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Nonopioid Mechanism of Morphine Modulation of the Activation of 5-Hydroxytryptamine Type 3 Receptors

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SUMMARY

The effect of morphine on the ion current mediated by 5-hydroxytryptamine (5-HT_3) receptors was investigated in rat nodose ganglion neurons and in *Xenopus* oocytes expressing the cloned 5-HT_3 receptor. Morphine reversibly inhibited the 5-HT-induced current and shifted the 5-HT concentration-response curve to the right in a parallel fashion, without reducing the maximal 5-HT response. IC_{50} values for morphine were 0.3 μM in nodose neurons and 0.32 μM in oocytes. The apparent K_d of morphine in nodose neurons was 0.903 μM. This effect of morphine was immediate, not dependent on membrane potential, and not prevented by the opioid receptor antagonists naltrexone and β-chlornaltrexamine. It is concluded that opioid receptors were not involved in the present study and that morphine acted at the agonist recognition site located on the 5-HT_3 receptor.

It was reported about 40 years ago that 5-HT released acetylcholine from nerve terminals by the activation of a neuronal 5-HT receptor and caused contraction of guinea pig ileum (1, 2). Because this response was inhibited by morphine, Gaddum and Picarelli (3) named the neuronal 5-HT receptor the 5-HT M receptor. However, a direct action of morphine on neuronal 5-HT receptors in guinea pig ileum was not supported by other studies that concluded that the effect of morphine on guinea pig ileum contraction was mediated by a nonselective inhibition of acetylcholine release (4, 5). More recent studies demonstrated that low concentrations of morphine and other opioid agonists did inhibit acetylcholine release in guinea pig ileum (6–8). In brain slice preparations, high concentrations of morphine are required to inhibit acetylcholine release (9). These studies confirm the inhibitory effect of morphine on acetylcholine release but, as suggested by Kosterlitz and Robinson (5), do not necessarily exclude the possibility of morphine interfering with the activity of a site different from the point of acetylcholine release.

Currently, the 5-HT M receptor is classified as the 5-HT_3 receptor, which is important in the central and peripheral nervous systems. This receptor is a ligand-gated ion channel (10) and mediates excitatory synaptic transmission in the central nervous system (11). Evidence suggests that some of the morphine-induced behavioral effects may also be produced by the inhibition of 5-HT_3 receptor function. It has been reported that 5-HT_3 receptor antagonists exhibit useful anxiolytic properties in both animal and human studies (12). On the other hand, morphine not only is used as an anxiolytic in patients but also is considered to produce pain relief in part by decreasing anticipatory anxiety (13, 14). It has also been reported that peripheral 5-HT_3 receptors mediate pain perception in humans and nociceptive responses in animals and selective 5-HT_3 receptor antagonists blocked the painful or nociceptive responses caused by 5-HT (12, 15–17). In addition, 5-HT_3 receptor antagonists blocked the morphine-induced reward of place preference in an animal model (18, 19), suggesting a possible involvement of 5-HT_3 receptors in morphine abuse. In the present study, the effect of morphine on the ion current mediated by 5-HT_3 receptors was investigated in rat nodose ganglion neurons and in *Xenopus* oocytes expressing the cloned 5-HT_3 receptor.

Materials and Methods

Experiments in nodose ganglion neurons. Male, adult, Sprague-Dawley rats (150–300 g) were killed by decapitation, and the nodose ganglion was rapidly extracted and placed in cold Dulbecco's modified Eagle's medium. The nodose ganglion was then minced with iridectomy scissors and digested in Dulbecco's modified Eagle's medium, containing 1.25 mg/ml collagenase (type IA), 0.8 mg/ml trypsin (type III), and 0.125 mg/ml DNAse (type IV), at 35° for 30–45 min, after which soybean trypsin inhibitor (Type II-S, 1 mg/ml; Sigma) was added. The solution containing the isolated neurons was then plated in Petri dishes.

Neurons were viewed using an inverted microscope and were superfused with extracellular solution at 1 ml/min. The extracellular solution contained 150 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM D-glucose; the pH was adjusted to 7.4 with NaOH. Sucrose was added to make the osmolarity 340 mOsmol/kg. Experiments were performed at room temperature. The whole-cell version of the patch-clamp technique was used with an Axopatch 1D amplifier. Unless stated otherwise, neurons were voltage-clamped at −50 mV. Patch electrodes (2–5 MΩ) were pulled from borosilicate glass and filled

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; MBS, modified Barth's solution; β-CNA, β-chlornaltrexamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

with an internal solution containing 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, and 2 mM ATP (magnesium salt); the pH was buffered to 7.4 with KOH and the osmolarity was adjusted to 310 mOsmol/kg with sucrose. Serotonin (5-HT) and other drugs were dissolved in external solution and applied through a fast superfusion system consisting of a series of fused silica tubes (200–300-μm diameter) glued together and held by a micromanipulator. These tubes were each connected to separate reservoirs containing either control or test solutions. The neuron under study was placed within 30 μm of the opening of these tubes and solution was allowed to superfuse the cell. After rapid sidewise movement of the superfusion system, a new solution superfused the neuron and the solution that had been superfusing the neuron was washed out. Ion substitution experiments showed that solution exchange was completed within 60 msec (data not shown).

Experiments in *Xenopus* oocytes. Mature, female, *Xenopus laevis* frogs (Xenopus I, Ann Arbor, MI) were anesthetized in ice-water with 0.15% tricaine (Sigma). Part of the ovary was removed through an incision in the ventral abdominal surface. Oocytes (stage 5 and 6) were defolliculated at room temperature (21–25°) by 2-hr treatment with 0.2% collagenase A (Boehringer Mannheim, Indianapolis, IN) in Ca^{2+}-free MBS. The denuded oocytes were then transferred to normal MBS, which contains 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.9 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 10 mM HEPES, pH 7.5. After a 24-hr incubation at 19° in normal MBS, oocytes were microinjected with 10–15 ng of mRNAs generated by *in vitro* transcription from the cDNA encoding the 5-HT_3 receptor of NCB-20 neuroblastoma cells (20) and were further incubated for 2–10 days in MBS, supplemented with 2 mM sodium pyruvate, 0.1 mg/ml streptomycin, and 10 units/ml penicillin, before electrical recording, which was performed at room temperature. Oocytes were voltage-clamped at −70 mV using the two-electrode voltage-clamp technique, with an Axoclamp IIA amplifier (Axon Instrument Co., Foster City, CA). Both electrodes were filled with 3 M KCl, and the resistance was 0.5–5 MΩ. Oocytes were placed in a 0.1-ml chamber and continuously superfused with MBS at a rate of 3–5 ml/min. Neurotransmitters and other drugs were dissolved in MBS and applied through a macropipette (1.5 mm) pointed directly at the oocyte (within 100 μm). Macropipettes were connected to different reservoirs containing appropriate solutions placed above the preparation, which permitted superfusion of the cells by gravity. The superfusion rate for applying agonist and/or drugs was 4 ml/min. A 5–10-min delay was employed between applications of 5-HT and other agonists, to avoid desensitization.

5-HT hydrochloride, 2-methyl-5-HT, *m*-chlorophenylbiguanide, MDL72222, naltrexone, β-CNA, and fentanyl were purchased from Research Biochemicals (Natick, MA). Morphine sulfate was from Merck.

Data analysis. Averaged values in the text and figures are mean ± standard error. Data were statistically compared by the use of analysis of variance or paired Student's *t* test, as appropriate. Statistical analysis of concentration-response data was performed using the nonlinear curve-fitting program ALLFIT.

Results

Effect of morphine on the 5-HT-induced current in nodose ganglion neurons. 5-HT activated an inward current in rat nodose ganglion neurons (21) (Fig. 1A). This current was blocked by the 5-HT_3 receptor antagonist MDL72222 with an IC_{50} value of 30 nM (Fig. 1C). Morphine (0.1–50 μM) applied together with 5-HT reversibly inhibited currents induced by 0.5 μM and 3 μM 5-HT, with IC_{50} values of 0.3 μM and 0.7 μM, respectively (Fig. 1, A and D). Significant suppression of 0.5 μM 5-HT-induced current was seen with 0.1 μM morphine. Morphine alone (1–3 μM, $n = 3$) did not produce a detectable membrane current in nodose ganglion neurons (data not shown).

The effects of other opioid agonists were also tested in iso-lated nodose neurons. The μ-opioid agonist fentanyl (0.25 μM) inhibited 5-HT (0.5 μM)-induced current by 48 ± 3.1% ($n = 4$) (Fig. 1B), whereas 1–5 μM [D-Ala^2,*N*-Me-Phe^4,Gly-ol^5]-enkephalin ($n = 4$) and 2 μM [D-Ala^2,D-Leu^5]-enkephalin ($n = 3$) had no significant effect on the 5-HT-activated current (data not shown).

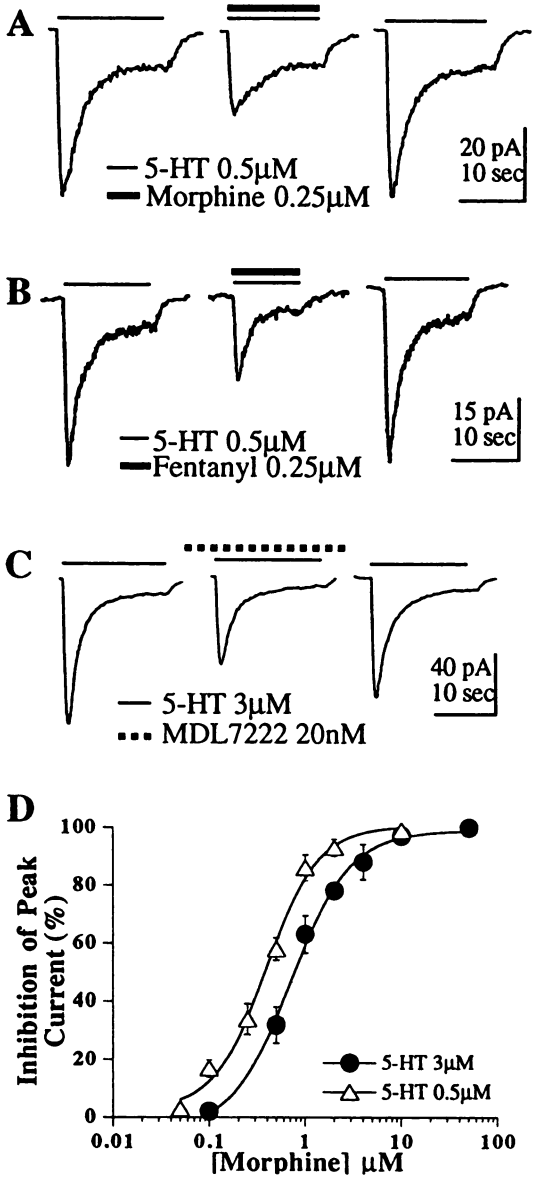

Fig. 1. A and B, Morphine (A) and fentanyl (B) (*thick bars*) inhibit the current induced by 0.5 μM 5-HT (*thin bars*). C, MDL72222 (*dashed line*) blocks the current activated by 5-HT. D, Concentration-response curves for morphine with 0.5 and 3 μM 5-HT are shown. The IC_{50} values with 0.5 and 3 μM 5-HT are 0.3 and 0.7 μM, respectively. Each *point* represents the average data from four to nine cells. Standard errors are also shown.

An increase of 5-HT concentration from 0.5 to 3 μM produced a parallel shift to the right of the morphine inhibition curve (Fig. 1D). On the other hand, morphine shifted the 5-HT concentration-response curve to the right in a parallel fashion and did not reduce the maximum 5-HT-induced current (Fig. 2A). The EC_{50} values for 5-HT were 2.26 μM in controls and 5.4 μM, 15 μM, and 151 μM with 1, 10, and 50 μM morphine, respectively. Hill coefficients were 1.3, 1.3, 1.34, and 1.24, respectively. A Schild plot of these data revealed a pA_2 value of 6.044, with a slope of 0.96 (Fig. 2B). The appar-

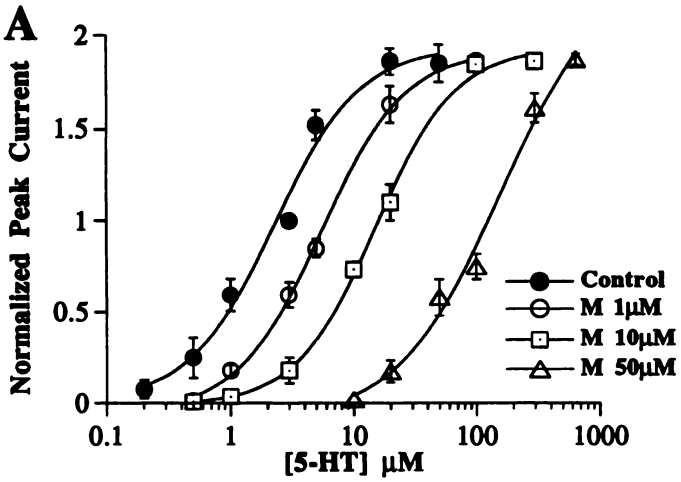

Fig. 2. A, 5-HT concentration-response curves in the absence and presence of morphine. Each *point* represents the average data from four to seven cells. Standard errors are shown. The EC_{50} is 2.26 μM in controls and 5.4 μM, 15 μM, and 151 μM with 1, 10, and 50 μM morphine (*M*), respectively. The Hill coefficients are 1.3, 1.3, 1.34, and 1.24, respectively. All currents were normalized to that induced by 3 μM 5-HT. B, Schild plot of the morphine effect at different concentrations.

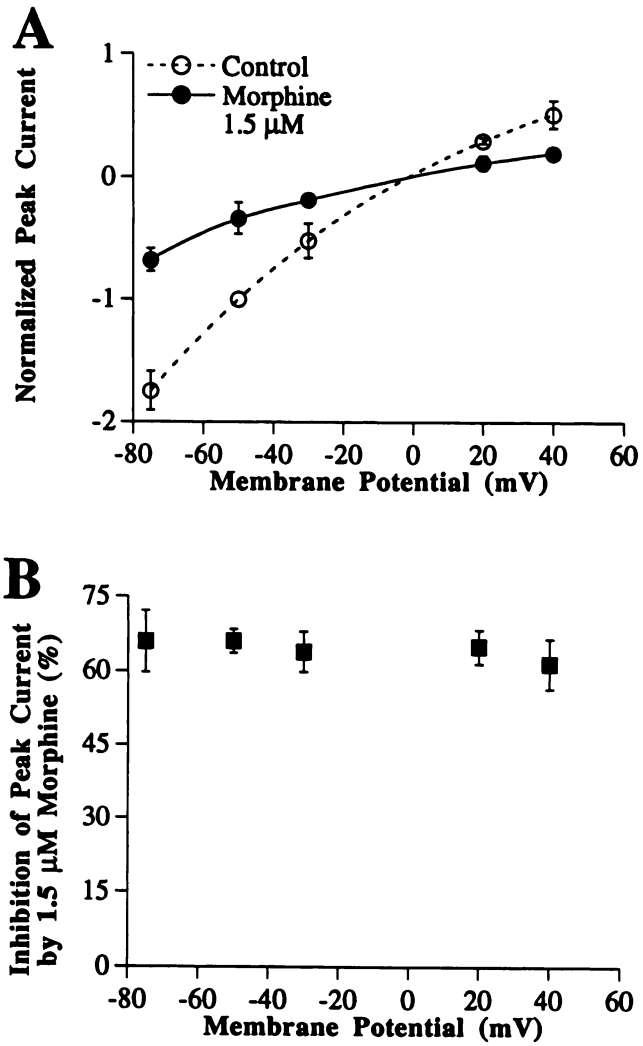

Fig. 3. A, Current-voltage relation curves for 5-HT (3 μM) in the absence and presence of 1.5 μM morphine. Currents at different membrane potentials were normalized to that at −50 mV. Data were from five cells. Standard errors are shown. B, Average data for the effect of morphine at different membrane potentials (n = 5).

ent K_d was 0.903 μM. These results suggest a competitive inhibition of 5-HT$_3$ receptors by morphine.

The effect of morphine on the 5-HT-induced current was not affected by membrane potential (−75 mV to +40 mV) (Fig. 3). The 5-HT current-voltage relation curves in the absence and presence of 1.5 μM morphine are shown in Fig. 3A. The average data for the morphine effect at different membrane potentials are shown in Fig. 3B. No significant difference was found (n = 5, p > 0.1). The average reversal potential of the 5-HT-induced current was 1.1 mV.

Effect of opioid antagonists on the morphine action in nodose neurons. To investigate the possible involvement of opioid receptors, effects of the nonselective opioid receptor antagonists naltrexone (2 μM) and β-CNA (0.5 μM) were examined. Naltrexone and β-CNA did not prevent the inhibition of 5-HT-induced current by morphine (Fig. 4, A and B). In the absence and presence of 0.5 μM β-CNA or 2 μM naltrexone, the inhibition of 1 μM 5-HT-induced current by 0.25 μM morphine was 40.9 ± 1.4% (n = 8), 40 ± 3.4% (n = 5), and 44 ± 5.3%(n = 9), respectively (Fig. 4C). No significant difference was found (p > 0.2, paired t test). At the concentrations used, β-CNA or naltrexone alone had no effect on 5-HT-activated current (Fig. 4, A and B).

Effect of morphine on the cloned 5-HT$_3$ receptor expressed in *Xenopus* oocytes. The foregoing experiments (Figs. 1D, 2, and 4) suggest that morphine competes with 5-HT and acts at the agonist recognition site on the 5-HT$_3$ receptor. However, evidence from receptor binding studies showing that morphine acts directly on 5-HT$_3$ receptors is not available. Therefore, the effect of morphine was further investigated in *Xenopus* oocytes, which do not have opioid receptors and have been used as a model system to express the cloned opioid receptor genes (22, 23). The cloned 5-HT$_3$ receptors (20) expressed in *Xenopus* oocytes were activated by the selective 5-HT$_3$ agonists 2-methyl-5-HT and *m*-chlorophenylbiguanide and were sensitive to the specific 5-HT$_3$ receptor antagonist MDL72222 (Fig. 5A). MDL72222 (20 nM) was applied for 5 min and then applied with 5-HT (Fig. 5A). Morphine inhibited the current induced by 1 μM 5-HT with an IC_{50} value of 0.32 μM (Fig. 5, B and C), and this effect was not prevented by 2 μM naltrexone (Fig. 5B). In the presence and absence of naltrexone, inhibition of 1 μM 5-HT-activated current by 0.4 μM morphine was 61.7 ±

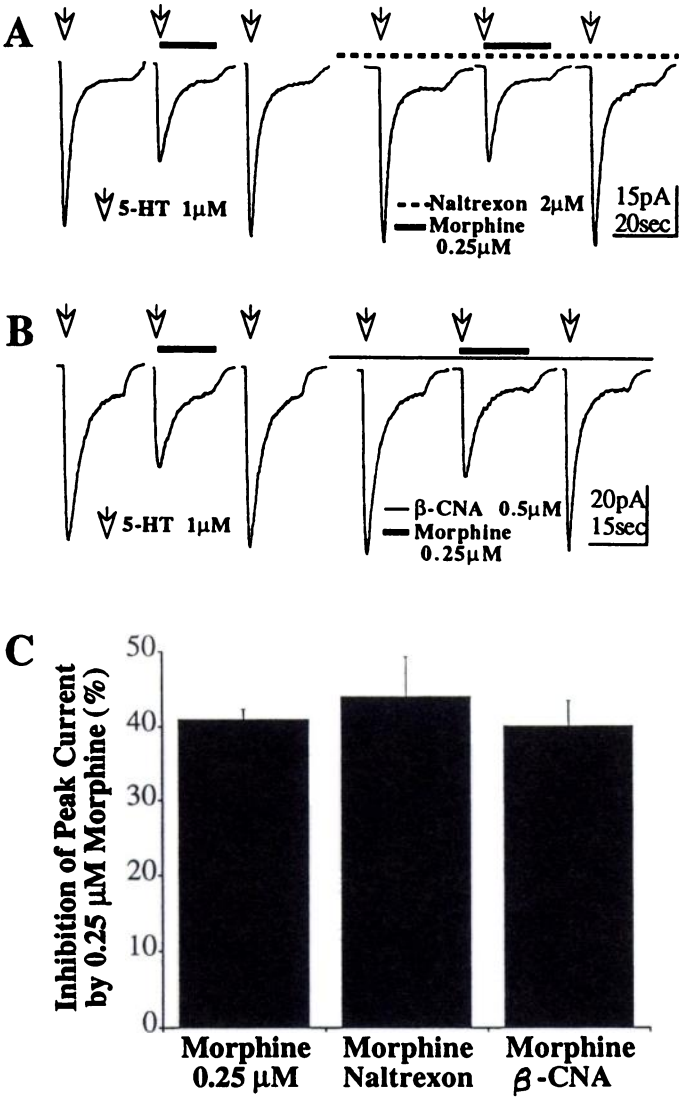

Fig. 4. A and B, Opioid receptor antagonists naltrexone (A) and β-CNA (B) did not block the effect of morphine on 5-HT-activated current. *Arrows*, 15-sec application of 1 μM 5-HT. *Thin bar* and *dashed line*, application of opioid receptor antagonists; *thick bars*, administration of 0.25 μM morphine. Recordings of A and B are from two different cells. C, Quantitative data on the morphine effect on the 5-HT current in the absence and presence of opioid receptor antagonists are shown. No significant difference was found ($p > 0.2$, $n = 5 - 9$).

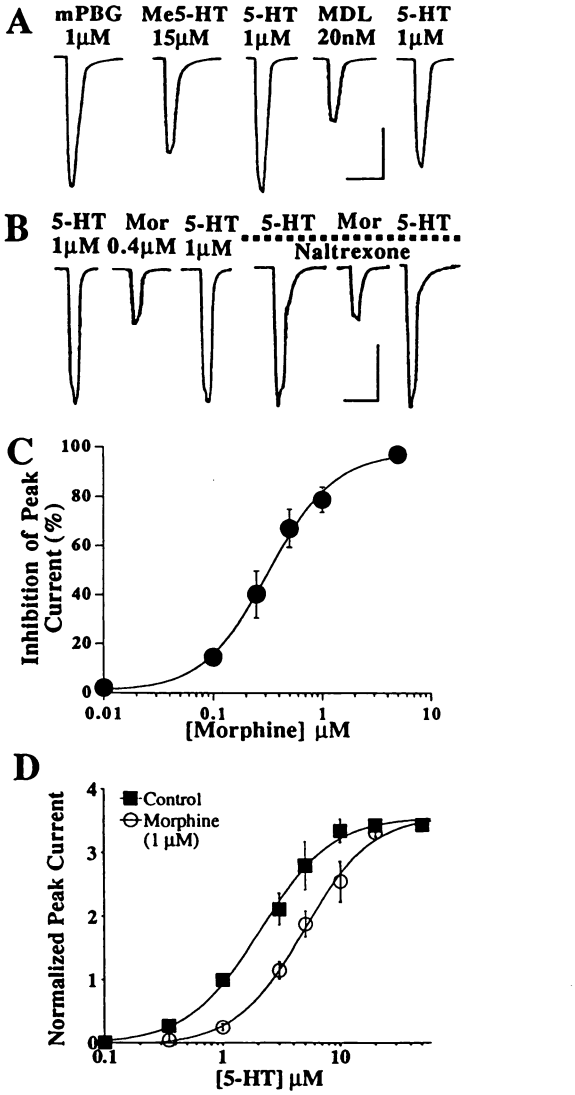

Fig. 5. Effect of morphine on the 5-HT-activated current in *Xenopus* oocytes expressing 5-HT_3 receptors. Oocytes were voltage-clamped at −70 mV, 2–10 days after injection of mRNA encoding the cloned 5-HT_3 receptor. Agonists were bath applied for 15 sec. A, Currents activated by *m*-chlorophenylbiguanide (*mPBG*), 2-methyl-5-HT (*Me5-HT*), and 5-HT. MDL72222 (*MDL*) (20 nM) preapplied for 5 min and then applied with 5-HT reversibly reduced 5-HT-activated current. B, Morphine (*Mor*) inhibition of 1 μM 5-HT-activated current; this inhibition was not affected by 2 μM naltrexone (*dashed line*). Naltrexone was preapplied for 5 min and then applied with 5-HT and/or morphine. C, Concentration-response curve for morphine effects on 1 μM 5-HT-induced current. The IC_{50} value is 0.32 μM and the Hill coefficient is 1.46. D, 5-HT concentration-response curves, with or without 1 μM morphine. Currents were normalized to that induced by 1 μM 5-HT. EC_{50} values are 2.05 and 4.9 μM and Hill coefficients are 1.41 and 1.49, respectively. Each point in C and D represents the average data from four to seven cells. Records in A and B were from two different oocytes. Calibration, 3 min and 250 nA.

5.2% and 59.2 ± 2.1%, respectively ($n = 7$) (Fig. 5C). No significant difference was found ($p > 0.5$). Morphine (1 μM) produced a parallel shift of the 5-HT concentration-response curve and increased the EC_{50} value from 2.05 μM to 4.9 μM (Fig. 5D). Morphine alone did not produce any detectable current (data not shown).

Discussion

The present study demonstrates for the first time that morphine acts directly on a ligand-gated ion channel. Morphine reversibly antagonizes the activation of 5-HT_3 receptors in nodose ganglion neurons and *Xenopus* oocytes expressing the receptor, in a concentration-dependent manner. The observations that naltrexone and β-CNA did not prevent this action of morphine and that morphine inhibited 5-HT_3 receptor activation in a preparation that does not have opioid receptors (22, 23) clearly indicate that the effect of morphine on 5-HT_3 receptors is not mediated by opioid receptors. Furthermore, the competitive relationship between morphine and 5-HT effects on the 5-HT_3 receptor-mediated current suggests that morphine acts at the agonist recognition site located on the 5-HT_3 receptor. These results support the hypothesis of Gaddum and Picarelli (3) that morphine blocks a neuronal 5-HT_3 receptor.

The effect of morphine on 5-HT_3 receptor-mediated current is

less potent than that of the selective 5-HT$_3$ receptor antagonist MDL72222. However, significant inhibition of 5-HT current was observed with 0.1 μM morphine, and the IC_{50} or K_d values of morphine for the 5-HT$_3$ receptor-mediated current are within the range of or close to the suggested effective morphine concentrations to achieve surgical analgesia (0.22–0.5 μM) (13) and satisfactory pain relief in cancer patients (0.06–1.3 μM) (13). In addition, the effective morphine concentrations are also below or close to that which inhibits guinea pig ileum contraction (3–5) and acetylcholine release (6, 7, 9). Therefore, it is likely that in some therapeutic uses of morphine the function of 5-HT$_3$ receptors is affected. However, the serotonin concentration reached in the synaptic cleft is not known. Millimolar concentrations of neurotransmitter have been suggested for central glutamate synapses (24). If this is also the case for serotonin, therapeutic concentrations of morphine may not affect the transmission mediated by 5-HT$_3$ receptors.

Because the major behavioral effects of morphine are prevented by opioid antagonists (13), the physiological significance of the morphine effect on 5-HT$_3$ receptors is not clear. However, there is evidence that some behavioral effects of morphine are not mediated by opioid receptors. It was shown that, in patients who received therapeutic doses of morphine, histamine release (which probably accounts for the flushed skin and urticaria) was increased and was not blocked by naloxone (13). Intrathecal administration of high doses of morphine caused a syndrome in rats called allodynia, which is not mediated by opioid receptors (25). Local injection of morphine into rat dorsal periaqueductal grey area can produce a fearful hyperactivity that is also not mediated by opioid receptors (26). From these observations, some speculations about the morphine effect on 5-HT$_3$ receptors may be worthwhile.

5-HT has been shown to be one of the most potent pain-producing agents and elicits strong pain at nanomolar concentrations (17). Other studies indicate that 5-HT$_3$ receptorsmediate not only the peripheral pain sensation in humans but also other forms of pain, such as migraine, angina, irritable bowel syndrome, and nociceptive responses to intravenousadministration of 5-HT (12, 15, 16). These painful or nociceptive responses can be blocked by different 5-HT$_3$ receptor antagonists, including ICS205930, MDL72222, MDL72422, BRL43694, and zatosetron (12, 17). The morphine antagonism of 5-HT$_3$ receptor activation observed in the present study reveals the possibility that, in addition to the analgesia caused by the activation of opioid receptors, morphine may produce pain relief by its inhibition of 5-HT$_3$ receptors. Similarly, the proaversive effect (26) and part of the anxiolytic effect of morphine may also be correlated with its 5-HT$_3$ receptor antagonism. Although the block of morphine-induced reward by 5-HT$_3$ receptor antagonists suggests an involvement of 5-HT$_3$ receptors in the reinforcing property of morphine (18, 19), further investigation is needed to support the correlations described above and the conclusion that the action of morphine on 5-HT$_3$ receptors contributes to the behavioral effects of morphine.

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