basic protein (MBP) T line cells provided these T cells are activated before injection (25). Because T cell activation is required for encephalitogenicity, KTX was tested on MBP T cells to block Kv1.3 current, MBP-induced proliferation, activation assessed by cytokine production and Ca\(^{2+}\) rise, and acquisition of encephalitogenic properties. Contrary to another study (26), we established that the immunosuppressive action of KTX is potent. Also, we found that KTX improves ongoing EAE by at least 50%.

Materials and Methods

Animals

Female inbred Lewis rats 8–10 wk of age and guinea pigs were purchased from Charles River Breeding Laboratories (St. Aubin les Elbeuf, France) and from Iffa Credo (L’Arbresle, France).

Cell lines

The MBP-specific encephalitogenic T cell line, called PAS, had been established from guinea pig MBP-primed Lewis rat lymph nodes. For Ag stimulation, PAS T cells (3 × 10\(^3\)/ml) were incubated for 2–3 days with 10 \(\mu\)g/ml of MBP and 15 × 10\(^3\)/ml syngeneic irradiated (2500 rad) thymocytes as Ag-presenting cells (APC) (RPMI 1640 140 Dutch modification medium supplemented with 1% syngeneic rat serum and additives. PAS T cells, which produce limited demyelination, had been characterized as cytototoxic, MHC class II-restricted CD4 T cells as Ag-presenting cells (APC) in RPMI 1640 Dutch modification medium supplemented with 1% syngeneic rat serum and additives. PAS T cells, which produce limited demyelination, had been characterized as cytototoxic, MHC class II-restricted CD4 T cells that are encephalitogenic in vivo (27, 28). The leukemic human T cell clone Jurkat JH6.2 was a gift from Dr. J. Nunez (U119 Institut National de la Sante et de la Recherche Mèdicale, Marseille, France) and maintained as described (29). The murine CTL-L cell line was a gift from Dr. A.M. Schmitt-Verschulst (Centre d’Immunologie, Marseille-Luminy, Marseille, France) and was grown in RPMI 1640 supplemented with 10% FCS, 50 \(\mu\)M/ml human rIL-2, and additives. The murine connective tissue fibroblast cell line L-M was a gift from Dr. A. Fontana (University Hospital, Zurich, Switzerland) and was maintained as described (30).

Reagents

MBP was extracted from guinea pig frozen CNS by the method of Deibler et al. (31) and purified by C18 reverse-phase HPLC with a Millipore/Reagents Waters Associates system (Milford, DE). The purity was assessed by electrospray mass spectrometry. KTX was synthesized using the solid phase method previously described (32). Synthetic charybotoxin (ChTX) and α-dendrotoxin (α-DTX) were purchased from Latoxan (Rosans, France); synthetic margatoxin (MgTX) was obtained from Alomone Labs (Jerusalem, Israel).

RT-PCR analysis of Kv1.3 mRNA levels

Total RNAs were extracted from 10\(^5\) lymphocytes (0, 24, 48, and 72 h after antigenic stimulation), using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. A DNA digestion was performed for 1 h at 70°C with 70 U/ml of RNase-free DNase I. After reverse transcription (RT) buffer (Life Technologies) containing 1000 U/ml of RNase inhibitor (Rnasin; Promega, Madison, WI), from heat inactivation of DNase (75°C for 5 min), DNase-treated RNA (500 ng in 10 \(\mu\)l) was reverse-transcribed (33) for 16 h at 37°C by adding 4 \(\mu\l of 5\times RT buffer (Life Technologies), 4 \(\mu\l of 5\times RT mix (containing 2.5 M deoxynucleotide triphosphate and 24 \(\mu\l\) random hexamers), 1 \(\mu\l\) Rnasin, and 1 \(\mu\l\) RT (Superscript II; Life Technologies). Amplifications of cDNA for Kv1.3 and GAPDH were performed by PCR with Hotstar Taq polymerase (Qiagen, Chatsworth, CA), using a Perkin-Elmer thermal cycler. Primer locations (referring to published sequences in GeneBank) were as follows: Kv1.3 (accession number X16001) upper primer 782-802; lower primer 988-968; GAPDH (accession number X02231) upper primer 591-610; lower primer 1042-1023. Conditions of linearity of PCR were determined in preliminary experiments (in terms of amounts of cDNA and of cycle number). After a hot start (94°C for 1.5 min), cycling conditions were 94°C (45 s), 62°C (1 min), 68°C (1.5 min), with each series of cycles followed by a 4-min elongation step (68°C). Amplification products were analyzed by electrophoresis on 2% agarose gels containing 5 \(\mu\l\) ethidium bromide. Images of the gels were numerized (using a Syngene gel imager) and then processed with Molecular Analyst software (Bio-Rad) for quantification of the signal. The ratio of the intensity of Kv1.3 band to the intensity of the GAPDH band was used to determine the relative variations of Kv1.3 transcripts after stimulation. Negative controls included PCR amplification of nonreversed-transcribed RNAs.

Electrophysiological recordings

Whole-cell recordings in PAS and Jurkat JH6.2 T cells were performed according to Mourre et al. (11). KTX was delivered to the bath via a pneumatic pico-pump system (WPI, Aston, U.K.). Dose-response curves were determined by successive additions of KTX at increasing concentrations. Experimental points were fitted to the theoretical hyperbolic curve by the equation: 

\[ \frac{[T]/[IC_{50}^2]}{[T]/[IC_{50}^2] + ([T]/[IC_{50}^2])^y} \]

in which \(y\) is the fraction of unblocked current, \(IC_{50}\) is the concentration of toxin inducing 50% block, and \(n\) is the Hill coefficient. Kinetics of the Kv1.3 blockade induced by KTX was determined as previously described for different toxins (34).

Lymphocyte proliferation assay

The PAS T cells were used after 4–6 days of IL-2-dependent expansion. They were seeded in flat-bottom 96-well plates (Nunc, Naperville, IL) in 200 \(\mu\l of culture medium supplemented with 1% homologous rat serum, in the presence of APC. K\(^+\) channel blockers were added to the cells 30 min before the Ag. The cells were cultured for 3 days and were pulsed with \([\text{H}]\)thymidine 16–18 h before being harvested. The proliferative response of T cells was assessed by a \(\beta\) scintillation counter measuring the \([\text{H}]\)thymidine incorporated.

IL-2 and TNF-αβ bioassays

The PAS T cells (3 × 10\(^3\)) and APC (2 × 10\(^3\))/0.2 ml were cultured in 96-well plates. KTX was added 30 min before the Ag. Supernatants (SNs) were collected 7 and 22 h later and assayed for their biological activities. The IL-2-dependent CTL-L cell line was used to assay the IL-2 activity, using mouse rIL-2 as the standard. TNF activity was determined using TNF-αβ-sensitive L-M cells as previously described (30). TNF-α and TNF-β activities were distinguished using a polyclonal TNF-α-specific rabbit anti-rat Ab (Genzyme, Cambridge, MA).

Adaptive transfer of EAE and treatment by KTX

One to four million 48-h Ag-activated PAS T cells were injected i.p. into syngeneic rats. The rats were treated by s.c. injections of the indicated doses of KTX in 1 ml of PBS starting the day of the first clinical signs of EAE. Control rats received PBS. The rats were weighed and observed daily. Rats without loss of weight and clinical signs of EAE were consid- ered negative. They were not included in the group of rats from which the mean maximal severity of clinical EAE was calculated.

Measurement of DTH reaction

Ear thickness was measured with a pressure-sensitive micrometer before and 48 h after the intradermal injection of MBP or saline on day 2 after EAE induction.

Statistical analysis

Statistical analysis was conducted using the Mann-Whitney U test. Mean differences between groups were considered significant at values of \(p < 0.05\).

Results

The MBP T cell line constitutively expresses Kv1.3 mRNA

As shown in Fig. 1, PAS lymphocytes express significant levels of Kv1.3 mRNA (time 0 after activation). A signal was readily detectable after amplification of a minute amount of cDNA (roughly corresponding to 250 pg of total RNA at time 0 after activation). The amplified fragment for Kv1.3 had the expected size and comi tated with the amplified fragment from cDNA obtained from rat
inactivated upon depolarization. The voltage for half-activation after 2 days of Ag stimulation. Channels rapidly activated and (upper traces) shows the Kv currents recorded in a PAS T cell. Experimental points were fitted to hyperbolic curves with 1/\(k_{\text{on}}\) increased linearly with the KTX concentrations, but 1/\(k_{\text{off}}\) was constant and independent from the KTX concentrations. Corresponding rate constants were 22.1 \times 10^6 \text{ M}^{-1} \text{s}^{-1} and 4.8 \times 10^{-3} \text{s}^{-1}. The \(K_{\text{p}}\) determined by the ratio \(k_{\text{off}}/k_{\text{on}}\) was 4 \text{nM}, close to that obtained in the precedent dose-response experiment.

**Kv blockers inhibit Ag-induced but not Jurkat JH6.2 T cell proliferation**

To determine whether blockers of Kv current would inhibit T cell proliferation, as was reported for mitogenically stimulated lymphocytes (15, 39, 40), we performed Ag-dependent T cell proliferation assays. PAS T cells were cultured with Ag and various concentrations of three \(K^+\) channel blockers. KTX inhibited the proliferation of the PAS T cells in a dose-dependent manner with an IC\(_{50}\) of 2.8 nM (Fig. 2A). High inhibition rates (70–95%) were observed from 5.1 to 0.1 nM KTX. KTX was nontoxic, as demonstrated by trypan blue exclusion after a 72-h culture of T cells with 100 nM KTX. Higher concentrations of the blocker did not further increase the inhibition (data not shown). Similar results were obtained with another Kv1.3 blocker, MgTX (data not shown). ChTX, a Kv and calcium-activated potassium channel blocker (1 nM, Fig. 1B). The blockade progressively decreased when the KTX perfusion was removed. However, patch clamping was difficult to maintain on PAS T cells, probably because of the extreme motility of the membranes, which rarely makes the seal stable for more than 10 min. Therefore, we further characterized the properties of the blockade of Kv1.3 induced by KTX in Jurkat JH6.2 cells, a human T cell leukemia clone constitutively expressing Kv1.3.

FIGURE 1. Semiquantitative RT-PCR of Kv1.3 mRNA levels in quiescent and activated lymphocytes. A, Kv1.3 amplification of various quantities of lymphocyte cDNA (expressed as picograms of initial RNA) demonstrates a linear profile at 32 cycles (no star), whereas plateau is reached at 37 cycles (two stars). Amplification of rat genomic DNA (1000 and 250 pg) gave a fragment of similar size (mainly visible at 37 cycles; two stars). PCR performed on nonreverse-transcribed lymphocyte RNA remained blank. B, PCR for GAPDH and Kv1.3 were performed on lymphocyte cDNA (equivalent to 250 pg of total RNA) at different times after activation. PCR were run in duplicate (indicated as a and b). For each transcript, PCR were prepared from a single reaction mixture. After adding the target cDNA, each reaction mixture was divided into three (GAPDH) or two (Kv1.3) aliquots. These aliquots were sequentially withdrawn from the thermocycler after 28 (no star), 33 (one star), and 38 (two stars) cycles in the case of GAPDH and after 32 (no star) and 37 (one star) cycles in the case of Kv1.3. Linearity of PCR as a function of target input was respected until the 33rd cycle for GAPDH and the 32nd cycle for Kv1.3 (data not shown in B).
 additions directly to the CTL-L and L-M cell cultures. Fig. 3A shows that KTX inhibited IL-2 production in a dose-dependent manner. Complete inhibition of IL-2 production was achieved at 100 nM KTX. In all the experiments, IC$_{50}$ was <12.5 nM, a value similar to those reported by other authors studying the ChTX inhibition of IL-2 production in anti-CD3- or PHA-stimulated human peripheral blood T lymphocytes (16, 18, 40). Because IL-2 production is necessary for PAS T cell proliferation, its addition may restore the proliferation inhibited in the presence of KTX, as described in the case of ChTX (15). To test this hypothesis, T cells were stimulated with MBP and cultured in the presence of IL-2 (0.5, 4, 20, or 50 U/ml) and KTX at various concentrations (data not shown). Exogenous IL-2 (4 U/ml) did not increase the T cell proliferation in

FIGURE 2.  
KTX blockade of Kv1.3 current in MBP-activated PAS and Jurkat JH6.2 T cells.  
A.  PAS T cells.  
Top left, Superimposed Kv1.3 currents induced by 150-ms depolarizing pulses at −50 to 40 mV (in 10-mV steps) from a holding potential of −80 mV. Leak and capacitative currents were subtracted.  
Top right, The voltage dependence of the activation was determined from fit of g/g$_{\text{max}}$ with a Boltzmann function.  
Bottom, Effects of 1 nM KTX on the Kv1.3 currents induced by the same pulse protocol as that described above. The test curve was performed 8 min after the application of KTX and 7 min after the wash.  
B.  Jurkat JH6.2 T cells.  
Top left, Superimposed Kv1.3 currents induced by 150-ms depolarizing pulses at −50 to 20 mV (in 10-mV steps) from a holding potential of −80 mV.  
Top right, KTX blockade of the Kv1.3 current recorded at 20 mV induced by 1 and 10 nM KTX applied successively. The two recordings were obtained from different cells.  
Bottom left, Fraction of the unblocked current plotted vs the KTX concentrations. The experimental points were fitted to a hyperbolic curve corresponding to a Hill coefficient n = 1.02 ± 0.1. Points are the means ± SE of three to nine experiments.  
Bottom right, Plot of 1/t for blockade (1/t$_{\text{on}}$) and recovery (1/t$_{\text{off}}$) as a function of KTX concentrations. Points are the means ± SE of four to five experiments.

FIGURE 3.  
Effects of Kv blockers on MBP-induced and Jurkat JH6.2 T cell proliferation.  
A.  Dose response of Kv blockers on MBP-specific T cell line.  
PAS T cells (2.5 × 10$^3$/well) were activated with MBP (10 µg/ml) and APC (2 × 10$^5$/well) in the presence of the channel blocker peptides, KTX (■), ChTX (○), or α-DTX (×).  
B.  KTX at active concentrations on PAS T cells does not block the proliferation of a leukemic cell line.  
Cells were cultured for 72 h. [3H]thymidine (1 µCi/well, sp. act. 2 Ci/mmol; Amersham, Little Chalfont, Buckinghamshire, U.K.) was added to each well for the final 18 h of culture. The data are mean percentages ± SD from three independent experiments normalized to [3H]thymidine uptake in the absence of blockers. Each condition was performed in quadruplicate wells.  
SD for each point was <10%. Without stimulus, cpm values were <400; with MBP, cpm values ranged from 100 to 250 × 10$^3$. Jurkat JH6.2 cpm values ranged from 100 to 150 × 10$^3$. blocker, also inhibited the T cell proliferation (IC$_{50}$ = 3.8 nM). As expected, α-DTX, a blocker of Kv1.1, Kv1.2, and Kv1.6 (41), all absent in lymphocytes, had no effect on the PAS T cell proliferation.

To determine whether KTX was a general inhibitor of cell proliferation, and could therefore also block the growth of continuously dividing cells expressing Kv1.3, KTX activity was tested on the growth of Jurkat JH6.2 T cells. KTX, like ChTX, at active concentrations on MBP-activated T cells, had no effect on the proliferation of Jurkat JH6.2 cells (Fig. 2B). Therefore, K$^+$ blockers inhibit the proliferation of primary T cells, which need an external stimulus to proliferate, but not of transformed cells.

KTX reduces IL-2 and TNF production by MBP-activated T cells

We assayed the IL-2 and TNF contents in culture SNs of T cells stimulated with MBP and APC in the presence of various concentrations of KTX. CTL-L and L-M cells were used to measure the IL-2 and TNF levels, respectively. Control SNs, prepared with APC maintained in culture for the same period with 100 nM KTX, had no effect in either biological assay, nor did KTX (100 nM)
The absence of KTX (Fig. 3B). The T cell proliferation was inhibited by 59% with 100 nM KTX in the presence of IL-2, as compared with 88% in the absence of IL-2. This partial restoration of the proliferation was not enhanced by IL-2 in amounts up to 50 U/ml (data not shown). This finding might be accounted for by the lack of IL-2R up-regulation that we found when T cells were MBP activated in the presence of KTX (data not shown). KTX also inhibited TNF production. TNF levels of SNs without KTX, harvested at 7 and 22 h of culture, were within the 15- to 25-ng/ml range in two independent experiments. The biological activity of TNF was consistently reduced by 50% in the SNs produced in the presence of 100 nM KTX (Fig. 3C).

**KTX inhibits the Ag-induced rise in [Ca\(^{2+}\)]** of MBP-activated T cells

One of the earliest events following stimulation is a rise in [Ca\(^{2+}\)]. To investigate the effects of KTX on this rise, we measured the [Ca\(^{2+}\)], of PAS T cells stimulated by activated macrophages pulsed with MBP. After 10 min of T cell activation, a measurable, sustained rise in [Ca\(^{2+}\)], was recorded (Fig. 3D). This rise was 50% lower in the presence of 300 nM KTX and became equivalent to that recorded in T cells stimulated with purified protein derivative (PPD), an irrelevant Ag. Hence, KTX inhibited the specific sustained rise in [Ca\(^{2+}\)].

**KTX inhibits DTH reaction to MBP**

To test the in vivo effects of Kv1.3 blockade, we examined the ability of KTX to inhibit DTH reaction. Indeed, EAE is associated with DTH reaction to myelin Ag, here MBP. Rats were injected with MBP-activated PAS T cells and challenged for DTH reaction 48 h later; they received MBP intradermally into one ear and PBS into the other one, as control. DTH response was measured 48 and 72 h later. KTX was administered twice a day (16 μg s.c.) from the day of adoptive transfer until the measurements of induration.
KTX inhibited the DTH response by 37–71% (Table I). Of note, KTX-treated rat CNS displayed no differences in the extent of cell infiltrations compared with PBS-treated rat CNS except when the former developed a very mild disease not exceeding a score of 0.5 (data not shown).

**KTX in vitro blocks the acquisition of encephalitogenic properties during T cell activation**

The concentration of KTX that blocks >80% [3H]thymidine incorporation (100 nM) found in preliminary experiments was assayed in vitro to determine whether it would block the encephalitogenicity. The Kv blocker was present throughout the 2-day activation culture of PAS T line cells and washed off along with MBP before inoculation into naive syngeneic rats. Pretreatment of T cells with KTX clearly attenuated the severity of adoptively transferred EAE in 15 rats compared with 16 rats injected with untreated T cells (Table II, experiments 1 and 3). When KTX was also given in vivo to Lewis rats during the period of the disease induction, the beneficial effects became more pronounced, whereas the untreated rats died from EAE (Table II, experiment 2). Histopathological examinations of CNS of rats (Table II, experiment 3) indicated that KTX-treated rat CNS exhibited as many EAE lesions as PBS-treated rat CNS (data not shown).

**KTX reduces the clinical severity of ongoing EAE**

To explore the in vivo efficacy of the Kv blocker for the treatment of a neurological autoimmune disease, adoptive EAE was transferred into Lewis rats with MBP-activated PAS T cells. Acute EAE developed 4–5 days later, lasted for 4–5 days (moderate EAE), or rapidly evolved to moribund state (lethal EAE). As soon as a clinical sign was detected, rats were treated with various doses of KTX for 2 or 3 days. KTX administration reduced the severity of the disease, prevented its mortality, and shortened its duration (Table III). The beneficial effects of KTX were dose dependent. In experiment A, when the highest dose (32 μg) was administered, both the clinical score and duration of EAE were significantly reduced (p < 0.001 and p < 0.002, respectively). When 8 μg was given, differences between clinical severities of the KTX-treated animals (1.9 ± 1.3) and the control rats (2.7 ± 1.2) were nearly significant (p = 0.1). The lowest dose (0.8 μg) displayed no effects. In experiment B, in which 8 μg was given, five KTX-treated rats developed discrete EAE, whereas five control rats were largely paralyzed and died (p < 0.004). Comparison of the s.c. and i.v. routes of KTX administration and of the volumes of KTX in saline injected (0.1 and 1 ml) did not reveal significant differences (data not shown). Taken together, these results prompted us to treat animals with the following conditions: 16 μg of KTX twice a day, in 1 ml PBS, s.c., as early as rats became sick. Of note, KTX, inhibitor of Kv1.1 and Kv1.3, injected into rats at 32 μg per day for 3 days, did not cause overt toxicity or neurological impairment. Pathological studies of CNS confirmed the absence of effects of KTX (data not shown). Blood biochemistry and hematology of KTX-treated rats were within the normal range of control animals (data not shown). In addition, KTX s.c. injected at high doses of up to 10 mg/kg is reported to induce few detectable symptoms in mice (32).

Lastly, brains and spinal cords of T cell recipients contained multiple inflammatory foci without notable discrepancy between

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### Table I. Inhibition of DTH to MBP is associated with inhibition of EAE in KTX-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence</th>
<th>Maximal clinical score</th>
<th>Mean Δ ear swelling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>8/8</td>
<td>1.75 ± 1.3</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>KTX</td>
<td>8/8</td>
<td>1.20 ± 1.1 (p = 0.02)</td>
<td>33 ± 10 (p = 0.035)</td>
</tr>
<tr>
<td>KTX</td>
<td>6/6</td>
<td>1.5 ± 0.5</td>
<td>47 ± 9</td>
</tr>
<tr>
<td>KTX</td>
<td>2/5</td>
<td>0.4 ± 0.5 (p = 0.01)</td>
<td>13.5 ± 14 (p = 0.01)</td>
</tr>
</tbody>
</table>

* Lewis rats were treated with KTX (16 μg s.c. twice a day) from day 0 to day 5.

### Table II. KTX in vitro blocks the acquisition of encephalitogenic properties during T cell activation

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Addition to Culture</th>
<th>Incidence</th>
<th>Clinical EAE</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS*</td>
<td>11/11</td>
<td>2.2 ± 0.9</td>
<td>5.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>KTX*</td>
<td>9/9</td>
<td>1.0 ± 0.0 (p &lt; 0.005)</td>
<td>3.7 ± 0.7 (p &lt; 0.05)</td>
</tr>
<tr>
<td>2</td>
<td>PBS*</td>
<td>5/5</td>
<td>6.0 ± 0</td>
<td>(death)</td>
</tr>
<tr>
<td></td>
<td>KTX*</td>
<td>5/5</td>
<td>1.6 ± 0.1</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PBS*</td>
<td>4/5</td>
<td>0.8 ± 0.4 (p &lt; 0.05)</td>
<td>2.4 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>PBS*</td>
<td>5/5</td>
<td>3.1 ± 0.6</td>
<td>5.4 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>KTX*</td>
<td>6/6</td>
<td>1.1 ± 0.4 (p &lt; 0.05)</td>
<td>4.5 ± 0.5 (p = 0.05)</td>
</tr>
</tbody>
</table>

* MBP-T cells were activated with KTX (100 nM) or PBS as control for 48 h. Rats were injected with MBP-T cells i.p. (day 0). The same number of blast cells was injected in each rat within the same experiment.

In addition, these two groups of animals received 8 μg of KTX s.c. from day 0 to day 6. Control rats received PBS s.c.
KTX-treated and untreated groups, as revealed by histological examination (data not shown). As KTX was given after the cell infiltration had developed, this infiltration was unaffected as expected.

**Discussion**

We tested KTX, a high affinity blocker of Kv1.1 and Kv1.3, to investigate both the pertinence of its use for treating autoimmune demyelinating diseases and the role of Kv in lymphocyte functions. This study shows that KTX inhibited Kv1.3 current and activation of encephalitogenic T cells, as reflected by the inhibition of the rise in [Ca^{2+}]. IL-2, and TNF production and by the large decrease in T cell proliferation induced by the Ag. KTX had no effect on the proliferation of leukemic T cells although they express Kv1.3. This finding underlines the specific effects of KTX on the T cell-induced activation process. Moreover, KTX was found to block the acquisition of encephalitogenic properties in vitro.

Finally, we provide evidence that KTX attenuated the severity of clinical adoptive EAE by 50% at least, and the DTH reaction to MBP. This is to our knowledge the first report relating the potential of a Kv blocker with restricted specificity in attenuating an autoimmune disease of the CNS. First, we will look at the in vitro results and then at how KTX may act in vivo.

We found that Kv1.3 mRNA transcription in rat encephalitogenic T line cells, specific for MBP, is constitutive and slightly increases by 50% upon activation. This finding is in good agreement with the moderate increases in the peak K^{+} current density (5- to 10-fold after T cell activation) described in mouse and rat T cells (42, 43). Next, electrophysiological characterization of K^{+} channel MBP T cells indicated that, with respect to current activation and inactivation, they are similar to the n type K channels in T lymphocytes (43 and 44, respectively) and to the Kv1.3 expressed in heterologous systems (36–38). Moreover, current blockade by three peptides known to block Kv1.3 (ChTX, KTX, and MgTX) strongly support the conclusion that the rat T cells used in this study expressed Kv1.3.

We then confirmed that KTX binds to lymphocyte Kv1.3 with an IC_{50} of 2 nM. Blockade and unblockade kinetics of Kv1.3 indicated that one molecule of KTX is sufficient to block one Kv1.3 (45). The IC_{50} value correlates well with the value reported for the KTX blockade of Kv1.3 in human T (26) and B cells (46). Kv and the calcium-activated potassium channel are thought to be involved in establishing the T cell membrane potential and providing the electrical driving force for Ca^{2+} influx and the subsequent cell activation (13). Yet, there are conflicting results on K^{+} involvement in the activation, probably because of differences between the T cell activation conditions, the donors, and the functional T cell assays, and possibly also because of the participation of other channels in sustaining the requisite membrane potential (15, 16, 26, 40, 47). In this study, we eliminated circumstantial variability of this kind by using an encephalitogenic rat T cell line reactive to MBP to explore several functions. We found that the IC_{50} value of KTX inhibiting T cell proliferation was similar to that found inhibiting Kv1.3 currents. This similarity suggests that the effects of the toxin on mitogenesis were mediated by these currents, as proposed for ChTX (16, 47). Our in vitro results revealed three particular features. First, the reproducible marked inhibition of the T cell proliferation observed here contrasts with that described by other authors (15, 16, 26). In particular, KTX, known to be a potent selective blocker of the Kv currents in mammalian cells (11, 26, 37), was suggested to be a weak inhibitor of T cell proliferation and to slightly reduce steady-state [Ca^{2+}], without affecting peak [Ca^{2+}], in comparison with ChTX (26). That we found KTX to be a potent inhibitor of T cell proliferation and activation might be accounted for by differences between experimental protocols but also by the origin and the concentrations of the serum added to the culture medium. Note that low serum concentrations favor the antiproliferative action of Kv blockers (15, 48). Second, we establish that rat T cells are adequate targets for inhibiting T cell activation by a Kv1.3 blocker; this is not the case for mouse T cells (23). Rat MBP T cells have been reported to be good targets for less specific K^{+} blockers. Thus, 4-AP blocks their proliferation and the acquisition of their encephalitogenicity (49) and the nonpeptidyl blocker, 5,8-diethoxysporalen (H37, Ref. 22) inhibits whole-cell K^{+} currents, their proliferation, and secretion of IFN-γ after activation. Thus, one can now test Kv blocker effects in vivo in well-known experimental rodent models. Third, the KTX inhibition of the proliferation of MBP-specific T cells was associated with a marked reduction in TNF production. To our knowledge, this is the first time the effects of K^{+} channel blocker on TNF production have been documented (see Fig. 2C).

It is well known that T cell activation is required for migration into CNS and encephalitogenicity (17, 50, 51). We show that KTX, added to the culture at the beginning of T cell activation, prevented T cells from becoming encephalitogenic. Therefore, it is highly probable that the decrease observed in encephalitogenicity correlated with the drop in Th1 type cytokine production and the inhibition of proliferation caused by the KTX-impairment of Ca^{2+} signaling. Immunosuppression can be obtained through the regulation of the Ca^{2+} signaling pathways in T lymphocytes. Yet, to be of interest, the mechanism of action of new compounds should differ from that of the current immunosuppressors such as cyclosporin A. KTX fulfills this requisite. First, KTX does not cross the membrane (52). Next, the mechanism whereby KTX interacts with T cell activation involves a decrease in the Ca^{2+} influx, which limits the Ca^{2+-} dependent production of IL-2, and ultimately the cell proliferation. Therefore, KTX intervenes earlier in the activation cascade than do cyclosporin A and FK-506. Lastly, because of the restricted tissue distribution of Kv1.1 and Kv1.3, KTX and related blockers might not have such toxic side effects as many of the

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### Table III. Therapy of EAE by KTX

<table>
<thead>
<tr>
<th>Treatment of Recipients(^{a}) ((\mu g) KTX)</th>
<th>Maximal clinical score</th>
<th>Incidence</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Moderate EAE</td>
<td>2.7 ± 1.2</td>
<td>21/21</td>
<td>4.9 ± 2.0</td>
</tr>
<tr>
<td>8</td>
<td>2.6 ± 1.3 ((p = 0.9))</td>
<td>8/8</td>
<td>4.6 ± 0.5 ((p = 0.4))</td>
</tr>
<tr>
<td>32</td>
<td>1.9 ± 1.3 ((p = 0.1))</td>
<td>10/10</td>
<td>3.2 ± 2.1 ((p = 0.1))</td>
</tr>
<tr>
<td>B Lethal EAE</td>
<td>1.4 ± 1.0 ((p = 0.001))</td>
<td>15/16</td>
<td>2.9 ± 1.6 ((p = 0.002))</td>
</tr>
<tr>
<td>8</td>
<td>6.0 ± 0</td>
<td>5/5</td>
<td>3.6 ± 0.5 until death</td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.2 ((p = 0.004))</td>
<td>5/5</td>
<td>2.1 ± 1.1</td>
</tr>
</tbody>
</table>

\(^{a}\) Lewis rats were injected with encephalitogenic MBP-activated T line cells (1.5 and 3 million for moderate EAE; 4 million for lethal EAE), i.p. (day 0). They were treated with KTX s.c. when the first clinical signs occurred, for 2 or 3 days. Control rats received PBS s.c.
current immunosuppressive drugs, such as cyclosporin A, FK-506, and rapamycin (14). Overall, these data show that KTX is a potent immunosuppressive agent and that Kv1.3 is a relevant target for immunosuppression.

In vivo, several mechanisms may account for the symptomatic improvement of EAE by KTX. KTX may exert two kinds of actions: one neurological, the other immunosuppressive. We provide evidence that KTX exerted immune suppression in vivo because KTX treatment decreased DTH response in Lewis rats. KTX effects could come into play at the level of migration of T cells from the blood vessels into tissues including CNS. However, we did not find evidence of major differences between the inflammatory lesions of CNS of rats injected with KTX-treated T cells and untreated T cells, and between the levels of adhesion molecule expression, in particular, very late antigen (VLA)-4, of T cells activated in the presence and in the absence of KTX (data not shown). Hence, our results are in agreement with neither this hypothesis nor with the results of Levite et al. (53). They demonstrate that elevated levels of (K+) in induced T cell adhesion to fibronectin-coated wells is inhibited by specific Kv1.3 blockers and that Kv1.3 is physically and functionally linked to β integrin moieties. Alternatively, the fact that KTX interfered with T cell functions, resulting in the peripheral immunosuppression observed, could be due to an inhibition of de novo Ag activation. We further show that KTX down-regulated the production of IL-2 and TNF by encephalitogenic T cells, and TNF-α and TNF-β play a role in the inflammation and in the formation of demyelinating lesions in EAE (54). Because blocking TNF-α prevents EAE (55, 56), both of these KTX-induced effects would interfere in vivo with the development of EAE. KTX could also inhibit the production of other proinflammatory cytokines and/or enhance that of contra-inflammatory ones such as TGF-β, which is increased by cyclosporin A in human T cells (57). Interestingly, TGF-β mediates suppression in EAE (58). On the basis of these data, KTX may play an important role on the immune system in inhibiting EAE.

Besides, KTX may exert an action on the CNS during EAE. No single animal model mimics all the features of human demyelinating diseases; rather, the available models reflect specific facets. Here, we focus on acute EAE in Lewis rats. Acute EAE can be considered to roughly mimic the initial attack of MS (59). Besides, adoptive EAE was chosen because highly pathogenic T cell lines can be easily established from this species and this strain. In that model, clinical signs run in a more synchronous course, which makes the results of drug treatment easier to analyze. Moreover, note that the encephalitogenic T cell line used here produces demyelination, although limited. Demyelination resulting from adoptive transfer of MBP T cells has already been reported (60–62). Paralysis has been shown to parallel the slowing of conduction in the CNS (61). A broad spectrum K+ blocker such as 4-AP restores synaptic transmission, which makes the results of drug treatment easier to analyze. Moreover, the prompt onset of at least some of the beneficial effects of 4-AP therapy in patients supports the notion of direct effects on the CNS, whatever the mechanism may be. Note that in contrast to 4-AP, immunosuppression is obtained by similar KTX concentrations in vivo and in vitro (10–50 nM).

In conclusion, we confirm that Kv1.3 is a relevant target for immunosuppression and we demonstrate that KTX is a potent candidate drug for treating autoimmune pathologies. Moreover, because KTX can also block Kv1.1, it may play a role directly at the neurological level, a role that could be reflected in its beneficial symptomatic effects in EAE. This role has to be investigated. Our studies point the way to future work to develop selective Kv1.3 and Kv1.1 blockers for treating autoimmune-demyelinating neurodegenerative diseases.

Acknowledgments

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References


