Journal of Insect Physiology 103 (2017) 98–106

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Physiological characterization and regulation of the contractile properties of the mosquito ventral diverticulum (crop)

Travis L. Calkins, Andrew DeLaat, Peter M. Piermarini*

Department of Entomology, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, OH, USA

A B S T R A C T

In adult dipteran insects (flies), the crop is a diverticulum of the esophagus that serves as a food storage organ. The crop pumps stored contents into the alimentary canal for digestion and absorption. The pumping is mediated by peristaltic contractions of the crop musculature. In adult female mosquitoes, the crop (ventral diverticulum) selectively stores sugar solutions (e.g., nectar); proteinaceous blood meals by-pass the crop and are transferred directly to the midgut for digestion. The mechanisms that regulate crop contractions have never been investigated in mosquitoes. Here we provide the first physiological characterization of the contractile properties of the mosquito crop and explore the mechanisms that regulate crop contractions. Using an *in vitro* bioassay we found that the isolated crop spontaneously contracts in Ringer solution for at least 1 h and its contractions are dependent on extracellular Ca^{2+}. Adding serotonin (5-hydroxytryptamine, 5-HT) or a membrane-permeable analog of cyclic adenosine monophosphate (cAMP) to the extracellular bath increased the frequency of crop contractions. On the other hand, adding benzethonium chloride (BzCl; a chemical that mimics the effects of myosuppressins), H-89 or Rp-cAMPS (inhibitors of protein kinase A, PKA), or carbenoxolone (an inhibitor of gap junctions) reduced the frequency of the unstimulated, spontaneous and/or 5-HT-stimulated crop contractions. Adding aedeskinin III did not detectably alter crop contraction rates. In addition to pharmacological evidence of gap junctions, we demonstrated that the crop expressed several mRNAs encoding gap junctional proteins (i.e. innexins). Furthermore, we localized immunoreactivity for innexin 2 and innexin 3 to muscle and epithelial cells of the crop, respectively. Our results 1) suggest that 5-HT and myosupressins oppositely regulate contractile activity of the mosquito crop, and 2) provide the first evidence for putative roles of cAMP, PKA, and gap junctions in modulating contractile activity of the dipteran crop.

1. Introduction

The alimentary canal of adult dipteran insects possesses a diverticulum of the foregut referred to as the crop or ventral diverticulum. The crop consists of 4 main structures: 1) luminal cuticle, 2) a simple epithelium, 3) an anastomosed network of visceral muscle, and 4) nerves that derive from the corpus cardiacum (Stoffolano and Haselton 2013). Food storage is the quintessential physiological function of the crop; it receives imbibed liquid meals and stores them for later digestion and absorption by the midgut. The release of food from the crop to the midgut is mediated by peristaltic contractions of the crop musculature. In mosquitoes, the crop is the primary storage organ for imbibed sugar (e.g., nectar) before it is pumped into the midgut for digestion. In contrast, blood, which is only ingested by adult females, by-passes the crop and is received directly by the midgut for immediate digestion (Day, 1954).

Previous studies in blow flies (*Phormia regina*), house flies (*Musca domestica*), and fruit flies (*Drosophila melanogaster*) have shown that contractions of the crop are influenced by an array of physiological, neuroendocrine, and genetic factors. In *P. regina*, extracellular Ca^{2+} is essential for crop muscle contraction, and hemolymph osmolality modulates the rate of contraction (Gelperin, 1966; Liscia et al. 2012; Solari et al. 2013). Moreover, the volume of liquid within the crop influences the contraction rates in both *P. regina* and *M. domestica* (Holling, 1976; Stoffolano et al. 2014b). Neuropeptides, such as adipokinetic hormone (AKH), dromyosuppressin (DMS), drosulfakinin, and FMRFamide, and biogenic amines, such as serotonin (5-hydroxytryptamine, 5-HT) and octopamine, modulate crop contraction rates in *P. regina* and/or *D. melanogaster* (Duttlinger et al. 2002; Liscia et al. 2012; Palmer et al. 2007; Solari et al. 2017; Stoffolano et al. 2013, 2014a). Notably, nerves associated with the crop contain DMS, FMRFamide, insulin-like peptide, 5-HT, and AKH, suggesting the potential for direct neural control of crop contractions (Cao and Brown, 2001; Duttlinger et al. 2002; Haselton et al. 2004; Lee and Park, 2004;

* Corresponding author at: Ohio Agricultural Research and Development Center, The Ohio State University, Department of Entomology, 224 Thorne Hall, Wooster, OH 44691, USA.
E-mail address: Piermarini.1@osu.edu (P.M. Piermarini).

http://dx.doi.org/10.1016/j.jinsphys.2017.10.012
Received 1 August 2017; Received in revised form 20 October 2017; Accepted 23 October 2017
Available online 28 October 2017

Liu et al. 2011; McCormick and Nichols, 1993). In *D. melanogaster*, mutation of the *drop-dead* (*drd*) gene elevates the spontaneous rates of crop contractions, but the mechanism behind this stimulation is unclear (Peller et al. 2009).

In contrast to the aforementioned flies, the contractile activity of the mosquito crop has not been previously investigated. Although a previous study has shown that the crop of *Aedes aegypti* is innervated with neurons containing FMRFamide, small cardioactive peptide b, and 5-HT immunoreactivities (Moffett and Moffett, 2005), the basic contractile properties of the mosquito crop and its regulation by physiological and neuroendocrine factors are unknown. Here our goal was to characterize the physiology and regulation of crop contractions in adult female *A. aegypti*, an important vector of arboviruses that cause Zika, dengue, chikungunya, and yellow fevers in humans. Utilizing an *in vitro* assay we show that the isolated mosquito crop spontaneously contracts in Ringer solution for at least one hour and its contractions are dependent upon the presence of extracellular Ca^{2+}. Moreover, we show that crop contraction rates are stimulated by 5-HT and a membrane-permeable analog of cAMP (8-Bromo-cAMP), inhibited by benzethonium chloride (BzCl; a chemical that mimics the effects of myosupressins) and inhibitors of protein kinase A (PKA), and unaffected by aedeskinin III (AKIII; a myoactive and diuretic neuropeptide). Furthermore, we provide the first pharmacological, molecular, and immunochemical evidence for gap junctions in the dipteran crop, suggesting that they contribute to the propagation of the contractile signal in the crop visceral musculature.

2. Materials and methods

2.1. Mosquito rearing

A. aegypti mosquitoes were obtained as eggs through the Malaria Research and Reference Reagent Resource Center (MR4) as part of the BEI Resources Repository (Liverpool strain; LVP-IB12 F19, deposited by M.Q. Benedict). In brief, mosquitoes were reared in an environmental chamber set to 28°C and 80% relative humidity with a 12 h:12 h light:dark cycle, as described previously (Piermarini et al. 2011). Larvae were fed daily with pulverized Tetramin flakes (Melle, Germany) and adults were fed 10% sucrose *ad libitum*.

2.2. Crop dissection

At 3–10 days post-emergence, adult female mosquitoes were removed from rearing cages and immobilized on ice. Only females with visibly distended abdomens (i.e., obvious signs of recent sugar feeding) were used. After removing the legs, the body was submerged in mosquito Ringer solution (150 mM NaCl, 3.4 mM KCl, 1.7 mM $CaCl_2$, 1.8 mM $NaHCO_3$, 1 mM $MgCl_2$, 5 mM Glucose, and 25 mM HEPES; pH 7.1; 330 mOsmol/kg) at room temperature. The head was removed with forceps (Dumont #5; Fine Science Tools, Inc., Foster City, CA) under Ringer solution and the thorax was gently teased away from the abdomen, exposing the crop and its attachment to the foregut of the alimentary canal. The foregut and anterior midgut were then compressed with forceps (anterior and posterior to the crop, respectively), allowing for the crop to be isolated from the alimentary canal with minimal leakage of crop contents. Crops that visibly lost volume and/or expelled contents to the bath were discarded.

2.3. Chemicals

The pharmacological agents tested (listed as final assay concentrations) included: 1) 1 nM, 10 nM, or 100 nM serotonin (5-HT; Thermo Fisher Scientific, Waltham, MA); 2) 1 mM 8-Bromo-cAMP (Tocris, Minneapolis, MN); 3) 25 μM benzethonium chloride (BzCl; Thermo Fisher Scientific); 4) 10 μM aedeskinin III (AKIII; synthesized by the Nachman laboratory; Zubrzak et al. 2007); 5) various concentrations (1–1000 μM) of carbenoxolone (CBX; Sigma-Aldrich, St. Louis, MO); 6) 10 μM H-89 (Tocris); and 7) 250 μM Rp-cAMPS (Enzo Life Sciences, East Farmingdale, New York). 5-HT is a biogenic amine that stimulates crop contractions in *P. regina* and *D. melanogaster* (Liscia et al., 2012; Solari et al., 2017). 8-Bromo-cAMP is a membrane-permeable analog of cAMP used to elevate intracellular concentrations of cAMP (e.g., Goino et al. 2014). BzCl is a chemical that mimics the actions of myosupressins (Lange et al. 1995; Nachman et al. 1996). AKIII is a myoactive neuropeptide that stimulates contractions of mosquito hindgut visceral muscle (Veenstra et al., 1997). CBX is an inhibitor of gap junctions (Rozental et al. 2001). H-89 inhibits the phosphorylation mechanisms of activated PKA and several other kinases (Lochner and Moolman, 2006); it has been widely used in insect studies to inhibit PKA (e.g., Beyenbach et al., 2009; Bhattacharya et al., 1999; Fechner et al., 2013; Gioino et al., 2014; Paluzzi et al., 2013; Tiburcy et al., 2013). Rp-cAMPS is an analog of cAMP that occupies the cAMP-binding sites of PKA, thereby preventing its activation (Lochner and Moolman, 2006). Stock solutions of the chemicals were prepared at 100-times the final assay concentrations in dH_2O (5-HT, BzCl, CBX, and AKIII) or dimethylsulfoxide (8-Bromo-cAMP, H-89, and Rp-cAMPS).

2.4. In vitro crop contraction assays

To measure crop contraction rates, we used an *in vitro* assay similar to that of Haselton et al. (2006) for the blow fly. In brief, immediately after isolation, crops were transferred by glass pipette directly into a single well of a 96-well microtiter plate (USA Scientific, Ocala, FL) containing 100 μl of mosquito Ringer solution, and the contraction rate (contractions per minute) was determined by eye under a stereoscope as described below. Our initial observations demonstrated that the contraction rates of crops were stable between 5 and 20 min post-transfer (red box in Fig. 1A). Thus, all measurements and experiments were performed within this 15 min window.

To test putative inhibitors and agonists of crop contraction rates, each crop served as its own control. After transferring a crop to a well, the number of contractions per minute was counted by eye at 5, 7, and 9 min post-transfer. These contraction rates were averaged together and referred to as the 'control' contraction rate. At 10 min post-transfer, 1 μl of Ringer solution was removed from the well and replaced with 1 μl of a stock treatment solution (see *Chemicals* above). After gentle mixing via pipetting, the number of contractions per minute was counted by eye at 2, 4, and 6 min after adding the treatment solution (i.e., 12, 14, and 16 min post-transfer). These rates were averaged together and referred to as the 'treatment' contraction rate. The addition of dH_2O or dimethylsulfoxide (DMSO) at a 1% final assay concentration did not significantly affect contraction rates (Supplemental Fig. 1).

In some experiments, we tested the effects of 10 nM 5-HT on crops that were pre-treated with 250 μM CBX, 10 μM H-89, or 250 μM Rp-cAMPS. For these experiments, 1) contractions per minute were counted at 5 and 7 min post-transfer for the control period, 2) CBX, H-89, or Rp-cAMPS was added at 8 min post-transfer and contractions per minute were counted at 10 and 12 min post-transfer for the treatment period, and 3) 5-HT was added at 13 min post-transfer and contractions per minute were counted at 15 and 17 min post-transfer for the 'treatment + 5-HT' period. As with the single treatment assays, each time a new compound was added, 1 μl of Ringer solution was removed from the well and replaced with 1 μl of treatment solution.

To determine the effects of extracellular Ca^{2+} on contraction rates, the crops were dissected—and the control contraction rates were measured—in a nominal Ca^{2+} Ringer solution. For the 'treatment', the Ca^{2+} concentration was increased to 1.7 mM (the normal concentration in Ringer solution) by adding 1 μl of 170 mM $CaCl_2$ dissolved in dH_2O.

2.5. RNA isolation and qPCR

Following dissection, crops (50 per replicate) for RNA isolation and