Opioid receptor independent effects of morphine on membrane currents in single cardiac myocytes

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Summary

We have examined the effects of morphine, a μ-opioid receptor agonist, on various membrane ionic currents in rat ventricular and human atrial myocytes, using patch-clamp techniques in the whole-cell configuration. Morphine produced a concentration-dependent reduction in peak transient sodium current. When the sodium current ($I_{Na}$) was evoked at 5-s intervals the estimated IC50 for morphine was approximately 30 μmol litre$^{-1}$. Morphine 10 μmol litre$^{-1}$ inhibited $I_{Na}$ with a 5-mV shift in the potential-dependent inactivation curve to negative potentials and retarded the $I_{Na}$ recovery rate from the inactivated state. Use-dependent $I_{Na}$ block was not observed when $I_{Na}$ was elicited at frequencies varying from 0.2 to 20 Hz. Morphine did not significantly affect the inward calcium current ($I_{Ca}$), transient outward current ($I_{to}$) or the inwardly rectifying potassium current ($I_{K1}$) at a concentration of 30 μmol litre$^{-1}$. The inhibitory effect of morphine on $I_{Na}$ could not be prevented or reversed by treatment with the opioid antagonist naloxone. Therefore, we suggest that morphine can directly inhibit the Na$^+$ inward current and bind to inactivated Na$^+$ channels. (Br. J. Anaesth. 1998; 81: 925–931).

Keywords: analgesics opioid, morphine; ions, sodium; ions, calcium; model, heart; receptors, opioid

Morphine, a μ-opioid receptor agonist, has been used clinically for the treatment of acute pulmonary oedema and relief of myocardial infarct. In addition, it has been shown to modify cardiac function. In isolated mammalian cardiac tissue, morphine produces a negative inotropic and chronotropic effect. The investigation of human preparations conformed to the principles outlined in the Declaration of Helsinki. Specimens of human right atrial appendages were obtained with consent from patients (25–60 yr) after repeated i.v. injections of morphine. Despite numerous studies on the cardiac effects of morphine, the mechanism of action related to the cardiac inhibitory effect of this agent is still controversial and remains to be clarified. Moreover, there are at present no detailed accounts of the effect of morphine on membrane ionic currents in isolated cardiac myocytes.

Therefore, we designed these experiments to examine the ionic mechanisms responsible for the inhibitory action of morphine in single rat and human cardiac myocytes. The mode of action of morphine on the sodium channel was studied by direct measurement of the sodium current ($I_{Na}$) using a voltage-clamp technique.

Materials and methods

Isolation of rat ventricular myocytes

Animal use in this study conformed with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985). Rat ventricular myocytes were isolated using a technique described previously. Briefly, rats weighing 250–350 g were anaesthetized with pentobarbital sodium 50 mg kg$^{-1}$ i.p. The hearts were excised and retrogradely perfused in a Langendorff apparatus with Ca$^{2+}$-free, HEPES-buffered Tyrode solution consisting of (mmol litre$^{-1}$): NaCl 137.0, KCl 5.4, MgCl2 1.1, dextrose 22.0, N-[2-hydroxyethyl] piperazine-N’-2-ethanesulphonic acid] (HEPES) 10.0, with pH adjusted to 7.4 with NaOH. The perfusate was oxygenated and maintained at 37 ± 0.2°C. After 5 min, the perfusate was changed to the same solution containing collagenase 0.5 mg ml$^{-1}$ (Type I, Sigma Chemical Co., St Louis, MO, USA) and protease 0.1 mg ml$^{-1}$ (Type XIV, Sigma). After 15–25 min of digestion, the residual enzymatic solution was cleaned with Ca$^{2+}$-HEPES-buffered Tyrode solution 0.05 mmol litre$^{-1}$. The ventricular tissue was then separated and small pieces of tissue were agitated gently. Isolated single cells were stored in Ca$^{2+}$-HEPES-buffered Tyrode solution 0.2 mmol litre$^{-1}$ at room temperature (25–27°C) for later use. Cells were used within 10 h of isolation.

Isolation of human atrial myocytes

The investigation of human preparations conformed to the principles outlined in the Declaration of Helsinki. Specimens of human right atrial appendages were obtained with consent from patients (25–60 yr).
during the surgical procedure when starting extracorporeal circulation. Single atrial cells were isolated by a procedure modified slightly from those described previously. Briefly, the tissue specimens were placed in chilled oxygenated Ca\(^{2+}\)-free, HEPES-buffered solution and were brought to the laboratory from the operating room. Samples of these tissue specimens were washed three times in the same solution and then gently stirred for 30 min in a similar solution containing collagenase 400 iu ml\(^{-1}\) (Type I, Sigma) and protease 4 iu ml\(^{-1}\) (Type XXVII, Sigma) at 35 ± 0.2°C. Thereafter, the partially digested samples were transferred to a fresh enzymatic solution containing collagenase 400 iu ml\(^{-1}\). Meanwhile, continuous microscopic examination of the enzymatic solution was performed to assess the number and quality of isolated cells. Finally, the isolated cells were washed and stored in Ca\(^{2+}\)-HEPES-buffered Tyrode solution 0.2 mmol litre\(^{-1}\) at room temperature for later use. Cells were used within 5 h of isolation.

**ELECTROPHYSIOLOGICAL RECORDING**

Transmembrane currents were measured using the tight-seal, whole-cell recording technique. Single myocytes were placed in a recording chamber (1-ml volume) attached to an inverted microscope (Nikon, Diaphot, Japan) and perfused with Ca\(^{2+}\)-HEPES-buffered Tyrode solution 1.8 mmol litre\(^{-1}\). The heat-polished glass pipettes were filled with internal solution and had tip resistances of 1–2 M\(\Omega\). A Dagan 8900 patch/whole-cell clamp amplifier fitted with 100 M\(\Omega\) feedback resistor in the headstage was used to clamp the myocytes. Most experiments were performed at room temperature. The total series resistance for the pathway between the pipette interior and the cell membrane was estimated from the cell capacitance and capacitance current decay. The capacitive transient during a step change in potential was partially compensated with analogue circuitry. Series resistance was compensated by at least 60–80% and was usually less than 1 M\(\Omega\). The maximal expected voltage decrease across the uncompensated series resistance was less than 8 mV for the largest current recorded and therefore was not corrected.

During measurement of \(I_{\text{Na}}\) and \(I_{\text{Ca}}\), the K\(^+\) currents were blocked by addition of Cs\(^+\) 1 mmol litre\(^{-1}\) to the bathing medium and internal dialysis of the cells with Cs\(^+\) containing pipette solution consisting of (mmol litre\(^{-1}\)): CsCl 130.0, K\_EGTA 15.0, tetraethylammonium chloride (TEA Cl) 15.0, HEPES 10.0, MgATP 5.0, titterated to pH 7.2 with CsOH. When the cells were kept in normal Tyrode solution, the inward sodium current elicited by depolarization to −40 mV was larger than 20 nA. Under these conditions, space and voltage control of membrane potential was unsatisfactory. To improve the clamp efficiency, we chose smaller atrial and ventricular cells, used larger suction pipettes (1–2 M\(\Omega\)) and perfused with Ca\(^{2+}\)-HEPES-buffered Tyrode solution 0.2 mmol litre\(^{-1}\) at room temperature for later use. Cells were used within 5 h of isolation.

\(I-V\) curves for \(I_{\text{Na}}\) highly skewed in shape and peaked at −40 mV (instead of −20 mV or −30 mV), the results were excluded.

Increasing concentrations of morphine (3–300 \(\mu\)mol litre\(^{-1}\)) were added cumulatively to the recording chamber at 10-min intervals. The inhibitory effects of morphine on \(I_{\text{Na}}\) were also tested in the presence of naloxone 10\(^{-7}\)–10\(^{-6}\) mol litre\(^{-1}\) added either 10 min before morphine or acutely during the response. To evaluate species differences, we also constructed concentration–response curves for morphine in atrial cells from human hearts. However, the mechanism of the Na\(^+\)-channel blocking action of morphine 10 \(\mu\)mol litre\(^{-1}\) was investigated only in ventricular cells from rat hearts. For measurement of \(I_{\text{Ca}}\) contamination of \(I_{\text{Na}}\) was excluded by stepping the membrane potential to −40 mV to inactivate \(I_{\text{Na}}\); \(I_{\text{Ca}}\) was then activated by a second step depolarization to 0 mV. During measurement of \(I_{\text{Na}}\) and \(I_{\text{Ca}}\), contamination by \(I_{\text{Na}}\) and \(I_{\text{Ca}}\) was prevented by addition of tetrodotoxin 30 \(\mu\)mol litre\(^{-1}\) and CoCl\(_2\) 1 mmol litre\(^{-1}\) to the bathing medium, respectively. The internal pipette solution contained (mmol litre\(^{-1}\)): KC\(_1\) 120.0, MgCl\(_2\) 5.0, MgATP 5.0, K\_EGTA 15.0, HEPES 10.0, titterated to pH 7.2 with KOH. Under these conditions, depolarization of membrane potentials to values positive to −40 mV from −80 mV resulted in rapid activation of \(I_{\text{Na}}\) which then decayed exponentially to steady state. \(I_{\text{Na}}\) was elicited by hyperpolarization of membrane potential to values more negative than −80 mV.

**DRUGS AND DATA ANALYSIS**

Morphine sulphate and tetrodotoxin were purchased from Sigma Chemical Co. (St Louis, MO, USA). All values are presented as mean (SEM). Repeated-measures analysis of variance and paired Student’s \(t\) tests were applied where appropriate, and values of \(P<0.05\) were considered significant. Concentration–response curves were fitted using commercial software (SigmaPlot, Jandel) and the following Hill equation: relative values, \(y = [1/(1 + (IC_{50}/D)^{nH})]\), where IC\(_{50}\) = concentration for half-maximal inhibition, \(D = \) concentration of morphine and \(nH = \) the Hill coefficient. The SigmaPlot curve fitter uses the Marquardt–Levenberg algorithm to find the coefficients (variables) of the independent variables \((x\) values) that give the best fit between the equation and the data. This algorithm seeks the values of the variables that minimize the sum of the squared differences between the observed and predicted values of the dependent variable \((y\) value). The inactivation curves of \(I_{\text{Na}}\) were fitted by the Boltzmann equation: \(I_{\text{Na}}\_{\text{inactiv}} = I_{\text{Na}}\_{\text{max}} / [1/(1 + \exp [(V-V_{1/2})/\sigma])], \) where \(V = \) preconditioning voltage, \(V_{1/2} = \) voltage at which half-maximal inactivation is obtained and \(\sigma = \) slope factor.

**Results**

**EFFECTS ON INWARD SODIUM CURRENT (\(I_{\text{Na}}\))**

Figure 1A (left) shows the time course of the inhibitory action of the sodium current by morphine 10–100 \(\mu\)mol litre\(^{-1}\). Depolarizing pulses of 15 ms duration to −20 mV from a holding potential of −80 mV were applied every 5 s. Application of...
morphine induced a dose-dependent decrease in $I_{Na}$ and its effect reached steady state at approximately 10 min. Inhibition of $I_{Na}$ by morphine was not prevented by pretreatment of the rat ventricular cell with an opioid receptor antagonist (naloxone 0.1 μmol litre$^{-1}$) or reversed by naloxone after steady-state inhibition of $I_{Na}$ by morphine was reached (fig. 1A, right). Morphine-induced inhibition of $I_{Na}$ was reversible after washout of morphine. Although morphine is a preferentially selective agonist for μ-opioid receptors, it can bind to σ or κ opioid receptors at concentrations greater than 0.1 μmol litre$^{-1}$. By blocking σ or κ opioid receptors with higher concentrations of naloxone (0.5–1 μmol litre$^{-1}$),$^{16}$ the possible contribution to the inhibitory action of morphine on $I_{Na}$ via activation of these two receptors was excluded. In the presence of these concentrations of naloxone, the extent of $I_{Na}$ inhibition was similar to that observed in cells pretreated with naloxone 0.1 μmol litre$^{-1}$ (data not shown). The inhibitory effect of morphine on peak $I_{Na}$ in rat ventricular myocytes was well fitted to the Hill equation. The calculated IC$_{50}$ and n$_H$ for morphine on $I_{Na}$ were 30.4 (6.0) μmol litre$^{-1}$ and 0.9 (0.1) ($n=6$), respectively. Similar studies in human atrial cells also found marked inhibition of $I_{Na}$ by morphine. The average IC$_{50}$ value for inhibition of $I_{Na}$ in human atrial cells was calculated as 25.7 (4.5) μmol litre$^{-1}$ ($n=5$). n$_H$ for morphine in human atrial cells was 0.9 (0.1) (fig. 1B).

MODE OF INHIBITION OF $I_{Na}$ BY MORPHINE

Figure 2A shows the representative $I_{Na}$ recorded from a single rat ventricular cell. $I_{Na}$ was elicited by depolarizing pulses to various test potentials (up to 50 mV) from a holding potential of −80 mV. The threshold for current activation was between −60 mV and −50 mV, and the peak current was obtained at −30 mV. Further depolarization decreased $I_{Na}$ with a reversal potential at approximately +50 to +60 mV. After 10-min exposure to morphine, peak $I_{Na}$ was decreased markedly without appreciable change in the time course of the decay phase of $I_{Na}$. Morphine decreased the peak $I_{Na}$ to approximately 65% of control (35% reduction). The time constant of $I_{Na}$ inactivation determined at −20 mV in the control group was similar to that in the morphine-treated group (1.3 (0.1) ms and 1.4 (0.2) ms, respectively; $n=6$). Figure 2B shows the current–voltage ($I$–$V$) relationship. Morphine blocked $I_{Na}$ without changing the threshold potential, peak potential or reversal potential.

Voltage-dependent steady-state inactivation of $I_{Na}$ was examined by a pulse paradigm (inset in fig. 3A) in which a 15-ms test pulse to −20 mV was preceded by a 1-s preconditioning pulse to levels of −120 to −40 mV. Figure 3A shows a family of currents elicited by test pulses from different preconditioning potentials in controls, after morphine and after washout of morphine. Figure 3A shows the steady-state inactivation
curves of $I_{Na}$ from 10 experiments. $V_{0.5}$ for the steady-state inactivation curves in the controls and in the presence of morphine 10 μmol litre$^{-1}$ were -72.2 (3.6) mV and -77.7 (3.2) mV ($P < 0.001$ vs control values), respectively. The slope factors were 5.1 (0.3) mV and 5.3 (0.3) mV, respectively. This shift in the steady-state inactivation curve of $I_{Na}$ was associated with retardation of $I_{Na}$ recovery from inactivation.

Figure 4 shows a typical result from one cell. Recovery of $I_{Na}$ from inactivation was studied using a two-pulse protocol (fig. 4A, inset). $I_{Na}$ was elicited by depolarization to $-20$ mV and separated by intervals of 10–350 ms. Each double-pulse sequence was followed by a 5-s rest period. The time course of recovery of $I_{Na}$ was well fitted by two exponential equations. In controls, the time constants of the fast recovery component ($\tau_f$) and the slow recovery component ($\tau_s$) were 14.9 (0.5) ms and 178.9 (22.1) ms ($n = 5$), respectively. After exposure to morphine 10 μmol litre$^{-1}$, the $\tau_f$ of $I_{Na}$ was prolonged to 17.8 (0.8) ms. The $\tau_s$ of $I_{Na}$ was, however, unaffected (169.8 (20.3) ms).

Figure 5 illustrates the effect of varying the stimulus frequency on $I_{Na}$ by morphine 10 and 30 μmol litre$^{-1}$. $I_{Na}$ was activated by depolarizing pulses to $-20$ mV from a holding potential of $-80$ mV. The stimulation frequency varied between 0.2 and 20 Hz. As shown in figure 5, there was no use-dependent block of $I_{Na}$ by morphine.
EFFECT OF MORPHINE ON POTASSIUM CURRENTS AND INWARD CALCIUM CURRENT

Figure 6A (top) shows traces of K⁺ currents in a rat ventricular cell elicited by depolarizing and hyperpolarizing pulses. Application of depolarizing pulses to potentials more positive than −20 mV resulted in generation of a transient outward current which then decayed to a steady-state level. On depolarization to −60, −40 and −20 mV, a constant amplitude of outward current via inward rectifying potassium channels was obtained. On hyperpolarization to potentials more negative than −80 mV, varying amplitude inward current via inwardly rectifying potassium channels was observed. Compared with inhibition of \( I_{K}\), the peak transient outward current, steady-state outward current and potassium current via inward rectifying potassium channels were unaffected by morphine 30 μmol litre⁻¹ (fig. 6A, bottom).

Figure 6A is a typical experiment that shows the time course for the effect of morphine 30 μmol litre⁻¹ on the inward calcium current (\( I_{Ca} \)) in a rat ventricular cell. These data in the presence of naloxone revealed that morphine 30 μmol litre⁻¹ had no direct effect on \( I_{Ca} \). Similar results were found in three other experiments.

![Figure 6A](image)

**Figure 6A**: Effect of morphine on K⁺ currents. Top: Families of current traces elicited by a series of 180-ms long depolarizing or hyperpolarizing pulses from a holding potential of −80 mV were obtained in the absence and presence of morphine 30 μmol litre⁻¹. Bottom: The \( I-V \) relationship of K⁺ currents measured at the peak and end of the voltage pulses before (α, Δ) and after (●, △) exposure to morphine 30 μmol litre⁻¹ (mean (SEM), n = 5).

**Figure 6B**: Time course of the effects of morphine on \( I_{Ca} \). Cells were depolarized to 0 mV from −40 mV for 120 ms. Current traces of \( I_{Ca} \) corresponding to the letters in the graph are shown in the inset.

**Discussion**

We have demonstrated that morphine produced reversible inhibition of \( I_{Ca} \) in rat ventricular and human atrial myocytes. However, even at concentrations of up to 30 μmol litre⁻¹, morphine failed to affect Ca²⁺ and K⁺ currents.

**MECHANISMS OF THE NA⁺-CHANNEL BLOCKING ACTION OF MORPHINE**

According to the results of our study, the inhibitory action of morphine on \( I_{Ca} \) was not antagonized by naloxone. Thus we suggest that morphine inhibition of \( I_{Ca} \) was not caused by stimulation of opioid receptors. As the extent of suppression of peak \( I_{Ca} \) by morphine (fig. 2) correlated well with the negative shift of the voltage-dependent inactivation curve of \( I_{Ca} \) (fig. 3) and use-dependence \( I_{Ca} \) block by morphine was not observed under a higher frequency (20 Hz) of depolarization pulses, these findings suggest that morphine may inhibit \( I_{Ca} \) by binding to inactivated Na⁺ channels. In addition to the negative shift of the voltage-dependent inactivation of \( I_{Ca} \) retardation of the fast recovery of \( I_{Ca} \) from inactivation was observed in cells treated with morphine. In spite of the retardation of fast recovery of \( I_{Ca} \) from inactivation, the time constant of the slow recovery of \( I_{Ca} \) from inactivation and inactivation time constant of \( I_{Ca} \) remained unchanged after the cells were treated with morphine. In view of the effects on \( I_{Ca} \), the interaction of morphine with cardiac Na⁺ channels can be depicted (fig. 7), where D = drug; R and O = resting and open states of the channels, respectively; and \( I_1 \) and \( I_2 \) = fast and slow recovery states of inactivated channels, respectively. In the absence of drug, the major fraction of Na⁺ channels in the inactivated state recovered with a fast time constant on repolarization to a holding potential of −80 mV. Therefore, most Na⁺ channels exist in the \( I_1 \) state under depolarized conditions. In the presence of morphine, the time constant of the fast recovery of Na⁺ channel from inactivation was increased but the time constant of the slow recovery from inactivation was unaffected; these results indicate a preferential binding of morphine to Na⁺ channels in the \( I_2 \) state.

![Figure 7](image)

**Figure 7**: Interaction of morphine with cardiac Na⁺ channels, where D is the drug; R and O are the resting and open states of the channels, respectively; \( I_1 \) and \( I_2 \) are the inactivated channels in fast and slow recovery states, respectively; RD is the binding of morphine to resting state channels; and ID is the binding of morphine to inactivated channels in fast recovery state.
As the time course of inactivation of \( I_{Na} \) at \(-20\) mV was unchanged in the presence of morphine, the rate of transformation of Na\(^+\) channels from O state to I\(_c\) state may be unaffected and binding of morphine to channels in the O state may be insignificant or not fast enough to affect the time course of \( I_{Na} \) inactivation. This consideration is further substantiated by the absence of frequency-dependent inhibition of \( I_{Na} \) by morphine (fig. 5).

According to previous studies, the potency of block of cardiac Na\(^+\) channels by class I antiarrhythmic agents is different when the channels exist in three different states (resting, open and inactivated states). Most class I antiarrhythmic agents have lower affinity for the channels in the resting state; their affinity increases when channels are in the open or inactivated state. As \( I_{Na} \) elicited from a holding potential of \(-110\) mV was reduced by morphine to a less extent than \( I_{Na} \) elicited from a holding potential of \(-80\) mV (fig. 3A), the binding affinity of morphine to resting state Na\(^+\) channels may be lower than that to inactivated Na\(^+\) channels.

**SIGNIFICANCE OF INHIBITION OF \( I_{Na} \)**

In view of the insignificant direct inhibition of the inward calcium current and potassium outward current (i.e. in the presence of naloxone, fig. 6), inhibition of the inward sodium current would shorten action potential duration in some cardiac tissues, such as Purkinje fibres, in most mammalian hearts and rat or guinea pig or human atria. Reduction of Na\(^+\) influx results in less intracellular Na\(^+\) accumulation. Intracellular Na\(^+\) activity has a positive correlation with intracellular Ca\(^{2+}\) activity.

Lower intracellular Na\(^+\) activity enhances Ca\(^{2+}\) extrusion via the Na\(^+\)-Ca\(^{2+}\) exchange mechanism which leads to reduction of intracellular calcium and negative inotropic effect. Further, shortening of the action potential by inhibition of \( I_{Na} \) should decrease calcium influx via non-inactivating calcium channels.

This effect also contributes to negative inotropism. The 40% inhibition of \( I_{Na} \) of human atrial cells with morphine 10 \( \mu \)mol litre\(^{-1}\) in this study (fig. 1b) is comparable with 52% inhibition of contractile force of human atrial strips by Llobel and Laorden. With regard to the antiarrhythmic activity of morphine, activation of central opioid receptor, antagonism of \( \beta \)-adrenergic stimulation of cardiac tissues via activation of cardiac \( \delta \) opioid receptor and activation of cardiac K\(_{ATP}\) channels were proposed as possible mechanisms responsible for the antiarrhythmic activity of morphine. In addition to these mechanisms, direct inhibition of \( I_{Na} \) by morphine may contribute partly to the antiarrhythmic activity of \( I_{Na} \) in inactive state.

In summary, morphine inhibited Na\(^+\) channels primarily by binding to inactivated Na\(^+\) channels. This effect may contribute to the negative inotropism and antiarrhythmic activity of morphine in cardiac tissues.

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