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Contryphan-Vn: a modulator of Ca^{2+}-dependent K^+ channels☆

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Abstract

Contryphan-Vn is a D-tryptophan-containing disulfide-constrained nonapeptide isolated from the venom of *Conus ventricosus*, the single Mediterranean cone snail species. The structure of the synthetic Contryphan-Vn has been determined by NMR spectroscopy. Unique among Contryphans, Contryphan-Vn displays the peculiar presence of a Lys–Trp dyad, reminiscent of that observed in several voltage-gated K^+ channel blockers. Electrophysiological experiments carried out on dorsal unpaired median neurons isolated from the cockroach (*Periplaneta americana*) nerve cord on rat fetal chromaffin cells indicate that Contryphan-Vn affects both voltage-gated and Ca^{2+}-dependent K^+ channel activities, with composite and diversified effects in invertebrate and vertebrate systems. Voltage-gated and Ca^{2+}-dependent K^+ channels represent the first functional target identified for a conopeptide of the Contryphan family. Furthermore, Contryphan-Vn is the first conopeptide known to modulate the activity of Ca^{2+}-dependent K^+ channels.
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Keywords: Contryphan-Vn; Solution structure; Cockroach dorsal unpaired median neurons; Rat fetal chromaffin cells; Ca^{2+}-dependent K^+ channels

The Contryphan family of conopeptides, first discovered in fish-hunting cone snail's venom [1], is remarkable for its richness in post-translational modifications, including proline hydroxylation, C-terminus amidation, tryptophan bromination, as well as leucine and tryptophan isomerization (Table 1), the more so considering they are among the smallest (8–9 amino acids) peptide components of cone snail's venom [1–6]. These disulfide cyclized bio-active peptides display a well-defined structure in solution, suggesting that their robust structural scaffold could be easily engineered to perform different functions [7–9]. Contryphans characterized so far exist in solution as a mixture of two conformers, due to *cis–trans* isomerization around the N-terminal Cys–Pro peptide bond [8]. The *cis* isomer is the most abundant and is thus thought to be the functionally relevant conformer [8]. The thermodynamic equilibrium between the two conformers mainly depends on the presence of a stabilizing electrostatic interaction between an acidic amino acid residue within the intercysteine loop and the N-terminal ammonium group [7,8]. The biological activity of Contryphans, as assayed through intra-cerebro-ventricular injection in mice, is that of causing a stiff-tail syndrome at low doses,

☆ *Abbreviations:* BgK, *Bunodosoma granulifera* voltage-gated K^+ channel (blocker); COSY, correlation spectroscopy; DUM, dorsal unpaired median (neurons); HSQC, single quantum correlation spectroscopy; NOE, nuclear overhauser effect; ROESY, rotating frame exchange spectroscopy; TOCSY, total correlation spectroscopy.

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Table 1
Amino acid sequence alignment of Contryphans

Conopeptide	Amino acid sequence[a]	Species	Ref.
Contryphan-R	G-CO<u>W</u>EPWC*	*Conus radiatus*	[1]
Contryphan-R/Tx	G-CO<u>W</u>EPWC*	*Conus textile*	[3]
Contryphan-P	G-CO<u>W</u>DPWC*	*Conus purpurascens*	[4]
Bromocontryphan	G-CO<u>W</u>EP<u>W</u>C*	*C. radiatus*	[2]
Contryphan-Sm	G-CO<u>W</u>QPWC*	*Conus stercusmuscarum*	[4]
Contryphan-Tx	G-CO<u>W</u>QPYC*	*C. textile*	[3]
Contryphan-Vn	GDCP<u>W</u>KPWC*	*Conus ventricosus*	[6]
Leu-Contryphan-Tx	--CV<u>L</u>YPWC	*C. textile*	[3]
Leu-Contryphan-P	G-CV<u>L</u>LPWC	*C. purpurascens*	[5]

The star (*) indicates C-terminus amidation. Disulfide connectivity is indicated above the amino acid sequences.
[a] D-Amino acids are underlined; O indicates hydroxyproline; W indicates 6-Br-Trp.

while inducing seizures and death at higher doses. However, the molecular target(s) of Contryphans as well as their specific pharmacological effect(s) remain still unknown [1,5].

Contryphan-Vn is a D-tryptophan-containing disulfide-constrained nonapeptide recently isolated from the venom of the temperate Mediterranean cone snail *Conus ventricosus* (Table 1) [6], a worm-hunting species phylogenetically older than those from which Contryphans have been so far characterized (all tropical Indo-Pacific fish-hunting and mollusk-hunting species) [10]. Contryphan-Vn displays a high sequence identity with the eight previously characterized Contryphans, but is unusual for the presence of the positively charged Lys6 residue within the intercysteine loop (Table 1) [6]. The presence of the intercysteine Lys6 confers to Contryphan-Vn a distinct surface electrostatic potential [6]. Analysis of the homology-modeled structure of Contryphan-Vn [6] indicates that this conopeptide displays the Lys6–Trp8 dyad, which is reminiscent of that found in several K^+ channel blocker toxins [11], suggesting a possible role of Contryphan-Vn as a K^+ channel modulator [6].

Here, the structural and functional characterization of Contryphan-Vn is reported. The analysis of the solution structure of Contryphan-Vn, determined by NMR spectroscopy, confirms the presence of the Lys6–Trp8 dyad, with Lys6 being involved in a strong salt bridge with Asp2. Electrophysiological experiments carried out on dorsal unpaired median neurons isolated from the cockroach nerve cord and on rat fetal chromaffin cells evidence composite and diversified effects in invertebrate and vertebrate systems which altogether point out a role of Contryphan-Vn in modulating voltage-gated and Ca^{2+}-dependent K^+ channel activities. Voltage-gated and Ca^{2+}-dependent K^+ channels represent the first functional target identified for a conopeptide of the Contryphan family. Moreover, Contryphan-Vn is the first conopeptide known to modulate Ca^{2+}-dependent K^+ channels.

Experimental procedures

Contryphan-Vn synthesis. Synthetic Contryphan-Vn for NMR spectroscopy and electrophysiological studies was prepared by the standard Fmoc chemistry on an automated peptide synthesizer (Pioneer, Applied Biosystems, Foster City, USA), as previously reported [6].

Contryphan-Vn solution structure determination. NMR experiments were performed using a 400-MHz Bruker Avance spectrometer (Bruker Biospin, Billerica, MA). The sample was typically 3.0 mM Contryphan-Vn in 90% H_2O/10% ^2H_2O, or 100% ^2H_2O at 25 °C and pH 3.5. Spectra were processed on Silicon Graphics (Mountain View, CA) workstations by NMRPipe software [12] and analyzed using NMRView [13]. All the proton resonances were assigned by 2D spectra: TOCSY (mixing time 60 ms) to identify the spin systems [14], ^1H–^{15}N HSQC [15], ^1H–^{13}C HMQC [16], and ^1H–^{13}C HSQC–TOCSY [17] to assist with cross-peak assignment, and ROESY [18], with a spin-lock time of 0.05, 0.08, 0.12, and 0.15 s, according to the sequential assignment method [19]. All the heteronuclear correlation experiments were carried out at natural abundance. Acquisition of a phase-sensitive COSY [20] enabled the measure of all backbone $^3J_{HNC\alpha H}$ and some $^3J_{HC\alpha C\beta H}$ and the identification of some stereospecific assignments [21].

The structural restraints used to determine the solution structure of Contryphan-Vn were obtained from the internuclear distances evaluated by the ROESY cross-peak volumes. These were measured from spectra obtained with 0.15 s of mixing time in H_2O and ^2H_2O, and converted and grouped into distance bounds: $1.8\,\text{Å} < d < 2.5\,\text{Å}$ for strong NOEs, $1.8\,\text{Å} < d < 3.4\,\text{Å}$ for medium NOEs, and $1.8\,\text{Å} < d < 6.0\,\text{Å}$ for weak NOEs [19]. The final NMR distance restraint set consisted of 70 NOEs. Moreover, the measured coupling constants allowed the determination of six backbone and three side-chain dihedral constraints [21].

A series of structures was then calculated for the major form (i.e., the *cis* form) of Contryphan-Vn (see below) using the NMR data as restraints for molecular simulations in a conventional XPLOR [22] simulated annealing protocol in vacuo [23]. Starting from the linear template structure and different randomized initial velocity distributions, structures were subjected to an all-hydrogen force field (including covalent geometry, planarity, hard sphere, van der Waals, empirical NOE, and *J*-coupling energy terms, but no Lennard-Jones, electrostatic, hydrogen bonding, and empirical dihedral angle terms). Simulated annealing was performed using a first phase consisting of 10,000 steps (2.0 fs time step) at 3727 °C, setting the initial van der Waals weight to a very low value (0.003) to allow atoms to pass each other in the early stages of simulation. Successive 7000 steps followed to gradually cool the system to −173 °C. A set of 50 structures with the least number of violations (zero or a single violation lower than 0.5 Å) were selected. Among these, PROCHECK-NMR [24] identified the best 20

structures with the greatest number of backbone torsion angles in most favorable regions of the Ramachandran plot and none in disallowed regions (Gly1 and D-Trp5 were not included in the analysis). The atomic coordinates and the NMR restraints of Contryphan-Vn have been deposited in the Protein Data Bank (Accession code 1N3V).

Contryphan-Vn bioactivity on cockroach dorsal unpaired median neurons. Electrophysiology experiments were performed on the somata of dorsal unpaired median (DUM) neurons isolated from the midline of the terminal abdominal ganglion of adult male cockroach (*Periplaneta americana*) nerve cord. Isolation of DUM neuron cell bodies was performed under sterile conditions using enzymatic digestion and mechanical dissociation, as previously described [25]. Briefly, the dorsal median parts were incubated for 35 min at 37 °C in sterile cockroach saline solution containing *Clostridium histolyticum* collagenase (Type IA, 1.5 mg/mL; Worthington Biochemical, Lakewood, NJ). The ganglia were rinsed twice in normal saline solution and mechanically dissociated by repetitive gentle suctions through fire-polished Pasteur pipettes. The DUM neurons, suspended in normal saline solution (containing 200 mM NaCl, 3.1 mM KCl, 4.0 mM $MgCl_2$, 5.0 mM $CaCl_2$, 50 mM sucrose, and 10 mM Hepes; pH 7.4) supplemented with 5% (v/v) fetal calf serum (Gibco, Cergy Pontoise, France), 50 IU/mL penicillin (Gibco, Cergy Pontoise, France), and 50 μg/mL streptomycin (Gibco, Cergy Pontoise, France), were allowed to settle on poly-D-lysin hydrobromide (MW 70,000–150,000; Sigma Chemical, St. Louis, MO) coating the bottom of 35-mm tissue culture petri dishes. The DUM neurons were chosen as previously described [26] and used for recordings 24 h after dissociation.

The whole-cell patch clamp technique [27] was used to record action potentials, membrane potentials (current-clamp mode), and K^+ currents (voltage-clamp mode), in the absence and presence of Contryphan-Vn (20 μM), Cd^{2+} (cadmium chloride; 1.0 mM), and iberiotoxin (10 nM; Sigma Chemical, St. Louis, MO). Signals were recorded with an Axopatch 200A patch clamp amplifier (Axon Instruments, Union City, CA) and filtered at 5 kHz (−3 dB, 4-pole low-pass Bessel filter). Patch electrodes were pulled from borosilicate capillary tubes (Clark Electromedical Instruments, Edenbridge, UK) using a PP83 puller (Narashige, Tokyo, Japan) and had resistances ranging from 0.8 to 1.2 MΩ when filled with the pipette solution (for composition see below). For current-clamp experiments, depolarizing and hyperpolarizing current pulses were applied from a programmable stimulator (SMP 310; Biologic, Claix, France). Signals were displayed on a digital oscilloscope (310; Nicolet Instrument, Madison, USA) and stored on a digital tape recorder 1202 (Biologic; Claix, France). For voltage-clamp recordings, the pClamp package (version 6.0.3; Axon Instruments, Union City, USA) was used for data acquisition and analysis. Data were then stored on the hard disk of an IBM Pentium 100 computer (IBM, White Plains, NY) connected to a 125-kHz labmaster DMA data acquisition system (TL-1-125 interface; Axon Instruments, Union City, CA).

For current-clamp recordings, the patch electrode was filled with an internal solution containing 160 mM potassium aspartate, 10 mM KF, 10 mM NaCl, 1.0 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1.0 mM ATP-Mg, 10 mM EGTA, and 10 mM Hepes (pH 7.4). Outward K^+ currents were recorded with an extracellular solution containing 100 mM NaCl, 70 mM Tris–HCl, 3.1 mM KCl, 4.0 mM $MgCl_2$, 5.0 mM $CaCl_2$, and 10 mM Hepes (pH 7.4). The Na^+ current was blocked by adding 100 nM tetrodotoxin (Sigma Chemical, St. Louis, MO) to saline solution. The composition of the pipette solution was the following: 135 mM KCl, 25 mM KF, 9.0 mM NaCl, 3.0 mM ATP-Mg, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.0 mM EGTA, and 10 mM Hepes (pH 7.4). Liquid junction potential between the pipette and the superfusing solution was always corrected before the seal formation (>2 GΩ). Capacitance and leak currents were substracted using P/6 protocol provided by the pClamp software (V.5.5 I., Axon Instruments, Union City, CA).

All experiments were carried out at 20 °C. Data, when quantified, were expressed as means ± SE.

Contryphan-Vn bioactivity on rat fetal chromaffin cells. Primary cultures of rat fetal (E19) chromaffin cells were performed as previously reported [28,29]. Whole-cell current recordings were done using the perforated patch-clamp technique [30], in the absence and presence of Contryphan-Vn (20 μM) and Cd^{2+} (cadmium chloride; 0.5 mM). The whole-cell configuration was achieved by perforating the area under the patch through the addition of amphotericin B (24 μg/mL; Sigma Chemical, St. Louis, MO) to the patch electrode solution. Patch electrodes were pulled from a glass capillary tubes (Blu-Tip, Oxford Labware, Sherwood-Davis & Geck, St. Louis, MO) using a programmable micropipette puller (model P-87; Sutter Instruments, Novato, CA) and coated with sticky wax (S.S. White, Gloucester, UK) to reduce capacitance. The tip of the patch pipette had a resistance of 2–4 MΩ when filled with the pipette solution (for composition see below). Membrane currents were recorded with a RK 400 patch-clamp amplifier (Biologic, Claix, France). Pulse generation and acquisition of signals were performed through a TL-l Labmaster DMA board (Scientific Solutions, Mentor, OH). The pClamp software (V.5.5 I., Axon Instruments, Union City, USA) was used for data acquisition and analysis. The leak-current was not subtracted.

The recording chamber was filled with a solution containing 135 mM NaCl, 5.0 mM KCl, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 10 mM glucose, 1.0 μM tetrodotoxin, and 10 mM Hepes (pH 7.4). The pipette solution for perforated patch recordings had the following composition: 105 mM potassium gluconate, 30 mM KCl, 5.0 mM NaCl, 0.1 mM $CaCl_2$, 24 μg/mL amphotericin B, and 10 mM Hepes (pH 7.2). The recorded cells were superfused through a microflow system.

The effect of Contryphan-Vn on membrane Ca^{2+} channel activity in rat fetal chromaffin cells was recorded with Ba^{2+} as a charge carrier.

All experiments were carried out at 20 °C. Data, when quantified, were expressed as means ± SE.

Contryphan-Vn binding to human voltage-gated K^+ channels. Membranes of transformed HEK 293 cells overexpressing human voltage-gated K^+ channels Kv1.1 or Kv1.2 were incubated for 2 h in 250 μL with 40 pM ^{125}I-BgK(Trp5 → Tyr/Tyr26 → Phe), a radiolabeled variant of the *Bunodosoma granulifera* voltage-gated K^+ channel blocker (BgK) [11,31], in the absence and presence of increasing concentrations of Contryphan-Vn (20–800 μM). ^{125}I-BgK(Trp5 → Tyr/Tyr26 → Phe) was synthesized as previously reported [11,32]. At the end of the incubation period, samples were filtered through Whatman GF/C glass-fiber filters (Whatman, Clifton, NJ) presoaked with 0.5% (w/v) polyethylenimine (Sigma Chemical, St. Louis, MO). Filters were rinsed three times with 3.0 mL of ice-cold buffer (150 mM NaCl and 20 mM Tris–HCl; pH 7.4). Duplicate samples were run for each point and the data were averaged. All binding assays were carried out at 20 °C in a medium consisting of 100 mM NaCl, 5.0 mM KCl, 0.1% (w/v) bovine serum albumin (Sigma Chemical, St. Louis, MO), and 20 mM Tris–HCl (pH 7.4).

Results

The Contryphan-Vn structure

The RP-HPLC profile of Contryphan-Vn A shows a pronounced asymmetry, suggesting the existence of an equilibrium between two families of stable conformers, probably due to *cis–trans* isomerization around the Cys3–Pro4 peptide bond (the *cis* conformer supposed to be the major one) [6], as already observed in other Contryphans [7,8]. The presence in the NMR spectrum of Contryphan-Vn of two separate sets of resonances with different intensities (data not shown) confirms this

hypothesis, with Pro4 being essentially in the *cis* configuration (the *cis–trans* ratio is approx. 7:1).

Collection of NOEs, evaluation of their intensities, and application of restrained molecular dynamics protocols in vacuo generate a final set of 20 structures for Contryphan-Vn (Fig. 1, panel A, and Table 2). The simulations converge into the single and well-defined backbone conformation (Fig. 1, panel B). The backbone of the solution structure of Contryphan-Vn (PDB code: 1N3V; present study) is very similar to those already reported for Contryphan-R (PDB code: 1QFB; [7]) and Contryphan-Sm (PDB code: 1DFY; [8]), the calculated root mean square deviations of averaged backbone loop coordinates being 0.78 and 0.92 Å, respectively. As other members of the Contryphan family, Contryphan-Vn exhibits a disulfide-bridged seven-residue cycle with a constrained backbone conformation. Two chain reversals are observed: a type IV turn from Gly1 to Lys6 and a type I β-turn from Lys6 to Cys9. The Pro7–Trp8 hydrophobic cluster (involving and protecting Lys6 and Asp2 in the majority of the conformational subfamilies) is present on one side of the backbone ring. The presence of this cluster is expected to play an important role for the thermodynamic stability and hence for the physiological function of Contryphan-Vn. From this viewpoint, the analysis of the three-dimensional structure of Contryphan-Vn confirms that Lys6 and Trp8 are at a proper distance (≈6.0 Å; Fig. 1, panel B) to form a functional dyad shared by several K^+ channel blockers [11,33], suggesting a functional role for Contryphan-Vn as a K^+ channel modulator [6].

The restrained molecular dynamics simulations show the existence of a persistent salt bridge between Asp2 and Lys6 (Fig. 1, panel B). The presence of specific NOEs between the β-CH_2 of Asp2 and the β-CH_2 and γ-CH_2 of Lys6 confirms that a very stable interaction occurs between the side chains of these two residues, leading to a strong reduction of the mobility of Trp8. The latter feature is not observed in other Contryphans, although a salt bridge is formed in Contryphan-R and Contryphan-P by the protonated N-terminus Gly1 residue and an anionic intercysteine loop residue (Glu5 or Asp5 in Contryphan-R and Contryphan-P, respectively; [7]).

Table 2
Structural statistics for the 20 structures of Contryphan-Vn

Root mean square deviations from experimental distance restraints (Å)	0.08 ± 0.02
No. of experimental distance restraints	74
Intra	21
Sequential	33
Medium	17
Long	3
No. of experimental *J*-coupling restraints	9
Root mean square deviations from idealized geometry	
Bonds (Å)	0.004 ± 0.001
Angles (deg)	0.7 ± 0.2
Impropers (deg)	0.34 ± 0.01
Energies (kcal/mol)[A]	
E_{NOE}[a]	0.7 ± 0.2
E_{COUP}[b]	5.3 ± 0.8
$E_{bond} + E_{angle} + E_{improper}$	24 ± 2
Pairwise root mean square deviations (Å)[B]	
Loop backbone (N, C_α, C)[c]	0.41 ± 0.17
All heavy[d]	1.40 ± 0.20

[A] Force constants for calculation were:
[a] 30 kcal/mol for the square-well potential for NOE.
[b] 200 kcal/mol for the harmonic potential for *J*-coupling restraints.
[B] Root mean square deviations calculated for:
[c] Residues 3–9.
[d] All peptide heavy atoms.

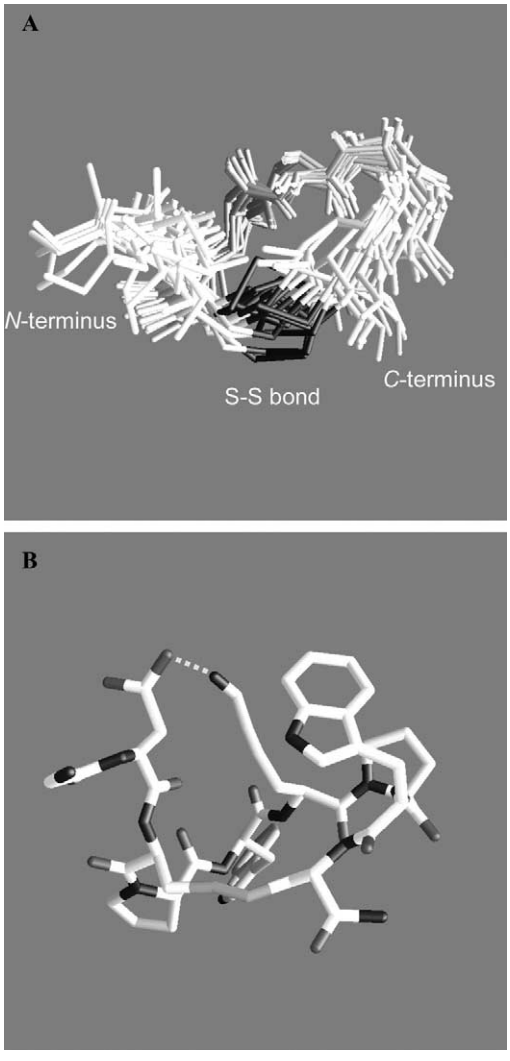

Fig. 1. The three-dimensional structure of Contryphan-Vn (PDB code: 1N3V). (A) Superimposition of the backbone of the final set of 20 structures obtained by restrained molecular dynamics simulations. (B) The average structure of Contryphan-Vn. The dotted line represents the hydrogen bond between the Asp2 and Lys6 side chains.

Contryphan-Vn bioactivity on dorsal unpaired median neurons isolated from the cockroach nerve cord

The somata of dorsal unpaired median (DUM) neurons isolated from the cockroach (*P. americana*) nerve cord maintained in short-term culture are capable of generating spontaneous or triggered overshooting Na^+-dependent action potentials [34]. DUM neurons are also characterized by a membrane potential depending on the external concentration of both K^+ and Na^+ [34,35]. Evoked action potentials recorded under control condition and in the presence of 20 μM Contryphan-Vn are illustrated in Fig. 2. Contryphan-Vn causes a depolarization of the membrane potential (10.1 ± 2.3 mV, $n = 3$) and an increase of the action potential duration ($34.1 \pm 11.0\%$, $n = 3$) (Fig. 2, panel B). Furthermore, when artificial hyperpolarization is imposed upon the DUM neuron cell body to bring the resting potential back to the initial value, a reduction of the post-hyperpolarizing phase is revealed and becomes more evident throughout Contryphan-Vn application (Fig. 2, panels C and D). The DUM neuron input resistance also increases by about 20% during application of Contryphan-Vn (Fig. 2, panel E), indicating a loss in ionic conductance. The membrane depolarization, the prolongation of the action potential duration, and the change in conductance might be accounted for by the blockade of the Ca^{2+}-dependent K^+ current. Up to date, five different types of depolarization-activated K^+ currents have been characterized in DUM neurons and identified as delayed rectifier and transient A-type currents as well as Na^+- and Ca^{2+}-activated K^+ currents [34]. Their specific functions

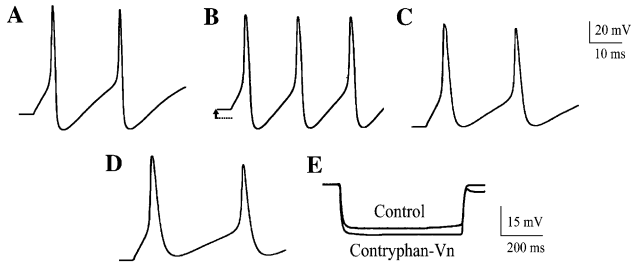

Fig. 2. Effect of Contryphan-Vn on the membrane potential, input membrane resistance, and triggered action potentials recorded from an isolated DUM neuron. Action potentials elicited by a depolarizing current pulse (0.8 nA, 50 ms in duration) recorded prior (panel A) and following (panel B) the application of Contryphan-Vn (20 μM). Within the first 5 min, Contryphan-Vn induces a depolarizing shift of the resting membrane potential (panel B, arrow), which is responsible for an increase of action potential discharge frequency. When the depolarizing shift of the resting membrane potential is compensated by steady state injection of negative current (cells were held at −55 mV), a reduction of the post-hyperpolarization is observed (panel C) within 15 min of Contryphan-Vn (20 μM) application. Then, the action potential discharge frequency progressively decreases (panel D). Superimposed membrane potential traces recorded in response to a hyperpolarizing current pulse (500 ms in duration and 0.9 nA in amplitude) in saline solution (panel E) before (control) and 15 min after the application of Contryphan-Vn (20 μM). For further experimental details, see text.

in the generation of the different phases of the spontaneous electrical activity of the DUM neuron were also elucidated [34]. In this context, we demonstrated that the Ca^{2+}-dependent K^+ current participated in the repolarization and post-hyperpolarization of action potentials.

Thus, the effect of Contryphan-Vn on the global outward K^+ current in voltage-clamp mode has been also investigated in the presence of the Na^+ channel blocker, tetrodotoxin. Contryphan-Vn decreases both peak and late components of the global outward K^+ current by $35.1 \pm 7.3\%$ ($n = 5$) and $27.8 \pm 6.2\%$ ($n = 5$), respectively (Fig. 3, panel A). A similar effect has been previously reported in DUM neurons with the Ca^{2+}-dependent K^+ channel blockers iberiotoxin and charybdotoxin, and with the inorganic Ca^{2+} channel blocker Cd^{2+} (cadmium chloride) [25]. These results suggest that Contryphan-Vn acts preferentially on Ca^{2+}-dependent K^+ currents. Accordingly, Contryphan-Vn (20 μM) partially reverses the blockade of the global outward K^+ current induced by Cd^{2+}, as it increases the peak current amplitude by $24.1 \pm 8.8\%$ ($n = 5$) (Fig. 3, panels B, C, and D). By contrast, in the presence of both Cd^{2+} and iberiotoxin, the amplitude of the global outward K^+ current, which only included delayed rectifier and transient A-type currents, is not affected by addition of Contryphan-Vn (Fig. 3, panel E).

As the effect of Contryphan-Vn seems to disappear when DUM neuron membrane is artificially hyperpolarized in current clamp mode (data not shown), the voltage-dependent effects of Contryphan-Vn have also been studied. The effect of Contryphan-Vn (20 μM) was tested on K^+ current at different membrane potentials in the presence or absence of Cd^{2+} (Fig. 4). At very negative potential (−100 mV), Contryphan-Vn has little or no effect (Fig. 4). By contrast, the effect is detectable in the −80 to −50 mV potential range (see Fig. 3, panel B, and Fig. 4). In DUM neurons, the Ca^{2+}-dependent K^+ current may be separated into a transient and a maintained (non-inactivating) current [25,34]. Interestingly, following application of Contryphan-Vn, the transient component of the Ca^{2+}-dependent K^+ current is completely blocked at a holding potential of −50 mV (Fig. 4). This result confirms that Contryphan-Vn does not alter the transient A-type current existing at −80 mV (Fig. 3, panel B), but totally inactivates at a holding potential of −50 mV [25]. Furthermore, both the transient and the maintained components of the Ca^{2+}-dependent K^+ current are reduced by Cd^{2+} (Fig. 4). Remarkably, Contryphan-Vn reverses the Cd^{2+} effect by acting preferentially on the maintained component of the Ca^{2+}-dependent K^+ current (Fig. 4).

Contryphan-Vn bioactivity on rat fetal chromaffin cells

Contryphan-Vn increases the global outward K^+ current induced by a depolarizing pulse to +60 mV from

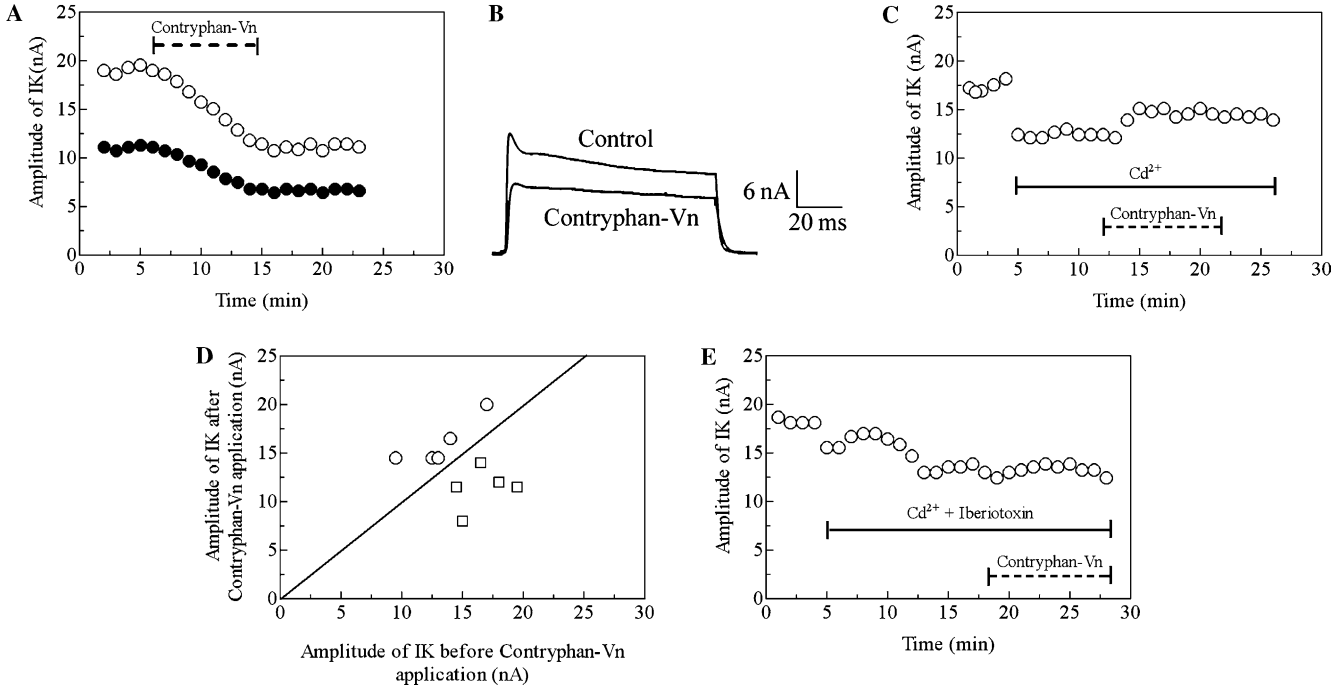

Fig. 3. Effect of Contryphan-Vn on the global outward K^+ current recorded from an isolated DUM neuron. Time course of inhibition by Contryphan-Vn of the global outward K^+ current (*I*K) measured at the beginning (peak current, open circles) and at the end of the depolarizing pulse (maintained current, filled circles) (panel A). The horizontal dashed bar indicates the time of application of Contryphan-Vn (20 μM). The global outward K^+ current has been evoked by a 100-ms depolarizing pulse to +20 mV (panel B). Dependence of the amplitude of the peak global outward K^+ current (*I*K) as a function of time in the presence of Cd^{2+} (1.0 mM) (panel C). Contryphan-Vn (20 μM) increases the amplitude of the peak global outward K^+ current (*I*K), in the presence of Cd^{2+} (1.0 mM). The horizontal continuous and dashed bars indicate the times of application of Cd^{2+} and Contryphan-Vn, respectively. Opposite effects induced by Contryphan-Vn on the global outward K^+ current (*I*K) recorded in the absence (squares) and in the presence (circles) of Cd^{2+} (1.0 mM) (panel D). The straight line represents the theoretical correlation ($r = 1$) of the global outward K^+ current (*I*K) amplitude measured before and after application of Contryphan-Vn (20 μM). Cd^{2+} (1.0 mM) and iberiotoxin (10 nM) gradually reduce and then stabilize the amplitude of the global outward K^+ current (panel E). Contryphan-Vn (20 μM) does not affect significantly the global outward K^+ current (*I*K) amplitude in the presence of Cd^{2+} (1.0 mM) and iberiotoxin (10 nM). The horizontal continuous and dashed bars indicate the times of application of the Cd^{2+}-iberiotoxin mixture and of Contryphan-Vn, respectively. For further experimental details, see text.

the holding potential −60 mV on rat fetal (E19) chromaffin cells in primary culture (Fig. 5, panel A). The effect of Contryphan-Vn is rapidly reversed upon washing out the peptide from the external solution. Moreover, Contryphan-Vn increases current density the most when the negativity of the cell holding membrane potential is reduced (Fig. 5, panel B). This indicates that the increase in the global outward K^+ current caused by Contryphan-Vn is dependent on the holding membrane potential.

The effect of Contryphan-Vn on the global outward K^+ current intensity in rat fetal chromaffin cells has been also investigated in the presence of Cd^{2+}, which blocks voltage-sensitive Ca^{2+} channels. As shown in Fig. 5 (panels C and D), the global outward K^+ current density in rat fetal chromaffin cells is markedly reduced by the presence of Cd^{2+}, at all membrane potentials studied. Under these conditions, Contryphan-Vn does not significantly affect the global outward K^+ current component remaining after blocking Ca^{2+} entry into chromaffin cells. These results suggest that Contryphan-Vn affects the Ca^{2+}-dependent K^+ component of the global outward K^+ current. However, the membrane Ca^{2+} channel activity is essentially unaffected by Contryphan-Vn (data not shown), suggesting that the observed effect is mediated by Ca^{2+} release from cell internal stores. Accordingly, preliminary results show an increase of basal Ca^{2+} fluorescence levels in rat fetal chromaffin cells upon Contryphan-Vn application.

Contryphan-Vn binding to human voltage-gated K^+ channels

Contryphan-Vn has been tested for its ability to inhibit the binding of ^{125}I-BgK(Trp5 → Tyr/Tyr26 → Phe) to membranes from transformed HEK 293 cells overexpressing the human voltage-gated K^+ channels Kv1.1 or Kv1.2. Contryphan-Vn does not affect significantly the binding of ^{125}I-BgK(Trp5 → Tyr/Tyr26 → Phe) to human voltage-gated K^+ channels Kv1.1 and Kv1.2; a weak effect occurs only at Contryphan-Vn concentration higher than 600 μM (data not shown).

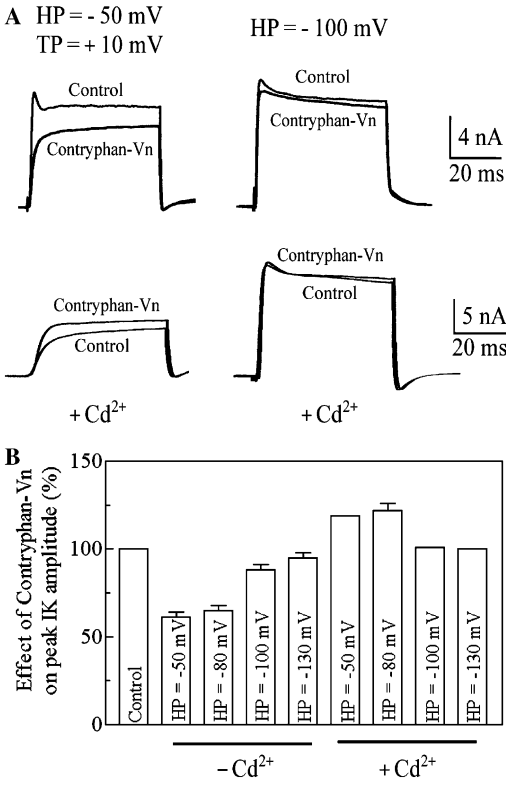

Fig. 4. Effect of Contryphan-Vn on the voltage dependence of the peak global outward K^+ current amplitude from an isolated DUM neuron. Representative superimposed global outward K^+ current traces evoked by a 100-ms depolarizing pulse from two different holding potentials (HP; indicated above current traces) recorded before (Control) and after application of Contryphan-Vn (20 μM), in the presence and absence of Cd^{2+} (1.0 mM) (panel A). Voltage dependence of the Contryphan-Vn-induced effect on the peak global outward K^+ current (panel B). Each histogram bar represents the mean percentage of the global outward K^+ current amplitude (±SE, $n = 2$–5). For further experimental details, see text.

Discussion

The analysis of the solution structure of Contryphan-Vn confirms that the 'Contryphan motif' represents a stable molecular scaffold whose structure is mainly determined by the size of the intercysteine loop and the presence and location in the sequence of the D-Trp and Pro residues [9]. Then, the substitution of other residues plays a minor role in the determination of the backbone conformation [9], while they may be relevant for the physiological function. From this viewpoint, the main structural characteristic of Contryphan-Vn is the presence of an intercysteine basic residue, Lys6, which participates in a strong salt bridge with Asp2 (Fig. 1, panel B). Both residues are not conserved in other known Contryphans (Table 1) and their presence confers a peculiar surface electrostatic potential to Contryphan-Vn [6], indicating that the molecular recognition properties of this conopeptide may be distinct from those of the other known Contryphans. Moreover, the Trp8–Lys6 dyad present in Contryphan-Vn (Fig. 1, panel B) is reminiscent of the functional dyad found in several K^+ channel blockers [11,33].

Contryphan-Vn modulates the global outward K^+ current in insect neurosecretory cells as well as in rat chromaffin cells through different and composite effects. In DUM neurons, Contryphan-Vn inhibits the global outward K^+ current, measured in the voltage-clamp experiments, as observed for the scorpion toxins charybdotoxin and iberiotoxin [25]. By contrast, Contryphan-Vn increases the current amplitude when Ca^{2+} permeation through the DUM neuron membrane is inhibited by the inorganic Ca^{2+} channel blocker Cd^{2+}. The opposite effects on the global outward K^+ current observed in the presence and absence of Cd^{2+} and the voltage dependence of these effects indicate that Contryphan-Vn exerts a complex action which is dependent on Ca^{2+} entry through the voltage-activated Ca^{2+} channels.

Electrophysiological experiments previously performed on DUM neurons indicated that two Ca^{2+}-activated K^+ currents can be separated according to their sensitivity to the steady-state holding potential [25]. At −80 mV, the Ca^{2+}-activated K^+ current displays a biphasic behavior characterized by a transient and a maintained component [25]. At −20 mV, the transient component disappears revealing only the maintained outward current [25]. Up to date, no specific Ca^{2+}-activated K^+ current blockers have allowed to discriminate between the fast transient and the late maintained component of the Ca^{2+}-activated K^+ current. Remarkably, Contryphan-Vn seems to be more potent when the membrane potential is held at the more depolarized potentials required for isolating the late component. This suggests that Contryphan-Vn affects differentially the two components of the Ca^{2+}-activated K^+ current via a distinct regulation of the intracellular Ca^{2+} level.

Interestingly, the results obtained with Contryphan-Vn assays on fetal rat chromaffin cells in primary culture demonstrate an increase of the Ca^{2+}-dependent K^+ outward current (Fig. 5A) and no effect on the calcium currents. These observations suggest that the effect observed is mediated by Ca^{2+} release from cell internal stores. These hypotheses are confirmed by the results obtained in the presence of Cd^{2+} (Fig. 5B) and by preliminary data showing an increase of basal Ca^{2+} fluorescence levels in rat fetal chromaffin cells (data not shown). However, a direct effect on Ca^{2+}-dependent K^+ channels cannot be discarded. Thus, though in DUM neurons and in rat fetal chromaffin cells the same components of the global outward K^+ current are affected by Contryphan-Vn, the effects elicited by this conopeptide are distinct and channel subtype specificity as well as mechanism of action may differ too. A deeper knowledge of Ca^{2+}-mediated regulation of K^+ channels may be the key to understand the molecular bases of the

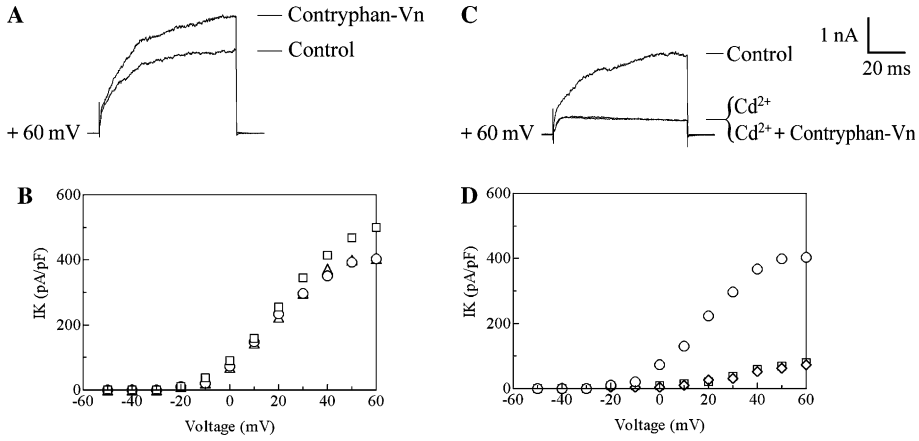

Fig. 5. Effect of Contryphan-Vn on the global outward K^+ current recorded from rat fetal chromaffin cells. Global outward K^+ current activated by a 60-ms depolarization to +60 mV from a holding potential of −60 mV, before (control) and after application of Contryphan-Vn (20 μM) to the external medium (panel A). Effect of voltage on the current density for the rat fetal chromaffin cell (panel B). Circles indicate data obtained in the absence of Contryphan-Vn. Squares indicate data obtained in the presence of Contryphan-Vn (20 μM). Triangles indicate data obtained after washing out Contryphan-Vn. Data shown in panels A and B refer to the same cell. Contryphan-Vn increases reversibly the global outward K^+ current. Global outward K^+ current activated by a 60-ms depolarization to +60 mV from a holding potential of −60 mV, before (control) and after application of Cd^{2+} (0.5 mM) or Cd^{2+} (0.5 mM) and Contryphan-Vn (20 μM) (panel C). Effect of voltage on the current density for the rat fetal chromaffin cell, in the presence of Cd^{2+} (panel D). Circles indicate data obtained in the absence of both Contryphan-Vn and Cd^{2+}. Diamonds indicate data obtained in the presence of Cd^{2+} (0.5 mM). Squares indicate data obtained in the presence of Contryphan-Vn (20 μM) and Cd^{2+} (0.5 mM). Data shown in panels C and D refer to the same cell. All records were taken at 10 mV voltage steps from a holding potential of −60 mV. For further experimental details, see text.

Contryphan-Vn physiological action on both systems investigated. In line with these considerations, binding experiments carried out on membrane preparations of transformed HEK 293 cells overexpressing human voltage-gated K^+ channels Kv1.1 and Kv1.2 channels do not evidence a competition of Contryphan-Vn for the BgK binding site. It is worthwhile to note that the molecular bases for BgK binding to human voltage-gated K^+ channels Kv1 channels have been recently demonstrated to rely on the electrostatic interactions between Lys25 residue of BgK and carbonyl oxygen atoms of residues from the channel selectivity filter, strengthened by solvent exclusion provided by neighboring aromatic residues [33]. Thus, given the degree of conservation of the filter region observed in prokaryotic and eukaryotic K^+ channels [36], it seems unlikely that the effect of Contryphan-Vn is due to binding to the selectivity filter of the K^+ channel system targeted by this conopeptide.

Conclusion

In this paper we show that Contryphan-Vn modulates the activity of voltage-gated and Ca^{2+}-dependent K^+ channels. This is the first report of a functional target for a peptide of the Contryphan family. Furthermore, Contryphan-Vn is the first conopeptide known to modulate the activity of Ca^{2+}-dependent K^+ channels. DUM neuron and chromaffin cell K^+ channel subtypes are low affinity molecular targets for Contryphan-Vn. However, the identification of the Ca^{2+}-dependent K^+ channel subtypes targeted by Contryphan-Vn would be of high relevance in defining the functional properties of this conopeptide and its potential pharmacological applications.

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References

[1] E.C. Jimenez, B.M. Olivera, W.R. Gray, L.J. Cruz, Contryphan is a D-tryptophan-containing *Conus* peptide, J. Biol. Chem. 271 (1996) 28002–28005.

[2] E.C. Jimenez, A.G. Craig, M. Watkins, D.R. Hillyard, W.R. Gray, J. Gulyas, J.E. Rivier, L.J. Cruz, B.M. Olivera, Bromocontryphan: post-translational bromination of tryptophan, Biochemistry 36 (1997) 989–994.
[3] E.C. Jimenez, M. Watkins, L.J. Juszczak, L.J. Cruz, B.M. Olivera, Contryphans from *Conus textile* venom ducts, Toxicon 39 (2001) 803–808.
[4] R. Jacobsen, E.C. Jimenez, M. Grilley, M. Watkins, D. Hillyard, L.J. Cruz, BM. Olivera, The Contryphans, a D-tryptophan-containing family of *Conus* peptides: interconversion between conformers, J. Pept. Res. 51 (1998) 173–179.
[5] R.B. Jacobsen, E.C. Jimenez, R.G. De la Cruz, W.R. Gray, L.J. Cruz, B.M. Olivera, A novel D-leucine-containing peptide: diverse conformational dynamics in the Contryphan family, J. Pept. Res. 54 (1999) 93–99.
[6] G. Raybaudi Massilia, M.E. Schininà, P. Ascenzi, F. Polticelli, Contryphan-Vn: a novel peptide from the venom of the Mediterranean snail *Conus ventricosus*, Biochem. Biophys. Res. Commun. 288 (2001) 908–913.
[7] P.K. Pallaghy, A.P. Melnikova, E.C. Jimenez, B.M. Olivera, R.S. Norton, Solution structure of Contryphan-R, a naturally occurring disulfide-bridged octapeptide containing D-tryptophan: comparison with protein loops, Biochemistry 38 (1999) 11553–11559.
[8] P.K. Pallaghy, W. He, E.C. Jimenez, B.M. Olivera, R.S. Norton, Structures of the Contryphan family of cyclic peptides: role of electrostatic interactions in *cis–trans* isomerism, Biochemistry 39 (2000) 12845–12852.
[9] P.K. Pallaghy, R.S. Norton, The cyclic Contryphan motif CPxXPXC, a robust scaffold potentially useful as an ω-conotoxin mimic, Biopolymers 54 (2000) 173–179.
[10] E. Rolán, G. Raybaudi Massilia, Evaluation of the character state polarity of *Conus* radular tooth characters, Bollettino Malacologico 38 (2002) 185–204.
[11] M. Dauplais, A. Lecoq, J. Song, J. Cotton, N. Jamin, B. Gilquin, C. Roumestand, C. Vita, C.L. de Medeiros, E.G. Rowan, A.L. Harvey, A. Ménez, On the convergent evolution of animal toxins: conservation of a diad of functional residues in potassium channel-blocking toxins with unrelated structures, J. Biol. Chem. 272 (1997) 4302–4309.
[12] F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, NMRPipe: a multidimensional spectral processing system based on UNIX pipes, J. Biomol. NMR 6 (1995) 277–293.
[13] B.A. Johnson, R.A.J. Blevins, NMRView: a computer programme for the visualization and analysis of NMR data, J. Biomol. NMR 4 (1994) 603–614.
[14] A. Bax, D.G. Davis, MLEV-17 based two-dimensional homonuclear magnetization transfer spectroscopy, J. Magn. Reson. 65 (1985) 355–360.
[15] S. Grzesiek, A. Bax, The importance of not saturating H_2O in protein NMR: application to sensitivity enhancement and NOE measurements, J. Am. Chem. Soc. 115 (1993) 12593–12594.
[16] A. Bax, R.H. Griffey, B.L. Hawkins, Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR, J. Magn. Reson. 55 (1983) 301–306.
[17] L. Braunschweiler, R.R. Ernst, Coherence transfer by isotropic mixing: application to proton correlation spectroscopy, J. Magn. Reson. 53 (1983) 521–528.
[18] A. Bax, D.G. Davis, Practical aspects of two-dimensional transverse NOE spectroscopy, J. Magn. Reson. 63 (1985) 207–213.
[19] K. Wüthrich, NMR of Proteins and Nucleic Acids, Wiley, New York, 1986.
[20] M. Rance, O.W. Sørensen, G. Bodenhausen, G. Wagner, R.R. Ernst, K. Wüthrich, Improved spectral resolution in COSY ^{1}H-NMR spectra of proteins via double quantum filtering, Biochem. Biophys. Res. Commun. 117 (1983) 479–485.
[21] G. Wagner, W. Braun, T.F. Havel, T. Schaumann, N. Gõ, K. Wüthrich, Protein structures in solution by nuclear magnetic resonance and distance geometry: the polypeptide fold of the basic pancreatic trypsin inhibitor determined using two different algorithms DISGEO and DISMAN, J. Mol. Biol. 196 (1987) 611–639.
[22] A.T. Brunger, X-Plor Version 3.1: A System for X-ray Crystallography and NMR, Yale University Press, New Haven, CT, 1993.
[23] J.G. Omichinski, P.V. Pedone, G. Felsenfeld, G.M. Clore, The solution structure of a specific GAGA factor-DNA complex reveals a modular binding mode, Nat. Struct. Biol. 4 (1997) 122–132.
[24] R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, Procheck: a program to check the stereochemical quality of protein structure, J. Appl. Crystallogr. 26 (1993) 283–291.
[25] F. Grolleau, B. Lapied, Separation and identification of multiple potassium currents regulating the pacemaker activity of insect neurosecretory cells (DUM neurons), J. Neurophysiol. 73 (1995) 160–171.
[26] B. Lapied, C.O. Malecot, M. Pelhate, Ionic species involved in the electrical activity of single aminergic neurons isolated from the sixth abdominal ganglion of the cockroach *Periplaneta americana*, J. Exp. Biol. 144 (1989) 535–549.
[27] O. Hamill, A. Marty, E. Neher, B. Sakmann, F.J. Sigworth, Improved patch-clamp technique for high-resolution current recording from cells and cell-free membrane patches, Pflügers Arch. 391 (1981) 85–100.
[28] S.J. Kim, W. Lim, J. Kim, Contribution of L- and N-type calcium currents to exocytosis in rat adrenal medullary chromaffin cells, Brain Res. 675 (1995) 289–296.
[29] R. Bournaud, J. Hidalgo, H. Yu, E. Jaimovich, T. Shimahara, Low threshold T-type calcium current in rat embryonic chromaffin cells, J. Physiol. 537 (2001) 35–44.
[30] R. Horn, A. Marty, Muscarinic activation of ionic currents measured by a new whole-cell recording method, J. Gen. Physiol. 92 (1988) 145–159.
[31] J. Racape, A. Lecoq, R. Romi-Lebrun, J. Liu, M. Kohler, M.L. Garcia, A. Ménez, S. Gasparini, Characterization of a novel radiolabeled peptide selective for a subpopulation of voltage-gated potassium channels in mammalian brain, J. Biol. Chem. 277 (2002) 3886–3893.
[32] S. Gasparini, J.M. Danse, A. Lecoq, S. Pinkasfeld, S. Zinn-Justin, L.C. Young, C.C. de Medeiros, E.G. Rowan, A.L. Harvey, A. Ménez, Delineation of the functional site of α-dendrotoxin: the functional topographies of dendrotoxins are different but share a conserved core with those of other Kv1 potassium channel-blocking toxins, J. Biol. Chem. 273 (1998) 25393–25403.
[33] B. Gilquin, J. Racape, A. Wrisch, V. Visan, A. Lecoq, S. Grissmer, A. Ménez, S. Gasparini, Structure of the BgK-Kv1.1 complex based on distance restraints identified by double mutant cycles: molecular basis for convergent evolution of Kv1 channel blockers, J. Biol. Chem. 277 (2002) 37406–37413.
[34] F. Grolleau, B. Lapied, Dorsal unpaired median neurons in the insect central nervous system: towards a better understanding of the ionic mechanisms underlying spontaneous electrical activity, J. Exp. Biol. 203 (2000) 1633–1648.
[35] B. Lapied, M. Stankiewicz, F. Grolleau, H. Rochat, E. Zlotkin, M. Pelhate, Biophysical properties of scorpion α-toxin-sensitive background sodium channel contributing to the pacemaker activity in insect neurosecretory cells (DUM neurons), Eur. J. Neurosci. 11 (1999) 1449–1460.
[36] Y. Jiang, A. Lee, J. Chen, M. Cadene, B.T. Chait, R. MacKinnon, The open pore conformation of potassium channels, Nature 417 (2002) 523–526.