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Morphine augments calcium-dependent potassium conductance in guinea-pig myenteric neurones

E. Cherubini, K. Morita[1] & R.A. North

Neuropharmacology Laboratory, Department of Nutrition and Food Science, Massachusetts Institute of Technology, 56-245, Cambridge, Massachusetts 02139, U.S.A.

1 Intracellular recordings were made from myenteric neurones removed from guinea-pig ileum and maintained *in vitro*.

2 Action potentials were elicited by passing brief depolarizing currents through the recording electrode. In AH cells they were followed by afterhyperpolarizations resulting from an increase in potassium conductance ($G_{K,Ca}$).

3 Morphine (1 nM – 1 μM), applied by superfusion, increased the duration of the afterhyperpolarization (and the underlying $G_{K,Ca}$) which followed from 1 to 30 action potentials. Morphine did not change the peak amplitude of the afterhyperpolarization.

4 This action of morphine occurred both in cells which showed no change in resting membrane potential or resistance and in cells which were hyperpolarized. It was prevented by naloxone (10 nM – 1 μM).

5 The possibility is proposed that morphine inhibits one of the mechanisms by which myenteric neurones control their free intracellular calcium concentration close to the plasma membrane.

Introduction

Opiates and opioid peptides increase the potassium conductance of neurones in the myenteric plexus (North & Tonini, 1977; Morita & North, 1981), the nucleus locus coeruleus (Pepper & Henderson, 1980; Williams *et al.*, 1982) and the substantia gelatinosa (Yoshimura & North, 1983). One of the principal factors which controls neurone potassium conductance and thus the membrane potential is the concentration of calcium ions in the cytoplasm (Meech, 1978). This is particularly true in the guinea-pig myenteric plexus AH neurones (Grafe *et al.*, 1980; Morita & North, 1981). These cells have a long-lasting afterhyperpolarization following the action potential which results from activation of potassium conductance by calcium entry during the action potential (Hirst & Spence, 1973; North, 1973; Hirst *et al.*, 1974; Morita *et al.*, 1982).

Opiate actions are known to be strongly calcium dependent in a variety of systems (Ross & Cardenas, 1979). A reduction in the extracellular calcium concentration usually increases the effectiveness of opiates. We had previously observed this inverse calcium dependency in myenteric neurones (Morita & North, 1981). We formed the hypothesis that the opioid induced changes in potassium conductance may result from changes in the levels of calcium in a cytoplasmic pool close to the inner surface of the membrane. In the present experiments we sought to test this hypothesis indirectly. Our strategy was to transiently increase the intracellular calcium concentration in this pool by 'injection' of calcium into the cell with one or more action potentials, and to observe the time course of the resulting changes in potassium conductance ($G_{K,Ca}$). A preliminary account of some of the results has been published (Tokimasa *et al.*, 1981).

Methods

Intracellular recordings were made from neurones lying within ganglia of the myenteric plexus of the guinea-pig ileum. The techniques for isolation of the ganglia, superfusion with physiological salt solutions, and intracellular recordings have been fully described (Nishi & North, 1973). The superfusing solution, heated so that its temperature over the tissue

[1]Present address: Department of Autonomic Physiology, Medical Research Institute, Tokyo Medical & Dental University, 2-3-10, Kanda-Surugadai, Chiyoda-ku, Tokyo, 101 Japan.

was 37°C, was of the following composition (mM): NaCl 117, KCl 4.7, NaH_2PO_4 1.2, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25 and glucose 11, gassed with 95% O_2 and 5% CO_2. Unless otherwise stated, recording electrodes were filled with potassium chloride (2 M), and cells were impaled under direct vision as they lay in a shallow bath on the stage of a microscope (Zeiss Nomarski, total magnification × 500). Both voltage recording and current injection were made through the same electrode by use of an active bridge circuit. Microelectrode resistance was nulled before cell impalement; the bridge balance was checked after withdrawal of the electrode using currents as great as those passed during the intracellular recording. Membrane potential and current injected were recorded on an oscilloscope and a pen recorder (pen response 50 mm (usually 50 mV) in less than 5 ms). Intracellular records of the action potential were electronically stored on a digitizing oscilloscope and then played back onto the pen recorder after expansion of the time base. Action potentials were evoked by passing brief depolarizing currents across the cell membrane. Cell input resistance was measured from the amplitude of small hyperpolarizations (50 – 80 ms duration) evoked by passing known currents. The conductance increase during the afterhyperpolarization was calculated from $(R/R') - 1$, where R is the input resistance at the resting potential and R' is the input resistance during the afterhyperpolarization. This is called the fractional conductance increase ($G_{K,Ca}$). The voltage-current relationship was linear in the potential range investigated (−55 to −75 mV). When morphine hyperpolarized the resting cell membrane, measurements of the afterhyperpolarization were made after restoring the original membrane potential by passing a small depolarizing current. Morphine sulphate (Mallinckrodt), normorphine hydrochloride (Dr A.E. Jacobsen), and naloxone hydrochloride (Endo) were applied by changing the superfusing solution to one which differed only in its content of the drug(s). Concentrations stated refer to these salts. Changes in calcium ion concentration were made without substitution.

Results

Afterhyperpolarization following one to three action potentials

The afterhyperpolarization which followed one or a few action potentials was prolonged by morphine in the concentration range of 10 nM – 1 μM (10 nM, 10 cells; 100 nM, 4 cells; 1 μM, 8 cells) (Figure 1). The magnitude of the effect was quite variable from cell to cell, but occurred whether or not morphine caused

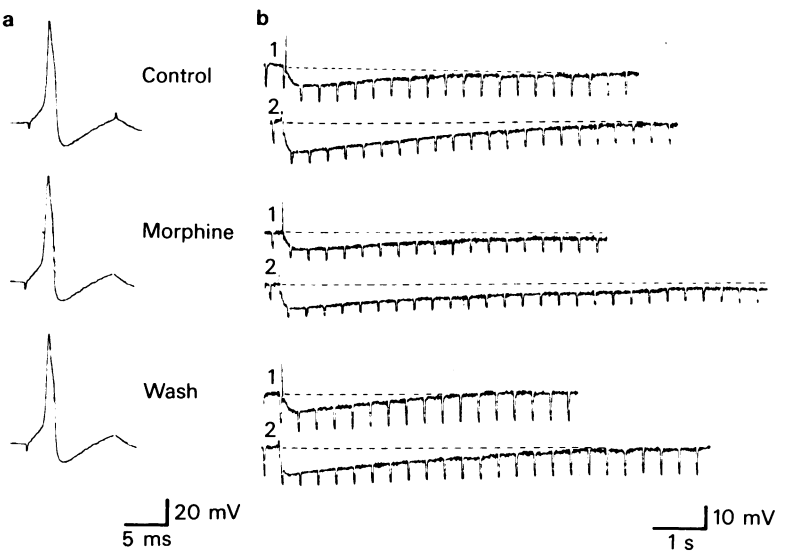

Figure 1 Morphine prolongs the afterhyperpolarization following one or two action potentials, but does not change action potential configuration. (a) Action potentials evoked by passing depolarizing current pulses before, during and after morphine (10 nM). (b) Afterhyperpolarizations which followed 1 and 2 action potentials before, during and after morphine. In each pair of records, the top trace (marked 1) is the afterhyperpolarization which followed the action potential shown in (a). In this cell, morphine also increased the resting input conductance and hyperpolarized the cell by 5 mV. The morphine effect on the action potential and afterhyperpolarization were determined after resetting the potential to its control level by passing a small depolarizing current.

Table 1 Effect of morphine on afterhyperpolarization following, 1, 2 and 30 action potentials

	One spike[a]		*Two spikes*[b]		*30 spikes*[b]	
	Control	*Morphine*	*Control*	*Morphine*	*Control*	*Morphine*
Peak amplitude (mV)	8.3 ± 1.4	10.1 ± 2.2	12.5 ± 2.1	13.8 ± 2.4	17.4 ± 0.8	17.4 ± 0.8
Time to 80% decay (s)	2.3 ± 0.6	3.9 ± 0.6*	3.1 ± 0.8	4.6 ± 0.7**	27.2 ± 4.8	47.7 ± 7.2***

[a]These are effects of 10 nM morphine (10 neurones). Similar but slightly larger effects were observed with 100 nM ($n = 4$) and 1 μM ($n = 8$). [b]These are effects of 1 nM morphine (10 neurones). Similar effects were observed with 10 nM ($n = 4$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ (paired t test). Morphine did not change resting potential in most of these cells; those cells which were hyperpolarized by morphine (up to 10 mV) were depolarized to their resting level by passage of current before evoking the action potentials.

any change in resting membrane potential (resting potential −58.0 ± 1.3 mV, mean ± s.e. mean, $n = 13$). Morphine did not change the peak amplitude of the afterhyperpolarization (Table 1). The development of the afterhyperpolarization following one to three action potentials was approximately exponential with time; the time constant (τ_1) was unaffected by morphine.

The decline of the afterhyperpolarization was also a single exponential (time constant τ_2) (Morita *et al.*, 1982). However, in morphine the decline appeared not to be a single exponential; it included a later slow decay phase which was not obvious in the absence of morphine (Figure 1). We measured the time required for the afterhyperpolarization to decline to 50% and 80% of its peak value. Morphine significantly increased the time to reach 80%, but had no effect on the time to decay to 50% (Table 1). This action of morphine was not observed after pretreatment of the tissue for 5 – 10 min with naloxone (100 nM – 1 μM). In several cells, we measured the fractional conductance increase ($G_{K,Ca}$) during the afterhyperpolarization and found this to be similarly affected by morphine. However, this gives little additional information because when the amplitude of the afterhyperpolarization is small (up to 10 mV), it is almost linearly related to $G_{K,Ca}$ (Morita *et al.*, 1982).

We considered the possibility that morphine directly affected the entry of calcium during the action potential. This seemed unlikely because it has been shown previously that changes in calcium entry (by increasing the number of action potentials, or by addition of cobalt) have marked effects on the peak amplitude of the afterhyperpolarization but do not alter its rates of rise or decline (τ_1 or τ_2) (North & Tokimasa, 1983). By contrast, morphine did not alter the peak amplitude. We also examined the effect of morphine (10 nM –1 μM, mostly higher concentrations) on the action potential in 19 neurones. Morphine caused no change in the configuration of the action potential in any cell (13 AH, 6 S cells) (Figure 1). Nine of these cells were hyperpolarized by morphine (12.0 ± 3.2 mV, mean ± s.e.); in those cases the action potential was observed after restoring the membrane potential to its resting level by passing current through the recording electrode. In ten of these neurones the action potential was also observed in the presence of tetrodotoxin; it was unaffected by morphine (1 μM). Morphine (100 nM – 1 μM) also had no consistent effects on the action potentials recorded from 15 neurones impaled with CsCl (1 M) filled electrodes; action potential durations were at a steady state (300 – 500 ms) when morphine was applied.

Afterhyperpolarization following 15–30 action potentials

The action of morphine (1 – 10 nM) was studied on the afterhyperpolarization which followed 15 or 30 action potentials (5 or 10 Hz for 3 s). The resting membrane potential of the 13 cells studied was −61.3 ± 1.4 mV (mean ± s.e.). The fractional conductance increase during the afterhyperpolarization ($G_{K,Ca}$) was calculated because the large amplitude of the afterhyperpolarization results in a significant reduction in driving force for the outward potassium current (Morita *et al.*, 1982).

Morphine prolonged the afterhyperpolarization and its underlying conductance increase. Morphine especially prolonged the late component (Figure 2). The results of several experiments are summarized in Table 1. The prolongation of the slow component was particularly obvious in the semilogarithmic plots of $G_{K,Ca}$ as a function of time (Figure 2). In most cells, the decline of $G_{K,Ca}$ could be fitted by the sum of two exponential functions (see Morita *et al.*,

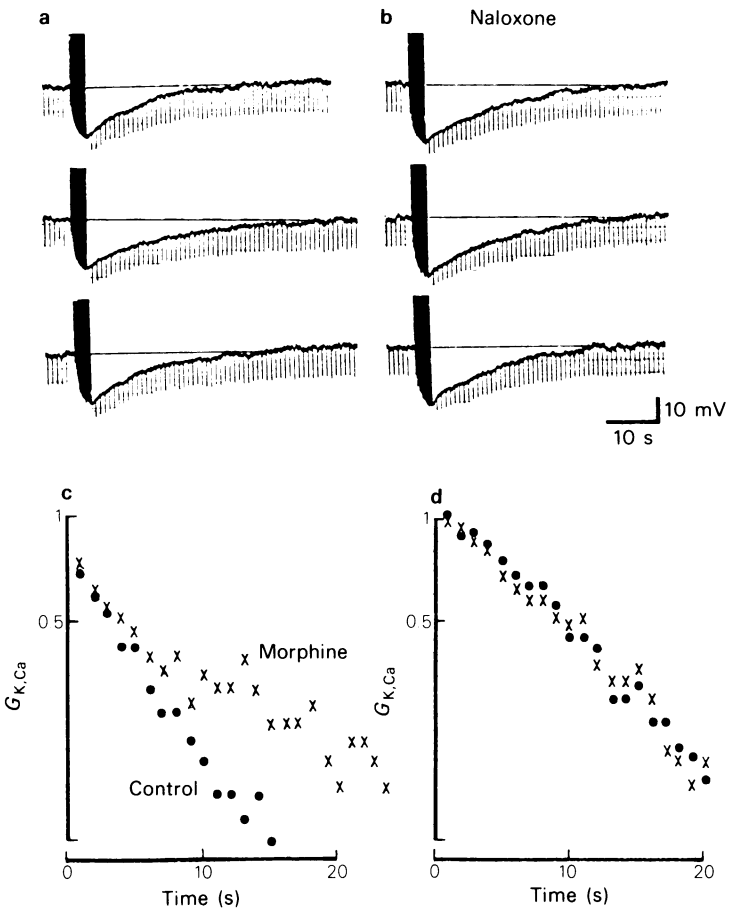

Figure 2 Morphine slows the late phase of the afterhyperpolarization following 30 action potentials. (a) Effect of morphine (1 nM) on afterhyperpolarization. Morphine caused no change in resting potential (−62 mV) or input resistance (28 MΩ). (b) One hour after (a) naloxone (10 nM) was added to the superfusing solution. Morphine was now ineffective. (c and d) $G_{K,Ca}$ measured from the electrotonic potentials in records (a) and (b) respectively; (●) control (x) morphine treated. Note the slowing of the late phase of $G_{K,Ca}$.

1982). The time constants of decay were termed $\tau_{2,fast}$ and $\tau_{2,slow}$. Control values were: $\tau_{2,fast} = 3.9 \pm 0.5$ s, $\tau_{2,slow} = 15.9 \pm 1.8$ s. After morphine (1 nM): $\tau_{2,fast} = 5.0 \pm 0.6$s, $\tau_{2,slow} = 25.0 \pm 3.7$s. The values are means and s.e. for 7 neurones; the value of $\tau_{2,slow}$ is significantly increased (paired t test, $P < 0.05$) by morphine but the value of $\tau_{2,slow}$ was unaffected. The prolongation of $\tau_{2,slow}$ by morphine was not observed in the presence of naloxone (1 – 10 n M) (Figure 2d).

The effect of morphine on the afterhyperpolarization was readily observed when the calcium concentration of the perfusing solution was reduced to half (1.25 m M) its control value (Figure 3). In contrast, doubling the calcium concentration to 5 mM increased the afterhyperpolarization duration (Morita *et al.*, 1982) but reduced or prevented the action of morphine.

Spontaneous oscillations

A small proportion of myenteric neurones (1–2%) showed spontaneous oscillations of membrane potential and conductance. The period varied among cells from 10–200 s but was most often about 1 min. These oscillations are strikingly similar to those induced by caffeine in bullfrog ganglion cells, which result from transient increases in the cytoplasmic calcium concentration (Kuba, 1980). Morphine (1 μ M) increased the amplitude of the oscillations in the 4 cells in which it could be tested.

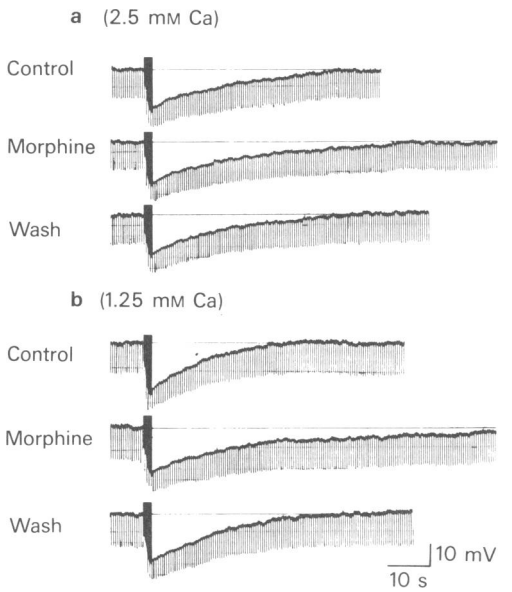

Figure 3 The effect of morphine in low calcium solution. (a) In 2.5 mM $CaCl_2$, morphine (10 nM) prolongs the afterhyperpolarization; resting potential −62 mV. (b) After changing to 1.25 mM $CaCl_2$, the neurone depolarized to −57 mV, hence the control afterhyperpolarization is slightly increased in amplitude although reduced in duration (see Morita *et al.*, 1982); morphine (10 nM) now caused a remarkable prolongation of the afterhyperpolarization.

Discussion

AH neurones of the guinea-pig myenteric plexus have a prominent afterhyperpolarization which is separated in time from the afterhyperpolarization which immediately follows spike repolarization. This afterhyperpolarization appears to be entirely due to an increase in potassium conductance resulting from calcium entering the neurone during the action potential (Nishi & North, 1973; Hirst & Spence, 1973; North, 1973; Morita *et al.*, 1982) In previous experiments, morphine was found to increase the resting membrane potassium conductance (Morita & North, 1981), thus raising the possibility that the potassium conductance may be increased as a result of a transient increase in the calcium concentration in a pool close to the inner surface of the plasma membrane. Such an idea was suggested by results of experiments on synaptosomes of rat brain, in which it was shown that morphine inhibited the uptake of ^{45}Ca ions (Guerrero-Munoz *et al.*, 1979).

In most of the present experiments we used low concentrations of morphine, and these often caused no detectable change in resting membrane potential or resistance. Whether or not the resting cell properties were altered, morphine delayed the return of the membrane potential to its control value when it was transiently hyperpolarized by calcium entry. In particular, morphine delayed the later part of the return to the resting potential, and had little or no effect on the initial decline of the afterhyperpolarization.

We considered several possible loci for the action of morphine. Firstly, does morphine alter calcium entry during the action potential? This possibility must be considered seriously because Mudge *et al.*, (1979), Higashi, (1982), Werz & Macdonald (1982) and Bixby & Spitzer (1983) have all shown effects of opioids on calcium action potentials. We found no evidence for such an action in myenteric neurones, there being no change in configuration of the calcium spike, and no change in the peak amplitude of the afterhyperpolarization. Secondly, does morphine increase the affinity of the potassium channel to intracellular calcium? This seems unlikely for one might then expect an increase in the afterhyperpolarization throughout its entire time course. In fact, what was observed was a prolongation of only the late part of the afterhyperpolarization. Thirdly, does morphine inhibit the removal of calcium from a pool close to the plasma membrane where its presence is associated with an increased potassium conductance? In earlier experiments (Morita *et al.*, 1982), it was observed that the decline in $G_{K,Ca}$ following 30 or more action potentials was double exponential, suggesting that two distinct processes might contribute to the removal of calcium from this pool. The present results indicate that the second of these processes may be particularly susceptible to inhibition by morphine (Figure 2). The physical identity of these processes might include the dissociation of calcium ions from the potassium channel, the sequestration of calcium into other cellular pools, or the extrusion of calcium across the plasma membrane.

Since morphine increases the potassium conductance of the resting membrane of some myenteric and other neurones (see Introduction), it is possible that this hyperpolarization also results from a transient increase in the cytosolic calcium concentration. This effect persists after removal of extracellular calcium. According to this scheme, morphine would interact with an extracellular cell surface receptor and this would lead to a release of calcium from intracellular stores. One might speculate that such an effect could not only increase potassium conductance in certain cells, but might also depress inward calcium currents in others. It must be emphasized that direct tests of this hypothesis are now required.

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