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Non-cholinergic, non-adrenergic inhibitory neurons in human internal anal sphincter muscle

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Non-cholinergic, non-adrenergic (NCNA) inhibitory nerves relax circular muscle strips from the human internal anal sphincter (IAS; Burleigh et al 1979; McKirdy 1981), thus confirming the predictions of Bennett & Whitney (1966) and Burnstock (1975). Electrical field stimulation of muscle strips obtained from any region of the human gut produces either biphasic responses or contractions (Bennett & Stockley 1975). Only the IAS (Burleigh et al 1979) and the lower oesophageal sphincter (Burleigh et al 1979) respond with just a relaxation, no cholinergic or adrenergic antagonist drugs being required to unmask or accentuate this reponse.

In the present study investigations have been made on the pharmacology of the NCNA inhibitory nerves particularly regarding the possible involvement of vasoactive intestinal peptide (VIP) which has been proposed as the transmitter released from NCNA inhibitory nerves of the lower oesophageal sphincter (Goyal et al 1980) and is found in nerve fibres supplying the IAS (Alumets et al 1978).

Methods

Sphincter muscle strips were obtained and set up as described previously (Burleigh et al 1979). Briefly, the strips were immersed in Krebs bicarbonate solution at 37 °C and gassed with 5% CO_2 in oxygen. Electrical field stimulation was produced using two parallel platinum wire electrodes immersed in the Krebs fluid; monophasic square wave pulses being delivered from a Grass S88 stimulator. Parameters of stimulation are indicated in the results section.

The compounds used were: morphine sulphate, leucine enkephalin, γ-amino butyric acid, substance P, bradykinin methionine lysine, vasoactive intestinal peptide, noradrenaline acid tartrate, isoprenaline sulphate, naloxone hydrochloride, carboxypeptidase-B DFP, α-chymotrypsin, tetrodotoxin. Where appropriate, significance of results was assessed using Student's paired *t*-test.

Results

IAS muscle showed a high degree of tone in-vitro. Muscle strips obtained from the proximal region of the sphincter showed spontaneous contractions and relaxations which occurred at a frequency of 11–12 min^{-1}. Tetrodotoxin (0·1–1 μg ml^{-1}, n = 14) caused small increases in tone of sphincter muscle, an effect usually seen only with proximal strips which had not been stored overnight at 4 °C (storage was sometimes used to increase the maximum number of experiments using tissue from one operation).

Compounds investigated as possible neurotransmitter candidates for the NCNA inhibitory nerves were: γ-aminobutyric acid (GABA), substance P, bradykinin and vasoactive intestinal peptide (VIP). GABA had no effect (10–100 × 10^{-6} M, n = 3) or gave small contractions of sphincter muscle (5 × 10^{-4} M, n = 3), substance P only produced contractions (7–33 × 10^{-6} M, n = 6), while bradykinin (15–190 × 10^{-9} M, n = 7) and VIP (0·6–750 × 10^{-9} M, n = 11) relaxed sphincter muscle.

Although there are no selective receptor blockers for bradykinin or VIP, their actions may be antagonized by proteolytic enzymes such as carboxypeptidase B-DFP (Al-Dhahir & Zeitlin 1981) or α-chymotrypsin (Mackenzie & Burnstock 1980). As α-chymotrypsin partially relaxed the muscle strips, noradrenaline (3 × 10^{-6} M) plus ascorbic acid (110 × 10^{-6} M) were added to the Krebs fluid to restore tone, an identical procedure being successfully used in a previous investigation (Burleigh et al 1979). The effect of these enzymes on relaxations to the peptides and stimulation of NCNA inhibitory nerves is recorded in Fig. 1. Carboxypeptidase B-DFP (16–20 units ml^{-1}) significantly reduced relaxations to bradykinin ($P < 0.05$) whereas relaxations to inhibitory nerve stimulation remained unchanged ($P > 0.9$). α-Chymotrypsin (0·5 units ml^{-1}) significantly reduced

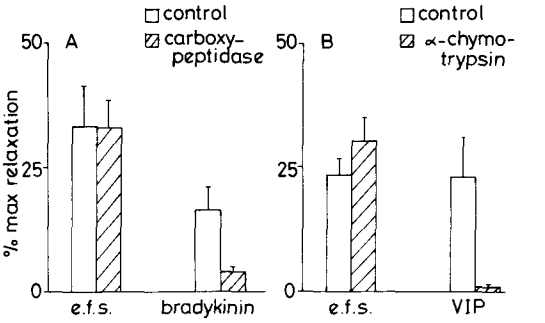

FIG. 1. (A) Effect of carboxypeptidase-B DFP (16–20 units ml^{-1}, (n = 7) on relaxations of sphincter muscle to bradykinin (15–190 × 10^{-9} M) and electrical field stimulation (e.f.s. 1 ms, 177 mA, 1–4 Hz, 30 s). (B) Effect of α-chymotrypsin (0·5 units ml^{-1}, n = 5) on relaxations to VIP (30–240 × 10^{-9} M) and e.f.s. (1 ms, 177 mA, 1–2 Hz, 30 s). Values expressed as mean ± s.e.m.

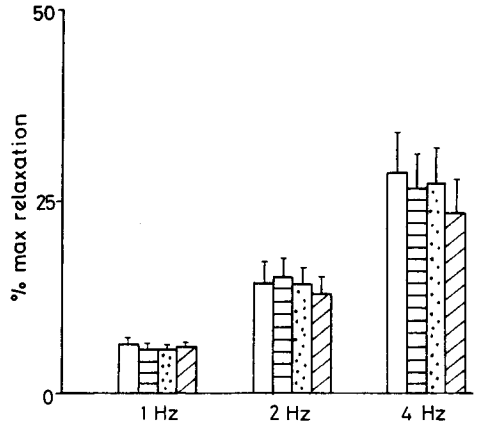

FIG. 2. Effect of morphine on relaxation of sphincter muscle to electrical field stimulation (1 ms, 177 mA, 30 s). Histogram shows % change in relaxation produced by morphine (open columns—control, cross hatched columns—$0{\cdot}3 \times 10^{-6}$ M, dotted columns—3×10^{-6} M, diagonally hatched columns—13×10^{-6} M) expressed as mean ± s.e.m. from 5 experiments.

relaxations to VIP ($P < 0{\cdot}05$) whereas relaxations to inhibitory nerve stimulation were increased, although not significantly ($P > 0{\cdot}05$).

The possibility that opioid receptors may modulate the activity of inhibitory nerves was investigated by testing morphine against relaxations produced by inhibitory nerve stimulation. From Fig. 2 it is apparent that morphine ($0{\cdot}3$–13×10^{-6} M, n = 5) had no significant effect on inhibitory responses ($P > 0{\cdot}5$). Furthermore, neither morphine ($0{\cdot}3$–13×10^{-6}, n = 5) nor leucine enkephalin (7–16×10^{-6} M, n = 3) increased the tone of sphincter muscle strips.

Discussion

The nature of the inhibitory neurotransmitter mediating responses to NCNA inhibitory nerve stimulation remains unknown. Human isolated internal anal sphincter (IAS) muscle provides a useful model to study these nerves as the presence of drugs to unmask or accentuate the inhibitory response is unnecessary. Compounds already discounted as putative neurotransmitters for the NCNA inhibitory nerves of IAS muscle are: noradrenaline, acetylcholine, prostaglandin E_2 and $F_{2\alpha}$, histamine, 5-HT and dopamine; VIP remained a possibility (Burleigh et al 1979), whilst evidence for or against such a role was lacking for GABA, substance P and bradykinin.

Relaxations to VIP were virtually abolished while those to electrical field stimulation were potentiated by α-chymotrypsin. Therefore it is unlikely that VIP functions as an inhibitory neurotransmitter in IAS muscle. Similar conclusions were reached by Mackenzie & Burnstock (1980) in their experiments using guinea-pig taenia coli.

Evidence obtained in the last 15 years or so strongly suggests that GABA is a central neurotransmitter mediating inhibitory actions of local interneurons in the brain (Otsuka 1973; Ryall 1975). Discovery of neurons that synthesize and take up GABA in about 3–5% of the myenteric plexus cells (Jessen et al 1979) raised the possibility of a peripheral neurotransmitter role for GABA in the gut. However, in the concentrations tested GABA did not exert an inhibitory effect on IAS muscle.

Substance P has been localized in a high number of nerve fibres in the human vagus nerve (Lundberg et al 1979) and also contracts circular and longitudinal muscle from the human colon (Couture et al 1981). Substance P was investigated as a possible inhibitory neurotransmitter in sphincter muscle as assumptions to its actions at that site cannot be made when one considers how other neurotransmitters exert opposite effects on IAS muscle compared with intestinal muscle. Experiments showed that the excitatory effects of substance P also extended to sphincter muscle.

Bradykinin mimics the NCNA response (relaxation followed by an after-contraction) of guinea-pig colon and rat distal colon to transmural electrical stimulation (Eaglesom & Zeitlin 1977; Al-Dhahir & Zeitlin 1981). On IAS muscle bradykinin produced only relaxation, as did electrical field stimulation. A role for bradykinin as the neurotransmitter of NCNA inhibitory nerves was not supported by the observation that the proteolytic enzyme carboxypeptide B greatly reduced responses to bradykinin without affecting those to electrical field stimulation. Similar results came from the studies using guinea-pig and rat colon.

Morphine greatly augments the tone of the anal sphincter (Jaffe & Martin 1980). Three mechanisms may be involved: (1) a direct effect on the smooth muscle (Burks 1981); (2) modulation of inhibitory intrinsic nerve activity (Shimo & Ishii 1978); (3) indirectly via actions on the central nervous system (Stewart et al 1978; Burleigh et al 1981). It would appear from the present study that the third mechanism is the most likely.

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Microsomal conjugation of fatty acids to codeine*

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In previous studies performed in our laboratory (Leighty 1973), long-retained unknown cannabinoic metabolites were detected in the liver, spleen, fat and bone marrow of rats 15 days after a single intravenous or chronic intraperitoneal injection of ^{14}C-Δ^8-tetrahydrocannabinol (^{14}C-Δ^8-THC) or ^{14}C-Δ^9-tetrahydrocannabinol (^{14}C-Δ^9-THC). Δ^8-THC and Δ^9-THC are psychoactive components in marihuana. These unknown metabolites comprised at least 80 percent of the cannabinoids detected in these tissues after 15 days. Subsequent studies (Leighty et al 1976) identified these long-retained cannabinoic metabolites as conjugates of palmitic, stearic, oleic, and linoleic acids.

An in-vitro rat liver coenzyme A fortified microsomal system was later developed in our laboratory that could produce the above fatty acid conjugated cannabinoids from the primary hydroxylated Δ^9-THC metabolite 11-OH-Δ^9-THC (Leighty 1979a) and other cannabinoids (Leighty 1980a).

The present studies were undertaken to determine if codeine, which has a secondary hydroxyl group in its structure, could also be conjugated to fatty acids in our in-vitro rat liver coenzyme A fortified microsomal system.

Materials and methods

Codeine (Mallinckrodt) and [1(m)-^{3}H]codeine (Amersham Searle) were obtained commercially. Palmitoylcodeine, to be used as a reference standard, was synthesized in our laboratory from codeine using palmitoylchloride in pyridine and standard synthesis procedures.

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For in-vitro conjugation to fatty acids 1 mmole of codeine or 100 μmoles of [^{3}H]codeine were incubated in a 37 °C metabolic shaker for 2 h in 25 ml of the rat liver coenzyme A fortified microsomal incubation mixture previously described (Leighty 1979a). The mixture was lyophilized and then extracted three times with 50 ml of chloroform. The solvent was removed on a flash evaporator and the residue resuspended in a small amount of chloroform. The chloroform suspension was then partially purified using thin-layer chromatography (t.l.c.) and high-pressure liquid chromatography (h.p.l.c.).

For t.l.c. aliquots of the chloroform suspension were spotted on silica gel plates (K1, Whatman) and developed in methanol–ammonia (99·5:0·5). Palmitoylcodeine and codeine were spotted on the same plate as reference standards and detected by spraying with acidified iodoplatinate. In this t.l.c. system, codeine has an R_F of 0·54 and palmitoylcodeine an R_F of 0·64. When the initial parent compound was [^{3}H]codeine, a small

Table 1. Diagnostic ions produced by CI and/or EI mass spectrometric analyses of palmitoylcodeine standard, codeine and a h.p.l.c. fraction of extract of microsomal system containing codeine.

	CI		EI	
Compound	(MH+)	Prominent fragment ions	(MH+)	Prominent fragment ions
Palmitoylcodeine standard	538	282	537	282, 229, 162, 124
Codeine			299	229, 162, 124
H.p.l.c. fraction of microsomal extract	538	282	537	282, 229, 162, 124