A novel peptide-processing activity of insect peptidyl-dipeptidase A (angiotensin I-converting enzyme): the hydrolysis of lysyl-arginine and arginyl-arginine from the C-terminus of an insect prohormone peptide

R. Elwyn ISAAC^{*1}, Liliane SCHOOFS[†], Tracy A. WILLIAMS[‡], Dirk VEELAERT[†], Mohammed SAJID^{*}, Pierre CORVOL[‡] and David COATES^{*}

^{*}Department of Biology, University of Leeds, Leeds LS2 9JT, U.K., [†]Zoological Institute, Katholieke Universiteit Leuven, Naamsestraat 59, 3000 Leuven, Belgium, and

[‡]INSERM Unit 36, Collège de France, 3 d'Ulm, 75005 Paris, France

Insect peptidyl-dipeptidase A [angiotensin I-converting enzyme (ACE)] is a soluble single-domain peptidyl-dipeptidase that has many properties in common with the C-domain of mammalian somatic ACE and with the single-domain mammalian germinal ACE. Mammalian somatic ACE is important in blood homeostasis, but the role of ACE in insects is not known. Immunocytochemistry has been used to localize ACE in the neuroendocrine system of the locust, *Locusta migratoria*. Staining was observed in five groups of neurosecretory cells in the brain and suboesophageal ganglion, in the nervi corpori cardiaci, the storage part of the corpora cardiaca and in the nervi corpori allati. In three groups of neurosecretory cells, ACE co-localized with locustamyotropins, suggesting a possible role for the enzyme in the metabolism of these neuropeptides. We demonstrate *in vitro* a novel activity of ACE that removes pairs of basic amino

acid residues from a locustamyotropin peptide extended at the C-terminus with either Gly-Lys-Arg or Gly-Arg-Arg, corresponding to a consensus recognition sequence for endoproteolysis of prohormone proteins by prohormone convertases. The low K_m and high k_{cat} values (K_m 7.3 and 5.0 μ M, k_{cat} 226 and 207 s^{-1} for the hydrolysis of Phe-Ser-Pro-Arg-Leu-Gly-Lys-Arg and Phe-Ser-Pro-Arg-Leu-Gly-Arg-Arg, respectively) obtained for the hydrolysis of these two peptides by insect ACE means that these peptides, along with mammalian bradykinin, are the most favoured *in vitro* ACE substrates so far identified. The discovery of this *in vitro* prohormone-processing activity of insect ACE provides a possible explanation for the intracellular co-localization of the enzyme with locustamyotropin peptides, and provides evidence for a new role for ACE in the biosynthesis of peptide hormones and transmitters.

INTRODUCTION

Mammalian peptidyl-dipeptidase A [angiotensin I-converting enzyme (ACE); EC 3.4.15.1] is a glycosylated ectoenzyme (150–180 kDa, depending on species) found mainly on the surface of endothelial, epithelial and neuronal cells [1–4]. The enzyme is composed of two highly similar protein domains (N- and C-domain, named after their positions relative to the N- and C-termini), each possessing a catalytic site that cleaves dipeptides from the C-terminus of oligopeptides [5]. Vascular endothelial ACE is well known for its role in the formation of the vasoconstrictor angiotensin II by removing His-Leu from the C-terminus of circulating angiotensin I, and hydrolysing Phe-Arg from the C-terminus of the vasodilatory peptide bradykinin [1]. The enzyme also inactivates the haemoregulatory peptide, N-acetyl-Ser-Asp-Lys-Pro (AcSDKP), by cleavage of the Asp-Lys peptide bond, mainly through the activity of the N-domain of endothelial ACE [6,7]. *In vitro* studies have shown that ACE can also function as an endopeptidase, cleaving a dipeptide-amide from cholecystokinin, a dipeptideamide and a tripeptideamide from substance P, and, rather surprisingly, an N-terminal tripeptide as well as the C-terminal tripeptideamide from luteinizing hormone-releasing hormone [8]. However, C-terminally blocked peptides are generally poorer substrates of ACE than angiotensin I, bradykinin and AcSDKP. A smaller single-domain form of mammalian ACE exists in male germ cells and is known as testicular or germinal ACE (M_r 100 000–110 000) [2,9,10]. The function of this enzyme, which is exclusively expressed in developing spermatids, is not known.

A soluble single-domain insect peptidyl-dipeptidase A with an M_r of 67 000 has recently been isolated from *Drosophila melanogaster* [11], the housefly *Musca domestica* [12] and the buffalo fly *Haematobia irritans exigua* [13]. The protein sequence predicted from the cDNA of *Drosophila* and *Haematobia* ACE shows a high level of similarity to both the N- and C-domains of human ACE [11,13–15] and the enzyme can convert angiotensin I into angiotensin II, cleaves bradykinin and is inhibited by inhibitors of mammalian ACE [12,13,16–18]. Thus the insect peptidyl-dipeptidase A is also known as ACE (*D. melanogaster* ACE gene is known as *Ance* or *Race* [11,15]).

The conservation of the properties of insect and mammalian ACE suggested that the insect enzyme may also be involved in the metabolism of peptide hormones. However, there are very few clues as to the nature of the peptide substrates of the insect enzyme, especially since there are no reports of insect homologues of the known *in vivo* substrates of mammalian ACE. As part of our search for ACE substrates in insects, we have undertaken immunocytochemical studies to identify any peptide hormones/neurotransmitters that might be co-localized with ACE in neuroendocrine tissues and neurosecretory cells of the central nervous system. For this study we turned our attention to locusts because of the large number (> 50) of bioactive peptides that have been isolated and characterized from these insects [19].

We now report the co-localization of insect ACE with peptides belonging to the locustamyotropin/leucopyrokinin/pheromone-biosynthesis-activating neuropeptide (PBAN)/diapause hormone family of peptides in the brain and suboesophageal ganglia of locusts [20–23]. The co-existence of ACE and locustamyotro-

Abbreviations used: ACE, angiotensin I-converting enzyme; PBAN, pheromone-biosynthesis-activating neuropeptide.

¹ To whom correspondence should be addressed.

tropin-like peptides within locust neurosecretory cells suggests a role for ACE in the intracellular processing of a locustamyotropin prohormone. In support of this hypothesis, we have identified a novel prohormone-processing activity of insect ACE that efficiently removes basic dipeptides from locustamyotropin oligopeptides possessing a C-terminal Gly-Lys-Arg and Gly-Arg-Arg extension.

EXPERIMENTAL

Materials

Immunochemical reagents were purchased from Sigma Chemical Co. Phe-Ser-Pro-Arg-Leu-Gly-Lys-Arg (FSPRLGKR) and Phe-Ser-Pro-Arg-Leu-Gly-Arg-Arg (FSPRLGRR) were synthesized at the Sussex Centre for Neuroscience, University of Sussex, Brighton, Sussex, U.K. Phe-Ser-Pro-Arg-Leu-Gly (FSPRLG) was obtained from the hydrolysis of FSPRLGKR by *Drosophila* ACE and was purified and quantified by reverse-phase HPLC. DNA polymerase for PCR was from Stratagene. DNA sequencing was conducted using Sequenase 2.0 kit from United States Biochemicals and the expression of the *D. melanogaster* ACE in *Pichia pastoris* was performed using the Invitrogen *Pichia* expression kit [17]. Preparation of antiserum to *M. domestica* ACE and locustamyotropin peptides has been described elsewhere [11,24].

Immunocytochemistry

Brain–corpora cardiaca–corpora allata–suboesophageal ganglia complexes were fixed *in situ* in Bouin–Hollande’s 10% sublimate (made from mixing 9 parts of solution A [1 l of distilled water containing 25 g of cupric acetate, 40 g of picric acid, 100 ml of formaldehyde and 10 ml of acetic acid with 1 part of saturated HgCl₂ solution in water). Fixed tissues were then treated and immunohistochemically stained using antisera recognizing locustamyotropin peptides and housefly ACE, by means of the peroxidase–antiperoxidase method as described previously [24]. Specificity of the antiserum to housefly ACE was controlled by immunoabsorption (1:2000 diluted serum) with either recombinant *Drosophila* ACE (1 μ M) or locustamyotropins I–IV (1 μ M).

Identification of peptide metabolites

HPLC-purified metabolites (FSPRLG and FSPR) were subjected to matrix-assisted laser-desorption time-of-flight MS using a matrix solution of 3,5-dimethoxy-4-hydroxycinnamic acid (10 mg/ml in aq. 30% acetonitrile/0.1% trifluoroacetic acid) and a VG Analytical Micromass ToFSpec instrument.

Expression of *Drosophila* ACE and a secreted form of the human ACE C-domain in *P. pastoris*

An *Eco*RI site was introduced by PCR into the *Ance* cDNA such that cleavage by *Eco*RI occurs after nucleotide 138 which removes the nucleotide sequence encoding the signal peptide [11]. The *Eco*RI site enabled an in-frame fusion with the prepro α -factor secretion signal present in the *P. pastoris* transfer vector pPIC9 (Invitrogen). The resulting construct was termed pPIC9AnCE [17]. All methods for the screening of recombinant colonies and for protein expression were carried out as described in the users’ manual for the *P. pastoris* expression system (Invitrogen). After expression of recombinant enzymes, yeast cells were removed by centrifugation (4000 *g* for 10 min at room temperature). Recombinant *Drosophila* ACE was the sole protein secreted into the culture medium as detected by SDS/PAGE and silver staining [17]. Secreted *Drosophila* ACE was subjected to chromatography

on a DEAE-cellulose column, and enzymically active fractions were concentrated as described previously [17].

Hydrolysis of peptides

FSPRLGKR and FSPRLGRR (4–40 μ M) were incubated with recombinant *Drosophila* ACE at 37 °C for 15–30 min in 0.1 M Mes buffer, pH 6.4 or 5.6, containing 10 μ M ZnSO₄ and 0–100 mM NaCl (final volume 240 μ l). Under these incubation conditions the extent of hydrolysis did not exceed 15% of the initial substrate, and reactions were linear with time. Reactions were terminated by the addition of 20 μ l of 8% (v/v) trifluoroacetic acid and the hydrolysis products were quantified by reverse-phase HPLC with UV detection (214 nm) using a Pharmacia Pep-S column and a linear gradient (3–30%) of acetonitrile in 0.15% trifluoroacetic acid.

RESULTS

Co-localization of insect ACE and locustamyotropin peptides in the central nervous system of the locust, *Locusta migratoria*

The distribution of insect ACE in the neuroendocrine system of *L. migratoria* was studied by immunocytochemistry of wax sections using an antibody raised to housefly ACE. ACE immunoreactivity was found in perikarya of five groups of neurosecretory cells in the brain and suboesophageal ganglion, in the nervi

Figure 1 Co-localization of ACE and locustamyotropins in the central nervous system of *L. migratoria*

Consecutive sections of the tritocerebrum (TRITO) of the brain (a,b) and suboesophageal ganglion (SOG) (c,d) stained with (a,c) antiserum to insect ACE and (b,d) antiserum to locustamyotropins. Magnification about 1000 $\times$.

Figure 2 Cleavage of Lys-Arg and Arg-Arg from the C-terminus of FSPRLGK/RR

HPLC analysis of the conversion of (a) Phe-Ser-Pro-Arg-Leu-Gly-Lys-Arg (peak 1), and (b) Phe-Ser-Pro-Arg-Leu-Gly-Arg-Arg (peak 3) to Phe-Ser-Pro-Arg-Leu-Gly (peak 2) by recombinant *Drosophila* ACE. Peptides (20 μ M) were incubated with recombinant *Drosophila* ACE (0.1 ng) at 37 °C for 15 min in 0.1 M Mes buffer, pH 6.4. Reactions were terminated and analysed by reverse-phase HPLC (214 nm) as described in the Experimental section. The UV-absorbing hydrolysis product (2) of both reactions was collected and identified as Phe-Ser-Pro-Arg-Leu-Gly ($[M+H]^+$ at m/z 676) by matrix-assisted laser-desorption time-of-flight MS.

corpora cardiaca, in the storage part of the corpora cardiaca and in the nervi corpori allati. Cells in three of the groups also stained with an antiserum recognizing the C-terminus of locustamyotropin I (Gly-Ala-Val-Pro-Ala-Ala-Gln-Phe-Ser-Pro-Arg-Leu-NH₂) and locustamyotropin II (Glu-Gly-Asp-Phe-Thr-Pro-Arg-Leu-NH₂) (Figure 1). Group I comprised three to seven cell bodies in the lateral edge of each posterior tritocerebral hemispheres of the brain, whereas groups II and III were in the ventral midline of the suboesophageal ganglion and consisted of an anterior group of four larger cells and a posterior group of up to 15 cells. The patterns of the intracellular staining with the two antisera were indistinguishable at the level of light microscopy and appeared to be granular in nature. The staining with the ACE and locustamyotropin antisera was blocked by preincubating the antisera with recombinant insect ACE and locustamyotropin peptides respectively. Preincubating anti-ACE antibody with locustamyotropins did not block the staining obtained with this antiserum.

Hydrolysis of Lys-Arg and Arg-Arg from the C-terminus of locustamyotropin peptides C-terminally extended with Gly-Lys-Arg and Gly-Arg-Arg

The co-localization of insect ACE and locustamyotropin-like peptides in neurosecretory cells of the locust brain and suboeso-

Table 1 Kinetic parameters for insect and human ACE

DrosACE is *D. melanogaster* ACE. hC-ACE and hN-ACE are the C- and N-domains of human somatic ACE respectively. Kinetic constants were determined by measuring the rates of conversions of FSPRLGKR and FSPRLGRR to FSPRLG, and FSPRLG to FSPR using the HPLC-based assays described in the Experimental section. The kinetic constants were obtained from triplicate data using a curve-fitting computer program (Pfit; Biosoft, U.K.). Data for the hydrolysis of angiotensin I and bradykinin by the N- and C-domains of human ACE are from [35] and [36] respectively.

Substrate	Enzyme	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
FSPRLGKR	drosACE	7.3	226	30.95
FSPRLGRR	drosACE	5.0	207	35.40
FSPRLG	drosACE	45.4	30.2	0.67
Angiotensin I	hC-ACE	18	34	1.89
Angiotensin I	hN-ACE	15	11	0.73
Bradykinin	hC-ACE	0.24	8	33
Bradykinin	hN-ACE	0.54	5	9

phageal ganglion, together with the granular appearance of the stained material, suggested that the enzyme may be involved in the intracellular processing of the prohormone to mature locustamyotropin-like peptides. The locustamyotropin prohormone has not been characterized, but the structure of the prohormones of locustamyotropin-related peptides of two other insects, *Bombyx mori* and *Helicoverpa zea*, is known from analysis of the cDNA of the diapause hormone and PBAN peptide. Both cDNAs consist of five copies of the Phe-Xaa-Pro-Arg/Lys-Leu sequence flanked at the predicted C-terminus by Gly followed by either single (Arg), or pairs (Lys-Arg or Arg-Arg) of, basic residues, which are potential cleavage sites for prohormone convertases [25–27]. The presence of multiple copies of peptide sequences flanked by pairs of basic residues is a very common feature of prohormones of insect peptides and it is likely that members of the locustamyotropin/leucopyrokinin/PBAN/diapause hormone family of peptides are derived from similar prohormone sequences [25,26,28–32]. Generally, the processing of a prohormone to biologically active peptide with an amidated C-terminus requires the removal of the basic residues to give a peptide intermediate with a C-terminal Gly, which then undergoes oxidation to generate a peptide terminating with an amidated residue [33]. The trimming of the Lys and Arg is normally catalysed by carboxypeptidase E [34]. We considered the possibility that insect ACE can serve as an alternative processing enzyme, removing pairs of basic amino acids (Lys-Arg or Arg-Arg) from the C-terminus of peptides generated as intermediates in the processing of locustamyotropins. To test our hypothesis, we synthesized two peptides FSPRLGKR and FSPRLGRR, both of which contain the core pentapeptide sequence for locustamyotropin I and are extended at the C-terminus by Gly and two basic residues. Recombinant *Drosophila* ACE at pH 6.4 cleaved the dipeptides Lys-Arg and Arg-Arg from the C-terminus of FSPRLGKR and FSPRLGRR respectively to yield FSPRLG (Figure 2). The *Drosophila* enzyme displayed high catalytic-centre activity ($k_{cat} > 200 s^{-1}$) and high affinity ($K_m < 10 \mu$ M) for these two substrates (Table 1). Our hypothesis that ACE takes part in the biosynthesis of locustamyotropin-like peptides predicts that the peptide FSPRLG, generated by removal of Lys-Arg and Arg-Arg, should be a less favoured substrate for ACE thus allowing the Gly-extended peptide to serve as a substrate for the next step in the biosynthetic pathway, namely α -amidation. FSPRLG is indeed a relatively poor substrate for *Drosophila*

ACE with a k_{cat}/K_m value of $0.67 \text{ s}^{-1} \cdot \mu\text{M}^{-1}$ for the conversion into FSPR, which is around 50 times lower than the k_{cat}/K_m for the cleavage of the paired basic residues from FSPRLGKR and FSPRLGRR (Table 1). Insect ACE was also active towards FSPRLGKR and FSPRLGRR at a pH of 5.6, with the rate of hydrolysis being around 60% of the rate of hydrolysis observed at pH 6.4. Insect ACE may be activated by Cl^- [12], but with respect to the hydrolysis of FSPRLGKR and FSPRLGRR by recombinant insect ACE, Cl^- (0–100 mM) had little effect.

DISCUSSION

We have provided evidence for the co-localization of ACE with locustamyotropin-like peptides in three groups of neurosecretory cells in the central nervous system of *L. migratoria*. The intracellular localization of ACE in these cells is quite different from the situation in the human and rat brain, where ACE is found associated with the plasma membrane [37,38]. Insect ACE is a soluble protein with a secretory signal peptide at the N-terminus. Thus when COS-7 cells were transfected with the *Drosophila* ACE cDNA, ACE protein entered the secretory pathway and over 90% of the enzyme was secreted into the medium as a soluble protein [11]. It is therefore possible that ACE in the neurosecretory cells of the locust brain is destined for the regulated secretory pathway and co-accumulates with locustamyotropins in secretory vesicles.

The locustamyotropin antibody used in this study recognizes epitopes of the C-terminal pentapeptide (Phe-Xaa-Pro-Arg-Leu-NH₂) and stains clusters of cells in the suboesophageal ganglia of four other insect species (*Periplaneta americana*, *Leucophaea maderae*, *Mamestra brassica* and *Neobellieria bullata*) [39]. These same cells also stain with an antibody raised to *H. zea* PBAN, a structurally related peptide that ends with the Phe-Ser-Pro-Arg-Leu-NH₂ C-terminal sequence. Insect peptides possessing the Phe-Xaa-Pro-Arg-Leu-NH₂ sequence are found in a wide range of species from several insect orders and they display diverse biological activities including stimulation of muscle contraction, pheromone biosynthesis, diapause induction and cuticular melanization [20,21,23]. ACE is also co-localized with locustamyotropin/leucopyrokinin/PBAN/diapause hormone peptides in the suboesophageal ganglion of *B. mori* and *Mamestra brassica* (L. Schoofs, D. Veelaert, A. De Loof and R. E. Isaac, unpublished work). Thus the relationship between ACE and the locustamyotropin/leucopyrokinin/PBAN/diapause hormone family of peptides appears to have been conserved in insects from orders that are phylogenetically distant, suggesting a conserved functional link between the enzyme and this family of neuro-peptides.

The present study has established that insect ACE is very efficient at removing Lys-Arg and Arg-Arg from locustamyotropin-like peptides extended at the C-terminus with either Gly-Lys-Arg or Gly-Arg-Arg, suggesting a possible function for ACE in the final stages of prohormone processing. This new activity of insect ACE is 17 and 50 times more efficient than the conversion of angiotensin I into angiotensin II by the N- and C-domains of human somatic ACE respectively (Table 1). The high k_{cat}/K_m values obtained for the hydrolysis of FSPRLGKR and FSPRLGRR matches the k_{cat}/K_m for the hydrolysis by mammalian ACE of bradykinin, the substrate most efficiently cleaved by the mammalian enzyme. These kinetic constants were determined at the slightly acidic pH of 6.4 and it was also shown that insect ACE was active at pH 5.6. Thus it appears that ACE would be capable of processing peptides in the weakly acidic environment of the mature secretory vesicle. Both the N- and C-

domains of mammalian somatic ACE are activated by Cl^- , but each domain responds differently to changes in Cl^- , and the extent of activation by Cl^- is substrate- and pH-dependent [36,40–42]. Housefly ACE is also activated by Cl^- when Bz-Gly-His-Leu and luteinizing hormone-releasing hormone are used as substrates [12]. In contrast, the rate of hydrolysis of basic dipeptides from the extended locustamyotropins did not increase in the presence of NaCl over a concentration range of 0–100 mM.

We have recently shown that human germinal ACE and the C-domain of human somatic ACE can hydrolyse pairs of basic residues from the C-terminus of Gly-Trp-Met-Asp-Phe-Gly-Arg-Arg, a peptide corresponding to residues 67–74 of preprogastrin and residues 79–86 of preprocholecystokinin and matching the C-terminal sequence of a human cholecystokinin- and gastrin-processing intermediate [43]. We also showed that human ACE C-domain displays a general hydrolytic activity towards a number of different peptide substrates with a common Lys/Arg-Arg C-terminal sequence and that these peptides are more efficiently hydrolysed than the physiological substrate, angiotensin I. Clearly the ability of ACE to process peptides by cleavage of basic dipeptides has been conserved during the course of evolution and this activity may have formed the basis for the evolutionarily ancient function of ACE.

Diapause hormone, PBAN and three other members of the locustamyotropin/leucopyrokinin/PBAN/diapause hormone family of *B. mori* have a common precursor with three dibasic sites (Lys-Arg or Arg-Arg) and three monobasic Arg sites as potential recognition sites for endoproteolysis by prohormone convertases [25]. The same basic sites are also present in the gene for PBAN of *H. zea* [26]. Evidence has recently been obtained by surgical removal of clusters of PBAN/diapause hormone-immunoreactive cells from the suboesophageal ganglion of *B. mori* pupae that the diapause-inducing activity resides in a posterior group of cells and that PBAN activity is differentially localized to a cluster of medial cells [44]. There is also developmental regulation of the post-translational processing of the PBAN/diapause hormone prohormone of *Bombyx*, since diapause hormone is present in pupae but very little is present in adult insects, the stage at which pheromonotropic activity is required [44,45]. ACE is a peptidyl-dipeptidase and does not display carboxypeptidase activity and therefore will only correctly process those intermediate peptides that end with pairs of basic amino acids. We must assume therefore that a carboxypeptidase E-like activity is necessary for the processing of intermediates with a single Arg at the C-terminus. It is possible that differential expression of ACE and carboxypeptidase E might provide a mechanism by which cells expressing the PBAN/diapause hormone gene can generate different mixtures of peptides from a single precursor molecule.

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REFERENCES

- 1 Soffer, R. L. (1976) Annu. Rev. Biochem. **45**, 73–94
- 2 Ehlers, M. R. W. and Riordan, J. F. (1989) Biochemistry **28**, 5311–5318
- 3 Erdos, E. G. (1990) Hypertension **16**, 363–370
- 4 Corvol, P., Michaud, A., Soubrier, F. and Williams, T. A. (1995) J. Hypertens. **13**, S3–S10
- 5 Wei, L., Alhencgas, F., Corvol, P. and Clauser, E. (1991) J. Biol. Chem. **266**, 9002–9008

- 6 Azizi, M., Rousseau, A., Ezan, E., Guyene, T.-T., Michelet, S., Grognet, J.-M., Lenfant, M., Corvol, P. and Menard, J. (1996) *J. Clin. Invest.* **97**, 839–844
- 7 Rousseau, A., Michaud, A., Chauvet, M.-T., Lenfant, M. and Corvol, P. (1995) *J. Biol. Chem.* **270**, 3656–3661
- 8 Hooper, N. M. (1991) *Int. J. Biochem.* **23**, 641–647
- 9 Sibony, M., Gasc, J. M., Soubrier, F., Alhencgela, F. and Corvol, P. (1993) *Hypertension* **21**, 827–835
- 10 Soubrier, F., Hubert, C., Testut, P., Nadaud, S., Alhencgela, F. and Corvol, P. (1993) *J. Hypertens.* **11**, 471–476
- 11 Cornell, M. J., Williams, T. A., Lamango, N. S., Coates, D., Corvol, P., Soubrier, F., Hoheisel, J., Lehrach, H. and Isaac, R. E. (1995) *J. Biol. Chem.* **270**, 13613–13619
- 12 Lamango, N. S., Sajid, M. and Isaac, R. E. (1996) *Biochem. J.* **314**, 639–646
- 13 Wijffels, G., Fitzgerald, C., Gough, J., Riding, G., Elvin, C., Kemp, D. and Willadsen, P. (1996) *Eur. J. Biochem.* **237**, 414–423
- 14 Cornell, M. J., Lamango, N. S. and Coates, D. (1993) *Biochem. Soc. Trans.* **21**, 243S
- 15 Tatei, K., Cai, H., Ip, Y. T. and Levine, M. (1995) *Mech. Dev.* **51**, 157–168
- 16 Lamango, N. S. and Isaac, R. E. (1994) *Biochem. J.* **299**, 651–657
- 17 Williams, T. A., Michaud, A., Houard, X., Chauvet, M. T., Soubrier, F. and Corvol, P. (1996) *Biochem. J.* **318**, 125–131
- 18 Lamango, N. S., Nachman, R. J., Hayes, T. K., Strey, A. and Isaac, R. E. (1997) *Peptides* **18**, 47–52
- 19 Schoofs, L., Veelaert, D., Broeck, J. V. and De Loof, A. (1997) *Peptides* **18**, 145–156
- 20 Nagasawa, H. (1992) *Experientia* **48**, 425–430
- 21 Nachman, R. J., Holman, G. M., Schoofs, L. and Yamashita, O. (1993) *Peptides* **14**, 1043–1048
- 22 Saito, H., Takeuchi, Y., Takeda, R., Hayashi, Y., Watanabe, K., Shin, M., Imai, K., Isobe, M. and Yamashita, O. (1994) *Peptides* **15**, 1173–1178
- 23 Teal, P. E. A., Abernathy, R. L., Nachman, R. J., Fang, N. B., Meredith, J. A. and Tumlinson, J. H. (1996) *Peptides* **17**, 337–344
- 24 Schoofs, L., Tips, A., Holman, G. M., Nachman, R. J. and De Loof, A. (1992) *Regul. Peptides* **37**, 237–254
- 25 Kawano, T., Kataoka, H., Nagasawa, H., Isogai, A. and Suzuki, A. (1992) *Biochem. Biophys. Res. Commun.* **189**, 221–226
- 26 Ma, P. W. K., Knipple, D. C. and Roelofs, W. L. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 6506–6510
- 27 Rouille, Y., Duguay, S. J., Lund, K., Furuta, M., Gong, Q., Lipkind, G., Oliva, A. A., Chan, S. J. and Steiner, D. F. (1995) *Front. Neuroendocrinol.* **16**, 322–361
- 28 Bendena, W. G., Donly, B. C., Fuse, M., Lee, E. H., Lange, A. B., Orchard, I. and Tobe, S. S. (1997) *Peptides* **18**, 157–163
- 29 Ding, Q., Donly, B. C., Tobe, S. S. and Bendena, W. G. (1995) *Eur. J. Biochem.* **234**, 737–746
- 30 Vandenbroeck, J., Veelaert, D., Bendena, W. G., Tobe, S. S. and Deloof, A. (1996) *Mol. Cell. Endocrinol.* **122**, 191–198
- 31 Veenstra, J. A., Pattillo, J. M. and Petzel, D. H. (1997) *J. Biol. Chem.* **272**, 10402–10407
- 32 Jansons, I. S., Cusson, M., McNeil, J. N., Tobe, S. S. and Bendena, W. G. (1996) *Insect Biochem. Mol. Biol.* **26**, 767–773
- 33 Bradbury, A. F. and Smyth, D. G. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 231–249, CRC Press, Boca Raton, FL
- 34 Fricker, L. D. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 199–229, CRC Press, Boca Raton, FL
- 35 Wei, L., Alhencgela, F., Soubrier, F., Michaud, A., Corvol, P. and Clauser, E. (1991) *J. Biol. Chem.* **266**, 5540–5546
- 36 Jaspard, E., Wei, L. and Alhenc-Gelas, F. D. (1993) *J. Biol. Chem.* **268**, 9496–9503
- 37 Pickel, V. M., Chan, J. and Ganten, D. (1986) *J. Neurosci.* **6**, 2457–2469
- 38 Defendini, R. A., Zimmerman, E. A., Weare, J. A., Alhenc-Gelas, F. and Erdos, E. G. (1983) *Neuroendocrinology* **37**, 32–40
- 39 Tips, A., Schoofs, L., Paemen, L., Ma, M., Blackburn, M., Raina, A. and De Loof, A. (1993) *Comp. Biochem. Physiol.* **106A**, 195–207
- 40 Bunning, P. and Riordan, J. F. (1983) *Biochemistry* **22**, 110–116
- 41 Bunning, P., Holmquist, B. and Riordan, J. F. (1983) *Biochemistry* **22**, 103–110
- 42 Shapiro, R., Holmquist, B. and Riordan, J. F. (1983) *Biochemistry* **22**, 3850–3857
- 43 Isaac, R. E., Williams, T. A., Sajid, M., Corvol, P. and Coates, D. (1997) *Biochem. J.* **328**, 587–591
- 44 Ichikawa, T., Shiota, T., Shimizu, I. and Kataoka, H. (1996) *Zool. Sci.* **13**, 21–25
- 45 Sonobe, H., Hiyama, Y. and Keino, H. (1997) *J. Insect Physiol.* **23**, 633–637