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Pharmacological characterization of the opioid receptor in the submucous plexus of the guinea-pig oesophagus

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1 The cholinergically mediated electrically-induced contractions of the submucous plexus-longitudinal muscularis mucosae preparation of the guinea-pig oesophagus were used to study the actions of opioid peptides and morphine.

2 The twitch contractions of the tissue (0.1 Hz, 0.5 ms, supramaximal voltage) were inhibited by all the opioid peptides and morphine in a concentration-dependent manner. The order of potency was dynorphin-(1–13) > α-neo-endorphin > β-endorphin > [D-Ala^2]-methionine-enkephalin >> α-endorphin, methionine-enkephalin, leucine-enkephalin and morphine.

3 The inhibitory actions of dynorphin-(1–13) (20 nM), α-neo-endorphin (100 nM) and β-endorphin (3 μM) were completely reversed either by naloxone (1 μM) or by morphine (100 μM). The K_e values of naloxone against dynorphin-(1–13) and α-neo-endorphin were 30 and 25 nM, respectively.

4 Increasing the concentration of calcium from 1.8 to 3.6 mM in Tyrode solution decreased the sensitivity of the tissue to dynorphin-(1–13) 7.4 times and to α-neo-endorphin 462 times.

5 The inhibitory actions of dynorphin-(1–13) (100 nM) and α-neo-endorphin (300 nM) were inversely related to stimulus frequency, being most active at low frequencies (0.1–1 Hz), and least active at high (30 Hz).

6 Exogenously applied acetylcholine produced concentration-dependent contractions of the isolated muscularis mucosae, with an EC_{50} of 72.6 ± 4.5 nM. The contractile response of the oesophagus to acetylcholine was not affected by the pretreatment of the tissue with dynorphin-(1–13) (100 nM), α-neo-endorphin (300 nM) or β-endorphin (3 μM).

7 It is concluded that the submucous plexus-longitudinal muscularis mucosae of the guinea-pig oesophagus is inhibited by opioid peptides acting at prejunctional opioid receptors, probably of the κ-subtype.

Introduction

The isolated ileum of the guinea-pig or its myenteric plexus-longitudinal muscle preparation is widely used as an *in vitro* model system to study the mechanism of action of narcotic analgesics (Paton, 1957). Characteristics of this preparation are (1) the extremely small concentrations of morphine required to inhibit the neurogenic contractions, (2) the reversibility of opiate actions by narcotic antagonists, (3) the stereospecificity for opiate actions and (4) the parallelism between the order of potency of drugs causing analgesia and their potency in inhibiting ileal response to cholinergic nerve stimulation (Cox & Weinstock, 1966; Gyang & Kosterlitz, 1966; Kosterlitz & Watt, 1968; Fennessy, Heimans & Rand, 1969). Hughes, Smith, Kosterlitz, Fothergill, Morgan & Morris (1975) demonstrated the existence of opiate-like pentapeptides, methionine-enkephalin and leucine-enkephalin, in brain tissue which mimic the inhibitory effect of opiates on the *in vitro* preparation.

By immunohistochemical studies, it has been demonstrated that the enkephalin-immunoreactive nerve fibres are found mainly in the myenteric plexus and smooth muscle layers of the guinea-pig oesophagus and small intestine (Alumets, Håkanson, Sundler & Chang, 1978; Jessen, Saffrey, Van Noorden, Bloom, Polak & Burnstock, 1980; Uddman, Alumets, Håkanson, Sundler & Walles, 1980).

As the isolated muscularis mucosae of the guinea-pig oesophagus contains a submucous plexus including cholinergic nerve cell bodies, independent of the myenteric plexus, it is a suitable preparation to examine the action of drugs on the submucous plexus (Kamikawa, Shimo & Uchida, 1982; Kamikawa & Shimo, 1982; 1983).

Methods

Male guinea-pigs (300 to 500 g) were killed by stunning, the oesophagus was excised and the isolated muscularis mucosae attached to the submucous plexus was prepared (Kamikawa & Shimo, 1979). Briefly, the excised oesophagus was pinned on a cork mat immersed in Tyrode solution. The outer striated muscle coat was cut longitudinally, and gently peeled away leaving an inner tube. The tube including longitudinal muscularis mucosae, about 15 mm long without a load, was immersed in a 10 ml organ bath filled with a modified Tyrode solution of the following composition (mM): NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaHCO_3$ 11.9, NaH_2PO_4 0.42, disodium ethylenediaminetetraacetic acid (EDTA) 0.03, ascorbic acid 0.12, choline chloride 0.02 and glucose 5.56 (pH 7.4). This solution was bubbled with 5% CO_2 and 95% O_2, and maintained at 37°C.

The preparation was suspended under a 0.3 g load and 60 min was allowed to elapse before experiments were started. During this equilibration period, the tissue was washed with fresh Tyrode solution every 10 min. Responses of the longitudinal muscularis mucosae were recorded by means of an isotonic transducer (MEC-1411) and a Nihon Kohden Polygraph (RJG-4004).

Electrical stimulation of intramural nerves in the muscularis mucosae was carried out transmurally by means of two coaxial platinum electrodes, the anode in the lumen and the cathode in the organ bath. To obtain stable twitch-like contractions, the stimulation parameters were 0.1 Hz, 0.5 ms and supramaximal voltage (approx. 40 V). In some experiments when the relationship between frequency of stimulation and response of the preparation was investigated, the isolated muscularis mucosae was transmurally stimulated with 30 pulses at different frequencies (0.1–30 Hz).

The inhibition of the twitch contractions by opioid peptides was measured as the percentage inhibition of the original twitch height obtained just before the peptide was applied to the bath. In experiments to obtain cumulative concentration-response curves, the interval between each curve was approx. 30 min. The concentration of an opioid peptide required to inhibit the twitch response by 50% (IC_{50}) was calculated from an individual concentration-response curve, and analysed statistically. The value of K_e as a parameter of opioid antagonist activity was determined by estimating the dose-ratio of agonist at a given concentration of antagonist: $K_e = a/DR - 1$, where a is the molar concentration of the antagonist and DR the ratio of IC_{50} of agonist in the presence and absence of the antagonist (Kosterlitz & Watt, 1968). Values in the text refer to the mean ± s.e.mean of n such determinations. Probability levels were determined by Student's t test, and were considered to be significant when $P < 0.05$.

Drugs used were morphine hydrochloride (Dainippon), acetylcholine chloride (Daiichi), naloxone hydrochloride (Endo), methionine-enkephalin, leucine-enkephalin, α-endorphin, human β-

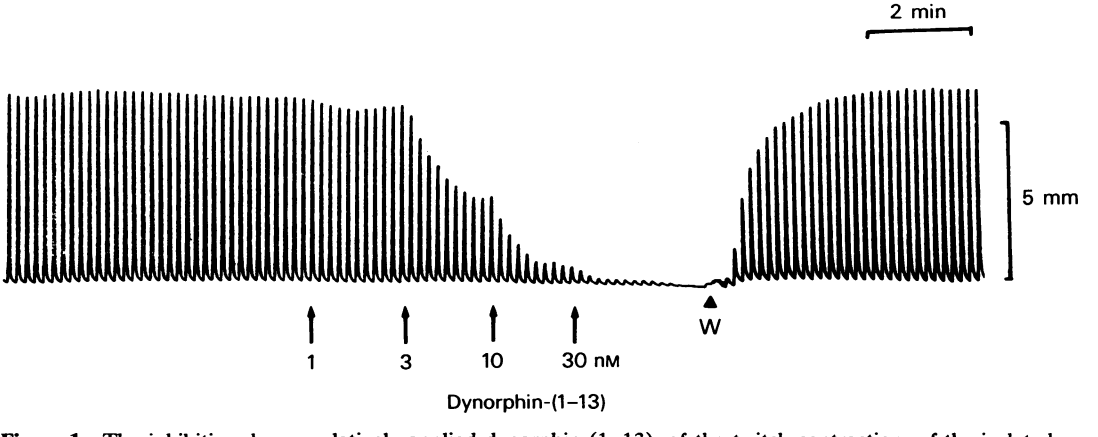

Figure 1 The inhibition, by cumulatively applied dynorphin-(1–13), of the twitch contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by electrical stimulation (0.1 Hz, 0.5 ms, supramaximal voltage). At W, the tissue was washed 3 times with fresh Tyrode solution. Vertical calibration shows 5 mm shortening of the tissue; horizontal calibration shows 2 min.

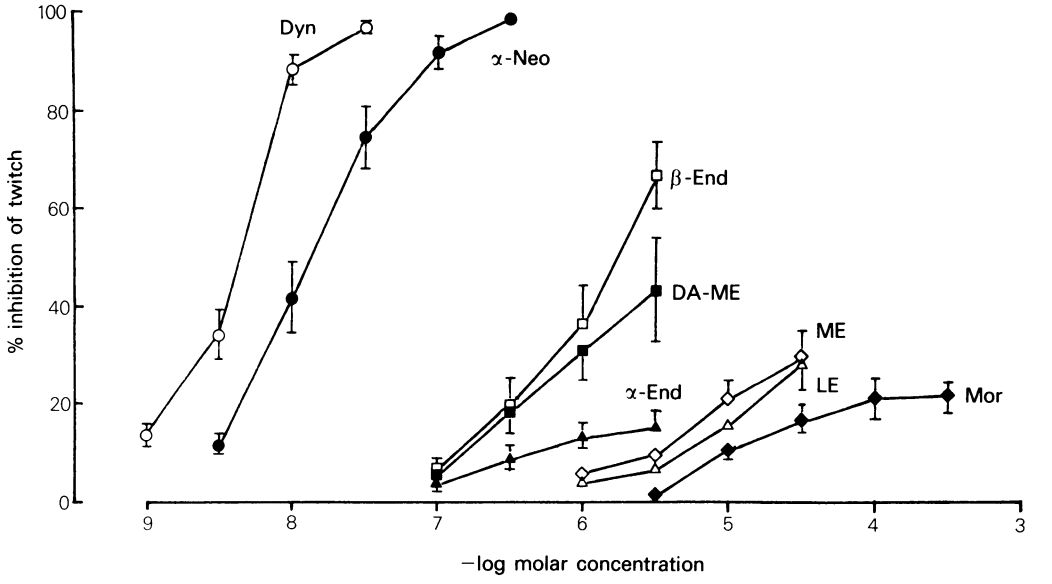

Figure 2 Cumulative concentration-response curves for the inhibitory actions of opioid agonists on the electrically induced twitch contractions of the isolated muscularis mucosae of the guinea-pig oesophagus: dynorphin-(1–13) (Dyn, ○, $n = 21$); α-neo-endorphin (α-Neo, ●, $n = 16$); β-endorphin (β-End, □, $n = 16$); [D-Ala^2]-methionine-enkephalin (DA-ME, ■, $n = 11$); α-endorphin (α-End, ▲, $n = 8$); methionine-enkephalin (ME, □, $n = 16$); leucine-enkephalin (LE, △, $n = 12$); morphine (Mor, ■, $n = 16$), Each point represents the mean response; vertical lines show s.e.mean. Concentrations of endorphins and enkephalins above 3 or 30 μM could not be prepared from the commercial peptides used here.

endorphin, dynorphin-(1–13), porcine α-neo-endorphin (Protein Research Foundation) and [D-Ala^2]-methionine-enkephalin (Peninsula, U.S.A.). All drugs were dissolved in 0.9% w/v NaCl solution (saline).

Results

All the opioid peptides tested inhibited the twitch-like contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by transmural electrical stimulation. However, their inhibitory potencies were variable and dynorphin-(1–13) was most potent. Above 1 nM, dynorphin-(1–13) inhibited the twitch response, and at 30 nM abolished the twitch contractions throughout the period of exposure (about 2 min), but the twitch was restored after washout (Figure 1). Figure 2 shows cumulative concentration-response curves for the inhibitory actions of opioid peptides. α-Neo-endorphin and β-endorphin produced similar inhibitions to that produced by dynorphin-(1–13), but were less potent. [D-Ala^2]-methionine-enkephalin, α-endorphin, methionine-enkephalin, leucine-enkephalin and morphine were much less effective and even at the highest concentrations examined did not reduce the twitch by 50%. The relative potency of α-neo-endorphin and β-endorphin compared with that of dynorphin-(1–13) (= 1) was 0.25 and 0.002, respectively (Table 1). The inhibition of twitch by all opioid peptides was completely reversed by naloxone (1 μM, $n = 21$). The K_e values of naloxone against dynorphin-(1–13) and α-neo-endorphin were 30 and 25 nM, respectively (Table 2). Interestingly, morphine itself, above 10 μM, produced a weak inhibition of the twitch which was completely reversed by naloxone (1 μM, $n = 4$), but 100 μM of morphine fully reversed the inhibitory actions of dynorphin-(1–13)

Table 1 The concentrations causing 50% inhibition of electrically-induced twitch contractions (IC_{50}) of the isolated muscularis mucosae of the guinea-pig oesophagus

Agonists	n	IC_{50} (nM, mean ± s.e.mean)
Dynorphin-(1–13)	21	5.4 ± 0.6
α-Neo-endorphin	16	21.4 ± 6.2
β-Endorphin	16	2600 ± 594
[D-Ala^2]-methionine-enkephalin	11	> 3000
α-Endorphin	8	> 3000
Methionine-enkephalin	16	> 30000
Leucine-enkephalin	12	> 30000
Morphine	16	> 300000

Table 2 The K_e values of naloxone and morphine against dynorphin-(1–13) and α-neo-endorphin in the isolated muscularis mucosae of the guinea-pig oesophagus

	K_e (nM, mean ± s.e.mean)	
	Naloxone	*Morphine*
Dynorphin-(1–13)	29.7 ± 7.4 (12)	12800 ± 3900 (6)
α-Neo-endorphin	24.7 ± 6.5 (12)	7800 ± 2300 (6)

Numbers in parentheses show numbers of observations. K_e values were determined by the method of Kosterlitz & Watt (1968).

(20 nM, $n = 4$) and α-neo-endorphin (100 nM, $n = 4$), as did 1 μM of naloxone. The K_e values of morphine against dynorphin-(1–13) and α-neo-endorphin were 12.8 and 7.8 μM, respectively (Table 2). Other weak agonists such as α-endorphin, methionine-enkephalin or leucine-enkephalin did not show any antagonistic activity in this preparation.

In the following experiments to characterize the opioid action in this preparation, dynorphin-(1–13) and α-neo-endorphin were mainly used as the agonists. Both inhibitory actions of dynorphin-(1–13) and α-neo-endorphin were fully reversible on washing, and repetitive applications produced consistent inhibitory responses.

To examine the role of the calcium ion on the opioid action, the twitch inhibitory actions of opioid peptides were observed in Tyrode solution with an increased calcium concentration (3.6 mM). In these conditions, the height of twitch contractions was almost equivalent to that in the normal Tyrode solution (1.8 mM Ca^{2+}). However, the increase in calcium concentration antagonized the inhibitory actions of dynorphin-(1–13), α-neo-endorphin and β-endorphin so that their concentration-response curves were shifted to the right and were displaced downwards relative to those in the normal solution (Figure 3). The antagonistic effect of calcium was more marked on the action of α-neo-endorphin rather than dynorphin-(1–13). The IC_{50} values of α-neo-endorphin (9.9 ± 4.0 μM, $n = 8$) and dynorphin-(1–13) (39.9 ± 13.6 nM, $n = 14$) in the high calcium medium were 462 and 7.4 times, respectively, higher than those in the normal medium.

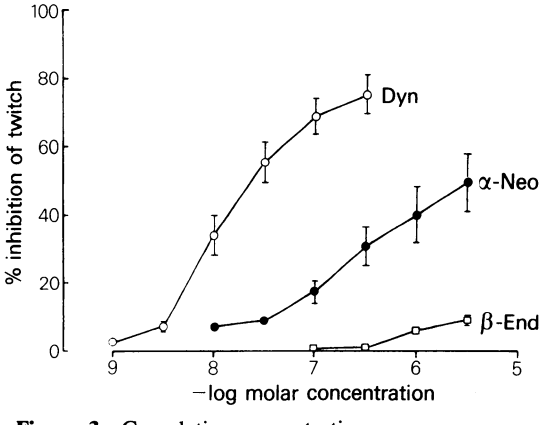

Figure 3 Cumulative concentration-response curves for the twitch inhibitory actions of dynorphin-(1–13) (Dyn, ○, $n = 14$), α-neo-endorphin (α-Neo, ●, $n = 12$) and β-endorphin (β-End, □, $n = 6$) examined in Tyrode solution with twice the normal concentration of calcium (3.6 mM). Each point represents the mean response; vertical lines show s.e.mean. The concentrations of Dyn and α-Neo to inhibit the twitch by 50% (IC_{50}) were 40 ± 14 and 9883 ± 3999 nM, respectively.

The isolated muscularis mucosae responded to contractions by electrical stimulations with varied frequencies (0.1–30 Hz), in a frequency-dependent manner (Figure 4). The contractile responses were also inhibited by dynorphin-(1–13) (100 nM) and α-neo-endorphin (300 nM), but the degree of inhibition was inversely related to the frequency of stimulation. The contractions to lower frequency stimulations (0.1–1 Hz) were abolished by these peptides while those to higher frequency stimulations (10–30 Hz) were inhibited only about 25% (Figure 4).

Exogenously applied acetylcholine, above 10 nM, produced a contraction of the isolated muscularis mucosae of the guinea-pig oesophagus, in a

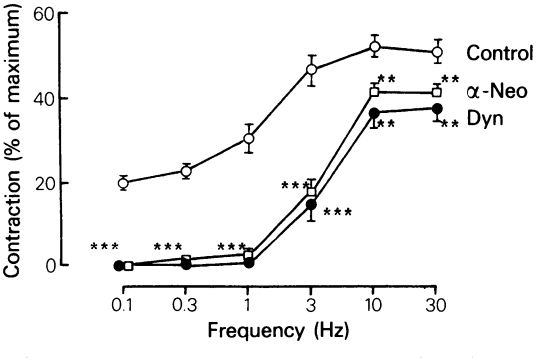

Figure 4 Inhibitory effects of dynorphin-(1–13) and α-neo-endorphin on contractions of the isolated muscularis mucosae of the guinea-pig oesophagus induced by electrical stimulations with varied frequencies. (○) Control ($n = 10$); (●) in the presence of dynorphin-(1–13) (Dyn, 100 nM, $n = 10$); (□) in the presence of α-neo-endorphin (α-Neo, 300 nM, $n = 10$). Electrical stimulation was carried out with rectangular pulses of various frequencies (0.1 to 30 Hz), pulse duration of 0.5 ms and supramaximal voltage. The total number of stimulating pulses was 30. Abscissa scale, stimulus frequencies (Hz). Ordinate scale, % maximal contraction induced by acetylcholine (3 μM). Each point represents the mean response; vertical lines show s.e.mean. $^{**}P < 0.01$; $^{***}P < 0.001$.

concentration-dependent manner, and at 3 μM produced the maximum contraction. The concentration of acetylcholine producing a 50% maximal contraction was 72.6 ± 4.5 nM ($n = 8$). The contractile response to acetylcholine was not affected by the presence of dynorphin-(1–13) (100 nM, $n = 8$), α-neo-endorphin (300 nM, $n = 8$) or β-endorphin (3 μM, $n = 6$).

Discussion

In the present experiments, the inhibition of electrically-induced contractions of the submucous plexus-longitudinal muscularis mucosae preparation of the guinea-pig oesophagus by various opioid peptides and morphine was concentration-dependent, and was fully reversed by washing. These inhibitory actions seem to be mediated by the opioid receptor that is located in cholinergic nerves of the submucous plexus and cause a prejunctional reduction of the acetylcholine release, since the electrically-induced contractions of this tissue are probably mediated by stimulation of intramural cholinergic nerves (Kamikawa & Shimo, 1979; Kamikawa *et al.*, 1982) and the inhibitory actions were completely reversed by naloxone, and were not accompanied with a significant inhibition of the acetylcholine-induced contractions. The order of potency was dynorphin-(1–13) > α-neo-endorphin > β-endorphin > [D-Ala^2]-methionine-enkephalin and much weaker α-endorphin, methionine-enkephalin, leucine-enkephalin and morphine. In other peripheral tissue preparations, such as guinea-pig ileum or mouse vas deferens, dynorphin-(1–13) was the most potent among endogenous opioid peptides and α-neo-endorphin was slightly less potent than dynorphin-(1–13) (Goldstein, Tachibana, Lowney, Hunkapiller & Hood, 1979; Kangawa, Matsuo & Igarashi, 1979; Wüster, Schulz & Herz, 1980; Vaught, 1981; Kangawa, Minamino, Chino, Sakakibara & Matsuo, 1981; Oka, Negishi, Kajiwara, Watanabe, Ishizuka & Matsumiya, 1982; Oka, Negishi, Suda, Sawa, Fujino & Wakimasu,1982; Yoshimura, Huidobro-Toro, Lee, Loh & Way, 1982). However, endorphins, enkephalins and morphine were more potent in those preparations than in the present preparation (Lord, Waterfield, Hughes & Kosterlitz, 1977; Kosterlitz & Hughes, 1978; Kamikawa & Shimo, 1978). The different orders of potency of different opioids can be explained by the concept of multiple opioid receptors, since the existence of heterogeneous populations of the opioid receptor has been postulated in both central and peripheral nervous structures (Lord *et al.*, 1977; Iwamoto & Martin, 1981; Wüster, Schulz & Herz, 1981). The guinea-pig ileum contains predominantly μ-receptors, where morphine acts as a potent agonist, while the mouse vas deferens contains the δ-receptor where leucine-enkephalin acts as a potent agonist. In addition, it has also been postulated that the rat vas deferens contains the ε-receptor which is specifically activated by β-endorphin (Schulz, Wüster & Herz, 1981) and the rabbit vas deferens contains the κ-receptor which is activated by ethylketocyclazocine, but not by morphine, enkephalins and endorphins (Oka, Negishi, Suda, Matsumiya, Inazu & Ueki, 1981). Much evidence indicates that dynorphin-(1–13) and α-neo-endorphin specifically activate the κ-opioid receptor in the guinea-pig ileum or the mouse vas deferens (Huidobro-Toro, Yoshimura, Lee, Loh & Way, 1981; Oka *et al.*, 1982 a, b; Chavkin, James & Goldstein, 1982; Yoshimura *et al.*, 1982). Therefore, the order of potency of opioid peptides observed in the present experiments suggests that the submucous plexus of the guinea-pig oesophagus contains predominantly the κ-opioid receptor. This is also supported by the antagonistic potency of naloxone. Naloxone is a relatively specific μ-receptor antagonist and its K_e value against μ-receptor agonists in the guinea-pig ileum was 1–3 nM (Lord *et al.*, 1977; Kamikawa & Shimo, 1978). However, the K_e value of naloxone against κ-receptor agonists in the same tissue was about 10 times higher, 10–40 nM (Oka *et al.*, 1982 a, b; Chavkin *et al.*, 1982; Yoshimura *et al.*, 1982). The K_e values of naloxone against dynorphin-(1–13) and α-neo-endorphin obtained in the present experiments (see Table 2) were close to that obtained in the guinea-pig ileum with κ-receptor agonists. Furthermore, in the guinea-pig oesophagus, morphine acts as a partial agonist, as the drug does in the rat vas deferens (Huidobro, Huidobro-Toro & Miranda, 1980; Ishii, Yamamoto, Muraki & Kato, 1981).

The opioid action in the present preparation also had the following characteristics: inhibition of the electrically-induced contraction inversely dependent on the stimulus frequency and antagonism by increasing the concentration of calcium in the medium. Antagonism of opioid actions by increasing the extracellular concentration of calcium has also been reported in the ginea-pig ileum (Opmeer & Van Ree, 1979; 1980). The present results indicate that calcium ion plays a significant role in the inhibitory action of opioid peptides on the twitch response of the muscularis mucosae. Although both concentration-inhibition curves of dynorphin-(1–13) and α-neo-endorphin in this preparation were shifted to the right by increasing the concentration of calcium, the degree of shift was more pronounced for α-neo-endorphin than for dynorphin-(1–13). The cause of the different degree of antagonism by calcium is not clear, but it may be that α-neo-endorphin acts on a different type of opioid receptor from that

for dynorphin-(1–13). Evidence indicating that α-neo-endorphin acts on an opioid receptor subtype other than κ- and μ-receptors in the mouse vas deferens, while the peptide acts as a κ-receptor agonist in the guinea-pig ileum, has been presented previously (Oka *et al.*, 1982a).

In conclusion, the submucous plexus-longitudinal muscularis mucosae of the guinea-pig oesophagus is a novel neuroeffector preparation involving only κ-opioid receptors, and therefore is a useful *in vitro* preparation to study the mechanism of action of opioids.

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