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Biological activity of the predicted red pigment-concentrating hormone of *Daphnia pulex* in a crustacean and an insect

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ABSTRACT

The elucidation of the genome of the waterflea *Daphnia pulex* made it possible to search for orthologue genes for the crustacean red pigment-concentrating hormone (named Panbo-RPCH after the species *Pandalus borealis* in which the red pigment-concentrating hormone was first identified); Panbo-RPCH is a member of the adipokinetic hormone (AKH)/red pigment-concentrating hormone (RPCH) peptide family. The information pointed to a putative mature RPCH octapeptide in *D. pulex* with the primary sequence of pGlu-Val-Asn-Phe-Ser-Thr-Ser-Trp amide (=Dappu-RPCH). Since Panbo-RPCH is endogenous in decapod crustaceans and in the green stink bug *Nezara viridula*, we assayed Dappu-RPCH in the shrimp *Palaemon pacificus* and in *N. viridula*. Here we show that this variant member of the AKH/RPCH family has no activity to concentrate the red, brown, yellow and blue pigments in the epithelium of the shrimp at physiological doses but is effective in mobilising lipids in the green stink bug *N. viridula*. Moreover, since Panbo-RPCH and Dappu-RPCH differ structurally at three positions, viz. Leu^2 to Val^2; Pro^6 to Thr^6; Gly^7 to Ser^7, we tested other members of the peptide family which have single or dual amino acid substitutions at the appropriate positions, for their chromatophorotropic action at physiological doses. These studies show unequivocally that a single change from Gly^7 to Ser^7 (as in the peptide Corpu-AKH) does not inflict any loss of biological activity, and the same is true for a single change from Pro^6 to Thr^6 (represented by the peptide Schgr-AKH-II). The change from Leu^2 to Val^2 (embodied in Manto-CC), however, is accompanied with a substantial loss of chromatophorotropic activity; combinations of Val^2 and Ser^7 (as in Anaim-AKH) or Val^2 and Thr^6 (as in Grybi-AKH) result in almost complete loss of biological activity. Dappu-RPCH with its three substitutions is not active at all in the shrimp at the tested concentration range of up to 30 pmol.

1. Introduction

Malacostracan crustaceans possess branched pigment cells, called chromatophores, which are located in the cuticular epidermis; these chromatophores contain pigment granules of various colours and are classified by the colour of the pigment granules, e.g. melanophores contain black or brown pigment, erythrophores contain red pigment, while xanthophores, leucophores and iridophores contain yellow, white and blue (iridescent) pigment granules, respectively (see Josefsson (1983) and Nery and Castrucci (2002)). Chromatophores may also contain more than one pigment and are then referred to as dichromatic or polychromatic when two or several pigments, respectively, are contained within the chromatophore (see Highnam and Hill (1977)). Some decapods display rapid, reversible colour changes as a result of bidirectional movements of pigment within the chromatophores in response to environmental cues, such as background colour and light intensity. This movement of pigment is controlled by antagonistic neuropeptide hormones, known as chromatophorotropins, which are synthesized in the eyestalks of decapods: the red pigment-concentrating hormone (RPCH) causes red, brown and yellow pigment granules to aggregate in the centre of the chromatophore and so result in a pale-looking cell and whole animal; the pigment-dispersing hormone (PDH), on the other hand, causes pigment granules to spread throughout the chromatophore and into the cellular processes, thus darkening the cell and the overall organismal appearance (see Rao (2001)).

Panbo-RPCH is the first invertebrate neuropeptide hormone ever to be fully characterised and sequenced: pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp amide (Fernlund and Josefsson, 1972); this octapeptide was first isolated from the prawn, *Pandalus borealis*, and subsequently from many other decapod crustaceans belonging to different infraorders (see Gäde and Marco (2006)). In all these species, the octapeptide structure of Panbo-RPCH is conserved. This is in stark contrast to the sister group, Insecta, where peptides, structurally similar to Panbo-RPCH, are not conserved across family groups and more than one structural variant may even be present

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in a single insect species (Gäde, 2009). These neuropeptides, produced in the corpora cardiaca in the insect head, are generically known as adipokinetic hormones (AKHs) since they mobilize energy substrates for intermediate metabolism (Gäde, 2004; Gäde and Marco, 2006). Six years ago, an AKH with the conserved amino acid structure of Panbo-RPCH was isolated and characterised from the green stink bug, *Nezara viridula*; homologous biological assays showed that Panbo-RPCH stimulated lipid mobilisation in the stink bug (Gäde et al., 2003). This result confirmed earlier data demonstrating that peptides of a very similar structure can elicit reciprocal actions in the two sister groups: injection of a crude extract of prawn eyestalks had an adipokinetic effect in locusts, and the AKH of a locust (a decapeptide) had a red pigment-concentrating effect in a prawn (Mordue and Stone, 1976). To date, there are no reports of AKH/RPCH eliciting a lipid mobilising effect in crustaceans (see Gäde and Marco (2006)).

Recently, the complete genome of a cladoceran crustacean, *Daphnia pulex*, was sequenced (www.wfleabase.org) and we identified *in silico* a putative precursor for an AKH/RPCH which may give rise to the following mature AKH-type peptide: pGlu-Val-Asn-Phe-Ser-Thr-Ser-Trp amide which we call here, in keeping with the rules of currently used nomenclature, Dappu-RPCH. This structural variant of Panbo-RPCH is, to date, only known from *in silico* analyses of expressed sequence tags (ESTs) of another daphnid species *Daphnia magna* (Christie et al., 2008) and was, interestingly, not found in a recent search of *D. pulex* ESTs (Gard et al., 2009). Since Dappu-RPCH is the only other structural variant of Panbo-RPCH known to occur in crustaceans, to date, and since daphnids do not have integumental chromatophores (own observations) we investigate here the comparative RPCH/AKH activity of Dappu-RPCH in a decapod crustacean and in a stink bug. For this purpose, (a) Panbo-RPCH, Dappu-RPCH and several insect AKHs with single or dual amino acid substitutions when compared with Panbo-RPCH and Dappu-RPCH were assayed in shrimps for a red pigment-concentrating effect, and (b) Dappu-RPCH was assayed in green stink bugs for an adipokinetic effect.

2. Materials and methods

2.1. Animals

Shrimps (*Palaemon pacificus*) were netted in the waters of the harbour of Kalk Bay near Cape Town (Western Province of the Republic of South Africa) in early May 2008 and late July 2008. Animals were maintained in rectangular glass tanks (volume of 20 l) housed in the Zoology departmental aquarium where the air temperature ranged between 16 and 20 °C and the tanks were filled with recirculating sea water at a temperature of 14 °C. Shrimps were fed *ad libitum* with a commercially available feed for aquarium fish (Tetra fish food flakes) and were supplied with artificial shelters and a light regime of 12 h light:12 h dark; a few days before use in experiments, shrimps were subjected to living on a black background under the same aquarium conditions described above.

A culture of the green stink bug, *N. viridula*, was kept under ambient austral summer conditions (17 h light: 7 h dark; 25 °C). Insects were supplied with water and food (green beans and shelled sunflower seeds) *ad libitum*, and newly-moulted adults of both sexes were separated from the rest of the culture and used for bioassays as 2- to 4-day-old adults.

2.2. Peptides

The following peptides were custom-synthesised either by Kevin D. Clark (Department of Entomology, University of Georgia, USA), or were purchased from Peninsula Laboratories (Belmont, CA, USA) or from PolyPeptide Laboratories s.r.o. (Prague, Czech Republic) and used in the study (see Table 1 for amino acid structure, species origin and reference): Panbo-RPCH, Ile^2-Panbo-RPCH, Dappu-RPCH, Manto-CC, Anaim-AKH, Corpu-AKH, Nepci-AKH, Schgr-AKH-II and Grybi-AKH. All peptides were supplied at greater than 95% purity and about 85% peptide content. Stock solutions of about 1 mM were prepared by weight, and were subsequently monitored for purity and peptide content by reversed-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection (276 nm excitation, 350 nm emission) using a Gilson RP-HPLC system (see Gäde (1985)) with a gradient of 43 to 53% B in 20 min (Nucleosil C18 column; Solvent A: 0.1% trifluoroacetic acid in water; solvent B: 60% acetonitrile in water with 0.1% trifluoroacetic acid; flow rate 1 ml min^{-1}). For injection into shrimps, peptide stocks were diluted with sterile sea water and 5 µl injected per shrimp. For injection into insects, peptide stocks were diluted with distilled water and 3 µl injected.

2.3. RPCH bioassay

Dark-adapted shrimps were fetched from the aquarium and kept in a black bucket in the laboratory in aerated sea water. Shrimps were individually removed from the water for a brief period (15 s) to inspect the chromatophores on the dorsal abdomen with a Nikon dissecting microscope (16× magnification) to determine the degree of pigment dispersion before injection. Staging of chromatophoral red pigment was done according to Hogben and Slome (1931). Five microliters sea water or peptide in sea water was injected through the lateral thoracic carapace of the shrimp with a 10 µl Hamilton glass syringe. Immediately after injection, the shrimp was put into a black rectangular basin (volume of 500 ml) filled with sea water at a temperature of 17.5 °C. The injected shrimps were kept individually in the dark basins for the duration of the experiment, maximally 30 min, and were taken out of the water (air temperature 20–22 °C) for a brief microscopic inspection every 5 min.

Table 1
Sequence and natural source of AKH/RPCH peptides used in the current study in pigment-concentrating and in lipid mobilising assays.

Peptide name	Peptide sequence*	Original source
Panbo-RPCH	pELNFSPGWamide	*Pandalus borealis* (Fernlund and Josefsson, 1972)
Ile^2-Panbo-RPCH	pE**I**NFSPGWamide	To date, no natural source known
Manto-CC	pE**V**NFSPGWamide	An unidentified Namibian species of the order Mantophasmatodea. (Gäde et al., 2005)
Anaim-AKH	pE**V**NFSP**S**Wamide	*Anax imperator* (Gäde et al., 1994)
Corpu-AKH	pELNFSP**S**Wamide	*Corixa punctata* (Gäde et al., 2007a)
Nepci-AKH	pELNFS**S**GWamide	*Nepa cinerea* (Gäde et al., 2007b)
Schgr-AKH-II	pELNFS**T**GWamide	*Schistocerca gregaria* (Siegert et al., 1985)
Grybi-AKH	pE**V**NFS**T**GWamide	*Gryllus bimaculatus* (Gäde and Rinehart, 1987)
Dappu-RPCH	pE**V**NFS**TS**Wamide	Deduced from the genome of *Daphnia pulex*

* Amino acid residues in bold text indicate a substitution with respect to the sequence of Panbo-RPCH.