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Cellular Mechanisms of Opioid Tolerance: Studies in Single Brain Neurons

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SUMMARY

Intracellular recordings of membrane potassium current were made from rat locus coeruleus *in vitro*. The effects of agonists at μ-opioid receptors were studied on neurons from rats that had been chronically treated with morphine; these were compared with actions on neurons from control rats. Tolerance to the opioid-induced increase in potassium conductance was observed, and this was more pronounced for normorphine than for [Met^5]enkephalin and [D-Ala^2, Mephe^4, Gly^5-ol]enkephalin: experiments with the irreversible receptor blocker β-chlornaltrexamine indicated that normophine had lower intrinsic efficacy than [Met^5] enkephalin and [D-Ala^2 MePhe^4, Gly^5-ol]enkephalin. This adaptation was not due to any change of the properties of the potassium conductance activated by μ-receptors because both full and partial agonists at α_2-adrenoceptors, which couple to the same potassium conductance, were unchanged in their effectiveness; nor was it associated with any change in the affinity of μ-receptors for the antagonist naloxone. Naloxone had no effect on the neurons other than simple competitive reversal of the action of the μ-receptor agonists. These results demonstrate that 1) the mechanism responsible for tolerance in locus coeruleus neurons is specifically associated with μ-receptors and/or their coupling to potassium channels, 2) the intrinsic efficacy of an opioid determines the degree of tolerance observed, and 3) tolerance and physical dependence can be dissociated at the cellular level.

Tolerance and physical dependence arising from chronic administration of opioids have been well documented in various experimental models. However, little progress has been made in identifying the cellular mechanisms underlying these phenomena. A basic requirement for identifying these mechanisms, which until recently has been lacking, is an understanding of the acute mechanisms of opioid action on single nerve cells. Thus, studies of tolerance and physical dependence have often been confounded by the measurement of biological effects that were removed from the primary site of opioid action because of the complexity of the tissue. Furthermore, quantification of opioid tolerance has often been complicated by the presence of multiple opioid receptor types in the tissue studied, by the sustained presence of opioids in assay systems, and by an inability to study tolerance in isolation from the associated signs of physical dependence that often occurs in multicellular preparations, when the agonist is removed.

The LC is a dense cluster of norepinephrine-containing cell bodies in the rat pons, with projections to diverse areas of the central nervous system (1). Opioid inhibition of LC neuronal firing is due to a membrane hyperpolarization that results from activation of an inwardly rectifying potassium conductance (2–4). Studies with agonists selective for μ-, δ-, and κ-receptor subtypes, together with determinations of μ- and δ-antagonist dissociation equilibrium constants (K_e), have demonstrated that LC neurons express only the μ-receptor subtype (4, 5). A guanine nucleotide binding protein (G protein) is involved in the coupling of μ-receptors to potassium channels. Thus, purified μ-receptors are associated with a G protein (6); pretreatment of animals with *Bordetella pertussis* toxin prevents the hyperpolarizing action of opioids on LC neurons (7), and intracellular application of a nonhydrolyzable derivative of guanosine triphosphate progressively reduces the opioid action (4). However, there is no evidence to implicate changes in intracellular levels of cyclic adenosine 3′,5′-monophosphate in the mediation of the opioid actions (3, 4). α_2-Adrenoceptor agonists also hyperpolarize LC neurons, and although the α_2-receptor can be fully distinguished from the μ-receptor with appropriate antagonists, both receptors converge on the same potassium conductance via a pertussis toxin sensitive G protein (3, 4).

This understanding of the mechanisms of acute opioid actions on LC neurons provided the starting point to localize the changes underlying tolerance to opioids. In the present experiments, the properties of μ-receptors, the potassium conduct-

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ABBREVIATIONS: CNA, β-chlornaltrexamine; DAGO, Tyr-D-Ala-Gly-MePhe-Gly-ol; LC, locus coeruleus; UK14304, 5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline.

ance to which they are coupled, and the coupling process itself were investigated *in vitro* in neurons removed from rats that had been chronically exposed to morphine.

Materials and Methods

Male Sprague-Dawley rats (160–180 g) were implanted subcutaneously with pellets that contained 75 mg morphine (base) under light ether anesthesia on alternate days for 7 days (1, 3, 5 , then 7 pellets), and brain slices were prepared 2–4 days later. Implantation of placebo pellets in several experiments resulted in responses determined electrophysiologically that were indistinguishable from those observed in tissue taken from naive animals. Alternatively, animals were treated with 20 mg/kg (base) morphine sulfate; two pellets were implanted on the following day, and slices were prepared on the next day.

Radioimmunoassay (kindly performed by Dr. M. A. Moskowitz) was performed with Abuscreen (Roche) using the method of Edwards *et al.* (8) to remove conjugated metabolites. Morphine recovery (corrected for extraction efficiency) from brain (minus pons/cerebellum) was 1.6 ± 0.3 μg/g fresh weight (n = 8). Assuming a uniform and similar distribution within the pons this corresponds to approximately 6 μM morphine in the slice on removal from the animal.

Slices of rat brainstem containing the LC were cut in a Vibratome (slice thickness 300 μm), transferred to a tissue bath (0.5 ml), and superfused with physiological saline at 37°C (1.5 ml/min). The physiological saline solution contained (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 11 glucose, and 24 $NaHCO_3$ and was gassed at 37°C with 95% O_2-5% CO_2. Drugs were applied to the slice by changing the solution to one that differed only in its content of the drug. Intracellular recordings were made with microelectrodes (30–60 MΩ, filled with 2 M KCl) using a single electrode voltage-clamp amplifier (Axoclamp II). Recordings of membrane voltage and current were plotted directly on chart recorder paper. Voltage-clamp experiments were conducted while continuously monitoring the potential at the headstage with a separate oscilloscope to ensure that the switch-clamp was set correctly. A more complete description of the methods used for this study has been published elsewhere (3). All data are presented as means ± standard error of mean. Student's two-tailed t test was used for statistical comparisons.

Drugs used were Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGO, Cambridge Research Biochemicals), kelatorphan (gift of B. Roques), β-chlornaltrexamine (Research Biochemicals), clonidine hydrochloride (Boehringer Ingelheim), cocaine hydrochloride, [Met^5]enkephalin, norepinephrine (Sigma), morphine pellets, normorphine sulfamate (National Institute on Drug Abuse), naloxone hydrochloride (Endo), prazosin hydrochloride, and UK14304 (Pfizer).

Results

Tolerance to opioid agonists. Outward currents induced by DAGO and normorphine in control and treated neurons are shown in Fig. 1, and the concentration-response relationships are plotted in Fig. 2 (*left*). After chronic treatment, the maximum outward current produced by DAGO (or by [Met]enkephalin) was unaffected (control 285 ± 19 pA, n = 38: treated 241 ± 25 pA, n = 27: both at −60 mV, t = 1.5, $p > 0.1$). However, the concentration-response curve was shifted right, giving a 2-fold tolerance. In contrast, the response evoked by a supramaximal concentration of normorphine in treated neurons was only about 60% of that which could be evoked in cells from control rats. Thus the degree of tolerance to normorphine ranged from about 6-fold at the foot of the concentration-response curve to infinite at 60% of the maximum response.

The degree of tolerance to normorphine was related to the duration of morphine treatment *in vivo*. After treatment with morphine for 2 days, tolerance to normorphine was less pronounced; the concentration-response curve was shifted to the right less than 2-fold, and the maximum response was reduced to 75 ± 4% (n = 7, t = 6.25, $p < 0.001$) of that which could be produced by DAGO.

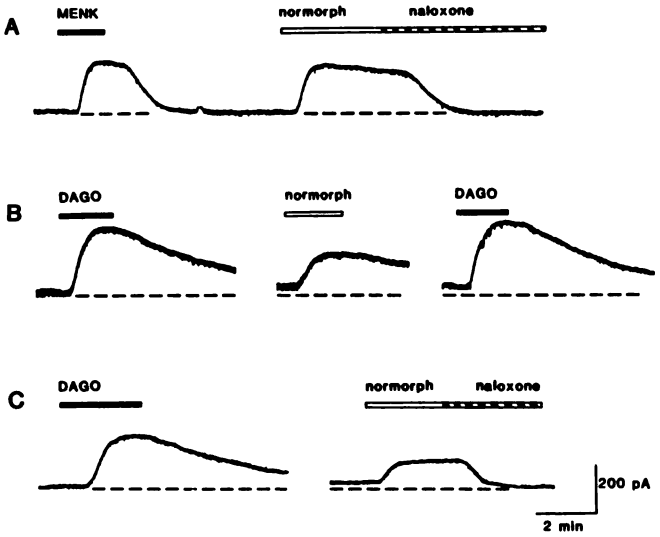

Fig. 1. Examples of membrane currents induced by opioids in locus coeruleus neurons from rats treated with morphine. A: single neuron from a control animal; supramaximal concentrations of [Met^5]enkephalin (MENK) and normorphine produced a similar outward current, and naloxone produced a simple reversal of the effect of normorphine. B: neuron from a morphine-treated animal; DAGO produced an outward current similar to that seen in control, as did [Met^5]enkephalin (not shown). However, the outward current produced by a supramaximal concentration of normorphine was only 60% of that produced by DAGO. After washout of the normorphine effect the DAGO was reapplied. C: neuron from a morphine-treated animal; response to supramaximal normorphine was 52% of that produced by DAGO and was completely reversed by naloxone (1 μM). *Bars*, periods of drug application; response delays were due to time for exchange for solutions before entering the tissue chamber. Holding potential of single-electrode voltage clamp was −60 mV throughout.

The observed tolerance to opioids was not complicated by the occurrence of physical dependence (see below) or the presence of residual morphine in brain slices. Preincubation of control slices with morphine in a concentration similar to that estimated to be present in slices prepared from treated animals (9) had no effect on subsequent responses to normorphine, i.e. the acute effect of morphine (10 μM, 1 hr preincubation) reversed fully within 1 hr of washout from control brain slices, and no reduction was observed in the responses to subsequent applications of normorphine (96 ± 3% of maximum current, n = 6) during the next 5 hr. Furthermore, the sustained reductions in the response to normorphine presented in Fig. 2 were observed for up to 6 hr after removal of tissue from treated animals; this was the maximum duration of these experiments.

Effect of reducing receptor reserve. Brain slices from either control or morphine-treated rats were exposed to CNA (30 nM) for 30 min, and the membrane currents induced by various concentrations of DAGO or normorphine were then measured. Results are illustrated in Fig. 3 and summarized in Fig. 2 (*right*). In tissues from rats not treated with morphine, outward currents caused by normorphine were reduced to a greater extent than those produced by DAGO, implying that normorphine has lower intrinsic efficacy than DAGO μ-receptors. Conversely, in tissues taken from morphine-treated rats,

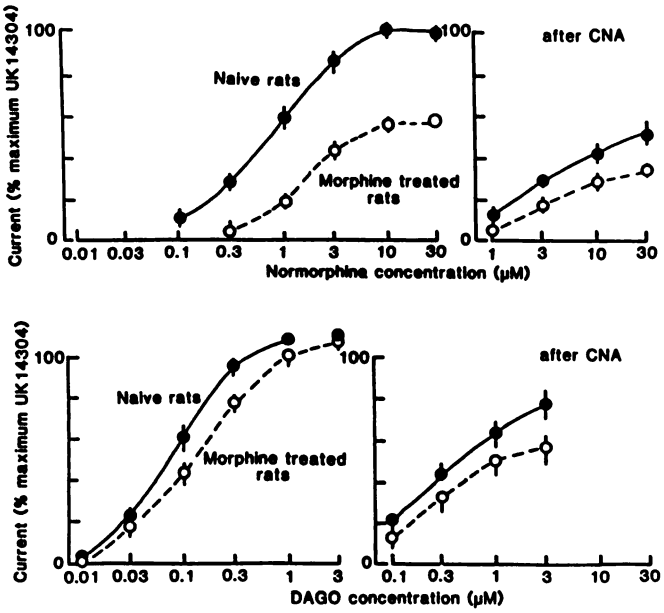

Fig. 2. Effects of agonists normorphine and DAGO on neurons from naive rats (*solid circles*) and rats treated chronically with morphine (*open circles*). *Points*, mean effect observed in *n* neurons; *vertical bar*, standard error that exceeded symbol size; *n* ranged from 2 to 12 (mean 6.1, standard deviation 2.5) for a total of 279 applications of drugs to neurons. Several concentrations were applied to most neurons. The *ordinate* is normalized with respect to the maximum current evoked by norepinephrine or UK14304 in the same cell (for *left panels*) and with respect to maximum current evoked by norepinephrine or UK14304 (for *right panels*). Maximum current evoked by DAGO, [Met^5]enkephalin, norepinephrine [30 μM in cocaine (10 μM) and prazosin (300 nM)], and UK14304 (1 μM) was not different in neurons not exposed to CNA and not different from maximum currents evoked by norepinephrine and UK14304 in neurons after CNA treatment. *Left panels*, responses in cells not exposed to CNA. Concentrations causing half-maximal effects (EC_{50}): DAGO, cells from naive rats: 91 ± 17 nM (n = 8); DAGO, cells from morphine-treated rats: 201 ± 48 nM (n = 11), $p < 0.05$; normorphine, cells from naive rats: 0.91 ± 0.22 μM (n = 8); and normorphine, cells from morphine-treated rats: 1.9 ± 0.17 μM (n = 7), $p < 0.01$. *Right panels*, responses in cells treated for 30 min with 30 nM CNA before testing the effect of agonists (see Materials and Methods). In naive rats, CNA pretreatment caused a greater depression of the effects of normorphine than of DAGO. Normorphine response was reduced to 53 ± 7% of the α_2-agonist response; DAGO response was reduced to 78 ± 7% of the maximal α_2-agonist response. This difference (53 ± 7% versus 78 ± 7%) indicates that morphine is a less full agonist than DAGO ($p < 0.05$). CNA shifted the concentration-response curves by approximately the same degree whether the cells were from naive or morphine-treated rats.

the outward currents induced by both DAGO and normorphine were both reduced by CNA treatment.

Effects of α_2-adrenoceptor agonists. It has been shown that agonists at α_2-adrenoceptors and agonists at μ-opioid receptors on the LC neurons increase the same potassium conductance (3). The outward currents induced by supramaximal concentrations of the α_2-adrenoceptor agonists UK14304 and noradrenaline were similar to those induced by DAGO, and these were not different between neurons from normal rats and neurons from rats that had been treated with morphine (t = 0.9, 23 df, $p > 0.2$). In control neurons, the maximum current evoked by clonidine was consistently lower than that caused by UK14304 or noradrenaline (Fig. 4), consistent with the lower intrinsic efficacy of clonidine reported in other tissues (10–12). However, the maximum current evoked by clonidine was not different between neurons from untreated rats and neurons from morphine-treated rats (t = 0.45, 11 df, $p > 0.5$) (Fig. 4).

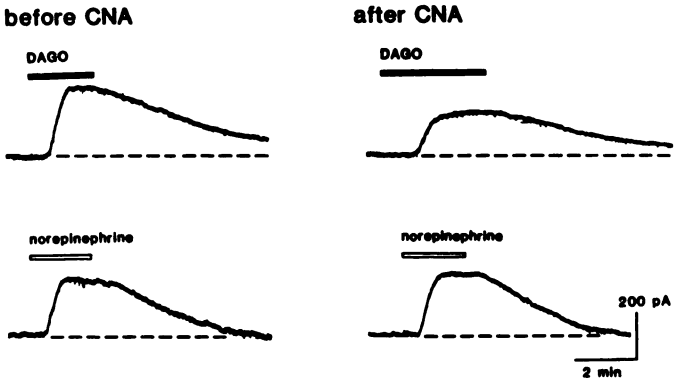

Fig. 3. Effects of pretreatment with CNA. Neuron from a control animal; before application of CNA supramaximal concentrations of DAGO and norepinephrine produced similar outward currents. After CNA treatment the response to DAGO was diminished to 65% of control; the norepinephrine response was unaffected, demonstrating that the potassium conductance could still be fully activated.

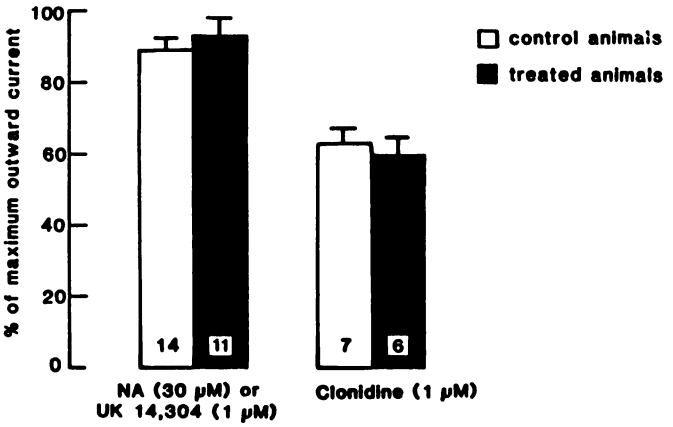

Fig. 4. The ability of α_2-adrenoceptor agonists to increase potassium conductance of LC neurons was unaffected by chronic morphine treatment. Responses to supramaximal concentrations of full agonists norepinephrine (30 μM, in cocaine and prazosin, see Fig. 2) or UK14304 (1 μM) were similar to those produced by DAGO and were unaffected by chronic treatment. Responses to supramaximal concentrations of clonidine (1 μM) did not differ between naive rats and rats treated with morphine but were less than the maximum possible current, demonstrating that clonidine is a partial agonist.

Membrane properties are unchanged by morphine treatment. The basic membrane properties of the cells taken from rats treated with morphine were not noticeably different from those of normal cells, at least during the first 6 hr after preparation of the slice. Since the solution used to superfuse the slice did not contain an opioid agonist, this indicates the lack of any obvious signs resulting from withdrawal of morphine. Spontaneous action potentials had amplitudes close to 80 mV, arose from a threshold potential of −55 mV, and the mean firing frequency was not obviously different between control tissue and morphine-treated rats. Similar findings were made when action potential frequency was measured *in vivo* (12) or with extracellular recording *in vitro* (13). Steady-state conductances determined by measuring currents resulting from a slow ramp voltage command (Fig. 5) were (at −60 mV to −90 mV) 8.3 ± 0.5 nS (n = 38) for control cells and 7.6 ± 0.5 nS (n = 27) for cells from treated rats. The additional conductance produced by DAGO was not different between the groups of rats. In 38 neurons from control rats DAGO caused a maximal

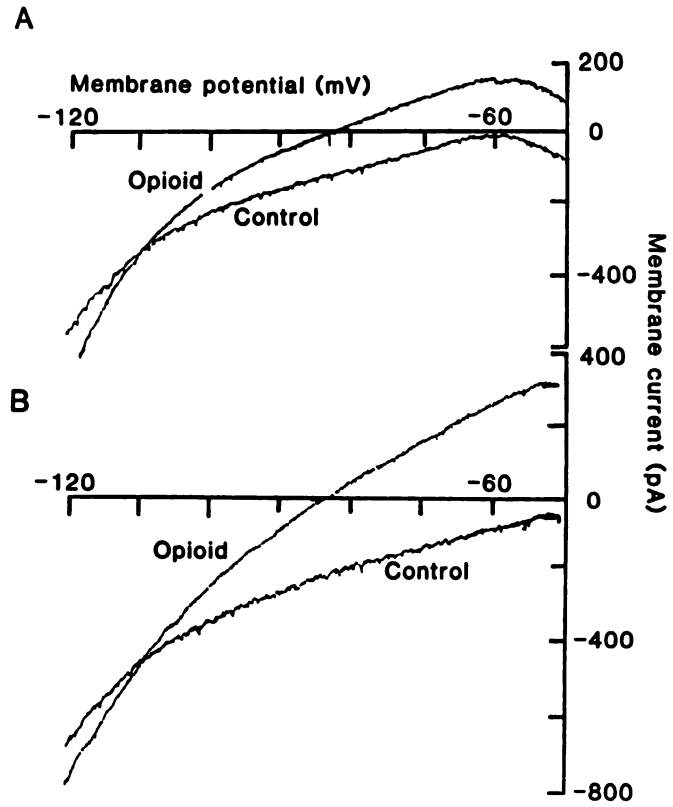

Fig. 5. Chronic treatment of rats with morphine does not change the passive properties of LC neurons. A and B are I/V relationships constructed by commanding the membrane with a slow voltage ramp. A, control cell. B, cell from morphine-treated rat. In both A and B, one record is the control I/V and the other is the I/V relation in the presence of [Met^5]enkephalin (10 μM) and kelatorphan (20 μM).

conductance increase of 3.4 ± 0.3 nS; the value was 2.8 ± 0.2 nS for 27 neurons from morphine-treated rats ($p > 0.15$).

Naloxone causes no signs of dependence. In the absence of opioid agonists in the superfusing solution, the antagonist naloxone (1 μM) had no effect on membrane current, or the frequency of spontaneous action potentials, of LC neurons from either control or morphine-treated animals. In the presence of an opioid agonist, naloxone (10 nM–1 μM) simply reversed the hyperpolarization, or outward membrane current (Fig. 1), whether the neuron had been taken from control or tolerant animals. Even when morphine (3 μM) was applied to the neuron for as long as 4 h, addition of naloxone only restored the control membrane potential and conductance. To quantify further the effects of naloxone in tolerant neurons, concentration-response relationships for DAGO or [Met^5]enkephalin were constructed in the presence of increasing concentrations of naloxone (one to four concentrations from 10 to 300 nM while recording from a single neuron; see Ref. 5). The PA_2 for naloxone determined by Schild analysis (15) or the method of Kosterlitz and Watt (16), and therefore the dissociation equilibrium constant (K_e), was similar in both groups [K_e versus [Met^5]enkephalin was 2.1 ± 0.5 nM ($n = 7$) in control rats and 2.4 ± 0.4 nM ($n = 4$) in treated rats: K_e versus DAGO was 1.7 ± 0.4 ($n = 3$) in control rats and 2.1 ± 0.2 ($n = 4$) in treated rats] (Fig. 6).

Discussion

The treatment regime used in the present experiments is known to cause both tolerance and dependence in various

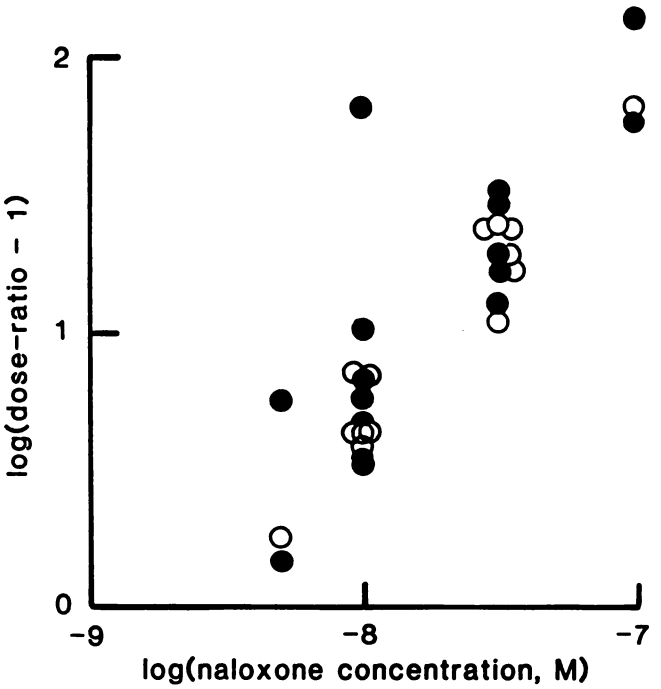

Fig. 6. Affinity of naloxone for μ-receptors is not changed by chronic morphine treatment. Dose ratios were measured from dose-response curves for [Met^5]enkephalin or DAGO before and after adding naloxone. Graph shows Schild plot of the results. *Solid circles*, control animals (K_e = 2.0 ± 0.4 nM, 10 cells); *open circles*, morphine-treated animals (K_e = 2.3 ± 0.2 nM, 8 cells).

experimental tests (9, 17). Thus the finding that naloxone was without significant effect on the properties of individual LC neurons indicates that tolerance is removed from dependence at the level of these individual cells. This implies that the response of the intact nervous system to withdrawal of morphine or application of naloxone requires the amplification provided by synaptic connections between neurons and does not simply result from the addition of serial effects on individual nerve cells. A similar dissociation of tolerance from dependence has been described in isolated tissues such as the mouse vas deferens (which has no network of interconnected neurons); this contrasts to the situation in the guinea pig isolated ileum that shows both tolerance and a striking sign of dependence (guinea pig ileum has an extensive neuronal network within its wall) (see Ref. 18 for review). An earlier report (13) that LC neurons in morphine-treated rats exhibit a withdrawal excitation when naloxone is applied should be interpreted with some skepticism; the excitation was rather small, probably no more than simply reversal of the effects of the circulating morphine, and might also have resulted from changes in afferent input to the nucleus rather than any direct action on the neurons of the LC. Extracellular recording in *in vitro* from LC neurons indicated that the cells were tolerant to the action of morphine and that naloxone did not excite them (14). The lack of any sign of dependence has an important practical consequence—it makes possible a more detailed study of the mechanisms underlying the tolerance that was observed, since the properties of the neurons and the responses to opioids remain essentially constant during 6 hr after removal from the morphine-loaded environment within of the rat brain.

The next finding worthy of discussion is that the degree of tolerance differs between normorphine and DAGO (or [Met]

enkephalin). The experiments with CNA in neurons from control rats indicate that normorphine acts on LC neurons with a lesser receptor reserve than DAGO. Morphine has also been shown to have lower intrinsic efficacy than many other agonists at μ-receptors in some peripheral tissues (9). The simplest interpretation of the findings in the morphine-treated rats is that a fraction of receptors have been lost (or uncoupled from the effector). This would provide for the findings that the maximum response evoked by normorphine was depressed, whereas the maximal effects of DAGO or [Met]enkephalin were unaffected. Analogous findings have been made by Chavkin and Goldstein (20) and Porecca and Burks (21), both of whom suggested that the degree of tolerance observed for the action of morphine was inversely related to the receptor reserve associated with its action under normal circumstances. The present findings extend those studies from peripheral tissues to the central nervous system and from multicellular preparations to single nerve cells; they also allow further inferences about the mechanism of the changes underlying tolerance at the molecular levels.

The findings should be interpreted in the perspective of what is known about the mechanism of the acute action of opioids on LC neurons. It is known that an inwardly rectifying potassium conductance increases as a result of opioids binding to μ-receptors, that agonists at α_2-adrenoceptors increase the same conductance, and that a G protein is involved in the transduction of these responses (4). In view of the failure of efforts to implicate cyclic adenosine 3′,5′-monophosphate and diacylglycerol in the transduction (3, 4), it is hypothesized that the receptors couple more directly with the potassium channel, perhaps by diffusion of G protein subunits within the membrane such as is known to occur in the case of the inwardly rectifying potassium conductance coupled to muscarinic receptors in cardiac tissue (22). In this context it can be said that the present results provide no evidence that the potassium channels themselves are changed as a result of chronic morphine treatment; the maximum conductance increased caused by DAGO and by noradrenaline was not different between the cells from control animals and cells from morphine-treated animals.

Because no change was found in the ability of the α_2-adrenoceptor agonists to increase the potassium conductance, even for the partial agonist clonidine, it may be concluded that morphine treatment does not cause any changes in the steps that are common to μ-opioid and α_2-adrenoceptor agonists. If the same G protein couples both receptors to the potassium channel (and there is no evidence on this except that responses to either agonist are blocked by pretreatment of rats with *Pertussis* toxin), then chronic morphine treatment might reduce the number of opioid receptors in the membrane, might reduce their affinity for agonists, or might impair their coupling to the G protein.

Whereas it might be thought that these two possibilities could be distinguished by analysis of the type introduced by Stephenson (23) and Furchgott (24), this is not so. Even for the simplest sequential model,

$$A + R \overset{K_1}{\rightleftarrows} AR \overset{K_2}{\rightleftarrows} AR^* \rightarrow E_a$$
$$\text{and } [R] + [AR] + [AR^*] = [R_t]$$

where $[AR^*]$ is the concentration of the transformed receptor that determines the size of the effect E_a, it is not possible to distinguish the affinity for the binding of the agonist (K_1) and the intrinsic efficacy of the agonist ($\epsilon = K_2$). (K_1 and K_2 are affinity constants, inverse of dissociation equilibrium constants). The usual method of analysis in which a fraction $(1 - q)$ of the receptors is irreversibly alkylated provides an estimate of agonist affinity that is K_1 ($K_2 + 1$) rather than K_1. In other words, changes in K_1 and in K_2 (or intrinsic efficacy) cannot be distinguished by these methods. (Ligand binding studies using agonists in circumstances in which the transition $AR \rightleftarrows AR^*$ can occur will also provide an estimate of K_1 ($K_2 + 1$) rather than K_1.)

If one assumes that a depletion of surface receptor binding sites underlies the observed tolerance, the Furchgott method of analysis of the pooled data predicts that a 70–80% reduction would be required to produce the observed right shift of normorphine concentration-response curve that was observed in the morphine-treated rats: opioid binding studies *in vivo* and *in vitro* have generally failed to find any reduction in the number of recognition sites (B_{max}) after chronic morphine treatment. Thus, although a reduction in the number of μ-receptors in the membrane cannot be excluded by the present results, the better interpretation is that the occupied receptors are less able to associate with the next step in the transduction process, presumed to be a G protein. This change in the receptor does not appear to be associated with any alteration in the affinity of the receptor for naloxone. This mechanism for tolerance is similar to that reported for homologous desensitization of δ-opioid receptors in neuroblastoma × glioma hybrid cells (NG108-15). In that system, desensitization of agonist-induced inhibition of adenylyl cyclase is not explained by the receptor internalization that occurs (25) but is correlated with an impaired ability of δ-agonists to stimulate GTPase activity of a *Pertussis* toxin sensitive G protein (26). Similarly, homologous desensitization of β-adrenoceptor stimulated adenylyl cyclase in rat lung membranes precedes internalization and appears to be due to sequestration of binding sites into membrane microdomains that are inaccessible to adenyl cyclase (27), a process that involves protein phosphorylation (28).

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