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The process of lipid storage in insect oocytes: The involvement of β-chain of ATP synthase in lipophorin-mediated lipid transfer in the chagas' disease vector *Panstrongylus megistus* (Hemiptera: Reduviidae)

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ABSTRACT

Lipophorin is the main lipoprotein in the hemolymph of insects. During vitellogenesis, lipophorin delivers its hydrophobic cargo to developing oocytes by its binding to non-endocytic receptors at the plasma membrane of the cells. In some species however, lipophorin may also be internalized to some extent, thus maximizing the storage of lipid resources in growing oocytes. The ectopic β chain of ATP synthase (β-ATPase) was recently described as a putative non-endocytic lipophorin receptor in the anterior midgut of the hematophagous insect *Panstrongylus megistus*. In the present work, females of this species at the vitellogenic stage of the reproductive cycle were employed to investigate the role of β-ATPase in the transfer of lipids to the ovarian tissue. Subcellular fractionation and western blot revealed the presence of β-ATPase in the microsomal membranes of the ovarian tissue, suggesting its localization in the plasma membrane. Immunofluorescence assays showed partial co-localization of β-ATPase and lipophorin in the membrane of oocytes as well as in the basal domain of the follicular epithelial cells. Ligand blotting and co-immunoprecipitation approaches confirmed the interaction between lipophorin and β-ATPase. *In vivo* experiments with an anti-β-ATPase antibody injected to block such an interaction demonstrated that the antibody significantly impaired the transfer of fatty acids from lipophorin to the oocyte. However, the endocytic pathway of lipophorin was not affected. On the other hand, partial inhibition of ATP synthase activity did not modify the transfer of lipids from lipophorin to oocytes. When the assays were performed at 4 °C to diminish endocytosis, the results showed that the antibody interfered with lipophorin binding to the oocyte plasma membrane as well as with the transfer of fatty acids from the lipoprotein to the oocyte. The findings strongly support that β-ATPase plays a role as a docking lipophorin receptor at the ovary of *P. megistus*, similarly to its function in the midgut of such a vector. In addition, the role of β-ATPase as a docking receptor seems to be independent of the enzymatic ATP synthase activity.

Abbreviations: β-ATPase, β-chain of the ATP synthase complex; Bodipy FL C16, 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid; Cameo2, C locus associated membrane protein homologous to a mammalian HDL receptor-2; CD36, cluster of differentiation 36; DAG, diacylglycerol; DAGTP, diacylglycerol transport protein; DIC, differential interference contrast; DiI, 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); HDL, high-density lipoprotein; JHBP, juvenile hormone binding protein; LDLR, low-density lipoprotein receptor; Lp, lipophorin; LpR, liphoporin receptor; LPL, lipoprotein lipase; LTP, lipid transfer particle; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SCRB15, scavenger receptor class B member 1 like protein 15.

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1. Introduction

Lipophorin is the main lipid carrier in the hemolymph of insects. Under most physiological conditions, lipophorin is a high-density lipoprotein and contains two structural apolipoproteins, apolipophorin I and II (Beenakkers et al., 1985; Canavoso et al., 2001; Van der Horst et al., 2009). Diacylglycerol is the major lipid transported by lipophorin but depending on the insect species and their developmental stage this lipoprotein can also carry other hydrophobic molecules such as fatty acids, hydrocarbons, sterols and phospholipids (Chino et al., 1969; Canavoso et al., 2001).

Lipophorin interacts with specific binding sites in the plasma membrane of the target cells and lipids are transferred mainly without endocytosis. Once the loading/unloading process is completed, the lipoprotein returns to the circulation for subsequent cycles of lipid mobilization (Chino and Gilbert, 1964; Chino and Downer, 1982; Arrese et al., 2001). In addition to this main pathway for lipid delivery, lipophorin can be either endocytosed by the oocytes as reported in several insect species (Kawooya and Law, 1988; Ziegler and Van Antwerpen, 2006) or endocytosed and resecreted, as in the fat body of *Locusta migratoria* (Van Hoof et al., 2005). The endocytic pathway is mediated by the lipophorin receptor (LpR) belonging to the Low-Density Lipoprotein Receptor (LDLR) family, which was first described in *L. migratoria* (Dantuma et al., 1999). Even though the presence of LpRs was reported in various insects, their physiological relevance for the transfer of neutral lipids to tissues remains elusive (Tufail and Takeda, 2009).

Other lipophorin receptor candidates include *Drosophila melanogaster* Dally and Dally-like, two membrane-associated heparan sulfate proteoglycans with lipophorin-binding capacity, although they appear to be more involved in cell signaling than in lipid transfer (Eugster et al., 2007). Cameo2 and SCRB15, two transmembrane proteins expressed in the silk gland of *Bombyx mori* function as non-endocytic lipophorin receptors that facilitate the selective uptake of carotenoids. However, the role of these CD36 family members in the general transfer of neutral lipids is still unknown (Sakudoh et al., 2013).

The multiprotein F_1F_o-ATP synthase complex was originally thought to be located exclusively in the inner membrane of mitochondria. This complex employs a transmembrane proton motive force to drive the chemical synthesis of ATP from ADP and inorganic phosphate (Walker, 2013). Notwithstanding, several lines of evidence confirmed the presence of ATP synthase in the plasma membrane of different mammalian cell types, mediating a variety of biological processes such as the metabolism of high-density lipoproteins, endothelial cell proliferation and antitumor activity (Vantourout et al., 2010). This surface or ectopic ATP synthase, as it was termed to distinguish it from its mitochondrial counterpart, was also described as a protein which binds different ligands in arthropods (Giot et al., 2003; Zalewska et al., 2009). Very recently, we have proposed that β-chain of the ATP synthase complex (β-ATPase) plays a role as non-endocytic lipophorin receptor in the anterior midgut of the triatomine *Panstrongylus megistus*, an important vector of Chagas' disease (Fruttero et al., 2014).

During oogenesis, lipids must be stored in growing oocytes as they are the main source of energy to sustain the development of the embryos. However, in the context of insect reproductive physiology, the process of lipid transfer to oocytes is still not completely understood (Ziegler and Van Antwerpen, 2006). Previous studies in vitellogenic females of the triatomines *P. megistus* and *Dipetalogaster maxima* allowed us to demonstrate that the storage of lipid resources by developing oocytes involves the convergence of non-endocytic and endocytic pathways of lipophorin to maximize the delivery of its lipid cargo (Fruttero et al., 2011; Leyria et al., 2014).

P. megistus belongs to the subfamily Triatominae, a group of hematophagous insects with relevance in public health as they are vectors of Chagas' disease (Schofield et al., 2006). The female of *P. megistus* takes large blood meals, rich in lipids and proteins. The blood meal triggers vitellogenesis, which in *P. megistus* can be broadly divided into early, mid and late vitellogenesis; the latter coincides with the oviposition period. During vitellogenesis, terminal oocytes remarkably enlarge due to internalization of yolk protein precursors and uptake of circulating lipids via lipophorin-mediated lipid transfer (Fruttero et al., 2011). In this work, we analyzed the role of the β-ATPase in the process of lipophorin-mediated lipid transfer to the ovarian tissue. Immunofluorescence, ligand blotting and co-immunoprecipitation approaches supported both, the binding and interaction between lipophorin and β-ATPase. Different *in vivo* assays directed to block such an interaction revealed the importance of β-ATPase in the process of lipid accumulation by developing oocytes. Finally, it was observed that blocking β-ATPase did not significantly impair lipophorin endocytosis.

2. Materials and methods

2.1. Chemicals

4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (Bodipy FL C16) and goat anti-rabbit IgG labeled with Alexa 568 antibody (Molecular Probes, Eugene, OR, USA), rabbit anti-ATP5B/β-chain of ATP synthase of human origin (catalog code sc-33618) and rabbit anti-cytochrome c of equine origin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); Sephadex G-25 PD-10 columns (GE Healthcare, Little Chalfont, UK); Tissue-Tek embedding medium Optimal Cutting Temperature (OCT) (Miles, Elkhart, IN, USA); Centricon devices (Millipore, Bedford, MA, USA); Enliten bioluminescence detection kit (Promega, Madison, WI, USA); Fluorsave (Calbiochem, Darmstadt, Germany); 3,3′-dithiobis(sulfosuccinimidyl propionate) (DTSSP) (Thermo Scientific, Rockford, IL, USA) and Color Prestained Protein Standard (New England Biolabs Inc, Ipswich, MA, USA) were from the indicated commercial sources. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Bovine Serum Albumin (BSA); Dimethyl sulfoxide (DMSO); dimethylpimelimidate (DMP); 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine (DiI); fluorescein isothiocyanate (FITC); Fetal Bovine Serum (FBS); oligomycin and all the other chemicals were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Insects

Experiments were carried out with insects taken from a colony of *P. megistus*, maintained at 28 °C, 70% relative humidity, 8:16 h light:dark photoperiod. Insects were fed on hen blood (Canavoso and Rubiolo, 1995), according to the recommendations of the National Institute of Parasitology (Health Ministry, Argentina) (Núñez and Segura, 1987). Standardized conditions of insect rearing were previously described (Fruttero et al., 2011). Briefly, fifth-instar females were separated from males before feeding. Newly emerged females were segregated individually and placed together with two recently fed males. Mating was checked by observation of the spermatophore. Mated females were maintained in individual containers until they were able to feed a blood meal (day 7 post-ecdysis). Experimental approaches were performed by sampling hemolymph and ovaries from females at early vitellogenesis, which in *P. megistus* correspond to days 3–4 after blood feeding (Fruttero et al., 2011).

2.3. Hemolymph and ovaries sampling

For lipophorin purification, the hemolymph was collected with a Hamilton syringe from immobilized females. Their legs were sectioned at the coxa and the hemolymph was collected in cold microtubes, in the presence of 10 mM Na_2EDTA, 5 mM dithiothreitol and protease inhibitors: 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 1 mM N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), 1 mM pepstatin A and 0.3 mM aprotinin (Fruttero et al., 2014). Samples were centrifuged at 10,000×*g* for 5 min at 4 °C to remove hemocytes and then stored at −70 °C, after protein determination (Bradford, 1976).

Ovaries from females at days 3–4 post-feeding were dissected out in cold phosphate buffered saline (PBS, 6.6 mM Na_2HPO_4/KH_2PO_4, 150 mM NaCl, pH 7.4), using a standard stereoscope and processed for cryostat sectioning as described in 2.10.

2.4. Lipophorin purification and fluorescent labeling

Lipophorin was isolated from the hemolymph by KBr gradient ultracentrifugation followed by a fractionation on Sepharose 6B as previously reported (Fruttero et al., 2009). For the *in vivo* functional experiments, the fate of the entire lipophorin was followed by labeling its phospholipid moiety with the non-exchangeable fluorophore DiI (Lp-DiI) (Via and Smith, 1986) as described previously (Fruttero et al., 2009). Lipophorin was also labeled on its exchangeable lipid fraction with Bodipy FL C16 (Lp-Bodipy-FA), a fluorescent palmitic acid analog (Martin-Nizard et al., 1987; Atella and Shahabuddin, 2002). In all the cases, fluorescently labeled Lp-DiI and Lp-Bodipy-FA were subjected to gel filtration on PD-10 columns to remove free fluorophores from the labeled ligands.

2.5. Preparation of anti-lipophorin antibody and labeling with FITC

The polyclonal anti-lipophorin antibody (anti-Lp) was obtained by inoculating New Zealand rabbits with purified lipophorin (1 mg/ml) as described previously (Canavoso and Rubiolo, 1995). The γ-globulin fraction was obtained by precipitation with ammonium sulfate.

For the immunofluorescence assays, anti-Lp antibody was labeled with fluorescein isothyocianate (anti-Lp-FITC, 5 mg/ml) in DMSO (Hermanson, 1996; Fruttero et al., 2009).

2.6. Immunodetection of β-ATPase in the ovarian tissue

The microsomal fraction, enriched in ovarian membranes and free of mitochondria, was obtained according to Zalewska et al. (2009) with minor modifications (Fruttero et al., 2014). Briefly, ovaries from five females at early vitellogenesis were homogenized in 20 mM Tris-HCl, 150 mM NaCl and 300 mM sucrose and the homogenate was centrifuged to remove cellular debris (1000×*g*, 10 min, 4 °C). The resulting supernatant was further centrifuged at 15,000×*g* (20 min at 4 °C) to pellet the mitochondrial fraction, and then subjected to an ultracentrifugation step at 100,000×*g* (60 min at 4 °C) to obtain the cytosolic and microsomal fractions. Total homogenate and subcellular fractions were subjected to Tris-Tricine-SDS gel electrophoresis as described elsewhere (Fruttero et al., 2014). The immunodetection of β-ATPase was performed by western blot, using a commercial rabbit anti-β-ATPase antibody (1:1000). Blots were also probed with a commercial rabbit anti-cytochrome c antibody (1:1000) to check for mitochondrial contamination of the microsomal fraction. The secondary antibody, Li-Cor IRDye 800CW polyclonal goat anti-rabbit IgG, was used at a dilution of 1:15,000 in blocking buffer and incubated at room temperature for 1 h. After washing as described, blots were scanned and analyzed with the Odyssey quantitative western blot near-infrared system (Li-Cor Biosciences, Lincoln, NE, USA) using default settings.

2.7. MTT assay

Evaluation of mitochondrial contamination was also assessed using the MTT colorimetric assay. Briefly, subcellular fractions obtained as described above, were incubated with the MTT reagent at a final concentration of 1 mg/ml, for 4 h at 37 °C. Thereafter, the residual formazan was dissolved in DMSO and the absorbance was measured at 570 nm. The microsomal and cytosol fractions were identified as possible sub-cellular sites of NAD(P)H-dependent MTT reduction (Berridge and Tan, 1993; Dhanjaland and Fry, 1997). For this reason, the MTT assay was conducted in the presence or absence of malonic acid, a selective inhibitor of succinate dehydrogenase, an enzyme responsible for MTT reduction in the mitochondria.

2.8. Ligand blotting

These experiments were carried out essentially as described in Fruttero et al. (2014). Briefly, microsomal fractions were fractionated by Tris-Tricine-SDS gel electrophoresis (8.5%), transferred to a nitrocellulose membrane and blocked (10 mM Tris, 150 mM NaCl, Tween 20 0.1%, pH 7.5, containing 5% non-fat milk and 3% BSA 3%). The blots were incubated with lipophorin (50 μg/ml) and probed with primary (anti-Lp 1:2000) and secondary antibodies (Li-Cor IRDye 800CW goat anti-rabbit IgG 1:15,000). The immunoreactions were visualized using the Odyssey quantitative western blot near-infrared system as described in 2.6.

2.9. Co-immunoprecipitation

Anti-BSA, anti-β-ATPase and anti-Lp antibodies (0.2 mg each) were covalently coupled to protein A Mag Sepharose beads (GE Healthcare, Little Chalfont, UK) by incubation for 1 h at room temperature. After washing twice with 50 mM Tris, 150 mM NaCl, pH 7.5 buffer (TBS), the beads were incubated with 200 mM triethanolamine pH 8.9 buffer containing 50 mM DMP for 60 min at room temperature. The beads were washed with 200 mM triethanolamine pH 8.9 buffer and blocked with 100 mM ethanolamine pH 8.9 for 15 min at room temperature. The elution buffer (0.1 M glycine, 2 M urea, pH 2.9) was added to remove the unbound antibody and then beads were washed several times with TBS. On the other hand, ovaries from females at early vitellogenesis were dissected out and then incubated at room temperature with 1.5 mM of the DTSSP crosslinker according to manufacturer's protocol. The reaction was stopped by incubating the organs in 20 mM Tris-HCl pH 8.5 for 15 min. Then, ovaries were processed as described in Section 2.6 and the obtained microsomal fraction (60 μg) was incubated with covalently coupled to protein A Mag Sepharose beads antibodies as indicated: anti-BSA antibody (control), anti-β-ATPase or anti-Lp antibodies (60 min at room temperature, with slow end-over-end mixing). The proteins bound to the beads were eluted with the elution buffer and protein A Mag Sepharose beads were removed using a magnetic rack. The eluted proteins, the input and the standards were subjected to western blot as described in Section 2.6.

2.10. Co-localization of endogenous lipophorin and β-ATPase in the ovarian tissue

Dissected ovaries were fixed in 4% paraformaldehyde in PBS for 50 min at room temperature, transferred to sucrose/PBS, embedded in OCT and snap frozen in liquid nitrogen (Fruttero et al.,

2011). Tissue sections of 12 μm were obtained with a Leica CM1510 cryostat (Leica Microsystems, Wetzlar, Germany) and placed onto poly-L-lysine-treated glass slides. Ovarian sections were incubated with 1% BSA and 5% FBS in PBS to block non-specific binding sites. The slides were sequentially incubated with the anti-β-ATPase (1:100), the anti-rabbit IgG labeled with Alexa 568 (1:400) and the anti-Lp-FITC (1:40) antibodies. Antibodies were diluted in 1% BSA in PBS. All incubations were performed inside a humid chamber at 37 °C for 1 h. Slides were rinsed twice with PBS for 5 min. Control experiments were carried out by omitting one of the following antibodies: anti-rabbit IgG coupled to Alexa 568, anti-β-ATPase or anti-Lp-FITC antibody. Slides were rinsed with distilled water, air-dried, mounted in Fluorsave and observed with an Olympus FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan) equipped with 488 and 543 nm lasers. Fluorescence and differential interference contrast (DIC) images were acquired and processed with FluoView FV1000 version 1.7.1.0 software.

2.11. The effect of in vivo β-ATPase blocking on lipid transfer from lipophorin to the ovarian tissue

For this set of studies, females at early vitellogenesis were injected with 5 μl of anti-β-ATPase antibody dialyzed against PBS (10 μg/insect). One hour after treatment with the antibody, insects were injected with 10 μl (15 μg/insect) of Lp-DiI or Lp-Bodipy-FA and dissected 3 h later. The ovaries were processed for cryostat sectioning and analyzed by laser confocal microscopy as described in Section 2.10. Insects were also injected with anti-BSA as controls. In order to prevent endocytosis of lipophorin, another set of assays was carried out with similar experimental protocols but keeping females at 4 °C.

To estimate the relative amount of Bodipy-FA transferred to the ovarian tissue, the ovaries were dissected out, weighed and subjected to lipid extraction (Folch et al., 1957; Fruttero et al., 2014). The corresponding fluorescence was measured using the Multi-Mode Microplate Reader Sinergy HT (BioTek Instruments, Winooski, VT, USA) with 485–505/528–548 nm excitation/emission filters. Results were expressed as arbitrary units of fluorescence/mg of tissue.

2.12. ATP synthase activity in the role of lipophorin-mediated lipid transfer to the oocytes

Experiments using oligomycin, an inhibitor of the H^+-ATP-synthase complex (Wolventang et al., 1994), were performed in order to evaluate if the enzymatic activity of ATP synthase plays any role in the process of lipophorin-mediated lipid transfer to the oocytes. To set up the assay conditions, females at early vitellogenesis were injected with different concentrations of oligomycin (treated insects) or with PBS (control insects). A dose of 3 μl containing 0.4 μg/female of oligomycin in PBS, which partially inhibited ATP synthase activity without compromising insect survival and other important functions such as vitellogenin uptake by the oocytes (data not shown), was used in subsequent experiments. Control and treated females were dissected 4 h after injection and the ATP content in the ovarian tissues was measured using a commercial bioluminescence detection kit (Enliten) according to the manufacturer's protocol. Briefly, weighed ovaries were homogenized in ice-cold 2.5% trichloroacetic acid (TCA) for 2 min. The supernatant was collected by centrifugation (5000×*g*, 4 °C, 5 min) and diluted 1500-fold with 100 mM Tris-acetate, 2 mM Na_2EDTA, pH 7.8. Then, 50 μl of the sample were placed in a 96-well microplate, followed by the addition of 50 μl of Luciferin/Luciferase mix. The luminescence intensity was registered with a 5-s delay and 10 s signal integration time, at 25 °C, using the BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT, USA). The ATP levels were calculated from a standard curve using the ATP stock provided by the commercial kit (Enliten). The amount of ATP was expressed as nmol ATP/mg of tissue.

To assess the effect of partial inhibition of ATP synthase activity on lipophorin lipid transfer to oocytes, vitellogenic females were injected with 3 μl of oligomycin in PBS (0.4 μg/insect) or with the same volume of PBS (controls). One hour after treatment, females were injected with 10 μl (15 μg/insect) of Lp-Bodipy-FA and dissected 3 h later. The ovaries were processed for cryostat sectioning and analyzed by laser confocal microscopy as described above. To estimate the relative amount of Bodipy-FA transferred to the ovarian tissue samples were processed as stated in Section 2.11.

2.13. Statistical analysis

For both, the MTT assays and the *in vivo* approaches directed to analyze the transfer of lipid to oocytes three independent experiments were carried out processing females individually (4–5 insects per time). Bodipy-FA fluorescence in ovarian lipid extracts was recorded in duplicate and the results, which passed normality tests, were expressed as mean ± SEM. Graphs and statistical tests were performed using GraphPad Prism 6.0 and GraphPad Instat 3.0. (GraphPad Software, San Diego, CA, USA). Student's *t*-test was used for comparisons and a P value <0.05 was considered statistically significant. Specific details about the analyses are stated in the legends to figures.

3. Results

If the ovarian β-ATPase plays any role as a lipophorin binding protein and/or lipophorin receptor, it will be localized in the plasma membrane of the cells. Therefore, we carried out subcellular fractionation of the ovarian tissue and the resulting fractions were probed against a commercial polyclonal anti-β-ATPase antibody. The ability of this antibody to cross-react with the β-ATPase of *P. megistus* was already demonstrated (Fruttero et al., 2014). The antibody recognized a band of 56 kDa compatible with the β-ATPase detected in the total homogenate, as well as in the mitochondrial and microsomal fractions (Fig. 1A). The presence of β-ATPase in the mitochondrial fractions was highly expected, taking into account its role in ATP synthesis. However, the expression of β-ATPase in the microsomal fraction is indicative of its localization in the plasma membrane of the cells of the ovarian tissue. To rule out the possibility of mitochondrial contamination, the microsomal fraction was also probed against an anti-cytochrome c antibody. No signal for such a mitochondrial marker was detected in this fraction (Fig. 1B, left panel). Moreover, complementary assays designed to evaluate mitochondrial contamination showed that although all subcellular fractions were able to reduce MTT, only the mitochondrial fraction was able to significantly decrease MTT reduction in the presence of malonic acid, a selective inhibitor of succinate dehydrogenase (Fig. 1B, right panel). Since it was previously demonstrated that succinate-dependent MTT reduction is largely restricted to mitochondria (Berridge and Tan, 1993), this set of results confirmed the lack of mitochondrial contamination of the microsomal fraction.

The morphological and histological organization of the ovarian tissue of *P. megistus* has been described elsewhere (Fruttero et al., 2011). Immunofluorescence assays were carried out to determine the localization of β-ATPase and to analyze a possible co-localization between β-ATPase and lipophorin which in turn, would be indicative of their interaction. The β-ATPase protein was mainly detected in the membrane of oocytes, close to the

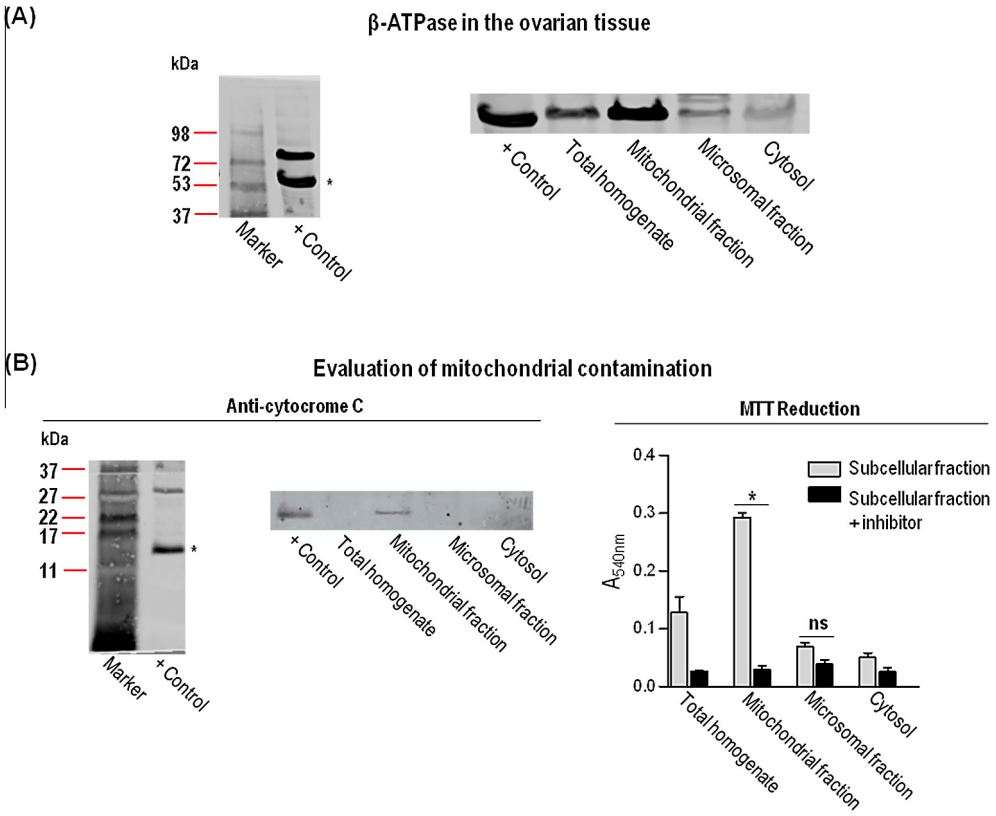

Fig. 1. (A) Immunodetection of β-ATPase in the ovarian tissue of *P. megistus*. Ovaries were homogenized and centrifuged as described in Materials and Methods. Subcellular fractions were probed with a polyclonal anti-β-ATPase antibody. Approximately 40 μg of proteins were loaded into each lane and a homogenate of rat brain was used as positive control (+ Control). The asterisk indicates β-ATPase. The western blot shown was a representative experiment of three independent assays. (B) Evaluation of mitochondrial contamination. Subcellular fractions were also probed with a commercial rabbit anti-cytochrome c antibody (1:1000) using a homogenate of rat brain as positive control (+ Control, left panel). The asterisk indicates cytochrome c. The MTT reduction assay was employed as an indication of mitochondrial contamination (right panel). The influence of a selective inhibitor of mitochondrial succinic dehydrogenase, malonic acid (20 mM), in subcellular fractions was also tested on MTT reduction as described in Materials and Methods. $^{*}P < 0.05$ (Unpaired *t* test); ns, not significant.

perioocytic space, as well as in the basal plasma membrane of the follicular epithelial cells (Fig. 2). It was also shown that in these tissue domains, β-ATPase partially co-localized with lipophorin (Fig. 2 merge).

We carried out biochemical approaches to obtain further evidence of binding and interaction between lipophorin and β-ATPase. Thus, ligand blotting assays showed a lipophorin-binding fraction of 56 kDa, compatible with β-ATPase (Fig. 3A). This fraction was absent in the control lane which was not incubated with lipophorin (Fig. 3A). Moreover, immunoprecipitation experiments indicated that endogenous lipophorin, which remains anchored to oocyte membrane, was present in the microsomal fraction and co-immunoprecipitated with β-ATPase (Fig. 3B, upper panel). Reciprocally, β-ATPase detected in the microsomal fraction co-immunoprecipitated with lipophorin (Fig. 3B, lower panel). Taken together, this set of results indicates an interaction between lipophorin and β-ATPase.

In vivo injections were employed to assess the role of β-ATPase in lipid delivery to the oocytes. Firstly, the fate of the injected anti-β-ATPase antibody in the ovarian tissue was analyzed by immunofluorescence using an antibody conjugated with Alexa 568 as secondary antibody. As shown in Fig. 4, the injected anti-β-ATPase antibody was detected in the perioocytic space, bound to the oocyte plasma membrane, as well as on the plasma membrane of the follicular epithelial cells (Fig. 4A). In order to establish if blocking the β-ATPase would affect the binding of lipophorin and lipophorin-mediated lipid transfer to developing oocytes, females at early vitellogenesis were injected with an irrelevant anti-BSA antibody (control) or with the anti-β-ATPase antibody. Thereafter, the insects were injected with fluorescently labeled lipophorin to follow either, the fate of the lipophorin particle (Lp-DiI) or its lipid cargo (Bodipy-FA). In comparison to controls, the anti-β-ATPase injection did not cause evident changes in the Lp-DiI signal, which was mainly visualized inside yolk bodies (Fig. 4B). On the other hand, when anti-β-ATPase-treated insects were injected with Lp-Bodipy-FA, the fluorescent signal found in lipid droplets corresponding to the lipid analog Bodipy-FA was significantly decreased in comparison to controls (Fig. 4C). Moreover, such a decrease in the fluorescent signal was also observed after total lipids from ovarian tissues were extracted and the Bodipy-FA was measured by fluorometry (Fig. 4D, $P < 0.05$). However, the signal of Lp-Bodipy-FA detected in the yolk bodies was similar in both, anti-β-ATPase-treated and control insects (Fig. 4C). Interestingly, under our experimental conditions, inhibition of ATP synthase activity with oligomycin rendering a 65% decrease in ATP content did not impair the transfer of fluorescent lipids from lipophorin to oocytes (Fig. 5A–C). Altogether, these results suggest that β-ATPase plays a significant role in lipophorin-mediated lipid transfer to the oocytes but not in lipophorin internalization by endocytosis. Such a role seems to be independent of the enzymatic activity of ATP synthase.

Further experiments were carried out keeping the insects at 4 °C to rule out the effect of internalization of lipophorin by endocytosis. Under this condition, in insects injected with either anti-BSA (control) or anti-β-ATPase antibodies followed by Lp-DiI injection, the fluorescent signal of Lp-DiI associated to the oocyte membrane was remarkably decreased in anti-β-ATPase-treated insects

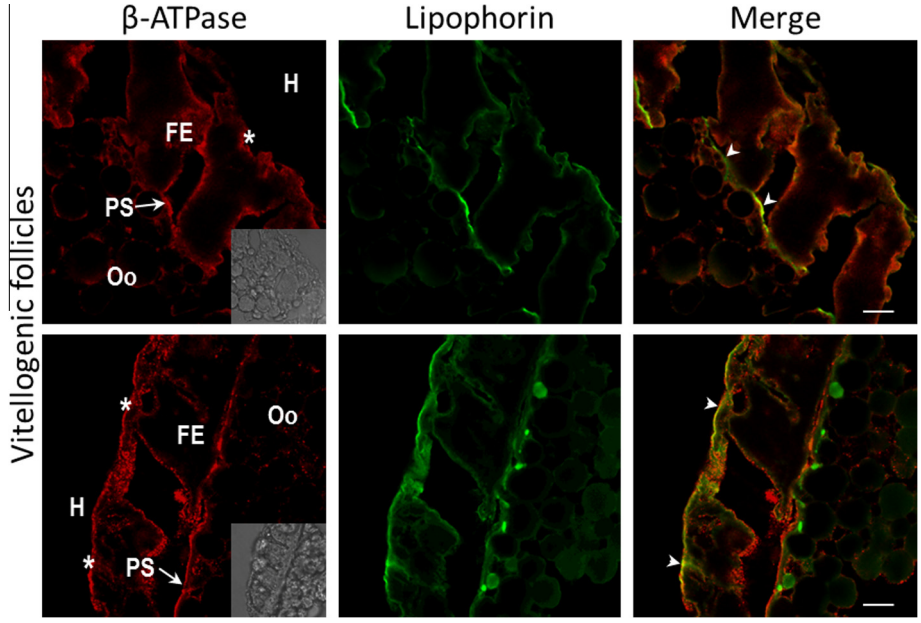

Fig. 2. Localization of β-ATPase and lipophorin in the ovarian tissue of *P. megistus* by immunofluorescence assays. Cryostat tissue sections were incubated with the anti-β-ATPase, anti-IgG labeled with Alexa 568 (red signal) and anti-Lp-FITC (green signal) antibodies as stated in Materials and Methods. In merged images, the partial co-localization of lipophorin and β-ATPase is indicated with arrowheads. The inserts show the corresponding DIC images. H, hemolymph; Oo, oocyte; FE, follicular epithelium; PS, perioocytic space; *, basal plasma membrane of the follicular epithelial cells. Bars. 5 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in separate experiments (n = 3).

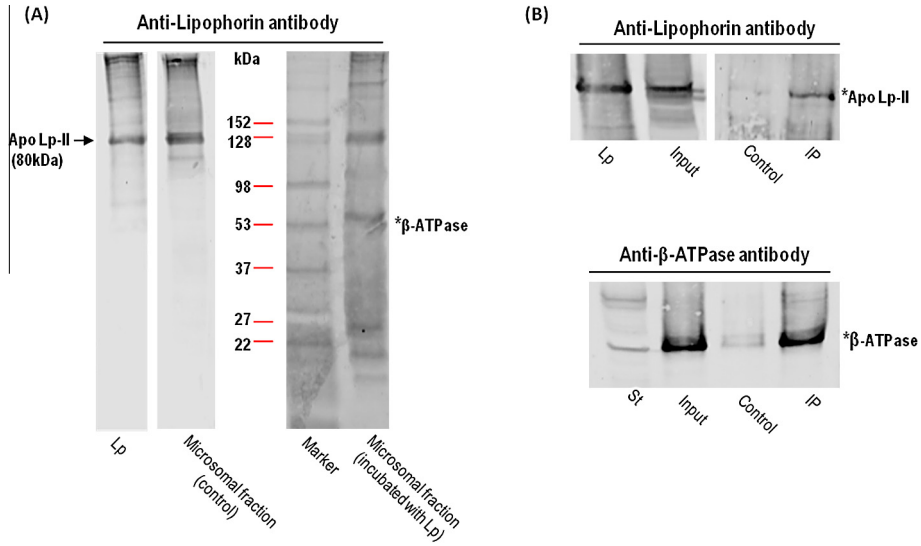

Fig. 3. Lipophorin (Lp) and β-ATPase interaction in the ovarian tissue. (A) Identification of Lp binding proteins in the microsomal fraction of ovarian tissue by ligand blotting. Microsomal fractions (40 μg) were subjected to electrophoresis under non-denaturing conditions, transferred to nitrocellulose and blocked. The nitrocellulose membranes were incubated with the anti-Lp antibody (control) or with Lp (50 μg/ml) and then with the anti-Lp antibody. Arrow indicates apolipophorin II (ApoLp-II) while the asterisk indicates a Lp binding band, which is absent in the control. The band is compatible with the β-ATPase molecular weight (56 kDa). (B) Co-immunoprecipitation of endogenous Lp and β-ATPase. Microsomal fractions of the ovarian tissue (60 μg, Input) obtained as stated in Materials and Methods were incubated with 0.2 mg of anti-BSA antibody (control) or 0.2 mg of anti-β-ATPase or anti-Lp antibodies, as indicated (IP). Samples were transferred to nitrocellulose and then probed against anti-Lp antibody (top panel) or anti-β-ATPase antibody (lower panel). In the lanes, the presence of Lp (as apo Lp-II) or β-ATPase as corresponding is shown by asterisks. IP lane, co-immunoprecipitation; Input lane, endogenous Lp or β-ATPase, as indicated; Lp and St lanes, purified Lp and homogenate of rat brain respectively, loaded as references.

in comparison to controls (Fig. 6). Likewise, in females injected with anti-β-ATPase or anti-BSA antibodies and subsequently with Lp-Bodipy-FA, the Bodipy-FA signal in the lipid droplets was significantly reduced in anti-β-ATPase-treated insects (Fig. 7A-B). Taken together, these set of results indicate that blocking β-ATPase with the antibody partially impaired lipophorin interaction with the ovarian tissue and to some extent, the lipophorin-mediated lipid transfer to the oocytes.

4. Discussion

Since the pioneer studies that proposed the functioning of lipophorin as a reusable shuttle (Downer and Chino, 1985), much knowledge has been gathered about the mechanism of lipid delivery to target cells (Van der Horst et al., 2009). Although it is clear that lipophorin receptors display essential roles during this physiological process, their precise function and mode of action is still

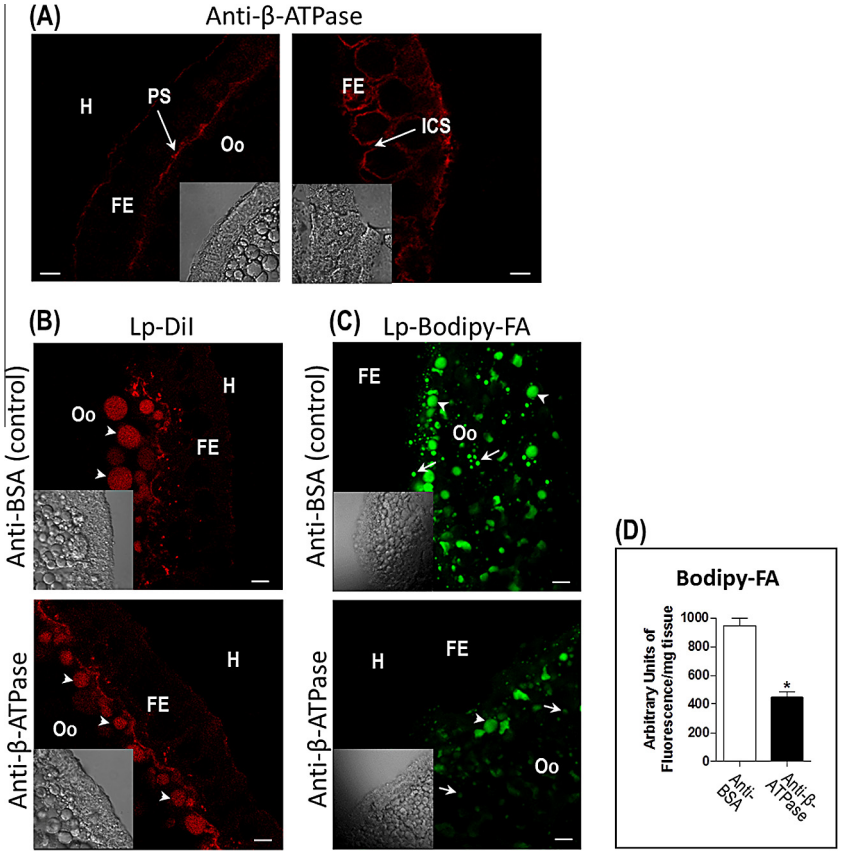

Fig. 4. The effect of β-ATPase blocking on lipophorin-mediated lipid transfer to the ovarian tissue of *P. megistus*. (A) The fate of the injected anti-β-ATPase antibody in the ovarian tissue was analyzed by immunofluorescence using an antibody conjugated with Alexa 568 as secondary antibody. Different sections of the tissue are shown. (B–C) The insects were injected with either, an irrelevant antibody (anti-BSA, control) or an anti-β-ATPase antibody as stated in Materials and Methods. One hour later, the insects were injected with Lp-DiI (red signal) or Lp-Bodipy-FA (green signal) and dissected out 3 h afterwards. Ovaries were processed for confocal laser microscopy. The inserts show the corresponding DIC images. H, hemolymph; Oo, oocyte; FE, follicular epithelium; PS, perioocytic space; ICS, intercellular space; arrowheads. yolk bodies; arrows. adiposomes. Bars: 10 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in separate experiments (n = 5). (D) Bodipy-FA fluorescence quantification. Total lipids from ovarian tissues of insects treated with anti-BSA or anti-β-ATPase and injected with Lp-Bodipy-FA were extracted and the fluorescence registered in a fluorometer was normalized by the weight of the tissue (n = 5). $^{*}P < 0.05$ (Unpaired *t* test).

not completely understood. In the fat body of *L. migratoria*, the LpR belonging to the LDLR family mediated both, lipophorin endocytosis and resecretion; however the inhibition of endocytosis did not affect the exchange of diacylglycerol between lipophorin and fat body cells (Dantuma et al., 1997). Additional evidence supported that endocytic LpRs may not play significant roles in the transfer of neutral lipids to target tissues. Thus, LpR knockdown did not alter the phenotype of the cockroach *Blatella germanica* (Ciudad et al., 2007). In the tsetse fly *Glossina morsitans*, the effect of knocking down the endocytic LpR was limited, causing only a delay in larval development (Benoit et al., 2011).

It has been established that endocytic LpRs mediate a minor pathway on lipid delivery to the oocytes (Ziegler and Van Antwerpen, 2006). Yet, it was reported in *D. melanogaster* that the expression of two LpRs was needed for the accumulation of neutral lipids in oocytes and imaginal disc cells by an endocytosis-independent mechanism. In particular, the product of the lpr2 gene appeared to stabilize lipophorin in the extracellular domain of the oocyte plasma membrane, hence facilitating the transfer of its lipid cargo upon lipolysis (Parra-Peralbo and Culi, 2011).

It was previously demonstrated in *P. megistus* that lipophorin specifically binds to β-ATPase and that such an interaction occurs in the plasma membrane of isolated enterocytes as well as in the sub-epithelial region of the midgut tissue (Fruttero et al., 2014). In the present work, β-ATPase was found in the microsomal subcellular fraction of the ovarian tissue whereas by immunofluorescence it was detected in the oocyte membrane, facing the perioocytic space. Moreover, the partial co-localization between β-ATPase and lipophorin as well as the results ensuing from ligand blotting and co-immunoprecipitation approaches are indicative of the interaction between these two proteins. All these findings are of relevance if β-ATPase functions as a docking receptor. On the other hand, it has been reported that the follicular epithelium may store large lipid droplets during vitellogenesis (Huebner and Anderson, 1972; Leyria et al., 2014). Interestingly, in *P. megistus* β-ATPase and lipophorin also co-localized in the basal plasma membrane of follicular epithelial cells, most likely due to their involvement in lipid supply.

The F_1F_O ATP synthase complex is composed by a F_O portion, the proton channel that is located inside the membrane and a soluble F_1 portion that harbors the catalytic activity (Walker, 2013). It has been demonstrated in *Galleria mellonella* that α- and β-ATPases of the fat body plasma membrane interacted with the juvenile hormone binding protein (JHBP) (Zalewska et al., 2009) while α-ATPase was described as a lipophorin binding protein in an interactomic study conducted in *D. melanogaster* (Giot et al., 2003). In several hemipterans, vectors of phytoplasmas, ectopic α- and β-ATPase of midgut and salivary gland cells were capable of binding antigenic proteins, thus controlling the transmission specificity of these phytopathogenic bacteria (Galetto et al., 2011). Plasma membrane β-ATPase was also involved in the acquisition of dengue

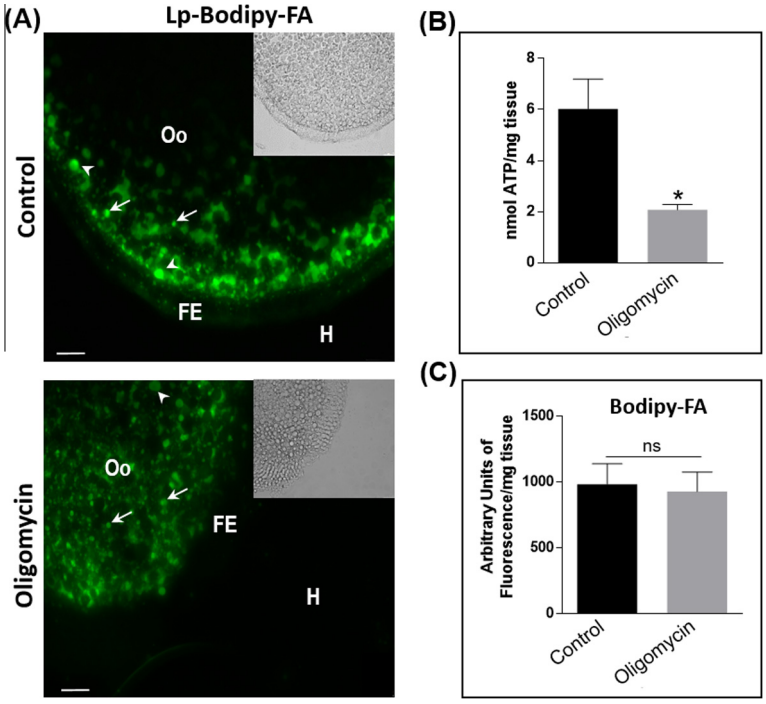

Fig. 5. Effect of ATP synthase activity on lipophorin-mediated lipid transfer to the ovarian tissue of *P. megistus*. (A) The females were injected with 3 μl of oligomycin (0.4 μg/insect) or with PBS (control insects). One hour later, the insects were injected with Lp-Bodipy-FA and dissected out 3 h afterwards. Ovaries were processed for confocal laser microscopy. The inserts show the corresponding DIC images. H, hemolymph; Oo, oocyte; FE, follicular epithelium; arrowheads, yolk bodies; arrows: adiposomes. Bars: 25 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in separate experiments (n = 5). (B) The effect of the partial inhibition of ATP synthase activity on lipophorin-mediated lipid transfer to oocytes was evaluated by measuring the amount of ATP in females treated with oligomycin (0.4 μg/insect) or with PBS (control) as indicated above. Insects were dissected 4 h after injection and the ATP content in the ovarian tissues was measured using a commercial bioluminescence detection kit as stated in Materials and Methods. The results are expressed as nmol of ATP/mg of tissue (n = 5). $^{*}P < 0.05$ (Unpaired *t* test). (C) Bodipy-FA fluorescence quantification. Total lipids from ovarian tissues of females treated with oligomycin or from controls were extracted and the fluorescence registered in a fluorometer was normalized by the weight of the tissue (n = 5). ns, not significant.

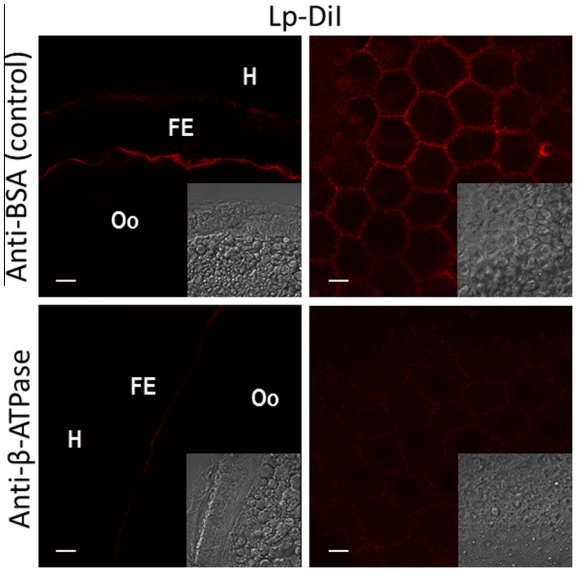

Fig. 6. The effect of β-ATPase blocking on lipophorin binding to the ovarian tissue of *P. megistus* in insects kept at 4 °C to prevent endocytosis. Insects were injected with an irrelevant antibody (anti-BSA, control) or anti-β-ATPase antibody. Thereafter, insects received an injection of Lp-DiI to trace the lipophorin particle as stated in Materials and Methods. Females were dissected out 3 h later and the ovaries were processed for confocal laser microscopy. Different tissue sections of insects from each condition are shown. The inserts show the corresponding DIC images. H, hemolymph; Oo, oocyte; FE, follicular epithelium. Bars: 10 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in separate experiments (n = 3).

virus (Paingankar et al., 2010) and chikungunya virus (Fongsaran et al., 2014) by the mosquito *Aedes* sp. These reports strengthened the attribution of plasma membrane β-ATPase as a binding protein and showed its multiple and relevant functions.

The first studies of the lipophorin interaction with the ovarian tissue in triatomines were performed in *Rhodnius prolixus*, describing that although this lipoprotein can be found in the cortical area of oocytes (Machado et al., 1996), it is not accumulated in the ovary (Gondim et al., 1989). Contrastingly, in vitellogenic females of the hematophagous *P. megistus* the storage of lipid resources in the oocytes is mediated by non-endocytic and endocytic pathways by which lipophorin maximizes the delivery of its lipid cargo. It was also shown that after internalization, lipophorin is stored in yolk bodies, co-localizing with vitellin (Fruttero et al., 2011). As demonstrated (Fruttero et al., 2011), upon Lp-Bodipy-FA injection, the label corresponding to the exchangeable Bodipy-FA was transferred to lipid droplets through oocyte plasma membrane while some of the label remained associated to endocytosed lipophorin and stored in yolk bodies. In the present work, we observed that treatment with anti-β-ATPase antibody clearly impaired the lipophorin binding to oocyte membrane, thus affecting the transfer of Bodipy-FA to lipid droplets. However, β-ATPase seems not to affect lipophorin endocytosis since its blocking did not induce significant changes in the pattern of Lp-DiI signal in yolk bodies. When experimental conditions were set at 4 °C to inhibit lipophorin endocytosis and consequently, the potential trafficking of labeled Bodipy-FA from yolk bodies to lipid droplets, blocking β-ATPase induced a decrease in the transfer of lipids. Overall, these results indicated that β-ATPase is functioning as a docking lipophorin receptor mediating the lipid transfer to oocytes.

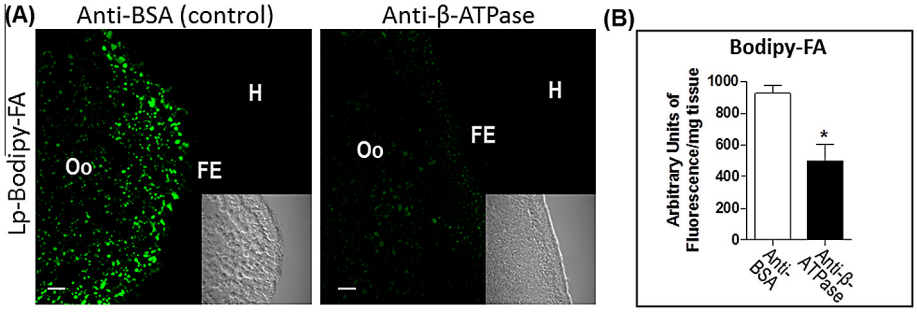

Fig. 7. The effect of β-ATPase blocking on lipophorin lipid transfer to the ovarian tissue of *P. megistus* in insects kept at 4 °C to prevent endocytosis. (A) Insects were injected with an irrelevant antibody (anti-BSA, control) or anti-β-ATPase antibody. Thereafter, insects received an injection of Lp-Bodipy-FA to trace the lipophorin lipid cargo as stated in Materials and Methods. Females were dissected out 3 h later and the ovaries were processed for confocal laser microscopy. The inserts show the corresponding DIC images. H, hemolymph; Oo, oocyte; FE, follicular epithelium. Bars: 10 μm. Similar results were observed after examination of 4–5 ovarioles per ovary in separate experiments (n = 3). (B) Bodipy-FA fluorescence quantification. Total lipids from ovarian tissues of insects treated with anti-BSA or anti-β-ATPase and injected with Lp-Bodipy-FA were extracted and the fluorescence registered in a fluorometer was normalized by the weight of the tissue (n = 5). $^{*}P < 0.05$ (Unpaired *t* test).

Considering the enzymatic nature of the ATP synthase complex, we wanted to know whether the synthase activity was involved in the lipophorin-mediated lipid transfer. When we employed oligomycin, a known inhibitor of ATP synthase, our results indicated that the partial inhibition of the enzymatic activity does not interfere with the transfer of lipids from lipophorin to the oocytes. These findings were similar to those of Martinez et al. (2003) and Fabre et al. (2006), in which the authors conclusively demonstrated that the cell surface ATP synthase activity is neither involved in lipid transfer from the High-density lipoprotein (HDL) to mammalian hepatocytes nor in the binding of HDL to β-ATPase, its receptor.

Currently, the role of β-ATPase as a receptor during hepatic HDL-cholesterol uptake in mammals is well understood (Martinez et al., 2003, 2004; Röhrl and Stangl, 2013). In comparison, much less is known about the function of plasma membrane ATP synthase on lipid metabolism of arthropods. It has been proposed that, in insects, lipid transfer at the cellular plasma membrane is highly selective, requiring not only receptors with high affinity for lipophorin but also factors such as lipoprotein lipases and the lipid transfer particle (LTP) which would be involved in the process (Arrese et al., 2001). In this context, recent findings depicted a complex picture of lipid transfer to target cells that include the participation of transmembrane proteins such as Cameo2 and SCRB15 (Tsuchida and Sakudoh, 2015) and the membrane-associated heparan sulfate proteoglycans Dally and Dally-like (Eugster et al., 2007). The circulating LTP would also participate in the process by binding to LpR and catalyzing lipid transfer between lipophorin and acceptor tissues (Rodríguez-Vázquez et al., 2015).

As reported previously for the midgut of *P. megistus* (Fruttero et al., 2014), experimental evidence in the present work allowed us to propose that in vitellogenic oocytes, β-ATPase operates as another lipophorin receptor. Although the precise mode of action is yet unknown, results from the *in vivo* β-ATPase blocking assays in the midgut and oocytes strongly suggested that the interaction of lipophorin with β-ATPase likely stabilizes its ligand at the surroundings of the plasma membrane, thus facilitating the lipid transfer. As summarized in the Fig. 8, the participation in the oocytes of different lipoprotein receptors together with other

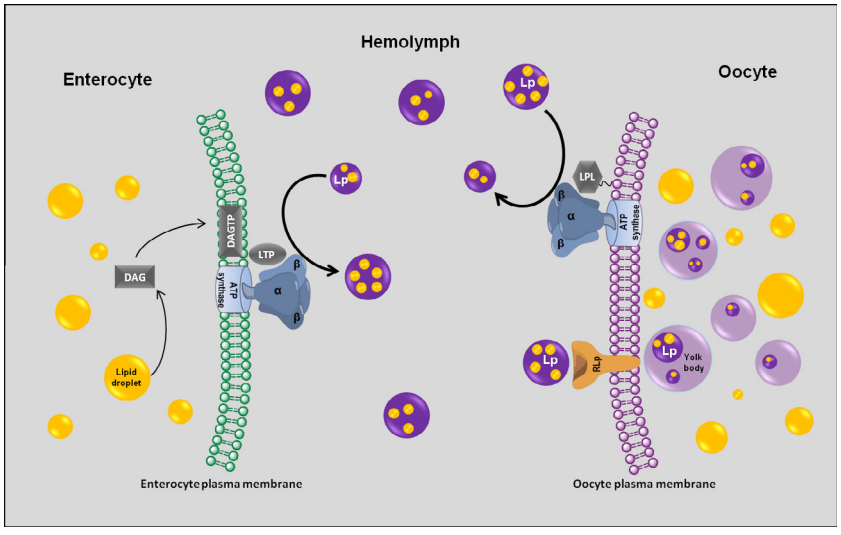

Fig. 8. The proposed model for the role of β-ATPase in lipophorin-mediated lipid transfer in the hematophagous insect *P. megistus*. In the midgut, enterocytes transiently store dietary lipids which in turn will be exported to the hemolymph via lipophorin (Lp). This process takes place at the plasma membrane of the cells and involves the interaction of Lp with a docking receptor identified as β-ATPase. The model implies the participation of a lipid transfer protein (LTP) as well as putative transporters for intracellular and membrane lipid trafficking such as a diacylglycerol transport protein (DAGTP). In fasting conditions, β-ATPase also participates in the transfer of lipids from Lp to the midgut (not shown). During vitellogenesis, the bulk of lipids are transferred from Lp to developing oocytes by its interaction with β-ATPase, which is located at the oocyte plasma membrane. This major pathway requires the hydrolysis of diacylglycerol (DAG) to fatty acids by a membrane-bound lipoprotein lipase (LPL), and its trafficking to lipid droplets for incorporation into the triacylglycerol pool. In developing oocytes however, lipids are also supplied by a minor endocytic pathway, which involves internalization of Lp by an unknown receptor (RLp) and its trafficking to yolk bodies to be stored together with vitellin.

molecules seems to be not mutually exclusive; moreover, they can modulate each other or function in coordination. Reinforcing this point of view, it was recently reported that the isoform LpR4 of *B. mori* interacted with β-ATPase and other proteins involved in lipid signaling, cell motility or protein kinase pathways (Gopalapillai et al., 2014).

In this work, our findings point to a function of β-ATPase which extends beyond the current knowledge of mechanisms implicated in lipid delivery to the insect oocytes. Undoubtedly, more studies will be necessary to fully understand the role of β-ATPase as a non-endocytic receptor of lipophorin. It would be interesting to know if the interaction between lipophorin and β-ATPase in the midgut and oocytes would require other protein(s) acting as a bridge (Fig. 8). Preliminary biophysical analyses suggest a direct binding between purified lipophorin and a recombinant β-ATPase from *P. megistus*.

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