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MODIFICATION OF BRAIN ACETYLCHOLINE RELEASE BY MORPHINE AND ITS ANTAGONISTS IN NORMAL AND MORPHINE-DEPENDENT RATS

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1 The spontaneous release of acetylcholine (ACh) from the cerebral cortex of control and morphine-dependent rats was investigated. The rate of resting output of ACh in morphine-dependent animals was lower than that in the control animals.

2 Administration of naloxone and nalorphine to morphine-dependent rats was followed by a significant rise in the release of cortical ACh. In control rats no such increase in the release of ACh occurred after similar injections of narcotic antagonists.

3 Injections of morphine produced a consistent decrease in the rate of spontaneous release of cortical ACh in the control rats, but similar injections in the dependent rats did not produce a decrease in the rate of cortical ACh release.

4 The relevance of these results with regard to development of the narcotic abstinence syndrome is discussed.

Introduction

Repeated administration of morphine and related narcotic analgesics leads to the development of tolerance and physical dependence. The physical dependence on these drugs is unique in that its presence can be easily demonstrated by the injection of a narcotic antagonist which precipitates an abstinence syndrome. It is known that this syndrome represents a state of hyperexcitability in the neurones of the central nervous system (CNS), and its development is associated with increased parasympathetic and sympathetic activity (Seevers & Deneau, 1963). However, the mechanisms which underly the development of the narcotic abstinence syndrome are complex and are only poorly understood. In view of the rapidity with which this syndrome develops after the administration of a narcotic antagonist to a dependent animal, it is probable that its development may be associated with a sudden increase in the release of an excitatory neurotransmitter, whose release has perhaps been previously suppressed by morphine.

Many investigators in the past have demonstrated that morphine impairs the release of acetylcholine (ACh) at cholinergic sites in the guinea-pig ileum (Paton, 1957; Schaumann, 1957), autonomic ganglia (Pelikan, 1960) and the neuromuscular junction (Pinsky & Fredrickson, 1971). Recently it was shown that injections of morphine and related agonists inhibit the release of ACh from cholinergic nerve endings in the cerebral cortex (Jhamandas, Pinsky & Phillis, 1970, 1971). In the latter study, it was found that if the narcotic antagonist naloxone was administered after a reduction in the release of ACh from the cortex produced by an injection of morphine, there was a rapid reversal of the action of morphine resulting in an increased output of ACh from the cerebral cortex. The investigation described here was carried out to test the possibility that the administration of a narcotic antagonist to morphine-dependent animals might result in an increased liberation of ACh from the cholinergic neurones in the CNS. It is conceivable that such an increased release of ACh may contribute to the development of the narcotic withdrawal syndrome precipitated by a narcotic antagonist (Paton, 1963; Crossland, 1970). In this paper we describe the changes in the spontaneous release of ACh from the cerebral cortex of normal and morphine-dependent rats after injection of naloxone and nalorphine in doses comparable to those which precipitate the withdrawal syndrome.

Methods

Production of dependence

Sprague-Dawley rats weighing 60-80 g were injected with morphine sulphate (20 mg/kg, i.p.) twice daily and given full access to food (Rockland

Complete Rat Diet) and water. The dose of morphine sulphate was tripled every five days until the animals were receiving 300 mg/kg. They were maintained on this dose for three weeks and subsequently used in the release experiments. Control animals were maintained under similar conditions but they received injections of 0.9% NaCl solution (saline, 0.5 ml, i.p.) instead of morphine.

Cortical acetylcholine release

The animal was anaesthetized lightly by intraperitoneal injection of 0.2 ml/100 g of a mixture of pentobarbitone sodium (30 mg/kg) and urethane (400 mg/kg). The trachea was cannulated in all animals and the femoral vein in some animals. The rat was placed in a stereotaxic head holder and the muscle, bone and dura overlying the cerebral cortex were removed on both sides. Two small cylindrical perspex cups of 2.5 mm^2 cross-sectional area were placed on the two parietal cortices. A paraffin seal between the cylinder surface and cortex prevented the leakage of the cup fluid. All exposed areas were covered with a 2-3 mm thick layer of 4% agar in saline. Both cups were filled with 0.2 ml of Ringer-Locke solution (mM): NaCl 154, KCl 5.63, $CaCl_2$ 2.16, $NaHCO_3$ 2.36, glucose 11; maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The solution, which also contained neostigmine bromide (50 μg/ml) and atropine sulphate (0.5 μg/ml), was left in contact with the cortex for 30 minutes. Collections were made at intervals of 10 min, three such collections were pooled for the assay of ACh. The collection during the first period of 30 min was rejected. Drugs were administered either intraperitoneally or intravenously and a 5-min wash period was allowed after each drug injection. The animals were maintained at 37° C by a thermal blanket.

Bioassay for acetylcholine

The solutions collected from the cerebral cortex were assayed on the hearts of *Mercenaria mercenaria* (Jhamandas *et al.*, 1971) obtained from Pacific Biomarine Supply Co., Venice, California.

Drugs

Drugs used were morphine sulphate, nalorphine hydrochloride, naloxone hydrochloride, atropine sulphate, and neostigmine bromide. All drug solutions were prepared by dissolving the drug in 0.9% w/v NaCl solution (saline). Weights of drugs are expressed as their salts.

Results

Resting release of acetylcholine

The resting output of ACh from the cortex varied considerably between different experiments. Preliminary experiments showed that it remained relatively constant in successive collections during any one experiment for at least 3 hours. When

Table 1 Effects of naloxone on the release of acetylcholine (ACh) from the cerebral cortex of control and morphine-dependent rats.

(a)

		Cortical ACh release during 30 min (ng/2.5 mm^2)			
Type of animal	*n**	*Pre-drug release†*	*Naloxone 1 mg/kg, i.p.*	*Naloxone 2 mg/kg, i.p.*	*Morphine 10 mg/kg, i.p.*
Control rats	4	5.45 ± 0.68	6.08 ± 0.45^{NS}	7.43 ± 1.00^{NS}	7.65 ± 0.68^{NS}
Dependent rats	6	3.33 ± 0.65	5.63 ± 0.95^{1}	7.47 ± 0.90^{3}	7.61 ± 0.90^{1}

(b)

Type of animal	*n**	*Pre-drug release†*	*Naloxone 0.2 mg/kg, i.v.*	*Naloxone 0.5 mg/kg, i.v.*	*Morphine 2.5 mg/kg, i.v.*
Control rats	6	3.42 ± 0.68	4.32 ± 0.99^{NS}	3.38 ± 0.72^{NS}	7.61 ± 1.13^{1}
Dependent rats	6	0.90 ± 0.05	2.88 ± 0.14^{3}	2.93 ± 0.99^{2}	3.69 ± 0.32^{3}

* *n* = number of cortical hemispheres.
† Control release represents release during 30 min period immediately preceding drug administration. Mean values ± s.e. Significance of difference from pre-drug values (*P*), ^{1} < 0.05; ^{2} < 0.01; ^{3} < 0.001; NS not significant. Drugs were injected at 30 min intervals. Post-drug collections began 5 min after injection.

drugs were investigated for their effect on ACh release, the total collection period did not exceed 3 hours. During a period of 30 min, pre-drug release from 22 cortices of control animals (5.12 ± 0.83 ng/2.5 mm^2, range 3.42-7.38) was significantly higher ($P < 0.05$) than the average resting release from 26 cortices of morphine-dependent rats (1.54 ± 0.47 ng/2.5 mm^2, range 0.77-3.33).

Effect of narcotic antagonists on acetylcholine release

Naloxone. In control rats, naloxone (1-2 mg/kg, i.p.) did not produce a significant rise in the release of ACh over the pre-drug levels (Table 1a). Morphine (10 mg/kg, i.p.) given after naloxone did not modify the rate of cortical ACh release. In morphine-dependent animals on the other hand, injection of naloxone in the same doses resulted in an increase in the output of ACh which was almost doubled. Morphine, 10 mg/kg, did not modify the release of ACh in these rats any further.

When naloxone was administered intravenously in smaller doses (0.2-0.5 mg/kg), the release of ACh in the control rats was not significantly altered (Table 1b). When morphine (2.5 mg/kg) was injected intravenously after naloxone, the rate of ACh release in the control rats was increased to twice the rate of the pre-drug release. In the dependent animals, intravenous administration of naloxone in the same doses resulted in a three-fold rise in the rate of release of ACh over the pre-drug values. Morphine (2.5 mg/kg, i.v.) after naloxone produced an additional increase in the rate of ACh release which was now four times greater than the pre-drug release.

Nalorphine. In control rats, nalorphine had no significant effect (Table 2). In morphine-dependent animals, nalorphine (5-10 mg/kg, i.p.) produced an increase in the release of ACh. Administration of morphine (10 mg/kg, i.p.) to control or dependent animals which had received nalorphine, had no significant effect on the output of ACh.

Effect of morphine on acetylcholine release

In control animals, intraperitoneal injection of morphine (10 mg/kg) reduced the ACh release to less than half the value of the resting release (Table 3a). Naloxone (1 mg/kg, i.p.) given after morphine not only restored the release of ACh but increased it to almost twice the pre-drug level. In the dependent animals, the injection of morphine (10 mg/kg, i.p.) did not reduce the already low rate of release of ACh. Naloxone (1 mg/kg, i.p.), given after morphine, however, significantly increased the release. When the morphine injection was repeated after naloxone treatment, there was no significant reduction in the release of ACh indicating a blockade of the morphine effect by naloxone.

Intravenous injection of morphine (2.5 mg/kg) in the control rats produced a marked decrease in the release of cortical ACh (Table 3b). Injection of naloxone (0.2 mg/kg, i.v.) after morphine restored the release of ACh to pre-drug levels. In the dependent rats, however, morphine failed to cause a reduction in the release of ACh. Administration of naloxone (0.2 mg/kg, i.v.) following morphine resulted in a rise in the release of ACh to a value almost three times higher than the pre-drug levels. After naloxone treatment, morphine (2.5 mg/kg, i.v.) caused only a small decline in the release of ACh to a level which was still twice as high as the pre-drug level.

Discussion

The rate of resting or pre-drug release of ACh from the parietal cortex in the control rats is similar to

Table 2 Effects of nalorphine on the release of acetylcholine (ACh) from the cerebral cortex of control and morphine-dependent rats.

		Cortical ACh release during 30 min (ng/2.5 mm^2)			
Type of animal	*n**	*Pre-drug release†*	*Nalorphine (5 mg/kg; i.p.)*	*Nalorphine (10 mg/kg; i.p.)*	*Morphine (10 mg/kg; i.p.)*
Control rats	4	6.26 ± 1.40	4.73 ± 0.77^{NS}	6.03 ± 1.80^{NS}	4.73 ± 0.90^{NS}
Dependent rats	6	1.13 ± 0.10	1.62 ± 0.18^{2}	2.12 ± 0.36^{1}	1.67 ± 0.36^{1}

* n = number of cortical hemispheres.

† Control release represents release during 30 min period immediately preceding drug administration. Mean values ± s.e. Significance of difference from pre-drug values (P), $^{1} < 0.05$; $^{2} < 0.01$; NS not significant. Drugs were injected at 30 min intervals. Post-drug collections began 5 min after injection.

the release of ACh from the cerebral cortex *in vivo* previously reported by Hemsworth & Neal (1968). Our experiments showed that chronic treatment with morphine caused a significant reduction in the resting output of ACh. Other investigators have shown that chronic administration of morphine to mice (Hano, Kaneto, Kakunaga & Moribayashi, 1964) and to rats (Large & Milton, 1970) does not lower the ACh content of the whole brain. Furthermore, Datta, Thal & Wajda (1971) reported that chronic treatment with morphine does not alter the activity of cortical or thalamic choline acetyltransferase, an enzyme responsible for the synthesis of ACh. On the other hand, morphine inhibits the activity of cholinesterase (Johannesson, 1962; Hein & Powell, 1967). It would therefore appear that the diminished rate of release of ACh observed by us was probably not due to a decreased synthesis or increased degradation of brain ACh. Such a decrease in release could be due to an impairment of the neuronal mechanisms responsible for the release from the cortical nerve endings. This possibility is supported by the experiments of Schuberth & Sundwall (1967) who showed that narcotic analgesics and ACh compete for membrane transport.

A significant observation was that the administration of narcotic antagonists, naloxone and nalorphine, to morphine-dependent animals led to an immediate sharp increase in the output of brain ACh in the 30 min period following their injection. This time period closely parallels the period during which the precipitated abstinence syndrome develops in a morphine-dependent rat. Kerr & Pozuelo (1971) have recently shown that in the morphine-dependent rat, injections of naloxone or nalorphine precipitate the withdrawal syndrome within 5 min and it persists for 25 to 30 minutes. Experiments on the withdrawal syndrome precipitated by naloxone (1 mg/kg, i.p.) showed a similar result (unpublished observations). Furthermore, the doses of nalorphine and naloxone that in our experiments produced an increase in the release of brain ACh in the dependent rats, corresponded very closely to those which precipitated the abstinence syndrome in morphine-dependent rats (Kerr & Pozuelo, 1971; Collier, Francis & Schneider, 1972). It therefore appears likely that an increased release of brain ACh occurs during precipitated narcotic abstinence in the rat and possibly in other species. The basic mechanisms which underly this rise in the release of ACh are still unknown, but it is possible that this effect might result from the displacement of morphine from the morphine receptor sites at the cholinergic nerves in the CNS and thus could cause a 'flooding' of the cholinoceptive sites.

Although morphine impaired the release of cortical ACh in normal rats the same dose of morphine administered to animals dependent on this drug did not impair the release of ACh. This observation suggests that tolerance has developed, a suggestion supported by the findings of Hano *et al.* (1964) in mice, and of Large & Milton (1970) in rats. These workers showed that, although acute injection of morphine in normal animals caused an increase in the ACh content of the whole brain, the injection of the same dose of morphine did not raise the content of brain ACh in morphine-

Table 3 Effect of morphine on the release of acetylcholine (ACh) from the cerebral cortex of the control and morphine-dependent rats.

(a)

		Cortical ACh release during 30 min (ng/2.5 mm²)			
Type of animal	*n**	*Pre-drug release†*	*Morphine (10 mg/kg; i.p.)*	*Naloxone (1 mg/kg; i.p.)*	*Morphine (10 mg/kg; i.p.)*
Control rats	4	3.06 ± 0.18	1.22 ± 0.41[2]	6.30 ± 0.32[2]	6.08 ± 0.23[2]
Dependent rats	4	0.77 ± 0.09	0.68 ± 0.09[NS]	1.94 ± 0.50[1]	1.67 ± 0.36[1]

(b)

Type of animal	*n**	*Pre-drug release†*	*Morphine (2.5 mg/kg; i.v.)*	*Naloxone (0.2 mg/kg; i.v.)*	*Naloxone (2.5 mg/kg; i.v.)*
Control rats	4	7.38 ± 0.54	1.44 ± 0.18[2]	7.34 ± 0.23[NS]	4.82 ± 0.22[1]
Dependent rats	4	1.58 ± 0.09	2.16 ± 0.14[NS]	4.28 ± 0.32[2]	3.15 ± 0.27[1]

* n = number of cortical hemispheres.

† Control release represents release during 30 min period immediately preceding drug administration. Mean values ± s.e. Significance of difference from pre-drug values (P), [1] < 0.05; [2] < 0.01; NS not significant. Drugs were injected at 30 min intervals. Post-drug collections began 5 min after injection.

dependent animals. It would seem that the increase of brain ACh levels resulting from the acute administration of morphine is predominantly a result of the decreased neuronal release.

In some experiments when morphine was administered intravenously after treatment with two doses of naloxone, it produced a rise in the release of cerebral ACh. In other experiments the same dose of morphine given intravenously before naloxone, produced a consistent fall in the output of brain ACh. The significance of this effect is not clear at present and further experiments are needed to establish its possible mechanism.

The present study shows that the interaction of morphine and the narcotic antagonists with the central cholinergic system provides a suitable model for the study of neurohumoral mechanisms that may be involved in narcotic tolerance and physical dependence. In some of the theories proposed to explain these phenomena there is a suggestion that chronic morphine administration may alter the synthesis or the function of a hypothetical excitatory neurohormone which participates in neuronal function (Goldstein & Goldstein, 1961; Shuster, 1961). The removal of morphine would lead to an unbalanced excess of this neurohormone and the production of the abstinence syndrome. The work reported here lends some support to these theories of dependence, in that alterations in the release of the neurotransmitter, acetylcholine, may have an important role to play in the development of narcotic tolerance and physical dependence.

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