pamil (*14*) at a concentration of 100 mg/kg per day [a dose that lowered arterial pressure by 29 ± 3 mmHg (mean ± SEM, $n = 10$)] by gavage from 6 days before to 14 days after balloon injury. At this dose no effect on myointimal proliferation was seen in the verapamil-treated rats [the cross-sectional area of neointima expressed as a percentage of media being 129 ± 18% ($n = 6$) in treated rats compared to 127 ± 13% ($n = 10$) in placebo-treated controls].

The present observations, taken together with data showing CE activity (*5*), angiotensinogen mRNA (*15*), and AII receptors in the vascular wall (*6*), support the hypothesis that there is a local angiotensin system that has a role in the myointimal proliferative response of the vascular wall to injury.

The processes that lead to formation of the neointima are complex and poorly understood, but initially quiescent SMCs must be activated either directly by injury or indirectly by factors released early during the response, such as interferon-γ from lymphocytes (*16*) or platelet-derived growth factor (PDGF) (*1, 17*). Blocking the activation of SMCs, however, is unlikely to be the principal mechanism for the suppression of neointima formation, as administration of cilazapril only during the initial phase of the vascular response was ineffective.

It is probable that CE inhibitors suppress the vascular response to injury by interrupting the conversion of angiotensin I (AI) to the active AII, and thus preventing modulation of SMC proliferation and matrix protein synthesis by AII. Inhibition of CE also increases bradykinin levels, since CE is one of the enzymes that metabolize bradykinin (*4*). Although unlikely, the possibility that bradykinin mediates the suppression of the vascular response to injury cannot be excluded. Bradykinin, however, is a mitogenic agent (*18*), and therefore it might be expected that increased bradykinin concentrations would augment the proliferative response. The observation that the *mas* proto-oncogene product is an angiotensin receptor with mitogenic activity (*19*) raises the possibility that the proliferative response to vascular injury is mediated through these receptors on SMCs and requires continued stimulation by AII.

It has been reported that AII neither stimulated the proliferation of cultured rat aortic SMCs nor augmented cell proliferation induced by PDGF (*20*). However, AII did increase SMC protein content by 20% after 4 days, and this effect was blocked by saralasin, an antagonist of AII. In contrast, others have found that AII stimulated the proliferation of human vascular SMCs cultured in serum-depleted medium (*21*). It is possible that AII is effective only when added at specific phases of the cell cycle, as has been shown for the AII-augmented proliferation of NIH 3T3 cells stimulated by epidermal growth factor (*22*).

Infusion of heparin suppresses neointima formation in the rat balloon model used for our study (*23*). The heparin administration must be started by 24 hours after injury and is equally effective whether administered continuously for 21 days or for 3 days (*10*). However, in contrast to our results with CE inhibitors, the maximal suppression of neointima formation achieved with heparin has been generally about 50% (*23*). It remains to be tested whether CE inhibition can act synergistically with heparin to prevent the proliferative response.

REFERENCES AND NOTES

1. R. Ross, *N. Engl. J. Med.* **314**, 488 (1986).
2. W. McBride *et al.*, *ibid.* **318**, 1734 (1988).
3. H. R. Baumgartner and A. Studer, *Pathol. Microbiol.* **29**, 393 (1966); A. W. Clowes, M. A. Reidy, M. M. Clowes, *Lab. Invest.* **49**, 208 (1983); A. W. Clowes and S. M. Schwartz, *Circ. Res.* **56**, 139 (1985).
4. D. J. Campbell, *J. Clin. Invest.* **79**, 1 (1987); V. J. Dzau, *Circulation* **77** (suppl. 1), 1 (1988); A. Pellicer *et al.*, *Science* **240**, 1660 (1988).
5. Y. Nakamura *et al.*, *J. Hypertens.* **6**, 105 (1988).
6. J. Penit, M. Faure, S. Jard, *Am. J. Physiol.* **244**, E72 (1983).
7. G. K. Owens and S. M. Schwartz, *Circ. Res.* **51**, 280 (1982); G. K. Owens, *ibid.* **56**, 525 (1985).
8. C. H. Hassall, A. Kröhn, C. J. Moody, W. A. Thomas, *FEBS Lett.* **147**, 175 (1982); I. L. Natoff *et al.*, *J. Cardiovasc. Pharmacol.* **7**, 569 (1985); J.-P. Clozel and F. Hefti, *ibid.* **10**, 350 (1987); J.-P. Clozel *et al.*, *J. Hypertens.*, in press.
9. Rats (rat-outbred Roche) were specific-pathogen free and from the Institute of Biomedical Research, Füllinsdorf, Switzerland.
10. A. W. Clowes and M. M. Clowes, *Lab. Invest.* **52**, 611 (1985).
11. C. C. Haudenschild and S. M. Schwartz, *ibid.* **41**, 407 (1979).
12. M. Gerold and H. Tschirky, *Arzneim. Forsch.* **18**, 1289 (1969).
13. M. A. Ondetti *et al.*, *Science* **196**, 441 (1977).
14. A. Fleckenstein, *Annu. Rev. Pharmacol. Toxicol.* **17**, 149 (1977).
15. L. A. Cassis *et al.*, *Hypertension* **11**, 591 (1988).
16. G. K. Hanson, L. Jonasson, J. Holm, M. M. Clowes, A. W. Clowes, *Circ. Res.* **63**, 712 (1988).
17. L. N. Walker *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7311 (1986).
18. N. E. Owen and M. L. Villereal, *Cell* **32**, 379 (1983).
19. T. R. Jackson, L. A. C. Blair, J. Marshall, M. Goedert, M. R. Hanley, *Nature* **335**, 437 (1988).
20. A. A. T. Geisterfer, M. J. Peach, G. K. Owens, *Circ. Res.* **62**, 749 (1988).
21. M. Campbell-Boswell and A. L. Robertson, *Exp. Mol. Pathol.* **35**, 265 (1981).
22. J. Norman *et al.*, *Am. J. Physiol.* **253**, F299 (1987).
23. A. W. Clowes and M. J. Karnovsky, *Nature* **265**, 625 (1977); J. R. Guyton, R. D. Rosenberg, A. W. Clowes, M. J. Karnovsky, *Circ. Res.* **46**, 625 (1980); R. L. Hoover, R. Rosenberg, W. Haering, M. J. Karnovsky, *ibid.* **47**, 578 (1980).

24. We thank M. M. Clowes and A. W. Clowes for helpful discussions; M. Broussé, C. Hungerbühler, M. Reichlin, F. Schodjai, S. Stäger, and P. Wyss for technical help; and D. Brütsch and J. Lobsiger for preparation of the manuscript.

3 January 1989; accepted 23 May 1989

The Periaqueductal Gray Matter Mediates Opiate-Induced Immunosuppression

RICHARD J. WEBER AND AGU PERT

The periaqueductal gray matter of the mesencephalon (PAG) subserves a variety of diverse autonomic functions and also appears to be a site for opiate action in the induction of immunosuppression. Microinjections of morphine into the PAG, but not into other opiate receptor–containing neuroanatomical sites, result in a rapid suppression of natural killer (NK) cell activity. The NK cell suppression can be blocked by prior peripheral administration of the opiate antagonist naltrexone. These findings demonstrate that certain central actions of opiates that produce changes in NK cell function are mediated through opiate receptors in the PAG and identify a brain region involved in opiate regulation of immune function.

OPIATES EXERT PROFOUND EFFECTS on immune function in vivo (*1*). Opiate addicts have increased susceptibility to infections (*2*), an effect related to deficits in immune function (*3*). Certain opiate agonists suppress antibody production (*4*), produce changes in the ability of leukocytes to respond to mitogenic stimuli (*5*), and decrease cytotoxic activity of natural killer (NK) cells (*6*). These immunosuppressive effects of opiates result in decreased survival in tumor-bearing animals (*7*) and increased susceptibility to bacterial and fungal infections (*8*) as well as to murine retroviral infections (*9*). Some in vivo effects of opiates on immune function are mediated indirectly through the central nervous system (CNS). For example, Shavit *et al.* (*10*) have shown that injections of morphine into the lateral cerebral ventricle suppress NK cell activity in rats. Conversely, peripheral administration of *N*-methyl morphine,

R. J. Weber, Laboratory of Medicinal Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892.

A. Pert, Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, MA 20892.

which does not cross the blood brain barrier, had no effect on NK activity in the same study. Although it appears that opiate agonists can act in the CNS to produce effects on immune function, the precise site of action is not known. We show here that microinjections of morphine into the periaqueductal gray matter (PAG), but not into other opiate receptor–containing neuroanatomical sites, result in rapid suppression of NK cell activity, which can be blocked by prior peripheral administration of naltrexone.

Male Fisher 344N rats (300 to 350 g) were implanted under chloral hydrate anesthesia with bilateral cannula guides constructed from 23-gauge stainless steel tubing. These guides were directed at regions 2 mm dorsal to the anterior hypothalamus [anterior-posterior (AP) 7.2, medial-lateral (ML) ±0.8, dorsal-ventral (DV) +2.7], arcuate nucleus–ventromedial hypothalamus (AP 5.7, ML ±0.5, DV +2.2), medial thalamus (AP 5.7, ML ±1.0, DV +5.8), medial amygdala (AP 6.2, ML ±3.5, DV +2.7), dorsal hippocampus (AP 5.7, ML ±2.0, DV +8.0), or the PAG (AP 1.2, ML ±0.5, DV +6.0). All coordinates are expressed as millimeters from interaural zero. One week after recovery from surgery the animals were injected bilaterally with morphine sulfate in pyrogen-free saline. Splenic NK cell activity was determined as described (Fig. 1).

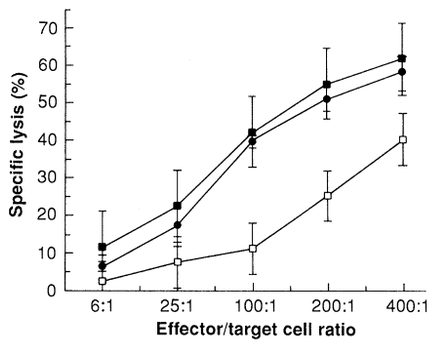

Fig. 1. Naltrexone blocks morphine suppression of NK cell activity. NK cell activity of splenocytes was determined in rats receiving the following treatments: PAG morphine (6.6 nmol in 1 μl of saline) plus saline intraperitoneal (□); PAG morphine (same dose) plus naltrexone (10 mg/kg) intraperitoneal (●); PAG saline (1 μl) plus intraperitoneal saline (■). The intraperitoneal injections were always made 5 min before PAG microinjections. Three hours after the injections, spleens were surgically excised, mechanically dissociated into single-cell suspensions, and separated on Ficoll-Hypaque density gradients (LSM, Litton Bionetics, Kensington, Maryland). Splenic leukocytes were washed twice and suspended in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). NK cell activity was assessed in a standard 4-hour chromium release assay with YAC-1, a murine lymphoma cell line as targets. YAC-1 cells were added to microtiter plates containing spleen cells at various concentrations to give effector/target ratios ranging from 400:1 to 6:1. Maximal release of ^{51}Cr was determined by incubating targets with 0.1*N* HCl. Spontaneous release was determined as the amount of radioactivity released in the presence of media alone and averaged 18% of the total amount released in the presence of 0.1*N* HCl. Data shown are the means ± SEM, $n = 5$ in each group. The percentage of specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximal release − spontaneous release) × 100. A repeated measures analysis of variance revealed a significant treatment effect [$F(2,12) = 27.7$; $P < 0.0001$] as well as a significant effect due to different effector/target cell ratios, as expected [$F(4,48) = 498.7$; $P < 0.0001$]. The group that received PAG morphine sulfate plus intraperitoneal saline was significantly different from the group that had been injected with PAG morphine sulfate plus intraperitoneal naltrexone and from the group that had been injected with PAG saline plus intraperitoneal saline (Newmann-Keuls multiple comparisons procedure, $P < 0.01$).

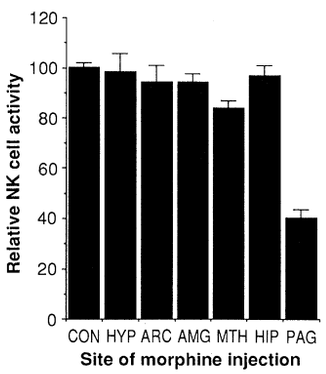

Fig. 2. Relative NK cell activity after central microinjection of morphine. Male Fisher 344N rats were anesthetized with a single subcutaneous injection of chloral hydrate (700 mg/kg) and implanted stereotactically with cannula guides constructed from 23-gauge stainless steel tubing. One week after surgery rats received injections aimed at the indicated structures (anterior hypothalamus, HYP; arcuate nucleus, ARC; medial amygdala, AMG; medial thalamus, MTH; dorsal hippocampus, HIP; and the PAG. A seventh group of animals served as an unanesthetized, noncannulated, uninjected control group (CON) to which the other six groups were compared. In a separate experiment, animals injected subcutaneously with chloral hydrate 7 days before determination of splenic NK cell activity did not differ significantly from unanesthetized controls (mean ± SEM: 54.5 ± 3.1 versus 55.3 ± 3.8; $n = 4$; $t = 0.258$; $P > 0.05$). Injections (6.6 nmol of morphine sulfate in 1 μl of saline) were made at the rate of 1 μl per 30 s through 30-gauge injectors that protruded 2.0 mm below the cannula guide tips. Three hours after administration of morphine sulfate the rats were decapitated; the brains were rapidly removed and prepared for subsequent histological examination, according to standard histological procedures to ascertain cannulae placement. All rats included in the study had injection sites in the appropriate structures. Spleens were removed and processed for measurement of NK cell activity, and the specific lysis (in percent) was calculated as described in the legend to Fig. 1. Data from four separate experiments were pooled, and relative NK cell activity at effector/target ratios of 200 to 1 is shown. Relative NK activity for each animal was determined in the following manner. Relative NK activity equals the specific lysis for each animal in a replication divided by the mean control group specific lysis for that replication multiplied by 100. Mean control group specific lysis was 58.1 with a range of 38.1 to 75.1. A one-way analysis of variance revealed a significant difference among the various treatment means [$F(6,69) = 21.2$; $P < 0.001$]. A Newman-Keuls test for critical differences among treatment means revealed that rats injected with morphine sulfate into the PAG were significantly different ($P < 0.01$) from all other treatment groups including the control.

Animals given morphine injections into the hypothalamus, arcuate nucleus, medial amygdala, medial thalamus, and dorsal hippocampus exhibited no effect on NK cell activity as compared to uninjected animals without cannulae, the most rigorous control group. Animals given morphine injections into the PAG, however, exhibited a dramatic suppression of NK cell activity as compared to control animals (Fig. 2). The effect is pharmacologically specific and mediated through an interaction of morphine with opiate receptors in the PAG, because the suppression of NK cell activity was blocked by prior intraperitoneal injection of the specific opiate antagonist naltrexone (10 mg per kilogram of body weight). Saline was administered into the PAG as a control for nonspecific mechanical stimulation of this brain region (Fig. 2). Naltrexone alone had no effect on NK cell activity. These findings suggest that certain central actions of opiates can induce changes in NK cell function, and these effects are mediated at least in part through opiate receptors in the PAG.

Although the PAG appears to be a major neural focus for the action of exogenous opiates in regulating immune function, it may also be that endogenous opiate action in this brain region is related to the ability of some forms of stress to alter immune function through opiate-dependent mechanisms. Opiate receptors and endogenous opiate peptides are present in the PAG, and endogenous opiates are released in the PAG during foot-shock stress (*11*). In addition, the PAG appears to be one of the primary sites of action of opiates in eliciting analgesia (*12*). Shavit *et al.* have demonstrated that certain forms of foot-shock stress in rats that produce an opiate-dependent analgesia (*13*) also suppress splenic NK cell activity and that both effects can be blocked by naltrexone. Stimuli associated with foot-shock stress may affect the PAG, possibly through spinoreticular pain pathways, and enhance the activity of endogenous opioid-containing neurons in this structure, resulting in

analgesic activity as well as suppression of immune function. The PAG has been implicated indirectly in modulating certain aspects of immune function. Electrical stimulation of this region enhances metastatic tumor growth (*14*), presumably through alterations in immune function, particularly in NK cell activity. Indeed, electrical stimulation of the PAG produces an opiate-mediated analgesia in a variety of species including humans (*15*). Therefore, it is likely that this region of the brain may be critically involved in the regulation of immune effector functions that are important in resistance to neoplasms and infectious diseases and that are controlled, at least in part, by endorphinergic circuitry.

The precise neural mechanisms underlying the ability of opiate action in the PAG to alter NK cell activity are not entirely clear. Signals from the CNS to the immune system are relayed primarily through the hypothalamic-pituitary-adrenal axis (HPA) or via sympathetic innervation of lymphoid organs. Thus, opiate action in the PAG could be translated into activation of the HPA axis through hypothalamic efferents (*16*), or enhanced opiate activity in the PAG could cause increases in peripheral sympathetic output, either of which could have an effect on NK cell activity (*17*).

REFERENCES AND NOTES

1. R. J. Weber and C. B. Pert, in *Central and Peripheral Endorphins: Basic and Clinical Aspects*, E. E. Muller and A. R. Genazzini, Eds. (Raven, New York, 1984), pp. 35–42; M. D. Yahya and R. R. Watson, *Life Sci.* **41**, 2503 (1987).
2. H. H. Hussey and S. Katz, *Am. J. Med.* **9**, 186 (1950); D. B. Louria, T. Hensle, J. Rose, *Ann. Intern. Med.* **67**, 1 (1967).
3. S. M. Brown, B. Stimmel, R. N. Taub, S. Kochwa, R. E. Rosenfield, *Arch. Intern. Med.* **134**, 1001 (1974).
4. R. J. Weber *et al.*, *Natl. Inst. Drug Abuse Res. Monogr.* **76**, 341 (1987); M. Gungor *et al.*, *Experientia* **36**, 1309 (1980).
5. H. U. Bryant *et al.*, *Life Sci.* **41**, 1731 (1987).
6. Y. Shavit *et al.*, *Brain Res.* **372**, 382 (1986).
7. J. W. Lewis *et al.*, *Nat. Immun. Cell Growth Regul.* **3**, 43 (1984).
8. E. Tubaro *et al.*, *J. Infect. Dis.* **148**, 656 (1983).
9. R. R. Watson *et al.*, *Life Sci.* **43**, v (1988).
10. Y. Shavit *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7114 (1986).
11. T. F. Seeger *et al.*, *Brain Res.* **305**, 303 (1984).
12. A. Pert and T. Yaksh, *ibid.* **80**, 135 (1974).
13. Y. Shavit *et al.*, *Science* **223**, 188 (1984).
14. R. H. Simon, E. J. Lovett III, D. Tomaszek, J. Lundy, *ibid.* **209**, 1132 (1980).
15. D. J. Mayer and J. C. Liebeskind, *Brain Res.* **68**, 73 (1974); H. Akil, D. J. Mayer, J. C. Liebeskind, *Science* **191**, 961 (1976); H. Akil, D. E. Richardson, J. Hughes, J. D. Barchas, *ibid.* **201**, 463 (1978).
16. J. E. Blalock, *J. Immunol.* **132**, 1067 (1984).
17. D. L. Felten *et al.*, *ibid.* **135**, 755s (1985).

18. We thank J. Alexander, D. Barba, A. Beckerman, A. Hagan, B. Ikejiri, M. Johnson, S. Lee, R. Plunkett, and T. Sullivan for experimental assistance; M. Hamilton for statistical assistance; and M. Herkenham and K. Rice for critical comments on the manuscript.

19 December 1988; accepted 21 April 1989

Effect of Serotonergic Afferents on Quantal Release at Central Inhibitory Synapses

ISABELLE MINTZ, TAKAHIRO GOTOW, ANTOINE TRILLER, HENRI KORN

Although most examples of modulation of synaptic transmission have been obtained from excitatory rather than from inhibitory connections, serotonin (5HT) is now shown to cause a presynaptic facilitation of release of the inhibitory neurotransmitter glycine. Brief local injections of this amine, or application of a 5HT uptake blocker, produce a long-lasting enhancement of both spontaneous and evoked inhibitory currents in the teleost Mauthner cell. Quantal analysis showed that the probability of release is increased. Focal recording indicated that 5HT acts directly on the inhibitory terminals, possibly reducing potassium conductances. Double staining with specific antibodies demonstrated a morphological substrate for this effect. Nerve endings that contain 5HT contact inhibitory terminals directly apposed to postsynaptic glycine receptors.

MODULATION OF TRANSMITTER RELEASE has been proposed as a key cellular mechanism for various forms of plasticity at excitatory synapses (*1, 2*). In contrast, although inhibitory networks do participate in long-lasting behavioral changes (*3*), studies of their presynaptic regulation are scarce and mainly involve peripheral systems (*4, 5*). To examine this question in a vertebrate brain, we chose the teleost Mauthner (M) cell, the command neuron of the escape reflex. The excitability of this cell is controlled by inhibitory afferents characterized morphologically (*6, 7*) and electrophysiologically (*8–11*). A quantal description of the spontaneous noise that these afferents produce permits straightforward access to their release parameters (*12*). We used this property to demonstrate that the inhibitory synapses receive presynaptic contacts that contain serotonin (5HT) and facilitate the release of glycine.

Experiments were conducted on curarized goldfish (*Carassius auratus*) anesthetized with 3-aminobenzoic acid ethyl ester (MS-222, Sandoz). Chloride-dependent inhibitory postsynaptic currents (IPSCs), produced by activity in two groups (*10*) of glycine-containing interneurons (Fig. 1A), were recorded at resting potential. Potassium acetate– or potassium chloride–filled microelectrodes (1.5 to 3 megohms) were used for single-electrode voltage-clamp recording (Axoclamp, Axon Instruments, Burlingame, California; sampling frequency, 18 to 25 kHz). Serotonin (2 to 10 m*M*, in a solution of 130 m*M* NaCl and 10 m*M* Hepes) was applied by pressure-pulses (30-ms duration) through a second microelectrode positioned 10 to 20 μm above the M cell and close to the recording site. As in other neurons (*13*), 5HT increased the inward rectification in the M cell (148% ± 34%, mean ± SD, $n = 20$). Once this occurred, the pipette was withdrawn to avoid leakage of the amine.

Evoked IPSCs were increased by 5HT. In an initial series of 14 experiments, it enhanced the collateral (Fig. 1B) and commissural (Fig. 1C) IPSCs by 136 ± 34% ($n = 14$) and 175 ± 77% ($n = 4$; that is, in all cases, when tested), respectively, because of an increase of glycine-activated conductances (Fig. 1B, inset).

In each experiment, the enhanced transmission was accompanied by an increase in inhibitory synaptic noise (Fig. $2A_1$), which dominates in the M cell (*14*). This augmentation started 2 to 5 min after a single 5HT application and generally persisted for at least 25 to 30 min. Its composition was systematically analyzed by comparing the amplitudes of individual components constituting noise on continuous recordings, according to a method (*12*) that distinguishes closely spaced overlapping events (Fig. $2A_2$). This analysis showed that 5HT did not alter the size of individual quanta, but that it increased the number of large responses that represent integral multiples of these basic units and that are produced, as are the evoked responses, by spike activities in presynaptic fibers (*12*) (Fig. 2, B_1 and B_2). Because the total number of responses was essentially the same in identical sampling periods (120 s in the illustrated case), these data indicate that 5HT increases the probability of release at the presynaptic terminal inhibitory active zones (*11*) close to the pipette.

Two sets of evidence demonstrate that endogenous 5HT acts in the same manner. First, topical application of 20 μ*M* imipramine, which blocks 5HT uptake, induced similar effects on the collateral and spontaneous IPSCs within 7 to 10 min, in seven

Laboratoire de Neurobiologie Cellulaire, INSERM U261, Département des Biotechnologies, Institut Pasteur, 25 Rue du Dr. Roux, 75724 Paris Cedex 15, France.