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Abstract: This paper investigates the effect of epinastine, a selective octopamine antagonist in invertebrates, in Caenorhabditis elegans. Specifically, its ability to block the inhibitory action of octopamine on C. elegans isolated pharynx was assayed. Isolated pharynxes were stimulated to pump by the addition of 500nM 5-hydroxytryptamine (5-HT), (113 ± 2 per 30 seconds, n=15). Octopamine inhibited the 5-HT-induced pumping in a concentration-dependent manner (threshold 1-5μM) with a 61 ± 11% inhibition with 50μM (n=5). Epinastine (0.1μM) antagonized the inhibitory response to octopamine ($P<0.001$; n=15). Tyramine also inhibited pharyngeal pumping induced by 5-HT but was less potent than octopamine. Tyramine, 1mM, gave an inhibition of pumping of 40 ± 5 % when applied at 100μM (n=5). A higher (10μM) concentration of epinastine was required to block the tryamine response compared to octopamine. It is concluded that epinastine selectively antagonizes the effect of octopamine on C. elegans pharynx. Further studies are required to test its selectivity for octopamine in other tissues and other nematodes.
Response to Reviewers: Referee 1.

Abstract. In pharmacological terms it is quite usual to talk about a threshold for a measurable effect. Note that the threshold is given as a range i.e. the effect comes in between 1 and 5 μM.

Line 22. reworded as suggested

Line 49. Information added as requested.

Line 78. They are a class of motorneuron, information added.

Line 81. Corrected.

Line 110. No, it was visual observed and counted. This is now clarified in the methods.

Line 133. This sentence has been corrected.

Line 134. Agree, and corrected.

Line 167. Stats added.

Line 171. The potentiation is statistically significant. The information has been added.

Line 193. The word 'parsimonious' has been removed

Line 241. Full stop added.

Line 262. Full stop added.

Line 336. Case corrected.

Line 372. Corrected.

Referee 2.

Thanks for pointing this out. For the sake of completeness a sentence referring to the biogenic amine-gated ion channels has been included in the introduction and the reference Ringstad et al 2009 added to the reference list.

Line 21. Agreed, and amended.

Line 40. Agreed, and amended.

Line 53. Corrected

Line 76. Agreed, sentence has been restructured.

Line 133. Corrected

The effect of a selective octopamine antagonist, epinastine, on pharyngeal pumping in *Caenorhabditis elegans*.

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Abstract

This paper investigates the effect of epinastine, a selective octopamine antagonist in invertebrates, in *Caenorhabditis elegans*. Specifically, its ability to block the inhibitory action of octopamine on *C. elegans* isolated pharynx was assayed. Isolated pharynxes were stimulated to pump by the addition of 500nM 5-hydroxytryptamine (5-HT), (113 ± 2 per 30 seconds, n=15). Octopamine inhibited the 5-HT-induced pumping in a concentration-dependent manner (threshold 1-5µM) with a 61 ± 11% inhibition with 50µM (n=5). Epinastine (0.1µM) antagonized the inhibitory response to octopamine ($P<0.001$; n=15). Tyramine also inhibited pharyngeal pumping induced by 5-HT but was less potent than octopamine. Tyramine, 1mM, gave an inhibition of pumping of 40 ± 5 % when applied at 100µM (n=5). A higher (10µM) concentration of epinastine was required to block the tryamine response compared to octopamine. It is concluded that epinastine selectively antagonizes the effect of octopamine on *C. elegans* pharynx. Further studies are required to test its selectivity for octopamine in other tissues and other nematodes.

Keywords

Caenorhabditis elegans; octopamine; tyramine, epinastine; pharyngeal pumping.

Introduction

Octopamine is a neurotransmittter in the majority of invertebrate phyla. It plays a key role as a transmitter or modulator of synaptic transmission both centrally and at neuromuscular junctions. In addition it can function as a neurohormone. Its occurrence has been comprehensively reviewed by Roeder (1999) where it has been identified in nematodes, annelids, molluscs, arthropods and vertebrates. Most of the research on octopamine has been undertaken to establish its roles in insects where it would appear to influence almost every system including muscle and endocrine function, sensory and cardiovascular systems and various insect behaviours including learning and memory (Farooqui 2007; Roeder 1999, 2005). Octopamine is synthesized in two stages, firstly tyrosine is decarboxylated to tyramine (by tyrosine decarboxylase) and then tyramine is β-hydroxylated to octopamine (by tyramine β-hydroxylase).

Octopamine receptors have been studied in molluscs and arthropods where their predominant effect is to activate adenylate cyclase (Chang et al. 2000; Evans 1981; Gerhardt et al. 1997; Han et al. 1998). Several octopamine receptors have been cloned from insects, including *Drosophila melanogaster, Apis mellifera* and *Periplaneta americana* (Bischof and Enan 2004; Grohmann et al. 2003; Han et al. 1998). There are also tyramine receptors in insects and these are negatively coupled to adenylate cyclase. They have been cloned from *D. melanogaster, Locusta migratoria, Bombyx mori, A. mellifera* and *Periplaneta americana* (Blenau et al. 2000; Ohta et al. 2003; Rotte et al. 2009; Saudou et al. 1990; Vanden Broeck et al. 1995). Many of the receptors respond to both octopamine and tyramine but there is a family of tyramine receptors in insects which are specific for tyramine and do not show cross-reactivity to octopamine (Cazzamali et al. 2005). There is now convincing evidence for tyramine as a transmitter in insects in its own right and this evidence has been reviewed by

Lange (2009). Frequently octopamine and tyramine have different or opposite effects on a system, providing further evidence for independent functioning of the two amines (Nagaya et al. 2002; Saraswati et al. 2004; Vierk et al. 2009). In addition to biogenic amine signaling through G-protein coupled receptor cascades, in the nematode *C. elegans* it has also been shown that tyramine and dopamine directly activate ligand-gated ion channels (Ringstad et al. 2009).

There is now good evidence for octopamine as a transmitter in molluscs where, for example, it is involved in the modulation of the buccal feeding network (Vehovszky et al. 2000). Epinastine, threshold 0.01 to 0.1μM, blocked both octopamine-induced hyperpolarization and synaptically evoked hyperpolarization of buccal motoneuron, B3 in *Lymnaea stagnalis*. Epinastine had no direct effect on the membrane potential of B3. There is also evidence that octopamine modulates *L. stagnalis* locomotion and this effect of octopamine is antagonized by epinastine. Octopamine receptors have been cloned from molluscs (Chang et al. 2000; Gerhardt et al. 1997).

In *Caenorhabditis elegans* there is a pair of head interneurons in the lateral ganglion, RICs, which contain both tyrosine decarboxylase and tyramine β-hydroxylase. A second pair of motoneurons, (the RIM motoneurons), regulate reversal frequency and head movements but only contain tyrosine decarboxylase (Alkema et al. 2005). The former are octopaminergic neurons while the latter are tryptaminergic. When octopamine is applied to *C. elegans*, pharyngeal pumping and egg-laying are reduced (Horvitz et al. 1982). The first tyramine receptor in *C. elegans* was characterized by Rex and Komuniecki (2002). This receptor, SER-2, has a higher affinity for tyramine than for octopamine and couples to a decrease in cAMP levels together with a rise in intracellular calcium. These authors also identified an

alternatively spliced isoform for SER-2, SER-2A. SER-2A has a lower affinity for octopamine and dopamine than does SER-2 (Rex et al. 2004). A second tyramine receptor, TYRA-2 has been identified in *C. elegans,* this receptor also has a greater affinity for tyramine compared with either octopamine or dopamine (Rex et al. 2005). Tyramine increases GTPγS binding in membranes from cells expressing TYRA-2 receptors.

Epinastine (3-amino-9,13b-dihydro-1h-dibenz(c,f)imidazo(1,5a)azepine HCl) is a non-sedating histamine H_1 receptor antagonist first used in vertebrates (Fugner et al. 1988) but subsequently found to be a highly selective antagonist for insect octopamine receptors (Roeder et al. 1998). Since then it has also been found to block molluscan and crustacean octopamine receptors (Vehovszky et al. 2000; Kaczer and Maldonado 2009). However, epinastine has not been examined as a potential octopamine antagonist in nematodes and this is the purpose of the present study.

Materials and Methods

Wild type N2 Bristol strain *C. elegans* were cultured in nematode growth medium agar plates at 20^{o} C as previously described (Brenner 1974). The agar plates were seeded with an OP50 *Escherichia coli* lawn. The experiments were performed on one day old adults that were transferred from these plates to a Petri dish containing Dent's saline (144mM NaCl, 10mM $MgCl_2$, 1mM $CaCl_2$, 6mM KCl and 5mM HEPES, pH 7.4) and bovine serum albumin and cut just posterior to the pharyngeal terminal bulb using a surgical blade while viewing under a binocular microscope. This cut induced the body wall muscles under the cuticle to contract, resulting in the isthmus and terminal bulb becoming exposed (Rogers et al. 2001).

The isolated pharynx was transferred using a pipette to a Petri dish containing 500nM 5-hydroxytryptamine (5-HT) in Dent's saline and pharyngeal pumping was counted by visual observation for 30 seconds, at 2 minute intervals, over a 10 minute period. The pharynx was then transferred to another Petri dish containing the drug under test plus 5-HT and the rate of pharyngeal pumping recorded for 30 seconds, every 2 minutes, for 10 minutes for different drug concentrations. The pharynx was finally transferred to another Petri dish containing 5-HT and the pumping rate monitored for a further 10 minute period as above. For experiments using epinastine, following incubation with 5-HT, the pharynx was transferred to a Petri dish containing varying concentrations of epinastine plus 5-HT for 6 minutes. The pharynx was then transferred to a dish containing 5-HT and the drug under test for a further 6 minutes and the pumping rate recorded as described above.

5-HT creatine sulphate, octopamine HCl and tyramine HCl were obtained from Sigma while epinastine HCl was a gift from Boehringer Ingleheim,

Data are expressed as the mean ± s.e.mean of 'n' determinations. Statistical analysis employed one-way Anova with Bonferroni's multiple comparison test or Mann Whitney test, as indicated. Significance level was set at $P<0.05$.

Results

The basal pumping rate of the cut pharyngeal preparation in the absence of 5-HT was less than 20 pumps per minute. In the presence of 500nM 5-HT the pumping rate became relatively stable after 2-4 minutes at around 100 pumps in 30s which equates well to the feeding rate observed in the intact animal in the presence of food (Figure 1A; Avery and Horvitz 1990). 5-HT (500nM) induced a pumping rate of 113 ± 2 pumps over a 30 second

period, n=15. The pharynxes were exposed to drugs by transferring them between dishes containing the different solutions and therefore the effect of this mechanical disturbance on pumping rate was determined. As can be seen from Figure 1A, there was a small, transient decrease in pumping rate of around 13% (indicated with arrow) when pharynxes were transferred between dishes (both containing 500nM 5-HT) but this rapidly returned to the control value. This is consistent with previous reports that mechanical disturbance causes a transient decrease in pharyngeal pumping (Chalfie et al. 1985).

The effect on pumping rate of applying octopamine is shown in Figure 1B. When the pharynx was transferred from 5-HT to 5-HT plus octopamine there was a rapid, concentration-dependent inhibition of pumping that was apparent when the first measurement was taken after 2 minutes in the drug. The pharynxes were exposed to octopamine for 10 minutes. In the presence of 10 and 20µM octopamine and lower concentrations (1 and 5µM, not shown), during this period of drug application the pumping rate gradually returned to the same level observed before the addition of octopamine. However, in the presence of the highest (50 µM) concentration of octopamine tested the inhibition persisted for the duration of drug application. At this highest concentration the effect of octopamine was still fully reversible and the pumping rate recovered when the pharynx was returned to 5-HT alone (Figure 1B).

The effect of epinastine on the response to octopamine was tested by pre-exposing the pharynxes to this drug at a range of concentrations before testing for octopamine inhibition. Epinastine inhibited the response to octopamine (10µM) with a threshold between 0.01 and 0.1µM (Figure 2). Epinastine at these concentrations had little direct effect on pharyngeal pumping over and above that expected from the mechanical disturbance (Figure 2).

The inhibitory effect on pumping rate of increasing concentrations of tyramine was also determined (Figure 3A). Unlike octopamine, the inhibitory effect was transient even at the highest concentration (1mM) tested suggesting that tyramine has less efficacy than octopamine on the pharyngeal system. The transient nature of the response to tyramine confounded the ability to accurately resolve the drug inhibitory effect from the inhibition caused by the mechanical transfer of the pharynxes. Nonetheless, the inhibitory effect in the presence of 50µM tyramine was greater than that observed by mechanical transfer alone (Figure 1A, 3B; reduction in pumps between 10 and 12 min time-point for control -22±1 pumps in 30s, for 50µM tyramine -61±15 pumps in 30s, $p<0.001$, unpaired Student's t-test, n=5) indicating that this amine has an effect on pumping. The inhibition was less than that observed for the same concentration of octopamine (Figure 3B). The effect of epinastine (0.1, 1.0 and 10µM) on the response to tyramine was tested, but only the highest concentration (10µM) elicited a significant block (Figure 3B) whilst in the presence of 1µM epinastine there was a slight potentiation ($p<0.001$; unpaired Student's t-test).

Discussion

The pharyngeal system provides a very useful assay for pharmacological studies on *C. elegans* (Franks et al 2006). Here we have employed this to investigate the pharmacology of octopamine and tyramine responses, particularly with respect to the antagonist epinastine.

5-HT was used in this study to provide both a reasonably consistent and elevated pumping rate of around 100 pumps per 30 seconds against which the inhibitory effects of octopamine and tyramine could be investigated. A concentration of 500nM induced a pumping rate which remained constant over the duration of an experiment, between 30 and 45 minutes in total. 5-HT-containing neurones occur in the pharyngeal nervous system and there are a number of

5-HT receptor subtypes in *C. elegans* (Chase and Koelle 2007) including SER-1 on pharyngeal muscle, SER-4 and SER-7 on pharyngeal neurones (Hobson et al. 2003, 2006; Tsalik et al. 2003). Thus 5-HT can modulate pharyngeal activity both through a direct effect on the muscle and indirectly via pharyngeal neurones though it has been proposed that SER-7 receptors, located on MCs, are required for the stimulation of pharyngeal pumping by 5-HT (Hobson et al. 2006).

Exogenous octopamine and tyramine both inhibited 5-HT stimulated pharyngeal pumping. This is consistent with earlier studies (Rogers et al 2001). An explanation of this effect, given that these molecules are all agonists at biogenic amine receptors, is that inhibitory transmitters octopamine and tyramine act as physiological, rather than pharmacological, antagonists of the excitatory 5-HT response. Since neither octopamine nor tyramine is present in neurones within the pharyngeal nervous system these amines must act as neurohormones to regulate pharyngeal pumping. The tyramine receptor, SER-2 is present on both pharyngeal muscle and neurones (Tsalik et al. 2003; Rex et al. 2004) while the proposed octopamine receptor, SER-3, (Suo et al. 2006) probably occurs in the pharyngeal system since the isolated pharynx preparation responds to octopamine (Rogers et al 2001).

Epinastine has been used as an antagonist of octopamine in a number of preparations where it acts in the low µM range (Unoki et al. 2006; Vehovszky et al. 2000). In other experiments where the antagonist is injected into the whole animal, diffused into a sensillum or applied transdermally, higher concentrations of epinastine have been used (Flecke and Stengyl 2009; Kaczer and Maldonado 2009; Ormshaw and Elliott 2006; Roeder et al. 1998). In the present study epinastine, 0.1µM, significantly reduced the inhibitory response to octopamine but only the higher concentration (10µM) significantly antagonized the effect of tyramine. In some of

the experiments there was an indication that higher concentrations of epinastine might have a small direct inhibitory effect on pharyngeal pumping and this direct effect might have masked an antagonism of the octopamine effect. It is not uncommon for vertebrate antagonists to possess some agonist activity on invertebrate receptors (Boyd et al. 1985).

The observation that epinastine was less effective at blocking the response to tyramine compared to the response to octopamine is consistent with the suggestion that these two agonists exert their inhibitory actions through distinct receptor pathways (Alkema et al 2005) and that epinastine is a selective octopamine antagonist. However, an alternative explanation is that tyramine exerts its effects in this preparation by acting as a weak agonist at octopamine receptors. Further studies employing receptor mutants are required to resolve this issue. Indeed, if tyramine were acting through specific tyramine receptors it might be expected to be effective at lower concentrations than observed in this study as tyramine receptors which are expressed on pharyngeal neurons (including MC and NSMs) have a greater affinity for tyramine than octopamine (Rex et al. 2005). Previously it has been shown that in *ser-2* mutants tyramine fails to inhibit pharyngeal pumping (Rex et al. 2004), suggesting that its action is through SER-2 receptors. In this regard, it would be interesting to test the effect of octopamine on *ser-2* mutant pumping.

Phentolamine is another compound which has been used to block octopamine responses in invertebrates (Evans 1981; Roeder 2005; Vehovszky et al. 2000) and also blocks octopamine inhibition of pharyngeal pumping in *C. elegans* (Carmaciu,Walker, Holden-Dye, unpublished). While octopamine inhibits egg-laying in *C. elegans*, phentolamine excites egg-laying, suggesting that phentolamine blocks the inhibitory action of endogenous octopamine (Horvitz

et al. 1982). Therefore in future experiments it would be interesting to see if epinastine also enhances egg-laying.

In conclusion, this study has provided evidence for epinastine as a selective antagonist for octopamine in *C. elegans* pharynx. This drug may therefore prove to be a useful tool for further characterization of octopamine receptors in nematodes.

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Figure legends.

Figure 1. The inhibitory effect of octopamine on 5-HT stimulated pharyngeal pumping. A. The number of pharyngeal pumps in 30s was counted every 2 minutes for the duration of the 34 minute experiment. Data are the mean ± s.e.mean of 5 determinations. 500nM 5-HT was present throughout. The arrows indicate the times at which the pharynx was transferred between dishes. It can be seen that transferring worms between dishes caused a transient mechanically induced inhibition of pharyngeal pumping rate. B. The experiment was performed in the same manner as for 'A' except the pharynxes were transferred to a dish containing both 500nM 5-HT and octopamine (at the concentration indicated) between time-point 10 and 12 min. Data are the mean ± s.e.mean of 5 determinations. Between time-point 22 and 24 min the pharynxes were transferred to another dish containing only 500nM 5-HT. The hatched area indicates measurements taken in the presence of octopamine.

Figure 2. The effect of epinastine on the pharyngeal response to octopamine. The number of pharyngeal pumps in 30s was counted every 2 minutes for the duration of the 36 minute experiment. Data are the mean ± s.e.mean of 5 to 15 determinations, as indicated. 500nM 5-HT was present throughout. The solid line indicates that epinastine is present at the concentration indicated. The hatched area indicates that 10µM octopamine is also present in the dish. Note the reduced inhibitory response to octopamine in the presence of 0.1µM epinastine.

Figure 3. The effect of tyramine and epinastine on pharyngeal pumping. A. The number of pharyngeal pumps in 30s was counted every 2 minutes for the duration of the 34 minute experiment. Data are the mean ± s.e.mean of 5 determinations. 500nM 5-HT was present

throughout. The hatched area indicates measurements taken in the presence of tyramine. B. A comparison of the effect of octopamine and tyramine. The % inhibition was determined from the pumping rate immediately before drug addition compared to the pump rate 2 min after drug addition. This therefore included a small (~10%) effect due to the mechanical disturbance of the pharynx as described in the results. ‘oct’ is 50μM octopamine; ‘tyr’ is 50μM tyramine; ‘tyr & 1μM epi’ is 50μM tyramine with 1μM epinastine; ‘tyr & 10μM epi’ is 50μM tyramine with 10μM epinastine. Data are mean±s.e.mean, n=5. $P<0.01$, Mann Whitney test.

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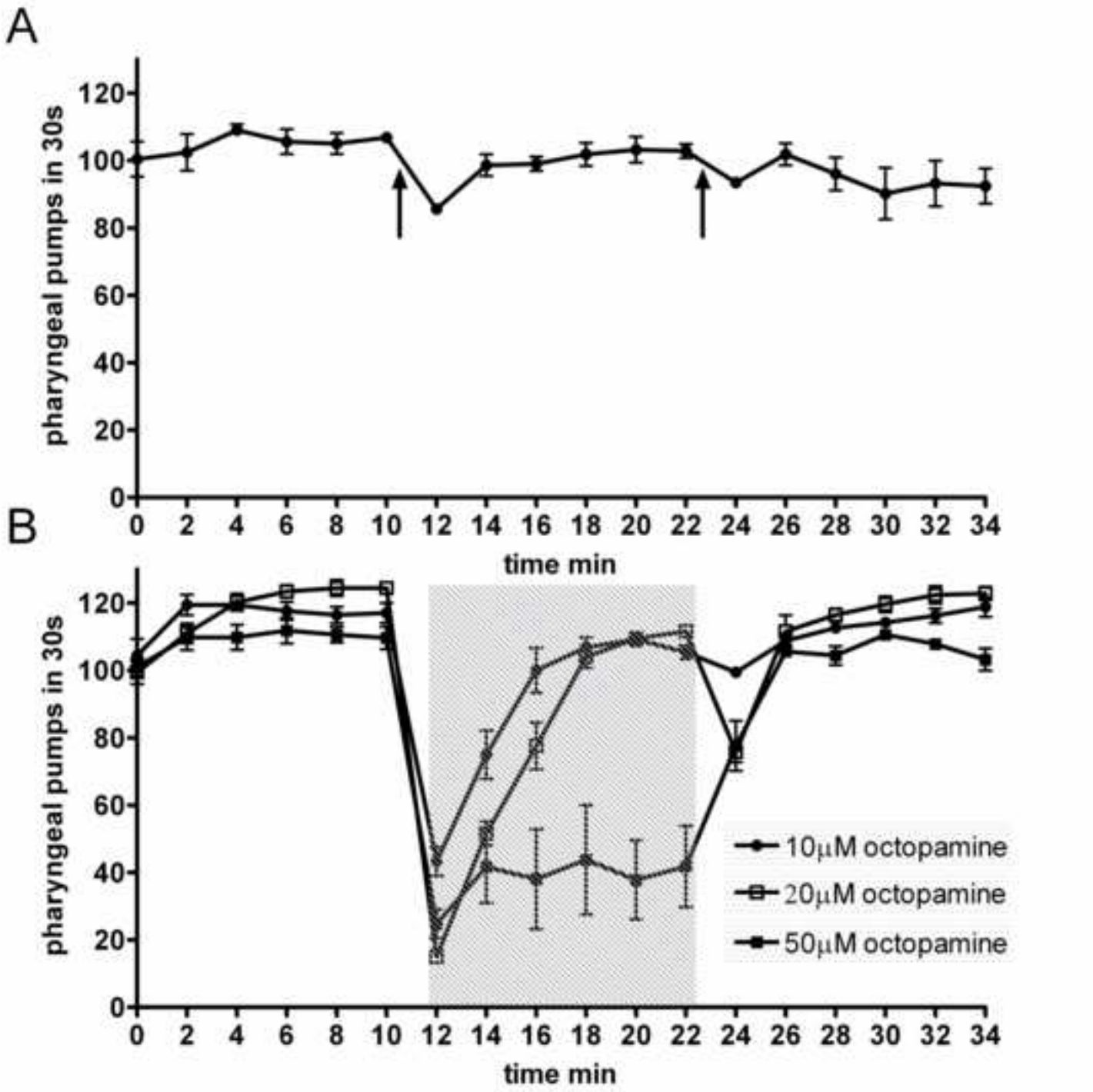

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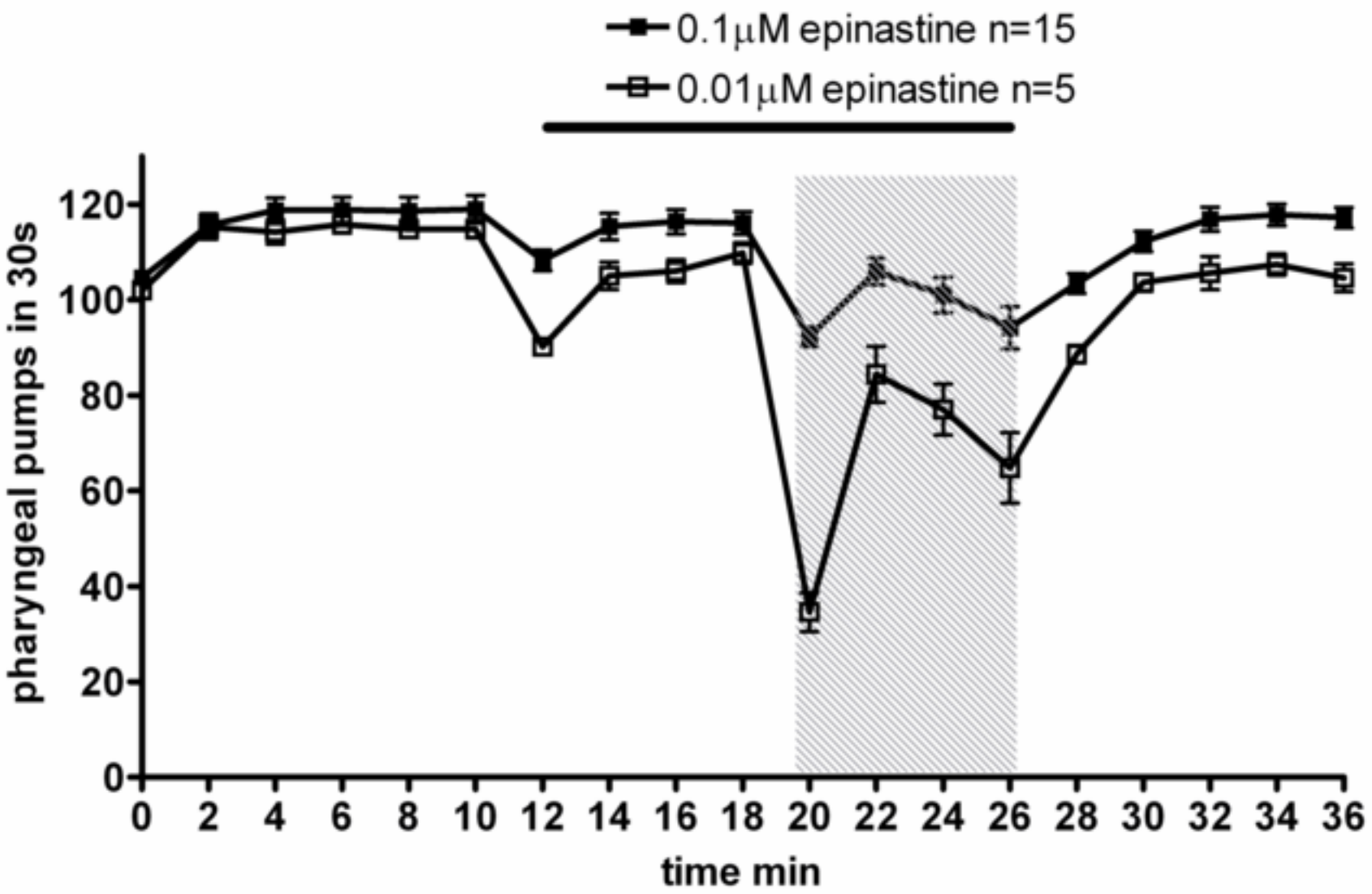

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