Opioid modulation of ferret vagal afferent mechanosensitivity

1,2,3Amanda J Page, 1Tracey A O’Donnell & 1,2,3L Ashley Blackshaw

**Short title:** Opioid receptors on vagal afferents

1Nerve Gut Research Laboratory, Department of Gastroenterology, and Hepatology, Hanson Institute, Royal Adelaide Hospital, 2Discipline of Medicine and 3Discipline of Physiology, School of Molecular and Biomedical Sciences, University of Adelaide.

Correspondence:
Amanda J. Page, Nerve Gut Research Laboratory, Level 1 Hanson Institute, Frome Road, Adelaide, SA 5000, Australia. (e-mail:
amanda.page@health.sa.gov.au).
Abstract

Despite universal use of opioids in the clinic to inhibit pain, there is relatively little known of their peripheral actions on sensory nerve endings, where in fact they may be better targeted with more widespread applications. Here we show differential effects of mu, kappa and delta opioids on mechanosensitive ferret esophageal vagal afferent endings investigated in vitro. The effects of selective agonists DAMGO, ICI 199441 and SNC 80 respectively on mechanosensory stimulus-response functions were quantified. DAMGO (10^{-7} – 10^{-5}M) reduced the responses of tension receptors to circumferential tension (1-5g) by up to 50%, and the responses of mucosal receptors to mucosal stroking (10-1000mg von Frey hair) by >50%. DAMGO effects were reversed by naloxone (10^{-5}M). Tension/mucosal (TM) receptor responses to tension and stroking were unaffected by DAMGO. ICI 199441 (10^{-6} – 10^{-5}M) potently inhibited all responses except TM receptor responses to tension, and SNC 80 (10^{-5} – 10^{-3}M) had no effect other than a minor inhibition of mucosal receptor responses to intense stimuli at 10^{-3}M. We conclude that mu- and kappa- opioids have potent and selective peripheral effects on esophageal vagal afferents which may have applications in treatment of disorders of visceral sensation.

Key words: vagal afferents, neuromodulation, opioid receptors
Introduction

Opioid receptors were identified more than 30 years ago as the primary targets with which morphine and its derivatives interact to exert their analgesic and other effects. The receptors along with their endogenous ligands, enkephalins, endorphins and other opioid peptides, are widely distributed in the central and peripheral nervous system, and play an important role in modulating endocrine, immune, cardiovascular, and gastrointestinal functions. Three major types of opioid receptors, mu, delta and kappa, have been defined pharmacologically. Both spinal and vagal sensory neurons express different numbers of mu, delta, and kappa-opioid receptors which are transported into the peripheral axons (1, 13, 15, 17, 31). The kappa opioid receptor (KOR) agonist fedotozine decreases sensitivity of rat visceral afferents to gastric and colonic distension (7). Despite robust effects of KOR ligands, mu and delta-opioid receptor agonists have not been found to affect mechanosensitivity in these models (20, 34), indicating selectivity of expression within rodent distension-sensitive afferents. The effect of opioid agonists in other species and on different classes of visceral afferents is unknown, for instance mucosal mechanoreceptors which only respond to mucosal stroking (22, 23). Such effects may have an important bearing on interpretation of clinical findings, such as the inhibitory effect of the mu opioid receptor (MOR) agonist morphine on triggering of transient lower esophageal sphincter (LES) relaxation by gastric distension in humans (27), and the effect of opioids on the perception of mechanical events in the human gut (10).
We chose the ferret as our species for investigation, based on a number of similarities with humans: the occurrence of vomiting and gastroesophageal reflux, the similarity of its upper gastrointestinal anatomy, and the fact that observations of pharmacology of ferret gastroesophageal vagal afferents \textit{in vitro} translates to effects on mechanical triggering of transient LES relaxations and thus gastroesophageal reflux in humans (22, 23). Using this \textit{in vitro} preparation, we investigated the effect of selective MOR, KOR and delta-opioid receptor (DOR) agonists on the sensitivity of esophageal vagal afferents to mechanical stimulation. We studied all of the mechanosensitive esophageal vagal afferent subtypes to determine if opioid actions are specific to a select sensory modality of vagal afferent fiber.
Methods

All studies were performed in accordance with the guidelines of the Animals Ethics Committees of the Royal Adelaide Hospital, and the Institute for Medical and veterinary Science, Adelaide, and also the Animal Ethics Committee of the University of Adelaide.

\textit{In vitro ferret gastro-oesophageal afferent preparation}

This preparation has been described in detail previously (21, 22, 24, 25). Briefly, ferrets (0.6-1.0Kg body weight) were deeply anesthetized with sodium pentobarbitone (50 mg.Kg, i.p.), and exsanguinated by cardiac puncture. The stomach and esophagus with attached vagal nerves were dissected in a modified Krebs' solution of the following composition (mM): 118.1 NaCl, 4.7 KCl, 25.1 NaHCO$_3$, 1.3 NaH$_2$PO$_4$, 1.2 MgSO$_4$.7H$_2$O, 1.5 CaCl$_2$, 1.0 citric acid and 11.1 glucose, bubbled with 95% O$_2$ and 5% CO$_2$. The preparation was then opened longitudinally along the esophagus and pinned out flat, mucosal side up, in a Perspex chamber and perfused at a rate of 12 ml/min with Krebs' bicarbonate buffer solution maintained at 34°C containing nifedipine (1 µM) to prevent smooth muscle contraction. The vagus nerve was drawn through a small hole into an isolated recording chamber filled with paraffin oil. Under a dissecting microscope, a small longitudinal incision was made in the nerve sheath. Using fine forceps, nerve fibres were teased back onto a platinum recording electrode.
**Characterisation of esophageal vagal afferent properties**

Location of receptive fields of all types of vagal afferent fibers was determined by mechanical stimulation throughout the preparation with a brush, then more accurately with a blunt glass rod. Accurate quantification of mechanical responsiveness was performed differently according to the primary adequate stimulus for the type of fiber. Mechanical thresholds of all types were determined using calibrated von Frey hairs. Mucosal receptors showed rapidly adapting responses to maintained pressure on the receptive field with a von Frey hair. This type of responsiveness was also seen in responses of tension/mucosal (TM) receptors to low-level mucosal mechanical stimulation. The most reproducible responses of these afferents to mucosal stimuli were evoked when the probe was moved at approx. 5 mm/sec across the receptive field. Because receptive fields of these afferents are small (1-3mm²), a single test at each intensity is likely to miss the centre of the receptive field on occasions. Therefore, we minimized error by measuring the mean response to the middle 8 of 10 standard strokes given at 1 sec intervals. Tension response curves were also obtained for all afferent fibres, which were used in combination with von Frey thresholds to determine whether the receptive fields of fibers were located in the mucosa, the muscle layer, or both. Tension stimuli were applied via a thread attached to an unpinned point adjacent to the mechanoreceptive field. The thread was attached to a cantilever (22). Each weight was applied as a step and maintained for 1 min, and the response was measured as the mean discharge evoked over this period. Because all responses to tension were similarly slowly adapting, this method of
assessment was considered representative of physiological responsiveness. The tension-response curves were produced by randomly applying weights to the cantilever system (1, 3 & 5g). A recovery period of at least 1 min was allowed between each tension stimulus. Although conduction velocity was not recorded in this study, our previous observations indicate that these are in the C- and A-delta range, with no functional differences according to conduction velocity (21, 22).

**Effect of opioid receptor agonists on the mechanosensitivity of vagal afferents**

After mechanical sensitivity of the esophageal vagal afferent had been established, the effect of the MOR agonist [D-Ala², NMe-Phe⁴, Gly-ol⁶]-enkephalin (DAMGO) on mechanical sensitivity was determined. DAMGO (0.1µM) was added to the superfusing solution and allowed to equilibrate for 20 mins, after which the tension-response and stroke-response curves were re-determined. This equilibration period was observed so as to ensure penetration of the drug into all layers of the tissue. This procedure was repeated for DAMGO at increasingly higher doses (0.3 – 1 µM).

The same procedure was carried out for the KOR agonist 2-(3, 4-Dichlorophenyl)-N-methyl-N-[(1S)-1phenyl-2-(1-pyrrolidinyl) ethyl] acetamide hydrochlorine (ICI 199441; 1 – 10 µM) and the DOR agonist (+)-4-[(αR) - α-((2S, 5R)-4-Allyl-2, 5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N, N-diethylbenzamide (SNC 80; 0.01 – 1 mM) in different groups of experiments.
Time control experiments were performed in which there was no significant change in the mechanical responses over a comparable duration.

**Effect of opioid antagonism and agonism on the mechanical sensitivity of ferret mucosal afferents**

After the mechanical sensitivity of the esophageal mucosal receptors had been established, the effect of the agonists DAMGO (1µM) or ICI 199441 (10µM) on mechanical sensitivity to mucosal stroking was determined in separate series of experiments. The maximal concentration of agonist was added to the superfusing solution and allowed to equilibrate for 20 minutes after which time the stroke-response curves were redetermined. The opioid receptor antagonist naloxone (10µM) was then added to the Krebs superfusing solution along with DAMGO (1µM) or ICI 199441 (10µM) and allowed to equilibrate for 20 minutes. Mechanical response relationships were then re-determined.

**Data recording and analysis**

Afferent impulses were amplified with a biological amplifier (DAM 50; World Precision Instruments, Sarasota, FL, USA), filtered (band-pass filter-932; CWE Inc., Ardmore, PA, USA) and monitored using an oscilloscope (DL 1200 A; Yokogawa, Tokyo). Single units were discriminated on the basis of action potential shape, duration and amplitude using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All data were recorded and analysed off-
line using a personal computer (IBM Thinkpad). Peristimulus time histograms and discharge traces were displayed using Spike 2 software. Data are expressed as means ± S.E.M., with n=number of individual afferents in all instances. Differences between stimulus-response curves were evaluated using two-way ANOVA. Differences were considered significant if p<0.05.

**Drugs**

Stock solutions of all drugs were kept frozen and diluted to their final concentration in Krebs solution on the day of the experiment. DAMGO, ICI 199441 and SNC 80 were all obtained from TOCRIS bioscience (Northpoint, Avonmouth, UK) and naloxone was obtained from SIGMA (Castle Hill, NSW, Australia).
Results

**Mechanosensory properties of esophageal vagal afferent fibers**

Three types of mechanosensitive fiber were observed using this *in vitro* preparation: those responding to circumferential tension but not low-intensity mucosal stimuli (tension receptors; n=19), those responding only to mucosal stroking (mucosal receptors; n=23), and those responding to both mucosal stroking and circumferential tension, which we have previously termed tension mucosal, or TM receptors (n=35) (22) All of the afferent fibers recorded had receptive fields in the esophagus or the gastroesophageal junction.

**Effect of DAMGO on the mechanosensitivity of esophageal vagal afferents**

The selective MOR agonist DAMGO (0.1 - 1µM) (12, 16) did not significantly affect the basal discharge of vagal esophageal mucosal, tension or TM receptors (data not illustrated). The effect of DAMGO on mucosal, tension and TM receptor sensitivity to mechanical stimulation is illustrated in Figure 1 and summarised in table 1. A typical response of a tension receptor to circumferential tension (3g) is illustrated in figure 1D. DAMGO (0.3 – 1µM) significantly and dose-dependently reduced the response of mucosal receptors to mucosal stroking with calibrated von Frey hairs (10 – 1000mg; Fig. 1A). DAMGO (0.1 - 1µM) significantly and dose-dependently reduced the response of tension receptors to circumferential tension (1, 3 & 5g; Fig. 1B &
D). DAMGO (0.1 - 1µM) did not significantly affect the response of TM receptors to either mucosal stroking with calibrated von Frey hairs (10 – 1000mg; Fig. 1Ci) or circumferential tension (1, 3 & 5g: Fig. 1Cii).

**Effect of ICI 199441 on the mechanosensitivity of esophageal vagal afferents**

The selective KOR agonist ICI 199441 (1 – 10µM) (3, 18) did not significantly affect the basal discharge of vagal esophageal mucosal, tension or TM receptors (data not illustrated). The effect of ICI 199441 on mucosal, tension and TM receptor sensitivity to mechanical stimulation is illustrated in Figure 2 and summarised in table 1. A typical response of a mucosal receptor to mucosal stroking with a 50mg von Frey hair is illustrated in figure 2D. ICI 199411 (10µM) significantly and dose-dependently reduced the response of mucosal receptors to mucosal stroking with calibrated von Frey hairs (10 – 1000mg; Fig. 2A & D). ICI 199441 (3-10µM) significantly and dose-dependently reduced the response of tension receptors to circumferential tension (3 & 5g; Fig. 2B). ICI 199441 (3 – 10µM) significantly and dose-dependently reduced the response of TM receptors to mucosal stroking with calibrated von Frey hairs (10 – 1000mg; Fig. 2Ci) but did not significantly affect the response of the same TM receptors to circumferential tension (1, 3 & 5g; Fig 2Cii).

**Effect of SNC 80 on the mechanosensitivity of esophageal vagal afferents**
The selective DOR agonist SNC 80 (0.01 – 1mM) (4, 30) did not significantly affect the basal discharge of vagal esophageal mucosal, tension or TM receptors (data not illustrated). The effect of SNC 80 on mucosal, tension and TM receptor sensitivity to mechanical stimulation is illustrated in Figure 3 and summarised in table 1. A typical response of a tension receptor to circumferential tension (3g) is illustrated in figure 3D. SNC 80 (1mM) only reduced the response of mucosal receptors to mucosal stroking with the 1000mg von Frey hair. SNC 80 (0.01-1mM) did not significantly affect the response of tension receptors to circumferential tension (1, 3 & 5g; Figure 3B & D). SNC 80 (0.01-1mM) did not significantly affect the response of TM receptors to either mucosal stroking with calibrated von Frey hairs (10-1000mg; Fig.3Ci) or circumferential tension (1, 3 & 5g; Fig. 3Cii).

Effect of opioid receptor antagonism on the inhibitory effect of DAMGO and ICI 199441

The effect of the non-selective opioid receptor antagonist naloxone on inhibition of ferret esophageal mucosal receptors by opioid agonists is shown in Figure 4. DAMGO (1µM) alone significantly reduced the response of mucosal receptors (n=6) to mucosal stroking with calibrated von Frey hairs (10-1000mg; Fig. 4A, C). When naloxone (10µM) was added to the Krebs superfusate along with DAMGO (1µM), the inhibitory effect of DAMGO was completely reversed to control values (Fig. 4A). A comparable reversal of the effect of ICI 199441 (10µM) was seen with naloxone (10µM, Fig. 4B).
Discussion

The present study provides the first evidence that visceral afferents are differentially affected by opioid receptor agonists. Our results confirm previous reports that KOR agonists reduce the sensitivity of rat vagal tension receptors (20). We further demonstrate that the KOR agonist reduced the mechanosensitivity of mucosal receptors and tension/mucosal receptor responses to mucosal stroking. This study provides the first evidence that activation of MORs inhibits visceral afferent mechanosensitivity, showing reduced sensitivity of a select population of esophageal vagal afferents by DAMGO. The MOR agonist reduced the sensitivity of mucosal and tension receptors but not the sensitivity of tension/mucosal receptors. MOR effects were reversed by the non-selective opioid antagonist naloxone. The DOR agonist SNC 80 had no substantial effect on any of the afferent types investigated in this study, indicating a lack of involvement of these receptors in esophageal vagal afferent function.

The effect of opioids are mediated by three distinct receptors; kappa-, mu- and delta opioid receptors. Mu- and delta-opioid receptor gene expression has been observed in rat nodose ganglia with a relatively high expression of MOR RNA compared to other ganglia (6). There is also evidence of axonal transport of MOR along the vagus nerve both towards the brain and towards the periphery (15). This expression of the MOR in nodose ganglia and transport to the periphery correlates strongly with our findings that the mu-receptor agonist DAMGO has an effect on the mechanosensitivity of mucosal
and tension sensitive vagal afferent mechanoreceptors. However, it has been shown that morphine, the MOR agonist, has no effect on the response of rat gastric mechanoreceptors to gastric distension (20). What, then, is the function of MOR expressed in rat nodose ganglia? This discrepancy between studies may be a regional difference, because all afferents recorded in the present study were located in the esophagus or the LES, and therefore a totally different anatomical population to the gastric afferents previously reported (20). However, it should be noted that the esophageal tension receptors on which we observed effects of DAMGO are otherwise indistinguishable in their pharmacology to gastric tension receptors in ferret (21, 25, 26), suggesting a species variation.

Our data are in contrast to those obtained by Eastwood & Grundy (11) who showed direct excitation of rat small intestinal afferents by DAMGO in vivo. We would expect the differences in findings are due to the release of other mediators by mu opioids in the small intestinal mucosa, which has many cells that possess opioid receptors (14, 19). The esophagus, on the other hand, has a squamous epithelium with no enteroendocrine cell population, where opioid effects are more likely to be direct on the afferent ending, such as has been suggested from behavioural studies in skin (8, 37). In the skin, about one third of unmyelinated cutaneous sensory axons are immunopositive for MORs and DORs (8). In addition, morphine has been shown to reduce the response of cutaneous afferents to mechanical, thermal and chemical activation (41, 42). Further down the intestine, mu-opioids do not affect the mechanically evoked discharge of afferent fibers innervating the rat colon (35,
However, it appears from less specific models of chemically-evoked visceral pain that μ-opioid analgesia may occur peripherally (9, 29). In our hands, the inhibitory effect of MOR agonists on mechanoreceptors is relatively selective for vagal esophageal mechanoreceptors - specifically mucosal receptors and tension receptors as opposed to tension/mucosal receptors.

DOR expression in rat nodose ganglia is relatively low compared to other ganglia (6). In the present study SNC 80, the DOR agonist, inhibited mucosal receptors only at the highest dose used and had no effect of the mechanosensitivity of tension receptors. This is in agreement with studies on gastric vagal mechanoreceptors in the rat, which were also unaffected by SNC 80 (20). It may be that the DOR detected within rat nodose ganglia (6) are associated with neurones other than those innervating the gastroesophageal region.

Systemic administration of KOR agonists attenuates responses to noxious colorectal distension (5). In addition, it has been shown that KOR agonists, but not MOR or DOR agonists, inhibit responses of mechanosensitive pelvic nerve afferent fibers to noxious colorectal or urinary bladder distension in the rat in vivo (34, 35, 39, 40). In the rat stomach it has been shown that KOR agonists reduce the response of gastric vagal afferents to distension (20). Our results confirm these findings in non-rodent species and further suggest that kappa opioids reduce the mechanosensitivity of mucosal and tension/mucosal receptors to mucosal stroking. However, ICI 199,441 did not affect the response of tension/mucosal receptors to circular tension. Selective
pharmacological effects on different properties of tension/mucosal receptors are also evident from our previous studies, such as with GABAB receptor agonists (21), suggesting receptors may be trafficked preferentially or act differently on endings of these fibres in mucosal or muscular layers. Fedotozine, a KOR agonist, exhibits some clinical activity as it increases the threshold at which gastric distension causes discomfort in healthy volunteers (7), relieves some symptoms associated with functional dyspepsia (28) and enhances the threshold at which colonic distension gives rise to discomfort in irritable bowel syndrome patients (10). Our results indicate a mechanism whereby some of the symptoms of functional dyspepsia from the upper GI tract, such as fullness, may be ameliorated clinically by KOR agonists.

MOR and KOR agonists have been shown to reduce the spontaneous discharge of afferent fibers innervating the inflamed knee joint of the cat (32) and also the spontaneous activity of saphenous afferent nerve fibers from injured tissue of rat hindpaw skin (2). In the absence of tissue injury, however, opioids do not depress the spontaneous discharge of nociceptors in skin and do not depress their responses to noxious stimuli (33, 36). In the present study none of the opioid receptor agonists studied significantly affected the spontaneous activity of the vagal afferents. However, the tissue used was non-inflamed so there is still the possibility that the spontaneous activity could be altered by opioid receptor agonists in the inflamed tissue.

Our findings have potential relevance to several aspects of visceral afferent function in human disease. Peripheral actions of MOR may underlie their
inhibition of triggering of transient LES relaxations by gastric distension (27), assuming actions on esophageal tension receptors are mirrored by those in the stomach. Kappa agonists remain to be investigated in this context. Peripherally restricted opioid agonists such as loperamide have been studied little in the context of effects on visceral afferent function. The results of the present study suggest this is a worthwhile area for further efforts in drug development, at least for disease in which the antisecretory and motor effects of mu opioids are not contra-indicated.

In conclusion, the present study provides direct evidence for the inhibitory modulation of primary afferent mechanotransduction by the G-protein coupled kappa- and mu-opioid receptors. The three types of esophageal vagal afferents we have identified show differential sensitivity to kappa- and mu-opioid receptor agonists.

**Acknowledgements**

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Table 1

A summary of the effect of selective opioid receptor agonists on mechanosensitivity of esophageal vagal afferents.

Maximum effect of the agonist is ↓↓ >50%, ↓ 25 – 50% or ↔<25% reduction in response to mechanical stimulation.

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Figure 1.

The effect of DAMGO on ferret esophageal vagal afferents.

Stimulus response functions of mucosal (A) and tension/mucosal (Ci) receptors to mucosal stroking. Stimulus response functions of tension (B) and tension/mucosal (Cii) receptors to circumferential tension. The responses are before (●) and after exposure to DAMGO (0.1µM (○), 0.3µM (■) and 1µM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (* p<0.05, **p<0.01, ***p<0.001). D, original recording of a tension receptor response to circular tension with a 3g weight in the absence (Di) and presence of DAMGO (0.1µM (Dii), 0.3µM (Diii) and 1µM (Div).
Figure 2.

The effect of ICI 199441 on ferret esophageal vagal afferents.

Stimulus response functions of mucosal (A) and tension/mucosal (Ci) receptors to mucosal stroking. Stimulus response functions of tension (B) and tension/mucosal (Cii) receptors to circumferential tension. The responses are before (●) and after exposure to ICI 199441 (1µM (○), 3µM (■) and 10µM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (* p<0.05, ***p<0.001). D, original recording of a mucosal receptor response to mucosal stroking with a 50mg von Frey hair in the absence (Di) and presence of ICI 199441 (1µM (Dii), 3µM (Diii) and 10µM (Div).
Figure 3.

The effect of SNC 80 on ferret esophageal vagal afferents.

Stimulus response functions of mucosal (A) and tension/mucosal (Ci) receptors to mucosal stroking. Stimulus response functions of tension (B) and tension/mucosal (Cii) receptors to circumferential tension. The responses are before (●) and after exposure to SNC 80 (0.01mM (○), 0.1mM (■) and 1mM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (* p<0.05). D, original recording of a tension receptor response to circular tension with a 3g weight in the absence (Di) and presence of SNC 80 (0.01mM (Dii), 0.1mM (Diii) and 1mM (Div)).
Figure 4.

The effect of naloxone on inhibition of ferret esophageal vagal afferents by DAMGO and ICI 199441.

Stimulus response functions of mucosal receptors to mucosal stroking (A, B).

A. Responses before (●) and after exposure to DAMGO (1µM, ○) and DAMGO (1µM) plus naloxone (10µM, ■). B. Responses before (●) and after ICI 199441 (10µM, ○) and ICI 199441 (10µM) plus naloxone (10µM, ■).

Asterisks indicate significant difference from control using a two-way ANOVA (***p<0.001). The hash symbols indicate significance from data obtained in the presence of agonist and naloxone using a two-way ANOVA (###p<0.001).

C, original recording of a mucosal receptor response to mucosal stroking with a 50mg von Frey hair in the absence (Ci) and presence of DAMGO (1µM (Cii)) and DAMGO along with naloxone (10µM (Ciii)).
References


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A

C (i)

(ii)

(iii)

B

von Frey hair (mg)

Impulses/stroke

n=6

von Frey hair (mg)

Impulses/stroke

n=6

50μV

0.5sec

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n=6

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n=6

50μV

0.5sec

n=6

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