J. Membrane Biol. 106, 243–252 (1988)

The Journal of
Membrane Biology

Charybdotoxin Blocks with High Affinity the Ca-Activated K^+ Channel of Hb A and Hb S Red Cells: Individual Differences in the Number of Channels

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Summary. We have investigated the effect of a purified preparation of Charybdotoxin (CTX) on the Ca-activated K^+ (Ca-K) channel of human red cells (RBC). Cytosolic Ca^{2+} was increased either by ATP depletion or by the Ca ionophore A23187 and incubation in Na^+ media containing $CaCl_2$. The Ca-K efflux activated by metabolic depletion was partially (77%) inhibited from 15.8 ± 2.4 mmol/liter cell · hr, to 3.7 ± 1.0 mmol/liter cell · hr by 6 nM CTX (n = 3). The kinetic of Ca-K efflux was studied by increasing cell ionized Ca^{2+} using A23187 (60 μmol/liter cell), and buffering with EGTA or citrate; initial rates of net K^+ efflux (90 mmol/liter cell K^+) into Na^+ medium containing glucose, ouabain, bumetanide at pH 7.4 were measured. Ca-K efflux increased in a sigmoidal fashion (n of Hill 1.8) when Ca^{2+} was raised, with a K_m of 0.37 μM and saturating between 2 and 10 μM Ca^{2+}. Ca-K efflux was partially blocked (71 ± 7.8%, mean ± SD, n = 17) by CTX with high affinity (IC_{50} 0.8 nM), a finding suggesting that is a high affinity ligand of Ca-K channels. CTX also blocked 72% of the Ca-activated K^+ efflux into 75 mM K^+ medium, which counteracted membrane hyperpolarization, cell acidification and cell shrinkage produced by opening of the K^+ channel in Na^+ media. CTX did not block Valinomycin-activated K^+ efflux into Na^+ or K^+ medium and therefore it does not inhibit K^+ movement coupled to anion conductive permeability.

The V_{max}, but not the K_m-Ca of Ca-K efflux showed large individual differences varying between 4.8 and 15.8 mmol/liter cell · min (FU). In red cells with Hb A, V_{max} was 9.36 ± 3.0 FU (mean ± SD, n = 17). The V_{max} of the CTX-sensitive, Ca-K efflux was 6.27 ± 2.5 FU (range 3.4 to 16.4 FU) in Hb A red cells and it was not significantly different in Hb S (6.75 ± 3.2 FU, n = 8). Since there is larger fraction of reticulocytes in Hb S red cells, this finding indicates that cell age might not be an important determinant of the V_{max} of Ca-K^+ efflux.

Estimation of the number of CTX-sensitive Ca-activated K^+ channels per cell indicate that there are 1 to 3 channels/per cell either in Hb A or Hb S red cells. The CTX-insensitive K^+ efflux (2.7 ± 0.9 FU) may reflect the activity of a different channel, nonspecific changes in permeability or coupling to an anion conductive pathway.

Key Words RBC K channels · charybdotoxin · Ca-K channel · Hb S RBC · human erythrocyte

Introduction

Human red cells poses a K^+-selective, channel-activated by cytosolic calcium, which was first described by Gardos [15] as a marked increased in K^+ permeability produced by external Ca in ATP-depleted cells. Further studies have shown the presence of at least 2 types of Ca-activated (CaK) K^+ channels in a variety of cells; one type of channel, such as the one in muscle membranes [7, 21] has a high conductance in the order of 200 pS ('maxi-channels'); others, such as the one in human red cell membranes has a low conductance in the order of 20 pS [16, 17]. The cellular functions of this channel in human red cells is still undefined. Studies in Hb S red cells have proposed that it may play a pathophysiological role in the deoxygenation-stimulated K^+ efflux and in the formation of dense cells [3].

The discovery by Miller et al. [26] that the small protein Charybdotoxin (CTX) present in the venom of the scorpion *Leiurus quinquetriatus* blocked "maxi" Ca-K channels from muscle membranes indicated that this toxin was a good ligand for this protein. The purified preparation of CTX obtained by Smith, Phillips and Miller [32] has a molecular mass of 4.3 kDa [14] and retains full channel blocking activity upon iodination.

The present study was designed to investigate whether or not a purified preparation of CTX blocks the Ca-K efflux from Hb A and S red cells. We found that the Ca-K efflux is only partially blocked (57–83%) by CTX with very high affinity (0.8 nM), a finding which may permit quantification of these K^+ channels in human red cell membranes. This study also indicates that the V_{max} of CTX-sensitive, Ca-K

efflux showed a wide range of variation in red cells of subjects with Hb A as well as in those with Hb S. Estimation of the number of Ca-K channels in human red cells indicate that there are between 1 and 3 channels per red cell.

Materials and Methods

Material

Blood from subjects with genotype Hb AA was obtained with informed consent at the Brigham and Women's Hospital. Blood from subjects with genotype Hb SS was obtained with informed consent by Dr. Ronald Nagel at the Heredity Clinic of the Jacobi Hospital, Bronx, NY. Subjects were identified as having Hb AA or SS by two electrophoretic methods (cellulose acetate at pH 8.6 and agar electrophoresis at pH 6.4) and by a solubility test [11].

Red Cell Preparation

Two to three ml of blood obtained in heparinized vacutainer tubes were centrifuged to remove plasma and buffy coat, and washed twice with preservation solution when red cells were to be used the next day. The cells used the same day were washed 4 times with choline washing solution containing (in mM) 149 choline chloride, 0.15 $MgCl_2$ and Tris-MOPS, pH 7.4 at 4°C; the cells were suspended at hematocrit 50 vol/vol in the washing solution for measurements of K^+ content and hemoglobin by optical density at 540 nm. Red cells to be used the next day or shipped to Boston (Hb S red cells) were preserved overnight in cold preserving solution containing (in mM): 135 KCl, 15 NaCl, 10 Tris-MOPS, pH 7.4 at 4°C.

K^+ Efflux Measurements

Red cells of subjects with Hb S are heterogenous in age and K^+ content; for this reason, Hb A and Hb S red cells were loaded to contain 90 mmol of K/liter cell by means of the nystatin method as previously described [4, 5]. To activate the K^+ channel, cell calcium was increased by metabolic depletion and incubation in 1.0 mM $CaCl_2$ media and with the Ca ionophore A23187. Net K^+ or ^{86}Rb efflux was determined. When cell calcium was increased by metabolic depletion (Gardos effect), the red cells were depleted of ATP following one of the procedures described by Lew [23]. Briefly, the depletion media contained (in mM): 140 KCl, 10 NaCl, 10 Tris-MOPS, pH 7.4 at 37°C, 0.15 $MgCl_2$, 5 inosine, 5 iodoacetamide. After 1 hr of incubation, 1.5 mM $CaCl_2$ was added and the incubation continued for another hour. At the end of the depletion period, the cells were centrifuged and washed 5 times with choline washing solution. K^+ efflux was measured incubating the cells in a media containing (in mM): 145 NaCl, 0.15 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 0.1 ouabain, 0.1 bumetanide, 1.0 $CaCl_2$, at 2% hematocrit with and without CTX. Triplicate samples were taken at 0, 30 and 60 min. Control cells were incubated in the same media containing 10 mM glucose.

When the A23187 ionophore was used to increase cell calcium, washed red cells were incubated in Na^+ media containing (in mM): 140 NaCl, 10 Tris-Mops, pH 7.4 at 37°C, 0.1 ouabain, 10 glucose and 0.1 bumetanide, 1 EGTA or 1 Tris-citrate, total $CaCl_2$ varied between 0.4 to 50 μM, 60 μmol/liter of A23187, at 1% hematocrit; a 1.9 mM ionophore stock solution in DMSO (kept protected from light) was added to the media just before the initiation of the efflux measurements. In the K^+ media, 75 mM Na^+ was replaced by 75 mM KCl. The transport reaction was initiated under dim light by adding the cells to pre-warmed media; duplicate samples of 1.0 ml were taken every minute for 5 min (unless otherwise indicated), pipetted into 1.5 ml cold Eppendorf tubes containing 0.4 ml of dibutyl phtalate oil and centrifuged for 10 sec in a Fisher centrifuge. Afterwards, the supernatants were removed for K^+ measurements by atomic absorption in a Perkin Elmer spectrophotometer Model 303A. K^+ efflux was calculated from the slope of the regression line of K^+ concentration *vs.* time taking into account the volume of red cells used.

For studying the effect of valinomycin on ^{86}Rb efflux, the cells were loaded with K^- and the isotope using the nystatin procedure as previously described [5]. The loaded cells were added at time zero to a pre-warmed efflux media containing (in mM): 140 NaCl (or 75 NaCl + 75 KCl), 10 glucose, 0.1 ouabain, 0.1 bumetanide, 0.15 $MgCL_2$, 10 Tris-MOPS, pH 7.4 and 0.1 μmol/ml RBC valinomycin. Duplicate samples were pipetted into 1.5 ml cold Eppendorf tubes containing oil at 0.5, 1, 2, 3 and 4 min and centrifuged for 10 sec for separation of the efflux media. For ^{86}Rb efflux measurements, a 0.8 ml aliquot of the media was counted in a gamma counter. Aliquots of 1 : 50 dilution of the initial cell suspension were counted for determination of the initial specific activity for calculation of the efflux in mmol/liter cells per min.

Calcium Measurements

The total Ca concentration of the flux media was measured by atomic absorption spectrophotometry (Perkin Elmer, Model 303A) using Ca standards (EM Sciences, Cherry Hill, NJ) in Na media.

Ionized Ca (Ca^{2+}) concentrations were buffered between 0 and 0.73 μM with EGTA. A custom made computer program was used to calculate the Ca^{2+} concentrations using the dissociation constant and correcting for ionic strength at pH 7.4 and 0.15 mM $MgCl_2$ as previously described [9]. Ca^{2+} concentrations between 1 and 10 μM were buffered with citrate and calculated from the dissociation constant [9]. The Ca^{2+} concentrations of the flux media were determined in the laboratory of Dr. Simon Levy (Department of Physiology, Boston University School of Medicine) using Ca^{2+}-selective microelectrodes made as described previously [22]. These electrodes were based on the newly developed Ca^{2+} sensor with submolar detection limit described by Schefer et al. [30]. A good agreement was obtained between the calculated and measured Ca^{2+}.

Ca uptake was measured using ^{45}Ca as previously described by Lew and Ferreira [24] and Escobales and Canessa [10]. In these experiments, the reaction was started by the addition of packed cells in triplicate to the medium containing 5 μCi of ^{45}Ca (Amersham Corp.). The radioactivity was counted after protein precipitation of the cell lysates with 5% trichloroacetic acid. Aliquots of the cell lysate were used to determine the red cell volume from the optical density of hemoglobin.

Measurements of Cell pH and Membrane Potential

In human red cells the membrane potential is close to the Cl^- equilibrium potential and therefore an estimation of the mem-

brane potential can be obtained from the Cl^- distribution ratio [12, 20] and the Nernst equation. Due to the operation of an electroneutral anion exchanger in parallel to the Jacob-Stewart cycle [18], the following ionic distribution ratios are observed:

$$e^{-FE_m/RT} = \frac{Cl_i^-}{Cl_o^-} = \frac{(HCO_3)_i^-}{(HCO_3)_o^-} = \frac{OH_i^-}{OH_o^-} = \frac{H_o^+}{H_i^+} \quad (1)$$

where $_i$ and $_o$ represent the internal and external ion concentrations, respectively, and E_m, F, R and T have their usual meanings. Therefore, the Cl_i/Cl_o ratio and E_m were estimated from pH_i and pH_o measurements using the following equations:

$$pH_i = \log \frac{Cl_i^-}{Cl_o^-} + pH_o \quad (2)$$

$$E_m = -58 \log Cl_i^-/Cl_o^-. \quad (3)$$

Cellular pH was measured in cell lysates. The cell suspension used for efflux measurements was separated by centrifugation over phtalate oil to separate the cells; the bottom of the tubes containing the packed red cells were cut into unbuffered distilled water containing 0.02% Acationox. Since under these conditions the buffering capacity of red cells is more than 200 times that of the medium, the pH measurements of the cell lysate represent the internal pH.

Charybdotoxin Preparations

The venom of *L. quinquetriatus* was obtained as lyophilized powder from Laxotan Scorpio Farm, Rosans, France, and was stored at −20°C. CTX was purified and assayed by Dr. Christopher Miller from the Department of Biochemistry at Brandeis University as previously described [26, 32]. In the majority of the experiments, we used the SPC 25 sephadex fraction, which was only 55% pure but represented an approximately 300-fold purification over the water extractable part of the crude venom. The concentration of the CTX preparations was determined by Dr. C. Miller and were between 1–4 μM. Concentrations of CTX stock solutions were determined by absorbance at 280 nm, using an extinction coefficient of 10,500 M^{-1} cm^{-1}, approximately 1.7-fold lower than that determined previously [32].

Control experiments were performed with the C8 fraction, which was 90% pure. CTX is an extremely stable molecule, which maintained its activity stored in isotonic NaCl solution at 1 to 4 μM.

Results

The Inhibition of the Gardos Effect by Charybdotoxin

The depletion of cell ATP, by incubation with inhibitors of glycolysis, leads to a large increase in K^+ permeability, which depends on the presence of Ca^{2+} in the media. As reported by Lew [23], ATP cell content drops to less than 5% of control values when incubated for 2 hr in 5 mM iodoacetamide, to block glycolysis, and 5 mM inosine to trap inorganic phosphate. At the same time, Ca^{2+} uptake and K^+ efflux increases. We investigated the effect of CTX on the Ca-K efflux from Hb A red cells following this protocol [23]. Table 1 shows the effect of metabolic depletion of K^+ efflux into Na^+ media containing ouabain and bumetanide in red cells of 3 subjects; K^+ efflux increased from 2.23 ± 0.45 (mean ± SD, n = 3) in glucose fed cells to 18 ± 2.8 mmol/liter cell · hr in the depleted cells. Addition of 6 nM CTX did not produce significant changes in K^+ efflux from glucose fed cells (Δ CTX = 0.37 ± 0.37 mmol/liter cell · hr) but markedly inhibited from 18.0 to 4.85 ± 0.13 mmol/liter cell · hr in depleted cells. The mean value of CTX-inhibitable K^+ efflux was 12.2 ± 1.4 mmol/liter · hr, which represented at 77 ± 2.6% inhibition of the Ca-activated component.

Table 1. The effect of Charybdotoxin on the Ca-activated K^- efflux in metabolically depleted cells

Subjects	K^+ efflux, mmol/liter cell · hr Total	+ CTX	CTX sensitive	% inhibition
(1)				
Fed cells	2.8 ± .10	1.9 ± .05	0.8	
Depleted	21.3 ± .06	6.7 ± .20	14.6	
Δ	18.5	4.8	13.8	74.1
(2)				
Fed cells	1.9 ± .10	1.7 ± .05	0.2	
Depleted	16.2 ± .17	4.8 ± .08	11.4	
Δ	14.3	3.1	11.2	78.4
(3)				
Fed cells	2.0 ± .06	1.9 ± .17	0.1	
Depleted	16.6 ± .46	5.0 ± .21	11.6	
Δ	14.6	3.1	11.5	78.8

Fed cells were incubated in a glucose media containing 1.5 mM $CaCl_2$; depleted cells were incubated in media containing inosine and iodoacetamide as described in Materials and Methods. The efflux media contained (in mM) 145 NaCl, 0.15 $MgCl_2$, 10 Tris-MOPS, pH 7.4 at 37°C, 0.1 ouabain and 0.1 bumetanide wt/vol 6 nM CTX.

The Effect of Cell Calcium and CTX on K Efflux

The kinetics of Ca^{2+}-activated K^+ efflux was studied using high concentrations of ionophore A23187 (60 μmol/liter cell) to rapidly increase cytosolic Ca^{2+} in most of the cells. As previously shown by Escobales and Canessa [10], in the presence of high ionophore concentrations, Ca^{2+} equilibrates relatively rapidly across the red cell membrane.

The time course of net K^+ loss into Na^+ medium was followed (Fig. 1) at an external Ca^{2+} of 4.0 μM in the absence and presence of 6 nM CTX. In this subject, the rate of Ca-K efflux was linear with time up to 4 min; in the presence of 6 nM CTX, the

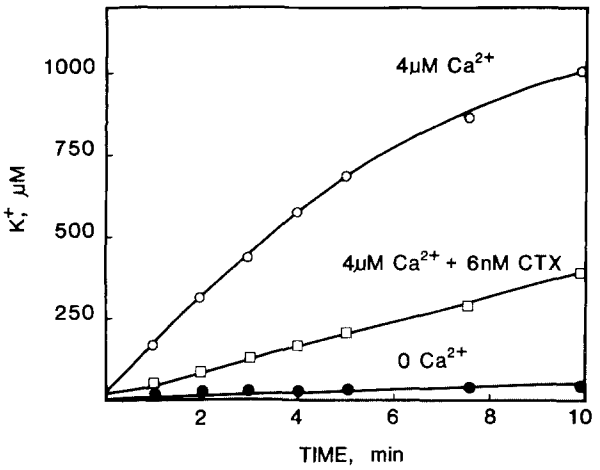

Fig. 1. The effect of Charybdotoxin on Ca-activated K^+ efflux from Hb A red cells. The time course of K^+ appearance in the medium is depicted in the presence of 0 Ca^{2+} (●—●), 4 μM Ca^{2+} (○—○), and 4 μM Ca^{2+} plus 6 nM CTX (□—□). The cells were incubated at 1% hematocrit in a medium containing (in mM): 140 NaCl, 10 Tris-MOPS, at pH 7.4, 0.1 ouabain, 0.1 bumetanide, 10 glucose and 60 μmol/liter cell of A23187

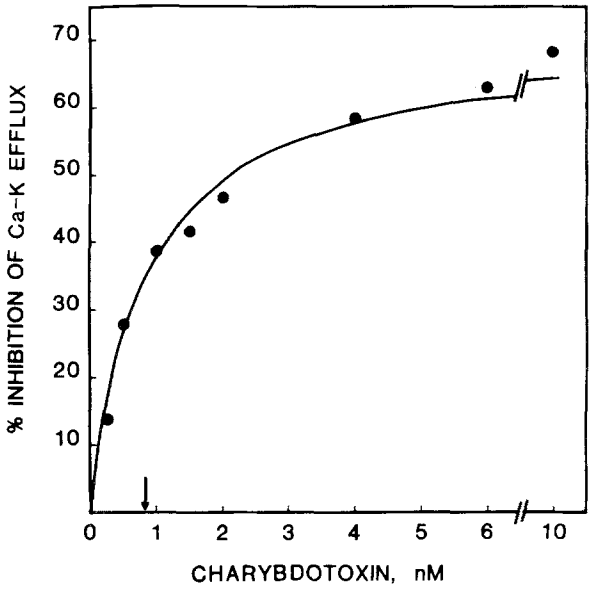

Fig. 2. Dose-response curve for inhibition of Ca-activated K^+ efflux by Charybdotoxin in a subject with Hb A red cells. The experimental points were fitted by a nonlinear fitting program for a hyperbola, which gave an IC_{50} value of 0.86 ± 0.11 nM and a maximal inhibition of 69.8 ± 0.5%. Ca^{2+} was 4 μM and other experimental conditions were similar to Fig. 1

K^+ efflux is rapidly inhibited and the rate is linear up to 20 min. In red cells of a subject with low efflux values (5 mmol/liter cell · min), the Ca-K efflux was linear up to 10 min.

The dose response of the Ca-K efflux (4 μM Ca^{2+}) *vs.* CTX concentration (Fig. 2) indicated that the toxin blocked the channel with high affinity. A nonlinear fitting of the experimental points to an hyperbola gave an IC_{50} value of 0.86 ± 0.11 nM and a maximum inhibition of 69.8 ± 0.5% in this subject. Titrations performed in another 2 subjects gave half maximal inhibitory concentrations of 0.7 and 1.1 nM, (a mean value of 0.88 nM) and no significant differences in the inhibition between 5, 10 and 20 nM CTX. We also performed a titration of the Ca-K efflux channel using fraction C8 of the scorpion venom, which is 90% pure CTX as estimated by Smith et al. [32]; the IC_{50} for inhibition was similar to that of the SPC preparation, which is only 50–60% pure in CTX.

Figure 3*A* and *B* show the dependence of K^+ efflux on external ionized calcium in red cells of 2 subjects with Hb A. The K^+ channel was activated when other routes of K^+ transport (such as the Na pump, Na-K-Cl and K : Cl cotransport) were inhibited by ouabain, bumetanide and isotonic media. As shown in Fig. 3*A*, K^+ efflux increased sigmoidally up to 9.0 mmol/liter cell · min when Ca^{2+} was increased up to 10 μM. The dependence of K^+ efflux on Ca^{2+} was obtained using a computer program for nonlinear fitting of hyperbolic and sigmoidal kinetics. For an hyperbolic function (n of Hill = 1.0, the calculated V_{max} was 10.3 ± 0.63 mmol/liter cell · min, the K_m for Ca^{2+} (K_m-Ca) 0.81 ± 0.17 μM. A better fitting was obtained for a sigmoidal dependence on ionized calcium with a n of Hill of 1.8 ± 0.28; the V_{max} of Ca-K efflux was 8.69 ± 0.37 mmol/liter cell · min with a K_m of 0.37 ± 0.11 μM. In the presence of 6 nM CTX, K^+ efflux was inhibited to 3.0 mmol/liter · min, which represented a 70% inhibition of the total Ca-K efflux in this subject. The inhibition by 10 and 20 nM CTX was not significantly higher than at 6 nM. Thus, as previously observed in the Gardos experiments (Table 1), a substantial component of Ca-K efflux was not inhibited by CTX. The nonlinear fitting of the CTX-insensitive flux gave a V_{max} of 3.04 ± 0.22 mmol/liter cell · min and a K_m of 0.52 ± 0.12 μM, with a Hill coefficient of 1.0. The CTX-sensitive Ca-K efflux from the red cells of this subject was estimated from the differences between the curves and yielded a V_{max} of 7.2 mmol/liter cell · min and a K_m of 0.97 μM.

As a comparison, Fig. 3*B* shows the kinetics of Ca-K efflux in the absence and presence of CTX in red cells of a subject with low fluxes. The total Ca-K efflux was about a half of that observed in the subject of Fig. 3*A* (5 mmol/liter cell · min); however, CTX inhibited 90% of the Ca-K efflux yielding 4.5 mmol/liter cell · min of CTX-sensitive component. The K_m-Ca to stimulate K^+ efflux and the Hill coefficient were similar to that of the subject with high fluxes (Fig. 3*A*).

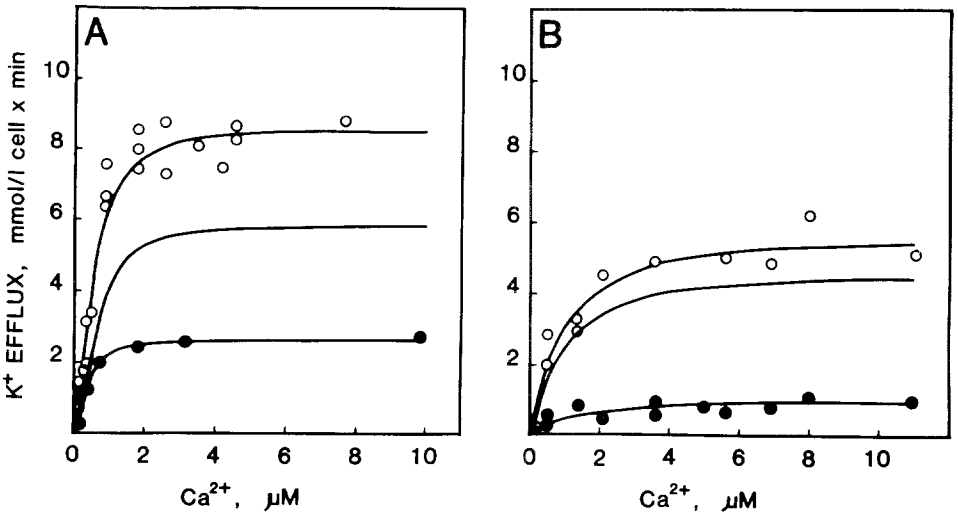

Fig. 3. The dependence of K^+ efflux from Hb A red cell from Ca^{2+} in the absence (○—○) and presence of 6 nM Charybdotoxin (●—●) in a subject with high V_{max} (*A*) and low V_{max} (*B*). These two curves were fitted by a computer program for nonlinear fitting for sigmoidal kinetics. The CTX-sensitive K^+ efflux was calculated as the difference between the two fitted curves in the absence and presence of CTX. Experimental conditions as in the legends of Fig. 1. Notice that in (*A*), the Ca-K efflux is much higher (10.3 ± 0.63 mmol/liter cell · min) than in (*B*) (5 mmol/liter cell · min); the % inhibition by CTX is lower (70%) in (*A*) than in (*B*) (90%). The dispersion of values after approaching saturation with Ca^{2+} higher than 2 μM represents several repetitive measurements

The free ionized cytosolic Ca concentration can be estimated from the total Ca concentration and the ionized fraction, alpha = r^2/m can be calculated from the r = Cl_i/Cl_o ratio and m = the slope of the dependence of total cell Ca from external Ca^{2+} as described by Lew and Ferreira [24]. Because under our experimental conditions, the external K^+ concentration (4 to 200 μM) was not clamped and therefore substantial hyperpolarization may occur, cytosolic Ca^{2+} was not calculated.

The Effect of Charybdotoxin on ^{45}Ca Uptake and Cell pH

We also tested the effect of CTX on ^{45}Ca uptake under similar experimental conditions. As shown in Table 2, the toxin inhibited about 20% of ^{45}Ca influx driven by A23187 at saturating external Ca^{2+} concentrations (10 μM). In the absence of external K^+, opening of the K^+ channel may hyperpolarize the membrane, H^+ redistribution will occur and cell pH will decrease. As shown in Table 2, the pH of cells incubated for 3 min in Na^+ medium with A23187 and Ca^{2+} became acid upon opening of the K^+ channels; inhibition of the Ca-activated K^+ efflux by CTX was accompanied by alkalinization. Calculated E_m from the Cl_i/Cl_o ratio indicated that upon opening of the Ca-K channels, membrane hyperpolarization occurred (−29 mV); inhibition by CTX was accompanied by return to normal E_m (−8.5 mV). Because A23187 facilitates Ca^{2+} influx mediating a Ca_o/H_i exchange, changes in cell pH may also influence ^{45}Ca uptake. Thus, hyperpolarization and cell acidification produced by opening of the K^+ channel in K-free media should increase A23187-mediated ^{45}Ca influx and blocking of the Ca-K channel by CTX should produce cell alkalinization, which in turn may decrease A23187-mediated Ca_o/H_i exchange. This may account for the 20% inhibition by CTX of ^{45}Ca uptake. However, under our experimental efflux conditions, Ca-K efflux is independent of Ca^{2+} concentration because the external media was saturated 5 μM Ca^{2+}; thus, the partial inhibition by CTX of ^{45}Ca uptake cannot account for the 72% inhibition of the Ca-K efflux.

Table 2. The effect of Charybdotoxin on cell pH and ^{45}Ca uptake on A23187 + Ca^{2+}-treated human red cells

	Basal	+ Ca^{2+}	Ca^{2+} + CTX
Call pH			
Na media	7.2	6.9	7.2
K media	7.2	7.2	7.2
E_m, mV	−8.5	−29.0	−8.5
^{45}Ca uptake			
(μmol/ml) · 10^2	—	4.75 ±0.50	3.5 ±1.8

K^+ efflux was activated using 60 μmol/ml cells of A23187, 5 μM Ca^{2+} and inhibited by 5 nM Charybdotoxin. All measurements were performed after 3 min incubation. Cl_i/Cl_o ratio and E_m were calculated as described in Materials and Methods. External pH was 7.4.

Table 3. The effect of Charybdotoxin on Valinomycin and Ca^{2+}-activated K^+ efflux in human red cells

	K^+ Efflux, mmol/liter cell · min	
	Na medium	K medium
Basal	0.02	0.015
+ Valinomycin	8.20	14.80
+ Valinomycin + CTX	7.70	14.90
Δ CTX	0.50	1.10
B:		
A23187 + EGTA	0.10	0.08
A23187 + 4 μM Ca^{2+}	15.20	9.70
% + CTX	3.20	2.70
Δ CTX	12.00	7.00
% inhibition	78.90	72.00

Na medium had 150 mM NaCl; K^+ medium was 75 mM Na and K^+. Valinomycin was 0.1 μmol/ml; 5 nM CTX.

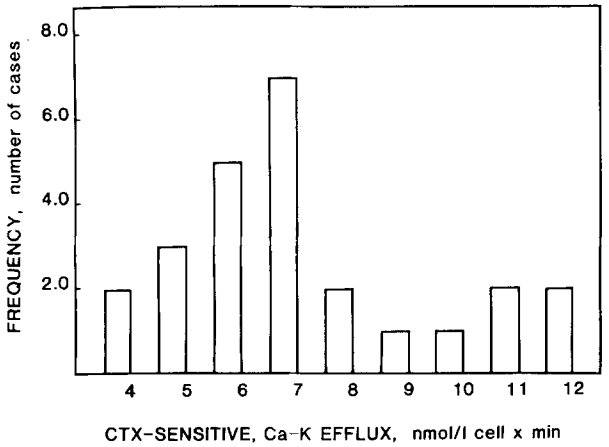

Fig. 4. Frequency distribution of the V_{max} of Ca-K efflux, CTX sensitive in red cells of 25 subjects with Hb A and S

Charybdotoxin Does Not Inhibit Valinomycin-Induced K Efflux

In order to exclude that CTX-inhibited, Ca-activated K^+ efflux by blocking a conductive anion movement, we investigated its effect on Valinomycin-stimulated K^+ efflux. As shown in Table 3, Valinomycin increased K^+ efflux into Na^+ medium up to 8.2 mmol/liter cell · min, but 5 nM CTX did not inhibit this flux component. The Valinomycin-stimulated K^+ efflux into 75 mM K^+ medium was significantly higher (14.8 mmol/liter · min) than into Na^+ media (8.2 mmol/liter · min); the reduction in K^+ is most likely produced by membrane hyperpolarization occurring in Na^+ media when enough K^+ ionophore molecules are incorporated into the membrane and make it behave as a K^+ electrode. Also CTX did not inhibit the Valinomycin-induced K^+ efflux into 75 mM K^+.

Table 4. Maximal rates of Charybdotoxin-sensitive and insensitive Ca-activated K^+ efflux from Hb A and S red cells

	K^+ Efflux, mmol/liter cell · min			
	Total	+ CTX	Δ CTX	Inhibition %
Hb A, n = 17				
Mean	9.36	2.69	6.27	71.0
SD	±3.05	±0.91	±2.50	±7.8
Range	4.80	0.80	3.40	57.0
	15.80	4.4	12.60	83.0
Hb S, n = 8				
Mean	9.86	2.65	6.75	73.9
SD	±2.75	±1.80	±3.20	±15.9
Range	5.60	0.20	4.90	57.5
	14.40	4.70	16.40	98.0

K^+ efflux was measured at 5 μM Ca^{2+} using 60 μmol/liter cell of A23187; the cells were incubated in Na^+ media as described in Materials and Methods with and without 6 nM CTX.

In contrast, Ca-activated K^+ efflux from red cells of this subject was much larger than the Valinomycin-stimulated K^+ efflux and blocked 79% by CTX (Table 3, section B). Table 3 also shows a comparison of CTX inhibition of Ca-activated K^+ efflux into Na^+ and K^+ media in which E_m and cell volume are kept constant. Ca-stimulated K^+ efflux decreased 64% when the K^+ chemical gradient was reduced indicating that opening of Ca-K channels may not move E_m too close to E_K as in the case of incorporation of Valinomycin into the membrane. CTX inhibition decreased from 79 to 72% when the cells were incubated in 75 mM K^+ instead of Na medium in this subject, but in another sample there was no significant differences in the percentage of inhibition by CTX. These results indicate that CTX blocks Ca-activated K^+ efflux by inhibition of the K^+ channel and not by blocking of conductive anion movement.

Individual Differences in the V_{max} of Ca-Activated, CTX-Sensitive K Efflux

We have determined the V_{max} of CTX-sensitive, Ca-K efflux in red cells of several individuals with Hb A and S. Ca-activated K^+ efflux was activated with saturating Ca^{2+} concentrations (5 μM) and inhibited by 6 nM CTX, a concentration 8 times higher than its K_m (0.8 nM). Table 4 shows results obtained in red cells of 17 subjects with Hb A and in 8 subjects with Hb S. The V_{max} of Ca-K efflux varied between 4.8 to 15.8 mmol/liter · min in Hb A red cells and between 5.6 and 14.4 in Hb S cells, but the mean values in both groups were not significantly different. Figure 4 shows the frequency distribution of

the V_{max} of Ca-K efflux in the red cells of the 25 subjects studied; 17 of the 25 subjects had V_{max} of CTX-sensitive, Ca-K efflux equal or less than 7 mmol/liter cell · min. The CTX-sensitive component of the Ca-K efflux varied between 3.4 and 12.6 mmol/liter cell · min in Hb A and Hb S red cells. In the 17 subjects with Hb A, CTX inhibited a 71 ± 7.8% (mean ± SD) of the Ca-K efflux, a value not significantly different from that observed in Hb S red cells.

We performed repetitive measurements of the V_{max} of Ca-activated, CTX-sensitive K^+ efflux in 4 subjects, which had a variation of 4% between measurements. In 3 subjects, the measurements were repeated one year apart and gave a similar percentage of variation.

In contrast to the individual differences in the V_{max} of CTX-sensitive, Ca-K efflux, the K_m for Ca^{2+} to activate K^+ efflux was similar in subjects with Hb A ($n = 3$) and S ($n = 2$).

Estimation of the Number of Ca-Activated K Channels in Human Red Cells

Because our results showed that CTX was a specific and high affinity blocker of Ca-activated K^+ movement, we have made an estimation of the number of channels present in human red cells (N_K) from the following expression:

$$N_K = \frac{I_K}{g \cdot P_o \cdot (E_m - E_K)} \quad (4)$$

where N_K = number of K^+ channels per cell, g = unitary conductance of Ca-activated K^+ channel in pS, P_o = open-time probability for the channel, I_K = current per cell in pA, E_m = membrane potential in mV and E_K = K^+ equilibrium potential in mV.

To calculate I_K, we used the mean value of the V_{max} of Ca-activated, CTX-sensitive K^+ efflux of 6.3 mmol/liter cell · min, which is equal to 10.5×10^{-5} mol/liter cell · sec; considering that there are 1.15×10^{13} cells in a liter of red cells, the flux was 9.13×10^{-18} mol/cell · sec. This net K^+ efflux was converted to 0.79 pA/cell using the Faraday constant. The E_K was calculated from:

$$E_K = -58 \log \frac{0.74\ K_i}{K_o} = -140 \text{ mV} \quad (5)$$

where 0.74 = the activity coefficient for K^+ at 140 mM K^+.
K_i = cell K^+ concentration, 140 mM
K_o = external K^+ concentration, mM; a value of 0.4 mM was used as determined in Fig. 1.

E_m was estimated from $E_{Cl} = -29$ mV (Table 3); the conductance of K^+ channel determined by Grygorczyk et al. [16, 17] of 20 pS and open-time probability of 0.5 for that $E_m = -29$ mV, N_K was estimated to be 0.79 channel/per cell. Because this value is limited strongly by our estimation of E_m on the basis of the Cl_i/Cl_o ratio, we also calculated the number of channels per cell, assuming that E_m has become closer to E_K, as −100 mV. For this limiting value, the number of Ca-activated, CTX-sensitive K^+ channels increased to 2.6 channels per cell.

Discussion

The present experiments provide evidence that the polypeptide CTX discovered by Miller et al. [26] blocks with high affinity the Ca-K channel of human red cells. CTX-inhibited K^+ efflux was activated by an increase in cytosolic calcium produced either by the ionophore A23187 or by metabolic depletion. Under both conditions, CTX concentration 8 times higher than its IC_{50} (0.86 nM) inhibited only partially (73 and 77%) the Ca-K efflux; this kinetic property indicates that the toxin is a very specific blocker of Ca-K channels. Our findings are not in agreement with recent studies using the crude venom of *L. quinquestriatus* [1] to inhibit the Ca-K channel. Abia et al. [1] reported 92% blocking of total ^{86}Rb influx with an IC_{50} 3 μg/ml using low A23187 concentrations (0.01 μmol A23187 per ml of red cells) and high total unbuffered calcium concentration (100 μM) to activate the channels. It is most likely that under those conditions, Ca^{2+} might not have been homogenized in all cells. Our studies show that CTX inhibited only partially (72%) but with high affinity (0.88 nM) Ca-K efflux into K-free media. On the basis that the molecular mass of CTX is 4.3 kDa [14], the IC_{50} of the purified preparation is 0.004 ng/ml, thus 6 orders of magnitude lower than the affinity for crude scorpion venom to block K^+ channels [1]. Castle and Strong [6] also reported high IC_{50} values (8 μg/ml) for inhibition of A23187-induced K^+ loss from erythrocytes using crude *L. quinquestriatus* venom.

CTX inhibition of the Ca-K channel of human RBC has an IC_{50} 3 orders of magnitude lower than quinine (IC_{50}, 5 μM) [28], and the Di-S-C2 carbocyanine dye (IC_{50}, 0.7 μM) [31]. We have observed (*data not shown*) that quinine also inhibits the basal K^+ efflux in agreement with other nonspecific actions of this drug.

Studies carried out by Miller et al. [26] and Smith et al. [32] indicated that CTX blocks the high conductance, Ca-K channel of mammalian skeletal muscle with an IC_{50} of 4 nM. As shown by these authors, the polypeptide induces long closed states

in that channel, which are interpreted as blocked events. The inhibition of the low conductance K^+ channel of the human red cells by CTX occurs with an affinity (0.8 nM), 4–5 times higher than that of the "maxi" channel; this suggests that both channels might be different not solely in the length of their conductive pathway, but also in the nature of the external sites interacting with the toxin. On the other hand, the muscle and red cell channels are insensitive to Apamin, a peptide that blocks Ca-K channel from nerve and liver cells [29].

Our study of the activation kinetics of the K^+ channel by Ca^{2+} indicated that this process followed a sigmoidal dependence on external Ca^{2+} (in the presence of A23187) and had very low values for the K_m-Ca. To our knowledge, the kinetic properties of Ca-K efflux were not previously determined. We found that the K_m-Ca is less than 1 μM Ca^{2+}, a value much lower than that previously reported by Lew and Ferreira [24] using a lower A23187 concentration (10 μmol/liter cell). Not only were very low Ca^{2+} concentrations needed to activate the K^+ channel, but the process also followed sigmoid kinetics with a Hill coefficient significantly higher than 1 ($n = 5$). This parameter may reflect the presence of more than one site for Ca activation in the channel protein, as we have previously reported for the "maxi" Ca-K channel of skeletal muscle [27].

Another important finding of this study is the observation that the V_{max} of CTX-sensitive, Ca-K efflux has a wider range of variation in red cells of individuals with Hb A or Hb S (Table 4); however, no significant differences were found in the V_{max} between subjects with Hb S or Hb A. Because red cell of patients with Hb S posses a larger fraction of reticulocytes [3],[1] our findings suggest that the interindividual differences of this pathway might be a more important determinant of the number of channels per cell than the cell age.

We have made an estimation of the number of Ca-K channels present in red cells using the V_{max} values of CTX-sensitive, Ca-K efflux assuming homogenous distribution. These calculations yielded values of 0.8 channels per cell, and a variation between individuals between 0.6 and 2 channels per cell for $E_m = -29$ mV and from 2 to 6 channels/cell if E_m approached E_K. Grygorczyk et al. [17] estimated the number of Ca-activated K^+ channels in one subject between 1 and 55 using their values of unitary conductance and measurements of the total K^+ efflux into 100 mM KCl media at Ca^{2+} 10 μM and A23187; these authors observed two components of Rb efflux and calculated that the numbers of K^+ channels may vary between 1 and 55, if they were present in different cells. In our experiments, other routes of K^+ movement were blocked by bumetanide and initial rate of the CTX-sensitive Ca-K efflux were measured using a K-free media, which assures maximal flux through this channel [33]. Our estimation of the number of Ca-K channels render values between 0.8 to 3 channels per cell, which are significantly lower than those calculated by Grygorczyk et al. [17] but in the same order of magnitude. However, in studies on the ^{86}Rb release from inside-out vesicles, a mean number of 100–200 channels per cell was calculated by Lew, Muallen and Seymour [25] and of 142 channels per cell by Alvarez and Garcia-Sancho [2]. Our results indicate that the assumptions made in those studies to estimate the number of channels give 2 orders of magnitude over-estimation of the number of K^+ channels in intact human red cells.

The small number of Ca-activated K^+ channels per cell has several implications. First, it can be compared with the 10^6 anion exchangers per cell present in human red cells [13]; thus, even though ionic channels can transport 10^6 to 10^7 ions per second, it is unlikely that the opening of the few Ca-activated K^+ channels can move E_m closer to E_K. As shown in Table 3, Valinomycin-stimulated K^+ efflux into Na^+ media was significantly lower than into K^+ media, most likely because incorporation of the ionophore hyperpolarize the membrane in the absence of K^+. In contrast, Ca-K efflux markedly decreased when Na^+ was replaced by K^+ indicating that the decrease in the K^+ gradient is more important that the hyperpolarization produced by K-free media. These data also support the conclusion that opening of the scarce number of Ca-K channels does not bring E_m close to E_K (−29 mV) in human red cells (Table 2).

Second, the small number of Ca-activated, CTX-sensitive K^+ channels indicates that red cell membrane is not a good source for attempting to purify this protein.

Third, a small number of Ca-activated, CTX-sensitive K^+ channels is present as well in Hb S red cells; this raises questions about its proposed pathophysiological role in the generation of dense cells in sickle cell anemia. Bookchin, Ortiz and Lew [3] have proposed that the increase in Ca^{2+} influx produced by deoxygenation may produce transient opening of the Ca-K channel, cell shrinkage and acidification, which could lead to the formation of dense cells. Our studies suggest that since CTX is a high affinity ligand of Ca-K channels, it can be used as a specific tool to identify the ion transport path-

[1] A preliminary report of this paper was presented at the 41st Annual Meeting of the Society of General Physiologists at the Marine Biological Laboratory, Woods Hole, Mass, September, 1987 [8].

way involved in the deoxygenation-induced K^+ efflux of Hb S red cells and to define the role of interindividual differences in the number of channels in this process.

The finding that CTX inhibited only a fraction of the Ca-K efflux also raises the question of the nature of the pathway for K^+ efflux, which is activated by Ca^{2+} but not inhibited by CTX. We can tentatively propose several explanations:

i) It may reflect nonspecific changes in membrane permeability; this appears unlikely considering that the CTX-insensitive component was detected at very low Ca^{2+}.

ii) It may reflect K^+ movement coupled to the anion conductive pathway activated by the membrane hyperpolarization produced by opening of the Ca-K channel [19]. Because Valinomycin-activated K^+ efflux was not inhibited by CTX, it is possible that the anion conductive pathway can account for the CTX-insensitive pathway. However, CTX inhibition of the channel did not increase when E_m was maintained constant (Table 3).

iii) It may represent the activity of a different subtype of Ca-K channel as also suggested by Grygorczyk et al. [17].

In summary, the present findings indicate that CTX can be an appropriate ligand to (i) quantify and identify the density of Ca-K channels because of its high affinity; (ii) determine individual differences on the number of Ca-K channels; and (iii) define the ionic pathways of deoxygenation induced K^+ efflux from Hb S red cells.

This work was supported by NIH Grants HLBI 35664 and 2106. We are very grateful to Dr. Christopher Miller for providing us purified CTX preparations and valuable discussion; to Dr. Ronald Nagel for providing the Hb S red cells and valuable discussion; to Dr. S. Levy for providing us the computer program for calculations of free calcium and measurements with the Ca electrode; and to Dr. Osvaldo Alvarez from the University of Chile for providing the program for curve fitting.

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Received 17 June 1988; revised 19 August 1988