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Title: K^+ absorption by locust gut and inhibition of ileal K^+ and water transport by FGLamide allatostatins.

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Running Head: FGLa/ASTs inhibit ileal K^+ transport

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SUMMARY

The Scanning Ion-Selective Electrode Technique (SIET) was utilized for the first time in *Locusta migratoria* to characterize K^+ transport along the digestive tract and to determine the effect of two locust FGLamide allatostatins (FGLa/ASTs) on K^+ transport: a previously sequenced FGLa/AST from *Schistocerca gregaria* (Scg-AST-6; ARPYSFGL-NH_2) and a newly sequenced FGLa/AST from *L. migratoria* (Locmi-FGLa/AST-2; LPVYNFGL-NH_2). Regional differences in K^+ fluxes along the gut were evident, where K^+ efflux *in vitro* (or absorption into the hemolymph *in vivo*) was greatest at the anterior ileum, and lowest at the colon. Ileal K^+ efflux was inhibited by both Scg-AST-6 and Locmi-FGLa/AST-2, with maximal inhibition at 10^{-10} and 10^{-11} M, respectively. Both FGLa/ASTs also inhibited cAMP-stimulated K^+ efflux from the ileum. Locmi-FGLa/AST-2 also inhibited efflux of water across the ileum. Locusts are terrestrial insects living in dry climates, risking desiccation and making water conservation a necessity. The results suggest that FGLa/ASTs may be acting as diuretics by increasing K^+ excretion and therefore increasing water excretion. Thus, it is likely that FGLa/ASTs are involved in the control of hemolymph water and ion levels during feeding and digestion, to help the locust deal with the excess K^+ load (and subsequently fluid) when the meal is processed.

KEYWORDS

Locusta migratoria, peptide, FGLa/AST, SIET, ion, ileum, hindgut, second messenger, cAMP, sequencing, MALDI-TOF

ABBREVIATION

FGLa/AST	allatostatin family with C-terminal ending of FGLamide
Locmi-FGLa/AST-2	*Locusta migratoria* allatostatin-2 (LPVYNFGLa)
ITP	Ion transport peptide
Scg-AST-6	*Schistocerca gregaria* allatostatin-6 (ARPYSFGLa)

INTRODUCTION

The ability of insects to regulate water and ion levels in varying environmental conditions is fundamental to their survival and depends on the coordinated activities of ion and osmoregulatory organs. Terrestrial insects rely primarily on the actions of the Malpighian tubules and hindgut to achieve ion and osmoregulation. The Malpighian tubules actively secrete ions from the hemolymph into their lumen, which is followed by water through the process of osmosis (see Schooley et al., 2011). The fluid produced by the Malpighian tubules enters the hindgut where in terrestrial insects both ions and water can be selectively reabsorbed as required to maintain homeostasis (Coast at al., 1999). Plant feeding insects need to cope with excess K^+ and Mg^{2+} from their diet (Dow, 1986; Dow et al., 1984). It has been shown that adult locusts fed on grass have 1.6 times more K^+ in their hemolymph relative to starved locusts (Hoyle, 1954). Likely as a result of this plant feeding strategy the Malpighian tubules of locusts produce a KCl rich fluid that also contains NaCl (Hanrahan and Phillips, 1983). As this fluid enters the hindgut the epithelium has the capacity to reabsorb large quantities of KCl and NaCl (Phillips, 1964) along with water (Audsley et al., 2013). The latter is of particular significance for terrestrial insects that have high surface to volume ratios and lose water through spiracular evaporation rendering them vulnerable to desiccation (Loveridge, 1968).

The ion transport mechanisms of the Malpighian tubules and the locust hindgut have been extensively studied and models proposed (see Coast, 2007; Phillips and Audsley, 1995; Audsley et al., 2013). Transport of cations in the Malpighian tubules is driven by an apical V-type H^+-ATPase which provides protons in the luminal space that are used by cation/H^+ exchangers to move Na^+ and K^+ into the lumen. On the basal side, Na^+, K^+ and Cl^- enter the cells through $Na^+/K^+/Cl^-$ co-transporters relying on a Na^+ gradient maintained by the actions of Na^+/K^+-ATPase (Coast, 2007). The ion transport mechanisms in the locust hindgut are, for the most part, consistent between the ileum and rectum where a unique Cl^- pump drives Cl^- into the cell from the lumen. K^+ follows through apical channels and Na^+ is co-transported with amino acids or exchanged for NH_4^+. Cl^- and K^+ exit the basal side into the hemolymph through channels and Na^+ exits through the Na^+/K^+-ATPase (Phillips and Audsley, 1995; Audsley et al., 2013). A number of neurohormonal factors that regulate the ion transport mechanisms of the Malpighian tubules and locust hindgut have been discovered and their actions characterized (Coast, 2007). Diuretic hormones that increase ion transport and fluid secretion by the Malpighian tubules are generally from the corticotropin releasing factor (CRF), calcitonin (CT) and kinin (K) families of peptides and include CRF-related and kinin-related peptides in the locust (Kay et al., 1991; Johard et al., 2003; Coast, 2007). Antidiuretic hormones that inhibit transport and fluid secretion by

Malpighian tubules are from the Capability (CAPA) family of peptides or related to the mealworm anti-diuretic factor (ADF); however, none of these have yet been identified in the locust (Paluzzi et al., 2008; Eigenheer et al., 2002; Coast, 2007). In the locust, the ion transport peptide (ITP), isolated from the desert locust *S. gregaria,* stimulates ion and fluid reabsorption across the isolated ileum through a cAMP second messenger cascade (Audsley et al., 1992a; Audsley et al., 1992b; Audsley et al., 2013). The chloride transport stimulating hormone (CTSH), which has yet to be fully characterized, stimulates rectal ion and fluid reabsorption, also utilizing cAMP (Spring et al., 1978; Phillips et al., 1980). In larval mosquitoes, proctolin, neuropeptide F, and FGLa/ASTs (the family of allatostatins with the C-terminal FGLamide) decrease the transepithelial voltage across the anterior midgut suggesting that these factors alter ion transport activities in the midgut (Onken et al., 2004). In addition, allatotropin and extended FLRFamides, inhibit ion transport across the midgut of *Manduca sexta* (Lee et al., 1998). More recently, an allatostatin A (an FGLa/AST) was shown to modulate K^+ fluxes at the *Drosophila* midgut (Vanderveken and O'Donnell, 2014).

Studies utilizing immunohistochemistry and peptide isolation have shown that FGLa/ASTs are widely distributed within a number of tissues in a variety of insect species. For instance, FGLa/ASTs are distributed in the central and stomatogastric nervous systems; in neurohaemal organs; in neurons that project to peripheral targets including the gut; and in gut endocrine cells (see Bendena et al., 1997; Skiebe et al., 2006; Robertson and Lange, 2010; Clark et al., 2008). The widespread distribution of FGLa/ASTs in various tissues and across insect species is of particular interest since FGLa/ASTs are present in species where these peptides do not have an allatostatic function; the function for which they were originally discovered (Woodhead et al., 1989). In locusts, as well as several other insect species, FGLa/ASTs are widely known for their gut myoinhibitory effects (Veelaert et al., 1996; Robertson et al., 2012). In addition, neurophysiological studies indicate that FGLa/ASTs are involved in the neuromodulation of feeding and digestion, where they affect the frontal ganglion and ventricular ganglion rhythmic motor patterns responsible for gut contraction (Zilberstein et al., 2004; Robertson et al., 2012). Thus, the widespread distribution of FGLa/ASTs coupled with the widespread myoinhibitory role on visceral muscle, suggests that the ancestral function of FGLa/ASTs is not inhibition of juvenile hormone synthesis but is likely a control of digestive processes. FGLa/ASTs may act as brain/gut peptides involved in inhibition of gut muscle contraction and alteration of ion transport. The present study was aimed at investigating whether FGLa/AST can modulate the ion transport functions of the gut. Locmi-FGLa/AST-2 (a novel peptide for *L. migratoria*) was isolated and sequenced from extracts of the brain/retrocerebral complex of *L. migratoria*. The scanning ion-

selective electrode technique (SIET) was utilized to assess the effects of FGLa/ASTs on the K^+ transport properties of the ileum. In addition, an *in vitro* ileum preparation was used to monitor the effects of Locmi-FGLa/AST-2 on water efflux.

RESULTS

FGLa/AST sequence determination

A novel FGLa/AST of *L. migratoria* was isolated and sequenced from a 30% acetonitrile-eluted Sep-Pak fraction from extracts of adult brain and retrocerebral complexes using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Analysis revealed an ion mass (*m/*z) in agreement with the monoisotopic mass ($[M+H]^+$) of a previously identified FGLa/AST within several insects, LPVYNFGLa (Figure 1A; 921.5). This ion mass was further analyzed with tandem MS (MALDI-TOFF MS/MS) to confirm the amino acid sequence to be LPVYNFGLa (Figure 1B). We have termed this peptide Locmi-FGLa/AST-2 since this matches the peptide sequence from *S. gregaria* and termed Scg-AST-2 (Clynen and Schoofs, 2009). In addition, we confirmed and completed the partial sequences reported by Clynen and Schoofs (2009) for *L. migratoria*, namely Locmi-FGLa/AST-5 (GRLYSFGLa) and Locmi-FGLa/AST-10 (APAEHRFSFGLa).

Characterization of K^+ fluxes along the locust gut

Regional differences in the magnitude of K^+ flux along the locust gut were evident (Figure 2). Six regions along the length of the locust gut were chosen for measurement: posterior foregut, gastric caecum, midgut, anterior ileum, colon, and rectum, and all regions showed a net mean K^+ efflux (Figure 2A). The anterior ileum had a significantly higher average rate of K^+ efflux than the other gut regions, which were not significantly different from one another (Figure 2B; p=0.001; one-way ANOVA). Like the ileum, the midgut consistently moved K^+ out of the gut lumen, and had the second highest average rate of K^+ efflux (1.92±0.91 nmol/cm^2/sec). The posterior foregut had the third highest average rate of K^+ efflux of 1.55±0.45 nmol/cm^2/sec and also consistently moved K^+ out of the gut lumen. The remaining gut regions exhibited inconsistency in the direction of K^+ movement across the gut basolateral membrane (Figure 2A, B). For instance, the colon had the lowest rate of K^+ efflux (0.45±0.2 nmol/cm^2/sec), but some preparations transported K^+ out of the lumen while others moved K^+ into the gut lumen. The rectum also exhibited site-specific alterations in the direction of K^+ transport and had an average rate of K^+ efflux of 1.5±0.5 nmol/cm^2/sec. The average rate of K^+ efflux was 1.03±0.4 nmol/cm^2/sec at the gastric caecae, and interestingly, as the electrode was moved from the tip of the gastric caecum toward the junction with the gut, there was an increase in the flux of K^+.

Time length controls for [K^+] gradients

Viability of the ileal preparation over the course of the experiments was tested by measuring K^+ fluxes over a total of 1.5 hours on individual control preparations. The average rate of K^+ efflux (movement of K^+ from the gut lumen to the saline bath) did not significantly decrease over the duration of the experiment, which was between 1 and 1.5 hours (Figure 3; p=0.8872; one-way ANOVA). Individual preparations varied in the rate of K^+ efflux, where three of the seven preparations had higher than average rates. In these preparations, baseline K^+ fluxes were greater than 10 nmol/cm^2/sec and remained above average for the duration of the trial.

The effect of FGLa/ASTs on K^+ transport

The anterior ileum showed the highest rate of K^+ efflux across the gut and as a result this area was chosen to assess potential effects of FGLa/ASTs on K^+ transport. The rate of baseline K^+ efflux for individual preparations used to assess effects of FGLa/AST ranged from 4.3±0.6 nmol/cm^2/sec to 9.85 ± 2.9 nmol/cm^2/sec. The effects of two FGLa/ASTs (Locmi-FGLa/AST-2 and Scg-AST-6) were determined relative to measurements made following a saline change (control) on the same preparation. Scg-AST-6 significantly decreased K^+ efflux across a range of doses from 10^{-14} to 10^{-7} M (Figure 4A). A dose of 10^{-10} M caused the greatest inhibition (34.1 ± 8.5%). Only partial recovery of the inhibitory effect of Scg-AST-6 was achieved upon washing (data not shown), indicating that the effects of the peptide are difficult to reverse.

The endogenous FGLa/AST, Locmi-FGLa/AST-2, inhibited K^+ efflux in a dose-dependent manner (Figure 4B). The effects of Locmi-FGLa/AST-2 were most potent across the range of 10^{-14} to 10^{-10} M with greatest inhibition observed with a dose of 10^{-11} M. Over these doses, Locmi-FGLa/AST-2 reduced K^+ efflux by 23 to 36% of initial fluxes in saline. The peptide was less effective at higher doses from 10^{-9} to 10^{-7} M (Figure 4B).

The effect of FGLa/ASTs on cAMP-stimulated K^+ transport

Membrane permeable cAMP, 8-bromo-cAMP, stimulated K^+ efflux at the locust ileum relative to saline (Figure 5A). The K^+ efflux remained stimulated with a second application of the same dose of 8-bromo-cAMP indicating that in subsequent experiments any inhibitory effects on K^+ flux after application of an FGLa/AST was due to the effects of the peptide and not due to a decrease in the effects of 8-bromo-cAMP over time. At 10^{-10} M, both Scg-AST-6 and Locmi-FGLa/AST-2 significantly inhibited cAMP-induced K^+ efflux (Figure 5B, C).

The effect of FGLa/AST on water transport

The ileum transports water from lumen to hemolymph and this can be monitored in vitro as a decrease in ileum weight over time. The *L. migratoria* ileum absorbs water at a rate of 3.03 µl/hour and this was significantly decreased to 1.61 µl/hour in the presence of 10^{-10} M Locmi-FGLa/AST-2 (Figure 6).

DISCUSSION

We have isolated and sequenced three FGLa/ASTs from the locust, *L. migratoria*, one of which is novel to *L. migratoria* and designated as Locmi-FGLa/AST-2, and two of which have been partially sequenced previously and designated Locmi-FGLa/AST-5 and Locmi-FGLa/AST-10 (see Clynen and Schoofs, 2009). The novel *L. migratoria* Locmi-FGLa/AST-2 is C-terminally amidated with the amino acid sequence, LPVYNFGL-NH_2. The amino acid sequence for Locmi-FGLa/AST-2 has been identified in a number of different insect species, but not previously in *L. migratoria*, and is 100% conserved across the species in which it has been characterized (see Zandawala et al., 2012). The significance of this highly conserved FGLa/AST is not clear; however, it may serve an important fundamental physiological process. There have been nine members of the FGLa/AST family identified in another locust species, *S. gregaria*, including a peptide with the sequence identical to that found for Locmi-FGLa/AST-2 (Veelaert et al., 1996). In the locust, *L. migratoria*, four FGLa/ASTs with sequences similar to those for *S. gregaria* were obtained; however Locmi-FGLa/AST-2 was not identified (Clynen and Schoofs, 2009). In all species the gene encoding for FGLa/ASTs codes for a number of individual FGLa/AST peptides that are processed together and so it is likely that additional members of the FGLa/AST family remain to be discovered in *L. migratoria*. The FGLa/AST family has been found in most insect species studied to date, and although these peptides have multiple physiological functions, the high degree of conservation of this gene and its peptide products across arthropods suggests that FGLa/ASTs modulate or control essential life processes. Some of these processes include gut functions involved in feeding and digestion as well as ion and water homeostasis. In fact, multiple studies have demonstrated the association of FGLa/ASTs with the gastrointestinal tract of arthropods (see Stay and Tobe, 2007; see Audsley and Weaver, 2009).

In addition to modulating gut contraction in the locust (see Roberston et al., 2012), we now show that FGLa/ASTs also alter ion and water transport across the ileum. The scanning ion-selective electrode technique (SIET) has been used to successfully detect net flux of various ions, including H^+, K^+, Na^+, Ca^{2+}, Cl^- and ammonia in a variety of animal and plant transporting tissues (Shih et al., 2008;

see Tong et al., 2007; Nawata et al., 2010; Del Duca et al., 2011; Rheault and O'Donnell, 2004). The application of SIET to specifically measure K^+ flux in insects has been reported in studies of cockroach blood-brain barrier (Kocmarek and O'Donnell, 2011), the Malpighian tubules of *Drosophila melanogaster* (Rheault and O'Donnell, 2004 and 2001), and the anal papillae of the mosquito and midge (Donini and O'Donnell, 2005; Nguyen and Donini, 2010). In the present study, SIET was utilized to measure spatial patterns of K^+ flux adjacent to the basolateral membrane of the isolated locust gut. Regional differences in the efflux of K^+ exist, where the chief site of K^+ efflux was found to be the anterior hindgut (ileum). The regional differences in K^+ efflux were expected and presumably relate to the morphology and/or function of each region. For instance, developmentally, the foregut arises from ectoderm and is lined by relatively impermeable cuticle, making storage a primary function of this region and absorption difficult (Dow, 1986). The hindgut also originates from ectoderm, but the cuticular lining of the hindgut is far more permeable than the foregut (Maddrell and Gardiner, 1980), allowing this region to carry out ion and water transport (Peacock, 1985; Peacock, 1986; Hanrahan and Phillips, 1983)). The epithelial cells of the colon possess short apical microvilli with mitochondria residing at their base but not in close association (Peacock, 1985). The basal membrane exhibits irregular folding which results in canaliculi (Peacock, 1985). This ultrastructure suggests that the colon is not a major site of ion and fluid reabsorption. On the other hand, the epithelial cells of the ileum possess extensive apical and basal infoldings that are closely associated with numerous mitochondria indicative of an epithelium that carries out transport (Peacock, 1986). Using an everted cannulated sac of the locust rectum, Goh and Phillips (1978) determined that the absorption rate of ions (Na^+, Cl^-, K^+) and water did not significantly change after the first hour and remained steady for four hours. The SIET measured K^+ fluxes at the ileum utilizing the semi-intact gut preparation showed that these fluxes remained consistent for at least 1.5 hours, which was the maximum length of time required to carry out the experiments. These studies confirm that the semi-intact gut is a viable and suitable preparation for assessing the effects of neuropeptides on gut ion transport. The endogenous FGLa/AST tested here, Locmi-FGLa/AST-2, inhibits K^+ efflux at the ileum of *L. migratoria* in a dose-dependent manner. Scg-AST-6 also inhibits K^+ efflux at the ileum with a maximal effect achieved with 10^{-10} M. Inhibiting K^+ efflux would be expected to inhibit uptake of osmotically-coupled water. Indeed, Locmi-FGLa/AST-2 at 10^{-10} M inhibited uptake of water by 47% over 60 min. The water uptake rates found here (3µl/60 min) are the same as those observed by Leichleitner et al. (1989) in *S. gregaria*, and confirms the viability and robustness of this *in vitro* ileum preparation from *L. migratoria*. FGLa/ASTs have previously been implicated in the modulation of ion transport in insects. In larval *A. aegypti*, FGLa/ASTs decreased the transepithelial voltage of the isolated gut (anterior stomach or midgut)

suggesting a modulatory effect on ion transport across the mosquito midgut (Onken et al., 2004). In larval *D. melanogaster*, FGLa/AST decreased K^+ absorption at the midgut and was associated with K^+ secretion at the ileum (Vanderveken and O'Donnell, 2014). Thus, one might anticipate that the action of FGLa/ASTs is not restricted to the hindgut of the locust but may affect midgut K^+ absorption as well; however, this remains to be tested. Much of the work on reabsorption in the hindgut and the associated solute and water transport processes of the ileum and rectum has been completed within the desert locust, *S. gregaria* (Phillips et al., 1986; Phillips et al., 1996). For both the ileum and rectum, the driving force for the uptake of ions from the lumen into the hemolymph is a unique apical membrane associated electrogenic Cl^- pump, which is stimulated by an elevation in the second messenger cAMP (Phillips et al., 1996; Audsley et al., 2013). Chloride uptake is further increased by the cAMP-stimulated opening of basolateral Cl^- channels, which stimulates passive transport of K^+ to maintain electrical neutrality (Audsley et al., 2013). Cyclic AMP-stimulated opening of apical K^+ channels also enhances this passive transport, and as a result of overall increases in ion transport, water reabsorption increases. The ITP binds to a G-protein coupled receptor bound to the basolateral membrane and acts via cAMP to induce the transport processes outlined here, leading to an increase in the reabsorption of salts (KCl and NaCl) and fluid (King et al., 1999; Audsley et al., 2013). The mode of action of the FGLa/ASTs on the ileum remains to be discovered; however, the FGLa/ASTs can counter the stimulatory effects of membrane permeable cAMP suggesting that they may be antagonistic to ITP and therefore function as diuretics.

The effects of the FGLa/ASTs were seen at low concentrations. Studies in other insects have revealed FGLa/AST titers in the hemolymph of maximum 1 – 2 nM (Yu et al., 1993). Thus, the range of doses that inhibit K^+ fluxes are certainly in the range for the FGLa/ASTs acting as neurohormones on the ileum. Higher doses were less effective and suggestive of desensitization of receptors that are sensitive to very low concentrations of FGLa/ASTs.

When locusts feed, they assume a load of solute and fluid. Diuretic hormones would then be released to increase secretion of Malpighian tubules and to also decrease the recovery of K^+ and water from the ileum, thereby leading to kaliuresis. The release of diuretic hormones in response to feeding has been directly demonstrated for *L. migratoria* diuretic hormone (Locmi-DH) (Patel et al., 1995) as well as serotonin, which acts as a DH in *Rhodnius prolixus* (Lange et al., 1989; Maddrell et al., 1991). The release of multiple diuretic hormones (e.g. Locmi-DH and Lom-K) may overlap and can act synergistically to maximally stimulate tubule fluid secretion (Coast et al., 1999). In addition, the rate of fluid and ion recycling between the Malpighian tubules, hindgut, and hemolymph can be increased by increasing recovery of ions and water from the ileum (Phillips and Audsley, 1995). At first it would

seem counter intuitive to increase Malpighian tubule secretion only to recover the fluid by reabsorption at the hindgut; however, this increase in fluid recycling increases the rate of clearance of solutes from the hemolymph (especially toxic compounds), allowing these substances to be concentrated for excretion and minimizing water loss (Phillips and Audsley, 1995; Coast et al., 1999). The experiments performed in this study were completed on locusts that had fed 1 to 10 hours prior and had a fresh bolus of food within their hindgut, and therefore would have an increased K^+ load as well as excess fluid. Thus, in this state, FGLa/ASTs may be released to act as DHs, in conjunction with Locmi-DH, to help deal with the excess K^+ ions and fluid gained from a recent meal.

Life on land takes ionoregulation to the extreme due to the intermittent access to ions from food and water, compounded by the need to conserve water. The ability of insects to conserve water under desiccating conditions may be the primary reason for the evolutionary success of terrestrial insects. Terrestrial plant feeding insects such as the locust must cope with varying K^+ content in the hemolymph (Dow, 1986). One way for locusts to cope is through modulation of ionoregulation by peptides, such as the FGLa/ASTs. Overall this work provides new insights into the multifunctional roles of the FGLa/ASTs, specifically acting as brain/gut peptides important in the modulation of digestive processes in this insect pest species.

MATERIALS AND METHODS

Animals

Locusta migratoria were raised in a long-standing colony at the University of Toronto Mississauga. The colony was kept on a 12 h light: 12 h dark cycle at 30ºC and 50% humidity. Locusts were kept in crowded conditions and were fed fresh wheat seedlings and bran *ad libitum*. Two to four week old adult male locusts were used for all physiological experiments, while both males and females were used for peptide sequencing. Male locusts were provided with food for 1 h, at which point the food was removed and the locusts were then used for experimentation within one to ten hours post-feeding. Only locusts that had a fresh food bolus contained within the hindgut were used for the experiments.

Chemicals

Locmi-FGLa/AST-2 (*L. migratoria* FGLamide/AST-2; LPVYNFGL-NH_2) was synthesized by Bio Basic (Markham, Ontario, Canada) and Scg-AST-6 (*S. gregaria* FGLamide/AST-6; ARPYSFGL-NH_2) was custom synthesized by the Insect Biotech Canada Core Facility (Queen's University, Kingston, ON, Canada). Before use, each FGLa/AST was reconstituted in double distilled water, yielding a 10^{-3} M stock solution that was divided into aliquots and frozen at -20ºC. Immediately prior to use, working

dilutions of each peptide were made in physiological saline (150 mM NaCl, 10 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM $NaHCO_3$, 5 mM pH 7.2 HEPES, 90 mM sucrose, and 5 mM trehalose) using a 10 µL aliquot of the appropriate FGLa/AST. Membrane permeable cyclic adenosine monophosphate (8-bromo-cAMP; Sigma Aldrich, MO, USA) was used at 4 mM.

Tissue extraction and purification for protein sequencing

The brain and retrocerebral complex of 100-200 adult *L. migratoria* were dissected in physiological saline and immediately placed in an ice-cold mixture of methanol: acetic acid: distilled water (90:9:1 by volume). Samples were then sonicated and centrifuged at 4ºC for 20 minutes at 9,400 g. The supernatant was concentrated using a Speed-Vac concentrator (Savant, Farmingdale, NY, USA) and then diluted in 1 mL 0.1% trifluoroacetic acid (TFA) and applied to a Sep-Pak C_{18} cartridge to remove salts and other impurities. Before application of the solution, the cartridge was equilibrated with 8 mL each of methanol, double distilled water, double distilled water containing 0.1% TFA, and 2 mL of 0.1% TFA in water containing 10 µg of bovine serum albumin (BSA; Sigma-Aldrich). Eluents from the column using 30% acetonitrile (ACN) containing 0.1% TFA, 50% ACN containing 0.1% TFA, and 90% ACN containing 0.1% TFA were collected. The collected extracts were then dried using a Speed-Vac concentrator for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF).

Mass spectrometry sequencing

The Sep-Pak eluted extracts were analyzed using MALDI-TOF MS (Q Star; Applied Biosystems Inc, Sciex, Concord, ON, Canada) at the Advanced Protein Technology Centre (Hospital for Sick Childrens, Toronto, ON, Canada). Peaks of interest in the MALDI-TOF analysis were subjected to further analysis using tandem mass spectrometry (MALDI-TOF MS/MS) to determine the amino acid sequence of each peptide using an Applied Biosystems Procise Model 494 sequencer (Foster City, CA, USA).

Construction of ion-selective microelectrodes for Scanning Ion-Selective Electrode Technique (SIET)

To measure [K^+] at the basolateral surface of the locust gut, K^+-selective microelectrodes were constructed. Glass capillary tubes (TW 150-4, World Precision Instruments, Sarasota, Florida, USA) were pulled on a Sutter P-97 Flaming Brown pipette puller (Sutter Instruments, San Rafael, California, USA) into micropipettes. The micropipettes were then baked at 300ºC for 30-40 minutes and then

vapour-silanized with dimethyltrimethylsilylamine (Fluka, Bachs, Switzerland) for one hour and then cooled before use. A micropipette was backfilled with 100 mM KCl and then frontloaded with K^+ ionophore (potassium ionophore I cocktail B; Fluka, Bachs Switzerland). The microelectrode was left to condition for at least 15 minutes before use and was calibrated, before and after each preparation, using 300 mM KCl and 30 mM KCl (with 270 mM NaCl) standard solutions.

SIET

The SIET system used in this study was as follows, and has been described previously (Nguyen and Donini, 2010; Jonusaite et al., 2013). Briefly, the K^+ microelectrode was fit onto an Ag/AgCl wire electrode holder, which was connected to a headstage. The headstage was connected to an IPA-2 Ion/Polarographic amplifier (Applicable Electronics, Forestdale, Massachusetts). A reference electrode was made by filling a capillary tube with 3M KCl containing 3% agar. The reference electrode was connected to the headstage with an electrode holder containing a silver pellet and filled with 3M KCl. The reference electrode was allowed to rest within the saline bath surrounding the locust gut preparation.

Measurement of [K^+] gradients at the gut

An *in vitro* preparation for the measurement of [K^+] gradients at the basal surface of the locust gut was developed as follows. The entire locust gut was isolated and the Malpighian tubules removed. The gut transferred to a Sylgard-coated dish with 1000 µl of fresh physiological saline. To minimize movement of the gut it was necessary to insert minutin pins at the foregut and the posterior end of the hindgut. The microelectrode was moved to a target site that was 20-30 µm away from the gut and a voltage was recorded. After recording the voltage at the target site, the microelectrode was moved 100 µm away to obtain a second voltage reading. The sampling protocol used a wait time of 4 s after microelectrode movement and a recording time of 1 s. At each target site this sampling protocol was repeated four times and the voltage difference between the two sites was used to calculate a voltage gradient by the Automated Scanning Electrode Technique software (ASET; Science Wares, East Falmouth, Massachusetts). The voltage difference and the gradient reported for each of the sites was an average of the four readings taken.

To obtain background voltage readings, the K^+ microelectrode was moved 1920-2560 µm away from the preparation, and the same sampling protocol was employed. These background voltage signals were then subtracted from those recorded adjacent to the locust gut. To achieve reliable voltage

readings it was important that the gut moved only minimally. The preparation was visually monitored during every recording and measurements made while the gut moved were discarded.

Using this sampling protocol, voltage recordings were made along the whole locust gut to determine if regional differences exist for K^+ transport. Six regions were chosen: posterior foregut, anterior lobe of the gastric caecae, the middle of the midgut, the anterior ileum, the middle of the colon, and the middle of the rectum. For each region, three target sites were chosen, each 640 µm apart, encompassing an area of each region of 1920 µm in length.

Effect of FGLa/ASTs on K^+ transport

To determine the effect of Scg-AST-6 and Locmi-FGLa/AST-2 on K^+ transport the anterior ileal region was chosen because of the magnitude of K^+ flux at this region and the ileums' role in ion and fluid reabsorption. Baseline voltage recordings were completed for each of six target sites on the anterior ileum using the sampling protocol above. Afterward, a saline change was completed and the bath was mixed manually with a pipette for 30 seconds. After a two minute wait period, voltage recordings were then obtained as above for the baseline recordings. A dose of FGLa/AST (either Scg-AST-6 or Locmi-FGLa/AST-2) was then added to the preparation, the bath was manually mixed for 30 seconds, and voltage recordings were completed at the six sites after a 2-minute wait period. The preparation was then washed for 5 minutes and voltage recordings were obtained after a two minute wait to determine the reversibility of the FGLa/AST effect. Background voltage recordings were taken after the baseline, saline, and wash voltage readings and the electrode was calibrated before and after the experiment.

Effect of FGLa/ASTs on cAMP-stimulated K^+ transport

The second messenger cAMP has been shown to increase ion transport at the anterior ileum (Audsley et al., 2013), thus we wanted to determine if FGLa/ASTs inhibited this cAMP-stimulated K^+ transport. The sampling protocol outlined above was employed with a few minor changes as follows. Baseline voltage recordings were completed for each of six target sites on the anterior ileum, afterward a saline change was completed, and the bath was mixed manually with a pipette for 30 seconds. After a two minute wait period, voltage recordings were then obtained at each of the six target sites. A 4 mM dose of 8-bromo-cAMP was then added to the preparation, the bath was manually mixed for 30 seconds, and voltage recordings were completed at the six sites after a 2-minute wait period. All saline was then removed from the preparation and 4 mM 8-bromo-cAMP and 10^{-10} M of one FGLa/AST were added simultaneously. The bath was then manually mixed for 30 seconds and voltage readings were

taken after a two-minute wait period. The preparation was then washed for 5 minutes and voltage recordings were obtained after a two-minute wait to determine the reversibility of the FGLa/AST effect. Background voltage recordings were taken after the baseline, saline, and wash voltage readings and the electrode was calibrated before and after the experiment.

Controls

Time-course controls were completed to ensure that the K^+ gradient was sustained over the duration of an experiment. The experimental protocol was the same as explained above, but a saline change was completed instead of adding a dose of FGLa/AST. In addition, cAMP controls were completed, to ensure that cAMP-stimulation remained consistent. For these controls, the experimental protocol was utilized as above, but there was no wash between subsequent 8-bromo-cAMP additions. After the first 4 mM 8-bromo-cAMP dose was added, the bathing saline was removed and saline containing 4 mM 8-bromo-cAMP was added and voltage recordings commenced after a two minute wait period.

To ensure that the FGLa/ASTs or 8-bromo-cAMP did not affect the electrode, the electrode was calibrated in the calibration solutions alone and then calibrated in the same calibration solutions containing either FGLa/AST or 8-bromo-cAMP. It was found that neither peptide nor 8-bromo-cAMP affected the electrode.

Calculation of K^+ fluxes

The voltage gradients obtained from the ASET software program were converted into concentration gradients using the equation:

$$\Delta C = C_b \times 10^{(\Delta V/S)} - C_b \qquad (1)$$

where ΔC is the concentration gradient between the two points measured at the locust gut ($\mu mol \cdot cm^{-3}$); C_b is the background K^+ concentration ($\mu mol \cdot cm^{-3}$); ΔV is the voltage gradient (μV) obtained from the ASET software; and S is the Nernst slope of the electrode obtained during calibration. The concentration gradient was then converted into a K^+ flux using Fick's equation:

$$J = D(\Delta C)/\Delta X \quad (2)$$

where J is the net flux of K^+ in $pmol \cdot cm^{-2} \cdot sec^{-1}$; D is the diffusion coefficient of K^+ (1.92E-05 cm^2/s) (Harned and Nuttall, 1949); ΔC is the concentration gradient ($\mu mol \cdot cm^{-3}$); and ΔX is the distance between the two points measured in micrometers.

Ileum absorption assay: water transport

The ileum was dissected from locusts in the fed state as described above. The anterior and posterior ends of the ileum were each ligated with silk thread and the ileum was placed in physiological saline for 15 min. Each ileum was then gently blotted and weighed on a Mettler AE240 balance. Once weighed, the tissues were placed in a microcentrifuge tube with 1000µl of saline or saline containing 10^{-10} M Locmi-FGLa/AST-2. The tissues were incubated for 60 min at room temperature, then gently removed, blotted and weighed. The difference in weight (initial – final) was calculated, and the rate of absorption (µl/min) was calculated assuming a specific gravity of 1. The results are expressed as mean ± S.E. (n=15).

Statistics

Data were analyzed as the average difference in K^+ flux between saline and the treatment and expressed as mean ± standard error. A paired two-tailed Student's t-test was used to determine significance between the saline and experimental K^+ flux for each dose of each FGLa/AST. A paired one-tailed Student's t-test was utilized to determine significance of cAMP-stimulated K^+ transport relative to saline and the effect of either FGLa/AST on cAMP-stimulated K^+ transport. For the time-length and cAMP controls, a one-way ANOVA was used to determine significance. An unpaired Student's t-test was utilized to determine the significance of the effect of Locmi-FGLa/AST-2 on ileal water transport.

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FIGURE CAPTIONS

Figure 1: Sequencing of Locmi-FGLa/AST-2 from extracts of brain/retrocerebral complexes of adult *Locusta migratoria*. (A) MALDI-TOF MS spectrum showing the presence of LPVYNFGL-NH_2 (theoretical *m/z* of 921.5). (B) The sequence was deduced using tandem MS. Prominent b-type fragment ions are labeled. M, mass number; Z, atomic number.

Figure 2: Potassium efflux across *Locusta migratoria* gut. (A) Locust gut schematic illustrating average regional differences in K^+ efflux. The length of the arrows indicate the rate of K^+ flux and the direction of the arrows indicate the net direction of movement of potassium across the gut measured at the basolateral side. (B) Mean ± standard error of the rate of K^+ efflux at each gut region. N= 4-6 preparations. ($p=0.001$; one-way ANOVA; different letters indicate significance; $p<0.05$)

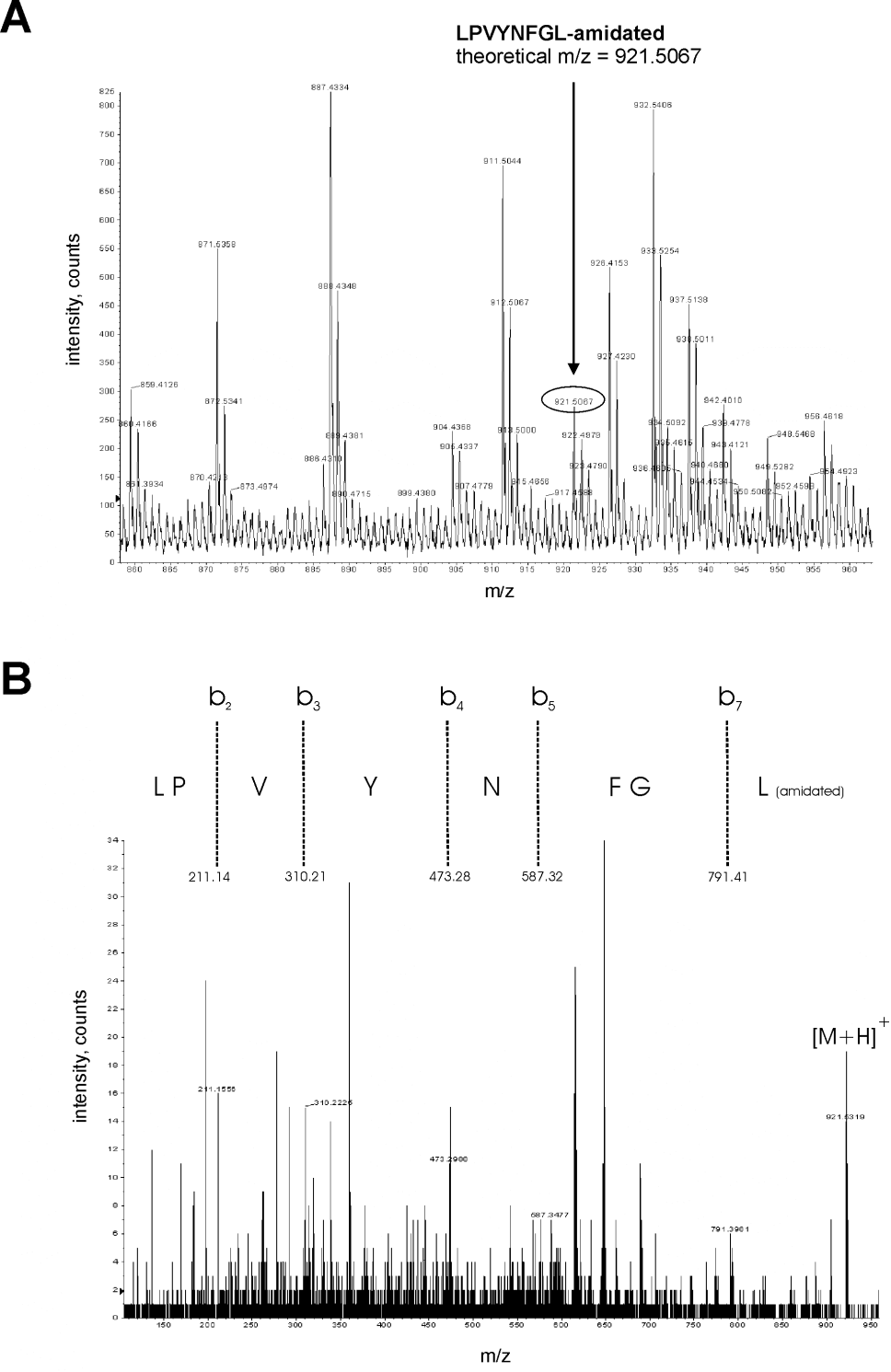
Figure 3: Time length controls illustrating rate of K^+ efflux from the anterior ileum. Baseline indicates voltage readings taken immediately after the preparation is set up. Scan 1, scan 2, and scan 3 correspond to approximately 20 minutes, 40 minutes, and one hour after baseline readings were completed. These scans were completed 2 minutes after a saline wash (to correspond to the saline change, addition of FGLa/AST, and wash in the experimental protocol). The average K^+ efflux of seven preparations indicated by filled circles does not significantly change over the duration of the experimental protocol ($p=0.8872$; one-way ANOVA; $p<0.05$ indicates significance). Average values are expressed as mean ± S.E. of the 7 individual preparations shown.

Figure 4: Effect of FGLa/ASTs on K^+ efflux from the anterior ileum. (A) Scg-AST-6 decreased K^+ efflux (*$p=0.025$; **$p<0.001$; ***$p=0.0006$; paired two-tailed Student's t-test between saline and Scg-AST-6 values for each dose; $p<0.05$ indicates significance). (B) Dose-dependent inhibition of K^+ efflux by Locmi-FGLa/AST-2 (*$p<0.05$; **$p=0.004$; paired two-tailed Student's t-test between saline and Locmi-FGLa/AST-2 values for each dose; $p<0.05$ indicates significance). Bars represent the average difference between saline and dose of FGLa/AST (average difference = flux in saline – flux in FGLa/AST, where greater positive differences represent greater inhibition of fluxes by the peptides). Averages are expressed as mean ± S.E. of 4-10 individual gut preparations.

Figure 5: cAMP-induced K^+ efflux from the anterior ileum (A) and FGLa/ASTs inhibition of the cAMP-induced K^+ efflux (B, C). (A) Saline indicates the readings taken after a saline change, while cAMP 1 and cAMP 2 indicate the readings taken following bath application of 4 mM 8-bromo-cAMP. cAMP significantly increased K^+ efflux (different letters indicate significance; $p=0.05$, paired one-

tailed Student's t-test between saline and each cAMP treatment). Bars represent the mean ± S.E. of 6 preparations. (B) Scg-AST-6 significantly decreased cAMP-induced K^+ efflux (different letters indicate significance between treatments; p=0.04; paired one-tailed Student's t-test between cAMP and Scg-AST-6+cAMP treatments; p<0.05 indicates significance). (C) Locmi-FGLa/AST-2 significantly decreased cAMP-induced K^+ efflux (different letters indicate significance; p=0.007, paired one-tailed Student's t-test between cAMP and Locmi-FGLa/AST-2+cAMP treatments; p<0.05 indicates significance). Bars represent the mean ± S.E. of 9 preparations for Scg-AST-6 and 7 preparations for Locmi-FGLa/AST-2.

Figure 6. Locmi-FGLa/AST-2 (10^{-10}M) significantly (p=0.0263, unpaired two tailed Student's t-test) inhibits the rate of absorption of water across the ileal sac *in vitro* over 60 min. Mean ± S.E. (n=15).


A
LPVYNFGL-amidated
theoretical m/z = 921.5067
intensity, counts
m/z
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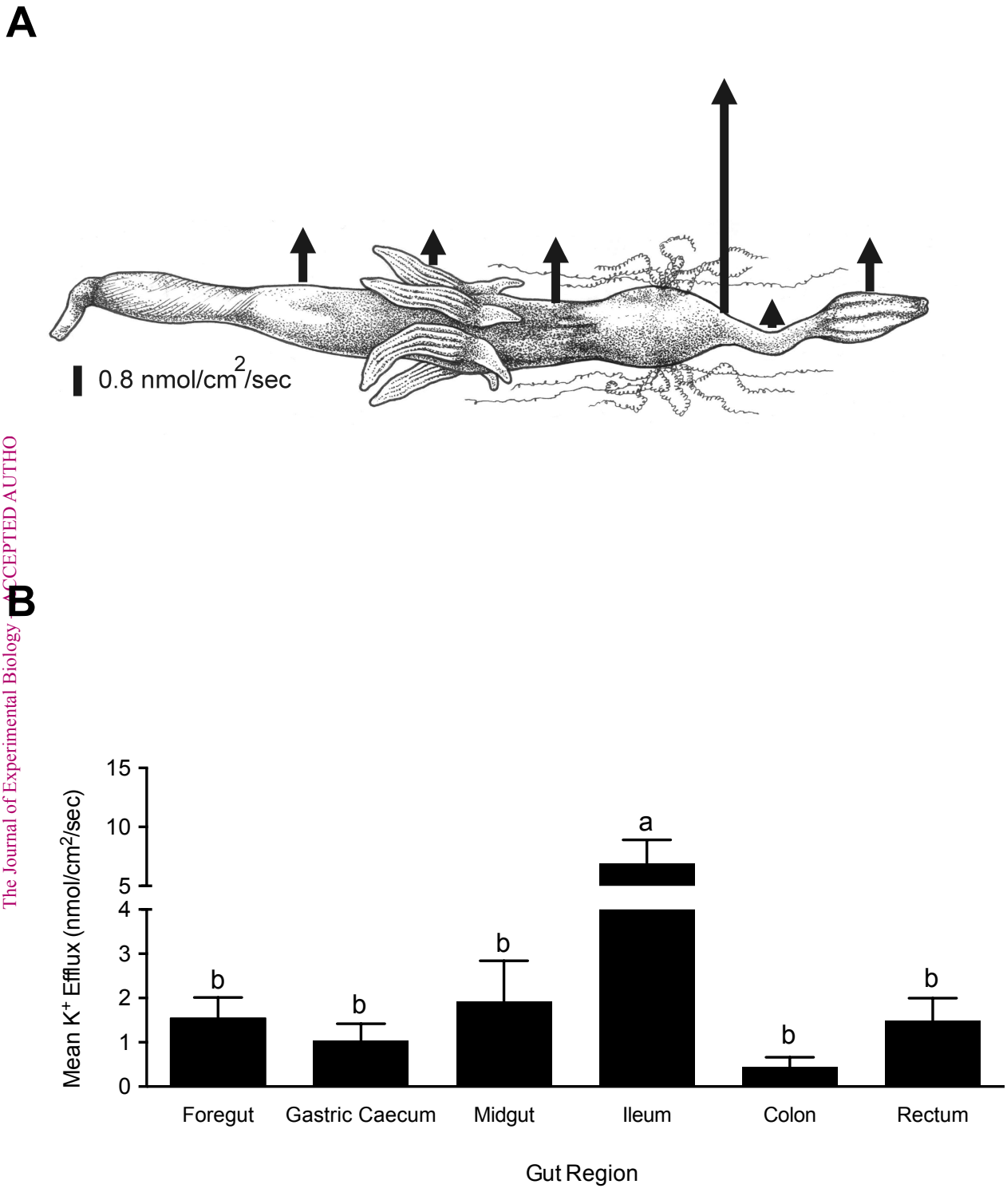

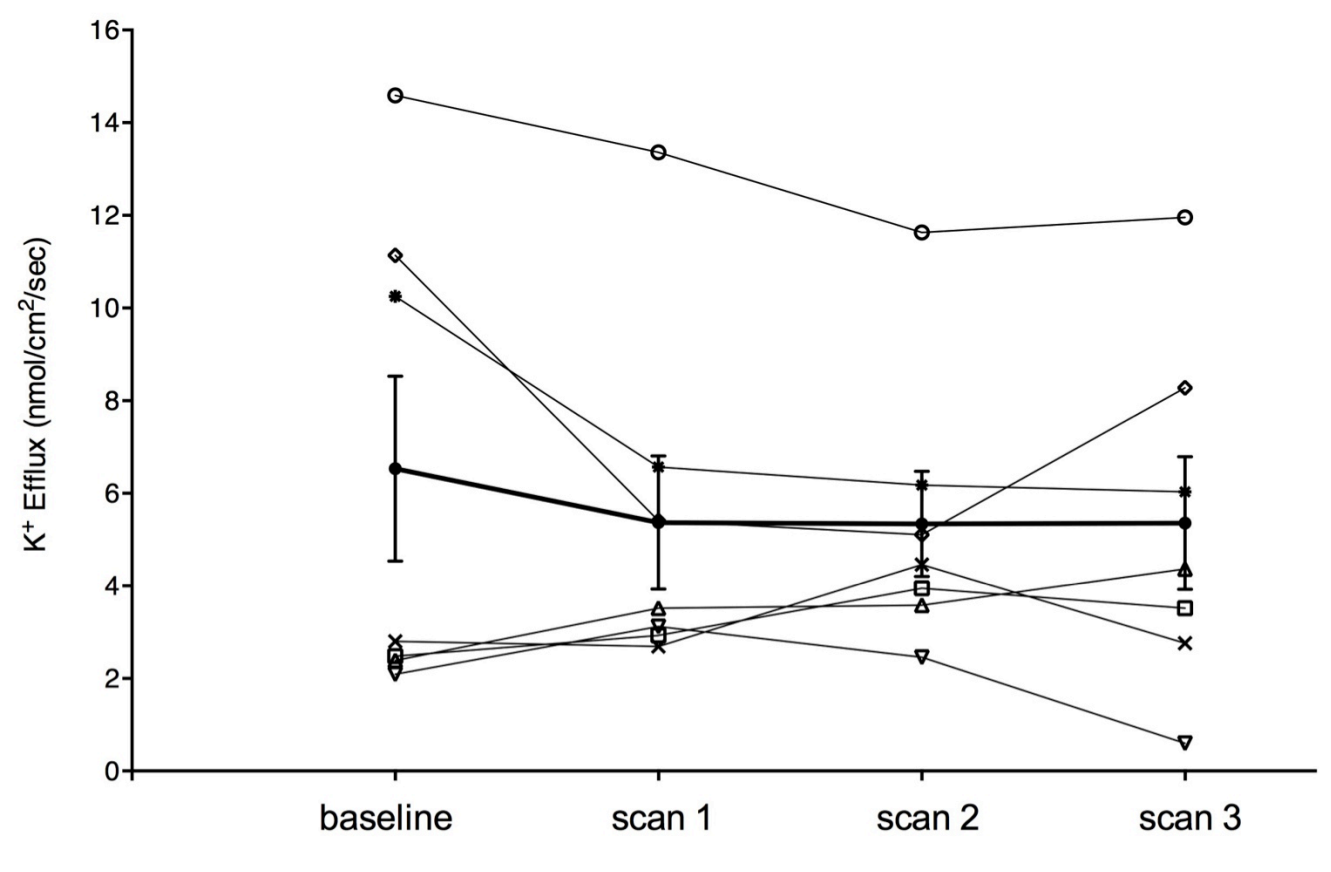
16
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8
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4
2
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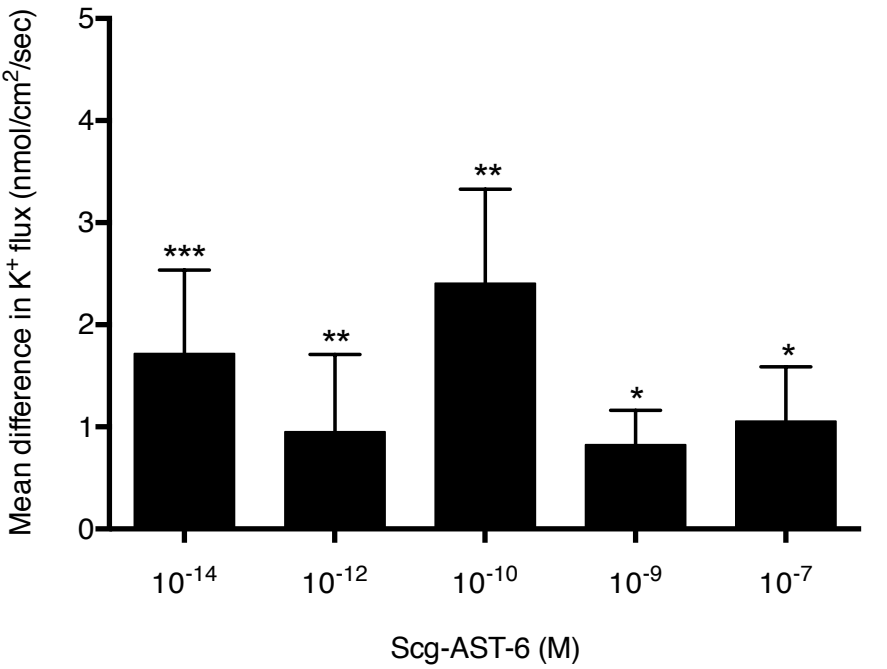
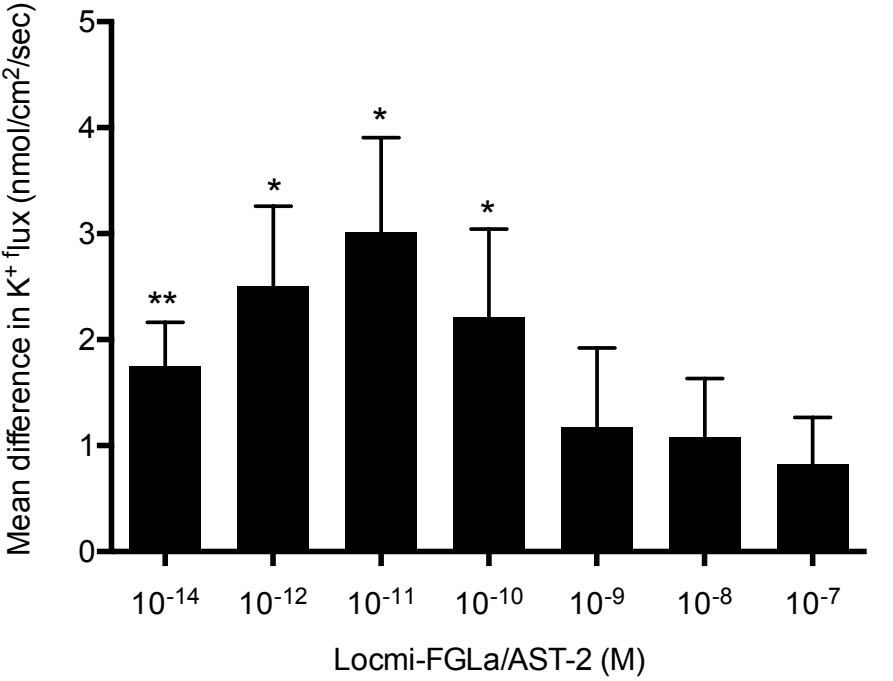
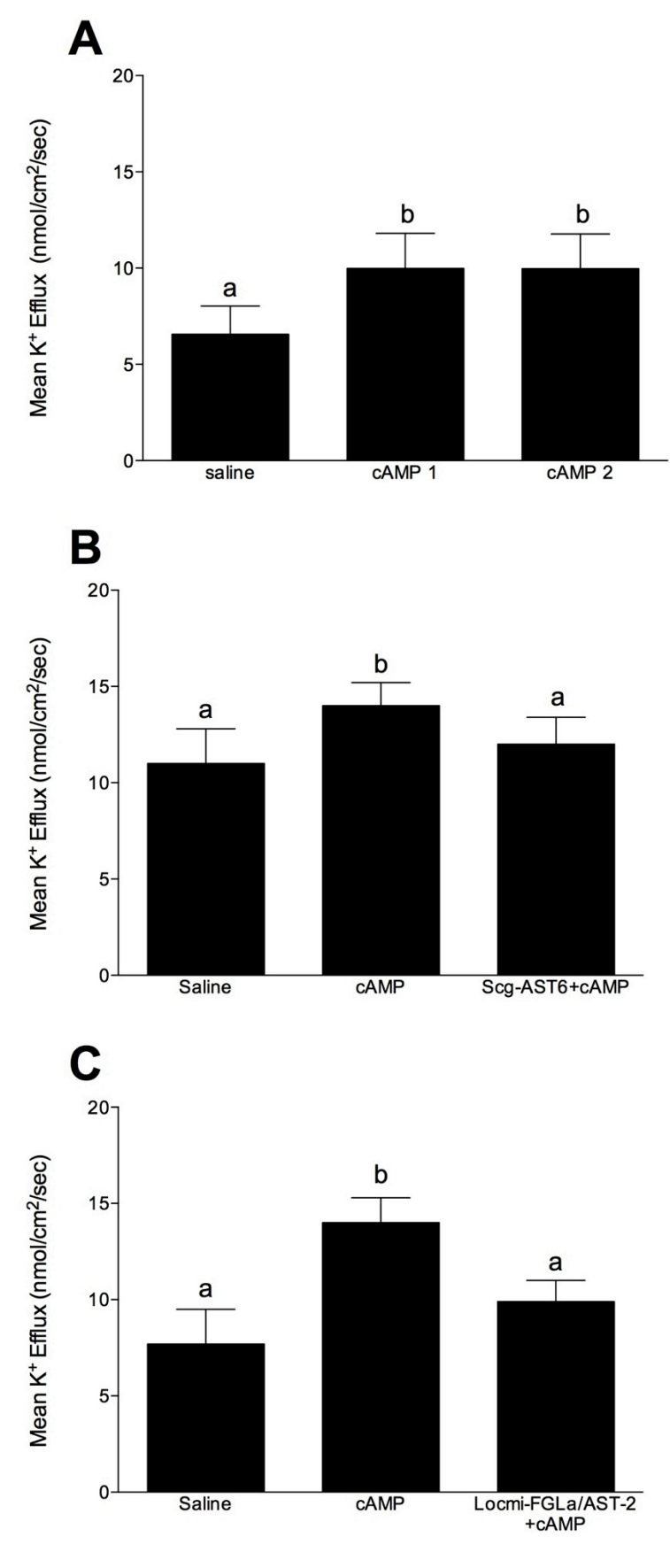
K+ Efflux (nmol/cm2/sec)
baseline
scan 1
scan 2
scan 3

A



B




A
Mean K+ Efflux (nmol/cm2/sec)
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15
10
5
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a
b
b
saline
cAMP 1
cAMP 2
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Mean K+ Efflux (nmol/cm2/sec)
20
15
10
5
0
a
b
a
Saline
cAMP
Scg-AST6+cAMP
C
Mean K+ Efflux (nmol/cm2/sec)
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15
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Saline
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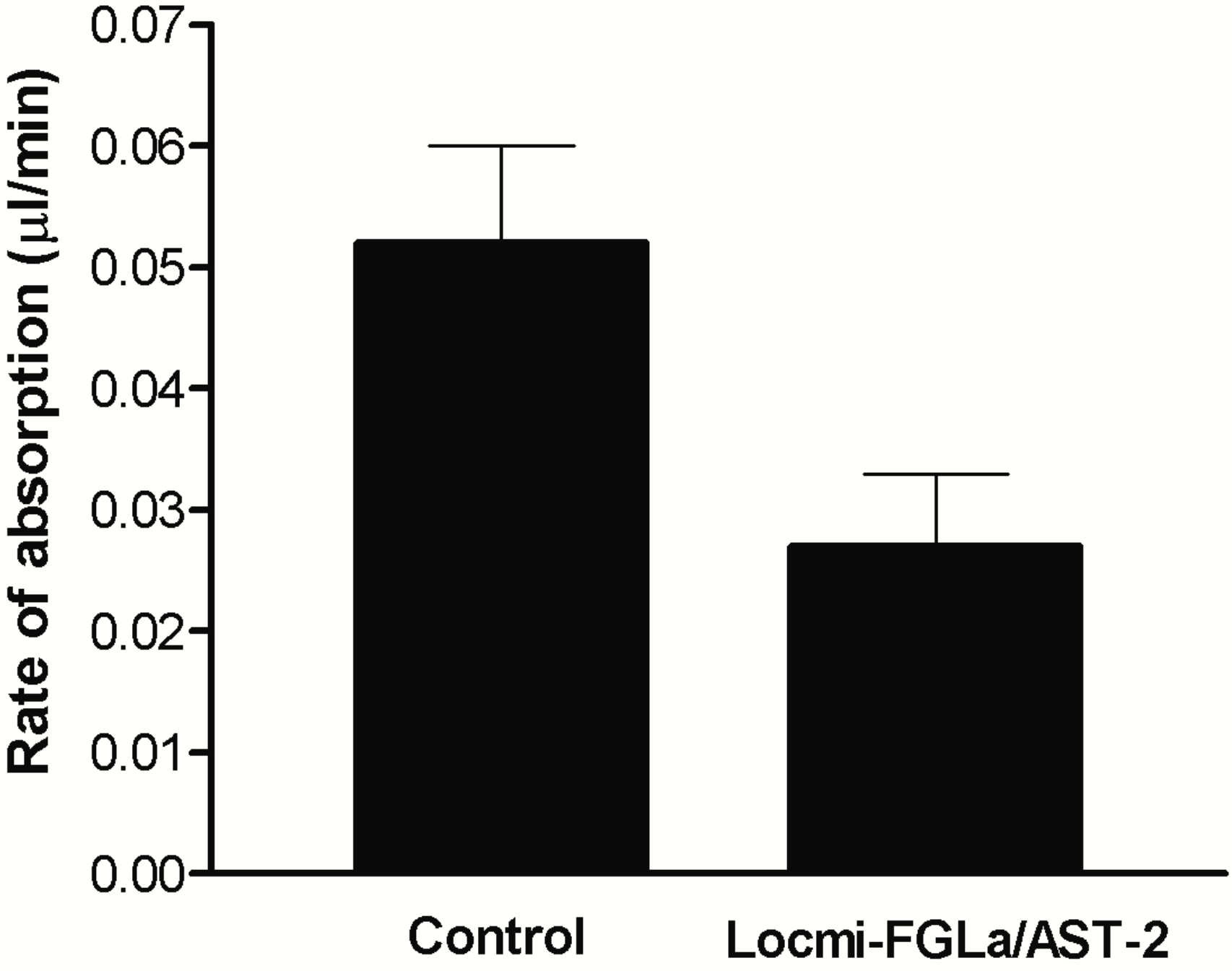
Locmi-FGLa/AST-2
+cAMP


Rate of absorption (µl/min)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Control
Locmi-FGLa/AST-2