Cellular Depolarization of Neurons in the Locus Ceruleus Region of the Guinea Pig Associated with the Development of Tolerance to Opioids


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ABSTRACT

These experiments were designed to test two hypotheses: 1) the tolerance induced by morphine pellet implantation in guinea pigs will result in subsensitivity of cells in the locus ceruleus (LC), not only to morphine, but to another agonist acting on a different receptor and transduction system, namely the γ-amino butyric acidA receptor agonist, muscimol; and 2) The non-specific (heterologous) tolerance would be associated with a partial depolarization of the tolerant cells and a decrease in the contribution of electrogenic Na+/K+ pumping. Extracellular recording from LC neurons in brain slices from animals implanted with either morphine or placebo pellets established that the tolerant preparations were subsensitive to both morphine and muscimol. Immunocytochemical analysis identified the α3-subunit as the primary isoform of the Na+/K+ pump in the cells under investigation. Whole-cell patch clamp recording of neurons in brain slices demonstrated that, with electrodes containing 20 mM Na+ (approximating [Na]i), tolerant cells were significantly depolarized by a mean of 6.7 mV. Dialysis with antibody specific for the α3-isofom from patch pipettes produced depolarization of both control and tolerant cells. However, the depolarizing effect of the antibody was less in tolerant cells, suggesting a lesser degree of electrogenic Na+ pumping. Furthermore, the presence of antibody reduced the membrane potentials of tolerant and placebo cells to equal values, suggesting that the diffusion potentials were not different. In contrast, antibody specific for the α1-subunit isoform in the pipettes had no effect on membrane potential in either control or tolerant cells. In conclusion, both hypotheses were supported.

The cellular basis of tolerance to, and dependence upon, opioids has long been a subject of great interest. Tolerance and dependence must be the consequence of changes in the function of the cells upon which the opioid acts (Collier, 1965) as stressed in recent reviews (Koob and Bloom, 1988; Johnson and Fleming, 1989; Nestler, 1992; Nestler et al., 1993; Fleming and Taylor, 1995). The associated sensitivity changes appear firmly established in non-neural tissue and given extensive attention in opioid tolerance and dependence in neurons (see reviews by Johnson and Fleming, 1989; Fleming and Taylor, 1995). The associated sensitivity changes appear and disappear gradually over periods of several days, in contrast to the rapidly developing and disappearing phenomenon of receptor uncoupling. Chronic adaptive supersensitivity/subsensitivity has been linked to alterations in three separate cellular functions: 1) changes in receptor density, 2) changes in membrane potential and the Na+/K+ pump, and 3) changes in the receptor-initiated transduction system, firmly established in non-neural tissue and given extensive attention in opioid tolerance and dependence in neurons (see reviews by Fleming and Westfall, 1988; Johnson and Fleming, 1989; Nestler et al., 1993; Fleming and Taylor, 1995).

Adaptive changes in sensitivity to agonists acting through separate receptors and transduction systems (nonspecific supersensitivity and subsensitivity) have been associated with a partial depolarization of the cell membrane in skeletal muscle (see review by Fleming and Westfall, 1988), smooth muscle of the guinea pig vas deferens (Fleming and Westfall, 1975; Hershman et al., 1995), the rabbit saphenous artery (Abel et al., 1981), canine colon (Rogers et al., 1993), and
neurons of the myenteric plexus (Taylor et al., 1988; Leedham et al., 1992; Kong et al., 1997).

The possibility that tolerance of neurons to opioids might be accompanied by altered sensitivity to nonopioid agonists has been investigated in two series of studies, which have thoroughly examined the issue with quite contrasting outcomes. Implantation of morphine pellets in rats readily induces both tolerance and dependence in locus ceruleus (LC) neurons, which can be demonstrated either in vivo or in slices (Aghajanian, 1978; Andrade et al., 1983). The tolerance induction is specific for opioids; that is, it is not accompanied by either subsensitivity to the α₂-adrenoceptor agonist, clonidine, or by supersensitivity to the stimulatory effect of glutamate. Consequently, the mechanistic studies have concentrated upon μ-receptor interactions or associated transduction processes, particularly the involvement of G proteins and cyclic AMP-dependent protein kinase (Nestler and Tallman, 1988) and supersensitivity to the excitatory effects of nicot ine, 5-hydroxytryptamine, and potassium chloride (Johnson et al., 1978). This nonspecific change in excitability is the result of a partial depolarization of S-neurons in morphine-tolerant myenteric ganglia, with no change in the action potential threshold (Leedham et al., 1992; Kong et al., 1997). Electrophysiological experiments indicated that opioid-receptor interactions or associated transduction processes were unchanged from control (Meng et al., 1997).

The differences in the results between the LC neurons of rat and those of the guinea pig neurons suggest basic differences in the mechanism of the development of tolerance/dependence among populations of neurons in different brain regions, between species, or with differing schedules of opioid treatment. The importance of studies on opioid tolerance in the LC of the guinea pig comes from two issues. First, the results obtained in the rat LC cited above and reviewed by Nestler (1992) show striking differences from opioid tolerance studied in guinea pig neurons from other regions. A direct comparison with guinea pig LC is therefore needed. Second, the LC has been suggested to have a major role in opioid dependence and withdrawal (e.g., see review by Redmond and Krystal, 1984).

**Experimental Procedures**

**Animals.** Male English short hair albino guinea pigs were purchased from Hilltop Laboratory Animals, Inc. (Scottdale, PA). Guinea pigs were kept in the central animal facility for a week before implantation.

**Morphine Implantation.** Animals weighing 240 to 360 g were anesthetized with Telazol (15 mg/kg of body weight) i.p. and implanted subcutaneously with pellets in the flanks (one pellet/100 g of body weight) containing either morphine (75 mg/pellet) or lactose (75 mg/pellet). The animals were allowed to recover and were returned to the animal quarters until the day of the experiment (7 days after implantation).

**Brain Slice Preparation.** On the day of the experiment, animals were anesthetized with pentobarbital (50 mg/kg) and sacrificed via decapitation in accordance with procedures approved by the Animal Care and Use Committee. All efforts were used to minimize animal suffering. The method for preparing brain slices containing the locus ceruleus in the guinea pig was similar to that described by Henderson et al. (1982), Williams et al. (1984), and Leonard and Llinas (1990). The pontine region of the brain stem was rapidly removed from the skull and cut into a 1-cm block. The tissue block was mounted on a Teflon stage with cyanoacrylate glue, and the back was supported by a piece of 3% agar block. Coronal brain slices of 250- to 300-μm thickness were cut using a Campden vibrating tissue slicer (Campden Instruments LTD, Leicester, UK) in 32°C artificial cerebrospinal fluid (aCSF) with the following composition in mM: 126 NaCl, 5.0 KCl, 1.3 MgSO₄, 1.2 NaH₂PO₄, 2.4 CaCl₂, 26 NaHCO₃, and 10 dextrose. The aCSF was saturated with 95% O₂ and 5% CO₂. Slices were maintained in oxygenated aCSF at room temperature for at least 2 h before transferring to the recording chamber. No opioids were used during this equilibration period for reasons extensively discussed in the review by Johnson and Fleming (1989). Extracellular neuronal activity was monitored using single-barreled glass micropipettes filled with 3 M NaCl. Individual action potentials were amplified, filtered (−40 dB/decade at 500 and 10,000 Hz), and monitored on an oscilloscope. Activity from an individual neuron was separated from background noise via a window discriminator, converted to constant-voltage pulses, and led to a ratemeter to obtain a constant record of neuronal activity. In parallel, the signals were led to a digital computer through a Digidata 1200 analog/digital conversion interface where activity was monitored in real time using pCLAMP software (Axon Instruments, Union City, CA) and stored for subsequent analysis and frequency determination.

Neurons were exposed to drugs and neurotransmitters by changing the aCSF bathing the slice from one without the chemical agent to one containing a single concentration of the agent. The time for complete exchange of bathing fluid was approximately 3 min. For construction of cumulative concentration-response curves, a stable level of neuronal activity was achieved before the addition of the next higher concentration. Cumulative concentration-response curves were completed within 30 min.

**Whole-Cell Patch Clamp Experiments.** Brain slices were placed in a recording chamber (RC-22, Warner Instrument Corp., Hamden, CT) and perfused with aCSF saturated with 95% O₂, 5% CO₂. The perfusate was maintained at 32°C during experiments, except for those experiments using Na⁺/K⁺-ATPase α₂- or α₁-subunit antibodies, in which the perfusate was maintained in 37°C. The patch electrode was placed on an individual neuron under direct observation using an Olympus BX50WI microscope equipped with a 40× water immersion objective and an infrared charge-coupled device camera (CCD-300-RR, Dage MTI, Michigan City, IN) coupled to a Sony monitor. The area of recording was limited to the triangular region just below the lateral border of the fourth floor of the ventricle. Patch electrodes were prepared from borosilicate glass tubing (1.2 mm o.d. × 0.6 mm i.d.) with a Flaming/Brown micro pipette puller (P-87, Sutter Instrument Co., San Rafael, CA). The electrode resistance measured between 2 to 5 MΩ when filled with a pipette solution of the following composition, in mM: 145 K gluconate, 3.0 MgATP, 1.1 EGTA, and 1.0 HEPES; pH was adjusted to 7.2 with 6 N KOH. Osmolarity measured between 270 and 290 mosmol/l. In sodium-containing pipette solutions, NaCl replaced K gluconate, in an equimolar amount. Whole-cell patch clamp recordings were obtained using an Axoclamp-2A amplifier (Axon Instruments, Union City, CA) in either the bridge current clamp mode or the voltage clamp mode. In the voltage clamp mode, neurons were clamped at −60 mV. Neuron input resistance was estimated by injecting a 300-ms, 50-pA hyperpolarizing current pulse into the neuron and the deflection of the membrane potential measured. Recording traces were digitized and stored for subsequent analysis. Neurons with a seal resistance less than 5 GΩ or without apparent spontaneous synaptic activity
were discarded from the data pool. Drugs were applied to the bath by switching perfusion from regular aCSF to aCSF containing drug. Na+/K+-ATPase α1- or α3-subunit monoclonal antibody was applied intracellularly by dialysis from a pipette solution containing the antibody in a 1:10 dilution. During these experiences, 3 × 10^-7 M tetrodotoxin was added to the aCSF to prevent firing of action potentials. In the antibody experiments, membrane potential was measured twice in each patch cell. The first time point was immediately after whole-cell patching, before significant dialysis of antibody could occur. The second time point was 60 to 90 min after whole-cell patching, allowing adequate time for the antibody to enter the cell. In experiments in which the neuron was labeled with neurobiotin (0.3%), neurobiotin was added to the pipette solution and the slice was fixed as described below at the end of the experiment.

Tissue Fixation. Tissues were fixed in picric acid-paraformaldehyde fixative buffer (Stefanini et al., 1967) for 1.5 h, rinsed three times in PBSA (0.1 M phosphate-buffered saline, pH 7.8) and then stored in PBSA at 4°C (Dey et al., 1981).

Immunohistochemistry. The procedure used was a modification of a protocol supplied by BD PharMingen, Inc. (San Diego, CA). All incubations and rinses were performed on a rocking platform with gentle rocking. Fixed tissues were placed in a staining net and incubations and rinses were performed on a rocking platform with rinses in PBSB/TX at room temperature, the tissues were transferred to 1.5-ml microcentrifuge tubes containing 600 μl of a solution consisting of PBSB/TX, 0.5% IgG-free bovine serum albumin, 1.7 μg/ml Texas Red streptavidin, and 7.5 μg/ml anti-α,β-subunit antibody. Controls lacking primary antibody were processed simultaneously. Tubes were gently mixed at 4°C for 2 days. Tissues were returned to the staining net and rinsed three times for 15 min each in PBSB/TX at room temperature. The tissues were then placed in 1.5 ml microcentrifuge tubes containing 600 μl of a solution consisting of a 1:200 dilution of fluorescein isothiocyanate-conjugated donkey anti-mouse IgG in donkey serum/PBSB/TX and incubated for 1 h in the dark at room temperature. After a final series of three 15-min rinses in PBSB/TX at room temperature, the tissues were transferred from the staining net to slides and mounted with Fluoromount.

Confocal Microscopy. Images (200×, 8-bit, 1024 × 1024 pixels) were captured using a Zeiss LSM 510 confocal microscope equipped with a Fluar 20×/0.75 ultraviolet objective. A pinhole size of 100 μm was used. Texas Red was illuminated using the 543-nm line of the HeNe1 laser and detected with a 560-nm long-pass filter. Fluorescein was illuminated using the 488-nm line of the argon laser and detected using a 505- to 530-nm band-pass filter. Texas Red was illuminated using the 543-nm line of the HeNe1 laser and detected with a 560-nm long-pass filter. Fluorescein was illuminated using the 488-nm line of the argon laser and detected using a 505- to 530-nm band-pass filter.

Materials. Telazol was obtained from Fort Dodge Animal Health (Fort Dodge, IA). Morphine and lactose pellets and morphine sulfate were obtained from K. H. Davis (Research Triangle Park, NC) through the National Institute on Drug Abuse. Pentobarbital was purchased from Abbott Laboratories (North Chicago, IL). Cyanoacrylate glue (Crazy Glue) was obtained from Elmer Products, Inc. (Columbus, OH). NaCl, NaHCO3, MgCl2, HEPES, K gluconate, ATP, donkey serum, IgG-free bovine serum albumin, [D-Ala2,N-Me-Phe4,Gly-ol3]-enkephalin (DAMGO), and agar were products of Sigma Chemical Co. (St. Louis, MO). KCl, KOH, NaH2PO4, CaCl2, MgSO4, dextrose, EGTA, paraformaldehyde, and Triton X-100 were purchased from Fisher Scientific (Fair Lawn, NJ). Borosilicate glass capillaries were obtained from Frederick Haer & Co. (Bowdoinham, ME). Sylgard 184 was purchased from Dow Corning Co. (Midland, MI). Tetrodotoxin was a product of Calbiochem Co. (La Jolla, CA). The mouse monoclonal anti-Na+/K+-ATPase (α1-subunit) antibody (clone 9A-5) and the mouse monoclonal anti-Na+/K+-ATPase (α3-subunit) antibody (clone XVIF9-G10) were purchased from Affinity Bioreagents, Inc. (Golden, CO). Neurobiotin and Texas Red streptavidin were obtained from Vector Laboratories, Inc. (Burlingame, CA). Picric acid was a product of Ricca Chemical Co. (Arlington, TX). Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG was a product of Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Fluoromount was purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL).

Statistical Analysis. Significant differences between the two different populations of neurons were determined using an analysis of variance followed by the appropriate post hoc test (usually Student’s t test) when required. Differences were considered significant when p < 0.05.

Results

The sensitivity of LC neurons to inhibition by agonists was examined with extracellular recording of the rate of action potential firing in brain slices. Figure 1A presents concentration-response curves for this inhibition by morphine in slices from guinea pigs implanted with placebo pellets (henceforth

Fig. 1. Cumulative concentration-response curves to the inhibitory effect of morphine and muscimol on spontaneous firing in locus ceruleus neurons from slices obtained from animals implanted for 7 days with placebo or morphine pellets. A, responses to morphine from placebo (18 cells from 10 animals) and tolerant (15 cells from 8 animals) preparations. B, responses to muscimol from placebo (15 cells from 10 animals) and tolerant (7 cells from 5 animals) preparations.
referred to as “control” slices) versus slices from guinea pigs implanted with morphine pellets (“tolerant” slices). The figure shows that the concentration-response curve was shifted approximately 10-fold to the right at the level of 40% inhibition in the tolerant preparations.

Figure 1B illustrates the concentration-response curves to the inhibitory effect of muscimol (1–1000 nM) in neurons, recorded extracellularly, from control and tolerant guinea pigs. There was significant subsensitivity at three of the four concentrations in the neurons from the morphine-treated guinea pigs. At the level of 40% inhibition, the subsensitivity to muscimol was approximately 10-fold, although, in contrast to morphine, the curves intersected at the maximum concentration. Thus, the subsensitivity of neurons in the LC region of the guinea pig following chronic morphine treatment is clearly nonspecific.

Basal spontaneous rate was measured in a large number of neurons (74 from control and 44 from tolerant animals). The mean control basal rate was 7.1 ± 0.5 versus 5.4 ± 0.4 Hz in the tolerant group (p < 0.05). Basal rates were measured also in important subgroups. The basal rate of the neurons recorded in Fig. 1A was 4.4 ± 0.5 Hz (control) and 3.9 ± 0.7 Hz in the tolerant group (p > 0.05). The basal rate of the neurons from which the data in Fig. 1B were derived was 7.8 ± 1.2 and 5.4 ± 0.8 in control and tolerant preparations, respectively (p > 0.05).

The whole-cell patch clamp technique was used to determine electrical properties of LC cells in control versus tolerant preparations. Recognizing that the absence of Na⁺ in the pipettes could suppress Na⁺/K⁺ pump activity due to equilibration of Na⁺/K⁺ free solution in the pipette with Na⁺ in the cell, we compared experiments conducted with no Na⁺, a moderate concentration (20 mM), and a high concentration (85 mM) Na⁺ in the pipettes. Table 1 illustrates the resting membrane potential in control versus tolerant cells at each of these pipette concentrations of Na⁺. The difference in membrane potential in 0 Na⁺ was small and not significantly different, consistent with low pump activity. With 20 mM Na⁺, the resting potential was greater in both groups, but the increase was greater in the control group. The tolerant cells, under these conditions, were significantly depolarized by 6.7 mV compared with control. Friedman and Haddad (1994) determined the [Na⁺] in CA1 neurons of the hippocampus to be 25 ± 1.5 mM. Thus, 20 mM Na⁺ in the patch pipette should approximate [Na]. With pipettes containing 85 mM Na⁺, the resting potentials were higher yet, presumably because the pump is highly active, and not different between control and tolerant groups.

The hyperpolarizing current induced by a submaximal concentration of the selective µ-opioid receptor agonist, DAMGO (1 μM), was tested in cells patched with pipettes containing 20 mM Na⁺. Figure 2 presents a tracing showing the hyperpolarizing effect of DAMGO, accompanied by the decrease in resistance typical of a drug which increases ionic conductance. The magnitude of the current induced by DAMGO was not different in tolerant cells (64.3 ± 22.9 pA, 12 cells) from that observed in control cells (47.8 ± 18.0 pA, 5 cells), reinforcing the fact that tolerance is not due to a reduction in the level of current activation induced by the opioid. Additional studies were performed to examine the membrane characteristics of another population of neurons in the guinea pig brainstem (i.e., hypoglossal neurons) that are normally unaffected by acute exposure to opioids. These cells were patched with pipettes containing 20 mM Na⁺. Comparing the resting membrane potential of hypoglossal neurons in slices from controls and animals implanted with morphine pellets revealed no significant difference between the two groups (resting membrane potential = −66.7 ± 0.91 mV (n = 53 neurons in seven control animals) versus −66.9 ± 0.89 mV (n = 58 neurons in six morphine-treated animals), as would be predicted.

A total of 36 LC cells from control preparations and 43 cells from tolerant preparations were patched. Immediately after patching, the neurons from the two groups differed significantly in membrane potential (control = −60.2 ± 1.3 mV, tolerant = −56.4 ± 0.8 mV, p < 0.05). Some of those cells were spontaneously active and some were not. The spontaneously active cells had lower membrane potentials than the quiescent cells (−51.6 ± 0.6 versus −61.2 ± 0.8 mV, p < 0.05). Consistent with this observation, the percentage of spontaneously active neurons from the population of tolerant preparations (42%) was significantly greater than the percentage of control neurons that were spontaneously active (19%, p < 0.05). Regardless of the level of spontaneous activity, 90% of the neurons encountered were acutely hyperpolarized by DAMGO, indicating that these were opioid-responsive neurons. Neurons that did not respond to opioid were excluded from the data analysis.

Immunohistochemical analysis indicated that these neurons, specifically including cells patched and labeled with neurobiotin, contained large endogenous amounts of the α₃- subunit isoform of the Na⁺/K⁺ pump (Fig. 3). Immunohistochemical analysis of the α₁-isoform (the most ubiquitous α-isoform) indicated that this subunit isoform was present in only very low amounts in these neurons (not shown).

Pipettes with 20 mM Na⁺, plus specific antibodies to the α₃-isoform of the Na⁺/K⁺ pump, were used to examine the possibility that the depolarized state of tolerant neurons was a function of differences in electrogenic Na⁺/K⁺ pumping.

Table 2 presents the results of experiments with this anti-

### Table 1

<table>
<thead>
<tr>
<th>Sodium Concentration</th>
<th>Resting Membrane Potential (mV)</th>
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<tr>
<td>0 mM Na⁺</td>
<td>Placebo: −55.5 ± 0.7 (58, 13)</td>
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<tr>
<td></td>
<td>Tolerant: −53.4 ± 0.8 (50, 10)</td>
</tr>
<tr>
<td>20 mM Na⁺</td>
<td>Placebo: −64.2 ± 1.6 (36, 4)</td>
</tr>
<tr>
<td></td>
<td>Tolerant: −57.5 ± 1.1 (45, 6)</td>
</tr>
<tr>
<td>85 mM Na⁺</td>
<td>Placebo: −77.5 ± 1.6 (28, 11)</td>
</tr>
<tr>
<td></td>
<td>Tolerant: −75.4 ± 1.7 (36, 5)</td>
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</table>

* p < 0.05 vs. placebo.

* Concentration of Na⁺ in the intracellular solution in the recording pipettes.
Opioid Tolerance and the Na⁺/K⁺ Pump in Locus Ceruleus

1 μM DAMGO

2 mV 1 min

Fig. 2. Tracing of recording from a whole-cell patched neuron in a control preparation. The vertical axis represents membrane potential. Vertical deflections represent the voltage response to hyperpolarizing current injection (50 pA, 300 ms, 0.17 Hz), which is used to determine membrane resistance. The hyperpolarizing effect of DAMGO is accompanied by a decrease in membrane resistance. Horizontal bar, duration of DAMGO application.

Discussion

Extracellular recording in neurons of the LC region of guinea pig brain established that chronic implantation of morphine pellets induced subsensitivity (tolerance) to the μ-opioid receptor agonist, morphine, and the γ-aminobutyric acidA-receptor agonist, muscimol. Interestingly, the basal spontaneous firing rate of neurons from tolerant preparations was modestly but significantly lower than that of control neurons, which would tend to reduce the observed difference in percentage of inhibition between the tolerant and control groups. Thus, guinea pig LC neurons exhibit nonspecific subsensitivity after morphine pellet implantation, just as do neurons from the myenteric plexus (Taylor et al., 1988) and the nTS (Malanga et al., 1997).

The electrophysiological properties and responses of LC neurons in brain slices were investigated using the whole-cell patch clamp technique (Hamill et al., 1981) with pipettes containing varying concentrations of Na⁺ (0, 20, and 85 mM). Since 20 mM Na⁺ is near the intracellular concentration of neurons (Friedman and Haddad, 1994), one would suspect that intrinsic activity of the Na⁺/K⁺ pump would most closely approximate the normal resting condition in cells recorded with pipettes containing that concentration. Pipettes with 0 Na⁺ should lower [Na⁺]i and suppress pump activity, while diffusion of Na⁺ into the cells from pipettes containing 85 mM Na⁺ would stimulate the pump well above normal. The results were consistent with those assumptions. Resting potentials were lowest with pipettes containing 0 mM Na⁺ and highest with pipettes containing 85 mM Na⁺. Since the pump contributes directly to the resting membrane potential (Thomas, 1972), it is proposed that electrogenic Na⁺/K⁺ pumping is primarily responsible for the observed differences in membrane potential.

The working hypotheses were as follows. First, neurons from the LC region of guinea-pigs implanted 7 days previously with pellets containing morphine (tolerant cells) would be somewhat depolarized relative to control cells. Second, the difference in resting potential would be due to a reduction in the contribution of electrogenic Na⁺/K⁺ pumping in the tolerant cells. Both of these hypotheses have been upheld in previous work in myenteric neurons (Leedham et al., 1992; Kong et al., 1997; Meng et al., 1997). The data reported here support those concepts in the LC region of the guinea pig. As seen in Table 1, with pipettes containing 0 Na⁺ (and, therefore, presumably low pump activity) there was only a small, insignificant difference in resting potential between control and tolerant cells. With pipettes containing 20 mM Na⁺, the statistically significant difference was nearly 7 mV. Resting potential increased as the Na⁺ concentration in the pipette increased in both control and tolerant cells, but the increase...
TABLE 2
Effect of α₁-isofrm antibody on membrane potential of locus ceruleus neurons in brain slices with whole-cell patching

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<thead>
<tr>
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<th>Depolarization Induced by Antibody</th>
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<tr>
<td></td>
<td>Without Antibodyb</td>
<td>With Antibodyc</td>
</tr>
<tr>
<td>Placebo</td>
<td>-63.4 ± 0.9 (18, 10)</td>
<td>-49.7 ± 1.4</td>
</tr>
<tr>
<td>Tolerant</td>
<td>-57.7 ± 2.3 (14, 8)*</td>
<td>-47.9 ± 2.1</td>
</tr>
<tr>
<td>Difference</td>
<td>5.7</td>
<td>1.8</td>
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*p < 0.05 vs. placebo.
*b Patch pipettes contained 20 mM Na⁺.
*c Recorded immediately after whole-cell patching.

TABLE 3
Effect of α₁-isofrm antibody on membrane potential of locus ceruleus neurons in brain slices with whole-cell patching

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| Placebo              | -63.4 ± 1.3 (16, 7)         | -61.5 ± 1.7                       | 1.9 ± 1.5
| Tolerant             | -56.9 ± 2.3 (17, 6)*        | -53.0 ± 2.9*                      | 3.9 ± 2.8
| Difference           | 6.5                         | 8.5                               | N.S.       |

*p < 0.05 vs. placebo.
*b Patch pipettes contained 20 mM Na⁺.
*c Recorded immediately after whole-cell patching.

The sodium pump is composed of two subunits, α and β, believed to form oligomers in a 1:1 M ratio (Lingrel et al., 1990). The α-subunit contains the ATP binding, phosphorylation, ouabain binding, and Na⁺ and K⁺ binding sites (Sweedner, 1989; Lingrel et al., 1990; Blanco and Mercer, 1998). The four known isoforms of the α-subunit are differentially distributed in tissues, have molecular weights of slightly greater than 100,000, are products of separate genes, and can be resolved and identified using electrophoresis and subunit-specific antibodies (Sweedner, 1989; Lingrel et al., 1990; Blanco and Mercer, 1998; Woo et al., 1999). The α₁-subunit isoform appears to be found predominately, and only, in neurons (Hieber et al., 1991; McGrail et al., 1991; Blanco and Mercer, 1998) while the α₁-subunit isoform is more ubiquitously distributed (Blanco and Mercer, 1998). In the current work, immunohistochemistry confirmed that the neurons in the LC region have large quantities of the α₁- but low amounts of the α₁-subunit isoform. Patched neurons, marked by injection of neurobiotin, expressed endogenous α₁,- but not α₁-subunit isoform.

Inhibiting the pump with an antibody selective for the α₁-subunit isoform protein (Table 2) reduced the resting potential in control and tolerant cells patched with pipettes containing 20 mM Na⁺. However, the depolarizing effect of the antibody was significantly greater in the controls, consistent with those cells having greater pump activity. Furthermore, the membrane potentials were nearly identical in the control and tolerant cells in the presence of the antibody, suggesting little, if any, difference in the diffusion potential. In contrast, cells exposed to antibody to the α₁-isofrm in the pipette showed no significant effect on membrane potential. Thus, the electrophysiological data are consistent with the immunohistochemical results and with the hypothesis that the Na⁻/K⁺ pump contributes less to the membrane potential of neurons in the LC region of tolerant versus control preparations.

The tolerance in guinea pig myenteric S neurons that develops from our implantation schedule is proposed to be due to the depolarized state of the neurons, not from alterations in receptors or transduction processes, because the hyperpolarizing effects of morphine and other inhibitory agonists on individual myenteric S neurons were not different between control and tolerant preparations (Meng et al., 1997). That finding is paralleled by results in neurons of the LC region reported here. The current induced by the µ-opioid agonist, DAMGO, was not significantly different between control and tolerant neurons. We conclude that in neurons in the LC region, just as in myenteric neurons, µ-receptor function and its signal transduction processes are not impaired by the implantation schedule used; rather, the set point for firing action potentials (resting membrane potential) is altered in these neurons.

Clear evidence exists that more than one mechanism of tolerance to opioids can exist in the same neuronal population [see reviews by Johnson and Fleming (1989) and Fleming and Taylor (1995)]. Two mechanisms have been demonstrated extensively in guinea pig myenteric neurons. One form is associated with very high exposure to agonist, has a rapid onset and offset, is specific for opioids, and has the characteristics of uncoupling of the µ-opioid receptor from its transduction process (Johnson and Fleming, 1989). A second form is associated with more moderate, long-term (days) exposure to opioid agonists, has a slow onset and offset, extends to nonopioid neuronal inhibitors, is associated with supersensitivity to excitatory agonists, and in myenteric neurons, is a function of a partial depolarization of the neuronal membrane, secondary to reduced electrogenic Na⁺/K⁺ pump-
ing (Johnson et al., 1978; Taylor et al., 1988; Leedham et al., 1989, 1992; Kong et al., 1997).

The nonspecific, membrane depolarization-associated form of tolerance was induced by a one-time subcutaneous implantation of one morphine pellet/100 g of body weight. Other researchers, using daily implantations of morphine pellets for 4 to 5 days, have reported tolerance in rat LC that is specific for opioids (Aghajanian, 1978; Andrade et al., 1988; Kogan et al., 1992). Those results have been associated with alterations in μ-opioid receptor transduction processes, specifically in G proteins and cyclic AMP-dependent protein kinase (Nestler and Tallman, 1988; Guitart and Nestler, 1989; Nestler et al., 1989). Those results led Nestler (1992) to suggest that the compensatory response of rat LC neurons to chronic administration of opioids involves up-regulation of the cyclic AMP system.

Recent work of Gintzler and associates (Wang and Gintzler, 1997; Chakrabarti et al., 1998) has demonstrated similar biochemical changes in guinea pig ileum (presumably in the myenteric neurons, although tolerance was not directly determined). Thus, guinea pig myenteric neurons may be capable of demonstrating all three types of adaptation following chronic exposure to opioids. Attempts to identify and separate these three mechanisms in other tissues have not been published.

The tolerance induced in three sets of guinea pig neurons (myenteric, Leedham et al., 1992; nTS, Malangta, 1997; LC region, the present data) clearly develops a nonspecific characteristic in response to a one-time implantation of pellets. In two of those sets of neurons, myenteric neurons (Kong et al., 1997) and the LC region (present results), evidence implicates the Na+/K+ pump and not the receptor-transduction system.

The membrane depolarization process occurs several days after a single implantation of morphine pellets. In contrast, the tolerance in the rat LC was induced with repeated implantation of morphine pellets, which would establish and maintain higher blood levels of morphine. It is tempting to speculate that the changes in the G protein and cyclic AMP system are slow adaptations to the uncoupling of the μ-opioid receptor, which occurs in response to higher levels of opioid agonist. In contrast, the depolarization form of adaptation follows long-term activation of the receptor-transduction processes.

In conclusion, the present work provides the first evidence that tolerance in central neurons can occur through a partial depolarization. Furthermore, as in myenteric neurons, a decrease in electrogenic activity of the Na+/K+ pump causes this depolarization.

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References


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