Eur Biophys J (1998) 27: 69–73

BIOPHYSICS LETTER

Marzia Pisciotta · Fredy I. Coronas
Lourival D. Possani · Gianfranco Prestipino

The *Androctonus australis* garzoni scorpion venom contains toxins that selectively affect voltage-dependent K^+-channels in cerebellum granular cells

Received: 10 February 1997 / Accepted: 7 August 1997

Abstract A purified peptide from *Androctonus australis* Garzoni venom (AaG) affects selectively a K^+-current recorded from cerebellum granular cells. This current is characterized by fast activating and inactivating kinetics similar to an I_A-type current. Addition of 2 μM peptide Aa1 (from *Androctonus australis*, toxin 1) to the external side of the channel suppressed completely and in a selective manner the I_A-type current, with an IC50 value of 130 nM, whereas in the same conditions, the other potassium current, identified as delayed rectifier (I_d), was not affected. Additionally, we show that another partially purified peptide (III-12) from the same venom was able to block reversibly both K^+-currents.

Key words Scorpion venom · Granular cells · Patch-clamp · Potassium currents

Introduction

Cerebellum granular cell membranes contain several types of potassium channels with different kinetics and pharmacology. We characterized the outward potassium currents from these cells using the patch-clamp technique in the whole-cell configuration. Two different currents were recorded: a fast transient, low-voltage activated current (I_A), blocked by 4-aminopyridine (4-AP) and a slowly rising and inactivating current. The latter has electrophysiological properties similar to the classical squid axon potassium current (I_d), (Robello et al., 1989).

The knowledge of the structure and function of potassium channels has been greatly increased by the discovery of specific natural peptide inhibitors used as probes at the molecular level (Carbone et al. 1982, Miller et al. 1985, Miller 1995). The neurotoxic peptides found in scorpion venoms, act specifically as high-affinity K^+-channel blockers (MacKinnon and Miller 1988, Goldstein and Miller 1993, Gross and McKinnon 1996); they have proven to be useful tools for channel purification (Prestipino et al. 1989, Noceti et al. 1995, Rehm et al. 1988), for structure-function analysis of voltage-gated and Ca^{++}-activated K^+ channels (Goldstein et al. 1994, Hidalgo and MacKinnon 1995; Crest et al. 1992; Knaus et al. 1995) and for the pharmacological classification of the different K^+-channel currents (Goldstein and Miller 1992; Giangiacomo et al. 1993; Garcia et al. 1994). For these reasons there is common interest in trying to isolate and characterize new venom peptides from scorpions, spiders and snails. Recently, it has been found that the *Androctonus mauritanicus* scorpion venom contains a toxin, P05, which is structurally and functionally similar to the leiurotoxin I (from the scorpion *Leiurus quinquestriatus*), a blocker of the apamin-sensitive Ca^{++}-activated K^+ channels (Sebatier et al. 1993). Similarly, kaliotoxin and agitoxin are peptides from scorpions of the species *Androctonus mauritanicus mauritanicus* (Crest et al. 1992) and *Leiurus quinquestriatus var. hebraeus* (Garcia et al. 1994; Gross and MacKinnon 1996) respectively, used to characterize several types of K^+-channels. In this communication we report the purification and physiological characterization of unknown peptides from *Androctonus australis* Garzoni scorpion venom capable of blocking, with different affinities, two distinct K^+ currents in cerebellum granular cells.

M. Pisciotta · G. Prestipino (✉)
Istituto di Cibernetica e Biofisica, Consiglio Nazionale delle Ricerche, Via de Marini 6, I-16149 Genova, Italy
(e-mail: presti@barolo.icb.ge.cnr.it)

F. J. Coronas · L. D. Possani
Instituto de Biotechnologia/UNAM, Avenida Universidad 2001, Cuernavaca 62271, Mexico

Materials and methods

Materials. Androctonus australis Garzoni (AaG) scorpion venom was purchased from Latoxan. The culture medium was from GIBCO Laboratories. Water double distilled on quartz was used for all experiments. Reagents and solvents used for chromatography and electrophysiological experi-

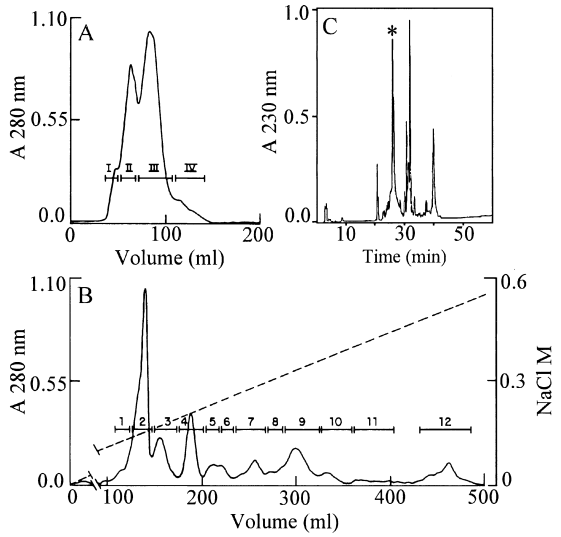

Fig. 1 A – C Fractionation of *Androctonus australis* Garzoni venom. **A**) The soluble venom (45 mg) from AaG was dissolved in 1.5 ml of 20 mM ammonium acetate buffer, pH 4.7, and applied to a Sephadex G-50-medium column (0.9×200 cm) equilibrated in the same buffer and run at 30 ml/hr. Tube-fractions of 2 ml each were collected. Four components, indicated by the horizontal bars were pooled according to absorbance at 280 nm (Fig. 1 a). This was repeated twice, with an overall recovery of the order of 90% of the material loaded into the column. **B**) The fraction number III (total 49 mg from two independent Sephadex G-50 applications) was further separated on a CM-cellulose column (0.9×30 cm), equilibrated and run in the presence of 20 mM ammonium acetate buffer, pH 4.7, and eluted with a salt gradient from 0 to 0.55 M NaCl (250 ml each side of the gradient-maker). Tube-fractions of 2.5 ml each, at a flow rate of 30 ml/hr were collected. Twelve sub-fractions were pooled as indicated by horizontal bars numbered 1 – 12. Sub-fraction III-5 was 4.5%, whereas sub-fraction III-12 was of the order of 7.2% of the material recovered. Overall column recovery was of the order of 87%. **C**) A sample of fraction III-5 (400 mg) from B, was applied to a C18 reverse column of a HPLC system (model Millenium 2010, from Millipore Co.) and separated using a linear gradient from 0.12% trifluoroacetic acid in the water, to 60% acetonitrile in the presence of 0.10% trifluoroacetic acid, during 60 min. Fraction labeled with asterisk (elution time 26.61) contained pure peptide Aa1 and constitutes about 27% of the sample recovered from the column

ments were analytical grade, obtained as previously described (Olamendi-Portugal et al. 1996).

Separation procedure. One hundred milligrams of venom from AaG was dissolved in 8 ml of double distilled water, centrifuged at 15,000 g for 15 min and the supernatant was lyophylized and kept at –20 °C until used. The various active fractions used in this work were obtained by chromatographic separations, as done for other scorpion venoms (Olamendi-Portugal et al. 1996). Briefly, the soluble venom was initially applied to a Sephadex G-50 column. The sub-fraction corresponding to the peptides of interest (range 4000 molecular mass) were further separated on carboxymethyl-cellulose (CM-cellulose) ion exchange resins, followed by high performance liquid chromatography (HPLC), as described in the legend of Fig. 1.

Cell culture. Experiments were performed on cerebellum granular cells in primary culture obtained from 8-day-old Wistar rats. Dissociated cell cultures were prepared by trypsin digestion and mechanical trituration, following the procedure of Levi et al. (1984). Cells were plated at a density of 2.5 10^6 per dish, on 35 mm plastic dishes or on glass coverslips, coated with 10 mg/ml poly-L-lysine and kept at 37 °C in humidified 95% air/5% CO_2 atmosphere. Experiments were performed 5 to 12 days after plating.

Patch-clamp measurements. Ionic currents were recorded using the whole-cell patch-clamp technique configuration (Hamill et al. 1981). Patch pipettes were made from borosilicate glass (CLARK Electromedical Instruments) and fire polished to obtain resistances between 2 – 3 MOhms. Cell responses were amplified and filtered at 2 kHz by an AxoPatch-1D (Axon Instruments). Both stimulation and data acquisition were performed with a 16-bit AD/DA converter (DigiData 1200, Axon Instruments) controlled by a computer PC 486. The whole-cell currents elicited by 150 – 200 ms-long voltage steps between –60 to 80 mV from –50 and –80 mV holding potentials (HP) were acquired at a sampling time of 200 µs. The capacitive transient component of recorded currents was analog-compensated. When the voltage error associated with the uncompensated series resistance was more than 10 mV the membrane potential was corrected off line. P/4 leakage substraction was performed on line. Data were stored on hard disk for subsequent analysis. The composition of the pipette filling solution was the following (in mM): 90 KF, 30 KCl, 2 $MgCl_2$, 2 EGTA, 5 NaCl, 10 HEPES, 30 Glucose, pH 7.35. The external standard solution, designed to suppress Na^+ and Ca^{++} currents, was (in mM): 135 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 0.3 tetrodotoxin, 0.2 $CdCl_2$, 10 HEPES, 10 Glucose, pH 7.35. Venom, sub-fractions and purified peptides were directly added in the chamber containing 200 µl of external solution.

The concentrations of sub-fractions were calculated by assuming the presence of peptides with the same molecular weight of 4000, similar to most known scorpion toxins blocking K^+-channels. All the experiments were carried out at room temperature (23±2).

Results and discussion

The soluble AaG venom applied into a Sephadex G-50 column was separated into four distinct sub-fractions. Number III (Fig. 1 a) contained peptides with molecular weights of the order of 4,000, similar to noxiustoxin and charybdotoxin (Possani et al., 1982; Miller 1995). For this reason fraction III was further separated on an ion exchange column. Figure 1 b shows the profile of the CM-cellulose column. The twelve distinct sub-fractions were all assayed for their effect in our cell culture system. Fraction III-5, because of its specific effect, was finally applied to a C18 column (Fig. 1 c), from which purified toxin Aa1 (compo-

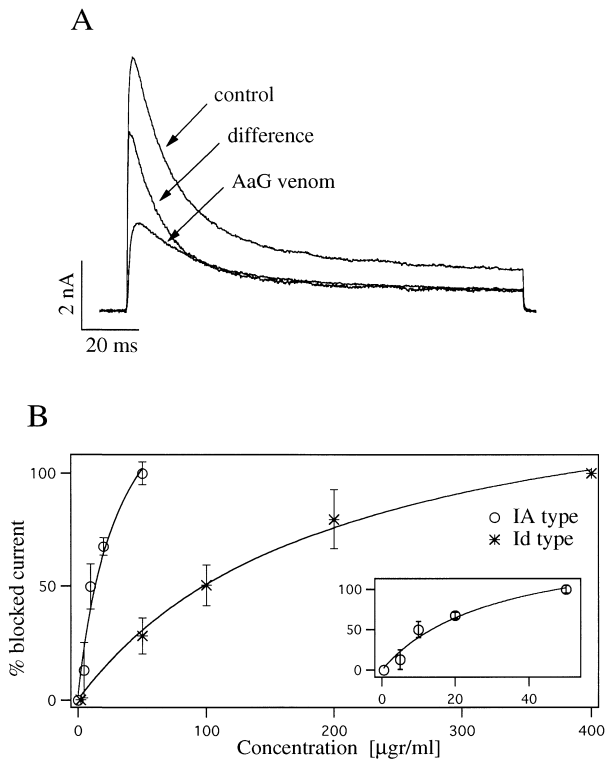

Fig. 2 A, B *Androctonus australis* Garzoni scorpion venom effects on I_K currents of granule cells. **A** Current recorded in control conditions and in the presence of 20 mg/ml AaG venom. A digital subtraction ("difference" line) between the two traces represents the current blocked by the venom. The traces were evoked by a 150 ms pulse to the test potential of +60 mV (HP = –80 mV). **B** Dose-response curves for transient and stationary currents vs. venom concentrations. Experimental points (mean values±SEM of 4–5 experiments) were fitted to the Michaelis-Menten equations $(I_{max}\text{-}I)/I_{max} = 1/(1+IC_{50}/C)$ that gave a half-effective doses of (15±10) µg/ml and (100±20) µg/ml for I_A and I_d currents respectively. The insert shows the dose-response curve for the peak in the 0.2–50 µg/ml concentration range

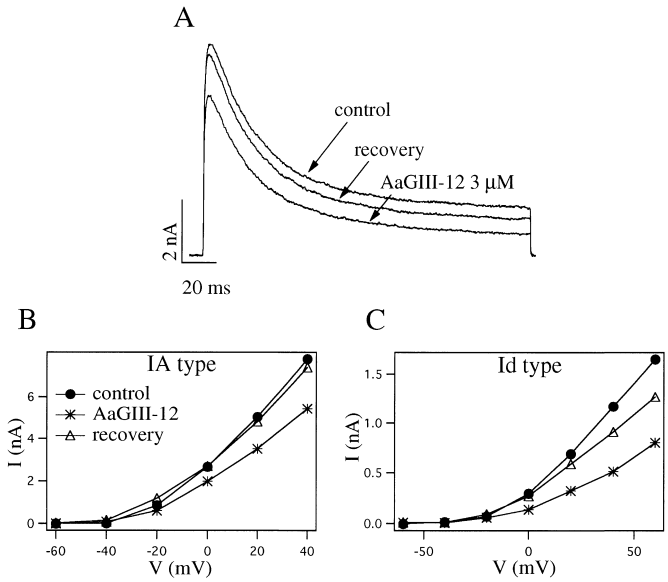

Fig. 3 A–C AaGIII-12 contains toxins that act on the I_A and I_d type potassium current. **A** Current elicited by a step potential of +60 mV in control conditions, in the presence of 3 µM AaGIII-12 fraction and after washout (HP = –80 mV). **B** Characteristic current-potential (I–V) plots in the presence of 3 µM AaGIII-12. The values were obtained from the peak current. **C**) I–V relationship measured at the end of 200 ms-long depolarizing test pulse (HP = –50 mV)

nent labeled with star, eluting at 26.61 min) was obtained in pure form and assayed.

Figure 2 a shows the effects of the AaG scorpion venom on the outward granular cell K^+ current recorded in whole cell configuration. The "difference" line represents the current obtained by subtracting the traces in control conditions and in the presence of 20 µg/ml of venom. It is evident from the figure that the venom depressed mainly the peak current (I_A). The peak current was reduced by ~65% while the stationary state by only ~14% (Fig. 2 b). To isolate the I_A type current we added 20 mM TEA in the external solution and depolarized the membrane at 60 mV from a holding potential (HP) of –80 mV. The conditions were mofidied to record the I_d component: TEA was substituted with 5 mM 4-AP and the HP was –50 mV, a value at which the I_A type is inactivated (Robello et al. 1989). The inhibition of AaG venom was dose-dependent for both transient and delayed components. The experimental points shown in Fig. 2 b were fitted to the Michaelis-Menten equation, which gave half-effective doses (IC_{50}) for I_A and I_d of (15±10) µg/ml and (100±20) µg/ml respectively.

The two apparent IC_{50} value differ from each other by approximately one order of magnitude; this finding suggests the presence of one or more peptides in the AaG venom that selectively affect the fast activating and inactivating K^+ current in the granular neurons. The purification of *Androctonus australis* Garzoni venom as described in Fig. 1 led to the separation of twelve sub-fractions. Among them, the components III-5 and III-12 were able to block the activity of K^+ channels. These fractions acted in a few minutes (1–3 min) and the block was never recovered completely, even after 15 min of washing. As illustrated in Fig. 3 a the addition to the external bath of 3 µM III-12 decreased both the I_A and I_d type currents. The current-voltage relationship (Fig. 3 b and 3 c) shows that the I_d type current block (~56%) was higher than the I_A block (~30%) at +40 mV membrane potential, indicating the presence of one or more peptides in the fraction III-12 with different affinities for the two current components. Furthermore, we studied the effect of the sub-fraction III-5 on the same potassium currents. Our results indicated that the fast component of the outward granular cells K^+ current was completely blocked by 600 nM of this fraction in a reversible manner, whereas the I_d component was unaffected (data not shown). In order to find which peptide in the fraction was capable of inhibiting the above mentioned K^+ currents we applied each one of the components isolated by HPLC (Fig. 1 c) to the channel-preparation. Figure 4 a shows the I_A current selectively blocked by toxin Aa1 (component labeled with star in the HPLC chromatogram). This peptide when re-applied to the HPLC system using a contin-

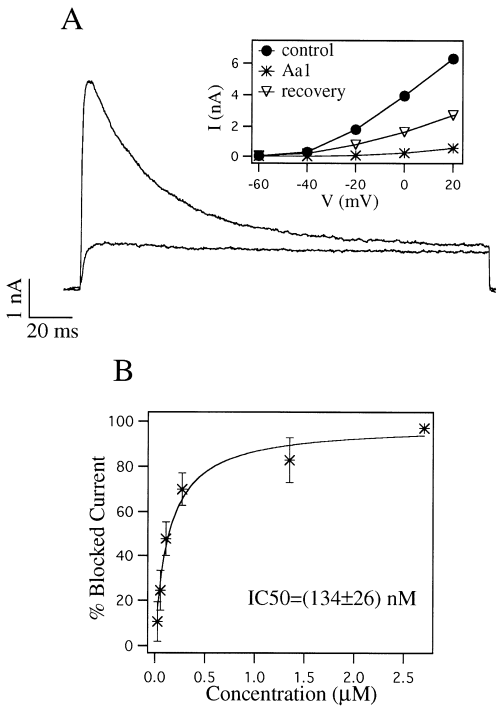

Fig. 4 A, B Aa1 toxin acts on the I_A type potassium current selectively. **A** Current recorded in control condition and in presence of 2 μM Aa1. In the insert: current-voltage relationship of the peak current in control conditions, in the presence of toxin 2 μM and after washout. **B** Dose-response curve for transient current vs. toxin concentrations applied. Experimental points were fitted to the Michaelis-Menten equation that gave a half-effective dose of (134±26) mM. Current was recorded at 40 mV from a HP of –80 mV

uous gradient system (data not shown) gave only one component (symmetrical peak) from which we assumed that it was pure. Attempts to sequence the toxin using a Pro-Sequencer model 6400/6600 from Millipore Co., consistently gave no sequence, from which we surmised that it was blocked at the N-terminal amino acid residue. Amino acid composition obtained from this material, after acid hydrolysis, confirmed that we were indeed working with a "bonafide" peptide. The dose-response curve for the fast activating current as a function of Aa1 concentration is represented in Fig. 4b. Experimental points were fitted to a Michaelis-Menten equation which gave a half-effective dose of (134±26) nM.

Since peptide Aa1 is blocked at the N-terminal region it is possibly similar to chary B dotoxin, iberiotoxin or leirustoxin-2, the only other K^+ channel blocking peptides from scorpions, reported thus far, to have a blocked amino terminus sequence. However, Aa1 is certainly none of them because it comes from the venom of the scorpion *Androctonus australis* Garzoni, whereas the other toxins are from *Leiurus quinquestriatus* venom. Furthermore, the kaliotoxin group of toxins, from the genus *Androctonus*, are not blocked at the amino-terminal amino acid (Crest et al., 1992). For these reasons we are confident that Aa1 must be a novel example of a very interesting scorpion venom peptide, with exquisite preference for the transient K^+ channels of the cerebellum granular cells.

Acknowledgements We would like to thank Mr. G. Gaggero and D. Magliozzi for their technical assistance, and Dr. M. Ottolia for helpful comments on this manuscript. This work was supported by the grant of European Communities CI1* CT94 0045 and by a grant from CNR/CONACYT for International cooperation.

References

Carbone E, Wanke E, Prestipino G, Possani LD, Maelicke A (1982) Selective blockage of voltage-dependent K^+ channels by a novel scorpion toxin. Nature 296: 90–91

Crest M, Jacquet G, Goia M, Zerrouk H, Benslimane A, Rochat H, Mansuelle P, Martin-Eauclaire MF (1992) Kaliotoxin, a novel peptide inhibitor of a neuronal BK-type Ca^{2+}-activated K^+ channels characterized from *Androctonus mauretanicus mauretanicus* venom. J Biol Chem 267: 1640–1647

Garcia ML, Garcia-Calvo M, Hidalgo P, Lee A, MacKinnon R (1994) Purification and characterization of three inhibitors of voltage-dependent K^+ channels from *Leiurus quinquestriatus* var. habraeus venom. Biochemistry 33: 6834–6839

Giangiacomo KH, Sugg EE, Garcia-Calvo M, Leonard RJ, McManus OB, Kaczorowsky GJ, Garcia ML (1993) Synthetic charybdotoxin-iberiotoxin chimeric peptides define toxin binding sites on calcium-activated and voltage-dependent potassium channels. Biochemistry 32: 2363–2370

Goldstein SA, Miller C (1992) A point mutation in a *Shaker* K^+ channel changes its charybdotoxin binding site from low to high affinity. Biophys J 62: 5–7

Goldstein SA, Miller C (1993) Mechanism of charybdotoxin block of a *Shaker* K^+ channel Biophys J 65: 1613–1619

Goldstein SA, Pheasant DJ, Miller C (1994) The charybdotoxin receptor of a *Shaker* K^+ channel: peptide and channel residues mediating molecular recognition. Neuron 12: 1377–1384

Gross A, MacKinnon R (1996) *Agitoxin* footprinting the *Shaker* potassium channel pore. Neuron 16: 399–406

Hamill OP, Marty A, Sakmann B, Neher E, Sigurth FT (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391: 85–100

Hidalgo P, MacKinnon R (1995) Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. Science 268: 307–310

Knaus HG, Koch ROA, Eberhart A, Kaczorowski GJ, Garcia ML, Slaughter RS (1995) ^{125}I Margatoxin, an extraordinarily high affinity ligand for voltage-gated potassium channel in mammalian brain. Biochemistry 34: 13 627–13 634

Levi G, Aloisi M, Ciotti M, Gallo V (1984) Autoradiographic localization and depolarization-induced release of acidic amino acids in differentiating granule cells cultures. Brain Res 290: 77–86

MacKinnon R, Miller C (1988) Mechanism of charybdotoxin block of a Ca^{++}-activated K^+ channel. J Gen Physiol 91: 335–349

Miller C, Moczydlowski E, La Torre R, Phillips M (1985) Charybdotoxin, a high affinity inhibitor of a single Ca^{++}-activated K^+ channel from mammalian skeletal muscle. Nature 31: 316–318

Miller C (1995) The charybdotoxin family of K^+ channel blocking peptide. Neuron 15: 5–10

Noceti F, Ramirez AN, Possani LD, Prestipino G (1995) Characterization of a voltage-dependent potassium channel in squid Schwann cells reconstituted in planar lipid bilayers. Glia 15: 33–42

Olamendi-Portugal T, Gómez-Lagunas F, Gurrola GB, Possani LD (1996) A novel structural class of K^+ channel blocking toxin from the scorpion *Pandinus imperator.* Biochem J (Great-Britain) 315: 977–981

Possani LD, Martin BM, Svendsen IB (1982) The primary structure of Noxiustoxin: a K^+ channel blocking peptide from the venom

of the scorpion *Centruroides noxius* Hoffmann. Carlsberg Res Commun 47: 285 – 289

Prestipino G, Valdivia HH, Lievano A, Darszon A, Ramirez AN, Possani LD (1989) Purification and reconstitution of potassium channel proteins from squid axon membranes. Febs 250: 570 – 574

Rehm H, Lazdunski M (1988) Purification and subunit structure of a putative K^+ channel protein identified by its binding properties of dendrotoxin I. Proc Natl Acad Sci USA 85: 4919 – 4923

Robello M, Carignani C, Marchetti C (1989) A transient voltage-dependent outward current in cultured cerebellar granules. Bioscience Reports 9: 451 – 457

Sebatier JM, Zerrouk H, Darbon H, Mebrouk K, Benslimane H, Rochat H, Martin-Eanclaire MF, Van Rietschoten J (1993) P05, a new leiurotoxin I-like scorpion toxin: synthesis and structure-activity relationships of the alpha-amidated analog, a ligand of Ca^{++}-activated K^+ channels with increased affinity. Biochemistry 32: 2763 – 2770