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The Antifungal Imidazole Clotrimazole and its Major In Vivo Metabolite are Potent Blockers of the Calcium-Activated Potassium Channel in Murine Erythroleukemia Cells

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Abstract. Clotrimazole (CLT), a member of the antifungal imidazole family of compounds, has been found to inhibit both calcium (Ca^{2+})-activated ^{86}Rb and potassium (K) fluxes of human red cells and to inhibit red cell binding of ^{125}I-charybdotoxin (ChTX) [11]. We have now used patch-clamp techniques to demonstrate reversible inhibition of whole cell $K_{Ca^{2+}}$ currents in murine erythroleukemia (MEL) cells by submicromolar concentrations of CLT. Inhibition was equivalent whether currents were elicited by bath application of the Ca^{2+} ionophore A23187 or by dialyzing cells with a pipette solution containing micromolar concentrations of free Ca^{2+}. The extent of inhibition of whole cell MEL $K_{Ca^{2+}}$ currents was voltage-dependent, decreasing with increasing test potential. We also determined the single channel basis of the CLT inhibition in MEL cells by demonstrating the inhibition of a calcium-activated, ChTX-sensitive K channel by CLT in outside-out patches. The channel was also blocked by the des-imidazolyl metabolite of CLT, 2-chlorophenyl-bisphenyl-methanol (MET II) [15], thus demonstrating that the imidazole ring is not required for the inhibitory action of CLT. Single $K_{Ca^{2+}}$ channels were also evident in inside-out patches of MEL cells. Block of K current by CLT was not unique to MEL cells. CLT also inhibited a component of the whole cell K current in PC12 cells. Channel specificity of block by CLT was determined by examining its effects on other types of voltage-sensitive currents. CLT block showed the following rank order of potency: K currents in PC12 cells > Ca^{2+} currents in PC12 cells ≫ Na currents in sympathetic neurons. These results demonstrate that direct inhibition of single $K_{Ca^{2+}}$ by CLT can be dissociated from inhibition of cytochrome P-450 in MEL cells.

Key words: Charybdotoxin — Clotrimazole — Cytochrome P-450 — Iberiotoxin — murine erythroleukemia cells — MEL cells — Pheochromocytoma cells — PC12 — Potassium channels — Gardos channel

Introduction

A voltage-insensitive, calcium (Ca^{2+})-activated potassium (K) channel [23, 32], also known as the Gardos channel [19], is thought to play a major role in volume regulation in normal [24] and sickled (SS) human erythrocytes [9, 11]. Its biophysical and pharmacological properties have been characterized in excised inside-out human red cell membrane patches, in which Ca^{2+}-activated K ($K_{Ca^{2+}}$) currents show inwardly rectifying properties with a unitary slope conductance ranging from 15–40 pS, depending on the ionic conditions used [21, 23, 32].

This current shares pharmacological properties with other $K_{Ca^{2+}}$ channels of intermediate conductance. It is insensitive to apamin, a bee venom toxin inhibitor of small-conductance $K_{Ca^{2+}}$ channels, but can be inhibited

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by more general K channel blockers such as barium, cesium, quinine and tetraethylammonium (TEA) [13, 22]. The scorpion venom toxin charybdotoxin (ChTX), a pore blocker of certain voltage-activated K currents as well as of $K_{Ca^{2+}}$ currents [12, 35], binds to both human and rabbit red blood cells where it potently inhibits Ca^{2+}-activated ^{86}Rb uptake and efflux and K efflux [10, 37, 52]. As a result of its activity, ChTX inhibits in vitro formation of dense cells, irreversible dehydration, and sickling of human sickle (SS) red blood cells [37, 52].

In addition to these traditional K channel blockers, a subset of antifungal imidazoles recently has been found to block K flux and ^{125}I-ChTX binding to normal and SS human red blood cells at nanomolar concentrations with clotrimazole (CLT) being the most potent imidazole tested [1, 8, 9, 11]. The combined results of ^{86}Rb flux and ^{125}I-ChTX binding studies raise the possibility that CLT inhibits Ca^{2+}-activated K transport by directly binding to the external surface of the Gardos channel.

CLT has long been used as an antimycotic drug to treat local and systemic fungal infections. Its mechanism of antifungal action has been attributed to its demonstrated inhibition of cytochrome P-450-dependent demethylation of lanosterol to ergosterol, the major lipid component of fungal cell membranes [28, 49]. It also inhibits cytochrome P-450 oxygenase activity in various types of mammalian cells [5, 36, 41, 42, 46]. In 1992, Garcia-Sancho and his colleagues reported the inhibition of K flux in human erythrocytes by CLT and other imidazoles in the micromolar and submicromolar range [1]. They attributed this inhibition of K transport to inhibition of cytochrome P-450 oxygenases, despite the absence of P-450 activity in red cell membranes [7, 14] and the inability of the P-450 inhibitor carbon monoxide to block K transport [1]. Further experiments showed that CLT also blocks Ca^{2+}-activated ^{86}Rb influx and, in the presence of the chloride transport inhibitor DIDS, lowers membrane potential in A23187-treated SS red cells [9, 11]. However, the ability of CLT to displace the specifically bound Gardos channel ligand, ^{125}I-ChTX, with potency equivalent to that of Ca^{2+}-activated ^{86}Rb influx blockade suggested that CLT acts directly upon the Gardos channel polypeptide [11], rather than via inhibition of cytochrome P-450 [1].

As a potent inhibitor of the $K_{Ca^{2+}}$ transport pathway, CLT blocks cell dehydration of SS and normal red cells in vitro [11] and when given orally to normal adults [8] and to patients with sickle cell anemia [9]. CLT has been shown in human clinical trials to attenuate red cell dehydration in sickle cell anemia at doses 10 times lower than those previously used to treat systemic fungal infections [9].

Because of the candidacy of CLT as a symptomatic treatment for sickle cell disease, and because CLT represents a novel class of inhibitors of K flux through $K_{Ca^{2+}}$ channels, the ability of CLT to block $K_{Ca^{2+}}$ current was tested in Friend murine erythroleukemia cells (MEL), a nucleated proerythroblastic cell line immortalized by integration of the Friend murine erythroleukemia virus [16, 17]. Upon induction with dimethyl sulfoxide, hexamethylene-bis-acetamide or other agents these developmentally arrested cells are induced to express both hemoglobin and the major erythrocyte membrane proteins of mature red blood cells [38, 39, 48].

Induced MEL cells have been shown to express three ion currents. A chloride (Cl) current and a stretch-activated cation current can be detected in cell-attached patches, but are not evident in whole cell recording conditions [4]. In addition, these cells have a $K_{Ca^{2+}}$ current that resembles the current of the Gardos channel in red cells [4, 6], with a similar unitary conductance, pharmacology and voltage-independence. MEL cells offer several advantages over human red cells for the recording of $K_{Ca^{2+}}$ currents, including a larger diameter, better adhesion to substrates, ability to withstand suction without collapse, and a greater estimated number of $K_{Ca^{2+}}$ channels/cell [4, 52]. In this paper we use whole-cell, outside-out, and inside-out patch clamp techniques to demonstrate that CLT potently blocks the intermediate conductance $K_{Ca^{2+}}$ channel in MEL cells. We additionally show that CLT blocks K currents in PC12 cells.

Materials and Methods

Cell Culture

Murine erythroleukemia (MEL) cells were cultured in suspension in Dulbecco's modified Eagle's medium (DMEM) containing 7.5% calf serum (Sigma), 7.5% fetal bovine serum (Hazelton), 4 mM glutamine (Sigma), and 1% penicillin-streptomycin (Gibco). Cells were maintained at 37°C, in a 5% CO_2 atmosphere and were split 1:5 every 3 to 5 days. Cells were plated on poly-L-lysine-coated coverslips (Sigma) for electrical recording and used the same day.

Superior cervical ganglia (SCG) were removed from the neck areas of ether-anesthetized 1-to-3 day-old Wistar rats (Charles River) following decapitation. Both ganglia were mechanically dissociated using standard methods [27]. The outer connective tissue surrounding each ganglion was teased away. Cells were separated by gently triturating ganglia 1–3 times through a 22 gauge 1-inch syringe needle. The dissociated cells were plated on poly-L-lysine, diluted 1:2 with double distilled water, and cultured at 37°C in DMEM, as above, in the presence of 2 μg/ml nerve growth factor isolated from mouse salivary glands [40]. Recordings were made within 48 hr after plating the cells.

PC12 cell stocks [20] were maintained in 25 cm^2 flasks (Corning) in DMEM, as above, and split 1:8 every 7–10 days. Recordings were made from cells that had been cultured for at least 4 days on collagen-coated coverslips (murine Type IV, Collaborative Research) and cultured in DMEM as above, but without nerve growth factor.

Whole Cell Patch-Clamp Recordings

The currents were recorded at room temperature (20°–22°C) in the whole cell patch configuration with a Dagan integrating patch-clamp

amplifier (≠3900) using standard techniques [25]. Currents were filtered at 1–5 kHz and digitized at 5 times the filter cutoff frequency. Recordings were saved and analyzed on a PDP-11/73 computer (INDEC). MEL whole cell recordings were not leak-subtracted because $K_{Ca^{2+}}$ channels were constitutively open under some of the recording conditions used. MEL cells were voltage-clamped at holding potentials characterized by minimal current flow. Holding potentials so chosen were dependent on the ionic conditions. All other whole cell currents had leak currents and capacitative transients subtracted from them. Current amplitudes of K and Ca^{2+} currents were measured at the end of the test pulse. Whole cell Na current amplitudes were measured at peak inward current. Statistical significance of current differences was calculated using Student's paired *t*-tests and *t*-tests for two means or repeated measures ANOVA. In the case of the latter, post-tests with *t*-tests corrected for multiple comparisons with the Bonferroni method were used; (Microsoft Excel 6.0, or Instat).

The salt solutions used for measuring K currents were varied to optimize the recording conditions for each cell type. Three different recording conditions were used to measure $K_{Ca^{2+}}$ currents in MEL cells.

(1) K currents activated in MEL cells by the Ca^{2+} ionophore A23187 were recorded in the following conditions (in mM); *Bath Solution:* 145 NaCl, 5.4 KCl, 10 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 5 Glucose, pH 7.40. *Pipette Solution:* 14 NaCl, 120 KCl, 10 HEPES, 10 ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), 4 $CaCl_2$, 2 $MgCl_2$, pH 7.35 (115 nM free internal Ca^{2+}). Free Ca^{2+} concentrations of the pipette solutions were calculated using the program Chelator [45].

(2) K currents activated in MEL cells by the Ca^{2+} ionophore A23187 in low Cl solutions were recorded in the following conditions (in mM); *Bath Solution:* 135 N-methyl-D-glucamine (NMG)-aspartate, 5.4 K-aspartate, 10 HEPES, 5 glucose, 1 EGTA, 2 $CaCl_2$, 0.15 $MgCl_2$, pH 7.4. *Pipette Solution:* 120 K-Aspartate, 10 HEPES, 1 EGTA, 0.15 $MgCl_2$, 0.15 $CaCl_2$, 4 ATP, pH 7.4 (22 nM free internal Ca^{2+}).

(3) For the recording of K currents in MEL cells in the absence of the Ca^{2+} ionophore, the following conditions were used in mM; *Bath Solution:* 135 NMG-aspartate, 5.4 K-aspartate, 10 HEPES, 5 glucose, 2 $CaCl_2$, 30 μg/ml bovine serum albumin (BSA; fatty acid free), pH 7.4. *Pipette Solution:* 130 K-Aspartate, 10 HEPES, 1 EGTA, 0.15 $MgCl_2$, 1.11 $CaCl_2$, 4 ATP, pH 7.35 (10 μM free internal Ca^{2+}).

Whole cell K currents in PC12 cells were recorded using the following conditions (in mM); *Bath Solution:* 145 NaCl, 5.4 KCl, 10 HEPES, 5 Glucose, 1.8 $CaCl_2$, 0.9 $MgCl_2$, 30 μg/ml BSA (fatty acid free), pH 7.40. *Pipette Solution:* 10 NaCl, 140 KH_2PO_4, 10 HEPES, 2 $MgCl_2$, 1 EGTA, 4 ATP, pH 7.40. Recording conditions which differed from these are noted in the text.

Whole-cell recordings of current flow through Ca^{2+} channels in MEL and PC12 cells were obtained with the following solutions (in mM); *Bath Solution:* 20 Ba-acetate, 135 NMG-aspartate, 10 HEPES, 30 μg/ml BSA (fatty acid free), pH 7.4. *Pipette Solution:* 135 Cs-aspartate, 10 EGTA, 5 $MgCl_2$, 4 ATP and 0.4 GTP, pH 7.40.

Whole cell Na currents in SCG neurons were recorded under the following conditions (in mM): *Bath Solution:* 88 NMG-aspartate, 50 NaCl, 2.4 KCl, 10 EGTA, 10 TEA, 1.8 $CaCl_2$, 0.9 $MgCl_2$, 30 μg/ml BSA (fatty acid free), pH 7.40. *Pipette Solution:* 135 Cs-Aspartate, 10 EGTA, 5 $MgCl_2$, 4 ATP, pH 7.40.

Stock solutions of the dihydropyridine agonist (+)-202-791 (gift from Dr. Hof, Sandoz), the Ca^{2+} ionophore A23187 (Sigma), clotrimazole (Sigma), and 2-chlorophenyl-bisphenyl-methanol (Metabolite II, gift from Drs. G. Kroll, Miles, and W. Ritter, Bayer) were made up in 100% ethanol or DMSO and then diluted at least 1,000-fold just prior to use. All drugs were introduced into the recording chamber by superfusion. Iberiotoxin (IbTX) and ChTX were purchased from Peptides International and TEA from Aldrich.

Single-Channel Patch Clamp

Single-channel currents were recorded using standard techniques [25]. All voltages refer to the cell interior referenced to the patch pipette. Currents were measured with a 10 GΩ headstage, low pass-filtered at 100–500 Hz (Axopatch 1-D, Axon Instruments, Burlingame, CA), digitized at 1–5 kHz, and stored on the hard-drive of an Hewlett Packard 486-DX computer. PCLAMP 6.0.3 software was used to control data acquisition via a Digidata 1200 interface and to analyze data (Axon Instruments, Burlingame, CA). Data were acquired continuously using FETCHEX subroutines. A digital filter was employed during subsequent analysis (FETCHAN subroutine) to further filter the data (100 Hz) for determinations of NP_o, the product of N, the number of open channels and P_o, the open probability (*see below*).

The single-channel pipette and bath solution compositions used in studies of outside-out patches in the absence of ionophore was the same as in whole cell protocol 3, except that Na aspartate was substituted for NMG aspartate in the bath. When Ca^{2+} ionophore was used, the solutions used were the same as in the whole cell protocol 2, but with Na aspartate substituting for NMG aspartate. After a cell-attached gigaseal patch was attained, whole cell configuration was attained by gentle suction. The outside-out configuration was attained subsequently by slowly withdrawing the micropipette from the cell body.

Cell-attached patches were excised to form the inside-out configuration to allow further study of calcium activation. In these experiments a high K aspartate solution was used in the pipette solution. Junction potentials were measured and subtracted for the calculation of changes in reversal potentials during selectivity experiments. The Goldman-Hodgkin-Katz equation was used for the determination of relative permeability.

Control recordings were made for the generation of *I-V* plots and the determination of NP_o. Drugs were tested at a maintained membrane potential of +25 mV, chosen because whole cell experiments indicated that voltage-dependent relief of CLT block was minimal at this potential. NP_o, the product of N, the number of observed open channels, and P_o, the open probability, was calculated as follows:

$$NP_o = \sum_{n=0}^{N} \frac{n(t_n)}{T}$$

where T was the record time, n was the number of channels open and t_n was the time during n channels are open. Therefore, NP_o can be calculated without any assumptions about the total number of channels in a patch or the open probability of a single-channel. NP_o was calculated from 30-sec records during control and experimental periods. Burst analysis was carried out to characterize the blocking effect of CLT in a single channel patch (*see* Sigurdson et al. [47])).

All experiments were conducted at 21°C. Mean values are reported ± standard error.

Results

Whole Cell $K_{Ca^{2+}}$ Currents in MEL Cells

Outward cation currents could be induced in MEL cells either by adding the Ca^{2+} ionophore A23187 (0.3 μM) to the bath (Figs. 1*A*, 2) or by raising the internal free Ca^{2+}

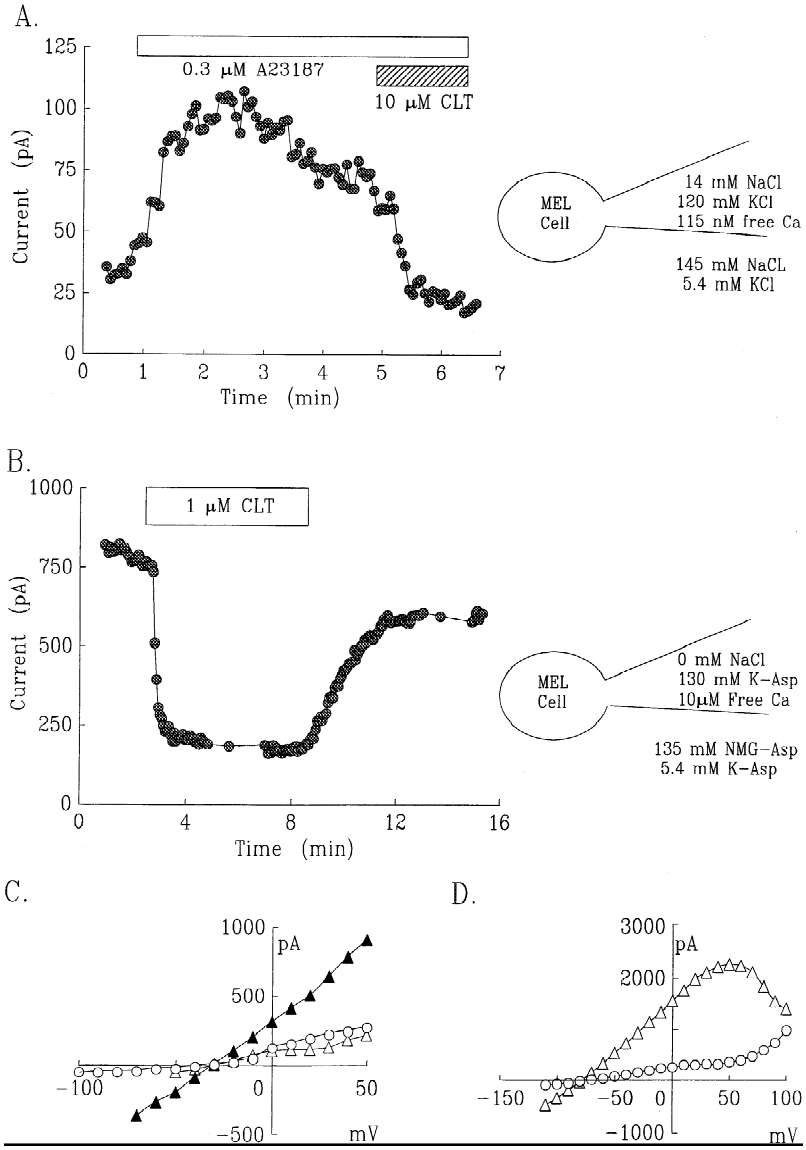

Fig. 1. Clotrimazole (CLT) inhibits whole cell K currents in Murine Erythroleukemia (MEL) Cells. MEL cells appear to express one type of K channel activity, a $K_{Ca^{2+}}$ channel of intermediate conductance [4]. (*A*) An example of the decrease in K currents over time with addition of 10 μM CLT to the bath in the presence of 0.3 μM A23187. Right inset summarizes recording conditions. Currents were measured from a cell held at −30 mV and stepped to +50 mV. (*B*) Plot of K current vs time demonstrates reversibility of the effects of 1 μM CLT when essentially fatty acid-free BSA (30 μg/ml) is included in the bath. In this experiment channels are constitutively activated by high internal Ca^{2+} (10 μM) concentration. Currents were measured from cells that were held at −80 mV and stepped to +30 mV. Right inset summarizes recording conditions. (*C*) Current-voltage relationships from the experiment shown in *A*. Holding potential, −30 mV. (*D*) Current-voltage relationships from the experiment shown in *B*. Holding potential, −80 mV. Open triangles, control; solid triangles A23187; open circles, CLT.

concentration (Figs. 1*B*, 2). At 115 nM free internal [Ca^{2+}], outward currents that could be further activated by the Ca^{2+} ionophore A23187 were present in both uninduced and induced MEL cells (*data not shown*). Therefore, uninduced cells were used for all subsequent recordings. We verified that this outward cation current in MEL cells is a $K_{Ca^{2+}}$ current. Addition of the Ca^{2+} ionophore A23187 (0.3 μM) to the bath significantly increased the relative outward current at a test potential of +50 mV by 460 ± 177% ($n = 8$, $P < 0.05$) as illustrated in the plot of current amplitude versus time (Fig. 1*A*), or by 2.3-fold when displayed as means of current amplitudes (Fig. 2, left set of bars, $P < 0.05$ by Wilcoxon paired sample test). When aspartate was substituted for the majority of internal and external chloride and *N*-methyl-D-glucamine (NMG) replaced external Na, addition of A23187 still produced an increase in relative current of 358 ± 142% or 2.2-fold ($P < 0.05$) when displayed as means of absolute currents (Fig. 2, middle set of bars). This increase did not differ significantly in magnitude ($P > 0.10$) from the A23187-elicited current measured in the presence of normal Cl (Fig. 2, middle set of bars). Furthermore, the current density, measured as pA/pF, in the presence of A23187 was not significantly different ($P > 0.10$) when aspartate was substituted for Cl. These data were consistent with the absence of detectable Cl current under identical whole cell recording conditions (*data not shown*). They are also consistent with the presence in induced MEL cells of Cl current recorded in the cell-attached configuration but absent in the whole cell configuration, as reported previously [4]. A similar current density also was observed when outward currents were induced by including 10 μM free Ca^{2+} in the pipette solution (Fig. 1*B* and Fig. 2, right set of bars; not statistically different, $P > 0.10$, from either of the above conditions). These data indicate that the iono-

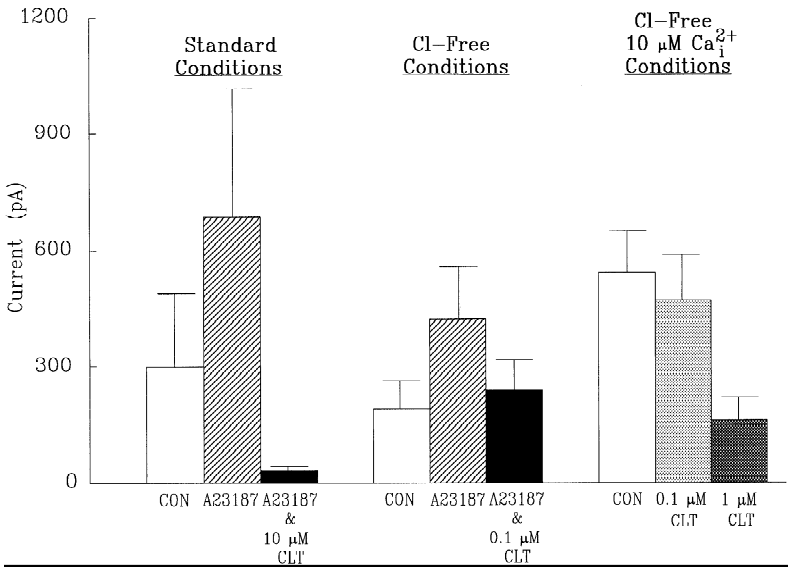

Fig. 2. Summary of the effects of CLT on $K_{Ca^{2+}}$ currents in MEL cells under different recording conditions. Left set of bars: 10 μM CLT significantly inhibited K currents activated by the Ca^{2+} ionophore A23187 (0.3 μM) using standard whole cell recording conditions ($P < 0.01$, two-tailed paired *t*-test). See Materials and Methods for complete description of solutions. Holding potential, −30 or −50 mV; test potential, +50 mV. Middle set of bars: when nominally Cl-free solutions were used in the presence of A23187 (0.3 μM), 0.1 μM CLT significantly decreased whole cell currents ($P < 0.05$, one-tailed paired *t*-test). Holding potential, −50 mV; test potential, +50 mV. Right set of bars: CLT also inhibited whole cell currents when nominally Cl-free solutions were used in the presence of a pipette solution containing 10 μM free Ca^{2+} concentration. $K_{Ca^{2+}}$ currents were significantly inhibited by 0.1 μM CLT and 1 μM CLT ($P < 0.01$, 2-tailed paired *t*-test). Holding potential, −80 mV; test potential, +30 mV. (n = 4–9 for each group). Open bars, control conditions; hatched bars, 0.3 μM A23187; black bars, A23187 plus CLT; gray bar, 10 μM free internal Ca^{2+} plus 0.1 μM CLT; dark, dotted bar, 10 μM free internal Ca^{2+} plus 1 μM CLT.

phore-induced current is carried by K ions and not by a Ca^{2+}-activated Cl current.

CLT Inhibits $K_{Ca^{2+}}$ Currents in MEL Cells

CLT inhibited whole cell outward currents in MEL cells under each of the three different recording conditions described above. First, current recorded in the presence of the Ca^{2+} ionophore A23187 was inhibited 96% following addition of 10 μM CLT ($n = 4$; Fig. 1*A*, Fig. 2 left set of bars). Second, despite the lack of evidence of a prominent Ca^{2+}-activated Cl current, we tested whether CLT was still an effective inhibitor of the whole cell current when the majority of Cl in the bath and pipette solutions was substituted with aspartate. In addition, the concentration of CLT was lowered 100-fold to 100 nM. Under these new conditions, the current recorded in the presence of ionophore was inhibited by CLT 43 ± 8% ($n = 4$; Fig. 2, $P < .05$), indicating that CLT inhibited a current other than a Cl current. This experiment also demonstrated that a submicromolar concentration of CLT was sufficient for substantial current inhibition. Third, CLT did not exert its effects on the $K_{Ca^{2+}}$ current indirectly by blocking the effects of the Ca^{2+} ionophore. When the $K_{Ca^{2+}}$ current was activated by dialyzing MEL cells with a high internal concentration of free Ca^{2+} (10 μM) in nominally Cl-free conditions, CLT was still able to significantly inhibit the outward current, sampled at a test potential of +30 mV, by 16 ± 5% ($n = 4$) at 100 nM and by 73 ± 5% ($n = 4$) at 1 μM (Fig. 1*B* and 2). The inhibition by CLT was readily reversible when BSA was included in the bath solution (*see* Fig. 1*B*). CLT also did not inhibit the $K_{Ca^{2+}}$ channel indirectly by blocking Ca^{2+} channels. No putative Ca^{2+} currents were detected in MEL cells when 20 mM Ba was used as the only charge carrier (I < 7 ± 1pA), nor was there an appearance of an inward Ba current when the dihydropyridine (+)-202-791 (1 μM), an agonist of L-type Ca^{2+} channels [30], was added to the bath (107 ± 9% of control level; $n = 4$; $P > 0.15$, one way paired *t*-test). Furthermore, when A23187 and CLT were applied together, no induction of outward K current was observed (*data not shown*). These data taken together indicate that the effects of CLT on whole cell cation current were attributable to inhibition of the $K_{Ca^{2+}}$ current.

The current-voltage relationships indicate that CLT inhibited both inward and outward flow of whole cell currents (Fig. 1*C* and *D*). However, when the inhibition of the currents was examined at test potentials greater than +50 mV, a dramatic reduction in the magnitude of inhibition by CLT could be observed in current-voltage relationships from individual recordings (Fig. 1*D*), as well as in a plot of the average inhibition of control current by μM CLT *vs.* test potential (Fig. 3). The voltage dependence of the magnitude of current inhibition by CLT in MEL cells may in part reflect the nonlinear behavior of control currents at positive potentials. This behavior is not seen in any other figure because Fig. 1*D* is the only figure showing whole cell currents measured at such elevated inside-positive clamped membrane potentials. However, in the presence of CLT at test potentials greater than +50 mV, the slope of the *I/V* relationship

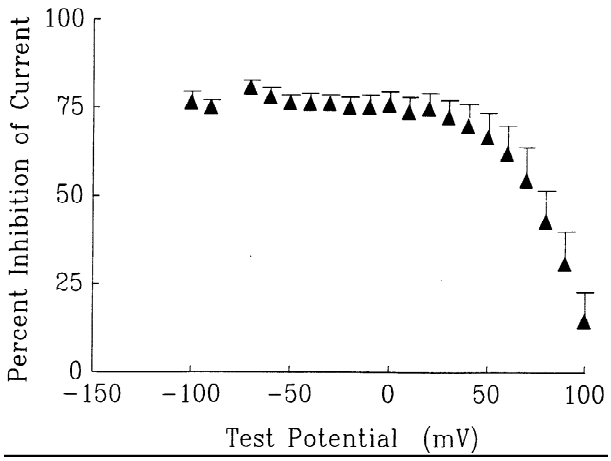

Fig. 3. Inhibition of whole cell K currents in MEL cells by CLT is dependent on voltage. Cells were held at a holding potential of −80 mV and stepped to various test potentials of 350 msec duration in the presence or absence of 1 μM CLT. After application of CLT, the whole cell current amplitude at a test potential of +30 mV was allowed to reach a new steady-state level before collecting sweeps for the current-voltage relationship. Maximal inhibition was reached usually within 60 sec after addition of CLT. Data are expressed as percent inhibition of the control current amplitude by 1 μM CLT and plotted for each test potential ± standard error of the mean. ($n = 5$ for each test potential).

increases, suggesting that at these positive test potentials CLT is less effective in inhibiting whole cell K currents.

Single Channel $K_{Ca^{2+}}$ Currents in MEL Cells: K Selectivity and Calcium Activation

We used single channel patch clamp techniques to determine the single-channel basis of the block by CLT of MEL whole cell currents. A K-selective channel could be observed in outside-out patches recorded with bath and pipette compositions similar to those used in the whole cell experiments. In asymmetric (K aspartate in, Na aspartate out) solutions, the mean slope conductance was 17.7 ± 2.3 pS ($n = 3$; Fig. 4), similar to that reported by Arcangeli et al. [4] in asymmetric solutions. The conductance of inside-out patches in symmetric K aspartate was 42.6 ± 4.4 pS ($n = 4$) and exhibited mild inward rectification (Fig. 5*B*), also similar to that reported previously in symmetric K solutions [4]. Outward currents at 0 mV holding potential were observed in outside-out patches in asymmetric solutions (K aspartate in, Na aspartate out), indicating that the channel was likely K selective. When the bath solution was changed from high Na aspartate to high K aspartate, the reversal potential changed 43 ± 8 mV ($n = 3$). When the same bath substitution was performed in the whole cell configuration the change in reversal potential was 51 ± 4 mV ($n = 3$), not significantly different from the result in the outside-out patch experiments. These changes in reversal potential indicated that the mean relative permeability of K:Na was 9:1 (*see* Fig. 4).

The channel activity was not voltage sensitive: NP_o was 0.64 ± 0.27 at +40 mV, 0.7 ± 0.15 at +25 mV, 0.52 ± 0.11 at −25 mV, and 0.49 ± .26 at −40 mV ($F = .22$, $P > 0.05$, $n = 3$, by ANOVA). The inside-out configuration was used to confirm that these K channels are Ca^{2+}-activated (Fig. 5). When the free Ca^{2+} concentration of the solution facing the cytoplasmic face of the patch was changed from 22 nM to 10 μM the NP_o significantly increased from 0.25 ± .07 to 1.01 ± .23 ($T = 3.644$, $P < 0.05$, $n = 4$). These results demonstrate that the unitary K currents that were detected are K-selective and are activated by increases in intracellular Ca^{2+}.

Outside-out patches were then used to study the effects of extracellular application of charybdotoxin, clotrimazole, and MET II on unitary $K_{Ca^{2+}}$ channel currents in MEL cells.

Clotrimazole and Charybdotoxin Inhibit Unitary $K_{Ca^{2+}}$ Currents in MEL Cells

To determine the single channel basis for the CLT inhibition of MEL whole cell K currents, outside-out patches from MEL cells exhibiting high $K_{Ca^{2+}}$ activity (due to 10 μM $[Ca^{2+}]$ in the pipette solution) were tested. In 5 patches CLT (1 μM) decreased NP_o from 0.55 ± 0.16 to 0.11 ± 0.09 ($T = 2.79$, $P < 0.05$, $n = 5$, by one-tailed *t*-test).

We next tested the ability of CLT to block unitary K channel activity elicited by the Ca^{2+} ionophore A23187 (0.3 μM). Outside-out patches from MEL cells having lower channel activity (with pipette $[Ca^{2+}]$ of 22 nM) were subsequently exposed to A23187, followed by A23187 + CLT (1 μM). The Ca^{2+} ionophore A23187 increased NP_o from 0.34 ± .12 to 0.69 ± .17 and CLT decreased NP_o to 0.15 ± .05 ($F = 8.91$, $P < 0.01$, $n = 6$, by one way ANOVA; Bonferroni *t*-tests: control *vs.* A23187, $T = 2.75$, $P < 0.056$; A23187 *vs.* CLT, $T = 4.15$, $P < 0.01$) (Fig. 6).

$K_{Ca^{2+}}$ channels from a variety of preparations and Ca^{2+}-activated K fluxes from erythrocytes have been reported to be ChTX sensitive [10, 12, 34, 37]. We tested the ChTX sensitivity of the MEL $K_{Ca^{2+}}$ channel in outside-out patches by superfusing 25 nM ChTX into the bath (Fig. 6*E*). In 6 patches ChTX decreased NP_o from 0.82 ± 0.17 to 0.20 ± .06 ($T = 4.25$, $P < 0.005$, $n = 6$).

Burst Analysis of CLT Inhibition

In most outside-out patches 2–3 channels were active, which limited the interpretation of kinetic data from the inhibitor studies. In a rare single channel patch, further analysis of inhibition by CLT was carried out (Fig. 7). Burst analysis indicated that the number of closing per

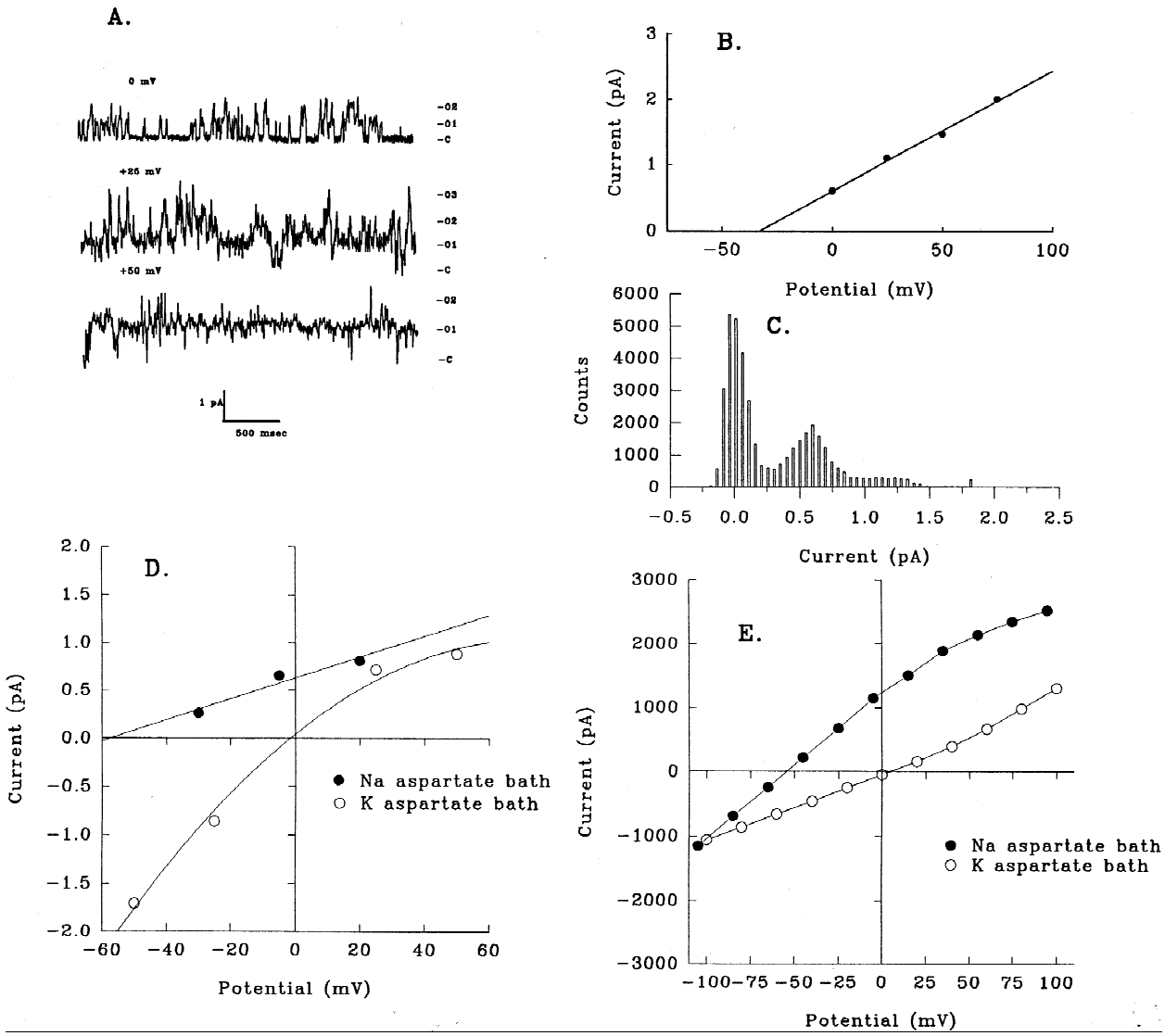

Fig. 4. K-selective single channel currents in outside-out patches from MEL cells. (*A*) Representative current traces recorded at the indicated potentials from an outside-out patch of MEL cell. C, closed state, O1, first opening, O2, second opening level. *Bath Solution:* 135 Na-aspartate, 5.4 K-aspartate, 10 HEPES, 5 glucose, 2 $CaCl_2$, 30 μg/ml bovine serum albumin (BSA; fatty acid free), pH 7.4. *Pipette Solution:* 130 K-Aspartate, 10 HEPES, 1 EGTA, 0.15 $MgCl_2$, 1.11 $CaCl_2$, 4 ATP, pH 7.35 (10 μM free internal Ca^{2+}). (*B*) Current voltage relationship constructed from traces in *A.* Slope conductance was 18 pS. (*C*) Representative amplitude histogram of current trace clamped at 0 mV. Gaussian fit of the data fitted peaks at 0, 0.56 ± .16, 1.18 ± .16, and 1.73 ± .09 pA, respectively, corresponding to C, O1, O2, and O3. (*D*) Representative experiment illustrating K selectivity of single-channel currents recorded from an MEL outside-out patch. The reversal potential changed from −2 mV in the K aspartate bath to −58 mV in the Na aspartate bath. By the Goldman-Hodgkin-Katz equation, the permeability ratio of K to Na is then 12:1. Lines fit by first order linear regression and second order linear regression for Na and K bath data, respectively. (*E*) K selectivity of whole cell currents recorded from an MEL cell. Solutions as in *A.* Reversal potential changed from +5 mV in K aspartate bath to −55 mV in Na aspartate bath, indicating a K:Na selectivity of 15:1.

burst at the optimal burst interval increased from 215 to 560 closings per burst, consistent with a flickery block by CLT that has been reported in carotid body cells [26]).

The Imidazole Ring is Not Required for CLT Inhibition of Unitary $K_{Ca^{2+}}$ Currents in MEL Cells

Brugnara et al. [8] reported that calcium-activated ^{86}Rb fluxes in erythrocytes were sensitive to two metabolites of CLT that lacked the imidazole ring, and thus were unable to inhibit cytochrome P-450. To determine if the imidazole ring is required for the inhibitory action of CLT on MEL $K_{Ca^{2+}}$ currents, MET II (10 μM) was added to the bathing solution of outside-out patches (Fig. 8). In the presence of MET II, control NP_o was reduced from 0.99 ± 0.08 to 0.13 ± 0.08 ($T = 10.99$, $P < 0.005$, $n = 4$). Therefore, since the imidazole ring is not required

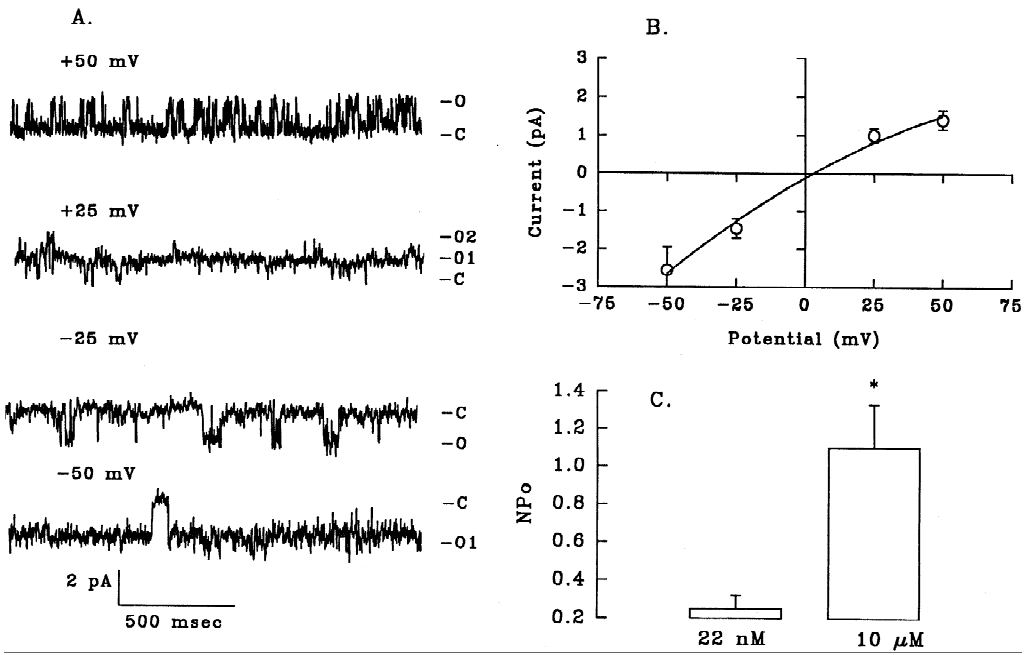

Fig. 5. MEL K channel is inwardly rectifying and activated by intracellular Ca^{2+}. Pipette solution was 135 K aspartate, 5 Na aspartate, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4. (*A*) Representative channel currents recorded at the indicated potentials in an inside-out patch in symmetrical K aspartate. Filtered at 500 Hz. (*B*) Current-voltage relationship of currents exhibited in *A*. Slope conductance (−25 to −50 mV) was 44 pS. (*C*) Substitution of 22 nM Ca^{2+} with a 10 μM bath significantly increased NP_o of MEL K channels in 4 paired experiments.

for inhibition of MEL $K_{Ca^{2+}}$ currents, inhibition of P-450 cannot be required for channel inhibition.

CLT Inhibits Whole Cell K Currents in PC12 Cells

To determine whether other cell types have whole cell K currents that can be inhibited by CLT, we examined whether CLT inhibits K currents in PC12 cells. Undifferentiated PC12 cells have at least 5 identifiable outward voltage-gated K currents [29]. One component is a $K_{Ca^{2+}}$ current that is insensitive to apamin, but blocked by TEA and ChTX [29, 44]. This $K_{Ca^{2+}}$ current can be activated by positive test potentials as well as a rise in the intracellular Ca^{2+} concentration. We verified that a component of the whole cell outward current of PC12 cells cultured under our conditions has the pharmacological profile of $K_{Ca^{2+}}$ currents. First, the majority of the whole cell current was inhibited by 25 nM ChTX. In addition, 100 nM iberiotoxin (IbTX), a more selective toxin blocker of $K_{Ca^{2+}}$ currents [18], inhibited the whole cell K current by 38 ± 9% (Fig. 9). From the plot of peak current *vs.* time (Fig. 10*A*) as well as from the individual sweeps (Fig. 10*C*) it can be seen that 5 μM CLT rapidly and reversibly inhibited the whole cell K current in PC12 cells by 76 ± 5% (Fig. 10*B; n* = 7). Whether CLT inhibited the same whole cell component as IbTX was not determined. However, the magnitude of inhibition is similar when submicromolar concentrations of CLT were used (Table). From the examination of the kinetics of the individual sweeps in this experiment (Fig. 10*C*), it appears that the current resistant to CLT has an increased rate of inactivation during the test pulse. However, in a separate series of experiments using lower concentrations of CLT that still blocked a large component of the current, the CLT-resistant inactivating current component was absent (Fig. 11*B*). PC12 cells have variable amounts of transient outward K current [29], and therefore this kinetic variability of the CLT-resistant current was not pursued. The current-voltage relationship indicates (Fig. 10*D*) that CLT inhibits whole K cell currents at all potentials tested.

Clotrimazole Preferentially Blocks K Currents

The potency of block by CLT was compared for whole cell K, Na and Ca^{2+} currents. While CLT inhibited voltage-activated Na currents in neonatal SCG neurons (Table, Fig. 11*A*) only at concentrations (10 μM) known to strongly inhibit cytochrome P-450 oxygenases [46], K and Ca^{2+} currents in PC12 cells were inhibited by lower concentrations of CLT. CLT blockade of the most sensitive portion of whole cell K currents was maximal at 10 nM (Table, Fig. 11*B*). Only in the presence of 5 μM (Fig. 10*A*) or 10 μM CLT (Table), did the K current decrease further to nearly complete inhibition. Na currents in

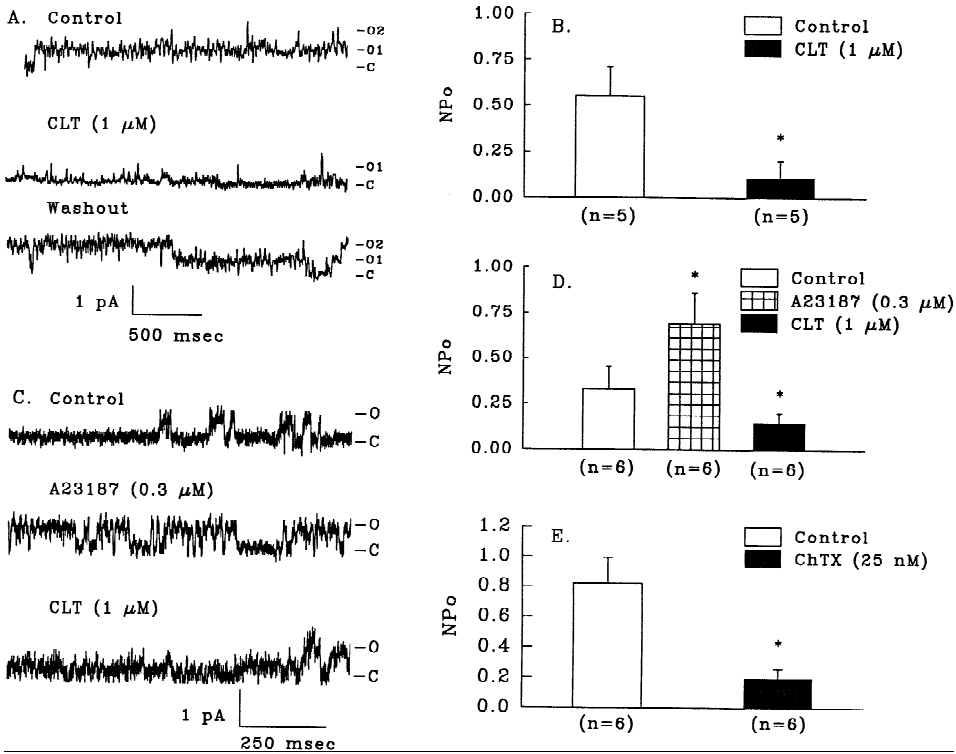

Fig. 6. Clotrimazole inhibits MEL K channels when first activated by pipette calcium or by A23187 in the bath. (*A*) Representative outside-out patch experiment illustrating reversible inhibition by clotrimazole of control K channel activity observed when 10 μM Ca^{2+} was included in pipette. V_m = +25 mV. (*B*) Mean NP_o of five paired MEL experiments as in *A*, illustrating significant inhibition by clotrimazole (*See results*). (*C*) Representative current traces from an outside-out patch illustrating control, A23187-activated, and clotrimazole-inhibited K channel activity. K currents activated in MEL cells by the Ca^{2+} ionophore, A23187, in low Cl solution. V_m = +25 mV. (*D*) CLT-inhibited, calcium-activated NP_o of 6 paired experiments as in *C*. (*E*) Charybdotoxin inhibits MEL K channel activity. Mean NP_o of 6 paired experiments demonstrating significant inhibition of NP_o by 25 nM charybdotoxin in outside-out patches. 10 μM Ca^{2+} was included in pipette solution. V_m = +25 mV.

SCG neurons were unaffected by CLT until the concentration was raised to 10 μM, when the currents were inhibited. Ca^{2+} currents in PC12 cells showed an intermediate sensitivity to block by CLT. They appear to be ~30 to 100-fold less sensitive to CLT than were the most sensitive component of K current (Table, Fig. 11*C*). Like the Na and K currents, the majority of the Ca^{2+} current was blocked by 10 μM CLT. This selectivity profile suggests that at 10 μM CLT, the additional inhibition of whole cell currents may be due to a different mechanism, perhaps to inhibition of cytochrome P-450 oxygenases. The rank order of sensitivity of cation currents to CLT was K currents > Ca^{2+} currents ⩾ Na currents (Table).

Discussion

CLT, a member of the antifungal imidazole family of compounds, represents a new class of potent, small molecule K channel blockers. Previously CLT has been found to inhibit ^{86}Rb and K fluxes across normal and SS human red cells as well as to inhibit the binding of ^{125}I-ChTX to these cells [8, 9, 11]. This report demonstrates with whole cell and single channel patch-clamp techniques the blockade by CLT of $K_{Ca^{2+}}$ currents in an erythroid cell type, the MEL cell. Moreover, submicromolar concentrations of CLT inhibit whole cell K currents in two additional cell types, PC12 (this work) and portal vein VSM cells which both display a component of $K_{Ca^{2+}}$ current. (A.R. Rittenhouse et al., 1997). CLT has been reported to inhibit additional ion fluxes across membranes, but with 10–100-fold higher IC_{50}s than reported for the inhibition of Ca^{2+}-activated K fluxes [1, 11]. Various imidazoles, including CLT, inhibit Ca^{2+} influx in GH_3 cells, thymocytes, platelets and neutrophils [2, 50]. CLT at micromolar concentrations also inhibits noninactivating, whole cell voltage-gated K currents in pulmonary artery VSM cells [54], and whole cell K currents in type I carotid cells [26].

Whole Cell $K_{Ca^{2+}}$ Currents in MEL Cells

While there has been extensive biophysical characterization of the large conductance $K_{Ca^{2+}}$ currents found in

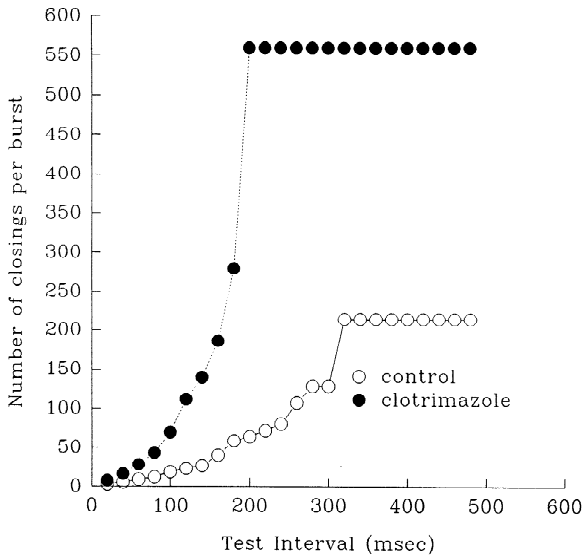

Fig. 7. Burst analysis indicates that the number of closures during a burst increases with CLT application. This display of number of closings per burst vs interburst interval illustrates that as the test interval increases, more events will be included in the burst (rising phase) and that when the interburst interval becomes identical to the true interburst interval, the number of closings per burst will be relatively insensitive to further changes in the test interval (a plateau is evident). (*See* Sigurdson et al. (47)). At the optimal interburst interval, the number of closings per burst was 215 in control and 560 after CLT application. CLT was tested in a single channel patch recorded in the outside-out configuration. Open circles, control; closed circles, CLT (1 μM).

muscle preparations both at the single channel and whole cell level [31], much less is known about the voltage-insensitive, intermediate conductance $K_{Ca^{2+}}$ currents found in erythrocytes. The small number of $K_{Ca^{2+}}$ channels present in the red, cell, between 1–200 functional channels/human red cell, or 120 ChTX binding sites/human red cell [21, 23, 33, 52] makes recording whole cell $K_{Ca^{2+}}$ currents technically difficult. This limitation can be ameliorated by recording $K_{Ca^{2+}}$ currents from MEL cells, with an estimated 500 functional channels/cell [4]. An additional feature of using the MEL cell is that all patch configurations can be obtained.

Using cell-attached and whole cell patch-clamp recording techniques, Arcangeli et al. [4] described three ionic currents in MEL cells. One of these was a $K_{Ca^{2+}}$ current of intermediate conductance, similar to the $K_{Ca^{2+}}$ currents described in human red blood cells [22, 23, 32]. Similar to $K_{Ca^{2+}}$ currents of intermediate conductance in human erythrocytes, K currents in MEL cells displayed a unitary conductance of 50 pS when 140 mM K was included in the bath and pipette solutions [4]. These K currents were voltage-insensitive, but were maximally activated by micromolar concentrations of free Ca^{2+} [4, 6]. In this latter recording condition a linear whole cell current-voltage relationship from −60 to 0 mV was observed. Under recording conditions with 10 μM free internal Ca^{2+} and nominally Cl free media, we found a similar linearity in the current-voltage relationship. However, with higher test potentials, the current appeared to saturate, making the overall current-voltage relationship inwardly rectifying. This inward rectification is similar to that observed in unitary current-voltage relationships of $K_{Ca^{2+}}$ currents in human red cells. Deviations from linearity are thought to be due to the relative amounts of K, Na, and Mg inside the cell *vs.* in the bath, as Na and Mg ions have been found to inhibit current flow through $K_{Ca^{2+}}$ channels in red cells [13, 22].

Comparison of CLT Effects on MEL Cell Currents with Effects on Red Blood Cell Fluxes

In addition to inhibiting K flux in red blood cells [8, 9, 11], CLT inhibits whole cell $K_{Ca^{2+}}$ currents in MEL cells. Despite the different recording conditions, CLT inhibited inward and outward K currents activated either by the Ca^{2+} ionophore A23187 or by recording with free internal Ca^{2+} concentrations in the pipette raised from 100 nM to 10 μM. CLT did not inhibit $K_{Ca^{2+}}$ currents indirectly by blocking Ca^{2+} channels, because their presence was undetectable even when (+)-202-791, the dihydropyridine agonist of L-type channels, was included in the bath solution.

Block of whole cell $K_{Ca^{2+}}$ currents in MEL cells by CLT is consistent with its inhibitory actions in flux studies in human erythrocytes. Submicromolar concentrations of CLT blocked ^{86}Rb influx into both normal and SS human red cells in vitro [8, 9, 11]. CLT maximally blocked A23187-induced ^{86}Rb influx 87% with an IC_{50} of 50 nM in 140 nM NaCl, 2 mM KCl, pH 8.0. Despite the different experimental conditions, the efficacy and potency of block by CLT of whole cell $K_{Ca^{2+}}$ currents and *^{86}Rb* influx at normal ionic strength are within several-fold of one another, consistent with block of the same pathway.

Mechanism of Action

The mechanism by which CLT inhibits $K_{Ca^{2+}}$ currents remains unknown. Three different mechanisms have been proposed to explain the actions of CLT. First, its ability to inhibit cytochrome P-450-dependent enzyme systems occurs by the interaction of imidazole nitrogens with the heme iron of cytochrome P-450. This interaction interferes with the binding of carbon monoxide to these hemoproteins, and presumably inhibits the binding of oxygen as well, thus inhibiting enzymatic activity [46, 49, 51, 53]. Alvarez et al. [1, 2] have proposed a mechanism for the inhibition of both Ca^{2+} and $K_{Ca^{2+}}$ currents by CLT involving the inhibition of cytochrome P-450 enzymes situated at internal Ca^{2+} stores, resulting in the

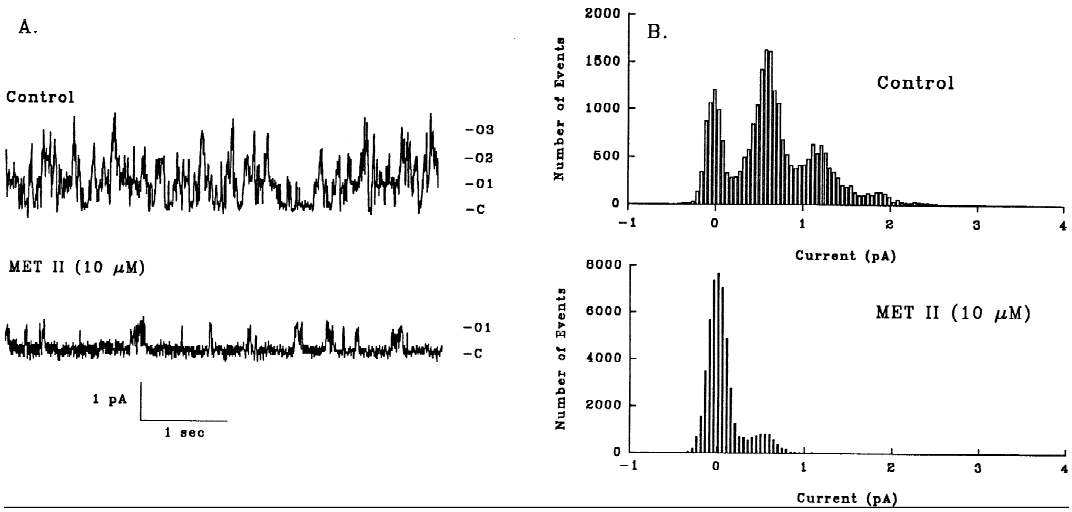

Fig. 8. The imidazole ring is not required for the inhibitory action of CLT. (*A*) Representative current records from an outside-out patch illustrating control channel activity and activity recorded after MET II was added to the bath. V_m = +25 mV, solutions as in Fig. 4. (*B*) Amplitude histogram of experiment in *A*, illustrating the decreased number of events in the 4 open levels and increased number of events in the closed state with MET II.

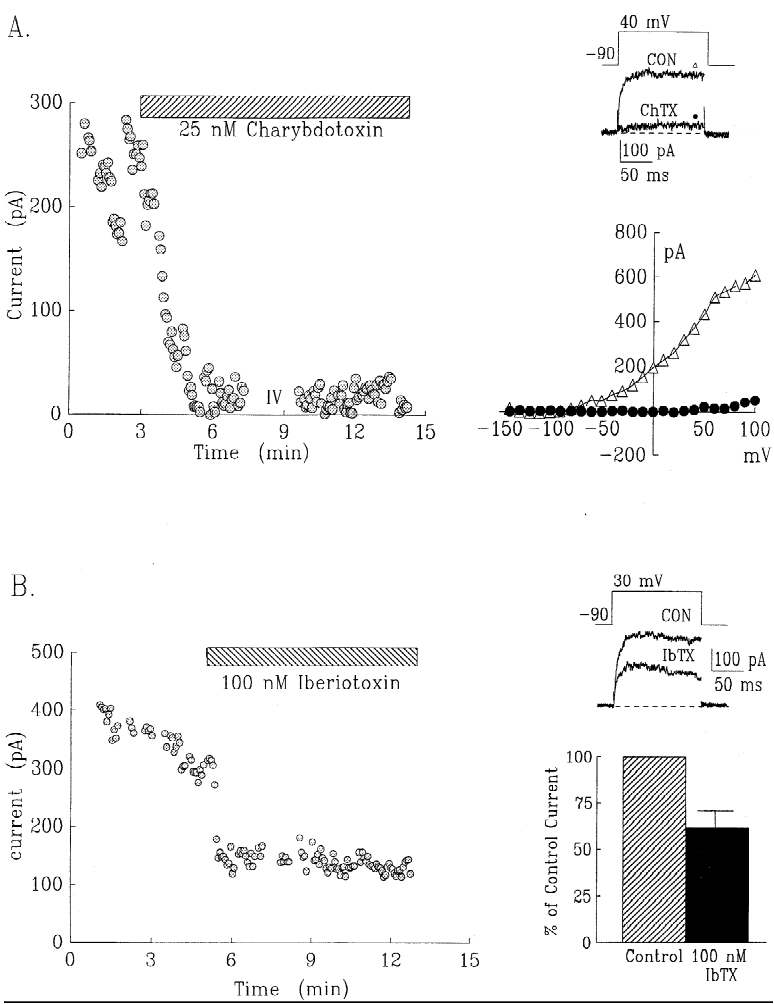

Fig. 9. Whole cell K currents in PC12 cells grown in 10% fetal bovine serum without NGF have a component of $K_{Ca^{2+}}$ current defined pharmacologically by its sensitivity to both charybdotoxin (ChTX) and iberiotoxin (IbTX). (*A*) The inhibition by ChTX can be observed in the plot of current amplitude vs time (left), in selected individual sweeps (upper right), and in current-voltage relationships (lower right) for control (open triangles) and toxin treatment (filled circles). (*B*) IbTX inhibits K current as observed in both the plot of current amplitude vs time (left) and in the individual sweeps (upper right) 38 ± 9% (lower right; $n = 8$; $P < 0.005$, 2-tailed paired *t*-test). Symbols above the individual sweeps show where current amplitude was measured. *Bath Solution:* 145 NaCl, 5.4 mM KCl, 10 mM HEPES, 5 mM Glucose, 1 mM EGTA, 0.7 mM $CaCl_2$, 0.15 mM $MgCl_2$ (155 nM free internal Ca^{2+}) pH 7.40. *Pipette Solution:* 140 mM K-aspartate, 10 mM HEPES, 0.15 mM $CaCl_2$, 1 mM EGTA, 0.15 mM $MgCl_2$ (23 nM free internal Ca^{2+}) pH 7.40.

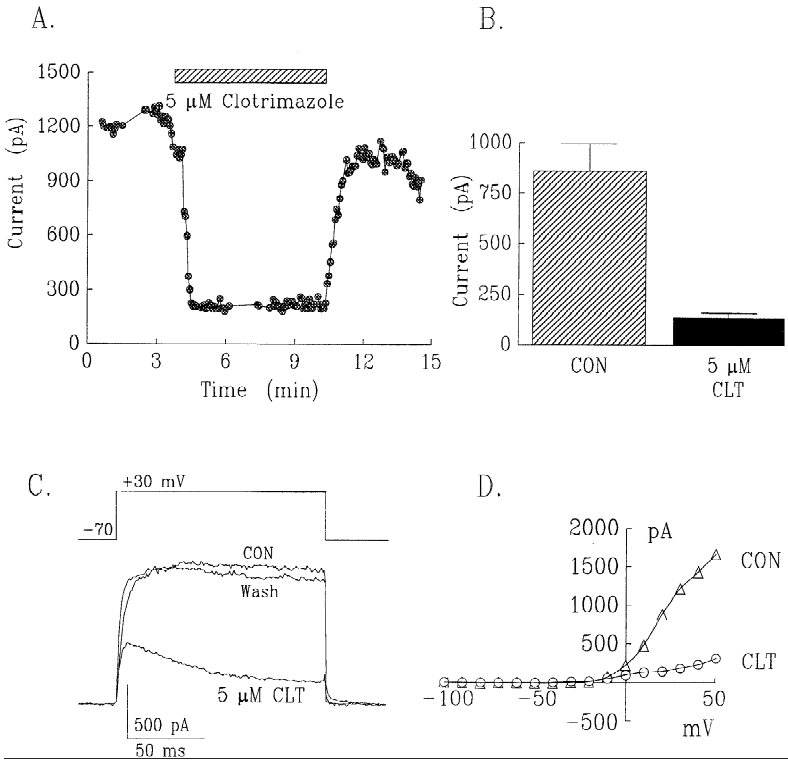

Fig. 10. Clotrimazole (CLT) reversibly inhibits whole cell K Currents in PC12 cells. 5 μM CLT reversibly (*A*) blocks the K currents in PC12 cells by 75 ± 5% (*B; P* < 0.005, 2-tailed paired *t*-test; *n* = 4), leaving a small component of inactivating current that can be observed in the individual sweeps (*C*) taken from the experiment shown in *A*. (*D*) Current-voltage relationships from experiment shown in *A*. No change in the threshold for activation was observed. *Bath Solution:* 145 NaCl, 5.4 mM KCl, 10 mM HEPES, 1.8 mM $CaCl_2$, 5 mM Glucose, pH 7.40. *Pipette Solution:* Same as Fig. 9. Holding potential = −70 mV. Test potential = +30 mV.

Table. Summary of the selectivity of clotrimazole for K currents. *n* = 3–10 cells for each concentration of CLT

Current type	Percent of control current			
	Clotrimazole concentration			
	10 nM	100 nM	1 μM	10 μM
I_{Na^+}	ND	104 ± 10	111 ± 10	22 ± 9
I_{K^+}	63 ± 9*	72 ± 8*	60 ± 3**	11 ± 1**
$I_{Ca^{2+}}$	92 ± 2*	88 ± 5*	65 ± 6**	8 ± 2*

Significance, *P < 0.01; **P < 0.001 for 2-tailed paired *t*-test. Na currents were measured in neonatal rat superior cervical ganglion neurons. K currents and Ca^{2+} currents were measured in PC12 cells.

inhibition of Ca^{2+} release and a consequent inhibition of both plasmalemmal currents. Interestingly, when they tested for inhibition of ^{42}K influx into red cells, by the P-450 monoxygenase inhibitor carbon monoxide, they found no effect. However, rather than suggesting a different mechanism of CLT action, they proposed that a novel carbon monoxide-insensitive hemoprotein may be involved in the regulation of the red cell $K_{Ca^{2+}}$ channel [1].

The inhibitory effects of CLT on noninactivating voltage-sensitive K currents in pulmonary artery cells have also been attributed to regulation of these channels by cytochrome P-450 oxygenases [54]. These authors propose that P-450 enzymes or their products bind directly to the channel proteins to inhibit K currents. However, the irreversible P-450 antagonist aminobenztriazole at 1 mM concentration did not produce irreversible inhibition of K currents, again raising the possibility that a different mechanism is involved. Aminobenztriazole was similarly inactive as an inhibitor of K and Ca^{2+} currents in type I carotid body cells [26].

Second, CLT acts as a competitive calmodulin antagonist in addition to its actions of binding to the heme-iron of various enzymes. Wolff et al. [51, 53] found that CLT inhibits calmodulin-dependent stimulation of nitric oxide synthase activity as well as calmodulin-dependent cyclic nucleotide phosphodiesterase activity by competing for the calmodulin binding site. They have proposed that in addition to disruption of oxygen binding to hemoproteins, CLT acts at a second site either by binding to calmodulin directly or by binding to proteins at a site that interferes with calmodulin binding. Thus, another possibility is that CLT inhibits $K_{Ca^{2+}}$ currents by decreasing a hypothetical direct calmodulin interaction with the channel or by inhibiting modulation of the channel by calmodulin-dependent enzymes.

Third, our data suggest that CLT acts to inhibit ion currents by direct interaction with the external face of the channel to inhibit K currents. This possibility is corroborated by the fact that submicromolar concentrations of CLT inhibit the binding of ^{125}I-ChTX to human red blood cells [11]. ChTX is known to bind to the external pore region of maxi-K channels with higher affinity for open *vs.* closed channels [3, 34]. CLT might in theory also inhibit channel opening via potent inhibition of cy-

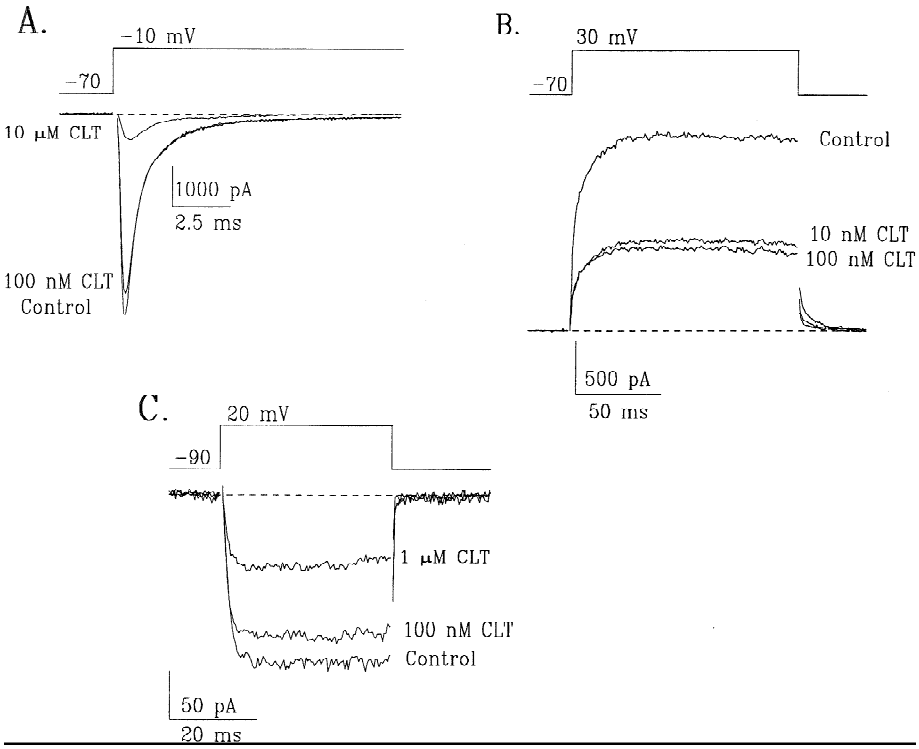

Fig. 11. Clotrimazole (CLT) preferentially blocks K currents. (*A*) CLT blocks Na currents in neonatal rat superior cervical ganglion neurons only at concentrations (10 μM) known to inhibit P-450 oxygenases. (*B*) The first component of CLT block of whole cell K currents in PC12 cells is maximal by 10 nM. Only at 10 μM CLT does the current decrease further (*see* Table). (*C*) Ca^{2+} currents in PC12 cells are approximately 30- to 100-fold less sensitive to block by CLT than are K currents.

tochrome P-450 or P-450 like enzymes. The lower number of open channels might then account for the observed shift in affinity of binding for ChTX, without a requirement for direct binding of CLT to the channel or to a related protein. However, a major in vivo metabolite of CLT, 2-chlorophenyl-bisphenyl methanol, also displaces ^{125}I-ChTX binding and inhibits Ca^{2+}-activated ^{86}Rb influx in normal red blood cells [8, 15]. This metabolite lacks the imidazole ring responsible for the inhibitory effects of antifungal imidazoles towards cytochrome P-450 enzymes [43].

We have identified single channels in MEL cells that are K-selective, calcium-activated, and ChTX sensitive with a conductance of 18 pS in asymmetric recording solutions. These characteristics are identical to those reported by others for the MEL cell $K_{Ca^{2+}}$ channel and for the Gardos channel of erythrocytes [4, 23]. The inhibition of unitary $K_{Ca^{2+}}$ currents by both CLT and MET II argues that CLT must have direct effects on the $K_{Ca^{2+}}$ channels, independent of inhibition of cytochrome P-450. In agreement with this last interpretation are the recent patch clamp studies of Hatton and Peers, who report that the related imidazole compound miconazole could directly block unitary $K_{Ca^{2+}}$ channels in the carotid body [26]. Furthermore, like the pore blocking activity of ChTX [3], the magnitude of inhibition by CLT of the whole cell current in MEL cells (Fig. 3) and in ferret portal vein VSM (A.R. Rittenhouse et al., 1997) decreased with increasing test potential, suggesting that CLT can be displaced from a binding site on a part of the channel.

However, the characteristics of the inhibition of whole cell currents by CLT in other cells (Table) leaves open the possibility that there are multiple mechanisms by which CLT inhibits ion currents. CLT had no effect on Na currents in SCG neurons until the concentration was raised to 10 μM, a concentration at which CLT inhibits cytochrome P-450 oxygenases. Likewise, the partial inhibition of both K and Ca^{2+} currents at low concentrations increased dramatically when the CLT concentration was raised from 1 to 10 μM, producing additional inhibition of whole cell K currents in PC12 cells. The results are consistent with an additional mechanism of inhibition at higher concentrations of CLT, and with lower potency blockade of additional types of cation channels by CLT.

Potential Use of CLT as a $K_{Ca^{2+}}$ Channel Inhibitor In vitro and In vivo

In summary, CLT shows promise as a potent small molecule ligand of $K_{Ca^{2+}}$ channels. CLT blocks K currents

more potently than Ca^{2+} currents and much more potently than Na currents. At least two types of $K_{Ca^{2+}}$ currents are inhibited by CLT at submicromolar concentrations: the intermediate conductance $K_{Ca^{2+}}$ channel in MEL cells and human erythrocytes and the large conductance, voltage-sensitive $K_{Ca^{2+}}$ channel (also known as the maxi-K channel) in portal vein VSM (A.R. Rittenhouse et al., 1997). As an experimental reagent, CLT has a higher potency than TEA, and is much less expensive and easier to work with than are the peptide toxins ChTX and IbTX. These properties are consistent with the ability of oral CLT treatment, via blockade of the erythroid $K_{Ca^{2+}}$ channel, to inhibit erythrocyte K depletion and dehydration in patients with sickle cell disease [9]. CLT and its metabolites are currently under investigation as novel chronic treatments for sickle cell disease.

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