Br. J. Pharmac (1987), **92**, 437–443

δ-Opioid receptors mediate inhibition of fast excitatory postsynaptic potentials in cat parasympathetic colonic ganglia

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1 The effects of opioids on synaptic transmission in cat sacral parasympathetic colonic ganglia were studied *in vitro*, using intracellular electrophysiological techniques. Electrical stimulation of the pelvic nerve evoked fast excitatory postsynaptic potentials (e.p.s.ps), which were blocked by hexamethonium and tetrodotoxin.

2 [D-Pen^2, D-Pen^5] enkephalin and [Met^5]enkephalinamide, δ-opioid receptor agonists, caused concentration-dependent, reversible depression of fast e.p.s.ps, but had no effect on depolarizations evoked by pressure ejection of the nicotinic agonist 1,l-dimethyl-4-phenyl-piperazinium. Cell transmembrane potential and membrane input resistance were also unaffected.

3 U-50,488H, a κ-opioid receptor agonist, had a very small depressant action while [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin, a μ-opioid receptor agonist, had no effect on fast e.p.s.p. amplitude. Neither compound affected cell transmembrane potential or membrane input resistance.

4 The inhibitory actions of [D-Pen^2, D-Pen^5] enkephalin were antagonized by both naloxone, an antagonist at each of the three opioid receptor types, and by ICI 174,864, an antagonist selective for δ-opioid receptors.

5 Naloxone and ICI 174,864 both also potentiated fast e.p.s.p. amplitude *per se* in 50% of cells tested.

6 It is concluded that exogenous opioids act at presynaptic δ-opioid receptors to inhibit sacral parasympathetic synaptic transmission in cat colonic ganglia *in vitro*. Furthermore, the effects of opioid anatagonists alone, suggest that endogenous opioids may also be released by preganglionic nerve stimulation and so regulate the release of acetylcholine in these ganglia.

Introduction

Opioids have been shown to inhibit neurotransmission in a wide variety of central and peripheral tissues, including the gastrointestinal tract. In a previous study we showed that opioids inhibit pelvic nerve-evoked contractions of cat distal colon longitudinal muscle *in vitro* (Kennedy & Krier, 1987a). This action was mediated via prejunctional δ-opioid receptors, located, at least in part, on neurones in the colonic myenteric plexus. However, it was not clear whether inhibitory opioid receptors were also present in the extramural parasympathetic colonic ganglia, which provide postganglionic innervation to the colon (de Groat & Krier, 1976; Krier & Hartman, 1984).

The aim of this study was to determine if opioids inhibit synaptic transmission in cat colonic ganglia *in vitro*, using intracellular electrophysiological recording techniques. Furthermore, agonists and antagonists selective for μ-, δ- and κ-opioid receptors were used to determine the class of any such opioid receptor present (Martin *et al.*, 1976; Lord *et al.*, 1977). Opioid receptor types in other mammalian prevertebral autonomic ganglia have not been elucidated using these compounds. Parts of this study have been communicated previously (Kennedy & Krier, 1987b).

Methods

Cats of either sex (2.5–4 kg) were anaesthetized with sodium pentobarbitone (25–35 mg kg^{-1}, i.p.) and exsanguinated. The large intestine was exposed and the left or right pelvic nerve and its neural connections with the extramural colonic ganglia and colonic nerve fibres were dissected from the underlying connective

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tissue. This preparation was placed in an organ bath (3.5 ml), pinned to a thin layer of Sylgard and overlying connective tissue removed from around the ganglia. The tissue was superfused continuously (6 ml min^{-1}) with a modified Krebs solution at 37°C, bubbled with 95% O_2, 5%CO_2, containing (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 134, HCO^-_3 15.5, $H_2PO^-_4$ 1.2 and glucose 11.5.

The methods used to record electrical potentials and to inject current were similar to those described previously (Krier & Szurszewski, 1982; Krier & Hartman, 1984). Intracellular recordings of transmembrane potential were made using conventional glass microelectrodes filled with 3 M KCl and with resistances of 30–80 MΩ. Bipolar platinum electrodes were placed on the pelvic nerve and rectangular pulses (0.5 ms, 0.2 Hz) applied via a stimulator to evoke fast excitatory postsynaptic potentials (e.p.s.ps) and action potentials. Fast e.p.s.ps were evoked while the cell was hyperpolarized by intracellular current pulses though the recording electrode (50–80 ms duration) to approximately −60 mV (mean = −60.1 ± 1.1 mV) in order to prevent the initiation of action potentials (see Konishi *et al.*, 1979). Synaptic potentials and transmembrane potential were displayed on an oscilloscope, recorded on magnetic tape by an FM tape recorder and placed on a digital computer by an analog-to-digital conversion program.

The amplitude of fast e.p.s.ps was continuously monitored before, during and after drug addition, using a signal averaging program which averaged 6–12 consecutive fast e.p.s.ps. A control period of 2–6 min (i.e. 24–72 individual fast e.p.s.ps) was obtained before drug addition. Drugs were applied to the tissue in the superfusate except for 1,1-dimethyl-4-phenylpiperazinium (DMPP), which was applied by pressure ejection from a micropipette (2 MΩ tip resistance, 20–50 ms pulse duration, 20 psi). The tip of the micropipette was positioned 50–100 μm from the impaled cell. Opioid agonists were added for 5–8 min. The amplitude of fast e.p.s.ps obtained in the presence of opioids was expressed as a percentage of those obtained during the control period.

Naloxone and ICI 174,864 were added to the bathing solution 10–12 min before the addition of opioid agonist. Antagonistic actions of these two drugs were measured in different cells from control responses to agonists as a 30 min dose cycle was needed to avoid desensitization. Subsequently, most cells could only be held long enough to apply an agonist once.

Conduction velocity of preganglionic fibres providing synaptic input to neurones in the colonic ganglia was estimated by subtracting the ganglionic delay (4.1 ± 0.5 ms, n = ms, n = 8, estimated by method of Krier & Hartman, 1984) from the measured latency of the evoked synaptic response and dividing by the conduction distance. Membrane input resistance was determined either from the amplitude of the steady-state electrotonic potential evoked by injection of a hyperpolarizing current pulse (50–80 ms duration, 0.2 Hz), or from the slope of the plot of the voltage-current relationship evoked by injection of a series of hyperpolarizing and subthreshold depolarizing current pulses (50–80 ms duration, 0.2 Hz) through the recording microelectrode. Membrane time constant was defined as the time taken for such a hyperpolarization to reach 63.2% of it final value. Membrane input capacitance was calculated as time constant/input resistance. All responses have been expressed as the mean ± s.e.mean, and were analysed by use of Student's unpaired t test. A probability of less than 0.05 was considered significant.

Drugs used were: 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP), hexamethonium bromide, [Met^5]enkephalinamide acetate, naloxone hydrocholoride, tetrodotoxin (Sigma Chemical Company); U-50,488H (*trans* 3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl cyclohexyl]-benzene acetamide methane sulphonate) (Upjohn Company); [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin and [D-Pen^2, D-Pen^5] enkephalin (Peninsula Laboratories); ICI 174,864 (ally l-Tyr-Aib-Aib-Phe-Leu-OH_2) (Cambridge Research Biochemicals Ltd) and sodium pentobaritone (Abbot Laboratories). Drugs were dissolved in distilled water or Krebs solution.

Results

Pelvic nerve-evoked fast e.p.s.ps

Intracellular recordings were made in 35 cells from 22 preparations. Mean transmembrane potential was −49.5 ± 0.5 mV (range = −45 to −60 mV). Mean membrane input resistance was 38.9 ± 2.5 MΩ (n = 29), mean time constant was 4.3 ± 0.3 ms (n = 29) and mean input capacitance was 124 ± 10 pF (n = 29). Electrical stimulation of the pelvic nerve trunk (0.2 Hz, 0.5 ms pulse duration) evoked fast e.p.s.ps and action potentials. Mean e.p.s.p. amplitude was 6.6 ± 0.4 mV (n = 31 cells). Threshold depolarization for initiation of an action potential was 9.6 ± 0.5 mV (n = 4) and mean action potential amplitude was 53.9 ± 1.2 mV (n = 14). Slow excitatory or inhibitory postsynaptic potentials were not seen, even when trains of stimuli were applied. Calculated conduction velocity of fibres providing presynaptic input to the ganglion cells was 0.74 ± 0.09 ms^{-1} (n = 29), confirming that synaptic input to neurones of the colonic ganglia is mediated entirely by slowly conducting preganglionic fibres (Krier & Hartman, 1984). Fast e.p.s.ps and action potentials were inhibited by tetrodotoxin (10^{-7} M;

$n = 4$) or by hexamethonium (10^{-4} M; $n = 2$), indicating that they were neurogenic and mediated by acetylcholine acting at nicotinic receptors.

Effects of opioid agonists on fast e.p.s.ps [Met^5]enkephalinamide (3×10^{-6}–3×10^{-5} M) depressed fast e.p.s.p. amplitude in a concentration-dependent, reproducible and reversible manner (9 of 9 cells) (Figure 1a, b). This was mimicked by [D-Pen^2, D-Pen^5] enkephalin (DPDPE) (10^{-6}–10^{-5} M) (8 of 9 cells), a δ-opioid receptor-selective agonist (Mosberg *et al.*, 1983; Corbett *et al.*, 1984) (Figures 1a and 3b, c). DPDPE was 3–4 times more potent than [Met^5]enkephalinamide (Figure 1a). Maximal depression of fast e.p.s.ps by both drugs was seen 2–5 min following onset on inhibition. Fast e.p.s.p. ampltiude returned to control values within 20–30 min without in drug-free solution (Figure 1b).

[D-Ala^2, MePhe^4, Gly-ol^5] enkephalin ($10-^5$ M), a μ-opioid receptor-selective agonist (Handa *et al.*, 1981; Cotton *et al.*, 1984; Corbett *et al.*, 1984), had no effect on fast e.p.s.p. amplitude ($n = 3$) (Figure 1a). U-50,488H (3×10^{-5} M), a κ-opioid receptor-selective agonist (Piercey *et al.*, 1982), had a very small

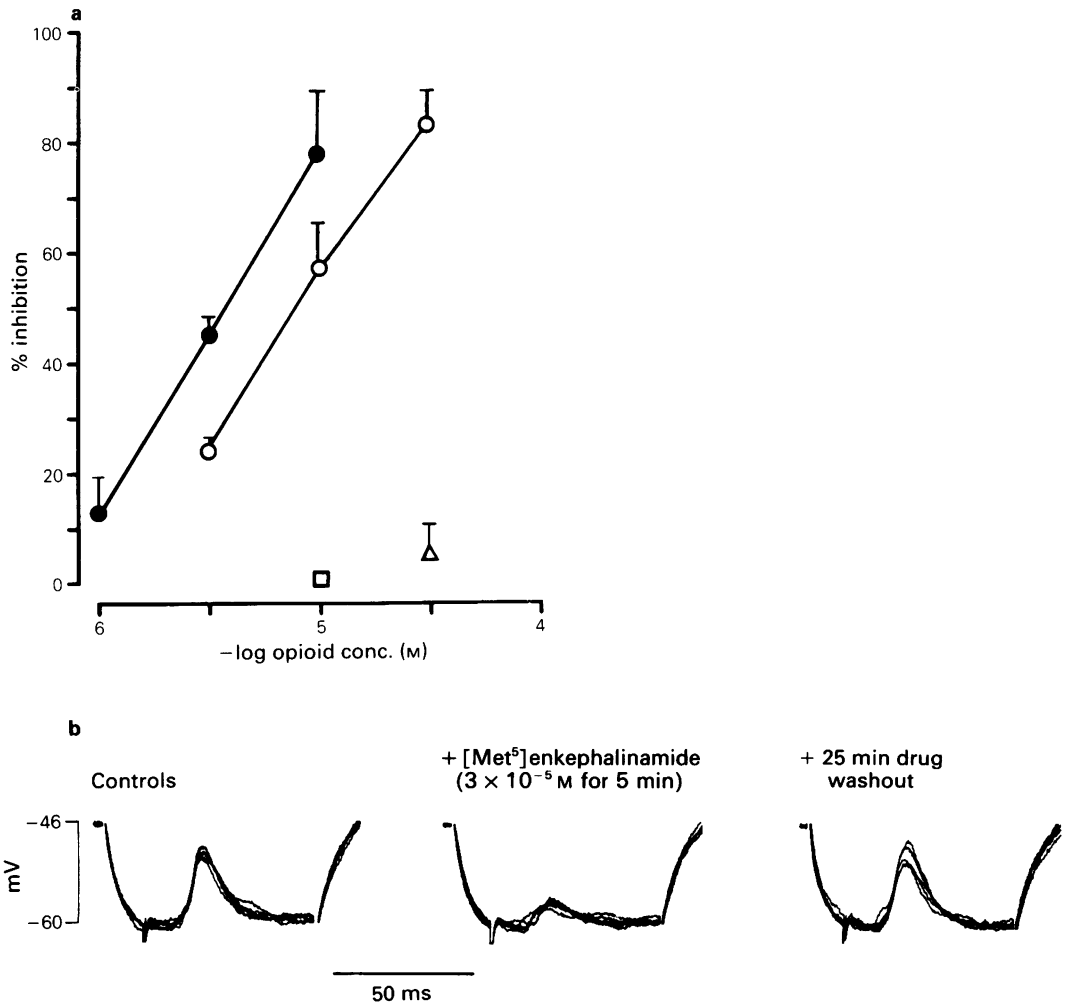

Figure 1 Cat colonic ganglia *in vitro*: (a) effect of [D-Pen^2, D-Pen^5] enkephalin (10^{-6}–10^{-5}) (●), [Met^5]enephalinamide ($3 \times 10^{-6-6}$–3×10^{-5} M) (○), [D-Ala^2, MePhe^4, Gly-ol^5] enkephalin (10^{-5} M), ([□]) and U-50,488H (3×10^{-5} M) (△) on pelvic nerve-evoked fast e.p.s.ps (0.2 Hz, 0.5 ms pulse duration). Vertical lines show s.e.mean, $n = 3$ for each point. (b) Inhibition of fast e.p.s.ps by [Met^5]enkephalinamide (3×10^{-5} M) and subsequent recovery on drug washout. Each frame shows computer-superimposed traces of 6 successive e.p.s.ps at same stimulation intensity.

depressant action of fast e.p.s.p. amplitude (5 ± 5% decrease) ($n = 3$) (Figure 1a). Thus, δ- but neither μ- nor κ-opioid receptor agonists potently inhibited nicotinic fast e.p.s.ps.

Effects of opioid agonists on transmembrane potential and membrane input resistance

In all of the cells described above, opioid agonists had no effect on transmembrane potential or membrane input resistance. Furthermore, in the absence of pelvic nerve stimulation, DPDPE (3×10^{-6} M) had no effect on membrane input resistance, when measured either from the steady-state amplitude of electronic potentials evoked by injection of a single hyperpolarizing current pulse (0.2–0.5 nA, 50–80 ms duration, 0.2 Hz ($n = 2$) (Figure 2a) or from the slope of the plot of the voltage-current relationship following a series of hyperpolarizing and subthreshold depolarized current pulses ($n = 2$) (Figure 2b). This suggests that opioids of unlikely to inhibit fast e.p.s.ps here by changing the electrophysiological properties of the postsynaptic membrane.

Effects of naloxone and ICI 174,864 Naloxone (10^{-6} M) and ICI 174,864 (10^{-6} M) (a selective antagonist at δ-opioid receptors (Cotton *et al.*, 1984; Corbett *et al.*, 1984; Kennedy & Krier, 1987a)) had no effect on transmembrane potential or membrane input resistance ($n = 5$ and $n = 3$, respectively). Fast e.p.s.p. amplitude was also unaffected by naloxone (10^{-6} M) in 3 of the cells and by ICI 174, 864 (10^{-6} M) in 1 of the cells. However, naloxone (10^{-6} M) increased fast e.p.s.p. amplitude by 100% in 1 cell, while in another cell fast e.p.s.p. amplitude was increased such that action potentials were evoked. Also, in 2 cells ICI 174,864 (10^{-6} M) increased fast e.p.s.p. amplitude by 26 and 75%, without initiating action potentials. These potentiating actions of naloxone and ICI 174,864 were seen within 1–2 min of the drug reaching the organ bath and potentiation was maintained for the duration of drug administration. The effects of washout of naloxone and ICI 174,864 were not studied.

In the presence of naloxone (10^{-6} M), the inhibitory actions of DPDPE (3×10^{-6} M^{-6} M) on fast e.p.s.ps were significantly antagonized ($P < 0.05$) (Figure 3a) (control = 44 ± 4% inhibition: test 6 ± 6% inhibition: $n = 3$ in both cases). This was seen whether naloxone had potentiated fast e.p.s.ps ($n = 1$) or not ($n = 2$). In the presence of ICI 174,864 (10^{-6} M), the inhibitory actions of DPDPE (3×10^{-6} M) on fast e.p.s.ps were abolished ($n = 3$) (Figure 3b,c). Similar to naloxone, antagonism was seen whether ICI 174,864 had potentiated fast e.p.s.ps ($n = 2$) or not ($n = 1$). Furthermore, in one cell in which potentiation was seen, pelvic nerve stimulation intensity was decreased such that fast e.p.s.ps were of a similar magnitude to control fast e.p.s.ps. Again, the inhibitory actions of DPDPE were antagonized.

Effect of DPDPE on nicotinic depolarizations

DPDPE may inhibit fast e.p.s.ps by altering the sensitivity of the postsynaptic cell to endogenously released acetylcholine. However, DPDPE (3×10^{-6} M) did not depress the amplitude of depolarizations evoked by pressure ejection of the nictonic agonist DMPP (10^{-2} M, 20–50 ms pulse duration, 20 psi)

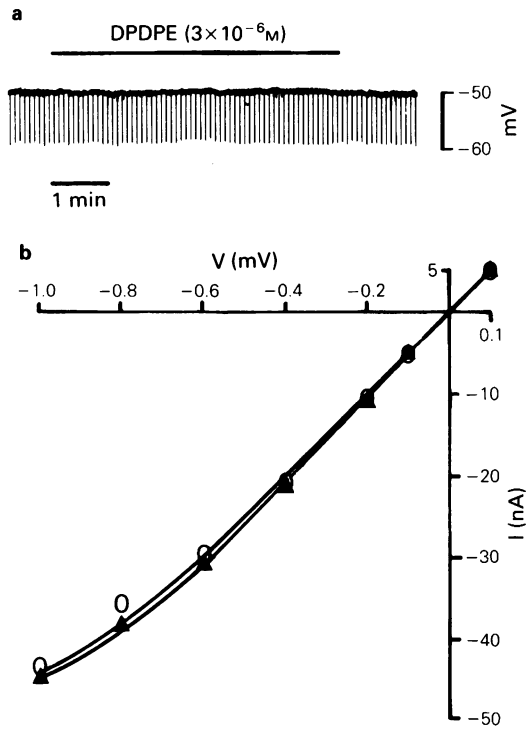

Figure 2 Cat colonic ganglia *in vitro*: effect of [D-Pen^2, D-Pen^5] enkephalin (DPDPE; 3×10^{-6} M) on transmembrane potential and calculated membrane input resistance. (a) Record of transmembrane potential and electrotonic potentials. Input resistance was calculated from the amplitude of the steady-state electrotonic potential evoked by injection of a hyperpolarizing current pulse (0.5 nA, 50 ms duration, 0.2 Hz) through the recording electrode. Input resistance was 20 MΩ in this cell. (b) Input resistance was calculated from the slope of the linear portion of the plot of the steady-state voltage-current relationship evoked by injection of a series of hyperpolarizing and subthreshold depolarizing current pulses (60 ms duration, 0.2 Hz). Membrane input resistance calculated for this cell was 51 MΩ in the absence (○) of opioid and 52 MΩ in its presence (▲).

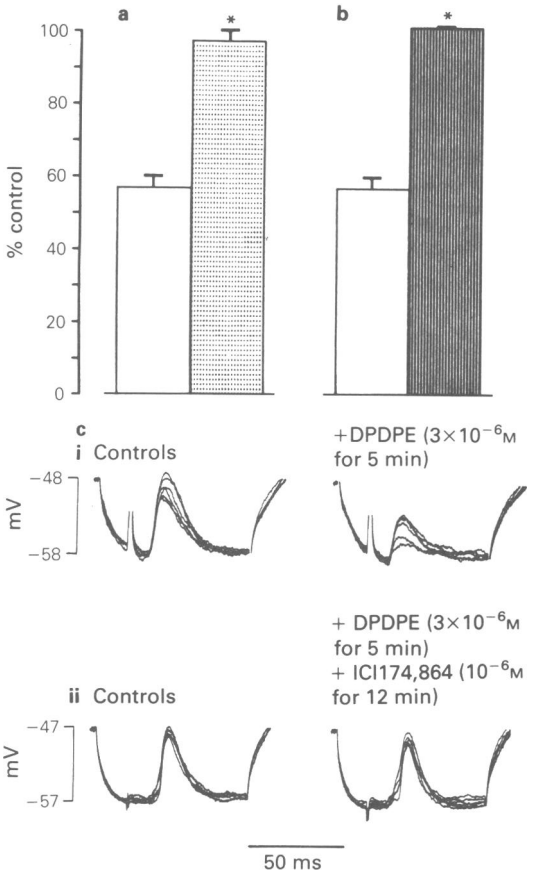

Figure 3 Cat colonic ganglia *in vitro*: effect of [D-Pen^2, D-Pen^5] enkephalin (DPDPE; 3×10^{-6} M) on pelvic nerve-evoked fast e.p.s.ps (0.2 Hz, 0.5 ms pulse duration) in the absence (open columns) and presence (shaded columns of (a) naloxone (10^{-6} M) and (b) ICI 174,864 (10^{-6} M). Vertical lines show s.e.mean; $n = 3$ for each column. Statistical differences are indicated by an asterisk ($^*P < 0.05$). (c) (i) Inhibition of fast e.p.s.ps by [D-Pen^2, D-Pen^5] enkephalin (3×10^{-6} M), (ii) antagonism of this inhibition by preincubation with ICI 174,864 (10^{-6} M). Each frame shows computer-superimposed traces of 6 successive responses at the same stimulation intensity. Responses in c (i) and (ii) are from two different cells.

($n = 2$) (Figure 4). Depolarization was abolished by hexamethonium (10^{-4} M) (n = 1) (Figure 4). These results are consistent with a presynaptic site of action for DPDPE in inhibiting fast e.p.s.ps.

Discussion

The results of this study show that exogenous opioids act at presynaptic δ-opioid receptors to inhibit sacral parasympathetic neurotransmission in cat colonic ganglia *in vitro*. Furthermore, since opioid receptor antagonists increased the amplitude of nicotinic fast e.p.s.ps in 50% of the cells tested, preganglionic nerve stimulation may also release endogenous opioids, which can subsequently modulate the release of acetylcholine in this ganglion.

Peripheral opioid receptors have been divided into three types, μ, δ and κ (Martin *et al.*, 1976; Lord *et al.*, 1977). In this study the receptors mediating inhibition of pelvic nerve-evoked fast e.p.s.ps appear to be of the δ type. The δ-selective agonist, DPDPE (Mosberg *et al.*, 1983; Corbett *et al.*, 1984), inhibited fast e.p.s.ps (and could abolish them at the highest concentration), whereas the κ-selective agonist, U-50,488H (Piercy *et al.*, 1982) and the μ-selective agonist, [D-Ala^2, MePhe^4, Gly-ol^5) enkephalin (Handa *et al.*, 1981; Cotton *et al.*, 1984) had little or no effect. Furthermore, the inhibitory actions of DPDPE were largely inhibited by naloxone and abolished by ICI 174,864, a δ-selective antagonist (Cotton *et al.*, 1984; Corbett *et al.*, 1984; Kennedy & Krier, 1987a).

δ-Opioid receptors have also been shown to be present in other neurones of the gastrointestinal tract, including guinea-pig caecum submucous plexus (Mihara & North, 1986) and cat colonic myenteric plexus (Kennedy & Krier, 1987a). δ-Opioid receptors have also been suggested to mediate inhibition of neurotransmission in other parasympathetic (Simonds *et al.*, 1983; Katayama & Nishi, 1984) and sympathetic (Konishi *et al.*, 1979; 1981) ganglia and of action potentials of neurones in sensory ganglia (Werz & McDonald, 1983), though this awaits confirmation with selective agonists and antagonists.

The present results suggest that the inhibitory opioid receptors are located presynaptically, acting to depress evoked release of acetycholine. Postsynaptic nicotinic receptors are unlikely to be affected by opioids since DPDPE had no effect on depolarization evoked by the nicotinic agonist DMPP. A change in the electrophysiological properties of the postsynaptic membrane is also unlikely since DPDPE and [Met^5]enkephalinamide did not alter transmembrane potential or membrane input resistance. The mechanism of action underlying presysnaptic inhibition was not studied further here, but δ-opioid receptors have been suggested to depress excitability in other systems by an increase in membrane potassium conductance (Werz & McDonald, 1983; North, 1986) or by a decrease in membrane calcium conductance (Tsunoo *et al.*, 1986).

Opioids inhibit pelvic nerve-evoked contractions of cat distal colon longitudinal muscle *in vitro* (Kennedy & Krier), 1987a). This was mediated by δ-opioid receptors, located, at least in part, in neurones of the colonic myenteric plexus. However, the contribution of opioid receptors in the extramural colonic ganglia to inhibition of contractile activity was not clear.

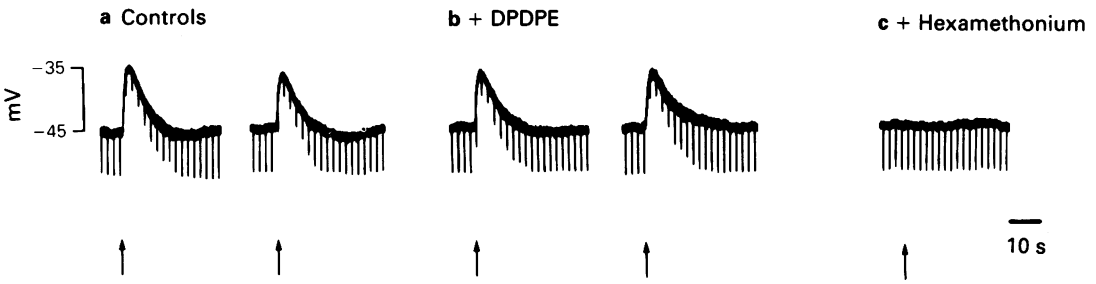

Figure 4 Cat colonic ganglia *in vitro*: depolarisation of transmembrane potential evoked by pressure ejection of 1,1-dimethyl-4-phenyl-piperazinium (DMPP, 10^{-2} M, 20 ms pulse duration, 20 psi) (↑), (a) in the absence of and (b) in the presence of [D-Pen^2, D-Pen^5] enkephalin (3×10^{-6} M for 5 min). (c) DMPP-induced depolarization was abolished by hexamethonium (10^{-4} M for 4 min).

The current study shows that although δ-opioid receptors are present in the extramural colonic ganglia and do mediate inhibition of ganglionic neurotransmission, they are unlikely to have been involved in the inhibition of contractile activity seen previously, since the effective concentration range for DPDPE in inhibiting contractions was 3×10^{-10}–10^{-8} M, whereas that for inhibiting fast e.p.s.ps was 10^{-6}–10^{-5} M.

Endogenous opioids could be important physiological inhibitory modulators of sacral parasympathetic neurotransmission in cat colonic ganglia. Firstly, enkephalin-like immunoreactivity has been shown in cat sacral preganglionic neurones (Glazer & Basbaum, 1980). Secondly, in the present study the opioid receptor antagonists naloxone and ICI 174,864, potentiated the amplitude of nicotinic fast e.p.s.ps. However, potentiation was seen in only 50% of the cells suggesting that opioid-release may be intermittent, as has been found in guinea-pig ileum (Clark & Smith, 1983). Alternatively, only some preganglionic neurones may release opioids at the low frequency of stimulation (0.2 hz) used here. A higher frequency of stimulation (50 Hz) has been shown to release opioids from preganglionic fibres providing input to neurones in the inferior mesenteric ganglion of guinea-pig (Konishi *et al.*, 1981). Further studies employing other concentrations of naloxone and ICI 174,864 and different parameters of pelvic nerve stimulation are required to quantify fully the potentiating actions of the opioid antagonists.

This work was supported by Research Grant AM 29920 from the National Institutes of Health. J.K. is a recipient of a Research Career Development Award (AM 01273). We thank Dr William H. Percy for advice and criticism, Lynn Sherer for technical assistance and Sharon Shaft for secretarial assistance.

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(Received April 10, 1987.
Revised June 4, 1987.
Accepted June 12, 1987.)