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Inhibition of red cell Ca^{2+}-dependent K^+ channels by snake venoms

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We have investigated the effects of several snake venoms on the Ca^{2+}-dependent K^+ channels of human red cells. A heat-resistant component of the venom of the snake *Notechis scutatus* irreversibly inhibited Ca^{2+}-dependent K^+ transport with a K_i value of 0.1–0.2 μg/ml. Metabolic changes of the cells modified the maximal effect of the venom. Binding of the venom required extracellular Ca^{2+} and was quick, but development of full inhibition required additional time. The effects of the venoms from *Notechis scutatus* and *Leiurus quinquestriatus* were additive, suggesting that both venoms act through different mechanisms. Venoms of the snakes *Vipera russelli russelli* and *Oxyuranus scutellatus* also inhibited Ca^{2+}-dependent K^+ transport with the same characteristics as the *Notechis scutatus* venom.

K^+-selective channels which are activated by the increase of the level of cytoplasmic Ca^{2+} have been described in a large variety of cells [1–5]. Single-channel conductance measurements have evidenced two different kinds of channels on the basis of their unitary conductance: large conductance channels (150–250 pS, Refs. 3–7) and small conductance channels (10–14 pS, Refs. 5 and 8). Apamin, a component of bee venom, has been reported to inhibit several small conductance channels [8] whereas charybdotoxin (CTX), a component of the venom of the scorpion *Leiurus quinquestriatus* (LQV), blocked large conductance channels [9,10]. The red channel is an exception because, even though it has small conductance (10–40 pS, Refs. 11–13), it is not inhibited by apamin but it is sensitive to charybdotoxin [14–16]. Another example of a small conductance channel sensitive to CTX has been reported recently [17]. The inhibition by CTX is reversible [9,15]. This makes this venom inadequate for counting channels in binding studies. In this paper we describe the effects of a new venom which inhibits irreversibly the human red cell channel by binding to a different place than charybdotoxin.

Methods

Freshly drawn blood anticoagulated with heparin was used in all the experiments. Red cells were washed twice with a solution containing 75 mM NaCl, 75 mM KCl, 0.1 mM EGTA and 10 mM Tris-HCl (pH 7.7), and once more with the final suspension medium (see below). The K^+ channels were activated in all the cases by increasing the intracellular Ca^{2+} concentration by incubating the cells with Ca^{2+}, and the divalent cation ionophore A23187 at concentrations giving maximum activation. Ca^{2+}-dependent K^+ transport was measured either as net K^+ loss using a light-scattering procedure [15,18] or as $^{42}K^+$ uptake under equilibrium exchange conditions. For scattering experiments cells were suspended at 2.5% haematocrit in a solution containing (mM): NaCl, 112.5; NaSCN, 37.5; $CaCl_2$, 0.5; potassium-Hepes, 10 (pH 7.5). The cell suspension was placed in an spectrophotometer cuvette under magnetic stirring and absorbance at 650 nm was recorded. The experiment was started by the addition of the ionophore A23187 to give a concentration of 2 μM in the suspension. For measurements of $^{42}K^+$ exchange cells were suspended at 20% haematocrit in a solution containing (mM): NaCl, 75; KCl, 75; $MgCl_2$, 0.2; $CaCl_2$, 0.5; potassium-Hepes, 10 (pH 7.5). The experiment was started either by the addition of tracer (about 0.5 μCi/ml) or A23187 (10 μM). Samples of 0.1 ml of the cell suspension were taken after different incubation periods and placed in 1.5 ml Eppendorf tubes containing 0.9 ml of ice-cold incubation medium containing 1 mM quinine-HCl and 0.4 ml of di-*n*-butylphthalate [19].

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NSV, *Notechis scutatus* venom; LQV, *Leiurus quinquestriatus* venom; CTX, charybdotoxin.

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The tubes were immediately spun at 12000 × *g* for 15 s, the upper aqueous layer and most of the oil were aspirated, the walls of the tube were cleaned with cotton swabs, and the cell pellet was extracted with 0.5 ml of 0.6 M perchloric acid. Radioactivity was measured by Cerenkow counting. All the experiments were performed at room temperature. ^{42}K was produced using a generator provided by Dr. H. Stirner, Technische Universität, München, F.R.G. Snake venoms were obtained from Latoxan, Rosans, France.

Results and Discussion

Table I shows that 3 our of 19 snake venoms tested inhibited Ca^{2+}-dependent K^+ transport without producing haemolysis. This paper will show only the results obtained with the venom from *Notechis scutatus* (NSV). Inhibition by the venoms from *Oxyuranus scutellatus* and *Vipera russelli russelli* presented very similar characteristics, suggesting that they are closely related (see below).

Fig. 1 shows the effect of different concentrations of NSV on the net K^+ loss induced by A23187 + Ca^{2+} in human red cells. Panel A shows the original light-scattering records. The curves were linearized as described previously [15,19] and the percentage of inhibition obtained in each case was plotted against the concentration of venom used (Fig. 1B). The estimated value for K_i from data presented in Fig. 1B was 0.1 μg/ml. This value is 10–100-times smaller than that reported for *Leiurus quinquestriatus* venom [15]. In another four experiments the K_i values obtained ranged between 0.1 and 0.2 μg/ml and were independent of variations of the maximal inhibition accomplished by the venom (see below). About 50% of the original inhibitory effect was preserved after heating the venom for 10 min at 90 °C.

TABLE I

Effect of several snake venoms (0.1 mg/ml) on Ca^{2+}-dependent K^+ transport in human red cells

Channel activity was measured by the light scattering procedure. The first ten venoms are 'blood clothing' and the last nine 'neurotoxic' venoms. n.d. stands for not determined.

Snake	Channel inhibition	Haemolysis
Bitis arietans	–	–
Cerastes cerastes	–	–
Echis ocellatus	–	–
Vipera russelli russelli	+	–
Agkistrodon bilineatus	–	–
Agkistrodon contortrix	–	–
Bothrops jararaca	–	–
Bothrops lanceolatus	–	–
Calloselasma rhodostoma	–	–
Oxyuranus scutellatus	+	–
Boulengerina annulata	–	n.d.
Bungarus multicinctus	–	n.d.
Dendroaspis viridis	–	+
Naja haje haje	–	±
Naja katiensis	n.d.	+ +
Naja melanoleuca melanoleuca	–	+ +
Naja massambica pallida	n.d.	+ + +
Walterinnesia aegyptia	–	–

Fig. 2 shows the effects obtained with maximally inhibitory concentrations of LQV, NSV or both venoms together. It is apparent that the inhibition effected by the simultaneous presence of both venoms was larger than any of the individual effects. This suggests that they can bind at the same time to the channel, and hence they bind to different sites and have a different mechanism of inhibition.

The inhibition by NSV had a peculiar dependence on the cell metabolic condition. Fig. 3 shows that the effect of a maximally inhibitory concentration of the venom

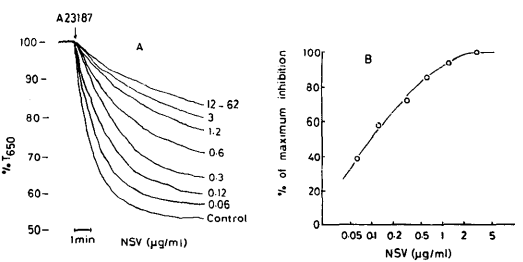

Fig. 1. Effect of different concentrations of the venom from *Notechis scutatus* (NSV) on Ca^{2+}-dependent K^+ transport, measured by the scattering procedure. (A) Crude records. Figures on the right refer to the concentrations of the venom in μg/ml. Cells were incubated with the venom for 1 h before the addition of the ionophore A23187. (B) Data from the same experiment plotted as percent inhibition versus the venom concentration.

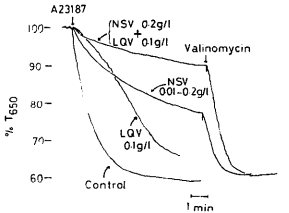

Fig. 2. Effects of the venoms of *Notechis scutatus* (NSV) and *Leiurus quinquestriatus* (LQV) on Ca^{2+}-dependent K^+ transport, measured by the scattering procedure. Cells were incubated for 30 min with each one of the venoms or with both venoms together before the addition of A23187. Valinomycin (2 μM) was added at the end to demonstrate that the failure of the cells to shrink was not due to loss of intracellular potassium nor to alterations of anion permeability.

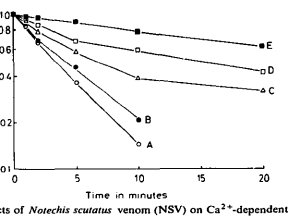

Fig. 4. Effects of *Notechis scutatus* venom (NSV) on Ca^{2+}-dependent $^{42}K^+$ uptake by human red cells. A: Control started with the ionophore A23187. B: Control preincubated during 30 min with A23187 and started with $^{42}K^+$. C: NSV (5 μg/ml) added at $t = 0$ with the ionophore. D: NSV added 30 min before the ionophore. E: NSV and ionophore added 30 min before starting the experiment with $^{42}K^+$. Data obtained at 40 and 60 min have been omitted for clarity, but they are discussed in the text.

depended on the time elapsed, since the cells were suspended in the incubation medium, prior to the addition of the venom. Just after suspension of the cells the inhibition was 68%, and increased to 83, 93 and 100% after 5, 10 and 20 h of incubation of the cells at room temperature, respectively. Progression of the inhibition was faster when the cell suspension was incubated at 37°C and not detectable after 24 h incubation at 4°C. In 15 different fresh blood samples and 2 bank blood samples stored in CPD-adenine the initial maximum inhibition by NSV was consistently 50–70%. The time course of the progress of inhibition varied, however, from batch to batch of cells. The presence of inosine in the incubation medium delayed the progress

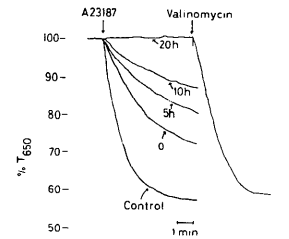

Fig. 3. Effect of preincubation of the cells on the subsequent inhibition by *Notechis scutatus* venom (NSV). Cells were preincubated at room temperature in a solution containing (mM): NaCl, 150; $CaCl_2$, 0.5, potassium-Hepes, 10 (pH 7.5), for the times indicated in the figure. Then 5 μg/ml of NSV were added and the incubation continued for 1 h. Immediately before the experiment, 1.2 ml of cell suspension were mixed with 0.4 ml of the same solution in which NaSCN replaced NaCl. Other details as in Figs. 1 and 2.

of the inhibition. These results suggest that metabolic changes in the cells may modify the interaction of the venom with the K^+ channels. It has been reported previously that the metabolic state of the cells could modify the properties of Ca^{2+}-dependent K^+ channels [20].

The development of full inhibitory effect required also some time of contact of the cells with the venom. Fig. 4 compares the inhibition produced by NSV when different periods of incubation of the cells with the venom were allowed prior to flux measurements. In the controls with no venom added the first-order rate constant for $^{42}K^+$ exchange remained constant at about 12 h^{-1} during the whole flux measurement period (curve A). When flux measurements were delayed 30 min with regard to the activation of the channels the rate constant for $^{42}K^+$ exchange was little modified (curve B). This is consistent with the previous finding that the K^+ channels are not inactivated with time [21,22]. When the venom was added coinciding with the activation of the channels (simultaneous addition of NSV and A23187, curve C) inhibition increased during the flux measurement period. The estimated value for the first-order rate constant was 7.8 during the first 2 min and decreased to about 1 after 20 min. When cells were incubated with the venom during 30 min before activation of the channels (curve D) the starting inhibition was larger ($k = 4.8$ h^{-1} during the first 2 min), although it still progressed during the flux measurement period ($k = 0.9$ after 20 min). Finally, when cells with activated channels were incubated with the venom for 30 min prior to the flux measurements (curve E) the starting inhibition was the largest ($k = 1.7$ h^{-1} during the first 2 min) and the relative increase of the inhibition during the flux measurement period was smaller ($k = 0.7$ after 20 min). These results suggest that the open channels are more

susceptible to the effects of NSV than the closed ones. This was confirmed by the experiment shown in Fig. 5. The initial rate of $^{42}K^+$ uptake was measured after several preincubation times with the venom, Ca^{2+} and either with or without the ionophore A23187. When the preincubation took place in the presence of A23187, the half-time for maximum inhibition decreased about 5 times (from 25 min to 5 min).

At variance with LQV [9,15], the inhibitory effect of NSV on Ca^{2+}-dependent K^+ transport was not reversed by washing. In fact, if the time of contact with the venom had been brief, the inhibition increased with time when the flux measurements were delayed after washing. This suggests that binding of the venom is quick and irreversible, but full inhibition only develops once the channels have suffered a conformational change which requires further time. The presence of free venom would not be required for the last stage.

Binding of NSV required the presence of Ca^{2+} in the external medium. If the venom was added to the cell suspension in the presence of EGTA no inhibition was detected after washing. On the other hand, if, after 2 min incubation with the venom and Ca^{2+}, Ca^{2+} was removed by addition of excess EGTA, the inhibitory effect was preserved after washing. This suggests that external Ca^{2+} is necessary for the initial binding. The existence of an external Ca^{2+} binding site in the channel protein has been postulated to explain the requirement of this cation for channel inactivation on removal of external K^+ [23]. Occupancy of this site could also facilitate the binding of the venom. An alternative possibility is that the binding of the venom was affected in these experiments through a decrease in intracellular Ca^{2+} concentration following incubation in Ca^{2+}-free medium. This would be in line with the facilitation by

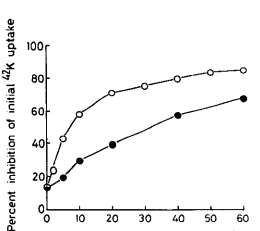

Fig. 5. Effects of *Notechis scutatus* venom (NSV) on the initial rate of Ca^{2+}-dependent $^{42}K^+$ uptake. Cells were preincubated at room temperature for the indicated times with 5 μg/ml of NSV (closed symbols) or with NSV and 10 μM A23187 (open symbols). Then either $^{42}K^+$ and A23187 or $^{42}K^+$, respectively, were added, and the uptake of radioactivity measured during 2 min. Results are expressed as percent inhibition, referred to the controls with no venom added.

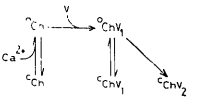

Fig. 6. Tentative model to explain the interactions of *Notechis scutatus* venom with the Ca^{2+}-dependent K^+ channels. The superscripts 'o' and 'c' refer to 'open' and 'closed' states. V stands for venom. For further explanations see text.

internal Ca^{2+} of the inhibition by NSV described above. Finally, the requirement of another cation susceptible to chelation by EGTA for binding of NSV cannot be excluded.

We have also studied some of the characteristics of the other two active snake venoms: *Vipera russelli russelli* and *Oxyuranus scutellatus*. Both venoms were irreversible inhibitors, needed preincubation to develop full inhibition and their effects were additive to those of LQV but not either between them nor to those of NSV. These similarities suggest that these other venoms are very closely related to NSV.

Fig. 6 proposes a provisional working hypothesis to explain the effects of the snake venoms studied here. Binding would take place quickly, only or mainly to channels in the open state. Binding of the venom would stabilize a new conformation in which the channel would still be able to open and close, but with a substantial decrease of unitary conductance (30 to 50% of the original state). Such low-conductance states have been detected in patch-clamp studies [6,13]. The stabilization of this state would explain the initial inhibition. Then the channels would evolve slowly to a second, irreversibly closed, conformation. Cell metabolic conditions could affect the speed of this transition.

The availability of these new venoms may be useful for the identification and characterization of Ca^{2+}-dependent K^+ channels in different cells. The irreversibility of the inhibition may also be useful in order to count the number of sensitive channels in a cell.

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