Hyperpolarizing Effects of Morphine, Clonidine and 2-Chloroadenosine in Myenteric Neurons Associated with Tolerance to Morphine

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ABSTRACT

Chronic treatment of guinea pigs with morphine produces non-specific sub sensitivities (tolerance) of the longitudinal smooth muscle myenteric plexus (LM/MP) preparation of the guinea pig ileum to morphine, clonidine and 2-chloroadenosine correlated with a partial depolarization of myenteric S neurons. The purpose of our investigation was to gain further evidence regarding the cellular mechanism of tolerance. Either morphine or placebo pellets were implanted s.c. in guinea pigs 7 days before the experiment. Subsensitivity was confirmed by a marked decrease of the inhibitory effect of 0.1 μM morphine and 0.3 μM clonidine on neurogenically induced twitches in longitudinal smooth muscle myenteric plexus preparations from the morphine-pretreated guinea pigs. Intracellular microelectrode recording established that only myenteric S neurons that were hyperpolarized by morphine exhibited the depolarized state (difference of 7.2 mV), which occurred without a change in the threshold for initiation of action potentials. S neurons that were hyperpolarized by superfusion with solution containing morphine, 0.1 μM, were acutely hyperpolarized an equivalent amount (6–8 mV) by clonidine, 0.3 μM, or 2-chloroadenosine, 0.1 μM. Morphine and clonidine, but not 2-chloroadenosine, reduced input resistance. The hyperpolarizations and changes in conductance were not different between tolerant and control preparations for any agonist. It is concluded that 1) the receptors for the three agonists are colocalized on selected S neurons, 2) the transduction process for the hyperpolarizing effect of 2-chloroadenosine is different than that for morphine and clonidine, 3) cross-tolerance among the agonists is not a function of altered receptors or signal transduction processes and 4) the depolarized state is associated with tolerance of myenteric S neurons.

Chronic changes in the activity of excitable cells induces homeostatic adaptations in responsiveness (adaptive super- and subsensitivity, Fleming and Westfall, 1988). Chronic exposure of neurons to opioids leads to such adaptive changes, demonstrable as tolerance and dependence (Collier, 1965, 1966; Johnson and Fleming, 1989; Fleming and Taylor, 1995). Many of the acute effects of opioids result from membrane hyperpolarization (Duggan and North, 1984; MacFadzean, 1988). Thus, continued exposure to opioids induces a chronic decrease in cell activity leading to adaptive changes in sensitivity (Johnson and Fleming, 1989; Fleming and Taylor, 1995). The guinea pig ileum, particularly the LM/MP preparation, has been used extensively to investigate tolerance to opioids at both the organ and cellular level (see review by Johnson and Fleming, 1989). Electrical stimulation of the LM/MP induces muscle twitches via activation of the motor neurons (Leedham et al., 1992; Fleming and Taylor, 1995). A number of agonists, including opioids, inhibit the neurogenic twitches of the LM/MP preparation by an action directly on myenteric neurons. Subcutaneous implantation of morphine-containing pellets induces a slowly developing tolerance (subsensitivity) to the inhibitory effects of μ-opioid receptor agonists, α-adrenoceptor agonists and 2-chloroadenosine (Schulz and Goldstein, 1973; Taylor et al., 1988; Leedham et al., 1989; 1991). Furthermore, the subsensitivity to inhibitory agonists is accompanied by supersensitivity to several excitatory agonists, including nicotine, 5-hydroxytryptamine and potassium ions (Schulz and Goldstein, 1973; Johnson et al., 1978). Work from this laboratory has established that such non-specific supersensitivity in other types of excitable cells is correlated with a partial depolarization of the cells, bringing the resting membrane potential closer to the threshold for activation (Fleming and Westfall, 1975; Goto et al., 1978; Abel et al., 1981).

Most of the cells in guinea-pig myenteric ganglia can be classified electrophysiologically either as “S” or “AH” neurons

ABBREVIATION: LM/MP, longitudinal muscle/myenteric plexus; PSS, physiological salt solution; nTS, nucleus tractus solitarius; LC, locus ceruleus; AMP, adenosine monophosphate.
(Nishi and North, 1973, Hirst et al., 1974). The cholinergic motor neurons that innervate the smooth muscle of the intestine are S cells (Furness and Costa, 1987). µ-Opioid-receptor agonists act on a select subpopulation of S neurons that includes the motor neurons (Fleming and Taylor, 1995) inducing hyperpolarization via activation of potassium channels (North and Tonini, 1977; North and Williams, 1983).

A partial depolarization of S neurons, without a change in the threshold for action potentials, coincides with the non-specific sensitivity changes in myenteric neurons from animals chronically exposed to morphine (Leedham et al., 1992). Consistent with the working hypothesis that it is the altered resting membrane potential, rather than changes in receptors or transduction processes, which underlies the tolerance, the magnitude of the acute hyperpolarization induced by morphine in individual S neurons was not different between placebo preparations and preparations from animals receiving morphine pellets. Further corollaries of the hypothesis are that 1) S neurons that are not acutely hyperpolarized by morphine will not become partially depolarized by chronic treatment with morphine, 2) the receptors for the hyperpolarizing effects of 2-chloroadenosine and clonidine are colocalized on the same neurons as the µ-opioid receptors and 3) the hyperpolarizing effects of clonidine and 2-chloroadenosine will not be different between naive and tolerant S neurons. The purpose of our work was to test these corollary hypotheses.

Methods

Implantation of morphine pellets. Adult male albino guinea pigs (Hilltop laboratory Animals, Inc., Scottsdale, PA) weighing 200 to 300g were used. Morphine (75 mg, as free base per pellet) or placebo (lactose) pellets were implanted s.c. in guinea pigs anesthetized with 0.1 ml/100g body weight of Innovar (0.05 mg/ml of fentanyl citrate and 2.5 mg/ml of droperidol) administered s.c. One pellet was implanted in each flank. Note that smaller animals and, thus, fewer pellets, were used than in previous studies (Johnson et al., 1978; Taylor et al., 1988; Leedham et al., 1992). With this method of implantation there is low morbidity and mortality (<1%) and sensitivity to morphine and clonidine (as determined in organ bath experiments similar to those of Taylor et al., 1988) was equivalent to that obtained previously with four pellets in larger animals. Guinea pigs were allowed to recover from the anesthesia and given access to food and water until the time of the experiment (7 days later). Animals implanted with placebo pellets and preparations or cells from these animals are, henceforth, termed “naive” or “placebo.” Animals implanted with morphine pellets and preparations or cells from these animals are termed “tolerant.”

Electrophysiology. Electrophysiological studies of myenteric neurons were done similarly to past studies in this laboratory (Leedham et al., 1992) and others (Cherubini and North, 1984; Johnson et al., 1987). Guinea pigs were killed by stunning and exsanguination 7 days after the implantation of morphine or placebo pellets. The terminal ileum was removed and approximately 10 cm of the distal end discarded. The adjacent 10 cm of ileum were removed and flushed with physiological saline. All tissues were kept free of morphine from the time of removal from the animal until the recording of responses. The continued presence of morphine has been shown to be associated with an acute desensitization phenomenon that complicates the long-lasting nonspecific tolerance (see discussion in Johnson and Fleming, 1989). Small pieces of ileum were pinned, mucosal side up, in the electrophysiologic recording chamber. The mucosa and circular muscle layers were carefully teased away. The exposed myenteric plexus with underlying longitudinal muscle was observed by means of an Olympus inverted microscope equipped with Normarski optics. Tissues were superfused continuously with PSS maintained at 36 ± 1°C, bubbled with 95% O2 and 5% CO2, at a flow rate of 2 to 3 ml/min. The composition of the PSS was as follows (in millimolar concentrations): NaCl, 117; KCl, 4.7; CaCl2, 2.5; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 25; and glucose, 11.5. Nicardipine (1–10 µM) was added to the PSS to reduce or prevent muscle contractions. Drugs were added by changing the perfusate from one without to one with the drug. The equilibration time, consequent to dead space in the superfusion system, was 1 to 2 min. Agonists were washed out promptly once a plateau of response had been achieved.

Procedures for intracellular recording and identification of S and AH neurons were based on those reported previously (Hirst et al., 1974; Cherubini and North, 1984; Johnson et al., 1987 and Leedham et al., 1992). Specifically, S neurons, the cells of importance to this study, were distinguished by the presence of repetitive somatic action potentials without afterhyperpolarizations, elicited by repetitive intracellular current injection and/or the presence of fast excitatory postsynaptic potentials, in response to transmural stimulation. Signals were amplified (Axoclamp IIa, Axon Instruments, Inc., Burlingame, CA) and displayed on a Tektronix model 5111 oscilloscope. A continuous record of the amplifier output was obtained via a digital computer interface board and the Axotape data acquisition software (Axon Instruments, Inc.). Input resistance was calculated from the amplitude of voltage responses (±20 mV) evoked by small hyperpolarizing electrotonic potentials.

Included in the results were cells that met all of the following criteria: 1) the cell was identified as an S neuron; 2) the Erest of the cell remained steady for at least 15 min; 3) the stable resting potential was at least -30 mV; 4) the peak amplitude of the action potential elicited by intracellular current injection surpassed 0 mV and 5) the impalement could be held at least long enough to apply morphine (0.1 µM) and determine the cell’s response, if any.

Nerve-muscle preparations. In parallel with the electrophysiological studies, LM/MP preparations from the same animals were regularly set up in organ bath experiments (for methods see Taylor et al., 1988; Leedham et al., 1989) to confirm the existence of tolerance in these experiments. One end of the muscle-nerve preparation was attached to a force displacement transducer (Grass, FT.03) and the signal led to a Grass polygraph (model 7) for measurement of tension development. An initial tension of 1g was placed on each preparation and a period of 1 hr for equilibration allowed during which the PBS was changed at 10- to 15-min intervals. The preparations were continuously stimulated via platinum ring electrodes using square wave pulses of supra-maximal voltage, 0.5 msec duration, delivered once every 10 sec from a Grass stimulator. The neurogenic contractions maintain a constant amplitude over several hours in the absence of drug treatment. Responses to agonists were measured as percent inhibition of the neurogenic contractions (“twitches”).

Drugs and statistical evaluations. Morphine sulfate, morphine pellets and placebo pellets were obtained from Mr. K. H. Davis, Jr. (Research Triangle Institute, Research Triangle Park, NC). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO). The concentrations of morphine (0.1 µM), clonidine (0.3 µM) and 2-chloroadenosine (0.1 µM) were chosen because, in organ bath experiments with the LM/MP preparation, these concentrations produce 50 to 70% inhibition of twitches in placebo preparations but minimal inhibition in tolerant preparations (Taylor et al., 1988). Statistical evaluations were made using Student’s t tests for unpaired samples and a probability level of 0.05 or less was accepted as significant.

Results

Multiple cells from 37 placebo guinea pigs and 36 guinea pigs implanted with morphine pellets (henceforth, termed
“tolerant” animals) were impaled. Neurons identified as AH were rejected. Morphine (0.1 μM) was applied to 44 S neurons from placebo animals, inducing hyperpolarizations in 23 (52%) of them. The same concentration of morphine was applied to 50 S cells from tolerant animals, inducing hyperpolarizations in 35 (70%) of them. The remaining cells were either unaffected by morphine or slightly depolarized. A few of the hyperpolarizing cells did not achieve stable membrane potentials and were excluded, leaving 18 placebo and 28 tolerant neurons for quantitative determinations.

Mean resting membrane potentials as well as the effects of morphine (0.1 μM) on S cells from those groups are presented in table 1. The cells from the tolerant animals were significantly depolarized by a mean of 7.2 mV (table 1), in comparison with cells from placebo animals. However, the threshold membrane potential for firing action potentials did not differ between S neurons from naive (-21.5 ± 1.9 mV) vs. tolerant preparations (-22.8 ± 1.5 mV). The magnitude of the hyperpolarizations and resistance changes induced by morphine, 0.1 μM were not significantly different between the groups (table 1). In contrast, S cells that did not hyperpolarize in response to morphine did not differ in resting potential between naive and tolerant preparations (48.9 ± 1.4 mV, n = 21 vs. 49.8 ± 1.1 mV, n = 15).

Clonidine (0.3 μM) was superfused during the recording from 8 placebo and 16 tolerant cells from among the cells included in table 1. All of those cells were hyperpolarized by both clonidine and morphine. Figure 1 presents tracings from typical experiments. Table 2 indicates the magnitude of the effects of morphine and clonidine when both were applied sequentally to the same cells. The responses to morphine and clonidine did not differ from each other nor did cells from placebo or tolerant preparations differ in response to either agonist. As expected, the hyperpolarizing effects of morphine and clonidine were accompanied by reductions in input resistance, in this respect also, placebo and tolerant cells did not differ significantly (compare resistance in tables 1 and 2).

2-Chloroadenosine was superfused during the recording from seven cells each from placebo and tolerant preparations from among the cells included in table 1. A comparison of the effects of morphine and 2-chloroadenosine is presented in table 3. The mean hyperpolarizing effects of the drugs did not differ from each other nor did placebo and tolerant preparations differ in the magnitude of the hyperpolarizing response to the two agonists. 2-Chloroadenosine had variable effects on resistance, causing small increases in resistance in some cells and small decreases in others. The mean effects on resistance, therefore, did not differ significantly from zero in either placebo or tolerant preparations.

Parallel organ bath experiments were conducted using LM/MP preparations from ilea from some of the same animals used for the electrophysiological experiments. Figure 2 demonstrates the subsensitivity to morphine and clonidine in preparations from animals receiving morphine pellets. In placebo preparations, morphine, 0.1 μM, and clonidine, 0.3 μM, induced similar degrees of inhibition of the twitch (70–80%). In contrast, in preparations from tolerant guinea pigs, either agonist induced only 10 to 15% inhibition of the twitch. 2-Chloroadenosine was not tested in the LM/MP preparations, since previous work (Taylor et al., 1988) had established that the subsensitivity to it was similar to the subsensitivity to morphine and clonidine.

**Discussion**

Adaptive supersensitivity in smooth muscle has been known for many years to be nonspecific in nature (see review by Fleming et al., 1973). That is, the adaptation process results in supersensitivity to several pharmacologically unrelated agonists. It has been established that nonspecific supersensitivity to excitatory agonists, in several smooth muscles, is the result of a partial depolarization, i.e., a reduction in the resting transmembrane potential difference (Fleming and Westfall, 1975; Abel et al., 1981; Rogers et al., 1993; Hershman et al., 1995).

Consideration of the specificity of adaptive changes in neurons is a relatively neglected area. The first evidence that the adaptation of myenteric neurons to long-term exposure to opioids resulted in nonspecific changes in sensitivity came from the work of Schulz and Goldstein (1973). They demonstrated that the implantation of morphine pellets in guinea pigs resulted in both tolerance (subsensitivity) to the inhibitory action of morphine in LM/MP preparations of the guinea pig ileum and supersensitivity to the stimulatory action of 5-hydroxytryptamine. Subsequent experiments with the LM/MP preparation (Johnson et al., 1978; Taylor et al., 1988) established that morphine pellets led to supersensitivity to three separate stimulatory agonists (5-hydroxytryptamine, nicotine and potassium) and subsensitivity to three separate classes of inhibitory agonists (μ-opioid receptor agonists, α2-adrenoceptor agonists and 2-chloroadenosine). The excitatory substances from that list are all known to depolarize myenteric S neurons whereas μ-opioid and α2-adrenoceptor agonists hyperpolarize those neurons (see review by Fleming and Taylor, 1995). The electrophysiologic effects of 2-chloroadenosine on myenteric neurons has not been investigated previously.

The postganglionic, cholinergic motor neurons to ileal smooth muscle are myenteric S neurons (see reviews by Furness and Costa, 1987; Johnson and Fleming, 1989 and Fleming and Taylor, 1995). Thus electrical or pharmacological stimulation of those neurons leads to contraction of the

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Resting Em (mV)</th>
<th>Resistance (MΩ)</th>
<th>Morphine Effects</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hyperpolariz (mV)</td>
</tr>
<tr>
<td>Placebo</td>
<td>−46.8 ± 0.8</td>
<td>113.1 ± 12.2</td>
<td>6.6 ± 0.5</td>
</tr>
<tr>
<td>Morphine</td>
<td>−39.6 ± 1.5</td>
<td>102.7 ± 8.6</td>
<td>5.8 ± 0.8</td>
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Values are means of 18 placebo cells and 28 cells from morphine-pretreated guinea pigs. * P < .05.
muscle in the LM/MP preparation and hyperpolarization of those neurons provides a plausible basis for inhibition of the electrically induced twitches of the muscle (North and Williams, 1983; North and Egan, 1983).

Given the above information, an hypothesis was developed that the adaptive mechanism involves a partial depolarization of myenteric S neurons such as to reduce the difference between resting potential and the threshold for firing action potentials. That hypothesis was strongly supported by previous work from this laboratory (Leedham et al., 1992). Myenteric S neurons from guinea pigs implanted with morphine pellets were depolarized by 7–9 mV relative to comparable neurons from placebo animals. In the same neurons, the threshold potential at which the neurons fired action potentials was not different in the two groups of preparations.

A corollary to the hypothesis is that the amount of hyperpolarization of individual neurons by an opioid such as morphine would be unchanged in tolerant myenteric ganglia. The

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine</th>
<th>Clonidine</th>
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<tbody>
<tr>
<td>Placebo</td>
<td>6.1 ± 0.7</td>
<td>7.3 ± 1.1</td>
</tr>
<tr>
<td>Morphine</td>
<td>7.5 ± 0.9</td>
<td>6.2 ± 0.9</td>
</tr>
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Values are means of 8 cells from placebo preparations and 16 cells from morphine-pretreated guinea pigs.

TABLE 3
Comparison of effects of morphine (0.1 μM) and 2-chloroadenosine (0.1 μM) in S cells from guinea pigs implanted with placebo or morphine pellets

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Morphine</th>
<th>2-Chloroadenosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>7.7 ± 0.4</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
<td>Morphine</td>
<td>8.1 ± 1.2</td>
<td>7.0 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means of seven cells each from placebo and morphine-pretreated guinea pigs.

Fig. 1. Effects of superfusion of morphine (MOR) and clonidine (CLON) on electrophysiological properties of myenteric S neurons. A, Morphine (0.1 μM) and clonidine (0.3 μM) each led to a membrane hyperpolarization of this S neuron from an animal implanted with placebo pellets. The resting membrane potential of this neuron before drug exposure was -53 mV. B, Clonidine and morphine each led to comparable membrane hyperpolarizations of a myenteric S neuron from an animal implanted with morphine pellets. The resting membrane potential of this neuron before drug exposure was -42 mV. The electrotonic potentials have been provided below the membrane potential tracings at the time during the recording session at which they were generated. The electrotonic potentials reveal that the hyperpolarizations induced by the agonists are associated with a reduction in cellular input resistance. The time scale represents 4 min for the membrane potential tracing and 2 min for the electronic potential insets that are presented.

Fig. 2. Effects of morphine and clonidine on neurogenic contractions of the LM/MP preparation of the guinea pig ileum. Preparations from guinea pigs implanted with either placebo or morphine pellets were exposed to morphine (0.1 μM) and clonidine (0.3 μM) as in the electrophysiological studies. The magnitude of the reduction in neurogenic contraction was calculated as percent inhibition from the amplitude prior to exposure to either agonist. Note that the effect of each agonist is significantly attenuated in preparations from animals pretreated with morphine, indicating the development of tolerance (subsensitivity). *Represents significant differences between placebo and morphine pellet-implanted preparations (P < .05).
tolerance (subsensitivity) would be the result of the lower starting point (resting membrane potential), not an altered response, per se. The experiments of Leedham et al. (1992) established that morphine, 0.1 \( \mu M \), produced similar hyperpolarizations in S neurons from placebo and morphine-pellet implanted guinea pigs. This finding was particularly important because 0.1 \( \mu M \) morphine produced 50 to 70% inhibition of the electrically induced twitch of LM/MP preparations from placebo animals but less than 10% inhibition in preparations from tolerant guinea pigs (Taylor et al., 1988). Thus, the overall hypothesis of these studies is that the ability of morphine, \( \alpha_2 \)-adrenoceptor agonists and 2-chloroadenosine to prevent excitation in morphine-tolerant myenteric neurons is reduced, not because of an altered response of the neurons, but because the preexisting depolarization of the neurons results in a lower total membrane potential (resting potential + agonist-induced hyperpolarization) in the presence of the agonists as compared to naive neurons.

Chavkin and Goldstein (1984) conducted experiments in the LM/MP that led to the conclusion that opioid-induced tolerance was associated with a reduction in \( \mu \)-opioid receptor reserve. There is no conflict between the above hypothesis and the conclusion of Chavkin and Goldstein (1984). There are three types of change in cellular function that could produce the result they obtained. 1) There could be a decrease in the density of receptors. 2) There could be a reduced transduction process after receptor occupation. 3) There could be an altered physiological parameter, such that the drug would need to occupy more receptors and initiate a greater magnitude of transduction to compensate for the altered parameter. Our data are inconsistent with 1) and 2) but completely consistent with 3). Due to the partially depolarized state of the tolerant neurons, the hyperpolarizing agent, e.g., morphine, would need to produce a greater hyperpolarization to achieve similar twitch inhibition as in naive preparations. Simply put, the inhibition is a function of the membrane potential in the presence of the drug (i.e., resting potential plus the hyperpolarizing effect of the drug). To achieve equal twitch inhibition, the inhibitory drug would need to activate a larger portion of available receptors to produce the greater hyperpolarization needed to compensate for the lesser resting potential.

Our experiments were undertaken to extend the findings of Leedham et al. (1992) and directly test the basic hypotheses. Somewhat smaller (younger) guinea pigs were used. Preliminary experiments established that the implantation of two pellets in the smaller animals produced tolerance in the LM/MP equivalent to that produced by four pellets in the larger animals. Routine experiments with morphine dose-response curves in LM/MP from the same idea used for electrophysiology confirmed that the animals receiving morphine pellets were tolerant.

The electrophysiological experiments concentrated on S neurons that were hyperpolarized by morphine. Those include the neurons that directly innervate the smooth muscle. Furthermore, based on the hypothesis, only neurons that are acutely inhibited by morphine should be the ones that are made tolerant by the chronic exposure to morphine. Of S neurons acutely exposed to morphine, 0.1 \( \mu M \), 52% of those in placebo preparations and 70% of those in tolerant preparations were hyperpolarized. The percentages agree well with those of Leedham et al. (1992), who reported 67 and 76%, respectively, and with the literature on control S neurons (see for example, Johnson and Pillai, 1990; Pillai and Johnson, 1991). Of particular importance, the percentages establish that tolerance is not associated with a decline in the fraction of S neurons that respond to morphine. An important new finding is that the resting membrane potential of S neurons, which were not acutely hyperpolarized by morphine, did not differ, in resting membrane potential, between naive and tolerant preparations. This observation is consistent with the hypothesis that only neurons acutely responsive to morphine will exhibit the adaptation associated with tolerance.

The remaining discussion emphasizes results obtained with S neurons shown to be hyperpolarized by morphine. The mean resting membrane potential in S neurons from placebo animals was 46.8 ± 0.8 and in S neurons from tolerant animals was 39.6 ± 1.5 mV, a significant mean depolarization of 7.2 mV. These numbers also compare favorably with those of Leedham et al. (1992); 52.1 ± 1.9, 43.4 ± 1.8 and 8.7 mV, respectively. Thus, the present results confirm the conclusion of Leedham et al. (1992) that tolerance is associated with a partially depolarized state of morphine-sensitive S neurons. The depolarization occurs without detectable changes in the calculated input resistance or the threshold for action potential generation (Leedham et al., 1992; our data). Morphine, 0.1 \( \mu M \), acutely induced a mean hyperpolarization of approximately 6 mV in both placebo and tolerant preparations, indicating that the effect of morphine on individual S neurons is not altered in the tolerant state. The importance of this concentration of morphine rests on the fact that it produced approximately 80% inhibition of the electrically induced twitch of control LM/MP preparations and only about 15% inhibition in tolerant preparations, as shown in figure 2. Again, this finding is nearly identical to that reported by Leedham et al. (1992).

An important characteristic of tolerance in the LM/MP is that the subsensitivity to \( \mu \)-opioid receptor agonists is accompanied by subsensitivity to the inhibitory effects of other agonists, including \( \alpha_2 \)-adrenoceptor agonists and 2-chloroadenosine (Taylor et al., 1988). \( \mu \)-opioid receptors and \( \alpha_2 \) adrenoceptors are known to be colocalized on S neurons and to induce hyperpolarization by increases in potassium conductance, probably via the same population of potassium channels (Surprenant and North, 1985). However, there have been no previous studies of the electrophysiological effects of 2-chloroadenosine on S neurons.

In our work, the colocalization of \( \alpha_2 \) adrenoceptors with \( \mu \) receptors on S neurons is reconfirmed. Clonidine (an \( \alpha_2 \)-adrenoceptor selective agonist), 0.3 \( \mu M \), was superfused during intracellular recording from a total of 24 S neurons which had been shown to hyperpolarize in response to morphine, 0.1 \( \mu M \). All 24 neurons hyperpolarized in response to clonidine by an amount similar to that induced by morphine. Furthermore, both drugs induced decreases in input resistance (i.e., increases in conductance) of similar magnitude. Neither the hyperpolarization nor the decrease in resistance induced by clonidine differed between placebo and tolerant S neurons. The concentrations of the two drugs were chosen on the basis that they produced equivalent inhibition of the electrically induced twitch in naive LM/MP preparations (i.e., from animals implanted with placebo pellets) and minimal
inhibition in preparations from morphine-tolerant prepara-
tions (Taylor et al., 1988; our data).

A total of 14 S neurons that were hyperpolarized by mor-
phine were exposed to 2-chloroadenosine, 0.1 μM. This con-
centration of the adenosine analog also inhibits the twitch approximately 60% in naive and 10% in tolerant LM/MP preparations (Taylor et al., 1988). All 14 neurons were hy-
erpolarized by 2-chloroadenosine to a magnitude equivalent
to that induced by morphine, 0.1 μM. Thus, the adenosine
receptors upon which this agonist acts are extensively co-
localized with mu-opioid receptors and, by inference, with
alpha2 adrenoceptors. The equivalent hyperpolarizations of
the same population of S neurons by morphine, clonidine and
2-chloroadenosine is consistent with the hyperpolarization of
these neurons being responsible for the inhibition of the
electrically-induced twitch in the LM/MP (North and Wil-
liams, 1983; North and Egan, 1983; Johnson and Fleming,
1989; Fleming and Taylor, 1995). Thus, an action of 2-chlo-
roadenosine in the LM/MP occurs at the level of the soma of
S neurons.

In contrast to the effects of morphine and clonidine, the
hyperpolarizations induced by 2-chloroadenosine were not
accompanied by significant changes in input resistance. This
indicates that, whatever is the mechanism by which 2-chlo-
roadenosine causes hyperpolarization of S neurons, it is not
by opening potassium channels. In other words, the trans-
duction process for 2-chloroadenosine is different from that of
morphine and clonidine. This is the first report of the effects
of 2-chloroadenosine on the electrophysiology of S neurons.

Although a hyperpolarization without a measurable decrease
in resistance is an unusual finding, such a dissociation has
been observed in other neurons and potential cellular mech-
anisms discussed (Siggins and Grul, 1986; Bloom, 1988).

When the effects of morphine, clonidine and 2-chloroad-
enosine were compared in tolerant vs. naive preparations,
there were no differences in the amount of hyperpolarization
or the induced changes in input resistance produced by any
one of the agonists. This finding strongly supports the con-
clusion of Leedham et al. (1992) that the tolerance (subsen-
sitivity) of the myenteric plexus to these agonists induced by
the implantation of morphine pellets is not due to changes in
the receptors or transduction processes through which they
act. Three different receptor systems and two clearly differ-
ent transduction processes are involved in the responses to
these three substances. However, subresponsivity of the elec-
trically induced twitch in the LM/MP to each of the three is
not accompanied by altered responses of the individual neu-
rons on which they act.

As already noted, the S neurons on which these agonists have
direct effects are partially depolarized without a change in the
threshold potential for firing action potentials (Leed-
ham et al., 1992, our study). Combined with the evidence that
receptors and transduction processes are not altered, the
hypothesis that the partially depolarized state is responsible
for the tolerant state in the LM/MP induced by implantation
of morphine pellets in the animals, is strongly supported.

The depolarization that accompanies nonspecific adaptive
changes in sensitivity of smooth muscle cells is related to
reduced electrogenic activity and reduced content of subunit
protein of the Na+K+ pump (Gerhoffer et al., 1979; Abel et
al., 1981; Hershman et al., 1993; Rogers et al., 1993; Hersh-
man et al., 1995). An investigation of a possible role of the
Na+, K+ pump in the adaptive depolarization in morphine-
tolerant guinea pig myenteric neurons is underway. (Kong et
al., 1997) Such studies will begin to address the molecular
level at which cellular adaptation occurs in response to
chronic drug treatment and provide insight into the cellular
basis for the development of tolerance.

In parallel studies, our laboratory has been characterizing
tolerance in neurons of the nTS of the guinea-pig as induced
by implantation of morphine pellets (Malanga et al., 1997).
Extracellular recording has established that spontaneous fir-
ing of nTS neurons, in slices from morphine-pretreated
guinea pigs, is subsensitive to inhibition by morphine,
clonidine, 2-chloroadenosine or muscimol and supersensitive
to excitation by potassium. Thus, the pattern of nonspecific
adaptation originally found in the LM/MP is found in the nTS
neurons of the same species.

Work has been underway in other laboratories on opioid
tolerance and dependence in the rat brain, with important
results particularly in the LC and nucleus accumbens. Mi-
croiontophoretic administration of morphine depresses the
firing rate of LC neurons. Opioids, acting on μ-opioid recep-
tors, hyperpolarize LC neurons by activation of a potassium
conductance (North and Williams, 1983; Williams and North,
1984). Agonists such as norepinephrine and clonidine also
hyperpolarize and inhibit LC neurons via coupling to the
same potassium channels as μ-opioid receptors (North and
Williams, 1983; Andrade and Aghajanian, 1985).

In the LC, μ-opioids are coupled separately by G-proteins
to changes in potassium conductance and to cyclic AMP (see
review by Johnson and Fleming, 1989). More recently, Alreja
and Aghajanian (1993) have presented evidence that met-
enkephalin suppresses a cAMP-dependent resting sodium
current in LC neurons. It should be noted that the suppres-
sion of the sodium current was demonstrated with concentra-
tions of met-enkephalin that were 100 to 200 times
greater than the maximum concentration required to in-
cease potassium conductance.

Implantation of morphine pellets in rats readily induces
both tolerance and dependence in LC neurons that can be
demonstrated either in vivo or in slices (Aghajanian, 1978;
Andrade et al., 1993; Kogan et al., 1992). In contrast to the
guinea pig LM/MP and nTS, the tolerance induced is specific
for opioids. That is, it is neither accompanied by subsensitiv-
ity to the α-adrenoceptor agonist, clonidine, nor by supersen-
sitivity to the stimulatory effect of glutamate.

There is evidence of an up-regulated cyclic AMP system in
the rat LC. Thus, chronic administration of opioids induces
increases in the α-subunits of the G-proteins, Gα and Gβ
(Nestler et al., 1989), adenyl cyclase (Duman et al., 1988),
cyclic AMP-dependent protein kinase (Nestler and Tallman,
1988) and several cyclic AMP-dependent phosphoproteins
(Guitart and Nestler, 1989). Nestler (1992) has proposed that
opioid-induced up-regulation of the cAMP system contributes
to opioid tolerance, dependence and withdrawal.

Preliminary work with the guinea pig LC in our laboratory
(Meng et al., 1996) indicates that tolerance in those neurons
is similar in character to the tolerance in the guinea pig
LM/MP and nTS. Thus it appears very likely that there is a
major species difference between the rat and guinea pig
regarding the characteristics and cellular mechanisms of tol-
erance and dependence.
Tolerance to Myenteric S Neurons

1997


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