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LIPASE ACTIVITY SENSITIVE TO DOPAMINE, GLUCAGON AND CYCLIC AMP IN THE HEPATOPANCREAS OF THE EURYHALINE BURROWING CRAB *NEOHELICE GRANULATA* (DANA, 1851) (DECAPODA, GRAPSIDAE)

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ABSTRACT

We studied the biochemical characteristics and modulation by dopamine, glucagon and cAMP of lipase activity in hepatopancreas of the euryhaline crab *Neohelice granulata* (Dana, 1851), considered to be an emergent model for biochemical, physiological and ecological research. Lipase activity was maximum at pH 8.5; it exhibited Michaelis-Menten kinetics (apparent K_m = 0.018 mM), was highest at 37°C but appeared to be cold- and heat-tolerant, since it was high at 4°C and at 45°C. Lipase activity was enhanced upon incubation of hepatopancreas with 10^{-4} M dopamine (about 100%), 2×10^{-3} M glucagon (about 250%) and 10^{-4} M cAMP (about 150%) suggesting a role of these chemical messengers in mechanisms of regulation of lipolytic activities and its direct effect on the hepatopancreas. The concomitant decrease in triglycerides content upon dopamine and cAMP treatment suggests a link between enhanced lipase activity by these messengers and triglycerides catabolism.

RESUMEN

El objetivo de este trabajo fue estudiar características bioquímicas y la modulación por dopamina, glucagón y AMPc in vitro de la actividad de lipasa en hepatopáncreas del cangrejo eurihalino *Neohelice granulata* (Dana, 1851) el cual es considerado un modelo emergente para la realización de estudios sobre bioquímica, fisiología y ecología. La actividad de lipasa fue máxima a pH 8.5, exhibió una cinética de Michaelis-Menten (K_m = 0.018 mM), el valor más alto de actividad fue registrado a 37°C y es aparentemente tolerante al frio y al calor ya que mantuvo alta actividad a 4°C y 45°C. La actividad de lipasa en hepatopáncreas se incrementó en presencia de dopamina 10^{-4} M (100%), glucagón 2×10^{-3} M (250%) y AMPc 10^{-4} M (150%). La concomitante disminución

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en el contenido de triglicéridos del hepatopáncreas en presencia de dopamina y AMPc sugiere la existencia de una relación entre el incremento de la actividad de lipasa y la posible movilización de reservas sugiriendo el rol de estos mensajeros químicos como componente de las vías de señalización involucrados en la regulación del metabolismo de lípidos.

INTRODUCTION

The hepatopancreas of decapod crustaceans is a multifunction organ playing a key role in digestion and absorption (Ceccaldi, 1989; Verri et al., 2001; Muhlia-Almazán & Garcia-Carreño, 2003; Zeng et al., 2010). It is the main site of triglyceride storage in most species (Allen et al., 2000; Muldford & Villena, 2000; García et al., 2002; Sánchez-Paz et al., 2006; Dima et al., 2009; Latyshev et al., 2009). Thus, level of lipase activity in the hepatopancreas and its potential modulation would play a central role in digestion, absorption and utilization of triglycerides. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a group of enzymes which naturally catalyse the hydrolysis of the ester bond of tri-, di-, and monoglycerides into fatty acids and glycerol (Casas-Godoy et al., 2012). Lipid storage is an evolutionary conserved process that exists in all organisms, from simple prokaryotes to humans (Birsoy et al., 2013). Lipases have a central physiological importance in all animals due to their role in the digestion of lipids into fatty acids for absorption and in the hydrolysis of triglycerides storages. However, information about the occurrence, biochemical characteristics and modulation of lipase activity in groups of ecological importance, such as euryhaline intertidal crabs, is lacking. To our knowledge, our recent work on *Cyrtograpsus angulatus* Dana, 1851 was the first to study biochemical characteristics of lipase activity in the hepatopancreas of a euryhaline intertidal crab and its modulation by dopamine (DA) injections, suggesting the role of this biogenic amine as primary chemical messenger involved in mechanisms of regulation of digestive and metabolic processes (Michiels et al., 2013).

DA is an important neurotransmitter and neurohormone in crustaceans (Clark et al., 2008; Christie, 2011) which plays various physiological roles, such as regulation of osmoregulatory mechanisms and of metabolic responses to different environmental conditions (Cheng et al., 2005; Lorenzon, 2005; Chiu et al., 2006; Genovese et al., 2006; Hsieh et al., 2006; Yeh et al., 2006; Chang et al., 2007; Liu et al., 2008; Avramov et al., 2013). Various chemical messengers, such as biogenic amines, insulin, glucagon, vasoactive peptide and gastrin, have been detected in the hepatopancreas, which appears also to be an important endocrine organ (Huang et al., 2005). In mammals, various primary chemical messengers such as catecholamines and peptide hormones (e.g., glucagon) and intracellular messengers (such as cAMP) are involved in the modulation of lipase activities (Birsoy et al., 2013; Bartness et al., 2014; Dashty, 2014; Geerling et al., 2014).

Neohelice granulata (Dana, 1851) is a euryhaline burrowing crab which is distributed in the intertidal areas of the southwestern Atlantic from southern Brazil to northern Argentinean Patagonia (Boschi, 1964; Botto & Irigoyen, 1979; Spivak et al., 1994; Iribarne et al., 1997, 2003; Spivak, 1997; Bortolus & Iribarne, 1999; Méndez-Casariego et al., 2011; Luppi et al., 2013). *N. granulata* is considered as an emergent animal model for biochemical, physiological and ecological research (Spivak, 2010). Works in our lab show the in vitro effect of dopamine on N-aminopeptidase activity in the hepatopancreas (data not shown) and of glucagon and cAMP on glucose release from the hepatopancreas (unpublished results) suggesting their role as chemical messengers involved in the modulation of digestive and metabolic function at the biochemical level. In this context, the aim of this work was to study the biochemical characteristics and modulation by dopamine, glucagon and cAMP of lipase activity in the hepatopancreas of *N. granulata*.

MATERIAL AND METHODS

Chemicals

pNPpalmitate (p-nitrophenylpalmitate), Tris-(hydroxymethylamino-methane) (Tris), ethyleneglicol N,N',N'-tetraacetic acid (EGTA), bovine serum albumin and dopamine (3-hydroxytyramine) cyclic AMP (cAMP) and glucagon were from Sigma (St. Louis, MO, U.S.A.); sucrose and trichloroacetic acid (TCA) were from Merck (Darmstadt, Germany); magnesium sulphate and Coomasie blue G250 were from Fluka (Seelze, Germany). All chemicals were of analytical grade. All solutions were prepared in glass-distilled water.

Animal collection and maintenance

The crabs were caught from the mudflat area of the Mar Chiquita coastal lagoon (Buenos Aires Province Argentina) (37°32′-37°45′S 57°19′-57°26′W). For all experiments salinity was measured in practical salinity units (psu). Only adult male crabs with a carapace width greater than 2.5 cm and in intermolt were collected. Captures were made at midday. Animals were transported to the laboratory in lagoon water on the day of collection. The crabs were maintained in natural seawater (35 psu) for at least 10 days prior to use, at the salinity at which *N. granulata* from the mudflat of Mar Chiquita coastal lagoon osmoconforms (Pinoni & López Mañanes, 2009; Asaro et al., 2011). The aquaria contained 36 litres of water, continuously aerated and filtered. A regime of 12 h light/12 h dark was applied and the temperature was kept at 22 ± 2°C. The water was continuously

filtered by means of an Atman filter (HF-0400). Aquaria were shielded by black plastic to reduce disturbance. Crabs were fed three times a week with commercial food (Wardley Cichlind T.E.N., Hartz, Secaucus, NJ, U.S.A.) (48% carbohydrates, 40% protein, 3% fat, 4% fibre) (about 0.07 g/individual), but they were starved 24-48 h prior to the experiments. No differences in the feeding behaviour occurred in the experimental conditions used.

Preparation of hepatopancreas enzyme extract

The crabs were cryoanesthesized by putting them on ice for about 20 min. The hepatopancreas was immediately excised, mixed with homogenizing medium (50 mM Tris/HCl pH 7.4; 4 ml per g hepatopancreas tissue) and homogenized (CAT homogenizer X120, tool T10) on ice. The homogenate was centrifuged at 10 000 $\times$ *g* for 15 min (Sorval, rotor SS34, refrigerated). The hepatopancreas from one individual was used for each preparation of enzyme extract. The supernatant was fractionated into 500-μl aliquots and stored at -20°C until use.

Biochemical assays

Lipase activity was determined by measuring p-nitrophenylpalmitate (pNPP) hydrolysis (Markweg et al., 1995) with some modifications (Michiels et al., 2013). The reaction was initiated by the addition of pNPP (final concentration 0.7 mM) to a reaction mixture containing a suitable aliquot of the corresponding sample (linearity zone on activity vs protein concentration plot) in 50 mM Tris-HCl buffer (pH 8.5)/4 μl Tween-80. Incubation was carried out at 37°C for 5 min. The reaction was stopped by addition of 500 μl of 0.2% TCA (w/v). The amount of p-nitrophenol (pNP) released was determined by reading the absorbance at 410 nm (Metrolab 330). Samples were incubated as described above but at varying pH (5.4-10.0) (50 mM phosphate buffer, pH 5.4-6.4; 50 mM Tris-HCl buffer, pH 7.2-8.5; 50 mM glycine buffer, pH 10.0), temperature (4-45°C) and pNPP concentrations (0.09-0.9 mM) of the reaction mixture for determining the effect of pH, temperature and pNPP concentration on lipase activity, respectively. The determination of enzyme activity was always performed with samples which had been stored at -20°C, without any previous thawing.

Protein was assayed according to the method of Bradford (1976), with bovine serum albumin as the standard.

Triglycerides (TG) were measured by the glycerol phosphate oxidase colorimetric method, using a commercial kit (TAG Wiener-Lab AA code 861110001). The sample was incubated with this reactant for 5 min at 37°C (Pinoni et al., 2011). The amount of glycerol released was determined by reading the absorbance of the coloured quinone complex at 505 nm.

Effect of dopamine (DA), cAMP and glucagon on lipase activity and triglyceride content in the hepatopancreas

The in vitro effect of DA, cAMP and glucagon was determined as described previously (López Mañanes, 2004). Sections of hepatopancreas were incubated in the absence or in the presence of 10^{-4} M DA, 10^{-4} M cAMP or 2×10^{-3} M glucagon in a medium (2 ml per g tissue) containing (in mM): 400 NaCl, 13 KCl, 10 $MgCl_2$ 8.8 H_3BO_3, pH 7.6 at 30°C. At 0 and after 30 (dopamine and cAMP) or 60 min (glucagon) of incubation, the medium was separated and tissue was homogenized in buffer (50 mM Tris-HCl, pH 7.4; 4 ml per g tissue). Lipase activity in the tissue was determined as described above. Lipase activity was also tested in the medium (indicator of released enzyme activity) (Resch-Sedlmeier & Sedlmeier, 1999; Lwalaba et al., 2010). Lipase activity was not detected in the medium throughout the experimental period, neither in the absence, nor in the presence of the agents tested. Triglycerides concentration was simultaneously determined in tissue extracts. Lipase activity or triglycerides concentration were not affected by incubation conditions used in the absence of agents throughout the experimental period.

Statistical analysis

The results of the effect of varying concentrations of pNPP on lipase activity were analysed by means of non-linear regression analysis (GraphPad Prism 4.0 software). The corresponding curves shown are those which best fit the experimental data. The K_m value (Michaelis-Menten constant) was estimated by analysis of data using a Lineweaver-Burk plot (GraphPad Prism 4.0 software). Statistical analyses were performed using the Sigma-Stat 3.0 statistical package for Windows operating system, which automatically performs a previous test for equal variance and normality. A *t*-test or repeated measures ANOVA were used to estimate the statistical significance of the differences and $P < 0.05$ was considered significant. An a posteriori Holm-Sidak test was used to identify differences.

RESULTS

Lipase activity in the hepatopancreas: effect of pH, substrate and temperature

Maximal activity was found at pH 8.5. The activity at pH 5.4-7.2 was about 50-70% of the activity at pH 8.5. At pH 10.0, lipase activity decreased markedly to about 27% of the activity at pH 8.5 (fig. 1a). The effect of pNPP concentration on lipase activity is shown in fig. 1b. Lipase activity in the hepatopancreas of *Neohelice granulata* exhibited Michaelis-Menten kinetics (apparent K_m = 0.018 mM). Fig. 1c shows the effect of temperature (4-45°C) on lipase activity.

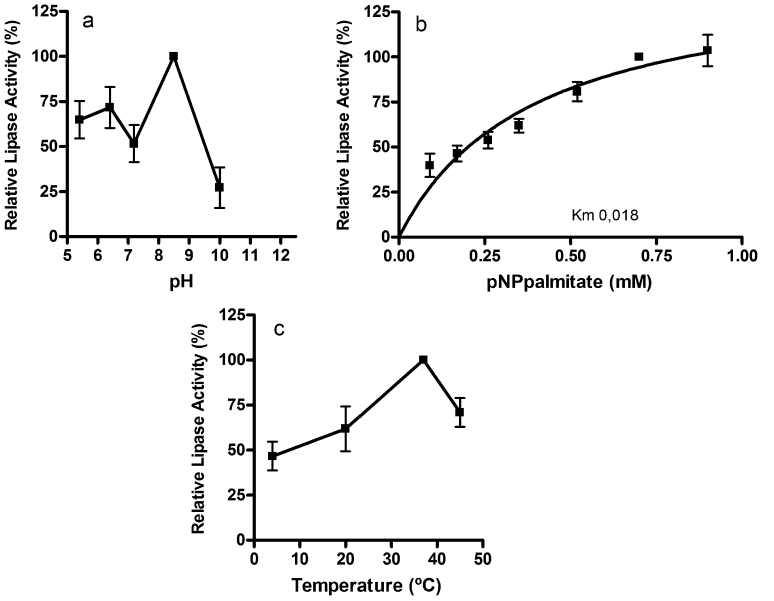

Fig. 1. Effect of pH, p-nitrophenylpalmitate (pNPP) concentration and temperature on lipase activity in hepatopancreas of *Neohelice granulata* (Dana, 1851). a, Effect of pH on lipase activity (relative to activity at pH 8.5) at 37°C in the presence of 0.7 mM pNPP; data are the mean ± SE for 5 individuals; b, effect of pNPP concentration on lipase activity (relative to the activity in the presence of 0.7 mM pNPP) at 37°C and pH 8.5; data are the mean ± SE for 5 individuals; c, effect of temperature on lipase activity (relative to the activity at 37°C) at pH 8.5 in the presence of 0.7 mM pNPP; data are mean ± SE for 3 individuals.

Lipase activity increased upon enhancement of temperature from 4 to 37°C. Maximal activity was found at 37°C. At 4°C and 20°C the activity was about 50 and 60%, respectively, of the activity at 37°C. At 45°C lipase activity was about 70% of the activity at 37°C.

In vitro effect of dopamine, glucagon and cAMP on lipase activity and triglycerides concentration in the hepatopancreas

10^{-4} M DA; 2×10^{-3} M glucagon and 10^{-4} M cAMP increased lipase activity in the hepatopancreas by about 100, 250 and 150%, respectively (fig. 2).

Triglyceride concentration in the hepatopancreas decreased upon incubation with 10^{-4} M DA and 10^{-4} M cAMP by about 55 and 70%, respectively. Glucagon did not affect triglyceride concentration (fig. 3).

DISCUSSION

In this work, we studied biochemical characteristics of lipase activity in the hepatopancreas of the euryhaline crab *Neohelice granulata* and its possible modulation

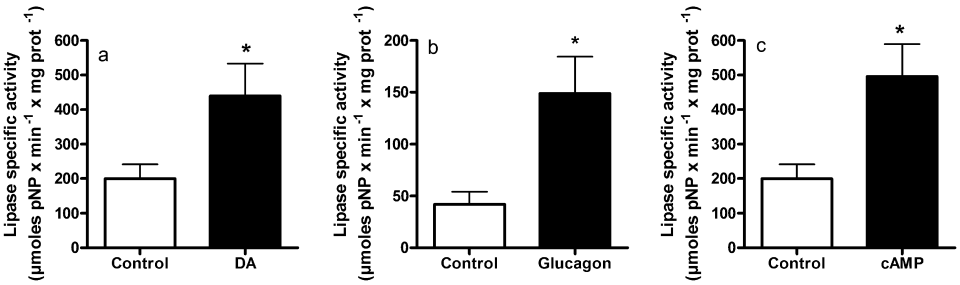

Fig. 2. In vitro effect of: a, dopamine; b, glucagon; and, c, cAMP; on lipase activity in the hepatopancreas of *Neohelice granulata* (Dana, 1851). Sections of the hepatopancreas were incubated with the corresponding agent and lipase activity was measured after incubation in tissue extracts in the presence of 0.7 mM pNPP at pH 8.5 and 37°C as described in Material and methods. Data are mean ± SE for 5 individuals. * Significantly different from the corresponding control ($P<0.05$).

in vitro by dopamine, glucagon and cAMP. The pH value for maximal lipase activity and the maintenance of activity (about 50-70% of maximal activity) throughout a wide range of pH (fig. 1a) is in agreement with that we previously found for lipase activity in hepatopancreas of *Cyrtograpsus angulatus* (cf. Michiels et al., 2013) and for the activity of the marine green crab *Carcinus mediterraneus* Czerniavsky, 1884 [now as: *Carcinus aestuarii* Nardo, 1847] (Cherif et al., 2007; Cherif & Gargouri, 2009; Smichi et al., 2012) and in hepatopancreas of *Cherax albidus* Sokol, 1988 (Coccia et al., 2011). The Michaelis-Menten kinetics of lipase activity of the hepatopancreas of *N. granulata* (fig. 1b) are in agreement with those found in *C. angulatus* (cf. Michiels et al., 2013) and with those reported in the crayfish *Procambarus clarkii* (Girard, 1852) (Hammer et al., 2003) and the prawn *Macrobrachium borellii* (Nobili, 1896) (Pasquevich et al., 2011). The high lipase activity at 37°C in the hepatopancreas of *N. granulata* (fig. 1c) is quite different from that described in *C. mediterraneus* (cf. Cherif & Gargouri, 2009; Smichi et al., 2012) but similar to that found in *C. angulatus* (cf. Michiels et al., 2013) and in

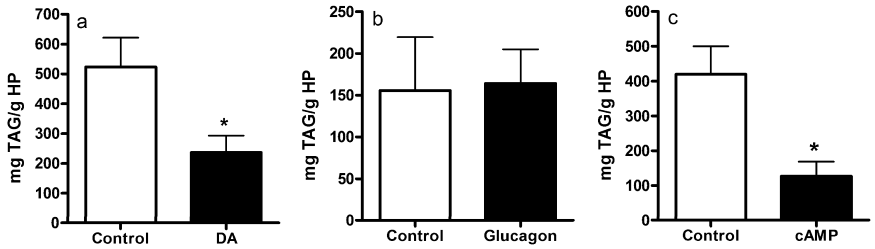

Fig. 3. In vitro effect of: a, dopamine; b, glucagon; and, c, cAMP; on triglyceride (TAG) concentration in the hepatopancreas (HP) of *Neohelice granulata* (Dana, 1851). Sections of the hepatopancreas were incubated with the corresponding agent and TAG concentration was measured after incubation in tissue extracts as described in Material and methods. Data are mean ± SE for 5 individuals. * Significantly different from the corresponding control ($P<0.05$).

C. albidus (cf. Coccia et al., 2011). Similar to the activity in *C. angulatus*, lipase activity in the hepatopancreas of *N. granulata* appeared to be high at low (4°C) and high (45°C) temperature (about 50 and 70% of maximal activity, respectively) (fig. 1c). This crab is commonly exposed to a wide range of temperatures (Luppi et al., 2013; personal observations), but whether lipase activity in the hepatopancreas is involved in biochemical acclimatisation to low and high temperatures requires further investigation. In the copepod *Calanus glacialis* Jaschnov, 1955 lipase activity in the hepatopancreas was related to a role in thermal acclimation (i.e., a higher digestion of lipids) (Freese et al., 2012).

The crustacean hepatopancreas is a multifunctional organ, and is the major site of nutrient absorption from digestive products (Wright & Ahearn., 1997; Verri et al., 2001; Muhlia-Almazán & García-Carreño, 2003). Lipids ingested are stored mainly as triglycerides (over 80-90%), with the hepatopancreas being the main site of storage in most species (Allen et al., 2000; Muldford & Villena, 2000; García et al., 2002; Sánchez-Paz et al., 2006; Dima et al., 2009; Latyshev et al., 2009). It has been proposed that when the internal reserves of a cell (i.e., triglycerides) must be mobilized, digestive enzymes (i.e., lipases) that are secreted during the digestive cycle could be activated intracellularly and finely regulated (Sanchez-Paz et al., 2006). However, the mechanisms involved (i.e., chemical messengers) are still to be established. Catecholamines, peptide hormones and intracellular messengers (such as cAMP) are important activators of lipases and lipolysis process in mammals (Birsoy et al., 2013; Bartness et al., 2014; Dashty, 2014; Geerling et al., 2014). In the digestive tract of various decapod crustaceans, endocrine epithelial cells have been reported as potential source of circulating peptides, some of which originally have been identified as belonging to the nervous system, while others are specific to the digestive system (Christie, 2011; McCoole et al., 2012; Nagur-Babu et al., 2012; Christie et al., 2013). DA, which is involved in the regulation of several functions and metabolic responses in decapod crustaceans (Nagur-Babu et al., 2012; Pan et al., 2014), has been also detected in the hepatopancreas (Huang et al., 2005). We have shown that injection of 10^{-4} M DA increases haemolymph glucose levels, inhibits alkaline phosphatase activity in chela muscle and increases lipase activity in the hepatopancreas of *C. angulatus*, suggesting to be a primary chemical messenger involved in the regulation of metabolic processes at the biochemical level (López Mañanes, 2004; Pinoni & López Mañanes, 2004; del Valle et al., 2012; Michiels et al., 2013). In *N. granulata*, DA appears to be a key primary chemical messenger having a role in regulatory pathways involved in the control of adaptive responses to salinity (Halperin et al., 2004; Genovese et al., 2006). The enhancement of lipase activity in vitro by 10^{-4} M DA (fig. 2) along with the results of our lab showing the in vitro stimulation of aminopeptidase-N activity (a peptidase with a key role in final steps of protein digestion) by DA

and the release of glucose from the hepatopancreas (unpublished results) suggests the role of DA as primary chemical messenger to modulate key components of digestive and metabolic process in this crab. DA is a pleiotropic compound that acts as a neurotransmitter and a hormone. The physiological actions of dopamine are known to be mediated by distinct but closely related membrane-bound G-protein-coupled receptors (Beaulieu & Gainetdinov, 2011). When coupled to Gαs proteins, D_1-like receptors can activate cAMP pathways. Enhanced cAMP levels activate hormone-sensitive lipase in mammals (Dashty, 2014), but to our knowledge, nothing is known about the role of dopamine on this activation. In this context, the observed in vitro effects on hepatopancreas of *N. granulata* support the idea of a direct effect of DA on this tissue, likely via activation of membrane DA receptors. *N. granulata* has been suggested to exhibit D_1- and D_2-like DA receptors in posterior gills (Genovese et al., 2006), but to our knowledge nothing is known about the occurrence of dopaminergic receptors in the hepatopancreas. The fact that no release of lipase from the hepatopancreas occurred throughout the experimental period suggests that a modulation of intracellular activity could have occurred, as was suggested for the hepatopancreas of decapod crustaceans (Sánchez Paz et al., 2006). The concomitant decrease in triglycerides content in the tissue upon DA treatment (fig. 3) suggests a link between increased lipase activity and a possible mobilization of these reserves. Since pathways involved in lipids catabolism are unknown in *N. granulata* (and to our knowledge in any other crab), further research is needed to test this hypothesis. Our results support the recently found reducing effects of DA on fat reserves in the nematode *Caenorhabditis elegans* (Maupas, 1900) revealing an ancient role for dopaminergic regulation of fat (Barros et al., 2014). Dopamine induces fat mobilization in some mammals (Thompson, 1984). In mammals, triglycerides catabolism implies the participation of several lipases (adipose triglyceride lipase, hormone sensitive lipase and monoglyceride lipase) (Watt & Spriet, 2010; Birsoy et al., 2013; Bartness et al., 2014; Dashty, 2014; Geerling et al., 2014). Increased cAMP levels lead to translocation and activation of hormone-sensitive lipase resulting in an enhancement of lipolysis (Dashty, 2014). The enhancement in vitro of lipase activity upon incubation with 10^{-4} M cAMP (fig. 2c) and the decrease in triglycerides content (fig. 3c) in the hepatopancreas of *N. granulata* suggests the occurrence of modulation of triglyceride catabolism by cAMP signalling pathways. In insects, in vitro experiments showed that the effect of adipokinetic hormone on lipolysis is mediated in part by cAMP, suggesting a role for cAMP-dependent protein kinase (PKA) in phosphorylation and activation of TAG lipase (Ogoyi et al., 1998).

In various mammals glucagon is known to enhance lipolysis by stimulating intracellular neutral lipase activity (Vaughan et al., 1964; Slavin et al., 1994;

Duncan et al., 2007). The increased lipase activity upon incubation in vitro with 2×10^{-3} M glucagon (fig. 2b) along with the fact that no release of lipase occurred suggests, similarly to that described for DA, a direct effect of glucagon on the hepatopancreas of *N. granulata* and the modulation of intracellular activity. Experimental work of our laboratory shows that glucagon induces in vitro the release of glucose from the hepatopancreas of this crab (unpublished results), further supporting the idea of the direct effect of this hormone. Unexpectedly, glucagon did not affect the triglyceride content in the hepatopancreas (fig. 3). Whether this can be attributed to differential mechanisms of activation (i.e., different lipases involved, differential transduction pathways, differential timing) remains to be investigated. In humans glucagon appears to contribute little to lipolysis in vivo and not to have an effect in adipose tissue in vitro (Bertin et al., 2001; Gravholt et al., 2001). Recently, six candidate genes for lipid digestion were identified in the hepatopancreas of the marine crab *Portunus trituberculatus* (Miers, 1876), including triacylglycerol lipase and pancreatic lipase-related protein 2 (Wang et al., 2014). Two classes of triacylglycerol lipases have been suggested to be present in the hepatopancreas of the prawn *Penaeus vannamei* Boone, 1931 [now as: *Litopenaeus vannamei* (Boone, 1931)], a lipase exclusively expressed in this tissue, suggesting its function as a digestive enzyme, and an intracellular lipase, probably involved in the mobilization of energy reserves (Rivera-Pérez & García-Carreño, 2011; Wang et al., 2014).

In conclusion, the results of this study show the enhancement of lipase activity in the hepatopancreas of *N. granulata* by DA, cAMP and glucagon, suggesting the presence of hormone-sensitive like lipase activity, the modulation of intracellular enzyme activity, possible links between DA and cAMP pathways with lipids metabolism (i.e., triglycerides catabolism) and a direct effect of DA and glucagon on the hepatopancreas.

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