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Novel peptides from assassin bugs (Hemiptera: Reduviidae): isolation, chemical and biological characterization

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Abstract Three novel peptides were isolated from the venomous saliva of predatory reduviids. They were identified by mass spectrometry and HPLC analysis and consist of 34–36 amino acid residues. They are relatively homologous to the calcium channel blockers ω-conotoxins from marine cone snails and belong to the four-loop Cys scaffold structural class. Ptu1, the shortest peptide, was chemically synthesized (sPtu1) and co-eluted with its native form. Circular dichroism spectra of the sPtu1 showed a high content of β-turns similar to that of ω-conotoxins GVIA and MVIIA. Electrophysiological experiments demonstrated that sPtu1 reversibly blocks the N-type calcium channels expressed in BHK cells.

Key words: Assassin bug; Conotoxin; Peptide; N-type calcium channel; Four-loop scaffold; Circular dichroism

1. Introduction

The venom of poisonous predators has been a great source of novel peptides with a notable potential for use in agriculture and in medicine. Such peptides have also been invaluable biochemical tools for the study of ion channels in animal cells. Toxic peptides targeting ion channels have been frequently isolated from snakes, scorpions, marine cone snails, spiders, and other animals [1,2]. Assassin bugs (Arthropoda: Insecta: Hemiptera: Reduviidae) are one of the largest and morphologically diverse families of true bugs feeding on crickets, caterpillars and other insects [3]. Some assassin bug species are bloodsucking parasites of mammals, even of humans [4]. The toxic saliva of the predatory assassin bugs contains a complex mixture of small and large peptides for diverse uses such as immobilizing and pre-digesting their prey, and defense against competitors and predators [5]. Reduviid venomous saliva as well as the venomous material of other insects is a new potential source of peptic material for the study and characterization of cell receptors and receptor subtypes that could lead to the development of novel agrochemicals and pharmaceuticals.

In this work, we describe for the first time the isolation, chemical characterization and biological activities of three novel peptides originating from the venomous saliva of assassin bugs. Because of the low quantities of material obtained from these insects, the peptides were identified by a non-conventional method based on mass spectrometry analysis of the crude material following liquid chromatography separation and chemical characterization of the most abundant low molecular weight components.

2. Materials and methods

2.1. Venom preparation

Crude assassin bug venomous saliva was obtained by electrical stimulation of field-collected *Peirates turpis*, *Agriosphodrus dohrni*, and *Isyndus obscurus* assassin bugs from Mt. Nijosan, Mt. Takayasuyama, and Mt. Sigisan respectively. The crude venomous saliva of each species was first diluted with 0.1% aqueous trifluoroacetic acid (TFA) and centrifuged at 14 000 rpm for 30 min at 4°C. The supernatant was stored at −20°C until further use.

2.2. Purification of assassin bug peptides

The extracted material from the saliva was fractionated by reversed-phase HPLC on a semi-preparative C_8 column (5C8MS, 10×250 mm, Nacalai Tesque, Japan). The column was equilibrated in 0.1% TFA, and eluted with a 60 min linear gradient of 0–60% acetonitrile containing 0.1% TFA (2 ml/min). Fractions were collected manually by monitoring the absorbance at 215 nm. The reversed-phase fractions were further purified by cation exchange chromatography on a TSK gel sulfopropyl column (SP-5PW, 4.6×75 mm, Tosoh, Japan). The reversed-phase fractions were separated using a linear gradient of 1 M acetic acid pH 2.9 to 2 M ammonium acetate in 1 M acetic acid pH 5.9, in 120 min (1 ml/min). Absorbance was monitored at 280 nm and the components were collected manually and dried under vacuum.

2.3. Mass spectrometry

Matrix-assisted laser desorption/ionization (MALDI) mass spectra were obtained on a Voyager Elite time-of-flight (TOF) spectrometer (PerSeptive Biosystems Inc., Framingham, MA, USA) equipped with a model VSL-337ND nitrogen laser (Laser Science, Newton, MA, USA). The experiments were performed as reported previously [6].

2.4. Biological activity

Invertebrate toxicity of assassin bug peptides was tested using crickets and cutworms. Lepidopteran larvae (*Spodoptera litura*, 2–3 mg)

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Abbreviations: Ptu1, Ado1, and lob1, peptides from the assassin bugs *Peirates turpis*, *Agriosphodrus dohrni*, and *Isyndus obscurus* respectively; sPtu1 and sAdo1, synthetic C-terminally carboxylated forms of Ptu1 and Ado1 respectively; GVIA, ω-conotoxin from *Conus geographus*; MVIIA, ω-conotoxin from *Conus magus*; TCEP, Tris-(2-carboxy-ethyl)phosphine; CDAP, 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CZE, capillary zone electrophoresis; CD, circular dichroism

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were injected into the pronotum with glass capillary pipettes containing up to 300 nl of diluted venom or fractions resuspended in water, and placed in 55 mm diameter plastic petri dishes for observation. Paralytic and lethal effects were observed at different time intervals up to 48 h. Crickets (*Grillus bimaculus*, 50–70 mg) were injected intrathoracically between the second and third pair of legs with 2 μl of peptide using a 10 μl microsyringe. Animal toxicity was also evaluated on goldfish and mice. Goldfish (2–3 g) were injected i.p. with 7 μg of assassin bug peptide previously diluted in deionized water. Male mice (C57BL/6, 20 g) were tested by i.c.v. and i.v. injection. Up to 7 μg of assassin bug peptide was diluted up to 5 μl with bovine serum albumin (BSA) solution (20 mg/ml in 0.9% NaCl) and injected with a 10 μl microsyringe fitted with a glass capillary mid-way between the left eye and the left ear. Negative controls were done with saline solution only and positive controls with ω-conotoxins GVIA or MVIIA (Peptide Institute, Osaka, Japan). Mice were placed in glass jars for observation of toxicity symptoms up to 48 h.

2.5. Sequence determination and enzymatic digestion

Assassin bug peptides were reduced with tributylphosphine (Nacalai Tesque, Japan) and were simultaneously alkylated with 4-vinyl-pyridine (Wako, Japan), in 0.5 M $NaHCO_3$ buffer (pH 8.3) for 2 h at 37°C in the dark. Hydrolysis of Ado1 and lob1 RA peptides with endoproteinase type XVII-B (Glu-C) from *Staphylococcus aureus* strain V8 was carried out as previously reported [7]. RA peptides and endoproteinase-digested fractions were analyzed by MALDI-TOF MS and sequenced by Edman degradation on a Shimadzu PPSQ-10 automated gas phase sequencer.

2.6. Peptide synthesis

Ptu1 was chemically synthesized by a solid phase method using Fmoc methodology on an Applied Biosystems 433 A peptide synthesizer. Fmoc-Leu-Wang resin (Watanabe Chemical Industries, Ltd., Japan) was used to provide a free carboxyl at the C-terminal of synthetic Ptu1-OH (sPtu1). Chemical synthesis, cleavage and deprotection of peptide resins were performed as previously described [8]. The crude synthetic peptide was dissolved in 20% aqueous acetonitrile, and the reduced peptide separated by RP-HPLC on a semipreparative C_8 column (5C8MS, 10×250 mm, Nacalai Tesque, Japan). The six free cysteine residues were allowed to oxidize by air exposure for 48 h at room temperature in 1 M aqueous guanidine hydrochloride containing 0.1 M ammonium acetate solution containing 1 mM reduced glutathione/0.1 mM oxidized glutathione.

2.7. Capillary electrophoresis

Capillary zone electrophoresis (CZE) analyses were performed on a Jasco system equipped with a UV detector and a 70 cm 20 mM sodium citrate buffer (pH 2.5) (Applied Biosystems, USA) was used for the analysis. Samples dissolved in migration buffer were applied hydrodynamically to the capillary and analyses were performed with a 20 kV constant voltage drop. Effluent was monitored at 210 nm.

2.8. Disulfide bridge determination

Partial reduction of Ptu1 with Tris-(2-carboxy-ethyl)phosphine (TCEP, Tokyo Kasei Kogyo Co., Japan) and cyanylation by 1-cyano-4-dimethylamino-pyridinium tetrafluoroborate (CDAP, Sigma Co., USA) followed by chemical cleavage of partially reduced peptides was performed as reported previously [9].

2.9. Circular dichroism (CD) measurements

CD spectra were obtained on a Jasco J-725 spectropolarimeter (Jasco, Japan). The spectra were measured from 260 to 190 nm in 60% trifluoroethanol, pH 7.1, at room temperature, with a 1 mm path length cell. The concentration of the peptides was 30 μM. Data from 10 separate recordings were averaged and analyzed by the method of Bohm et al. [10].

2.10. Whole cell patch clamp recordings

The whole cell Ca^{2+} channel currents were recorded from BHK-N101 cells (baby hamster kidney cell line, generously supplied by Dr. Y. Mori [11]) that stably express rabbit voltage-dependent N-type Ca^{2+} channel α_{1B} subunit, β_{1A} subunit, and α_2/δ subunit. Ba^{2+} (2 mM) was used as a charge carrier in the bath solution (137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 2 mM $BaCl_2$, pH 7.4, adjusted with NaOH) at room temperature. Ba^{2+} channel currents through P/Q-type Ca^{2+} channels were recorded from BHK-BI cells [12], and so were L-type Ca^{2+} channel currents from BHK6 cells, kindly supplied by Dr. Y. Mori. BHK6 cells were transiently expressed with rat brain α_{1C} subunit (rbCII) that was generously supplied by Dr. T.P. Snutch [13]. The resistance of the heat-polished microelectrodes was kept between 2 and 4 MΩ when filled with the internal solution (120 mM CsCl, 20 mM TEACl, 14 mM EGTA, 5 mM MgATP, 5 mM Na_2 creatine phosphate, 0.2 mM GTP, 10 mM HEPES, 0.2 mM cAMP; pH 7.3 adjusted with CsOH). Currents were measured using Patch/Whole Cell Clamp Amplifier (Nihon Kohden, Tokyo, Japan) or Axopatch 1D (Axon Instruments, Inc., Foster City, CA, USA), and A/D converter (Digidata 1200, Axon Instruments, Inc., Foster City, CA, USA). Voltage clamp protocols and data acquisitions were performed using pCLAMP6 software (Axon Instruments, Inc., Foster City, CA, USA). Ba^{2+} currents through Ca^{2+} channels were elicited by giving test pulses from a holding potential at −100 mV to 0 mV for 100 ms at 0.1 Hz. Ptu1 was diluted in the bath solution containing 0.1% BSA and applied to cells via a concentration clamp apparatus (Vibraspec, Inc., Philadelphia, PA, USA).

3. Results and discussion

3.1. Peptide characterization and structure

A preliminary MALDI-TOF MS screening of the assassin bug venomous saliva permitted us to detect molecular weight components from 1 to 30 kDa (data not shown). The venomous saliva was fractionated by reversed-phase HPLC and all the fractions were analyzed by MALDI-TOF MS (Fig. 1). The most abundant HPLC fractions from each assassin bug species contained components having molecular masses from 2 to 5 kDa and higher than 10 kDa. The HPLC fractions containing larger amounts of material with low molecular masses and a relatively high purity of components were selected for further purification and chemical characterization. HPLC fractions with molecular masses of 3615.3, 3781.2 and 3938.6 Da from the species *P. turpis*, *A. dohrni*, and *I. obscurus* respectively were chosen and subsequently purified by cation exchange chromatography. After cation exchange purification, each peptide was obtained with a purity higher than 98% as confirmed by MALDI-TOF MS and CZE (data not shown). The three peptides were designated Ptu1, Ado1, and lob1 according to the name of the genus and species of each reduviid. They were reduced and alkylated by TCEP and 4-vinyl-pyridine showing a 630 Da increase in their molecular weights indicating the presence of three disulfide bonds (data not shown). The data obtained from automated Edman sequencing of the reduced-alkylated peptides allowed the complete determination of all three sequences (Table 1). The reduced-alkylated peptides Ado1 and lob1 were also digested with Glu-C and their digested fragments analyzed by MALDI-TOF MS and Edman degradation to confirm their amino acid sequences. The calculated molecular masses of the peptides were compared to the molecular masses obtained from MALDI-TOF MS. Ptu1, Ado1 and lob1 have measured molecular weights of 3615.1, 3781.3 and 3938.5 Da respectively. Theoretical molecular weights calculated from sequence data and assuming a free C-terminal carboxylic acid and the pairing of the six cysteine residues into three disulfide bridges were respectively 3615.2, 3781.7 and 3938.4 (Table 1). Although the peptides Ptu1, Ado1, and lob1 belong to different Reduviidae genera, their amino acid sequence motif was well conserved with some point mutations (Table 1). The assassin bug peptides are basic (Ptu1, Ado1) and neutral (lob1) and are relatively homologous to the ω-conotoxins. Among other amino

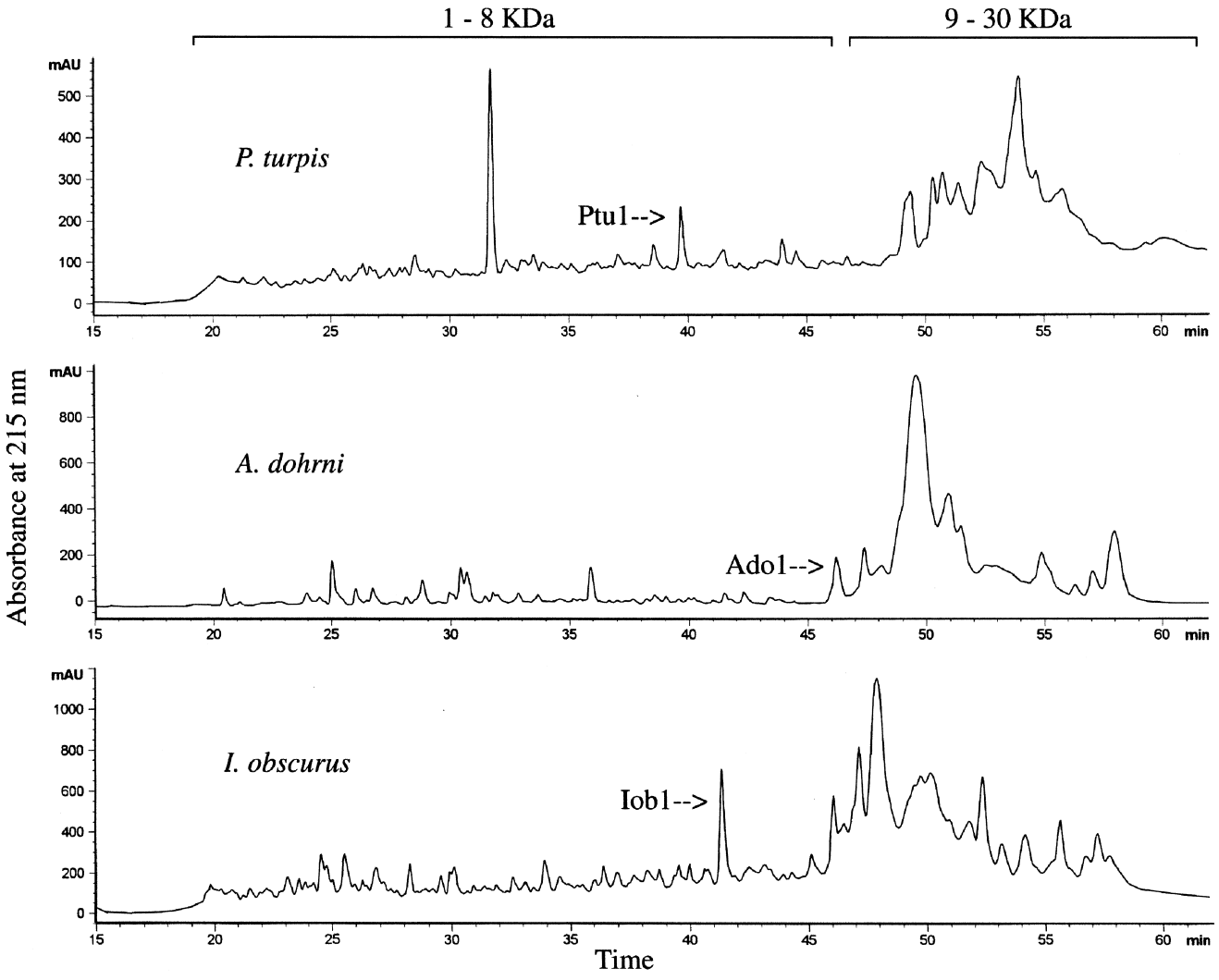

Fig. 1. MALDI-TOF spectra and HPLC chromatograms of the venomous saliva of *P. turpis*, *A. dohrni* and *I. obscurus*.

acid residues, ω-conotoxins and assassin bug peptides have well conserved the neutral residue Gly11 and the basic residues R43 or K43 (Table 1). The presence of aspartic and glutamic residues at the very end of the N-terminal tail of the assassin bug peptides is also noteworthy. Such a characteristic negatively charged N-terminal region has also been observed at the N-terminal tail of the calcium channel blocker ω-conotoxin PnVIB from *Conus pennaceus* [14] (Table 1). Such a negatively charged region does not seem to affect biological activity in molluscan neurons [14], but perhaps it is important for recognition of different known receptors on calcium channels. Although the homology of assassin bug peptides and conotoxins is low ($<44\%$), it is well known that ω-conotoxins with marked primary structure differences are usually very specific for the same type of calcium ion channel [15]. As an example, the homology between the N-type calcium channel toxins GVIA and MVIIA is 32%. The homology of GVIA and Ptu1 is 37%, and that of GVIIA and Ptu1 is 41%. Therefore, the primary structure homology of assassin bug to the ω-conotoxins is a substantial clue for investigating their biological activity in different types of calcium channels.

In order to carry out chemical and biological characterization, Ptu1, the shorter peptide, was synthesized in its free carboxylated form (sPtu1). The identity between the synthetic peptide and the native one was verified by MALDI-TOF MS and Edman degradation. In a co-injection experiment using capillary electrophoresis, pure native Ptu1 and pure sPtu1 co-eluted in a single peak indicating most likely their identical structural conformation. The sPtu1 peptide was subsequently used to determine the disulfide bridge pairing. sPtu1 was reacted in the presence of TCEP and CDAP and two partially reduced cyanylated protein isomers were obtained with molecular masses of 3667.1 Da each. After cleavage with 1 N NH_4OH followed by TCEP reduction and MALDI-TOF analysis, the identity of the cyanylated isomers was achieved demonstrating the links between Cys_5-Cys_{20} and Cys_{19}-Cys_{33}. The disulfide bond between C_{12}-C_{26} was inferred since all other cystine residues were accounted for. According to these results, the disulfide bonding pattern found for Ptu1 was similar to that described for ω-conotoxins and all other four-loop peptides.

Since the assassin bug peptides were homologous in their primary sequence and possessed a similar disulfide bridge pat-

Table 1
Alignment of the assassin bug peptides with the sequences of cone snail toxins [14,18–23]

Peptide	Sequence	pI/Target	Homology to Ptu1 (%)	Reference
	1 10 20 30 40 50			
Ptu1	--AEKDCIAP GAPCFG---T DKPCCNP--R AWCSSYA--- -NKCL----- ---CO2H	9.7/N-Ca2+	-	This work
Ado1	--ADDDCLPR GSKCLG---E NKQCCK---G TTCMFYA--- -NRCVGV--- ---CO2H	9.7	41	This work
Iob1	-GADEDCLPR GSKCLG---E NKQCCE---K TTCMFYA--- -NRCVGI--- ---CO2H	7.2	41	This work
ω-GVIIA	------CKSP GTPCSR---G MRDCCT---- -SCLLYS--- -NKCRRY--- ---CO2H	11.5/N-Ca2+	41	[18,19]
ω-GVIIB	------CKSP GTPCSR---G MRDCCT---- -SCLSYS--- -NKCRRY--- ---CO2H	11.5/N-Ca2+	44	[18,19]
ω-GVIA	------CKSP GSSCSP---T SYNCCR---- -SCNPYT--- -KRC--Y--- ---NH2	10.7/N-Ca2+	37	[20]
ω-MVIIA	------CKGK GAKCSR---L MYDCCT---- GSC-RS---- -GKC------ ---NH2	11.2/N-Ca2+	24	[21]
ω-MVIIB	------CKGK GASCHR---T SYDCCT---- GSC-NR---- -GKC------ ---NH2	11.1/Ca2+	28	[21]
ω-MVIIC	------CKGK GAPCRK---T MYDCCS---- GSCGRR---- -GKC------ ---NH2	11.7/P,Q-Ca2+	30	[22]
ω-SVIA	------CRSS GSPC-G---V TSICCG---- -RCYR----- -GKCT----- ---NH2	12.2/Ca2+	41	[23]
ω-SVIB	------CKLK GQSCRK---T SYDCCS---- GSCGRS---- -GKC------ ---NH2	11.2/N-Ca2+	23	[23]
ω-PnVIA	-----GCLEV DYFCGIPFAN NGLCCS---- GNCV------ -FVCTPQ--- ---CO2H	3.0/Ca2+	9	[14]
ω-PnVIB	---DDDCEPP GNFCGM-IKI GPPCCS---- GWCF------ -FACA----- ---CO2H	3.5/Ca2+	33	[14]
	------C--- ---C------ ---CC----- --C------- ---C------ ---			
	Loop1 Loop2 Loop3 Loop4			

The multiple sequence alignment was done with ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) using a BLOSUM amino acid substitution. Theoretical p*I* values for oxidized cysteine were calculated by the GPMAW software (ChemSW, California, USA). Homology values are relative to Ptu1 and were calculated by ClustalW at http://www2.ebi.ac.uk/clustalW/ using a BLOSUM matrix.

tern to the ω-conotoxins, the secondary structures of the synthetic peptides Ptu1, GVIA and MVIIA were analyzed by CD and compared (Fig. 2). Their secondary structure component values were α-helical 10.5%, 17.9%, 16.9%; antiparallel β-sheet 4.2%, 29.1%, 30.0%; parallel β-sheet 2.9%, 3.3%, 2.6%; β-turns 34.6%, 18.8%, 43.1%; and random coil 58.0%, 24.6%, 20.6% for Ptu1, GVIA and MVIIA respectively. The CD spectra of Ptu1 and MVIIA were quite similar from 200 to 260 nm with minima at around 205 nm typical of an unordered or random coil structure [16]. However, GVIA showed a different spectrum in the 200–260 nm range, a GVIA analog where Tyr27 was replaced by Ala27, Y27A-GVIA, showed that the phenolic ring of Tyr27 of GVIA has a dominant contribution to its CD spectrum and it was responsible for the positive band observed in the range from 195 to 205 nm [17]. Y27A-GVIA and MVIIA showed similar CD spectra in aqueous solution [17]. Both MVIIA and Ptu1 lack this aromatic chromophore at their C-terminus. Moreover, all three peptides seem to be full of β-turns, which is a common characteristic of the four-loop scaffold conotoxins. The most important CD spectral data seem to come from the antiparallel β-sheet analysis of Ptu1 and ω-conotoxins. That is, the antiparallel β-sheet fractions in GVIA and MVIIA scored 29.1% and 30.0%, respectively, while that in Ptu1 was only 4.2%. The antiparallel β-sheet fraction in Ptu1 seems to be replaced by a high content of unordered structure. The triple-stranded antiparallel β-sheets in the ω-conotoxin family and the low content of antiparallel β-sheet in Ptu1 may be an important factor for biological activity. However, a 3D structure determination of Ptu1 is required to confirm this suggestion.

3.2. Biological activity

The structural similarity between assassin bug peptides and

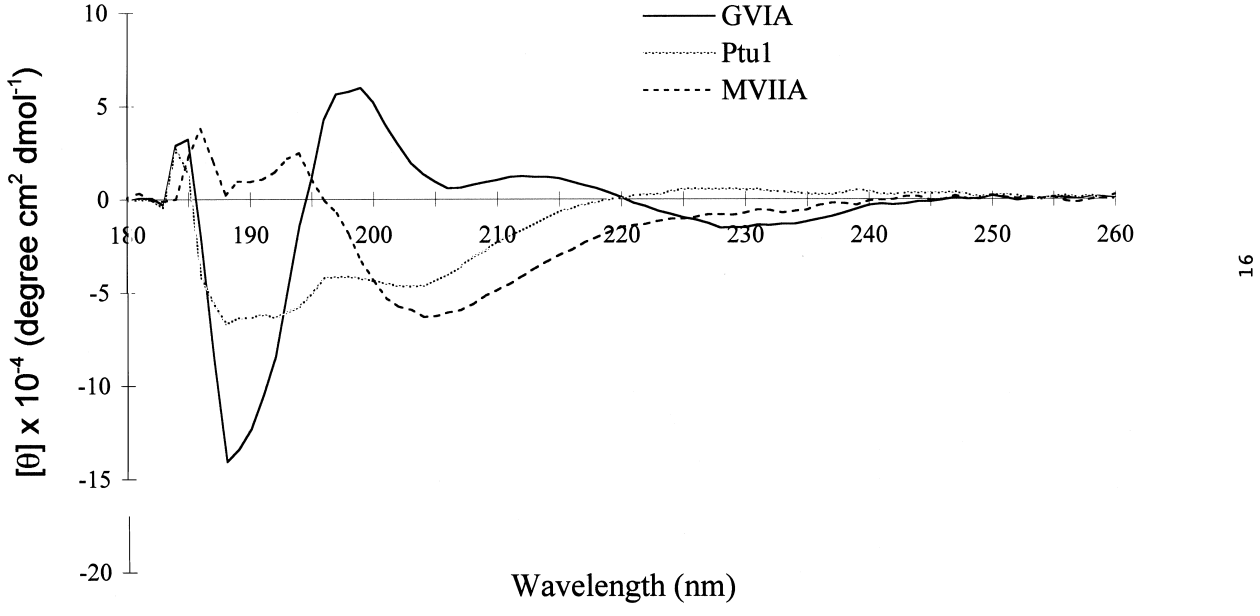

Fig. 2. CD spectra of Ptu1, GVIA, and MVIIA.

ω-conotoxins led us to test Ptu1 for toxicity towards different animals. The assassin bug peptides were assayed in vivo by direct injection into the body of goldfish (i.p.), mice (i.c.v.), cutworm larvae and crickets. However, Ptu1 showed no toxic symptoms in either vertebrates or invertebrates during a period of 48 h post-injection. Nevertheless, because of the relative identity of the assassin bug peptides with ω-conotoxins, electrophysiological experiments were carried out using different BHK cell lines expressing the N-, L-, or P/Q-type calcium channels in the presence and in the absence of Ptu1. Ptu1 produced a block of Ba^{2+} currents ($IC_{50} \approx 0.3$ μM) through the voltage-dependent N-type Ca^{2+} channel (Fig. 3A). In contrast to ω-conotoxin GVIA, which produces irreversible block of N-type Ba^{2+} channel currents ($IC_{100} < 0.1$ μM) in BHK-N101 cells [11], the block of Ba^{2+} channel current produced by Ptu1 was completely reversed on washout of the peptide (Fig. 3A), which was in contrast to the irreversible block by ω-CgTx-GVIA (Fig. 3D). Ptu1 at 300 nM blocked Ba^{2+} currents through the voltage-dependent N-type Ca^{2+} channel to 52.9 ± 6.9% of control (100%) (mean ± S.E.M., $n = 4$, see Fig. 3B). However, Ptu1 produced a merely slight but reversible block of Ba^{2+} currents through other voltage-dependent Ca^{2+} channels such as L-type Ca^{2+} channel and P/Q-type Ca^{2+} channel (76.4% and 76.3%, respectively, $n = 2$, Fig. 3C) from BHK-BI cells and BHK6 cells expressing the α_{1A} and α_{1C} subunits respectively. Thus Ptu1 blocked the N-type Ca^{2+} channel most selectively among high voltage-activated Ca^{2+} channels. Ado1 and lob1 were not tested for biological activity on calcium channels because of the small quantities of material left after the in vivo assays.

Despite Ptu1's low blocking capabilities in N-type calcium channels, it should be noted that most N-type calcium channel toxins isolated to date have IC_{50} values from picomolar to micromolar depending mainly on the electrophysiology techniques and the type of tissues or cells used [24,25]. Although ω-conotoxin GVIA is the most reliable parameter to compare

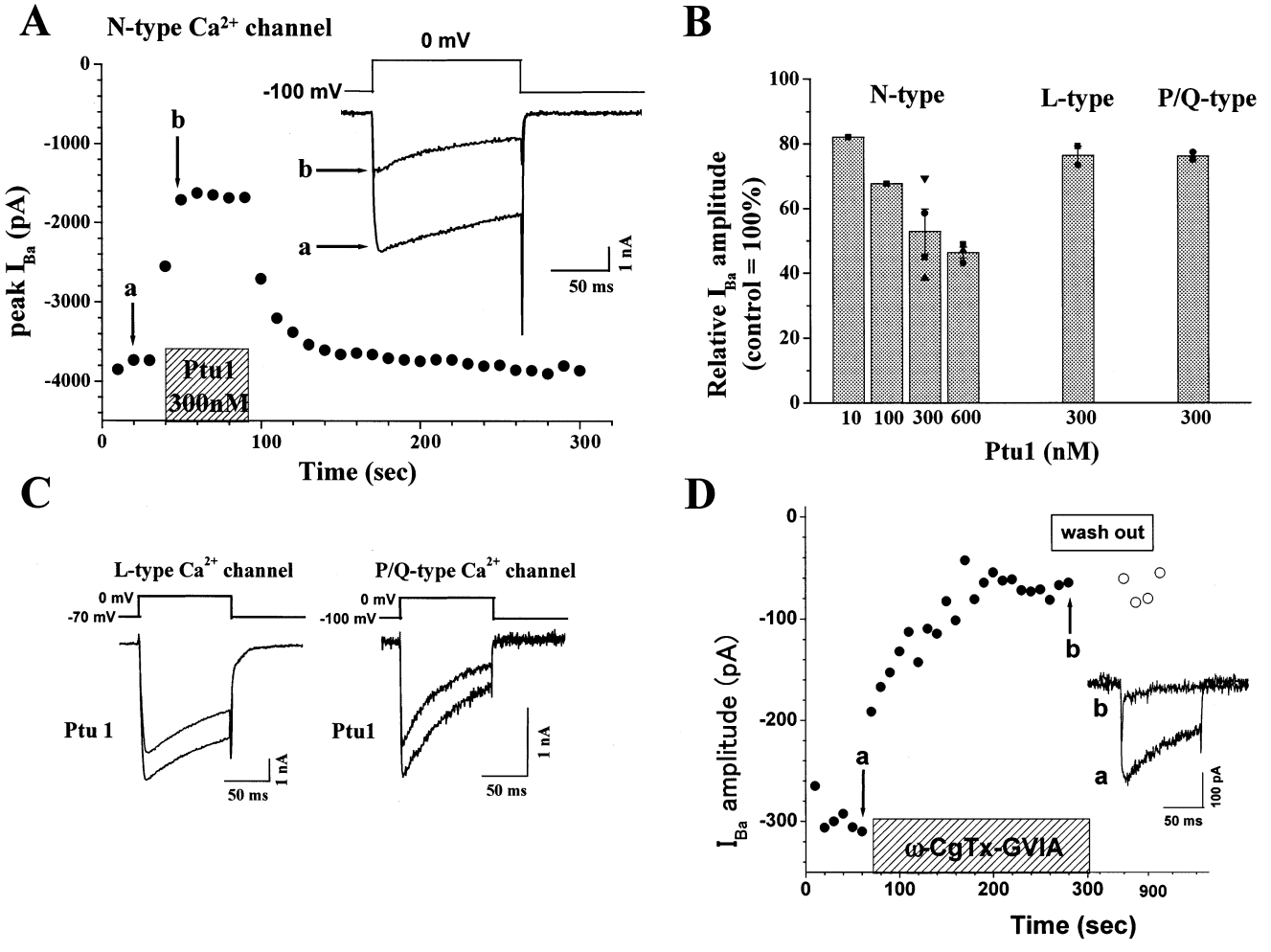

Fig. 3. Effect of Ptu1 on high voltage-activated Ca^{2+} channels expressed in BHK cells. Ca^{2+} channel currents were recorded with Ba^{2+} (2 mM) as a charge carrier. A: Effect of Ptu1 (300 nM) on Ba^{2+} currents through the voltage-dependent N-type Ca^{2+} channel α_{1B} subunit expressed in BHK-N101 cells together with β_{1A} subunit and α_2/δ subunit. Ba^{2+} currents were elicited by giving test pulses from a holding potential at −100 mV to 0 mV for 100 ms as 0.1 Hz. Ptu1 was rapidly applied via a concentration clamp apparatus during the period indicated. Ba^{2+} currents recorded before and after (indicated as a and b, respectively) application of Ptu1 were superimposed in the inset. B: Summary of the block of Ba^{2+} currents through high voltage-activated Ca^{2+} channels by Ptu1. C: Effects of Ptu1 on Ba^{2+} currents through L-type Ca^{2+} channel α_{1C} subunit expressed in BHK6 cells together with β_{1A} subunit and α_2/δ subunit (left) and through P/Q-type Ca^{2+} channel α_{1A} subunit expressed in BHK-B1 cells together with β_{1A} subunit and α_2/δ subunit (right). D: Irreversible block of N-type Ca^{2+} channel by ω-CgTx-GVIA at 10^{-7} M.

and categorize the potency of N-type calcium channel blockers, we do not rule out that Ptu1 may act on other tissue cells with similar or more potency than GVIA.

Ptu1 was not toxic to mice either when injected i.c.v. up to 7 μg/mouse or when injected i.v. at the same concentration. In addition, experiments using mast peritoneal cells from rats have shown that Ptu1 does not release histamine at concentrations up to 10 μg/ml (data not shown). In this respect, the non-toxic effects, the ion channel specificity and the fast reversible activity of Ptu1 could be an advantage over other calcium channel peptides (i.e. ω-conotoxins) for the design of novel drugs for the treatment of N-type calcium channel-related disorders. Further research should involve electrophysiological and binding experiments in vertebrate tissue cells using sPtu1 and sAdo1 for determining more acutely their biological targets as well as NMR studies for determining their three-dimensional structures.

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