mice was limited to the first 24 hours after ingestion. In man, but not in mouse, χ2236 was recovered for 4 days. One reason for the difference may relate to the different diet and intestinal flora of mouse and man. It appears that the plasmid enhanced survival in this disabled strain as well as in the nondisabled χ1666 strain. Our data do not preclude survival of χ1776 below the detectable level. Survival of χ1776 with its plasmid was still 1/10,000 that of the wild-type *E. coli* K-12. Neither *E. coli* strain was able to colonize the intestinal tract of the volunteers, and neither was detectable more than 6 days after ingestion. During 4 to 6 days in the intestinal tract, the plasmid remained nonmobilized, at least by available detection techniques.

The survival of χ2236 was unexpected and raised questions about the effective biologic containment of this system. The failure to detect transfer of the plasmid to endogenous hosts reaffirmed the biologic containment of the EK2 system. Furthermore, no transfer was detected from the EK1 system despite survival at higher titers and for longer periods of time. These results, which provide data on survival of an EK1 and EK2 system in mammals, support their safe use in recombinant DNA technology under routine laboratory conditions. These studies raise the possibility, however, that other plasmid vectors may also enhance survival of the bacterial host. However, as long as plasmid vectors are selected for their inability to be mobilized, this possibility should not pose a problem.

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References and Notes

1. R. Curtiss III, M. Inoue, D. Pereira, J. C. Hsu, L. Alexander, L. Rock, *Miami Winter Symp.* **13**, 99 (1977).
2. F. Bolivar, R. L. Rodriques, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, S. Falkow, *Gene* **2**, 95 (1977).
3. F. R. Blattner *et al.*, *Science* **196**, 161 (1977).
4. P. Leder, D. Tiemeier, L. Enquist, *ibid.*, p. 175.
5. L. Enquist, D. Tiemeier, P. Leder, R. Weisberg, N. Sternberg, *Nature (London)* **259**, 596 (1976).
6. S. B. Levy, N. Sullivan, S. L. Gorbach, *ibid.* **274**, 395 (1978).
7. R. Curtiss III provided the χ1776, χ2236, and χ1666. The D20-5 was prepared by transforming χ1666 with pBR322 DNA obtained from G. Sutcliffe.
8. Composition per liter: peptone (10 g), dipotassium phosphate (2 g), agar (15 g), eosin (0.4 g), methylene blue (0.065 g), lactose (10 g), NaCl (5 g), yeast extract (1 g), diaminopimelic acid (100 μg/ml), thymidine (20 μg/ml), biotin (1 μg/ml), and nalidixic acid (50 μg/ml).
9. Isolates from each volunteer were sent to J. Donch (Palo Alto), who used biochemical tests to confirm the identity of each isolate with the host χ1776, and to R. Clowes (Dallas), who used plasmid isolation and restriction enzyme analysis to determine that pBR322 was the only plasmid in the recovered organisms.
10. B. Medez, C. Tachibana, S. B. Levy, *Plasmid* **3**, 99 (1980).
11. C. Tachibana, B. Marshall, S. Schluederberg, S. B. Levy, *Abstr. Annu. Meet. Am. Soc. Microbiol.* (1980), p. 3961.
12. M. Grunstein and D. S. Hogness, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3961 (1975).
13. S. S. Hua and A. Markovitz, *J. Bacteriol.* **110**, 1089 (1972).
14. These studies were performed under the support of NIH contract NO1AI72529 and grant 5M01RR00054-18 to the New England Medical Center Clinical Study Unit. We acknowledge the help of T. Keller, S. Miller, J. Davis, S. Schluederberg, K. Meal, R. Chan, R. Cisneros, H. Lee, and the nursing staff and administration of the Clinical Study Unit. The colony hybridization studies were performed by C. Tachibana of our laboratory.

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Opiates and Opioid Peptides Hyperpolarize Locus Coeruleus Neurons in vitro

Abstract. *Intracellular recordings were made from locus coeruleus neurons in a brain slice preparation. Opiates and opioid peptides produced a dose-dependent, stereospecific, naloxone-reversible hyperpolarization of the neuronal membrane. This was associated with an increase in membrane conductance.*

The effects of opiates and opioid peptides on the firing rates of single neurons in the central nervous system have been widely documented (*1*). The most commonly observed response has been a decrease in the rate of neuronal firing. However, the extracellular recording techniques used could not elucidate the mechanisms by which the inhibition is produced nor whether it is mediated through a pre- or postsynaptic site. Therefore, to investigate the mechanisms underlying the inhibition of neuronal activity by opiates and opioid peptides, we recorded intracellularly from locus coeruleus neurons in vitro and studied the actions of these drugs on neuronal membrane properties.

The locus coeruleus is a homogenous group of catecholamine-containing cell bodies with projections throughout the central nervous system (*2*). This pontine nucleus provides an excellent site for studying the actions of opiates and opioid peptides, since it possesses a high density of opiate binding sites (*3*) and is innervated by nerve terminals that appear to contain enkephalin (*4, 5*). The locus coeruleus has been implicated as a site of opiate action because its destruction attenuates morphine-induced antinociception in rats (*6*). Previous electrophysiological studies of locus coeruleus neurons showed that opiates and opioid peptides depress both their spontaneous firing and the increase in firing following peripheral noxious stimulation (*7*). We now report that the application of opiates and opioid peptides to locus coeruleus neurons produces a stereospecific, naloxone-reversible membrane hyperpolarization that is associated with an increase in membrane conductance.

Thin slices of guinea pig pons were mounted in a recording chamber and superfused with Krebs solution at 37°C (*8*). Locus coeruleus neurons were impaled up to 9 hours after the slice was cut. Intracellular recordings were made from single cells for as long as 4 hours. The cells had stable resting membrane potentials of 45 to 72 mV. Drugs were applied to the tissue by changing the perfusion solution to one that differed only in its drug content.

Normorphine (100 n*M* to 3 μ*M*) caused hyperpolarization of the neuronal membrane in 19 of 28 locus coeruleus neurons tested (of the remainder, one was depolarized and eight were unaffected) (Fig. 1). The hyperpolarization was associated with a decrease in input resistance in 10 of the 14 neurons for which resistance measurements were obtained (Fig. 1, B and C). The degree of hyperpolarization was related to the concentration of the drug applied (Fig. 1A): at all concentrations tested, the effect began within 1 minute of exposure of the tissue to normorphine and was reversed when the tissue was washed with drug-free Krebs solution. The amplitude of the hyperpolarization produced by 1 μ*M* normorphine varied from 2 to 20 mV among neurons (mean, 9.8 ± 1.1 mV, $N = 19$), but for a given neuron the responses were reproducible. When neurons fired action potentials spontaneously, the hyperpolarization produced by normorphine was always associated with a marked reduction or abolition of the spontaneous firing.

The hyperpolarization of locus coeruleus neurons by normorphine was probably not the result of inhibition of the tonic release of an unknown excitatory neurotransmitter, because normorphine was still effective when the tissue had been perfused for up to 10 minutes with a solution containing no

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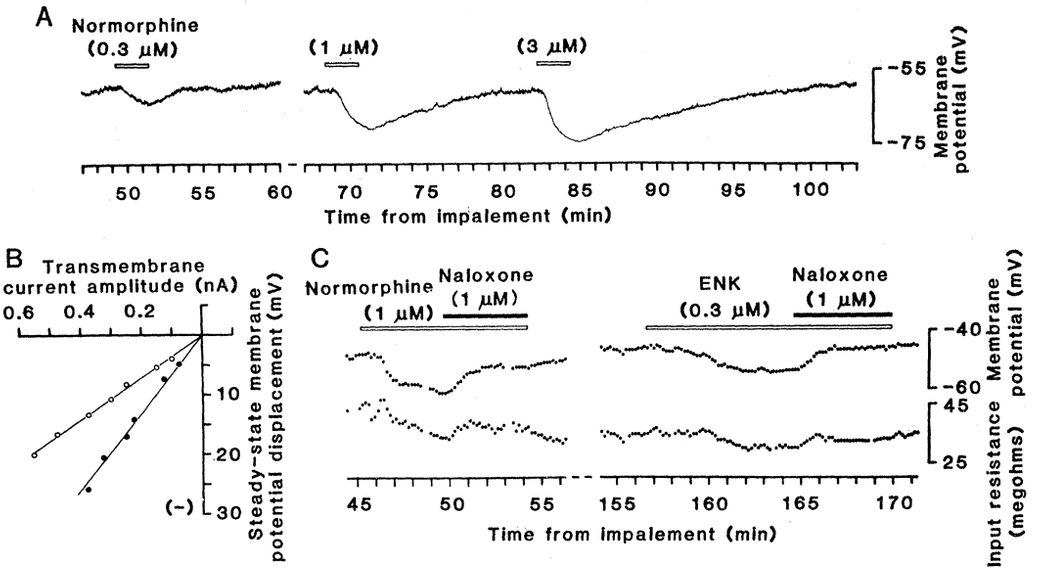

Fig. 1. Effects of normorphine and D-Ala^2-Met^5-enkephalin on locus coeruleus neurons. (A) Dose-response relation for the normorphine-induced hyperpolarization in a single locus coeruleus neuron. The duration of each normorphine application is indicated by the open bars. (B) Current-voltage relation for a locus coeruleus neuron before (●) and during (○) the hyperpolarization (12 mV from resting membrane potential) produced by normorphine (1 μM). (C) Naloxone-induced reversal of the membrane hyperpolarization produced by normorphine or D-Ala^2-Met^5-enkephalin in a single locus coeruleus neuron. The duration of normorphine or D-Ala^2-Met^5-enkephalin (*ENK*) application is indicated by the open bars; that of naloxone application, by the closed bars.

calcium and 6 mM magnesium (Fig. 2). A calcium-free solution blocks all excitatory postsynaptic potentials in this preparation (*9*).

D-Ala^2-Met^5-Enkephalin (100 to 300 nM) and D-Ala^2-D-Leu^5-enkephalin (300 nM) produced effects similar to those of normorphine on membrane potential, input resistance, and spontaneous firing in neurons to which the peptides and normorphine were applied (Fig. 1C).

Naloxone (100 nM to 1 μM) rapidly and completely reversed the hyperpolarization produced by normorphine (1 μM) or D-Ala^2-Met^5-enkephalin (300 nM) (Fig. 1C). In addition, the potent analgesic levorphanol (100 to 300 nM) hyperpolarized six of eight neurons, whereas an up to tenfold higher concentration of its (+)-enantiomer dextrorphan was without effect. The levorphanol effect was slower than that of normorphine. In terms of the amplitude of potential change caused by a given concentration, levorphanol was approximately ten times more potent. The reversal by naloxone and the stereospecificity of opiate action strongly suggests that the hyperpolarization of locus coeruleus neurons by opiates and opioid peptides is mediated via specific opiate receptors.

These results demonstrate that opiates and opioid peptides inhibit locus coeruleus neurons by a direct action at a postsynaptic site. The increase in membrane conductance produced by these drugs suggests that the hyperpolarization is due to increased permeability of the membrane to one or more ions—possibly chloride or potassium. It was not possible to determine whether the hyperpolarization of the postsynaptic membrane originated in the cell body or on its processes. However, it has been found that enkephalin-like immunoreactivity is located in rat nerve terminals that form axodendritic synapses with locus coeruleus neurons (*4*). A nonsomatic site of action would be similar to that reported for the hyperpolarizing action of enkephalin in myenteric neurons (*10*).

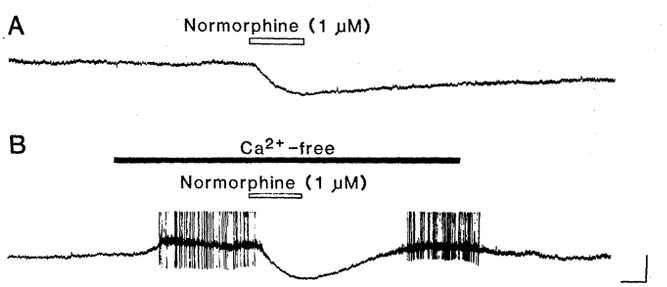

Fig. 2. Hyperpolarization of a locus coeruleus neuron by normorphine (A) in normal Krebs solution and (B) in Krebs solution containing no calcium and 6 mM magnesium. Although perfusion of the brain slice with Krebs solution containing no calcium for 5 minutes caused the neuron to depolarize and fire action potentials, it did not alter the amplitude of the hyperpolarization produced by normorphine (1 μM). The amplitude of the action potentials was attenuated by the recording system. Calibrations: vertical, 10 mV; horizontal, 1 minute.

The various actions of opiates and opioid peptides in the peripheral and central nervous systems might be mediated by a single mechanism similar to that observed in locus coeruleus neurons: a membrane hyperpolarization associated with increased conductance. Opiates and opioid peptides depress neuronal firing rates (*1*); a membrane hyperpolarization by these drugs could produce such an effect by moving the membrane potential below the threshold for action potential generation (Fig. 2). Also, opiates and opioid peptides inhibit neurotransmitter release at various sites (*11*). North *et al.* (*10*) suggested that a membrane hyperpolarization or conductance increase produced by opioid peptides at nonsomatic sites would result in a decrease in transmitter release, since such an action would prevent action potential propagation along nerve processes to the sites of transmitter release. Dendritic hyperpolarization has been proposed (*1*) as one explanation of the reduction in the rate of rise of synaptic potentials by opiates in spinal neurons in vivo (*12*). Finally, opiates increase the threshold for antidromic excitation of primary afferent neurons (*13*); hyperpolarization of primary afferent terminals would result in an increased threshold for excitation. Although a membrane hyperpolarization and the associated increase in neuronal conductance may account for some of the observed effects of opiates and opioid peptides, this mechanism does not appear to underlie the effects of these drugs on cultured spinal neurons and cultured dorsal root ganglion cells (*14*).

Our findings raise the possibility that, in the locus coeruleus, endogenous opioid peptides may mediate direct postsynaptic inhibition. However, further

evidence for the release of endogenous opioid peptides after physiological stimulation is required to establish their neurotransmitter role at this site.

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References and Notes

1. See review by R. A. North [*Life Sci.* **24**, 1927 (1979)].
2. D. G. Amaral and H. M. Sinnamon, *Prog. Neurobiol. N.Y.* **9**, 147 (1977).
3. S. F. Atweh and M. J. Kuhar, *Brain Res.* **129**, 1 (1977); C. B. Pert, M. J. Kuhar, S. H. Snyder, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3929 (1977).
4. V. M. Pickel, T. H. Joh, D. J. Reis, S. E. Leeman, R. J. Miller, *Brain Res.* **160**, 387 (1979).
5. R. Simantov *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2167 (1977).
6. M. Sasa, K. Munekiyo, Y. Osumi, S. Takaori, *Eur. J. Pharmacol.* **42**, 53 (1977).
7. S. J. Bird and M. J. Kuhar, *Brain Res.* **122**, 523 (1977); P. J. Guyenet and G. K. Aghajanian, *ibid.* **136**, 178 (1977); J. Korf, B. S. Bunney, G. K. Aghajanian, *Eur. J. Pharmacol.* **25**, 165 (1974); W. S. Young, S. J. Bird, M. J. Kuhar, *Brain Res.* **129**, 366 (1977).
8. Coronal slices (300 μm thick) of guinea pig pons were mounted in a recording chamber [P. A. Schwartzkroin, *Brain Res.* **85**, 423 (1975)] and superfused with Krebs solution (2 ml/min) at 37°C. The Krebs solution was of the following composition (m*M*): NaCl, 126; KCl, 5; NaH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 26; and glucose, 10; and was gassed with 95 percent O_2 and 5 percent CO_2. To prevent movement of the slice, a titanium electron microscopy grid was placed on top of it and held in position by a platinum loop attached to a micromanipulator. Viewed from above at a magnification of ×10 to ×40, the locus coeruleus could be seen as a dark gray area below the fourth ventricle and medial to the mesencephalic root of the trigeminal nerve. Recording microelectrodes, filled with 4*M* potassium acetate and having tip resistances of 50 to 100 megohms were positioned over this area and manually advanced into the tissue until a cell was impaled; then the position of the recording electrode was noted. At the end of each experiment the tissue was fixed in formaldehyde and stained with cresyl violet. The experiments reported here are those in which subsequent histological examination of the slice confirmed that the recordings had been made from neurons of the locus coeruleus.
9. C. M. Pepper and G. Henderson, unpublished observations.
10. R. A. North, Y. Katayama, J. T. Williams, *Brain Res.* **165**, 67 (1979).
11. G. M. Lees, H. W. Kosterlitz, A. A. Waterfield, *Agonist and Antagonist Actions of Narcotic Analgesic Drugs*, H. W. Kosterlitz, H. O. J. Collier, J. E. Villareal, Eds. (University Park Press, Baltimore, 1972), p. 142; K. Jhamandas, J. Sawynock, M. Sutak, *Nature (London)* **269**, 433 (1977); K. Starke, *Rev. Physiol. Biochem. Pharmacol.* **77**, 1 (1977).
12. W. Zieglgänsberger and H. Bayerl, *Brain Res.* **115**, 111 (1976).
13. B. R. Sastry, *Neuropharmacology* **18**, 367 (1979); E. Carstens, I. Tulloch, W. Zieglgänsberger, M. Zimmerman, *Pfluegers Arch.* **379**, 143 (1979).
14. J. L. Barker, J. H. Neale, T. G. Smith, Jr., R. L. Macdonald, *Science* **199**, 1451 (1978); A. W. Mudge, S. E. Leeman, G. D. Fischbach, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 526 (1979).
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Evidence for Lignin-Like Constituents in Early Silurian (Llandoverian) Plant Fossils

Abstract. *Chemical evidence is presented with previously reported morphological features for banded-tube cell types in the earliest known plant fossils associated with stream-deposited sediments. Phenolic aldehydes (p-hydroxybenzaldehyde, vanillin) and aromatic compounds from pyrolysis (2-methoxy-4-hydroxybenzaldehyde, methylsyringaldehyde) derived from cellular remains are interpreted as evidence for lignin or lignin-like degradation products. The presence of parallel-aligned banded tubes, with annular to spiral thickenings and occasional end walls, in conjunction with lignin-like constituents fulfill most of the morphological and chemical criteria for cell types that could have functioned as water-conducting cells.*

Parallel-aligned banded tubes with annular to spiral ribbing have been reported in early Silurian (Llandoverian) plant fossils found in stream-deposited sediments (*1*). The parallel alignment, possession of end walls, and ribbing in some of these cellular remains suggest a structural similarity to tracheids and a functional specialization related to water-transport tissues. Owing to the extreme age of this early Silurian material, which predates the earliest known vascular plants, the structural similarities between the banded tubes and tracheids may be misleading. Tracheids must be defined on the basis of their morphology and biochemistry, as well as their function. They are highly specialized cells, nonliving at maturity, more or less elongated, having a variety of potential pit types (*2*), and are chemically characterized by having lignified walls with secondary thickenings. Within the evolutionary context, the tracheid is the single most important anatomical criterion for affirming a vascular plant habit (*3*). Other features such as trilete spores, cuticles, sporopollenin, and stomata provide only inferential evidence for a vascular plant. Lignin, a necessary aspect of the tracheid definition, has been isolated from Paleozoic, Mesozoic, and Cenozoic fossil vascular plants (*4*-*6*). Its presence may be detected by alkaline oxidation with nitrobenzene or pyrolysis, which yields *p*-hydroxybenzaldehyde, vanillin, syringaldehyde, and various aromatic acids (*7*). We have chemically analyzed the Llandoverian plant fossils from the Massanutten Sandstone, Virginia (*1*), for phenolics and possible lignin decomposition products. The chemical data confirm the presence of low concentrations of what we interpret to be lignin-like constituents. The presence of these chemicals in early Silurian plant remains found in stream-deposited sediments suggests that these plants may have had cell types that were both structurally and biochemically similar to those of water-conducting cells.

Fossiliferous siltstone containing the carbonaceous plant remains was collected from the lower Massanutten Sandstone, Virginia, which is considered to be Albion (Llandoverian) in age (*1*). The fossils appear as mostly amorphous coalified remains showing little distinguishable megastructure. They were mechanically removed from freshly parted samples and washed in double-distilled water. Duplicate samples of the associated siltstone and a high-fired clay were tested with the plant samples so as to check for organic contaminants in the extraction or handling procedure. None were detected. The chemical constituents reported here are considered indicative of the plant assemblage's biochemistry. Organic solvent extractions (*4*), pyrolysis (*5*), and oxidation with alkaline nitrobenzene (*6*) of separate subsamples were performed, and the chemical constituents were identified by gas chromatography (GC) and by combined GC-mass spectroscopy (GC-MS) (*4*). Of particular interest is the isolation of significant amounts of vanillin and *p*-hydroxybenzaldehyde, both considered by-products of lignin degradation (Fig. 1A). Pyrolyzates (450°C) from the carbonaceous plant material contained indanone, naphthalenes, and benzofurans; and 600°C pyrograms yielded as their primary aromatic products benzene, alkyl-substituted benzenes, phenols, xylenols, and benzofurans (Fig. 1B). Organic solvent extractions with a mixture of benzene and methanol, 3:1 (by volume), yielded a consortium of constituents similar to those isolated from Devonian vascular or presumed vascular land plants: straight-chain fatty acids (C_{10} to C_{24}, maximum C_{17}); phenols, $C_nH_{(2n-18)}O$ (C_{10} to C_{22}, maximum C_{14}); the aromat-

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