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Agonists at μ-opioid, M_2-muscarinic and $GABA_B$-receptors increase the same potassium conductance in rat lateral parabrachial neurones

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1 Intracellular recordings of membrane potential and current were made from neurones in the lateral parabrachial nucleus in slices of rat brain *in vitro*.

2 The membrane was hyperpolarized by the opioid peptides Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL, 0.01–1 μM) and [Met^5]enkephalin (3–30 μM), though not by Tyr-D-Pen-Gly-Phe-D-Pen and U50488. In two experiments, naloxone competitively antagonized the effects of DAGOL and [Met]enkephalin with equilibrium dissociation constants of 0.8 and 3.2 nM, respectively.

3 Baclofen (0.3–30 μM) also hyperpolarized the neurones; this action was unaffected by naloxone.

4 DAGOL, [Met^5]enkephalin and baclofen caused outward currents at the resting potential. These currents reversed polarity at a membrane potential which changed with the logarithm of the extracellular potassium concentration.

5 Muscarine has been shown previously to increase the potassium conductance by an action at M_2-receptors: the potassium currents induced by maximal concentrations of muscarine, baclofen and [Met^5]enkephalin were non-additive, indicating that these agonists opened the same population of potassium channels.

6 Noradrenaline, UK14304, carboxamidotryptamine, dopamine, adenosine and somatostatin had little or no effect on membrane potential.

7 It is concluded that rat lateral parabrachial neurones express μ-opioid, γ-aminobutyric acid$_B$ ($GABA_B$), and M_2-muscarinic receptors: activation of any of these receptors increases the potassium conductance of the membrane and inhibits the neurones through hyperpolarization.

Introduction

The parabrachial nuclei are involved in the integration of autonomic information, with roles in cardiovascular, respiratory and gustatory function (Block, 1987; Cechetto, 1987; Travers *et al.*, 1987). Located in the dorsolateral pontine tegmentum, the nuclei are extensively interconnected with other regions involved in autonomic visceral-sensory and behavioural regulation, including the nucleus tractus solitarius, area postrema, ventrolateral medulla, intralaminar thalamic nuclei, hypothalamic nuclei and central nucleus of the amygdala (Fulwiler & Saper, 1984; Bystrzycka & Nail, 1985; Cechetto, 1987).

Immunoreactivity for a number of putative neurotransmitters, including peptides, has been localized in the parabrachial nuclei (e.g. Block, 1987), and a possible site of action of endogenous and exogenous opioids is suggested by the high density of somata and terminals containing immunoreactivity for products of proenkephalin (Murakami *et al.*, 1987) and prodynorphin (Watson *et al.*, 1983), along with the presence of opiate binding sites (Atweh & Kuhar, 1977). Indeed, it appears that there is a direct projection from the marginal zone of the dorsal horn to the parabrachial nucleus, which contains opioid peptides (Standaert *et al.*, 1986), strengthening the notion that it functions as a relay for at least some sensory modalities.

Extracellular recordings from respiratory related neurones in the parabrachial nuclei have shown that opioids usually produce inhibition of activity in the cat (Denavit-Saubié *et al.*, 1978), or excitation in the rabbit (Zhonghan & Jingru, 1981). However, in neither case is it known that the effect represents a

direct action of opioids on the parabrachial cells. One purpose of the experiments described here was to determine whether opioid receptors exist on the parabrachial neurones, and to find out what was the consequence of their activation. μ- and δ-opioid receptors often coexist on mammalian neurones with receptors for a number of other transmitters; agonists at all these receptors lead to an increase in potassium conductance (see North *et al.*, 1987). Thus, a secondary aim was to determine which, if any, of these other receptors were present on the parabrachial neurones.

Methods

Intracellular recordings were made from lateral parabrachial neurones by techniques similar to those previously described for rat locus coeruleus neurones (Williams *et al.*, 1984). Briefly, coronal slices (300 μm) of pons were completely submerged in a heated (37°C), flowing (1.5 ml min^{-1}) solution of the following composition (mM): NaCl 126, KCl 2.5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11; this was gassed with 95% O_2/5% CO_2. The lateral parabrachial nucleus was identified visually in the dorsolateral pons as the translucent region bordered by the lateral surface of the pons and the brachium conjunctivum. Intracellular recordings were made with microelectrodes (40–70 MΩ, filled with 2 M KCl) using a single electrode voltage clamp amplifier (Axoclamp 2A). Electrodes coated with Sylgard (Dow Corning) to within 300 μm of the tip were used in voltage-clamp experiments, and headstage voltage was continuously monitored. Recordings of membrane potential and current were plotted directly on chart recorder paper.

All data are presented as mean ± s.e.mean. Student's two-tailed t test was used for all statistical comparisons. Drugs were applied by changing the superfusion solution to one which differed only in its content of the drug.

The following drugs were used: adenosine (Sigma); baclofen (Ciba-Geigy); 5-carboxamidotryptamine (Glaxo); DL-muscarine HCl (Sigma); [Met^5]enkephalin (Sigma, Peninsula); naloxone hydrochloride ndo); N,N-bisallyl-Tyr-Aib-Aib-Phe-Leu-OH (Aib-aminoisobutyrate, ICI174864, ICI); quinpirole (Lilly); somatostatin (Peninsula); Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL, Cambridge Research Biochemicals); Tyr-D-Pen-Gly-Phe-D-Pen (Pen = penicillamine, DPDPE, Peninsula); U50488H (*trans*-(+)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methane sulphonate, Upjohn); UK14304 (5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline, Pfizer).

Results

Membrane properties

Parabrachial neurones had a resting potential of −67 ± 2 mV (range −56 to −85 mV, $n = 36$) and input resistance of 208 ± 18 MΩ (range 88 to 400 MΩ, $n = 24$). Most neurones did not fire spontaneously, but action potentials were occasionally evoked by spontaneously occurring synaptic poten-

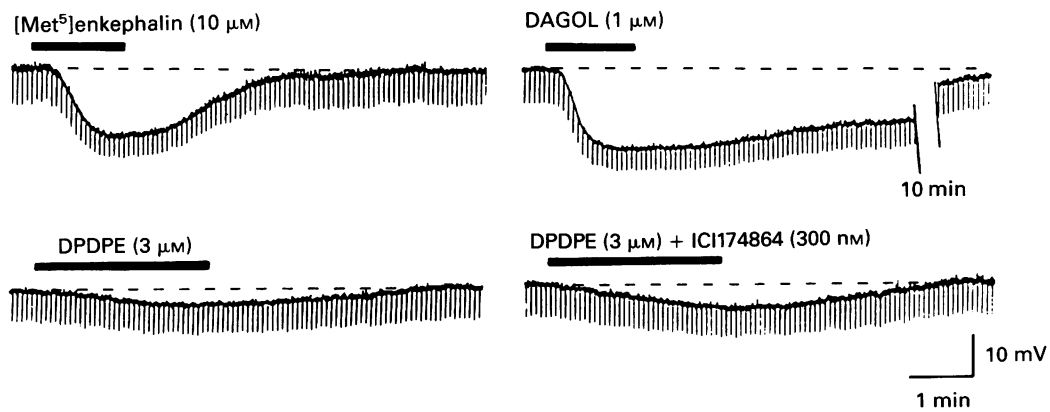

Figure 1 Hyperpolarizations induced by opioids in one neurone. Drugs were superfused during periods shown by solid bars. [Met^5]enkephalin (10 μM) and Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL; 1 μM) produced hyperpolarizations with associated decreases in input resistance. Tyr-D-Pen-Gly-Phe-D-Pen (DPDPE; 3 μM) produced only a small hyperpolarization which was not affected by ICI174864 (300 nM, applied 5 min before and then concomitant with the DPDPE). Resting membrane potential was −69 mV. Downward deflections are membrane potential responses to current pulses passed through the recording electrode (40 pA, 120 ms).

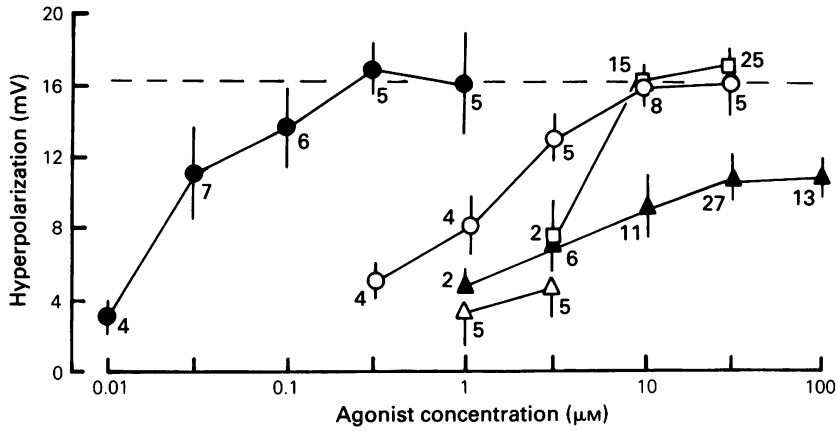

Figure 2 Concentration-effect curves for agonists that hyperpolarize parabrachial neurones. [Met^5]enkephalin (□), DAGOL (●) and baclofen (○) produced equivalent maximal hyperpolarizations (indicated by broken line). DPDPE (△) and U50488H (not shown) were essentially inactive. Hyperpolarizations in response to muscarine (▲) did not reach the same maximum amplitude as those caused by [Met^5]enkephalin, DAGOL and baclofen. Each symbol represents the mean of n (number beside symbol) observations and vertical lines indicate s.e.mean. For abbreviations used see legend of Figure 1

tials. Such spontaneous synaptic potentials were observed in 67% cells; they were reversibly blocked by calcium-free solutions. Fifteen % of cells fired action potentials sporadically (0.2–1 Hz) even in the absence of obvious spontaneous synaptic potentials. The action potentials, whether arising from synaptic potentials or evoked by brief depolarizing current pulses, had amplitudes of 70–85 mV, were 0.7–1.0 ms in duration at half-amplitude, arose from a threshold of about −55 mV, and were followed by after-hyperpolarizations of 8–25 mV amplitude and 100–300 ms duration.

Opioids act at μ-receptors

Almost all neurones (58 of 60 tested) were hyperpolarized by superfusion of [Met^5]enkephalin or by the μ-receptor selective analogue DAGOL (Figure 1). Hyperpolarizations induced by [Met^5]enkephalin were not affected by superfusion with a solution containing cobalt (4 mM, phosphate was omitted from the solution). DAGOL also hyperpolarized the cells, with an EC_{50} of 55 ± 16 nM, $n = 5$, Figure 2). DPDPE (1–3 μM), an enkephalin analogue more selective for δ-receptors, produced only small hyperpolarizations and these were not affected by ICI174864 (300 nM, Figures 1 and 2). U50488 (1–3 μM, $n = 4$, applied for up to 8 min) had no effect on membrane potential.

Figure 3 shows the results of an experiment in which the action of DAGOL was antagonized by naloxone; the antagonism was apparently competitive, and the slope of the Schild plot was 1.2. The

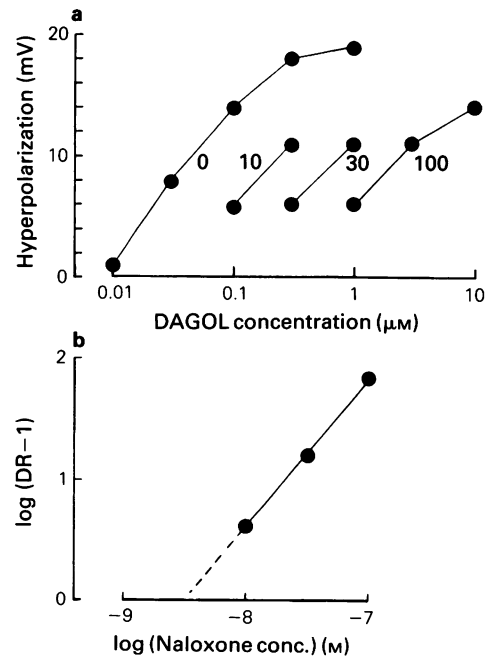

Figure 3 Naloxone antagonism of the hyperpolarization induced by Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL). (a) Increasing concentrations of naloxone (10, 30 and 100 nM as indicated) produced rightward shifts of the concentration-effect curve. (b) Schild analysis of these data provided a pA_2 for naloxone of 8.5 (line was fitted by eye, slope is 1.2).

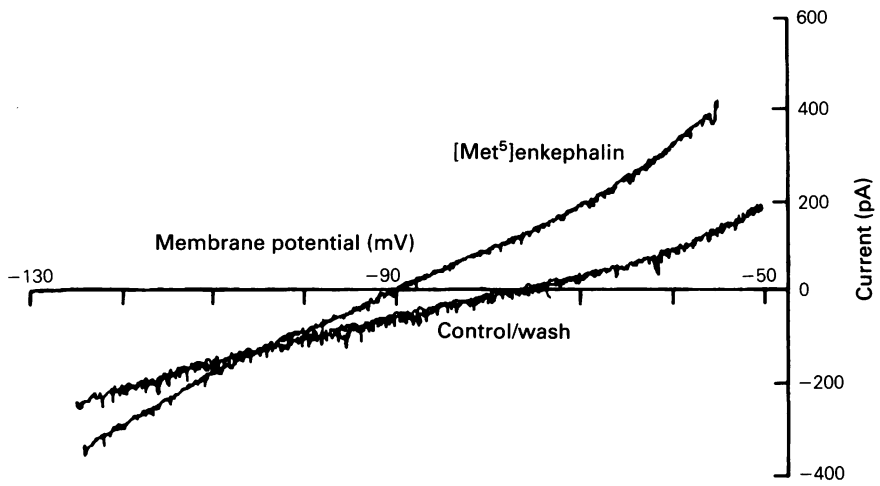

Figure 4 Steady-state current-voltage relationship of a parabrachial neurone in the absence (two superimposed traces, control and washout) and in the presence of superfused [Met^5]enkephalin (10 μM). Continuous plot of current response during a slow ramp depolarization (<1 mV s^{-1}) from -125 to -50 mV. [Met]enkephalin produced an outward current (130 pA) and increased the membrane conductance (from 7.5 to 10.1 nS) at the resting potential (-76 mV, zero current). The polarity of the additional current caused by enkephalin reversed at -104 mV (potassium concentration 2.5 mM).

dissociation equilibrium constant for naloxone (K_D) was 0.8 and 3.2 nM in two experiments of this kind. Naloxone (up to 300 nM) had no effect on resting membrane potential in the absence of opioid.

Opioids increase potassium conductance

[Met^5]enkephalin and DAGOL caused a reduction in the neurone input resistance (Figure 1) and an increase in conductance measured under the voltage clamp. Figure 4 shows the steady-state current-voltage relationship of a neurone before and after adding [Met^5]enkephalin. The current caused by the opioid reversed polarity from outward to inward at -111 ± 2 mV ($n = 10$, Figure 5). The reversal potential of this current (measured by subtracting control current from current in the presence of 10–30 μM [Met^5]enkephalin) was measured in four extracellular potassium ion concentrations. Figure 5b shows that the reversal potential (E_{rev}) was related to the potassium ion concentration ([K_O]) by $E_{rev} = m \log[K_O]$ (m ranged from 41–71 mV in different cells).

The current induced by opioids in normal extracellular potassium concentration was linearly dependent on membrane potential in the range -70 to -120 mV (Figure 5). The increase in slope conductance induced by [Met^5]enkephalin (10–30 μM) was determined at potentials negative (-120 mV) and positive (-70 mV) to the reversal potential. The increase in membrane conductance caused by the opioid was 5.0 ± 1.0 nS ($n = 15$) at -70 mV (1.8 fold increase compared to control conductance) and 6.1 ± 1.2 nS ($n = 15$) at -120 mV. These results indicate no significant rectification of the opioid current (paired $t = 1.97$, 13 d.f., $P > 0.05$). However, when the potassium concentration was raised to 10.5 mM, the opioid current (which now reversed at -74.0 ± 1.4 mV, $n = 6$) showed significant inward rectification. In 10.5 mM potassium, the conductance increase caused by the opioid was 9.6 ± 2.8 nS at -60 mV, and 16.7 ± 4.1 nS at -90 mV (paired $t = 4.0$, 4 d.f., $P < 0.02$).

Baclofen increases potassium conductance

Superfusion with baclofen, an agonist at γ-aminobutyric acid$_B$ (GABA$_B$)-receptors, hyperpolarized all 14 neurones tested. Under voltage clamp, baclofen caused an outward current when the cell was held close to its resting potential (Figure 6); this current reversed polarity at -107 ± 2 mV ($n = 4$) (potassium concentration 2.5 mM), which was not different from the reversal potential for the action of opioids, or from the reversal potential for the action of muscarine (see Egan & North, 1986). Effective concentrations of baclofen ranged from 300 nM to 30 μM, and the maximum effect observed was the same as the maximum effect of DAGOL (Figure 2).

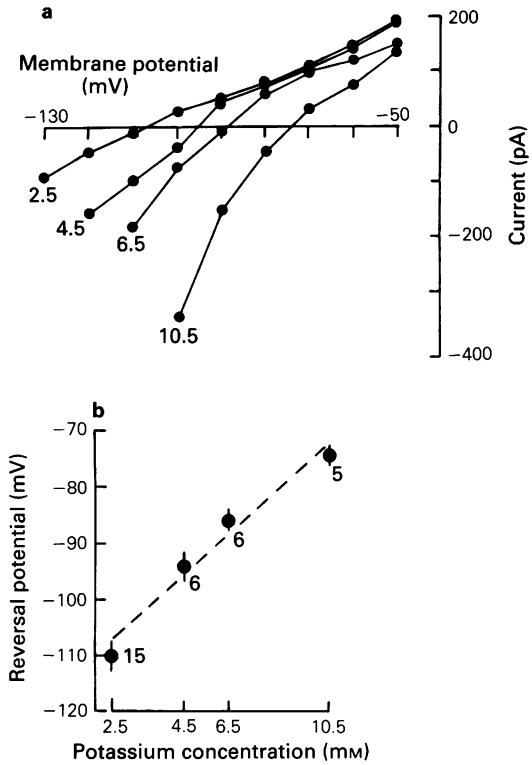

Figure 5 (a) Current caused by [Met^5]enkephalin (10 μM) in a single neurone as a function of membrane potential in four potassium concentrations (indicated in mM). (b) The reversal potential for the current induced by the opioid [Met^5]enkephalin as a function of the potassium concentration. The number of cells in which the measurement was made is indicated beside each point; [Met^5]enkephalin was used at either 10 or 30 μM. Vertical lines indicate s.e.mean.

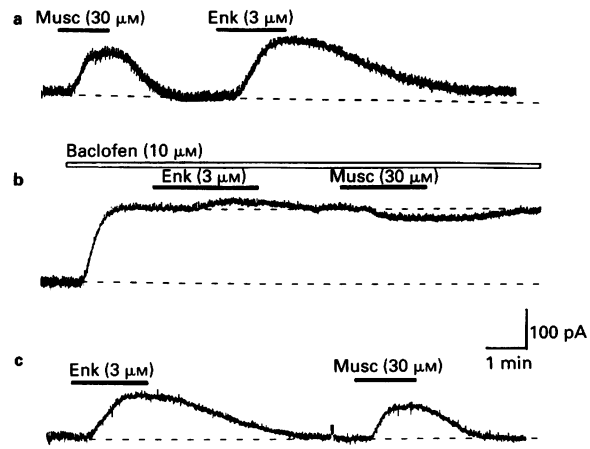

Figure 6 Outward currents induced by [Met^5]enkephalin or muscarine were occluded in the presence of a maximal concentration of baclofen. Solid bars indicate periods during which a superfusing solution contained muscarine (Musc; 30 μM) and [Met^5]enkephalin (Enk; 3 μM); open bar indicates period of application of baclofen (10 μM). (a) Initial applications of muscarine and [Met^5]enkephalin caused outward currents that were close to maximal in this cell. (b) Baclofen evoked an outward current, and concurrent application of [Met^5]enkephalin was almost ineffective; muscarine now caused a small inward current. (c) Twenty min after washing out baclofen, the effects of [Met^5]enkephalin and muscarine returned. Holding potential was −70 mV.

Muscarine, baclofen and opioids increase the same potassium conductance

Figure 6 illustrates an experiment in which application of [Met^5]enkephalin or muscarine evoked no additional outward current when they were applied in the presence of baclofen. In the presence of maximal concentrations of baclofen or muscarine (current (I) was 173 ± 24 pA, $n = 3$), [Met^5]enkephalin produced no additional outward current (I = 177 ± 31 pA, paired $t = 0.1$, 1 d.f.). In the presence of maximal concentrations of baclofen or [Met^5]enkephalin (I = 186 ± 29 pA, $n = 6$), muscarine produced no additional current (actually, a small inhibition of the outward current was observed: I = 152 ± 40 pA, paired $t = 3.3$, 4 d.f., $P < 0.05$). The outward current produced by the combination of muscarine and baclofen or muscarine and [Met^5]enkephalin was not significantly greater than that produced by muscarine alone (126 ± 14%, $n = 6$). These results indicate that activation of μ-opioid, M_2-muscarinic and $GABA_B$-receptors leads to the opening of a common population of potassium channels.

Lack of effect of some other agonists

A number of different receptor subtypes have been shown to be coupled to a potassium conductance having the properties described here. These include adenosine (A_1), adrenoceptors (α_2), dopamine (D_2), somatostatin and 5-hydroxytryptamine (5-HT_1) receptors (see North *et al.*, 1987). The following agonists selective for these receptors did not significantly hyperpolarize parabrachial neurones, even though robust responses to an opioid, muscarine or baclofen were observed in the same cell: UK14304 (k_2 3 μM, $n = 8$), 5-carboxamidotryptamine (5-HT_1, 300 nM, $n = 7$), quinpirole (D_2, 10 μM, $n = 4$), adenosine (A_1, 100 μM, $n = 5$), and somatostatin (300 nM, $n = 6$).

Discussion

Lateral parabrachial neurones are hyperpolarized by agonists at μ-receptors

The present results demonstrate that opioids act directly at μ-receptors to hyperpolarize these neurones. These actions are similar to those described for μ-receptors in a variety of neurones (see North *et al.*, 1987). Thus, the potency of DAGOL was similar to that described for locus coeruleus neurones (Williams & North, 1984; North *et al.*, 1987), and the δ-selective agonist DPDPE was essentially inactive at concentrations approximately 100 fold greater than its EC_{50} on neurones bearing only δ-receptors (North *et al.*, 1987). Furthermore, the equilibrium dissociation constant for naloxone as an antagonist of the response to DAGOL corresponds to those determined on single locus coeruleus neurones (Williams & North, 1984) and the guinea-pig ileum (Kosterlitz & Watt, 1968; Leslie *et al.*, 1980), and with the dissociation constant for naloxone as a displacer of [^{3}H]-dihydromorphine binding (Chang *et al.*, 1979, Leslie *et al.*, 1980).

The amplitude and consistency of hyperpolarizations observed in this study suggest that the parabrachial nuclei migth be an important site for analgesic, respiratory depressant and other autonomic actions of morphine. Although the neurones could not, of course, be identified functionally in these experiments, the opioid responsive neurones were impaled in regions thought to be involved in respiratory function (ventrolateral to the brachium conjunctivum) (Block, 1987; Cechetto, 1987; Travers *et al.*, 1987) in the cat. However, it should be noted that the functional roles of discrete parts of the nucleus are less clear-cut in the rat (Fulwiler & Saper, 1984).

μ-Opioid, M_2-muscarinic and $GABA_B$-receptors couple to the same potassium channels

It was shown previously that muscarinic agonists evoke a potassium conductance increase in parabrachial neurones and that the receptor was the M_2 type (Egan & North, 1986). In the present study 39 of 52 (75%) of the neurones were hyperpolarized by muscarine. Baclofen also increased the potassium conductance of the neurones, presumably by an action at $GABA_B$-receptors, as has been described in a variety of mammalian neurones (Gahwiler & Brown, 1985; Newberry & Nicoll, 1985; Steven *et al.*, 1985; Howe *et al.*, 1987; Osmanovic & Shefner, 1988). The occlusion by each other of the membrane currents caused by muscarine, baclofen and the opioids implies that the same population of potassium channels is affected.

It is possible that muscarine exerts an action on the parabrachial cells in addition to increasing the potassium conductance; the smaller maximal hyperpolarization evoked by muscarine as compared to DAGOL, [Met^5]enkephalin or baclofen (Figure 2), and the slight reduction by muscarine of the outward current induced by the opioids or baclofen, would both be consistent with an additional action inducing a small inward current. Such an effect of muscarine has been observed in the rat locus coeruleus (Egan & North, unpublished observations) and in guinea-pig papillary muscle (Pappano *et al.*, 1988).

It seems as though one receptor type for most of the major brain transmitters exerts its effects through the same population of potassium channels (see North *et al.*, 1987); different neurones express different combinations of these receptors that couple to the channels. In the rat, locus coeruleus cells have at least μ-opioid receptors and α_2-adrenoceptors (North & Williams, 1985), substantia nigra cells have dopamine D_2- and $GABA_B$-receptors (Lacey *et al.*, 1988) and hippocampus cells have 5-HT_1- and $GABA_B$-receptors (Andrade *et al.*, 1986). Our results indicate that the parabrachial neurones have yet another combination of receptors coupled to these potassium channels: μ-opioid, muscarinic-M_2 and $GABA_B$. Agonists at these three receptor types are in clinical use, and it should be borne in mind that any of their effects attributable to actions on these cells (such as depression of respiration) would be expected to be strongly additive.

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