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Duck-Billed Platypus Venom Peptides Induce Ca^{2+} Influx in Neuroblastoma Cells

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The duck-billed platypus, *Ornithorhynchus anatinus*, is one of the few venomous Australian mammals.[1] Envenoming by a male platypus causes immediate excruciating pain in humans, which evolves toward a long-lasting hyperalgesia. Several constituents of the venom fluid or its crural gland have been identified, including defensin-like peptides,[2] C-type natriuretic peptides (OvCNPs),[3] NGF, and hyaluronidase. OvCNP causes the relaxation of rat uterine smooth muscle, promotes histamine release from mast cells, and forms fast cation channels in lipid bilayers.[4] Recently, an L-to-D-peptide isomerase was identified by which D-Leu^2 in OvCNPs and D-Met^2 in defensins are formed.[5] Furthermore, the platypus genome project (completed in 2008) revealed that reptile and platypus defensin-like peptides have been co-opted independently from the same gene families.[6] Notably, only a few members of the most ancient mammals, such as shrews and a solenodon (*Insectivora*) as well as a platypus (*Monotremata*), have been shown to produce toxic venom.[7] Thus, identification of the unique bioactive substances in these venoms should help to clarify their evolutionary properties and ecological roles.

Recently, we reported that crude platypus venom potently induces Ca^{2+} influx in human neuroblastoma IMR-32 cells.[8] Guided by this bioassay, we have identified 11 novel peptides **1**−**11** (Figure 1). Here we describe the purification and characterization of these peptides found in the venom.

On the basis of the finding that platypus venom contains variously sized molecules, we first chose gel-permeation HPLC for purification (Figure 2). As expected, the low-molecular-weight components, especially fraction (fr.) **IX**, potently induced Ca^{2+} influx, and most proteins were eluted in fr. **II**−**V**.[9] Reversed-phase HPLC (RP-HPLC) purification of fr. **IX** gave heptapeptide **1** as a primary component (~200 ng/μL of venom fluid). With regard to fr. **VI**, five major peaks were detected between 30 and 80 min, from which hexapeptide **2**, peptides **5**−**8**, and peptides **9**−**11** were purified as major constituents. Furthermore, direct RP-HPLC purification of the crude venom afforded peptides **3** and **4**.[10]

Through the use of MALDI-TOF/TOF MS analysis, the primary structure of **1** was established to be H-His-Asp-His-Pro-Asn-Pro-Arg-OH. Similarly, **2**−**4** were found to be analogues of **1** that coincided with the 6−9 N-terminal residues of OvCNP. Meanwhile, peptides **5**−**9** coincided with the 132−150 part of OvCNP precursor peptide (preOvCNP), a translated sequence from the predicted mRNA in the platypus genome (GenomeNet Database, XM_001515107). Peptides **1**−**9** were all chemically synthesized, and their retention

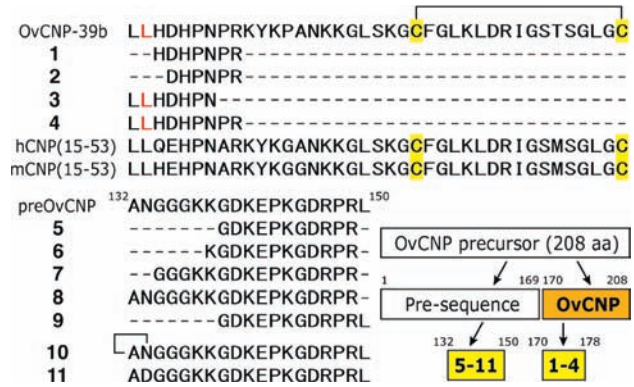

Figure 1. Sequence alignment of CNPs and the venom peptides **1**−**11**. Conserved cysteine residues are highlighted in yellow. D-Amino acids are indicated in red. A schematic representation of the position of venom peptides in preOvCNP is shown at the lower right.

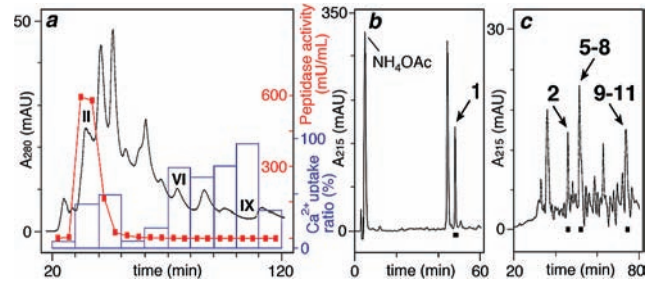

Figure 2. Purification of the venom peptides. (a) Gel-permeation HPLC of the venom fluid (5 μL). The Ca^{2+} influx effect (blue column) and peptidase activity (Pro-Phe-Arg-MCA, red ■) for each fraction are shown. (b, c) RP-HPLC traces obtained using a C_{30} semimicro column in which (b) 2/5 amount of fr. **IX** and (c) 1/20 amount of fr. **VI** were loaded; ■ markers show purified peptides.

times were identical to those of the natural compounds. Moreover, the Leu^2 residues in peptides **3** and **4** were D-form, not L-form, as in OvCNP-39b.[10] The y-ion fragment patterns in the MS/MS data suggested that the 12 C-terminal residues in peptides **10** and **11** were identical to those in **9**, but their molecular mass units were 17 less and 1 more than those of preOvCNP(132−150), respectively. As a result, we concluded that **10** had a cyclic amide structure at the Ala^1-Asn^2 moiety by deamidation,[11] while the amide moiety of the Asn^2 residue was hydrolyzed to an aspartic acid residue in **11**.

Synthetic heptapeptide **1** induced a significant increase in $[Ca^{2+}]_i$ in IMR-32 cells at 75 μM (Figure 3).[12] Nonapeptide **4** also showed weak Ca^{2+} influx at 500 μM, but hexapeptide **2** did not at the same

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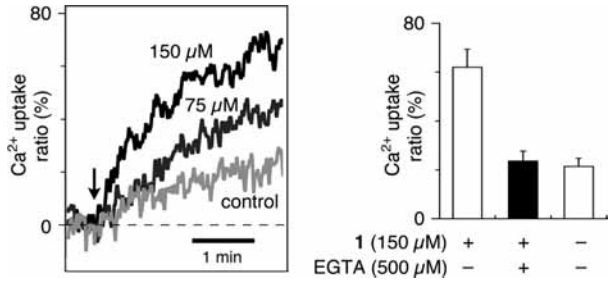

Figure 3. Effects of **1** on Ca^{2+} uptake in IMR-32 cells: (left) Increase in the f_{340}/f_{380} ratio monitored as the $[Ca^{2+}]_i$ uptake rate. The arrow indicates the time of sample injection. (right) Inhibitory effects of EGTA. Values are the mean ± standard deviation of duplicate experiments.

concentration. At a microscopic level, brief treatment with these three peptides at 500 μM did not significantly damage the plasma membrane in IMR-32 cells. Notably, unlike the fast and temporary increase in $[Ca^{2+}]_i$ caused by KCl, the calcium uptake caused by **1** was slow and continuous, which is identical to the result with crude venom.[8] Furthermore, ethylene glycol tetraacetic acid (EGTA) markedly inhibited the increase in $[Ca^{2+}]_i$ caused by **1**, which suggested that extracellular calcium was essential for the activity of **1**. These observations indicated that the Ca^{2+} influx effect of **1** was actually caused not by its high concentration, which could disrupt membranes, but rather by specific interactions with target molecules.

CNPs are vasorelaxant peptide hormones that are widely distributed in endothelium, myocardium, gastrointestinal, and genitourinary tracts as well as the central nervous system.[13] The amino acid sequences of mammalian CNPs, such as human CNP (hCNP-53, -22) and mouse CNP (mCNP-53), are highly conserved, and a disulfide bond between two cysteine residues in the C-terminus has been shown to be essential for their activities.[3] To the best of our knowledge, this is the first example of the isolation of N-terminal linear fragments of CNPs in any mammal.

Differentiated IMR-32 cells typically express L- and N-type voltage-gated calcium channels (VGCCs). To clarify whether the gradual increase in $[Ca^{2+}]_i$ caused by heptapeptide **1** is due to the direct activation of VGCCs, several bioassays were carried out. Heptapeptide **1** did not significantly bind to L- and N-type VGCCs, potassium channels (K_A and K_{ATP}, hERG), or a sodium channel at 10 μM. In addition, **1** did not show meaningful agonistic or antagonistic effects in guinea pig ileum specimens at 30 μM. Thus, we concluded that venom peptide **1** may specifically bind to other receptor channels or biomacromolecules,[14] such as GTP-binding regulatory proteins, as in the case of the direct activation of G_i proteins by mastoparan[15] or substance P.[16] Further studies will be required in order to clarify the target molecules and physiological roles of the unique compounds found in this study as well as their precise mechanisms of action that lead to neurotoxic symptoms.

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Supporting Information Available: General experimental procedures, HPLC and MS data, characterization data for new compounds, and complete ref 6a. This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Fr. **II** strongly hydrolyzed Pro-Phe-Arg-MCA, a substrate of tissue kallikrein, and this peptidase activity was markedly suppressed by treatment with 10 μM aprotinin. These results suggested that the platypus venom had trypsin- or kallikrein-type proteolytic activity.
(10) For details, see Figures S1 and S16 in the Supporting Information.
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