Br. J. Pharmac. (1986), **88**, 315–322

Opioids increase potassium conductance in submucous neurones of guinea-pig caecum by activating δ-receptors

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1 Intracellular records were made from neurones in the submucous plexus of the guinea-pig caecum.

2 [Met^5]enkephalin, [Leu^5]enkephalin, [D-Ala^2,D-Leu^5]enkephalin (DADLE) and [D-Ser^2,Leu^5]enkephalin-Thr (DSLET) hyperpolarized the membrane when applied in concentrations of 30 nM – 10 μM. Normorphine, [D-Ala^2, MePhe^4,Gly^5]enkephalin-ol (DAGO), [D-Ala^2,MePhe^4,Met(0)^5]enkephalin-ol (FK33824), dynorphin A and tifluadom had no effect at concentrations up to 10 μM.

3 The hyperpolarization resulted from an increase in the membrane potassium conductance.

4 Hyperpolarizations induced by [Met^5]enkephalin were antagonized competitively by naloxone and by N-bisallyl[aminoisobutyrate^{2,3}, Leu^5]enkephalin (ICI 174864). The Schild plots for these antagonisms had slopes not different from one, and the dissociation equilibrium constants among individual neurones were 5–50 nM for naloxone and 5–60 nM for ICI 174864.

5 The results indicate that the opioid receptors on guinea-pig submucous neurones which are coupled to potassium channels are of the δ-type.

Introduction

Opioid receptors of the δ-type were discovered as a result of their relative insensitivity to blockade by the antagonist naloxone (Lord *et al.*, 1977). They have a characteristic distribution throughout the nervous system which is distinct from that of the μ- or κ-type (see Akil *et al.*, 1984). Electrophysiological experiments have shown that opioids increase membrane potassium conductance in a variety of tissues (see Duggan & North, 1983). The results of experiments with selective agonists in cultured mouse dorsal root ganglia (Werz & Macdonald, 1983a, b) and measurements of naloxone dissociation equilibrium constants in neurones of rat locus coeruleus (Williams & North, 1984) indicate that the receptor involved is the μ-type. Activation of opioid κ-receptors, on the other hand, appears to reduce membrane calcium conductance by an action that is independent of potassium conductance (Werz & Macdonald, 1984a, b; Cherubini & North, 1985).

One tissue in which the opioid receptor has been characterized as the δ-type is the guinea-pig intestinal mucosa (Kachur *et al.*, 1980); in this case opioids act to reduce the mucosal short-circuit current, reflecting an inhibition of electrogenic chloride secretion. This net absorption of chloride by opioids is blocked by tetrodotoxin (Binder *et al.*, 1984), leading those authors to conclude that the δ-receptor was on the nerves rather than the mucosal cells themselves. In keeping with this is the failure to detect any binding of [^{3}H]-[Met^5]enkephalin on enterocytes of the rabbit ileum (Binder *et al.*, 1984). The nerves have their cell bodies in the submucous plexus, and are thus accessible for intracellular recording. The purpose of the present experiments was to investigate the effects of opioid peptides on the ion conductances of neurones of the guinea-pig submucous plexus and to determine the type of opioid receptor involved.

A preliminary account of the findings has been published (Mihara & North, 1985).

Methods

Adult male guinea-pigs were stunned and bled. A segment of caecum was removed and individual ganglia of the submucous plexus were dissected and pinned in a shallow tissue bath (see Surprenant, 1984; Mihara *et al.*, 1985). The ganglia were superfused with a solution of the following composition (mM): NaCl 117, KCl 4.7, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. This solution was gassed with 95% O_2 and 5% CO_2 and heated

before entering the tissue bath; the flow rate was adjusted (about 1.5 ml min^{-1}) so that the temperature in the tissue bath was 37°C. Intracellular recordings were made from microelectrodes filled with a solution of potassium chloride (or acetate), and having resistances of 70–100 MΩ. Membrane currents were measured in some experiments with a single electrode voltage-clamp amplifier (Dagan 8100).

Drugs were applied to the preparation by two methods. In those experiments in which precise knowledge of the drug concentration was not necessary (for example, determination of reversal potential), [Met^5]enkephalin was ejected from a pipette. The tip of this pipette (diameter 5–15 μm) was positioned about 10–20 μm laterally from the impaled neurone; the [Met^5]enkephalin (100 μM) was ejected by applying a brief pulse of pressure to the pipette (typically 100 kPa, 30 ms). In the majority of experiments, drugs were applied by dissolving them in known concentrations in the superfusing solution, and then changing the superfusing solution by means of a three-way tap. There was a delay of 20 s between turning the tap and the arrival at the tissue bath of the changed solution. Complete exchange of the bath solution, indicated by effects reaching their steady state, was usually complete within 10 s. Antagonist dissociation equilibrium constants (K_Ds) were determined by the method of Arunlakshana & Schild (1959); in these experiments, non-cumulative agonist concentration-response curves were constructed first before and then in the presence of various antagonist concentrations. Whenever possible, the sensitivity to agonist was determined again after washing out the highest concentration of antagonist used; this was not always possible because it required recording from a single cell for at least 5 h.

Compounds used were: dynorphin (Peninsula), β-funaltrexamine (the 6β-fumaramate methylester derivative of naltrexamine, Dr P. Portoghese, University of Minnsota), [D-Ala^2,D-Leu^5]enkephalin (DADLE), [D-Ala^2,MePhe^4,Met(0)^5]enkephalin-ol (FK33824), [D-Ala^2,MePhe^4,Gly^5]enkephalin-ol (DAGO), [Leu^5]enkephalin, [Met^5]enkephalin, [D-Ser^2,Leu^5]enkephalin-Thr (DSLET) (all from Peninsula), [N-bisallyl-Tyr^1,Aib^{2,3}Leu^5]enkephalin (ICI 174864) (Aib = aminoisobutyrate) (courtesy of ICI), morphine sulphate (Mallinckrodt), naloxone hydrochloride (Endo), noradrenaline bitatrate (Sigma), normorphine hydrochloride (National Institute on Drug Abuse), somatostatin (Peninsula) and tifluadom (Sandoz).

Results

The 225 neurones from which recordings were made in the present study had properties similar to those described in previous experiments on the guinea-pig ileum (Surprenant, 1984) and caecum (Mihara *et al.*, 1985). Recordings from 8 AH cells were omitted. Resting membrane potentials ranged from − 50 to − 68 mV (− 54.1 ± 0.67; mean ± s.e.mean, $n = 47$), input resistances ranged from 52 to 144 MΩ (72.9 ± 21.6; $n = 33$), and time constants ranged from 8 to 22 ms (12.4 ± 0.73; $n = 23$.

Some opioids hyperpolarize submucous neurones

Submucous plexus neurones were hyperpolarized when the superfusion solution was changed to one that contained [Met^5]enkephalin or [Leu^5]enkephalin (Figure 1). The hyperpolarization was rapid in onset, reaching its peak within 10–20 s of the arrival of the changed solution at the tissue, and the membrane potential reversed rapidly to its resting level when superfusion with the control solution was resumed (Figure 1). When membrane current was recorded under voltage clamp, superfusion with [Leu^5]enkephalin evoked an outward current having the same time course as the hyperpolarization. The peak amplitude of the hyperpolarization was dependent on the concentration of agonist applied (300 nM–10 μM), but never exceeded 35 mV. [Met^5]enkephalin caused hyperpolarizations of 9.1 ± 0.94 mV (range 3–16 mV, $n = 24$) at 300 nM, 13.0 ± 0.67 mV (range 4–30 mV, $n = 86$) at 1 μM, 22.9 ± 1.15 mV (range 15–32 mV, $n = 18$) at 3 μM, and 26.0 ± 2.4 mV (range 19–34 mV, $n = 6$) at 10 μM. [Leu^5]enkephalin caused hyperpolarizations of 10.5 ± 1.2 mV (range 4–19 mV, $n = 11$) at 100 nM, 15.7 ± 0.88 mV (range 4–26 mV, $n = 58$) at 1 μM, 22.0 ± 1.2 mV (range 17–27 mV, $n = 8$) at 3 μM, and 24.3 ± 1.6 mV (range 19–27 mV, $n = 4$) at 10 μM. The values given are the means with the s.e.mean. The peak currents (holding at − 60 mV) were typically 300 pA, implying a maximal opioid conductance of about 10 nS (calculated for a driving force of 30 mV, see below).

When the period of superfusion was continued for more than 2 min, the hyperpolarization often progressively passed off during the presence of the agonist. In the same cell, the hyperpolarization evoked by noradrenaline did not decline during superfusions of up to 30 min (see also Mihara *et al.*, 1985; North & Surprenant, 1985). In some neurones, repeated applications of [Met^5]enkephalin separated by 20–30 min caused hyperpolarizations of progressively declining amplitudes, even though the responses of the same neurone to noradrenaline and somatostatin (see below) did not decline with time. This was observed in 9 out of 86 neurones to which [Met^5]enkephalin was applied and 7 out of 58 neurones to which [Leu^5]enkephalin was applied.

The opioids which were effective in hyperpolarizing submucous plexus neurones were [Met^5]enkephalin, [Leu^5]enkephalin, DSLET and DADLE. The equi-

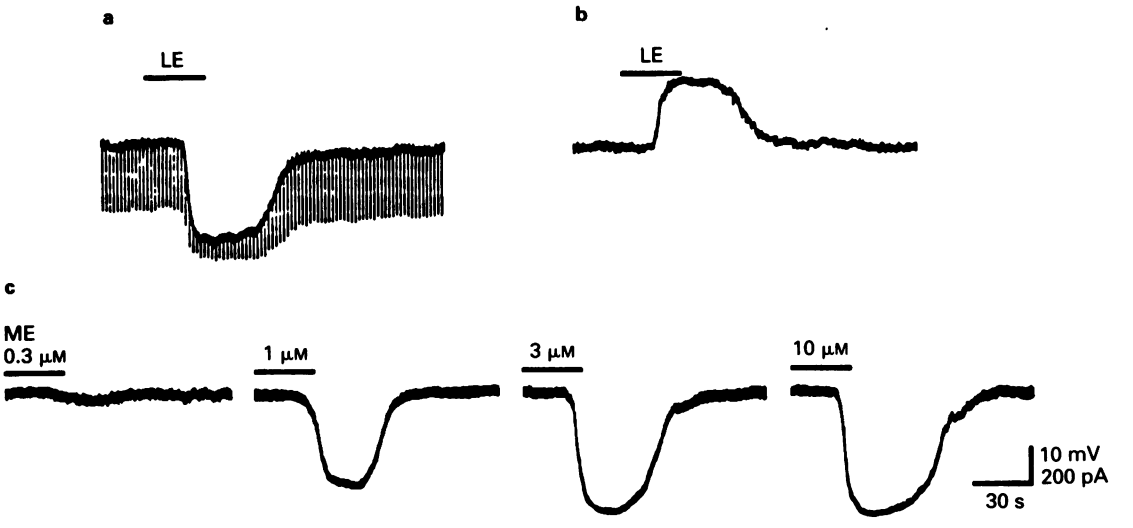

Figure 1 Hyperpolarization and outward current caused by [Leu^5]enkephalin and [Met^5]enkephalin. (a) Membrane potential; downward deflections are electrotonic potentials evoked by passing hyperpolarizing current pulses of 100 pA, 100 ms at 0.5 Hz. The superfusion solution contained [Leu^5]enkephalin (LE) (1 μM) during the period indicated by the bar (30 s). The apparent delay in the onset of the hyperpolarization in this and other records represents the time required for the changed solution to pass through a heat-exchanger before it reached the tissue. Resting potential − 55 mV. (b) The neurone was voltage-clamped at the resting potential and the application of LE repeated. This caused an outward current of about 400 pA. (c) Effect on membrane potential of four different concentrations of [Met^5]enkephalin (ME) in the same neurone.

effective concentrations of DADLE and DSLET were 3–10 times lower than those of [Met^5]enkephalin and [Leu^5]enkephalin, which were equipotent. Normorphine (up to 10 μM), tifluadom (up to 1 μM), FK33824 (up to 100 nM), DAGO (up to 1 μM) and dynorphin A (up to 500 nM) were ineffective on the same neurones which were hyperpolarized by [Met^5]enkephalin or [Leu^5]enkephalin (Figure 2). The numbers of submucous plexus neurones which were hyperpolarized by superfusion of agonists, in relation to the number tested were as follows: [Met^5]enkephalin, 102 of 111; [Leu^5]enkephalin, 70 of 74; normophine, 0 of 15; DADLE, 13 of 14; DAGO, 0 of 7; DSLET, 17 of 18; FK33824, 0 of 5; tifluadom 0 of 8; dynorphin A, 0 of 5. Pressure application of [Met^5]enkephalin hyperpolarized 23 of 25 neurones in this series of experiments.

Hyperpolarization results from potassium conductance increase

These experiments were performed with [Met^5]enkephalin as the agonist, and with the pressure pulse method of application (see Methods). The hyperpolarization (or outward current) evoked by [Met^5]enkephalin became smaller in amplitude as the membrane was hyperpolarized, and reversed its polarity at about − 90 mV. The amplitude of the [Met^5]enkephalin potential change was linearly related to the membrane potential at which it was evoked, implying a lack of voltage sensitivity to the underlying conductance change. The voltage dependence of the [Met^5]enkephalin hyperpolarization was also examined in solutions which contained different potassium ion concentrations. A typical experiment is shown in Figure 3; three other neurones gave similar results. The reversal potentials were − 145.3 ± 1.2 mV (n = 3) in 0.47 mM, − 92.2 ± 0.94 mV (n = 6) in 4.7 mM and − 56.3 ± 0.7 (n = 3) in 20 mM potassium. The reversal potentials for the [Met^5]enkephalin hyperpolarization were therefore the same as those for the inhibitory postsynaptic potential (i.p.s.p.) and noradrenaline hyperpolarizations (Mihara *et al.* 1985; North & Surprenant, 1985).

Opioid receptor is δ-type

The [Met^5]enkephalin hyperpolarization was reversibly blocked by naloxone and ICI174864, but not by idazoxan (1 μM). In some neurones, the recording was

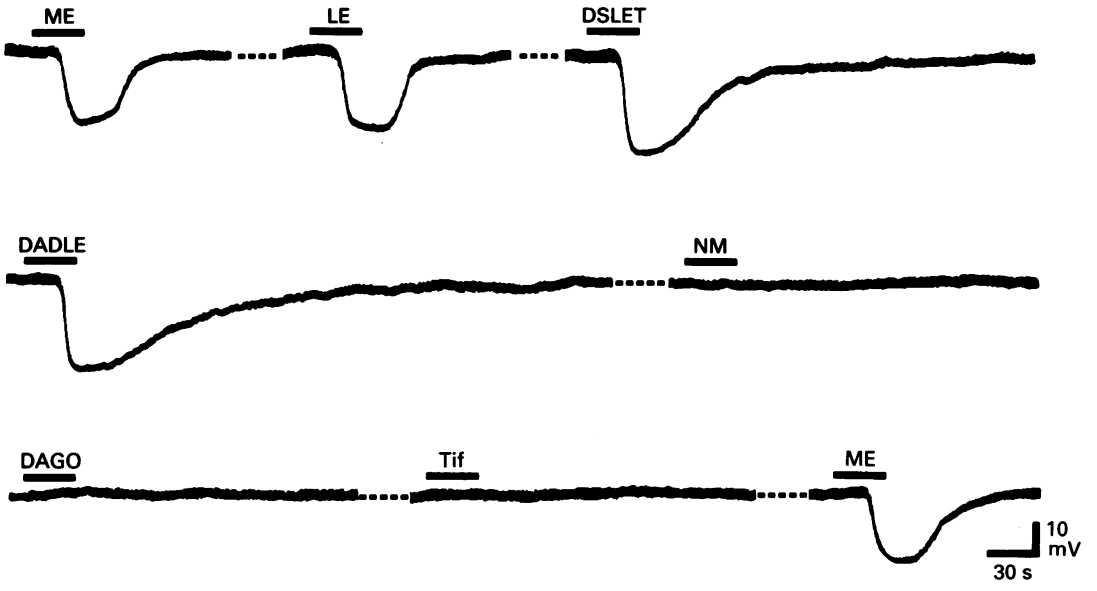

Figure 2 Comparison of the effects of various agonists on the membrane potential of a single neurone. The three traces are a continuous recording of membrane potential. During the periods indicated by the bars, the superfusion solution was changed to one which contained the substance indicated. Concentration of all compounds was 1 μM. ME = [Met^5]enkephalin; LE = [Leu^5]enkephalin; DSLET = [D-Ser^2,Leu^5]enkephalin-Thr; DADLE = [D-Ala^2,D-Leu^5]enkephalin; NM = normorphine; DAGO = [D-Ala^2,Gly^5]enkephalin-ol; Tif = tifluadom.

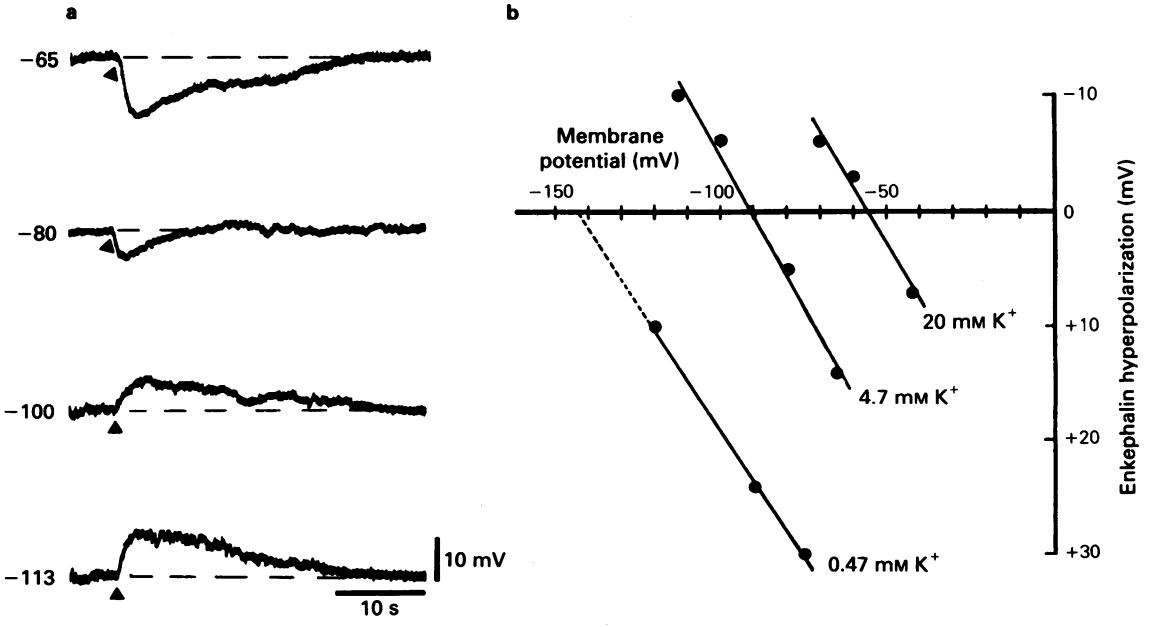

Figure 3 Effect of membrane potential on responses to [Met^5]enkephalin: (a) in each trace (resting potential indicated at left) the triangles indicate the times of application of [Met^5]enkephalin (a pressure of 140 kPa was applied for 30 ms to a pipette containing [Met^5]enkephalin (100 μM), the tip of which was placed about 10 μm from the neurone). The polarity of the [Met^5]enkephalin response reversed at about − 90 mV. (b) The experiment illustrated in (a) was repeated during superfusion with solutions that contained 20 mM and 0.47 mM potassium chloride.

maintained for sufficiently long to apply the agonist many times; an example is illustrated in Figure 4. Concentrations of [Met^5]enkephalin gave hyperpolarizations of 11, 24 and 26 mV. Further applications of [Met^5]enkephalin were then made in the continuous presence of various concentrations of ICI 174864 (open circles). After the final concentration (1 μM) of ICI 174864 was washed out, the control dose-response curve for [Met^5]enkephalin was determined again. The experiment was then repeated with naloxone as the antagonist. The Schild plots (Figure 4b) had slopes not different from one, and in this experiment provided estimates of K_Ds for ICI 174864 and naloxone of 50 and 8 nM respectively. The values for the K_Ds determined on individual neurones in such experiments were 6, 8 and 41 nM for naloxone and 8, 10 and 50 nM for ICI 174864.

β-Funaltrexamine (200 nM) had no effect on the membrane potential of submucous plexus neurones, but it antagonized the [Met^5]enkephalin hyperpolarization ($n = 3$). The antagonistic effect of β-FNA was reversed after 50 min of washing.

δ-Opioids, α_2-adrenoceptor agonists and somatostatin increase the same potassium conductance

Noradrenaline, acting on α_2-adrenoceptors, also hyperpolarizes submucous plexus neurones by increasing a membrane potassium conductance (North & Surprenant, 1985; Mihara *et al.*, 1985). In the present experiments, it was found that somatostatin had an action very similar to that of noradrenaline, except that the somatostatin hyperpolarization was unaffected by an α_2-adrenoceptor blocker such as idazoxan. The effective concentrations of somatostatin were 10 nM – 1 μM, and the hyperpolarization (which readily reached 20–30 mV in amplitude) persisted for as long as the superfusion solution contained the somatostatin. That is to say, desensitization was not apparent, and in this respect also the somatostatin hyperpolarization resembled that caused by noradrenaline.

The hypothesis was therefore tested that these agonists opened a population of potassium channels different from that opened by δ-opioid agonists. All experiments were carried out under voltage clamp. The outward current induced by a maximal (or close to maximal concentration) of two of the three agonists (noradrenaline, somatostatin and [Met^5]enkephalin) was observed by applying them for 30 s (this was sufficient for the effect to reach its peak amplitude). One of the agonists was then applied continuously for 5–20 min, and for 30 s within this period the superfusion solution was changed to one which contained both agonists (Figure 5). The outward current induced by one agonist could not be increased by concomitant addition of a second agonist; in other words, in a given

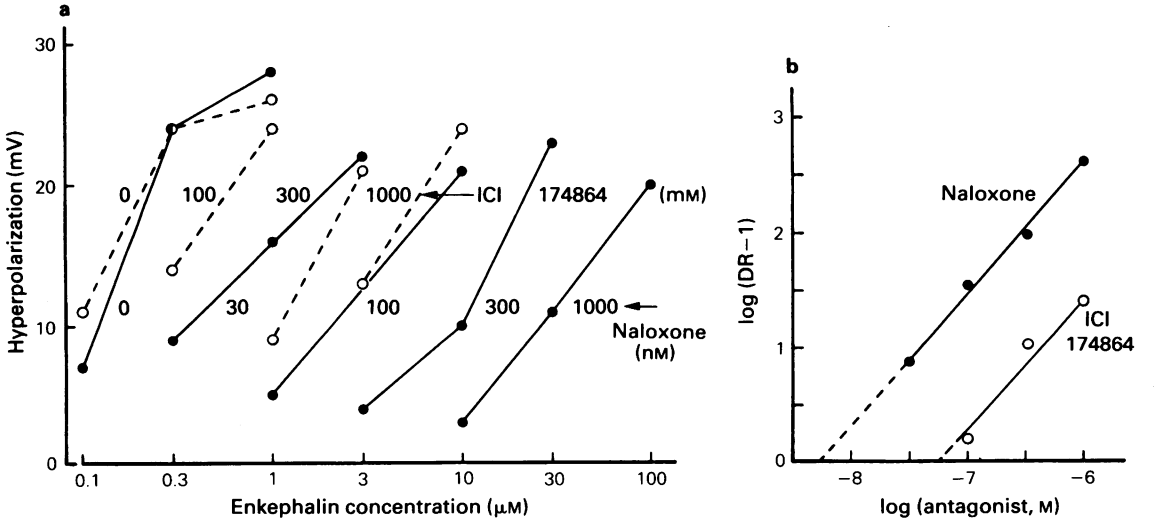

Figure 4 Determination of antagonist K_Ds on a single neurone: (a) hyperpolarization as a function of [Met^5]enkephalin concentration; (○) effects of [Met^5]enkephalin applied alone, and then in the presence of increasing concentrations of ICI 174864. The ICI 174864 (1 μM) was then washed from the tissue for 60 min, and the control sensitivity to [Met^5]enkephalin was restored (●, leftmost points). The effects of [Met^5]enkephalin were then tested in the presence of increasing concentration of naloxone. (b) Schild plots from the results in (a) at a response level of 20 mV. The antagonist K_Ds estimated for this neurone were 8 nM for naloxone and 50 nM for ICI 174864.

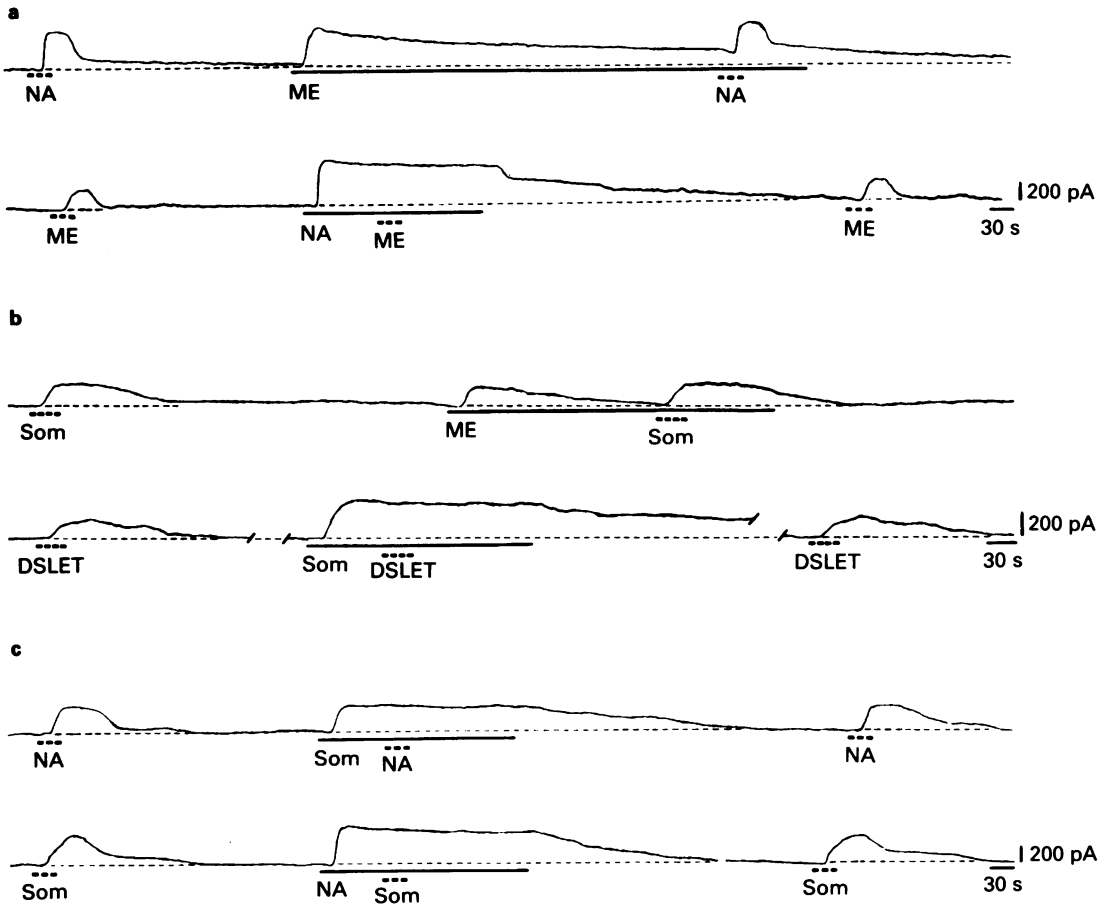

Figure 5 Interaction among outward currents induced by opioids, noradrenaline and somatostatin. (a) Noradrenaline (1 μM, NA) was applied for 30 s; it caused a current of about 400 pA. [Met^5]enkephalin (1 μM, ME) was next applied for 10 min; the current slowly declined. Concomitant application of NA and ME caused a current similar to that evoked by either agonist alone. The second trace shows that ME was no longer capable of causing an outward current when it was applied at the time of a large current evoked by NA. (b) A similar experiment in which the interaction was studied between somatostatin (1 μM, Som) and ME, and between [D-Ser^2,Leu^5]enkephalin-Thr (DSLET) and somatostatin. DSLET was without effect when it was applied during the somatostatin current. (c) A similar experiment in which the interaction was studied between noradrenaline and somatostatin.

neurone, the same peak current could be made to flow by any of the three agonists applied alone, and combinations of any two agonists failed to evoke currents larger than those produced by one agonist alone. The hypothesis that the three agonists act on separate conductances was rejected.

Discussion

The principal finding of the present experiments was that opioid receptors on submucous plexus neurones of the guinea-pig caecum were coupled to membrane potassium channels. The potassium conductance had properties which were not different from those previously described for that increased by μ-opioid receptors in rat locus coeruleus neurones (Williams & North, 1984). The defining criteria for the δ-opioid receptor in the present experiments were three, in declining order of importance. First, the K_D values for naloxone were different in the two tissues (18 *vs* 2 nM), and the K_D values for ICI 174864 were very different

(23 nM *vs* 7 μM); both antagonists acted as reversible competitive antagonists in both tissues up to dose-ratios of 30–1000. ICI 174864 has previously been shown to be highly selective for δ-opioid receptors (Cotton *et al.*, 1984). Second, normorphine, FK33824 and DAGO were without effect on guinea-pig submucous neurones at concentrations 30–100 times those which cause large hyperpolarizations of rat locus coeruleus neurones. These three agonists are known to be selective for μ-opioid receptors (Paterson *et al.*, 1983). Third, β-FNA, which acts as an irreversible antagonist in the rat locus coeruleus, behaved as a reversible antagonist in the guinea-pig submucous plexus cells. β-FNA is a selective μ-receptor alkylating agent (Ward *et al.*, 1982).

δ-Opioid receptors have also been shown to be coupled to potassium conductance in mouse dorsal root ganglion cells in culture, but on the basis only of agonist selectivity (the second criterion above) (Werz & McDonald, 1984a,b). The properties of the potassium conductance increased by opioids in those experiments appears to be slightly different from that in submucous (δ-receptors) or locus coeruleus (μ-receptors) neurones, being not responsible for the resting potential but contributing to action potential repolarization and the action potential after-hyperpolarization (see North, 1986). It is not clear whether this difference might be accounted for by the difference in species or stages of development or, perhaps, by some difference in the experimental conditions.

Coexistence of μ-opioid receptors and α_2-adrenoceptors has previously been shown for single neurones of the guinea-pig myenteric plexus (Surprenant & North, 1985) and for the rat locus coeruleus (North & Williams, 1985). Both μ-opioid agonists and α_2-adrenoceptor agonists act on their distinct receptors to increase a conductance which has the same properties. In the case of locus coeruleus, the conductance increased by μ-opioid and α_2-adrenoceptor ligands is apparently identical (North & Williams, 1985). In the present series of experiments, it has been found that, in a different population of neurones, both δ-opioid receptors and α_2-adrenoceptors open the same conductance and that somatostatin, acting on a third distinct receptor, affects the same conductance. This implies, at the very least, that the intracellular transduction processes initiated at these three separate receptors converge at some locus prior to channel activation. It may be speculated that the δ-receptor, the α_2-adrenoceptor and the somatostatin receptor exhibit only minor differences in their extracellular ligand binding domains, while being otherwise highly homologous. It is well known that δ-receptors, α_2-adrenoceptors and somatostatin receptors can all be shown, under various circumstances, to be associated with a regulatory guanine nucleotide binding protein, and it will be interesting to determine whether the increase in potassium conductance caused by the three agonists can be affected in parallel by agents which interfere with this association.

On the other hand, there was a difference apparent in the present experiments between the actions of opioids on δ-receptors and the effects of noradrenaline and somatostatin. This was the decline in the opioid outward current or hyperpolarization which was often observed during superfusions longer than a few minutes (Figure 5); such fading was not seen in the outward current or hyperpolarizations caused by noradrenaline and somatostatin. Superfusion with similar concentrations of [Met^5]enkephalin of rat locus coeruleus neurones causes a hyperpolarization which does not decline during periods of an hour or more (North & Williams, 1985), but there the receptors are of the μ-opioid and not δ-type (Williams & North, 1984). A related observation was the decline in response when the opioids were applied repeatedly, even though this was not seen with noradrenaline and/or somatostatin in the same neurone. Taken together, these results indicate that the progressive loss of the response is not occurring at the level of the potassium channel itself, but is a particular property of the δ-receptor type. This loss of the δ-opioid response may be important from the practical point of view, because it could result in underestimates of the effects of opioids on neurones with δ-opioid receptors (Surprenant & North, 1985).

The present results may have therapeutic implications, if the distribution of receptor subtypes on sets of neurones can be extrapolated across the species to man. Peripherally acting exogenous opioids have several effects on gastrointestinal function, the overall balance of which underlies their usefulness in the treatment of diarrhoea (see Gaginella, 1984). Inhibition of short-circuit current, which would indicate a movement of electrolytes (and water) from lumen to blood, is likely to be a useful action of an agent possessing activity at δ-receptors. On the other hand, drugs (such as morphine) which are essentially devoid of δ-agonist activity may have effects on motility, while not changing intestinal ion transport. The results also indicate that a combination of drugs acting on α_2-adrenoceptors and δ-opioid receptors may affect the secretory activities of the intestine while having insignificant effects on motility.

Supported by grants from the U.S. Department of Health and Human Services DA03160 and AM32979.

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(Received August 26, 1985.
Revised January 14, 1986.
Accepted Janaury 20, 1986.)