Neuropeptide regulation of biosynthesis of the juvenoid, methyl farnesoate, in the edible crab, *Cancer pagurus*

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The neuropeptide mandibular organ (MO)-inhibiting hormone (MO-IH), synthesized and secreted from the X-organ-sinus-gland complex of the eyestalk, regulates the biosynthesis of the putative crustacean juvenile hormone, methyl farnesoate (MF). Using radiolabelled acetate as a precursor for isoprenoid biosynthesis, farnesoic acid (FA), farnesol, farnesal, MF and geranyl geraniol were detected in MOs cultured for 24 h. Treatment of MOs with extract of sinus gland inhibited the final step of biosynthesis of MF, catalysed by FA *O*-methyltransferase. Additionally, treatment of MOs with purified MO-IH exhibited a dose-dependent inhibition of this final step of MF synthesis. The extent of this inhibition was dependent on the ovary stage of the MO-donor animal, being maximal in MOs from animals in the early stages of ovarian development. Assay of FA *O*-methyltransferase activity, using [^{3}H]FA in the presence of *S*-adenosyl-L-methionine, demonstrated that the enzyme was located in the cytosolic fraction of MOs and was inhibited by incubation of MOs with MO-IH prior to preparation of subcellular fractions. For cytosolic preparations taken from vitellogenic animals, both V_{max} and K_m were appreciably lower than for those taken from non-vitellogenic animals. Conversely, eyestalk ablation of early-vitellogenic animals, which removes the source of MO-IH *in vivo*, resulted in enhancement of the cytosolic FA *O*-methyltransferase activity. Although both V_{max} and K_m show an appreciable increase upon eyestalk ablation, the increased enzyme activity is probably reflected by the fact that V_{max}/K_m (an approximate indication of k_{cat}) has increased 5-fold. The combined evidence demonstrates that MO-IH inhibits FA *O*-methyltransferase, the enzyme which catalyses the final step of MF biosynthesis in MOs.

INTRODUCTION

Methyl farnesoate (MF) is a sesquiterpenoid secretion of the paired mandibular organs (MOs) of crustaceans [1,2]. Structurally, MF is similar to insect juvenile hormone (JH), JH-III, except that it lacks the C10-11 epoxide group of JH-III. Established physiological roles for JHs in insects include the regulation of metamorphosis, male and female reproductive development and secretion of ecdysteroids (moulting hormones) from prothoracic glands (a site of synthesis of ecdysteroids) [3]. By analogy to the functions of JHs in insects, and the correlation of MF titres in the crustacean haemolymph with ovarian development, it has been suggested that MF has an important role in the regulation of crustacean reproduction [4].

Increases in haemolymph MF titre have been positively correlated with ovarian development in the spider crab, *Libinia emarginata*, and the freshwater prawn, *Macrobrachium rosenbergii* [5,6]. Recently, it has been demonstrated that the MF titre in the haemolymph of the edible crab, *Cancer pagurus*, is maximal at the beginning of secondary vitellogenesis (i.e. when the ovaries begin to accumulate yolk proteins in oocytes) [7]. Furthermore, treatment *in vitro* of shrimp oocytes with physiological doses of MF caused an increase in oocyte diameter [8], an effect reminiscent of JH-stimulated patency of ovaries of insects for uptake of yolk proteins [9]. Early experiments injecting homogenates of MOs, or implanting MOs, into crustaceans demonstrated moult-acceleratory effects [10,11]. More recently, it has been demonstrated that incubation of Y-organs (a site of synthesis of ecdysteroids) with MF, or in the presence of MOs, causes an increase in secretion of ecdysteroids from Y-organs [12]. Thus, the foregoing evidence supports the hypothesis that MF is a key regulatory factor involved in growth and reproductive development in crustaceans.

With respect to the control of MF synthesis in *Cancer pagurus*, it has been demonstrated that a 78 amino acid neuropeptide, named MO-inhibiting hormone (MO-IH), a member of the crustacean hyperglycaemic hormone (CHH)/moult-inhibiting hormone (MIH)/vitellogenesis-inhibiting hormone (VIH) family of crustacean neuropeptides [13], negatively regulates synthesis of MF in MOs [14]. Two isoforms of MO-IH were isolated from *C. pagurus*, named MO-IH-1 and -2, which differed by one amino acid [14]. The present study was carried out to characterize the point(s) in the biosynthetic pathway of MF in MOs at which the inhibitory effect of MO-IH is exerted. Knowledge of the manner in which MO-IH regulates MF biosynthesis will enhance our understanding of what is clearly a complex system of interplay between three structurally distinct groups of hormones (ecdysteroids, neuropeptides and the isoprenoid, MF), which regulate the physiologically important events of growth and reproduction in crustaceans.

EXPERIMENTAL

Animals

Female edible crabs, *C. pagurus*, were obtained locally from commercial fishermen and stored in a recirculating sea water system under ambient light and temperature conditions prior to use. Generally, crabs were bilaterally eyestalk-ablated 24 h prior to dissection of MOs, unless otherwise stated.

Purification and quantification of MO-IH

MO-IH-1 and -2 were extracted and purified by a two-step HPLC system as described previously [13]. MO-IH-1 and -2 were

Abbreviations used: MF, methyl farnesoate; FA, farnesoic acid; MO, mandibular organ; JH, juvenile hormone; MO-IH, mandibular organ-inhibiting hormone; CHH, crustacean hyperglycaemic hormone; TBAS, tetra-*n*-butyl ammonium hydrogen sulphate.

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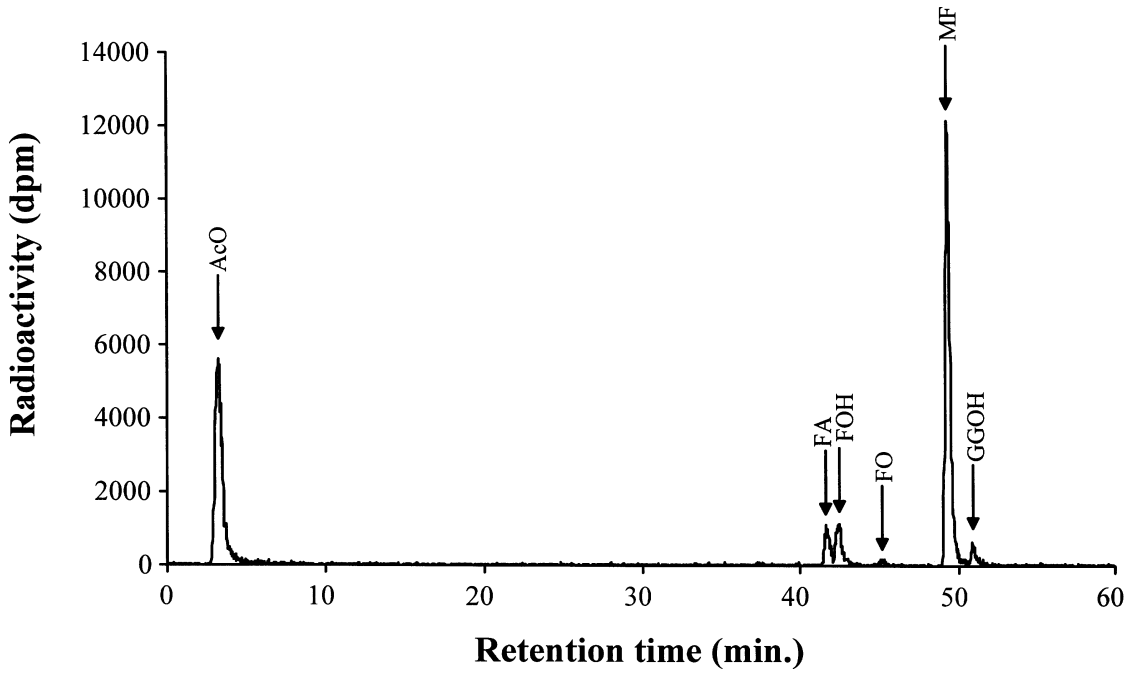

Figure 1 Reversed-phase HPLC analysis of metabolites of sodium [^{3}H]acetate extracted from MOs

Individual MOs were cultured in the presence of sodium [^{3}H]acetate (74 KBq/mmol) for 24 h as described previously [14]. Following incubation, mandibular organs were homogenized, radioactive products extracted into CH_3CN and analysed by reversed-phase HPLC (solvent system 1) with online radioactivity detection. The positions of elution of authentic compounds are given as: acetate, AcO; farnesoic acid, FA; farnesol, FOH; farnesal, FO; methyl farnesoate, MF; geranyl geraniol, GGOH.

quantified by amino acid analysis [14]. For dose-response experiments, the more abundant MO-IH-1 peptide was used.

MO assay

Culture of MOs was essentially carried out as described previously [14] using sodium [^{3}H]acetate (74 kBq/200 μl of culture medium/MO; 0.07–0.19 TBq/mmol; New England Nuclear) as a metabolic precursor for MF synthesis. MOs were cultured individually in microtitre plates and the numbers used for individual experiments are given in the relevant Tables and Figures. Following incubation, MOs were frozen rapidly in liquid nitrogen prior to extraction and analysis. MOs were homogenized in 700 μl of CH_3CN/4% $NaCl_{(aq)}$ (5:2, v/v), centrifuged (1000 ***g***, 10 min, 4 °C) and the CH_3CN layer retained. The homogenate was extracted a further two times with 500 μl of CH_3CN, the total CH_3CN extract combined and a portion analysed for radioactively labelled compounds by HPLC with an online radioactivity monitor (A500; Flo-one/Beta, Canberra Packard Ltd.). Chromatographic conditions were: Novapak C_{18} (Waters) 100 × 8 mm column; solvent A, 0.1% trifluoroacetic acid in water, solvent B, 0.1% trifluoroacetic acid in CH_3CN; linear gradient of 10–100% solvent B in solvent A over 45 min at 1 ml/min (HPLC solvent system 1) monitoring absorbance at 214 nm. Retention times were: acetate, 3.27 min; farnesoic acid (FA), 41.33 min; farnesol, 42.67 min; MF, 49.05 min; geranyl geraniol, 51.00 min. The identity of the labelled products was confirmed by co-chromatography with authentic compounds on the same HPLC column, eluting with a linear gradient of 4 mM tetra-*n*-butyl ammonium hydrogen sulphate (TBAS) ion-pair reagent in water to 1 mM TBAS in 99.5% CH_3OH in water over 30 min at a flow rate of 1 ml/min (HPLC solvent system 2). For this, a stock solution of 200 mM TBAS in water (pH 6.0) was prepared as described [15]. Retention times were: acetate, 4.10 min; FA, 37.47 min; farnesol, 40.27 min; MF, 41.50 min; geranyl geraniol, 50.51 min.

Preparation of subcellular fractions of MOs

Groups of 10 MOs were homogenized using a Potter-Elvehjem homogenizer in 600 μl of ice-cold Hepes buffer (0.037 M, containing 0.3 M sucrose, 0.01 M KF), pH 7.5 [16]. The homogenate was centrifuged (1100 ***g***, 5 min, 4 °C) and the supernatant re-centrifuged (12000 ***g***, 10 min, 4 °C). The resulting supernatant was removed, the pellet washed in the same buffer and re-centrifuged (12000 ***g***, 10 min, 4 °C) to obtain the mitochondrial fraction, and the original supernatant was centrifuged (150000 ***g***, 90 min, 4 °C) to obtain a microsomal pellet and cytosolic supernatant. Cytosol was dialysed against hypotonic Hepes (0.037 M, containing 0.01 M KF) prior to assay of FA methyltransferase activity. Each particulate fraction was resuspended in 200 μl of hypotonic Hepes buffer (0.037 M, containing 0.01 M KF), pH 7.5 [16] and the protein content estimated by the method of Bradford [17].

Synthesis of FA

FA was produced by alkaline hydrolysis of a known quantity of *all-trans*-MF (Dr. M. F. Feldlaufer, United States Department of Agriculture, Beltsville, U.S.A.) or [12-^{3}H]*all-trans*-MF (Professor G. D. Prestwich, Stony Brook, New York, U.S.A.) according to a published method [18], and purified as described previously [7], the purity being assessed by HPLC as described above.

O-Methyltransferase assay

Freshly prepared subcellular fractions from 10 MOs (nuclei/unbroken cells, mitochondria, microsomes and cytosol) were assayed for FA *O*-methyltransferase after resuspension of the particulate fractions in hypotonic Hepes buffer (0.037 M, containing 0.01 M KF), pH 7.5 [16]. For initial experiments, incubation mixtures contained subcellular fraction (30 μl), 2.4 μM [^{3}H]FA substrate (0.10 GBq/mmol; $>99\%$ purity by HPLC) and 30 μM *S*-adenosyl-L-methionine cofactor, in a final reaction volume of 50 μl, and were incubated for 1 h at 37 °C. Reactions were terminated by addition of 150 μl of CH_3CN followed by centrifugation (10000 ***g***, 10 min). A portion of the supernatant (generally 120 μl) was analysed for conversion of [^{3}H]FA substrate into [^{3}H]MF by HPLC with online radioactivity monitoring as described above.

In order to assess the effect of MO-IH-1 on FA *O*-methyltransferase activity, groups of untreated control and 10 nM MO-IH-1-treated MOs were cultured for 24 h, under conditions described above, prior to preparation of subcellular fractions for assay.

To investigate the time course of FA *O*-methyltransferase activity, incubation mixtures (50 μl), in hypotonic 0.037 M Hepes buffer (pH 7.5) containing 0.01 M KF, consisted of: cytosol (10 μl; approx. 20 μg of protein; 0.2 MO equivalents), 20 μM [^{3}H]FA substrate (0.85 GBq/mmol) and 150 μM *S*-adenosyl-L-methionine. Reactions were carried out at 37 °C for various times, terminated and the samples analysed as described above.

To determine the biochemical basis of the apparent increase in activity of FA *O*-methyltransferase in MOs from eyestalk-ablated crabs compared with that from eyestalk-intact crabs, the effect of increasing FA concentration on FA *O*-methyltransferase activity (K_m, V_{max}) was investigated. Incubations were set up as described above, except that the final concentration of FA was varied between 1 and 700 μM ([^{3}H]FA varied between 50000 and 100000 d.p.m./incubation). Samples were incubated at 37 °C for 3 min and reactions terminated by the addition of 150 μl of CH_3CN. Samples were analysed by reversed-phase HPLC with online radioactivity detection as described above.

RESULTS

Identity of isoprenoid products

Analysis of the isoprenoid-derived products of the metabolism of [^{3}H]acetate by MOs cultured *in vitro*, was readily achieved using reversed-phase HPLC. A typical chromatogram (solvent system 1) is shown in Figure 1. Using this system, major peaks of radioactivity co-chromatographed with authentic acetate, FA, farnesol, MF and geranyl geraniol, and their identities were corroborated by co-chromatography using HPLC solvent system 2. In some samples, small quantities of farnesal were also detected.

Time course of isoprenoid biosynthesis

The time course of incorporation of [^{3}H]acetate into FA, farnesol and MF was investigated to determine the optimum incubation period for subsequent experiments, and whether or not pairs of MOs from individual animals synthesized equivalent amounts of these farnesoid compounds. The results are given in Table 1 and show that up to 12 h, FA synthesis increases, whereas the synthesis of MF and farnesol remains low. Incubation periods greater than 12 h show a rapid accumulation of MF and increased farnesol synthesis, whereas FA synthesis decreases at 18 h, gradually increasing thereafter. Throughout the time course of incubation, left and right MOs synthesized similar quantities

Table 1 Incorporation of acetate into farnesoids

Pairs of MO (left, L, and right, R) were incubated for 2–24 h at 12 °C in culture medium supplemented with 74 kBq sodium [^{3}H]acetate. Radiolabelled products were extracted and analysed as detailed in the text. Crabs were bilaterally eyestalk ablated 24 h prior to experiments. Values are means (d.p.m. $\times 10^{-3}$) $\pm$ S.E.M.

	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Incubation time (h) ...	2		4		5		6		12		18		24			
Number of replicates	5		5		5		5		4		5		9			
FA	6.97 $\pm$ 2.51	6.30 $\pm$ 1.63	6.98 $\pm$ 3.50	5.56 $\pm$ 2.99	23.36 $\pm$ 10.03	18.37 $\pm$ 9.69	13.73 $\pm$ 4.79	17.50 $\pm$ 6.28	32.85 $\pm$ 5.28	36.90 $\pm$ 9.69	6.97 $\pm$ 1.78	7.43 $\pm$ 1.33	18.89 $\pm$ 4.22	18.87 $\pm$ 3.32		
Farnesol	1.40 $\pm$ 0.72	0.24 $\pm$ 0.24	0.85 $\pm$ 0.62	0.28 $\pm$ 0.28	0.87 $\pm$ 0.72	3.90 $\pm$ 1.74	1.87 $\pm$ 0.30	3.39 $\pm$ 1.36	3.10 $\pm$ 0.60	3.30 $\pm$ 0.74	3.27 $\pm$ 0.96	2.89 $\pm$ 0.51	12.62 $\pm$ 2.81	12.01 $\pm$ 3.03		
MF	–	–	–	–	0.45 $\pm$ 0.45	0.18 $\pm$ 0.13	0.52 $\pm$ 0.33	0.20 $\pm$ 0.20	3.29 $\pm$ 2.34	0.61 $\pm$ 0.37	83.88 $\pm$ 20.53	96.94 $\pm$ 22.19	171.22 $\pm$ 31.17	144.85 $\pm$ 30.60		

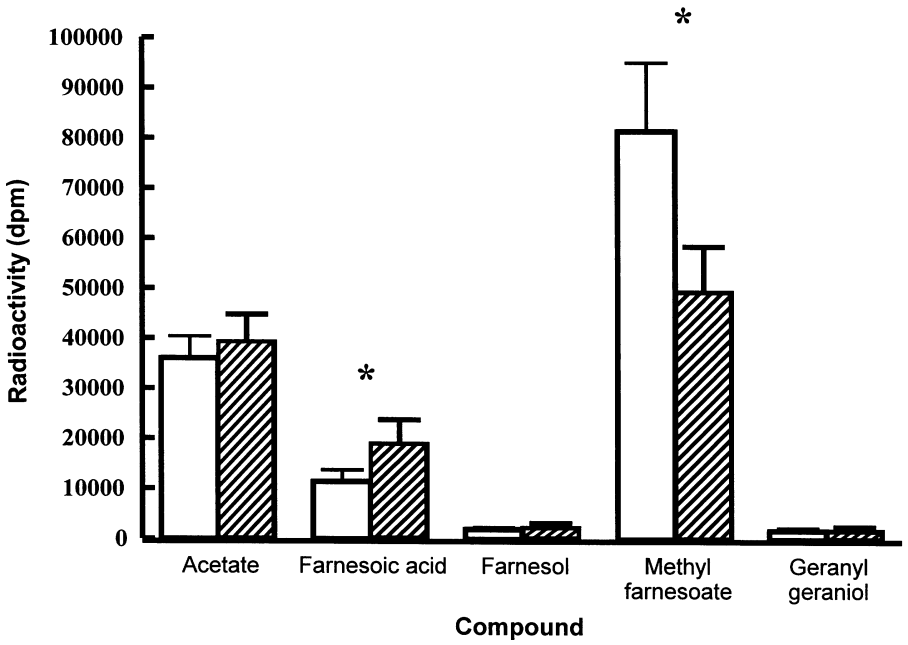

Figure 2 Comparison of the effects of sinus gland extract on the metabolism of sodium [^{3}H]acetate in MOs

Groups of control (untreated) and treated (extract equivalent to 0.25 sinus gland) MOs were cultured individually in the presence of sodium [^{3}H]acetate. Following culture, MOs were homogenized, radiolabelled products extracted into CH_3CN and analysed by reversed-phase HPLC with online radioactivity detection. Comparison of incorporation of [^{3}H]acetate into components of the isoprenoid pathway in treated (hatched bars) and untreated control (open bars) MOs is given. $n = 5$ pairs of MOs for each column; bars = S.E.M., * denotes statistically significant difference between treated and untreated control column values ($P < 0.05$, unpaired t test).

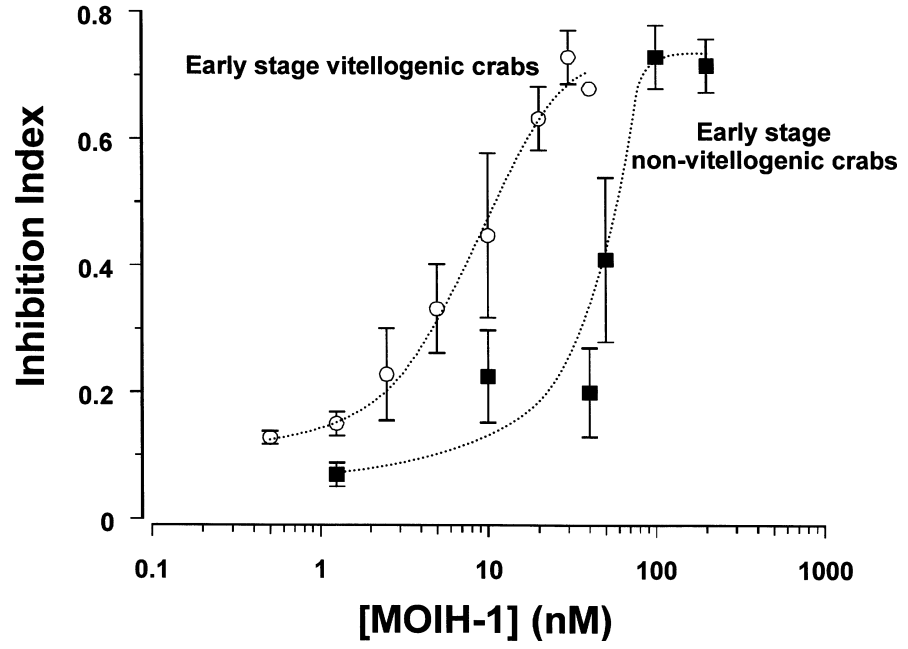

Figure 3 Dose-response relationship of inhibition of the final step of MF synthesis in MOs exposed to MO-IH-1

Groups of control (untreated) and MO-IH-1-treated MOs from non-vitellogenic crabs (■) or ones in early secondary vitellogenesis (○), were cultured individually in the presence of sodium [^{3}H]acetate. Following culture, MOs were homogenized, radiolabelled products extracted into CH_3CN and analysed by reversed-phase HPLC with online radioactivity detection. For definition of inhibition index, see the Results section. $n = 5$–18 pairs of MOs for each column; bars = S.E.M. Lines of best fit were calculated using a curve-fitting program (Fig. P, Elsevier Biosoft).

Table 2 Subcellular localization of FA *O*-methyltransferase activity and effects of eyestalk ablation and 10 nM MO-IH-1

For the ablation experiment, subcellular fractions were prepared from freshly dissected MOs. For the 10 nM MO-IH-1 treatment experiment, groups of untreated control and peptide-treated MOs were cultured separately for 24 h prior to preparation of subcellular fractions. Values are means ± S.E.M., nd = not detected.

	FA *O*-methyltransferase activity for MOs (fmol of MF formed/min)			
	Effect of ablation ($n = 4$)		Effect of 10 nM MO-IH-1 ($n = 3$)	
Subcellular fraction	Eyestalk-intact animals	Eyestalk-ablated animals	Control	Treated
Nuclei/cell debris	1.15 ± 0.02	11.7 ± 3.30	nd	nd
Mitochondria	0.18 ± 0.18	0.88 ± 0.18	nd	nd
Microsomes	0.98 ± 0.03	1.75 ± 0.75	nd	nd
Cytosol	35.0 ± 1.30	57.8 ± 3.70	10.6 ± 1.10	4.95 ± 0.34

of FA and MF, exhibiting statistically significant pairing correlation coefficients (0.75–0.98). However, comparison of the synthesis of farnesol in left and right MOs did not exhibit statistically significant pairing correlation coefficients at any of the time points. In subsequent experiments, incubations were for 24 h.

Neuropeptide regulation of isoprenoid biosynthesis

To determine the point in the biosynthetic pathway of MF at which the inhibitory effect of MO-IH is exerted, pairs of MOs (left and right) were incubated, with one gland being used for MO-IH treatment and the other as an untreated control gland. Initial experiments utilized crude extracts of sinus gland as a source of MO-IH. Sinus gland extract incubated with MOs, resulted in statistically significant suppression of incorporation of [^{3}H]acetate into MF and accumulation of FA, compared with untreated control MOs (Figure 2). Additionally, a slight, but not statistically significant, apparent increase in the level of farnesol in sinus gland extract-treated MOs compared with untreated control MOs was measured ($P > 0.05$, $n = 5$; unpaired t test). The levels of radiolabelled acetate substrate and geranyl geraniol within control and sinus gland extract-treated MOs did not vary significantly ($P = 0.45$, $r^2 = 0.67$, $n = 5$; paired t test). To allow for the possible variation in synthetic rates of pairs of MOs from different animals, the inhibitory response was normalized to take into account the ratio of MF to FA in each sample analysed, and expressed as an inhibition index, which was calculated as 1−[(MF/FA) in treated MOs]/[(MF/FA) in control MOs]. Further experiments clearly showed that sinus gland extract inhibited the final step of MF biosynthesis (i.e. inhibition of FA *O*-methyltransferase catalysing the methylation of the carboxylic acid group of FA to produce MF) in a dose-dependent manner (results not shown).

To further characterize this inhibitory response, MOs from crabs of two different vitellogenic stages of ovary development (early-stage vitellogenic and early-stage non-vitellogenic animals) were used and cultured in the presence of various concentrations of MO-IH-1. The results are given in Figure 3 and clearly demonstrate that MOs from animals of early vitellogenic stages are much more sensitive to the inhibitory effects of MO-IH-1 than are MOs from non-vitellogenic animals.

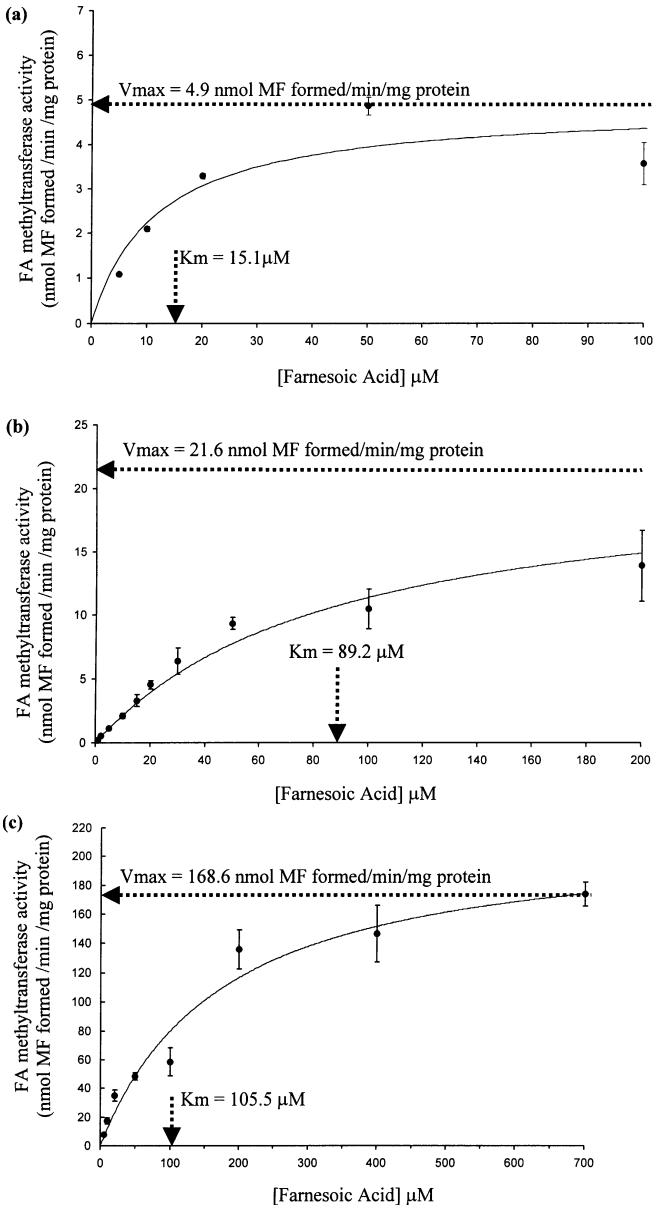

Figure 4 Kinetic analysis of cytosolic FA methyltransferase activity

FA methyltransferase activity was measured in batches of MOs from (**a**) eyestalk-intact early-vitellogenic animals, (**b**) eyestalk-intact non-vitellogenic animals, and (**c**) eyestalk-ablated animals. For enzyme assays, incubation mixtures (50 μl), in hypotonic 0.037 M Hepes buffer, pH 7.5, containing 0.01 M KF, consisted of cytosol (10 μl; approx. 20 μg of protein; 0.2 MO equivalents), 1–700 μM FA substrate (containing 50 000–100 000 d.p.m. [^{3}H]FA; 0.25 GBq/mmol) and 150 μM *S*-adenosyl-L-methionine. Reactions were carried out at 37 °C for 3 min, terminated by addition of CH_3CN and radiolabelled products analysed by reversed-phase HPLC with online radioactivity detection. Hyperbolic regression curve fitting was carried out using Hyper version 1.1s (Dr. J. S. Easterby, University of Liverpool, Liverpool, U.K.). Values are the mean of triplicate determinations from batches of pooled MOs; bars = S.E.M.

In view of a recent report that in two different species of crustacean, the spider crab, *L. emarginata*, and a crayfish, *Procambarus clarkii*, the CHH was responsible for the inhibition of MF synthesis in cultured MOs [19], MOs of *C. pagurus* were incubated in the presence of 100 nM *C. pagurus* CHH. Additionally, MOs of the shore crab, *Carcinus maenas*, were incubated in the presence of 50 nM *C. maenas* CHH to assess the

possible inhibitory effects of this peptide on MF synthesis. The results clearly demonstrate that 100 nM CHH has no significant inhibitory effect on MF synthesis in *C. pagurus* MOs *in vitro*. However, 50 nM *C. maenas* CHH was sufficient to profoundly inhibit MF synthesis in *C. maenas* MOs ($94 \pm 12\,\%$ inhibition; $n = 8$ pairs of MOs).

Subcellular localization of FA *O*-methyltransferase activity

To determine the subcellular localization of the enzyme catalysing the conversion of FA to MF, batches of MOs were homogenized and subcellular fractions prepared by centrifugation. The results given in Table 2 clearly demonstrate the cytosolic localization of the FA *O*-methyltransferase activity. Typical specific activities of the enzyme in the cytosol were in the region of 1.5–2.0 pmol of MF formed/min/mg of protein using cofactor and substrate concentrations used previously in the analysis of JH acid methyltransferase activity in JH biosynthesis in the tobacco hornworm, *Manduca sexta* [20]. Furthermore, the results also show that this activity is enhanced in MOs from animals that had been bilaterally eyestalk-ablated 24 h prior to MO dissection.

Determination of K_m and V_{max} of FA *O*-methyltransferase

Initial experiments demonstrated that, over a 90 min period, the rate of FA *O*-methyltransferase activity exhibited hyperbolic kinetics, being linear up to 5 min (results not shown). Additionally, using a 3 min incubation period, FA *O*-methyltransferase activity was linear over a protein concentration range of 50–500 μg/ml (results not shown). For subsequent kinetic analyses of FA *O*-methyltransferase activity, incubation times were 3 min and the protein concentration was in the range 160–220 μg/ml.

To determine whether the difference in FA *O*-methyltransferase activity following eyestalk removal could be correlated with changes in K_m and/or V_{max}, a series of incubations of dialysed MO cytosol with varying FA concentration were carried out. The results (Figure 4) show clearly that both V_{max} and K_m for FA were increased in samples from animals that had been bilaterally eyestalk-ablated 24 h prior to dissection of MOs and preparation of cytosol for FA *O*-methyltransferase assays, as compared with eyestalk-intact animals.

DISCUSSION

The combined results demonstrate clearly that the final step in the biosynthesis of MF in MOs is regulated by the action of the sinus gland-derived neuropeptide MO-IH-1. Initially, investigation of the isoprenoid products of culture *in vitro* of MOs in the presence of radiolabelled acetate demonstrated that only MF, FA, farnesol and geranyl geraniol were accumulated to an appreciable extent (Figure 1). Occasionally, a small peak which co-chromatographed with farnesal was also detected. Furthermore, the time course of incorporation of [^{3}H]acetate into isoprenoids in MOs showed poor incorporation of radiolabel into MF up to 12 h (Table 1). This may reflect the time taken for sufficient quantities of radiolabelled acetate to uniformly label the endogenous pools of acetate and FA. An endogenous pool of FA in crayfish MOs has previously been postulated, based on metabolic labelling experiments [21].

Comparison of incorporation of radiolabelled acetate into MF, FA and farnesol in left and right MOs from the same animals (Table 1) showed highly significant pairing correlation coefficients (0.75–0.98) for the incorporation into MF and FA in left and right MOs. However, pairwise comparison of the incorporation of the radiolabel into farnesol did not show significant pairing. This could be attributed to the fact that FA and MF are products of the isoprenoid biosynthesis in MOs that are unique to the farnesoid shunt branch of isoprenoid biosynthesis. Farnesol is an isoprenoid that will have other roles aside from being a metabolic precursor of FA and MF biosynthesis; thus it is conceivable that farnesol will be utilized to different extents in two separate MOs. The observed accumulation of FA in MOs suggested that the enzyme catalysing the methylation of FA to produce MF may be the rate-limiting step of MF biosynthesis.

The point in the biosynthetic pathway of MF at which MO-IH exerts control was investigated by analysing changes in the profile of radiolabelled compounds extracted from groups of MOs treated with MO-IH, compared with those compounds in untreated control MO incubations. Initial experiments, utilizing crude acetic acid extracts of sinus glands (containing MO-IH), demonstrated a dose-dependent inhibitory effect of sinus gland extract on incorporation of radiolabelled acetate into MF with a significant accumulation of FA (Figure 2). Apparent corresponding increases in the level of farnesol were also detected, but to a lesser extent. This evidence, strongly suggesting that the final step of MF biosynthesis, catalysed by FA *O*-methyltransferase, was regulated by MO-IH, was confirmed by treating MOs with purified MO-IH-1 (Figure 3). The results demonstrate the dose-dependent inhibition of the final step of MF biosynthesis by MO-IH-1. Furthermore, this inhibitory effect of MO-IH-1 on the methylation of FA to produce MF is entirely dependent upon the ovary stage of the MO donor animal. Animals not undergoing vitellogenesis appear to be relatively insensitive ($ED_{50} = 50$ nM MO-IH-1) to the inhibitory effects of MO-IH-1, whereas vitellogenic animals respond well ($ED_{50} = 7$ nM MO-IH-1). These results are consistent with our previous findings on the effect of sinus gland-extract treatment on MF synthesis in MOs from donor animals of different vitellogenic stages [14]. This enhanced sensitivity of FA *O*-methyltransferase activity to inhibition by MO-IH-1 in early ovarian development maybe explained by phenomena such as up-regulation of peptide receptor expression and/or sensitivity towards MO-IH, or modulation of signal-transduction pathways within MO cells. This early period of ovarian development, when MOs are more sensitive to the inhibitory effects of MO-IH-1 on MF synthesis, coincides with a peak in haemolymph MF titre in female *C. pagurus* [7], suggesting that circulating MO-IH concentration is low at this stage of ovarian development. The crabs used during the course of these experiments were bilaterally eyestalk-ablated 24 h prior to MO dissection and culturing. Such a period post-ablation would be presumed to be sufficient to significantly deplete circulating levels of MO-IH and increase the rate of biosynthesis of MF in MOs (see Table 2 and [14]). However, this period of time post-ablation does not appear to be sufficient to cause up-regulation of MO-IH receptors, as an ovary stage-dependent response is apparent.

It is apparent that MO-IH is, as yet, unique to *C. pagurus* and that CHH has no significant effect in our MF synthesis assay (see the Results section). However, an indirect involvement of CHH in MO-IH-mediated inhibition of MF synthesis has not been precluded, as it is clear for *C. maenas*, *P. clarkii* and *L. emarginata* that inhibition of MF synthesis in MOs appears to be effected by a CHH peptide [19]. This difference in the type of peptide used to regulate MF biosynthesis in MOs of crustaceans (an MO-IH or a CHH) may highlight a possible evolutionary divergence amongst crustaceans, with respect to control of MF biosynthesis in MOs.

In order to determine the subcellular localization of the *S*-adenosyl-L-methionine-FA *O*-methyltransferase, subcellular fractions of MOs were prepared by centrifugation and assayed

for FA *O*-methyltransferase activity. Of the fractions tested (nuclei/unbroken cells, mitochondria, microsomes and cytosol), only the cytosolic fraction possessed FA *O*-methyltransferase activity (Table 2), although some activity was observed in the nuclei/cell debris fraction, presumably owing to unbroken cells. Various *O*-methyltransferases occur in different subcellular compartments [22], but cytosolic localization of FA *O*-methyltransferase is consistent with the finding that JH acid *O*-methyltransferase activity in insects is located in the cytosol [23]. This enzyme activity was enhanced in MOs from animals that had been bilaterally eyestalk-ablated (thereby removing the source of MO-IH *in vivo*) 24 h prior to MO dissection and preparation of cytosol, compared with eyestalk-intact animals. The eyestalk-ablated animals exhibited an approx. 1.7-fold increase of cytosolic FA *O*-methyltransferase activity. A similar observation using crude sinus gland extracts has been recently reported for the analogous methyltransferase from MOs of the lobster, *Homarus americanus* [24]. Furthermore, comparison of the activity of FA *O*-methyltransferase in cytosol from batches of MOs from eyestalk-intact animals in early vitellogenesis (Figure 4a), and from eyestalk-intact non-vitellogenic animals (Figure 4b), clearly demonstrates a fundamental difference between two natural physiological states. That is, the K_m of FA *O*-methyltransferase for FA is much lower in MOs from early-vitellogenic animals, suggesting that the affinity of FA *O*-methyltransferase for FA is greater. Early-vitellogenic animals contain the highest measured levels of MF in the haemolymph of *C. pagurus* during ovarian development. Since no information is available on the amount of FA *O*-methyltransferase or the pool size of FA in MOs from animals at different stages of ovarian development, it is impossible to predict the true physiological significance of these results. Using the ratio of V_{max} to K_m as a approximate value for k_{cat} (turnover number), it is apparent that for eyestalk-intact non-vitellogenic and early-vitellogenic animals there is no discernible difference. This may suggest that levels of FA *O*-methyltransferase are varying throughout ovarian development. Also, if the previous results are compared with those for eyestalk-ablated animals (Figure 4c), it is clear that a marked increase in both V_{max} and K_m are observed. If the ratio of V_{max} to K_m is compared with the previous results, an approximate 5-fold increase is observed. These dramatic increases in V_{max} and K_m following ablation are consistent with measured increases in the rate of synthesis of MF in MOs *in vitro* [14]. In the current work, FA *O*-methyltransferase activity in the cytosol was inhibited by pre-incubation of MOs in the presence of 10 nM MO-IH-1 for 24 h prior to preparation of cytosol fractions for enzyme assay (Table 2). It is also apparent from the results that FA *O*-methyltransferase activity is significantly reduced on culturing MOs *in vitro* prior to preparation of subcellular fractions for FA methyltransferase activity assay. The foregoing evidence strongly suggests that, in crustaceans, cytosolic FA *O*-methyltransferase is a key site of regulation of the MF biosynthetic pathway in MOs by the neuropeptide MO-IH. Conversely, in cockroach corpora allata (a site of JH bio synthesis) it has been suggested that neither the cytosolic JH acid *O*-methyltransferase nor HMG-CoA reductase and HMG-CoA synthase [25] are key regulatory enzymes in JH-III biosynthesis [26]. Indeed, in that case, evidence has been obtained that transport of acetate from mitochondria into the cytoplasm of corpora allata is the point at which the inhibitory neuropeptide, allatostatin-1, exerts control of JH-III synthesis in cockroaches. The mechanism of down-regulation of the FA *O*-methyltransferase mediated by MO-IH remains to be elucidated.

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