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Regulatory mechanisms and the role of calcium and potassium channels controlling supercontractile crop muscles in adult *Phormia regina*

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ABSTRACT

Bioassays and electrophysiological recordings were conducted in the adult blowfly *Phormia regina* to provide new insights into the regulatory mechanisms governing the crop filling and emptying processes of the supercontractile crop muscles.

The cibarial pump drives ingestion. Simultaneous multisite extracellular recordings show that crop lobe (P5) distension during ingestion of a 4.7 μl sugar meal does not require muscle activity by any of the other pumps of the system. Conversely, pumping of fluids toward the anterior of the crop system during crop emptying is brought about by active muscle contraction, in the form of a highly coordinated peristaltic wave starting from P5 and progressively propagating to P6, P4 and P3 pumps, with P5 contracting with a frequency about 3.4 times higher than the other pumps. The crop contraction rate is also modulated by hemolymph-borne factors such as sugars, through ligand recognition at a presumptive receptor site rather than by an osmotic effect, as assessed by both behavioural and electrophysiological experiments. In this respect, sugars of equal osmolarity produce different effects, glucose being inhibitory and mannose ineffective for crop muscles, while trehalose enhances crop activity.

Finally, voltage and current clamp experiments show that the muscle action potentials (mAPs) at the P4 pump are sustained by a serotonin-sensitive calcium conductance. Serotonin enhances calcium entry into the muscle cells and this could lead, as an indirect modulatory effect, to activation of a Ca^{2+}-activated K^+ conductance ($I_{K(Ca)}$), which sustains the following mAP repolarization phase in such a way that further mAPs can be generated early and the frequency consequently increased.

1. Introduction

Neural control systems governing the regulation of meal size are subjects of great interest in both mammalian (Berthoud and Morrison, 2008; de Castro, 2010; Cegla et al., 2010; Gorissen et al., 2006; Lutz, 2012) and insect (Al-Anzi et al., 2010; Audsley and Weaver, 2009; Gordon and Scott, 2009; Reiher et al., 2011) systems. In particular, the adult queen blowfly, *Phormia regina* Meigen, has been instructive in identifying the sensory, motor, and integrative components underlying feeding (Dethier, 1976; Gelperin, 1966a, 1967, 1972; Haselton et al., 2009; Liscia et al., 2012; Stoffolano et al., 2010; Thomson, 1975a,b, ; Thomson and Holling, 1975a,b, so that *Phormia* is now the standard reference model for studies on feeding control systems in adult flies.

As an adult, *Phormia* possesses a diverticulated crop, which is an elongated ventral outpocketing of the foregut, consisting of a duct ending in a bilobed sac, the walls of which contain a complex muscular system of pumps and sphincters (Stoffolano and Haselton, 2013). The crop is used for the storage of ingested solutions of proteins or carbohydrates (Stoffolano and Haselton, 2013) before aliquots of these solutions are transported into the midgut for digestion. The crop outer wall comprises supercontractile muscles operating on the principles of hydrodynamics (Stoffolano and Haselton, 2013; Thomson and Holling, 1975b).

The adult crop system consists of 4 main structures: (1) epithelial cells producing the cuticular lining, (2) the cuticular intima itself, (3) a pair of crop nerve bundles emanating from the corpora

Abbreviations: mAP, muscle action potential; DMS, dromyosuppressin; AKH, adipokinetic hormone; ACCR, average crop contraction rate; 5-HT, serotonin.

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cardiaca and branching over the surface of the crop lobes (P5) and (4) the crop muscles of the duct and lobes. These muscles are anastomosed, supercontractile muscles connected by intercellular bridges. They form the various pumps and sphincters involved in the filling and emptying processes of the crop lobes (P5) (see Stoffolano and Haselton, 2013, for a review). Thomson (1975b), commenting on Gelperin's, 1966a paper, noted that because crop emptying is independent of both nervous and endocrine control, "...the process must be based on a myogenic mechanism."

Crop filling is mainly driven by the cibarial pump (Stoffolano and Haselton, 2013), while emptying, although lacking any direct nervous control (Gelperin, 1966a; Knight, 1962; Stoffolano et al., 2010), is under complex neuroendocrine modulation, also influenced by multiple neuropeptides such as dromyosuppressin (DMS) (Richer et al., 2000) and neurohormones such as serotonin (Liscia et al., 2012). It was recently shown (Liscia et al., 2012) that serotonin finely modulates the crop emptying rate by increasing contractions of pump 4 (P4). Pump P4 is part of the crop duct, rather than part of the crop lobes (P5) (Thomson and Holling, 1975a). In addition, P4 and P5 respond differently to chemical neuromodulators: adipokinetic hormone (AKH) shuts down the crop sac muscles, but stimulates the P4 pump (Stoffolano et al., 2010), and serotonin increases crop sac contractions and P4 pump activity. Extracellular calcium is essential for crop muscle activity, regardless of the presence of serotonin (Liscia et al., 2012). In spite of these studies, essential details on the basal mechanisms of crop filling and emptying are still lacking.

Previous work suggested that the hemolymph composition surrounding the crop lobes (P5) affects the rate of their contractions, but the only study to address this issue is that of Thomson (1975a). In his study, Thomson investigated whether it is the osmotic pressure of the hemolymph acting directly on the muscles or chemical recognition of specific hemolymph molecules by the crop muscles that regulates their activity. He concluded that the critical regulatory interaction was not osmotic, but rather a chemical response. Later, Amakawa (2001) showed that changing the hemolymph concentration of trehalose caused differences in the proboscis extension response in *P. regina*. Thus, hemolymph molecules can impact crop filling, emptying and feeding behavior.

Based on these considerations, in the present study we first determined, using extracellular recordings, the contribution of the P3, P4 and P6 pumps and lobe muscles (P5) in the basal mechanism underlying the filling and emptying processes of the crop system in the blowfly *P. regina*.

We then investigated whether the crop contraction rate depends on osmotic and/or neurochemical components of the medium bathing the crop.

Finally, by means of current and voltage clamp experiments we studied the myogenic origin of crop muscle excitability and determined which ionic channels and currents give rise to and sustain muscle action potentials at the P4 pump.

The present study provides new insights into the physiological mechanisms governing the filling and emptying processes of the adult crop, an important feeding organ in insects. Also, for the first time, our results assign a key role to a serotonin-sensitive calcium conductance and to a calcium activated potassium conductance for regulation of crop muscle excitability and suggest that serotonin interacts with these channels by enhancing their activity.

2. Materials and methods

2.1. Maintaining flies

Experiments were performed on adult virgin female blowflies, *P. regina*, 2-days post-emergence (Dethier, 1961), obtained from a colony maintained as previously described at 27 °C, 50% RH, and a photoperiod of 16L:8D (Stoffolano, 1974). Eggs were collected and placed in 473 ml plastic cups containing an artificial diet (Stoffolano et al., 2010). After several days of growth, the cups were placed in a container with sand; and, when the larvae were ready to pupate, they crawled into the sand. Pupae were then transferred to another 473 ml plastic cup, which was placed into a metal cage (20.3 cm on each side). When the adult flies emerged, they were fed a 0.126 M sucrose solution according to Liscia et al. (2012).

All flies emerging within 24 h were considered as one cohort. Insects were tested after being starved, but water satiated, for 24 h, in order to start electrophysiological experiments on insects with empty crops. Preparations in which the crop was not completely empty were discarded.

2.2. Electrophysiological recordings of crop muscle activity from whole insects

Flies were cold-immobilized at 20 °C until they became inactive (typically less than 5 min), and then restrained in soft dental wax to limit movements, according to the procedure used by Liscia et al. (2012). A section of cuticle was removed from the ventral surface of the abdomen in order to expose the crop lobes (P5), the P4 and P6 pumps, and the abdominal portion of the crop duct, so that each of these structures could be reached easily by the electrodes.(Fig.1). Dissections were performed in *Phormia* saline (Chen and Friedman, 1975). Care was taken to ensure that the central nervous system, all peripheral nerves, and muscles remained intact; preparations that failed to resume crop activity after dissection were discarded. In these experimental situations, the labella of flies were also visible and accessible for feeding the fly and thereby inducing crop filling.

Recordings of muscle activity (electromyograms, EMGs) from the surface of the P3, P4 and P6 pumps, and crop lobes (P5) were made *en passant*, either in single or double simultaneous recording configuration, by way of small borosilicate glass suction electrodes filled with *Phormia* saline. The electrodes had a long shaft that ensured the necessary flexibility to follow small muscle contractions. With this experimental arrangement (Fig.1), stable recordings were possible for more than 2-3 h.

Recordings were preamplified and band-pass filtered (0.1-1000 Hz), using an A-M Systems (Everett, WA, USA) four-channel differential AC amplifier (Model 1700), digitized with an Axon Digidata 1344A A/D converter (sampling rate, 10 kHz per channel), and stored on a PC for further analyses (pClamp 10.0 software, Axon Instruments).

In most cases, recordings of spike activity from the crop nerve were recorded and presented superimposed on muscle action potentials (mAPs). During electrophysiological recordings, movements of P3, P4 and P6 pumps, and crop lobes (P5) were simultaneously monitored electrophysiologically and video-recorded by way of a Moticam 2300 (3.0 M Pixel, USB 2.0) color digital camera coupled to the stereomicroscope (Zeiss, Stemi 2000-C); video information was stored on a computer as avi files and analyzed with Motic Images Plus 2.0 ML software.

2.3. Procedure for recording muscle action potentials during crop filling and emptying

To determine the physiological mechanisms underlying crop filling and emptying, mAPs were continuously recorded from the P3 and the P4 pumps, in each of 12 tested flies, during:

(a) the starved initial condition with empty crop (control),
(b) the phase of insect feeding with 4.7 μl of a 1 M sucrose solution placed on the labellum, which resulted in crop distension,