Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae

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**Ormerod KG, Hadden JK, Deady LD, Mercier AJ, Krans JL.** Action of octopamine and tyramine on muscles of *Drosophila melanogaster* larvae. *J Neurophysiol* 110: 1984–1996, 2013. First published July 31, 2013; doi:10.1152/jn.00431.2013.—Octopamine (OA) and tyramine (TA) play important roles in homeostatic mechanisms, behavior, and modulation of neuromuscular junctions in arthropods. However, direct actions of these amines on muscle force production that are distinct from effects at the neuromuscular synapse have not been well studied. We utilize the technical benefits of the *Drosophila* larval preparation to distinguish the effects of OA and TA on the neuromuscular synapse from their effects on contractility of muscle cells. In contrast to the slight and often insignificant effects of TA, the action of OA was profound across all metrics assessed. We demonstrate that exogenous OA application decreases the input resistance of larval muscle fibers, increases the amplitude of excitatory junction potentials (EJPs), augments contraction force and duration, and at higher concentrations (10^{-5} and 10^{-4} M) affects muscle cells 12 and 13 more than muscle cells 6 and 7. Similarly, OA increases the force of synaptically driven contractions in a cell-specific manner. Moreover, such augmentation of contractile force persisted during direct muscle depolarization concurrent with synaptic block. OA elicited an even more profound effect on basal tonus. Application of 10^{-5} M OA increased synaptically driven contractions by ~1.1 mN but gave rise to a 28-mN increase in basal tonus in the absence of synaptic activation. Augmentation of basal tonus exceeded any physiological stimulation paradigm and can potentially be explained by changes in intramuscular protein mechanics. Thus we provide evidence for independent but complementary effects of OA on chemical synapses and muscle contractility.

**OA** is synthesized de novo from the amino acid tyrosine via a two-step enzymatic conversion, first to tyramine (TA) and then to OA. TA was once considered only an intermediary of OA biosynthesis but has now been demonstrated to have its own independent effects on synaptic transmission and to function through independent receptors (*Drosophila*: Bayliss et al. 2013; Nagaya et al. 2002; *Caenorhabditis elegans*: Alkema et al. 2005; Pirri et al. 2009; *Acrididae*: Homberg et al. 2013; Kononenko et al. 2009; Vierk et al. 2009). The actions of OA and TA appear to vary considerably across arthropod preparations, in some cases even by sign (i.e., locust vs. fly: Evans and Siegler 1982; Nagaya et al. 2002; Saraswati et al. 2004; Walther and Zittlau 1988). Although the action of OA is typically profound, there is some disagreement as to the action of amines within *Drosophila* preparations due in part to the use of calcium-free, high-magnesium saline (Kutsukake et al. 2000; calcium-free HL-3 contains 20 mM Mg^{2+}), which is known to have anesthetic effects and suppress membrane excitability in many animals, including *Drosophila* (Chordata: Iseri and French 1994; Arthropoda, Crustacea: Katz 1936; Arthropoda, insect—*Drosophila*: Feng et al. 2004). Indeed, whereas some report that TA greatly attenuates neuromuscular transduction (Roeder 2005), others suggest that it has little or no action on the longitudinal muscle fibers of flies (Nagaya et al. 2002; Ormerod et al. 2012).

High levels of OA are found within insect central and peripheral nervous tissues, where it functions as a neurotransmitter and a neuromodulator (Roeder 1999). Circulating levels of OA in the hemolymph of insects are also observed during stressful situations, where OA plays a neuroendocrine role (Farooqui 2007). OA has been shown to affect a number of behaviors (e.g., locomotion, flight, egg laying, aggressiveness, and ovulation) and is associated with major nervous system functions, such as desensitization, learning, and memory (for...
suggestive of a long-term, intramuscular change by an unidentified tone far exceeded augmentation of evoked contractions and is caused by OA-induced augmentation of basal muscle tone in the absence of any synaptic activation, making it possible to examine modulatory actions of OA and TA on chemical synapses and direct effects on muscle fibers. We utilized several strategies to make such a distinction in the location of amine action, including investigation of 1) passive membrane properties (i.e., membrane resistance), 2) principal components of excitatory junction potentials (EJPs), and 3) force production. Force augmentation by OA was characterized with the following three components: 1) contractile force evoked via traditional electrical activation of the motor nerve (i.e., through the synapse), 2) basal muscle tone in the absence of any synaptic activation, and 3) local depolarization concurrent with synaptic block. We provide evidence that, in addition to its ability to augment muscle contractions and potentiate neuromuscular transduction, OA also augments evoked contractions downstream of chemical synapses. The OA-induced augmentation of basal tone far exceeded augmentation of evoked contractions and is suggestive of a long-term, intramuscular change by an unidentified factor.

Materials and Methods

Animals and Basic Preparation

Drosophila melanogaster Canton S (CS) flies, obtained from the Bloomington Drosophila stock center, were used for all experiments. Flies were reared at 21°C on a 12:12-h light-dark cycle and were provided with either a cornmeal-based medium (Boreal Laboratories, St. Catharines, ON, Canada) including dry yeast or a standard diet (after David 1962) consisting of 100 g of yeast, 100 g of glucose, 12 g of agar and 10 ml of propionic acid (mold inhibitor) combined in 1,220 ml of H2O. OA, TA, yohimbine, and cyproheptadine were acquired from Sigma Aldrich (St. Louis, MO).

Only early wandering stage third-instar larvae were selected. Animals were collected from the sides of their culture vials and placed dorsal side up onto a dissecting dish containing either of two hemolymph-like Drosophila salines, HL-6 and HL-3.1, the compositions of which have been published (Macleod et al. 2002 and Feng et al. 2004, respectively). All of the experiments outlined here were confirmed in both solutions except for evoked contraction recordings (only HL-3.1). A semi-intact larval body wall preparation (Paterson et al. 2010) was used for recording intracellular electrical signals and force (Fig. 1A).

Briefly, larvae were incised along the longitudinal axis and pinned open. The segmental nerves could be severed near their exit from the ventral ganglion, and the CNS and all gut organs were removed. The bath was continuously perfused (0.7 ml/min, dish volume ~300 μl) with oxygenated physiological saline, except in the case of application of a toxin (see Synaptically evoked force), in which case saline containing the toxin was directly applied and not recirculated, in an effort to avoid its residual remnants confounding future experiments. Experiments followed the same basic application routine: 10–15 min in control saline, application of amine, and washout for at least twice the duration of exposure to amine. In some experiments, muscle fibers 12 and 13, or fibers 6 and 7, were lesioned with fine dissection scissors. Unless noted, all body wall muscles were intact.

Intracellular recording. Intracellular recordings (Fig. 1A) were obtained with sharp microelectrodes, produced from thin-wall monofilament glass (WPI, Sarasota, FL) with a Flaming-Brown microelectrode puller (P-97, Sutter Instrument, Novato, CA). Intracellular recordings were made from longitudinal muscle fibers 6, 7, 12, and 13 across abdominal segments 3, 4, and 5. The anatomy and position of longitudinal muscles (see Fig. 1A) in these centralized segments are highly conserved and function to shorten body length during rhythmic contractions of locomotion. Intracellular data from homologous muscle fibers (i.e., muscle fibers 6 and 7, muscle fibers 12 and 13) were combined and are reported as such. Synaptic potentials were elicited by stimulating all segmental motoneurons via a glass suction electrode, a Grass S88 stimulator, and a stimulus isolation unit (Grass Technologies, West Warwick, RI). Single impulses were generated at 0.2 Hz, 0.5-ms pulse duration, and ~115% of the voltage needed to attain maximal compound EJP amplitude. Stimulus frequency and voltage are described in text for contraction recordings, as some experiments utilized direct stimulation of the muscle (see Synaptically evoked force).

EJPs were recorded with either an AxoClamp 2B (Molecular Devices, Sunnyvale, CA) or a Neurodata IR283A (Cygnus Technology, Delaware Water Gap, PA) intracellular recording amplifier. Three principal components were measured from these recordings: 1) maximum amplitude, 2) rise time constant (τrise; latency to reach ~63% of peak), and 3) decay time constant (τdecay; latency to decay 63% from peak). Current injection was required for input resistance measurements and accomplished with the single-electrode voltage-current-clamp technique.

Contractions

Synaptically evoked force. A force transducer was custom-designed and constructed with high-gauge factor silicon wafer strain gauges (Micron Instruments, Simi Valley, CA) and routed through an A-M (Micron Instruments, Simi Valley, CA) and routed through an A-M

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transducer and amplifier. The central nervous system (CNS) was eviscerated, recorded with an established method (see text) and an FT03 Grass tension concurrent with suction stimulation to presynaptic nerves.

A shorter than the length at which a decrease in force occurred. This sequentially increased until reaching the peak of the length-tension tal data, evoked contractions were monitored as muscle length was approached to isometric conditions.

The favorable resolution (amount of silicon deformation) and stiffness with whole body modulus of strain of the beam must be matched to the force generated. A polycarbonate beam (1.5 cm in length) was used to construct the transducer. The beam was placed in a Wheatstone bridge circuit made of silicon wafers. An amplifier was used to inject current ($I$) and record voltage from a single intracellular electrode. Either stimulating techniques, suction electrode and direct stimulating electrode, are iii: A hook placed upon the posterior portion of the preparation connects to the beam of the force transducer, which utilizes a custom full Wheatstone bridge circuit made of silicon wafers. iii: An amplifier was used to inject current ($I$) and record voltage from a single intracellular electrode. Either muscle fibers were injected with a series of currents (4, 6, 8, 10, 12 pA) and the voltage responses were recorded or membrane potential ($V_m$) was recorded concurrent with suction stimulation to presynaptic nerves. B: basal tonus was recorded with an established method (see text) and an FT03 Grass tension transducer and amplifier. The central nervous system (CNS) was eviscerated, and saline, with or without amines, was washed over the preparation.

Systems DC amplifier (model 3000; Sequim, WA) at its lowest differential setting (50×). This transducer was utilized in all experiments recording evoked contractions (Fig. 1Aii; after Paterson et al. 2010). Briefly, custom-designed silicon wafers were placed in a full Wheatstone bridge configuration around the weakest point of a 0.02-in. polycarbonate beam (1.5 cm × 5 cm), yielding a signal/noise limited resolution of ~600 nN. As with any force-sensing device, the modulus of strain of the beam must be matched to the force generated. This newest generation of force beam in our laboratory was designed with whole body D. melanogaster contractions in mind and provides favorable resolution (amount of silicon deformation) and stiffness (approach to isometric conditions).

Muscle fiber length was controlled with the following procedure (except during basal tonus recordings). Prior to acquiring experimental data, evoked contractions were monitored as muscle length was sequentially increased until reaching the peak of the length-tension curve. This peak was identified empirically as the muscle length just shorter than the length at which a decrease in force occurred. This method was utilized as one part of working toward the isometric condition. Additionally, video was acquired through the microscope (TCA 5.0 MP, 8 fps; Ample Scientific, Norcross, GA) while the muscle length was adjusted. Length change during contraction was measured as the difference between the animal’s length prior to and after contraction (analysis performed with ImageJ, National Institutes of Health, Bethesda, MD). Force data were rejected if a animal length exceeded 5% (~200 µm of a 4-mm larva; mean of 10 randomly selected videos was 3.62 ± 0.49% or ~140-µm total animal length change). However, given that much of the body wall tissue of these larvae can be modeled as a viscoelastic material, small changes in total animal length cannot ensure that a particular segment’s muscle fiber length does not change relative to the length of fibers in abutting segments.

Stimuli were delivered to segmental nerves through a glass suction electrode or directly to the muscles to evoke contraction. Stimulus duration was 700 µs during experiments measuring contractions but reduced to 200 µs during membrane potential ($V_m$) recordings. Duration was also reduced during direct muscle activation, and stimuli were delivered directly through the saline ~2 mm from the longitudinal muscles. Voltage was decreased an order of magnitude below that required for neuronal activation and then was increased progressively until contraction amplitude matched that of synaptically evoked contractions prior to changing to direct stimulation. In these latter experiments, a spider toxin [α-plectoxin-Pt1a (PLTX-II); Alamone Labs, Jerusalem, Israel] was applied to block synaptic transmission. PLTX-II is a 44-amino acid peptidyl toxin produced by Plectreurus tristis and is known to effectively block voltage-gated, presynaptic calcium channels (Branton et al. 1987; Leung et al. 1989).

It has been well documented that the larval D. melanogaster preparation exhibits decay in several physiological properties (Macleod et al. 2002; Stewart et al. 1994). Extensive work has been done to maximize preparation longevity with the use of hemolymph-like saline (Krans et al. 2010; Stewart et al. 1994). Moreover, scaling equations are routinely used to account for the progressively depolarized $V_m$ that often occur over time in larval body wall muscle (Martin 1976; McLachlan and Martin 1981; Stevens 1976). A descriptive model of decay in contraction force is necessary to quantify the change in force production at various times after dissection. Although a given contraction may be lower than initial peak values obtained immediately after dissection, it may actually correspond to an augmentation, given the normal decay in contractile physiology. We quantified this physiological “rundown” in peak force over 2 h of recording (Fig. 2). Decay in peak force evoked by equal trains of nerve stimulation—in which no change in saline composition was administered—was better fit by an exponential decay function than a linear function (Fig. 2, B and C; $R^2 = 0.95$ and 0.72, respectively; $P < 0.01$, both with Pearson’s correlation, $n = 10$). In a minority of cases, a logarithmic fit [e.g., peak force ($F_{pk}$) = −0.155 ln(t) + 1; not shown] was also an acceptable model, i.e., 2 of these 10 experiments dedicated to quantifying decay were marginally better fit with a logarithmic function than an exponential function. On the basis of these experiments, we chose to use an exponential fit. We evaluated 103 additional preparations for which some manipulation of the preparation saline was made but full reversal was attained, and in 87 of those 103 preparations (84%), $R^2 > 0.92$ with an exponential fit.

Basil tonus. The posterior end of each dissected third-instar larva was pinned down to a custom-made recording dish. The anterior of the larva was attached to a Grass FT03 tension transducer (Grass Instruments, Quincy, MA) with a custom metal rod with a bent minuten pin at the distal end. The minuten pin was inserted into the larva in a manner that ensured that muscle movements were parallel to the motion of the transducer spring. Care was taken to ensure that the preparation was not overstretched. The larva was raised slightly off the dish (~15°) to prevent friction and maximize contraction transduction. Contractions were amplified with a MOD CP122A amplifier.
from both linear (gray; Fpk = e^{-0.013 X}) provides a statistically significant fit of the decay in contraction amplitude (R^2 = 0.95, P < 0.01, Pearson’s correlation, n = 10). Gray x are individual Fpk data from all 10 animals; black squares are mean Fpk values across all 10 animals. C: residuals from both linear (gray; Fpk = -0.0076 + 1; x = 7.48) and exponential (black; Fpk = e^{-0.013 X}; x = 2.74) functions were examined to identify the simplest function that reasonably fit the decay in contraction amplitude.

(Glass Technologies). The signal was digitized with a DATAQ DI-158U data acquisition device and then viewed and analyzed with DATAQ acquisition software. Solutions were applied directly to the larva with a peristaltic pump (0.7 ml/min, volume of dish ~300 μl). Excess solution was removed with continuous suction. Baseline recordings were taken for at least 5 min prior to exchanging saline for experimental solutions.

Data Analysis

EJPs were averaged into 30-s time intervals (6 EJPs per interval) over each 15-min trial, and each time point was then averaged over the replicate trials for each condition. Likewise, 8–10 contractions were averaged every 5 min (with a 35- or 45-s intertrial pause), and contraction trials typically lasted ~2 h. Thus hundreds of total repetitions for each experimental condition were used in computing averages. However, the number of replicates (n) reported indicates the number of animals, not repetitions. SE is computed with the number of animals and is reported unless otherwise noted. Fit equations, correlation and Pearson’s values, and t-test probabilities were generated with the statistics toolbox in MATLAB (MathWorks, Natick, MA). SigmaPlot (Systat Software, San Jose, CA) was used to generate logistic equations (3 parameters plus intercept) and analyses of variance (ANOVAs). Formulas are given in figure legends where possible, whereas statistical findings are reported in text.

Some data have been reported previously in abstract form (Ormerod et al. 2012).

RESULTS

We first characterized the OA concentration ([OA]) and TA concentration ([TA]) dependence of EJP peak amplitude when evoked via neural stimulation. At bath concentrations of >10^{-7} M, OA augmented EJP amplitude significantly (Fig. 3; P < 0.01, t-tests) in a dose-dependent manner (P < 0.01; 1-way nonparametric ANOVA), and the effect was reversible. In contrast, the action of TA on EJP amplitude was not significant at concentrations <10^{-6} M in HL-6 saline (Fig. 3D; P > 0.05). TA did not significantly change τrise or τdecay at any concentration examined (data not shown). At concentrations ≥10^{-6} M, TA significantly reduced the amplitude of EJPs (Fig. 3D), albeit much less so than OA’s augmentation at a comparable concentration. Reversibility of amine modulation of EJP amplitude required approximately the same duration of washout as exposure at high doses (i.e., 10^{-4} M; Fig. 3C), but at all lower concentrations, EJP amplitude returned to control values in <5 min of washing in control saline (HL-6, perfusion rate = 0.7 ml/min). At 10^{-5} and 10^{-4} M [OA], the augmentation of EJP amplitude was significantly greater in muscles 12 and 13 than in muscles 6 and 7 (Fig. 3D; P < 0.01, t-tests). Specifically, the mean augmentation of EJP amplitude in muscles 6 and 7 at the two highest [OA] was +29.9% of control amplitude, whereas the mean augmentation of EJP amplitude in muscles 12 and 13 was +39.9%. In an attempt to ascertain whether the OA-mediated effects on EJP amplitude were occurring in part through nonselective activation of TA receptors, we coapplied OA near its EC_{50} (10^{-6} M; Fig. 3D) and a TA receptor antagonist, yohimbine (10^{-5} M). There was no significant difference between values recorded during OA application alone and those recorded during coapplication of OA and yohimbine (e.g., 10^{-6} M [OA] + 10^{-5} M [yohimbine]; Fig. 3A; P = 0.1). However, yohimbine did block the TA-induced reduction in EJP amplitude (Fig. 3B; 10^{-6} [TA] + 10^{-5} M [yohimbine]), as there was no statistical difference between control EJP values and those collected with coapplied yohimbine and TA (P > 0.1, t-test).

We further examined these findings in HL-6 saline containing three times more calcium (i.e., 1.5 vs. 0.5 mM), because external calcium concentration has been demonstrated to influence OA-mediated effects (Klassen and Kammer 1985) (Table 1). We examined several parameters of EJPs when recorded in saline containing OA, TA, and yohimbine and observed no significant differences in amine-driven changes when using the different concentrations of calcium ([Ca^{2+}]).
We next repeated these experiments with HL-3.1 saline since it is another commonly used saline for intracellular recording from this model preparation. We first observed that EJPs were significantly larger in HL-3.1 than HL-6 saline both with 0.5 and 1.5 mM [Ca\(^{2+}\)] \((P < 0.01, t\)-test). Despite the larger initial EJP values, neither of the physiological salines nor the different calcium concentrations significantly altered the percent changes in EJP amplitude that we observed upon application of OA, TA, or antagonist. For example, EJP amplitude was 32.3 ± 0.8 mV in 10\(^{-6}\) M [OA] and 27.9 ± 2.6 mV in control HL-3.1, yielding a 19% increase in amplitude (Table 1). This ~20% augmentation in HL-3.1 was not significantly different from that achieved in HL-6, containing 0.5 mM [Ca\(^{2+}\)] (Table 1; \(P = 0.26, t\)-test). Additionally, there was no significant change in EJP peak amplitude values recorded during bath application of OA compared with EJP values obtained during coapplication of OA and yohimbine 10\(^{-6}\) M and 10\(^{-6}\) M yohimbine (10\(^{-6}\) M [OA] (dashed line) augment EJP amplitude and duration. B: averaged EJP waveforms of 10\(^{-6}\) M tyramine concentration ([TA]; gray) and coapplication of yohimbine (10\(^{-5}\) M) with 10\(^{-6}\) M [OA] (dashed line) augment EJP amplitude and duration. C: averaged EJP waveforms of 10\(^{-6}\) M tyramine concentration ([TA]; gray) and coapplication of yohimbine (10\(^{-5}\) M) with 10\(^{-6}\) M [TA] (dashed line) fall within the 95% confidence interval (dotted line) of control saline (black). C: EJP amplitude is plotted over 15 min to demonstrate the rate of reversibility/washout. Horizontal black bars indicate when OA or TA was applied. Top: reversibility occurred with latency comparable to exposure duration when 10\(^{-4}\) M amine was applied, whereas reversibility of 10\(^{-6}\) M [OA] and 10\(^{-6}\) M [TA] (bottom) began immediately upon washout. D: EJP amplitude increased from control (dashed line) with OA application in a dose-dependent manner, whereas amplitude slightly decreased with TA application. The action on m 12/13 was greater than on m 6/7 at high doses of OA, whereas TA maintained a conservative effect on m 12/13 and m 6/7.

Table 1. Summary of excitatory junctional potentials in two salines

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|-----------------|-----------------|
| **EJP Values in HL-6, mV** | **EJP Values in HL-3.1, mV** |
| 0.5 mM [Ca\(^{2+}\)] | 1.5 mM [Ca\(^{2+}\)] | 0.5 mM [Ca\(^{2+}\)] | 1.5 mM [Ca\(^{2+}\)] |
| Control | 16.47 ± 2.2* | 21.34 ± 3.1 | 26.16 ± 2.99* | 27.9 ± 2.6 |
| 10\(^{-6}\) M OA (EC\(_{50}\)) | 20.18 ± 2.1 (22.5%) | 26.85 ± 2.2 (25.80%) | 31.89 ± 2.01 (22.27%) | 32.3 ± 0.8 (19%) |
| 10\(^{-6}\) M OA + 10\(^{-5}\) M YOH | 20.77 ± 3.88 (26.1%) | 26.63 ± 3.41 (24.8%) | 33.28 ± 2.23 (27.60%) | 34.4 ± 2.1 (25%) |
| 10\(^{-6}\) M TA | 14.83 ± 4.02 (−9.9%) | 19.52 ± 5.07 (−8.54%) | 25.57 ± 5.7 (−9.90%) | 25.4 ± 4.1 (−9.0%) |
| 10\(^{-6}\) M TA + 10\(^{-5}\) M YOH | 15.81 ± 3.57 (−3.98%) | 20.75 ± 4.10 (−2.77%) | 25.55 ± 6.75 (−2.33%) | 27.7 ± 5.7 (−0.6%) |

Values are mean ± SE excitatory junctional potential (EJP) amplitudes recorded from third-instar body wall muscle during application of octopamine (OA), tyramine (TA), and the antagonist yohimbine (YOH) in 2 different physiological salines (HL-3.1 and HL-6) and 2 different calcium concentrations ([Ca\(^{2+}\]): 0.5 mM and 1.5 mM); \(n_{\text{animals}} > 4\) for each metric. *Significant differences between salines.
yohimbine (Table 1; \( P > 0.1, t\)-test). Application of TA once again attenuated EJP amplitude in HL-3.1 saline as it did in HL-6 (i.e., \( 10^{-6} \) M [TA]; \( P < 0.01, t\)-test).

Cell-specific differences were also examined in saline containing 0.5 mM and 1.5 mM calcium (Table 2; \( n_{\text{animals}} \geq 8 \), all metrics). We chose to examine the cell-specific effects of amines in HL-6 because, in general, the action of amines on EJP amplitude was not different between the two salines (i.e., \% change was comparable and not statistically different between HL-3.1 and HL-6).

We measured voltage deflections to brief (~1 s) pulses of hyperpolarizing current steps (Fig. 4A) to estimate input resistance of the muscle cells and evaluate the action of the amines on muscle membrane properties. At concentrations \( > 10^{-5} \) M, OA significantly decreased input resistance (Fig. 4B; \( P < 0.01 \) for \( 10^{-5}–10^{-3} \) M [OA]) and did so in a dose-dependent manner (Fig. 4C; \( P < 0.05 \); 1-way nonparametric ANOVA). TA did not have a significant dose-dependent action on input resistance (\( P < 0.05 \); 1-way nonparametric ANOVA), although at the highest dose examined there was a statistical difference between input resistance estimated in TA and control saline (\( P < 0.05 \), \( t\)-test).

OA modulated several components of contraction force. Most notably, even at low doses, OA increased the peak amplitude of contractions elicited by stimulating the motor nerve at 25 Hz (Fig. 5A). Modulation of contraction force was significantly dependent on changes in [OA] (Fig. 5B; 1-way nonparametric ANOVA, \( P < 0.01 \)). The OA-dependent augmentation of force saturated above \( 10^{-4} \) M [OA] and yielded 32.3 ± 6.7% greater force than observed in controls (Fig. 5B). When combined with the TA receptor antagonist yohimbine (\( 10^{-5} \) M), [OA] at \( 10^{-4} \) M augmented force 29.34 ± 2.26%, which was not statistically different from OA alone (\( P > 0.05 \), \( t\)-test). Minimal augmentation was observed at concentrations of \( 10^{-8}–10^{-7} \) M [OA], ~8 ± 6%, which may be attributable to the modest scaling routine utilized to counteract physiological rundown (MATERIALS AND METHODS). The dose at which 50% of OA augmentation was achieved was estimated with a standard logistic equation and was \( 5.3 \times 10^{-6} \) M [OA].

Since high doses of OA induced a greater change in EJP amplitude among muscles 12 and 13 than muscles 6 and 7, we next evaluated the cell specificity of its action on contraction force by ablating either muscles 6 and 7 or muscles 12 and 13. Greater augmentation was observed when muscles 12 and 13 were left intact than when muscles 6 and 7 were left intact (Fig. 5B). Across all doses tested, augmentation of force in muscles 12 and 13 was 28.59 ± 1.88% greater than in muscles 6 and 7. The augmentation of force in muscles 12 and 13 was significantly greater than the value obtained with all fibers intact at \( 10^{-4} \) and \( 10^{-3} \) M [OA] (+6.04 ± 0.88%, \( P < 0.01 \)). Likewise, the augmentation of force observed at these concentrations in muscles 6 and 7 was significantly lower than that observed when all fibers were intact (~4.5 ± 0.64%, \( P = 0.0094 \)). The greater modulation of contraction in muscles 12 and 13 compared with muscles 6 and 7 corresponds well with the cell-specific effects of OA on EJP amplitude (Fig. 3D).

We hypothesized that the OA-induced augmentation of contraction force would shift the motoneuron frequency-force relationship to the left, yielding greater forces in OA than control saline from otherwise equivalent motoneuron trains. We tested this hypothesis by selecting a single concentration of OA (\( 5.5 \times 10^{-6} \) M, approximating the \( E_{C50} \) indicated above) and measuring force of contraction prior to, during, and after OA application. The three-parameter logistics equations util-

Table 2. Cell-specific effects of OA in HL-6 saline

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<th>0.5 mM [Ca\textsuperscript{2+}]</th>
<th>1.5 mM [Ca\textsuperscript{2+}]</th>
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<tr>
<td>MF 6/7 EJP control</td>
<td>15.8 ± 3.2</td>
<td>20.3 ± 3.6</td>
</tr>
<tr>
<td>MF 6/7 10\textsuperscript{-6} M OA</td>
<td>20.2 ± 2.8 (27.7%)</td>
<td>26.3 ± 3.8 (29.9%)</td>
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<tr>
<td>MF 12/13 EJP control</td>
<td>16.6 ± 5.8</td>
<td>22.7 ± 4.9</td>
</tr>
<tr>
<td>MF 12/13 10\textsuperscript{-6} M OA</td>
<td>20.0 ± 4.5 (20.0%)</td>
<td>28.1 ± 5.7 (23.7%)</td>
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EJP values (in mV) are means ± SE for HL-6 with 2 different [Ca\textsuperscript{2+}] (0.5 mM and 1.5 mM). MF, muscle fiber.
Fig. 5. Action of amines on synaptically evoked force production. A: averaged contractions (n = 8–10 repetitions each) driven by 25-Hz stimulation of the motor nerve for 1 s: prior to application of OA (black: control HL-3.1 saline) and during bath application of OA-containing saline (gray: [OA] 10^{-4} M), coapplication of yohimbine (10^{-5} M) with 10^{-5} M [OA] (dashed gray line), application of TA (10^{-5} M) (light gray), and coapplication of yohimbine (10^{-6} M) with TA (10^{-6} M) (dashed light gray). Cross hairs indicate the points used to compute time constants (\tau: latency from preceding force inflection: either onset of contraction or relaxation). B: OA-dependent augmentation of contraction: OA dependence of F_{pk} from synaptically evoked contractions (n = 33 total). The response of all 4 muscle fibers is shown with black squares (n = 33). Open squares indicate that m 12 and 13 were ablated while m 6 and 7 were left intact (n = 11). In contrast, gray circles indicate that m 12 and 13 were intact while m 6 and 7 were ablated (n = 12). C: force-frequency (motoneuron) relationship shifts left in OA-containing saline (5.5 \times 10^{-5} M) (light gray), and coapplication of yohimbine (10^{-6} M) (dashed light gray). Cross hairs indicate the points used to compute time constants (\tau: latency from preceding force inflection: either onset of contraction or relaxation). Inset: muscle contraction (raw recordings) at varying frequencies. D: time constants of contraction with TA application (circles) are not significantly different from control values. In contrast, time constants with OA application (squares) change in a dose-dependent manner consistent with increased contraction duration (n = 31 preparations). \tau_{rise} [OA] decreases, reaching peak force in less time, while \tau_{decay} [OA] increases, maintaining force longer (see text). Standard deviation is plotted in D.
A smaller than the slow, progressive increase in basal tonus, the observed was determined to be 8.8 mN (Fig. 6). To evaluate the magnitude of augmentation and compare it to that observed upon synaptic activation, we fit the change of [OA] at which 50% of the total augmentation in basal tonus was observed was determined to be 8.8 \times 10^{-7} M, whereas 90% of maximal augmentation was attained in 1.4 \times 10^{-6} M (OA). Octopaminergic augmentation of contractions was maintained in the absence of synaptic stimulation (Fig. 7). The persistent loss of contraction during axonal stimulation corresponded to a loss of synaptic depolarization of the muscle (Fig. 7A). The persistent loss of contraction during axonal stimulation was maintained in the absence of synaptic stimulation (Fig. 7B). However, contractions were recovered in basal tonus with a simple logistic equation (Fig. 6C). Indeed, there was no significant difference in the rise slope of logistic functions used to fit these two data sets—either with or without synaptic activation (Fig. 7C). Contraction data represent mean \pm SEM of 3–5 experiments performed on 3–5 preparations for each condition. OA may exist independently of synaptic activation. These experiments utilized the spider toxin PLTX-II which is a known presynaptic voltage-gated calcium channel blocker (Branton et al., 1987). An early work characterizing the action of the PLTX molecule was performed in 1987. Early work characterizing the action of the PLTX molecule was performed in 1987.
between $10^{-4}$ and $10^{-5}$ M [OA] in both conditions ($8.0 \times 10^{-5}$ M [OA]), and the force associated with synaptically driven contractions increased similarly, $\sim 32\%$. OA also augmented force significantly in directly stimulated muscles after block of neuromuscular synapses. The significant OA-induced reduction in input resistance and dramatic increases in muscle tonus, far exceeding synaptically driven changes in force production, provide additional evidence for an independent postsynaptic action of OA. Additionally, OA was found to consistently potentiate EJPs to a greater extent in some fibers than in others, thereby providing evidence for cell specificity. OA also significantly shifted the motoneuron frequency-force relationship to the left; 90% of maximum force was obtained in $5.5 \times 10^{-6}$ M [OA] at only $55\%$ of the stimulus frequency required in control saline. The greatly increased relaxation/decay time of contractions, taken together with augmented force, suggests a robust action on muscle contractile properties and work potential. The coapplication of the selective TA receptor antagonist yohimbine (Donini and Lange 2004) indicates that, even at high concentrations of OA application, the effects are unlikely to be confounded by TA receptor activation. We further tested this by using cyproheptadine, a blocker of amine receptors shown to only weakly antagonize OA receptors in locust but block TA receptors slightly better (Orchard and Lange 1986). Once more, the action of cyproheptadine, when coapplied with amine, was insignificant (data not shown).

OA elicited a dose-dependent decrease in muscle fiber input resistance, suggesting that OA opens ion channels and/or greatly activates exchanger rates at the muscle membrane (Fritz et al. 1979; Walther and Zittlau 1998). Given the significant reduction in input resistance, Ohm’s law predicts a concurrent reduction in EJP amplitude. However, application of OA demonstrated a substantial dose-dependent increase in EJP amplitude relative to control preparations, suggesting that the drop in input resistance is more than compensated by an increase in synaptic current. It has been shown previously that OA increases miniature end-plate potential (mepp) frequency (Evans 1981; O’Gara and Dewes 1990), but previous reports do not indicate an effect of OA on mepp amplitude (Evans 1981). Nonetheless, we observed a significant decrease in muscle membrane resistance ($\leq 20\%$) and an increase in postsynaptic potential amplitude. A plausible explanation is that OA increases EJP amplitude by increasing the amount of transmitter released per nerve impulse. Hidoh and Fukami (1987) reported that OA increased EJP amplitude in mealworm (Tenebrio molitor) larvae by roughly 40% at concentrations at or above $10^{-6}$ M. They also observed a significant increase in mepp frequency and no change in mepp amplitude following
OA application (Hidoh and Fukami 1987). These observations, coupled with a significant increase in quantal content, led them to speculate that an increase in intracellular Ca\(^{2+}\) was responsible for the increased EJP amplitude. More recently, OA has been shown to enhance transmitter release in Aplysia neurons via an increase in calcium entry at synaptic boutons (Jin et al. 2012). Our demonstration of unwavering EJP augmentation (\(\sim 25\%\) at \(10^{-6}\) [OA]) by OA in saline with either 0.5 (HL-6) or 1.5 (HL-3.1) mM [Ca\(^{2+}\)] supports a presynaptic effect consistent with these previously reported.

Of the principal parameters of contraction force altered by OA, the most noticeable initially was a significant increase in synthetically evoked force of contractions. High concentrations (i.e., \(10^{-4}\) M [OA]) generated \(\sim 30\%\) greater force than observed in control saline, which was comparable to the observed increases in EJP amplitude (\(\sim 35\%\)) at the same OA concentration. OA has previously been demonstrated to have an effect on twitch amplitude in a variety of arthropod species (O’Gara and Dewes 1990), although, interestingly, it is sometimes opposite in sign (Evans 1981; Evans and O’Shea 1978, 1979; Evans and Siegler 1982). OA potentiates striated muscle contractions in crayfish (Fisher and Florey 1983), lobster (Kravitz et al. 1980), and crab (Rane et al. 1984). The EC\(_{50}\) for force augmentation reported here (\(3.3 \times 10^{-6}\) M) is similar to what Evans (1981) reported for the effect of OA on twitch amplitude in locust (\(3.3 \times 10^{-6}\) M). OA application also significantly increased the relaxation time (decreased rate of decay) of contractions in the present study. Here again, the effects observed on EJPs translated well to force recordings; the \(\tau_{\text{decay}}\) for EJP was 22\% greater than control (\(10^{-5}\) M [OA]), and the \(\tau_{\text{decay}}\) for synthetically driven contraction force at the same concentration was 30\% greater than control. A number of previous arthropod investigations report a significant decrease in relaxation time (increase in the relaxation rate) of twitch amplitudes and muscle contractions, which is opposite in sign to our results (O’Gara and Dewes 1990; O’Shea and Evans 1979; Whim and Evans 1988, 1989). In most cases, however, OA application increases EJP amplitudes, which correlates well with increases in the force of twitch amplitude. The apparent differences in the rates of relaxation could be attributable to several factors: 1) variability of muscle type within a species (e.g., slow twitch vs. fast twitch; Atwood et al. 1965; Wiersma and Van Harreveld 1938) and across life stages (i.e., insect flight vs. larval muscles; Dudley 2000; Paterson et al. 2010), 2) developmental strategies (hemimetabolous vs. holometabolous; Hoyle 1983; Konopova et al. 2011), 3) the evolution of different intramuscular proteins [i.e., expression level of giant sarcomere-associated proteins (gSAPs), discussed below], or 4) variations in recording methodology (discussed below).

Although the OA-dependent changes in contraction force during synthetically driven recordings were significant, they were small compared with the effects of OA on basal tonus. Application of \(10^{-5}\) M OA-containing saline resulted in a 1.1-mN increase in the force generated by synaptic activation, compared with a 28-mN change in basal tonus without synaptic activation. Not only do these results highlight a profound postsynaptic effect, but the 28 mN of basal tonus augmentation is drastically larger than what has been observed under normal physiological stimulation paradigms (Paterson et al. 2010). This provides additional evidence that OA may be working on extrajunctional receptors or influencing other intramuscular properties (discussed below). To verify that the effects on basal tonus were independent of the synapse, we pharmacologically blocked the presynaptic contribution and directly depolarized muscles. Force was augmented \(\sim 22\%\) under these conditions (\(10^{-4}\) and \(10^{-3}\) M), suggesting that only about one-third of the 32\% augmentation of force via synaptic activation is attributable to larger EJPs. Previous reports on the effects of OA consistently demonstrate a reduction in basal tonus of skeletal muscle in locust and cricket (O’Gara and Dewes 1990; O’Shea and Evans 1979; Whim and Evans 1988, 1989). Similar to the effects on relaxation rate, the unexpected effects we report on basal tonus may be attributable to several factors (some biological factors are indicated above). However, a second explanation for these findings involves the recording apparatus utilized in most studies. Commercial force transducers (i.e., Grass FT03) often rely on a large spring constant that maintains a particular muscle preparation length. Our force beams are matched specifically to the tissue of larval D. melanogaster and thus utilize a far lesser modulus of elasticity of the force of the beam itself (and associated smaller spring constant). If internal muscle resistance is decreased by fight-or-flight hormones—hypothetically to empower greater contractions—then a large transducer spring constant could effectively mask increased force capabilities by simply resisting length change more effectively. Nevertheless, our basal tonus observations provide further evidence for an independent postsynaptic target of OA. Moreover, these results demonstrate that in the presence of a modulatory substance the EJP is not necessarily the sole indicator of force production, which suggests that caution should be taken in drawing conclusions about muscular force from electrophysiological data alone.

In addition to the dose-dependent increases in EJP amplitude following OA application, OA potentiated EJPs more strongly in some muscle fibers (12 and 13) than in others (6 and 7). Monastirioti et al. (1995) demonstrated differential OA expression within motoneuron subtypes innervating Drosophila larval body wall muscles with immunoreactivity. They concluded that OA-immunoreactive boutons innervated muscles 12 and 13 but not muscles 6 and 7. If the presence of OA-immunoreactivity is tightly correlated with the capacity to be modulated by exogenous OA, we would have predicted little or no increase in EJP amplitude for fibers 6 and 7. Thus the presence or absence of OA within synaptic boutons does not correlate well with the ability of the innervated muscle fibers to respond to exogenous OA application. This conclusion is consistent with the accepted view that OA acts as a neurohormone (e.g., during fight or flight) in addition to its function as a neurotransmitter. Our data indicate that the absence of OA-immunoreactivity in muscles 6 and 7 does not exclude them from modulation by OA but may indicate the presence of additional cellular machinery (i.e., receptors) in muscles 12 and 13 since that pair exhibited greater augmentation of EJP amplitude and decay time.

Our data support the idea that OA can and does act in a cell-specific manner in muscles. We thus sought to determine whether this cell-specific difference in EJP potentiation extends to force generation. Using an ablation technique, we eliminated muscles 6 and 7 from our recordings and examined OA-dependent force changes associated with synthetically driven contractions. With only muscles 12 and 13 intact, at
10^{-4} and 10^{-3} M [OA] we observed a 38 ± 5% increase in force, a 6.0 ± 0.9% increase over the augmentation observed with all muscles intact. Next, we eliminated muscles 12 and 13, leaving 6 and 7 intact, and observed a 28 ± 2% augmentation in force at 10^{-4} and 10^{-3} M [OA], a 4.5 ± 0.6% decrease compared with all fibers intact. These results demonstrate that the cell-specific effects on EJPs correspond to complementary cell specificity in force augmentation.

We demonstrated here that OA's metabolic precursor TA decreased the amplitude of EJPs, although modestly relative to the actions of OA, as previously shown (Nagaya 2002). Interestingly, TA's inhibitory effect at the synapse did not translate to a reduction in force production (Fig. 5) or basal tonus (Fig. 6). Additionally, coapplication of OA and the TA-selective antagonist yohimbine did not affect the amplitude or time constants of evoked muscle contractions. Thus both the synaptic and muscle-specific effects of OA appear to be independently modulated and independent of any nonspecific interaction with TA receptors.

It has been shown for a multitude of neuromodulators/neurotransmitters within the Drosophila CNS that signaling molecules often recruit specific subsets of neurons in order to produce/alter a specific behavior. Examples include effects of OA on male social behavior (Certel et al. 2010) and the roles of dopamine in stress (Neckameyer and Weinstein 2005) and of serotonin (5-HT) in sleep (Yu et al. 2008). While behaviors are controlled and coordinated centrally, the effector cells should be modulated in a manner that complements changes in motor output generated within the CNS. OA has provided evidence for a role in coordinating behavior, from the CNS (Certel et al. 2010) to the periphery (Fox et al. 2006; Saraswati et al. 2004). Here we demonstrate independent but complementary actions of OA at the peripheral level.

Putative Mechanism and Model of Octopamine Neuromuscular Modulation

Given that coapplication of the well-established TA receptor antagonist yohimbine (Orchard and Lange 1985; Saraswati et al. 2004) with OA yielded no significant deviation from octopaminergic augmentation, we offer the following explanation for the independent, complementary pre- and postsynaptic effects. Several decades of research support the hypothesis that different isoforms of OA receptors are localized pre- and postsynaptically. The original classification scheme for OA receptors, as suggested by Evans (1981), made a clear distinction between two main classes of OA receptors (OCTOPAMINE1 and OCTOPAMINE2) based mainly on pharmacological characterizations. OCTOPAMINE1 receptors typically yield increases in intracellular calcium, whereas OCTOPAMINE2 is thought to mediate the activation of adenylate cyclase and subsequently modified intracellular adenosine 3',5'-cyclic monophosphate concentration ([cAMP]). It is likely then, that a variant of the OCTOPAMINE1 receptor (potentially an isof orm of the OAMB receptor) is present at the presynaptic bouton. In the context of the present results, it seems plausible that a member of the OCTOPAMINE2 receptor is expressed postsynaptically. A subgroup of OCTOPAMINE2 was stated to be located postsynaptically—on the muscle—and to mediate an increase in the relaxation rate of tension (now synonymous with the β-adrenergic-like OA receptors; Evans and Maquiera 2005).

Therefore, the OA-induced changes postsynaptically are potentially attributable to the activation of a second messenger system. Drosophila possesses many cyclic nucleotide-gated channels, such as cAMP-dependent K^+ channel, that could account for the drop in input resistance (Delgado et al. 1991; Wicher et al. 2001). Interestingly, adenylyl cyclase activation typically results in cAMP-dependent phosphorylation of protein kinase A (PKA). PKA has been demonstrated to activate L-type calcium channels, which are speculated to be localized postsynaptically to larval body wall muscle in Drosophila (Basavappa et al. 1999). This activation of L-type calcium channels could also be responsible for the changes in input resistance and account for an enhancement in intracellular calcium concentrations, likely yielding increased force production.

However, our data also show a drastic increase in basal tonus, over 25 times the augmentation observed during synaptically driven contractions. An effect as prolonged as OA's effect on basal tonus could reflect changes in vital intramuscular proteins contributing to force production. Actin, myosin, and troponin/tropomyosin interactions cannot account for the prolonged, augmented state of contractility often observed in muscle physiology (e.g., Blaschko effect or catch tension; Krans 2010). Recent evidence provides support for the idea that elastic proteins of the muscle (gSAPs) that interact with actin/myosin may be responsible for such phenomena (arthropods: Hooper and Thuma 2005; Hooper et al. 2008; chordates: Nishikawa et al. 2012). If gSAP function is indeed similar across phyla, then any number of the gSAPs may form a cross bridge facilitating an indirect, long-lasting bond between actin and myosin resulting in the persistent effect on basal tonus observed here (Nishikawa et al. 2012). It is noteworthy that PKA has previously been demonstrated to phosphorylate titin (Kruger and Linke 2006) and that in chordate fibers calcium influx—here putatively augmented by PKA—increases titin stiffness (Labeit et al. 2003). Thus the downstream action of OA on OCTOPAMINE2 receptors being a change in [cAMP] provides a pathway for physiological modulation of the gSAPs, which is consistent with the exceptional change in basal tonus reported here and changes in work capacity reported elsewhere (Evans and Siegler 1982).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


ACTION OF OCTOPAMINE AND TYRAMINE ON LARVAL FRUIT FLY MUSCLE


