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In vivo RECEPTOR BINDING OF THE OPIATE PARTIAL AGONIST, BUPRENORPHINE, CORRELATED WITH ITS AGONISTIC AND ANTAGONISTIC ACTIONS

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1 In order to gain more insight into the mechanisms behind the actions of opiate partial agonists, an analysis of the dual agonist/antagonist properties of the partial agonist, buprenorphine, was made in conjunction with *in vivo* binding studies of the drug in the rat.

2 Buprenorphine revealed a bell-shaped dose-response curve for antinociception peaking at approx. 0.5 mg/kg subcutaneously. It antagonized morphine antinociception at doses which normally have agonistic effects and produced maximum antagonistic effects at doses above those having prominent agonistic activity. The withdrawal precipitating potency of buprenorphine as measured in highly morphine-dependent rats was present at doses above those having agonistic activity. The entire dose-response curve for buprenorphine was shifted symmetrically to the right by the opiate antagonist, naltrexone.

3 The dose-dependent occupation of receptors *in vivo* by buprenorphine seemed to be almost complete over the agonist dosage range; almost no further receptor occupation over the antagonist range was seen.

4 The possibility is discussed that site-to-site receptor interactions leading to cooperativity of effect may be the best explanation of these results.

Introduction

A wealth of information is now available about opiate receptors, in particular with regard to quantity, distribution and affinity. However, biochemical data concerning the connection between the occupation of these receptors and opiate effects is still very restricted. Binding studies alone have only limited usefulness in this context and, therefore, it is necessary to resort to other methods, such as behavioural studies, which, especially when done in conjunction with *in vivo* binding methods, can provide more insight into the translation of receptor occupation into pharmacological action.

Opiate partial agonists present an excellent opportunity for such studies because they show varying degrees of agonist and antagonist activity, depending upon dosage. The partial agonist, buprenorphine, is a particularly useful tool in this respect since it has unusually clear morphine-like agonist effects at rather low dosages and only minimal agonist effects at higher dosages (Cowan, Lewis & Mac Farlane 1977a; Cowan, Doxey & Harry 1977b). However, in contrast, the antagonist properties of the drug are less clearly established. Buprenorphine is unable to precipitate withdrawal in morphine-dependent rats and possesses only limited efficacy in antagonizing morphine-analgesia in rats and mice (Cowan *et al.*, 1977a). It is important to define more clearly the extent of the antagonist properties of buprenorphine and, in particular, the dose-range within which they occur, in order to establish the relationship between the agonist and antagonist effects of the drug, which, in turn, reflect the degree of activation of the opiate receptor upon occupation by the substance.

The purpose of this paper is to compare the dose-ranges of the agonist and antagonist actions of buprenorphine, in conjunction with a study of the binding of the drug *in vivo* over these dosages. The results should help clarify the nature of the biochemical mechanism responsible for the decline in the agonist action of buprenorphine at high doses.

Methods

Animals and chronic drug treatment

White, male Sprague-Dawley rats were housed six to a box and kept at least one week before the beginning of experiments. Animals weighed approx. 250 g (230–270 g) at the time of testing, except for *in vivo*

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binding studies and for the antinociceptive experiments accompanying them for which rats weighed 150 g. Smaller rats were used in the latter experiments in order to conserve [^{3}H]-buprenorphine. Experiments were also performed with rats made tolerant to buprenorphine by treatment twice daily for 4 days with the dose of the drug having peak antinociceptive activity (0.5 mg/kg, s.c.). On the 5th day, animals showed no antinociceptive response to even higher doses of the drug (see also Dum, Bläsig & Herz, 1981). To test the withdrawal-precipitating potency of buprenorphine, experiments were also performed in animals made highly tolerant-dependent on morphine. This was done by implanting pellets (containing morphine 75 mg as a base and carrier substances), under ether anaesthesia, subcutaneously into the back of the rat according to the following schedule: 1 on the first day, 2 on the fourth day and 3 on the seventh day. Withdrawal was precipitated on day 10 (see Bläsig, Herz, Reinhold & Zieglgänsberger, 1973).

Measurement of antinociception

The antinociceptive effect of buprenorphine was measured by a slightly modified 'vocalization test' (Hoffmeister & Schlichting, 1968) whereby vocalization was induced by an electrical constant current stimulator (rectangular pulses, from 0.1–2 mA, frequency 50 Hz, duration 10 ms, delivered for 2 s) attached to the root of the tail by means of a bipolar electrode. Before treatment, the threshold of vocalization was 0.4 mA.

Measurement of withdrawal

Morphine tolerant/dependent animals (see above) were challenged with subcutaneous injections of increasing doses of buprenorphine and observed immediately afterwards for 1 h in separate, clear acrylic boxes (base 17 × 22 cm, height 23.5 cm) for selected signs of withdrawal. The number of jumps, writhes and wet dog shakes were counted. Scores of 1, 2 or 3 were given for scream on touch, or diarrhoea if the sign occurred during the first, second or all three of the 10 min periods of the first 30 min of observation, respectively. The presence or absence of various other signs of withdrawal such as teeth chattering, chewing, rhinorrhoea, ptosis and eye twitching were noted (see Bläsig, Herz, Reinhold & Zieglgänsberger, 1973 for details).

Measurement of in vivo *receptor occupation by buprenorphine*

Receptor binding of buprenorphine was traced *in vivo* by measuring the decrease in the rat brain of the amount of highly labelled [^{3}H]-buprenorphine in the presence of unlabelled buprenorphine. In experiments measuring the dose-dependent decrease, the radioactivity in the cerebellum was used as a measure of the amount of opiate not specifically bound, since it is known that this structure has only a few opiate receptors and since no reduction in radioactivity took place in this structure in the presence of high doses of unlabelled buprenorphine. This method is able to measure binding of opiates to brain tissue *in vivo* which correlates with pharmacological effects and which shows characteristics similar to those measured *in vitro* (Höllt & Herz, 1978). Rats were killed by decapitation 60 min after the simultaneous injection (i.v.) of labelled drug with saline (0.9% w/v NaCl solution) or doses of unlabelled buprenorphine. Brains were removed immediately and were either dissected as described previously (Glowinski & Iversen, 1966) or, in the dose-dependent displacement studies, simply divided into cerebellum and remaining brain. Parts were weighed and combusted in a Packard Tri-Carb Sample Oxidizer. The radioactivity in the tissue parts was measured by scintillation counting in a Packard Tri-Carb Scintillation Spectrometer with 40% efficiency.

Drugs

Buprenorphine hydrochloride was dissolved, with the help of a sonifier, in distilled water and injected in a volume of 1 ml/rat. Naltrexone hydrochloride was dissolved in saline and injected in a volume of 0.5 ml (i.p.). Dosages are given in terms of the base. [^{3}H]-buprenorphine (28 mCi/μmol) and unlabelled buprenorphine were gifts from Dr Rance, Reckitt and Colman, Ltd (Kingston-upon-Hull); morphine hydrochloride was purchased from Merck (Darmstadt, F.R.G.); naloxone hydrochloride was a gift from Dr Ferster, Endo Laboratories (Garden City, N.Y., U.S.A.).

Results

Antinociceptive action of buprenorphine

As seen in Figure 1a, a bell-shaped dose-response curve for the antinociceptive action of buprenorphine was obtained in the vocalization test in the rat. This bell-shape was seen when the antinociceptive response was measured at one time point, at 1 h after subcutaneous injection of the drug. When the entire time course of buprenorphine action was measured, however, high doses of the drug were also seen to have antinociceptive activity but at other times. Figure 1b shows the time course of action of three representative doses of buprenorphine. Whereas low doses have only one peak of antinociception, high

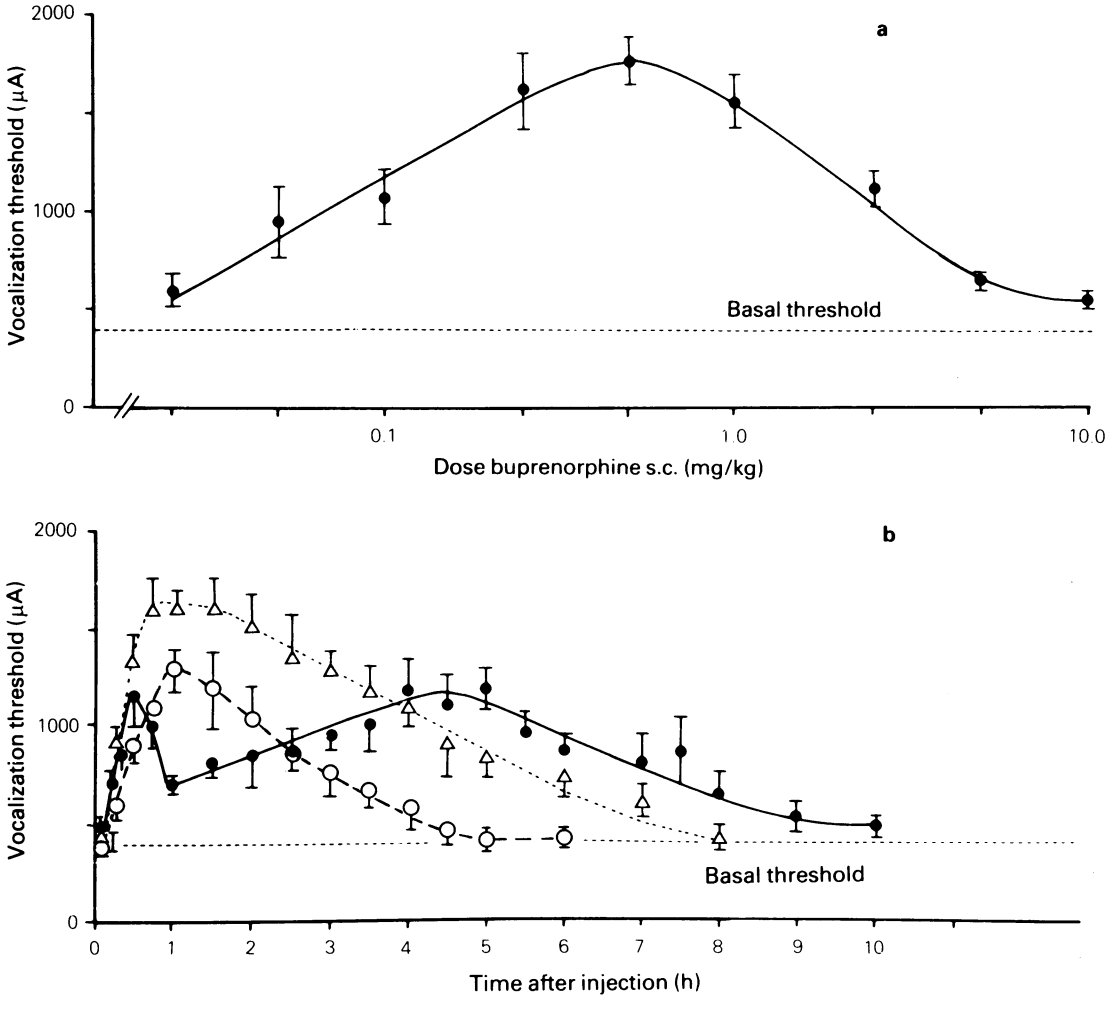

Figure 1 (a) The dose-response curve for buprenorphine antinociception in rats, measured 1 h after injection (s.c.) using the vocalization test. $n = 70$; vertical lines show s.e.mean. (b) The time courses of three representative doses of buprenorphine 0.1 mg/kg (s.c.) (○); 0.5 mg/kg (s.c.) (△); 5 mg/kg (s.c.) (●) $n = 8$/dose; vertical lines show s.e.mean.

doses have two, an early one, which is very sharp, and a much later one, which is less distinct and which appears at increasingly later times with increasing dose. In between the peaks, there is a dose-dependent decline in antinociception. The maximum antinociceptive effect of buprenorphine never exceeded, at any time point, that reached by 0.5 mg/kg, subcutaneously, at 1 h after injection.

The morphine-antagonizing action of buprenorphine

Lower doses of buprenorphine (0.01–0.05 mg/kg) were ineffective in reducing morphine antinociception, when injected up to 1 h beforehand, simultaneously with, or within 15 min after morphine injection (8 mg/kg, s.c., 45 min before test). To test the antagonist effect of higher doses of buprenorphine, which produce their own agonist effects, experiments were performed in animals chronically treated with 0.5 mg/kg (s.c.) buprenorphine, until tolerance developed, which took until the fifth day. The morphine dose-response curve was shifted by a factor of about 6 to the right in these animals (see Figure 2). An injection of 0.5 mg/kg (s.c.) buprenorphine 45 min before morphine, that is, 1.5 h before the antinociceptive test, shifted the morphine dose-response curve by an additional factor of 10 to the right. It must be emphasized that this effect is primarily due to antagonism and to the presence of residual pretreatment drug since the tolerance effect is ac-

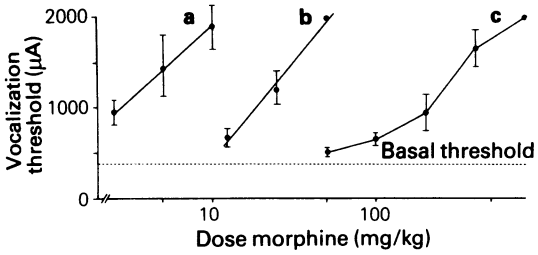

Figure 2 Buprenorphine antagonism of morphine antinociceptive effect in buprenorphine-pretreated rats, measured by the vocalization test. (a) Naive rats with no pretreatment; (b) rats pretreated with buprenorphine (0.5 mg/kg, s.c.) twice daily, 4 days, test made on day 5, 12 h after last buprenorphine injection; (c) rats pretreated in the same way with buprenorphine, test made on day 5, 1.5 h after the last buprenorphine injection. Animals were completely tolerant to the antinociceptive effect of the injection of buprenorphine. $n = 6$ rats/point; vertical lines show s.e.mean.

counted for in the morphine curve obtained in the same animals in the absence of buprenorphine. The antagonist efficacy of higher doses of buprenorphine could be tested in naive animals because such doses had little antinociceptive action of their own. The antagonist efficacy of this range of doses was very high: 10 mg/kg (s.c.), injected 45 min before morphine, completely blocked the antinociceptive effect of doses or morphine as high as 800 mg/kg (s.c.).

In order to test further the antagonist properties of buprenorphine, experiments were carried out to determine the withdrawal-precipitating capacity of the partial agonist in morphine-dependent rats. Rats were made highly tolerant/dependent on morphine by the implantation of morphine pellets (see Methods). As can be seen in Table 1, buprenorphine was able to precipitate withdrawal in these animals at doses above 1 mg/kg (s.c.), i.e. at doses above those having maximum antinociceptive efficacy. Screaming on touch, diarrhoea, writhing and wet dog shaking, which are characteristics of a low degree of withdrawal, were quite intense and continued for at least 1 h after injection of buprenorphine. Jumping, which is characteristic of more intense withdrawal, only occurred at low frequencies; it was not observed in all animals, and appeared only during the first 30 min (see Bläsing *et al.*, 1973 for a discussion of 'shift of signs' with intensity of withdrawal). A dose of 30 mg/kg seems to have the greatest withdrawal-precipitating potency, since jumping was almost exclusively initiated at this dose.

The antinociceptive effect of buprenorphine in the presence of naltrexone

Because buprenorphine has a long action time and dissociates only very slowly from its receptors (Hambrook & Rance, 1976; Cowan *et al.*, 1977a; Dum *et al.*, 1981), naltrexone was injected before buprenorphine to prevent rather than to reverse the buprenorphine effect. Naltrexone, rather than naloxone, was used because of its longer action time. An injection of 1.5 mg/kg (i.p.) naltrexone was found to shift the entire dose-response curve of buprenorphine symmetrically to the right. As a result, the agonist potency of some higher doses of buprenorphine was actually increased by the antagonist (see Figure 3).

Table 1 The precipitation of various signs of withdrawal by buprenorphine in morphine-dependent rats

Dose of buprenorphine (mg/kg)	*Number of counted signs*			*Score for checked signs*	
	Jumps	*Wet dog shakes*	*Writhes*	*Screaming on touch*	*Diarrhoea*
1	0 (0–0)	0.5 (0–2)	1.0 (0–5)	0 (0–1)	0.9 (0–2)
3	0 (0–0)	4.0 (0–14)	5.1 (0–18)	0.4 (0–2)	1.9 (1–3)
10	0 (0–1)	5.6 (1–10)	8 (0–25)	0.6 (0–3)	2.3 (1–3)
30	2.5 (0–11)	3.1 (0–7)	4.25 (0–16)	1.9 (0–3)	2.0 (1–3)
100	0.5 (0–2)	2.0 (0–6)	17.0 (5–33)	1.3 (0–3)	0.9 (0–2)

The average and the range of the number of counted signs and the average and the range of the checked signs precipitated within 0.5 h by different doses of buprenorphine (s.c.) in rats made highly dependent on morphine by the implantation (s.c.) of pellets containing morphine within 7 days; tests made 10 days after the first implantation. Scores of 1, 2 or 3 were given if the behaviour occurred during the first, the first and second or all three of the 10 min periods, respectively, of the 0.5 h observation time. $n = 8$/group.

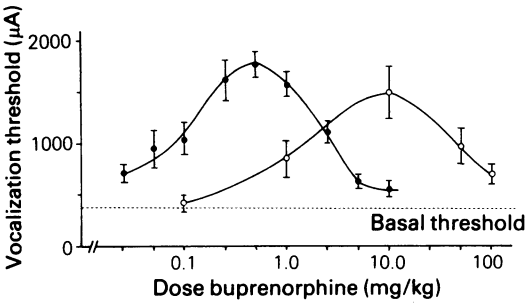

Figure 3 The shift of the dose-response curve of buprenorphine for antinociception in the rat by a single dose of naltrexone (1.5 mg/kg, i.p.), given 30 min before buprenorphine (s.c.). Vocalization test made 1 h after buprenorphine. 8 rats/point; vertical lines shows s.e.mean. (●) Buprenorphine injected alone; (○) buprenorphine injected after naltrexone.

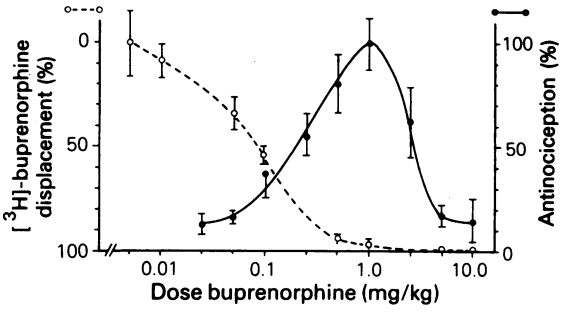

Figure 5 Comparison of reduction of receptor bound [^{3}H]-buprenorphine (○) in brain of rat by increasing doses of unlabelled buprenorphine to the antinociceptive effect (●) of the same doses of drug. Data normalized as to the percentage of maximum effect. Drugs injected (i.v.) 1 h before rats were killed or vocalization test for antinociception. $n = 8$ rats/point; vertical lines show s.e.mean. Slight difference from antinociceptive curve of Figure 1a probably results from differences in size of rats and in mode of injection.

In vivo *receptor occupation of buprenorphine in rat brain accompanying antinociceptive action*

The *in vivo* binding of [^{3}H]-buprenorphine in the rat brain was measured so that the receptor binding of the drug could be directly compared with the antinociceptive effect. As can be seen in Figure 4, the radioactivity found in the brain of the rats injected with [^{3}H]-buprenorphine was reduced in most brain parts by an injection of a high dose of unlabelled buprenorphine (10 mg/kg). The pattern of this reduction resembles that seen with other opiates using the same method (Höllt & Herz, 1978). There was no significant change in the level of radioactivity in the cerebellum, which has almost no opiate receptors, but considerable reduction in the hypothalamus, midbrain, brain stem, cortex and limbic structures was seen. In a second experiment, increasing doses of unlabelled buprenorphine were injected with [^{3}H]-buprenorphine in order to follow the gradual saturation of receptors by the drug. Since no change in the radioactivity in the cerebellum occurs with this procedure, the radioactivity in this structure was used as an internal standard for unspecific binding, controlling the pharmacokinetic effects (see Methods). A ratio of the radioactivity in the brain without cerebellum to that in the cerebellum was calculated. The maximum ratio that was observed, in the absence of unlabelled buprenorphine was about 3. The minimum ratio, found in the presence of 10 mg/kg (s.c.) unlabelled buprenorphine, was about 1.1. Figure 5 shows the correlation of the reduction of the ratio with the antinociceptive effect, both normalized. Suprisingly, a nearly complete saturation of receptors (about 95%) was measured at doses producing maximum antinociception, i.e. at the peak of the bell-shaped dose-response curve.

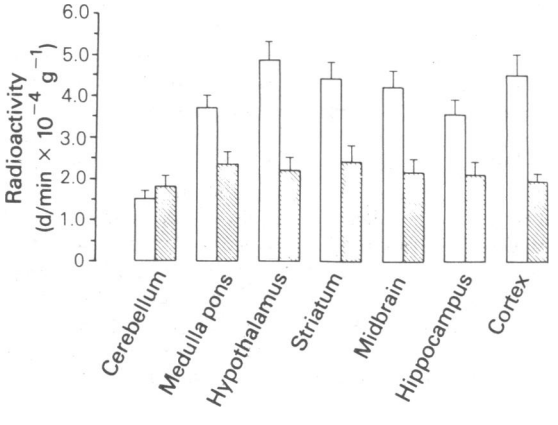

Figure 4 The reduction of radioactivity in different parts of the rat brain after injection of [^{3}H]-buprenorphine (5 μCi/rat) simultaneously with or without a large dose of unlabelled buprenorphine (10 mg/kg). Open columns = saline; hatched columns = unlabelled buprenorphine. Animals killed 1 h after injection (i.v.). $n = 8$/point; vertical lines show s.e.mean.

Discussion

The bell-shape of the dose-response curve of buprenorphine found in this study as well as in others (Cowan *et al.*, 1977a,b) implies that the drug has a range of concentrations at which it has a maximum antinociceptive effect, above or below which there is a reduction in activity. This is supported by changes found in the shape of the time course of the drug

effect with dose. As doses of buprenorphine were increased beyond the optimal range, the single peak of antinociception, found at about 1 h after injection, began to flatten and divide into two peaks, a sharper one occurring earlier, and a flatter one occurring at increasingly later times with increasing dosage. The occurrence of this second peak eliminates the possibility that the decrease in agonist effect at higher drug concentrations is the result of tachyphylaxis.

In between the two peaks, a dose-dependent decline in antinociceptive activity occurred. At the same time, the antagonist activity was found to increase, as measured both by the ability of the drug to block morphine antinociception and to precipitate withdrawal in highly tolerant-dependent rats. Thus, the bell-shaped dose-response curve reflects not just a decline in agonist activity at higher doses, but also a transformation of the activity of the drug into a primarily antagonist one. The antagonist efficacy becomes quite large, as 10 mg/kg buprenorphine can block the analgesic effect of doses of morphine as high as 800 mg/kg, although the maximum intensity of withdrawal precipitated is limited, as measured by the amount of withdrawal jumping. This may be due to some residual agonist action of the drug. The findings of Cowan *et al.* (1977a) that buprenorphine cannot precipitate withdrawal in rats may be explained by the fact that these authors used rats with a lower degree of tolerance/dependence.

The dual agonist-antagonist actions of buprenorphine both seem to stem from interactions with opiate receptors. As found for naloxone (Rance, Lord & Robinson, 1979), naltrexone was seen in this study to shift the entire dose-response curve, symmetrically, to the right. Thus opiate antagonists are able to affect receptors responsible for both the agonist and antagonist actions of buprenorphine. As also discussed by Rance *et al.* (1979), these findings also eliminate two possible explanations for the shape of the dose-response curve of the drug, namely that it is due to non-competitive auto-antagonism (Ariens, van Rossum & Simonis, 1957), and that it is due to a two-point attachment to the receptor (De Lean, Munson & Rodbard, 1979), because both theories predict a flattening, rather than a shifting in the dose-response curve in the presence of an antagonist. Since non-competitive auto-antagonism is a mechanism by which a drug would antagonize itself by binding at higher concentrations to a second receptor, blocking agonist action, the elimination of this explanation makes it unlikely that buprenorphine inhibits itself at higher doses by binding to a separate antagonist receptor.

Some aspects of dual action of buprenorphine can be explained in the context of the two state theory of the receptor, as it has been applied to explain the actions of partial agonist (see Ariens & De Miranda, 1979). This theory proposes that receptors exist in dynamic equilibrium between active and inactive forms, the former bound preferentially by agonists, the latter by antagonists and both bound to varying degrees by partial agonists. However, this theory predicts, in this simple form, that partial agonists would have monotonic increasing dose-response curves for agonist effects, since greater numbers of receptors, some of them in the activated form, would be occupied at higher drug concentration.

In an effort to gain more information about the buprenorphine-receptor interaction at a molecular level, the *in vivo* binding of the drug was measured. These experiments were able to trace a dose-dependent saturation of receptors by buprenorphine over the agonist range of the drug, so that about 95% saturation was reached at the dose causing maximal antinociceptive effect. Since buprenorphine has been reported to be an almost pure μ agonist (Martin, Eades, Thompson, Huppler & Gilbert, 1976), the majority of this binding is probably to these receptors, which have been associated with the production of antinociception, although they are important to other opiate effects as well (Schulz, Wüster & Herz, 1981). Almost no further receptor displacement could be measured over the antagonist range. A possible explanation for this would be that the receptor binding over this range is of a lower affinity. Such a possibility would not necessarily imply the existence of a separate population of receptors, especially as the data discussed above do not suggest the presence of separate antagonist receptors. A lower affinity for buprenorphine over the antagonist dose range might reflect a change in binding to an otherwise homogeneous group of receptors, at increasing occupancy, due to cooperativity.

Cooperative receptor interaction would also explain the unusual pharmacological action of buprenorphine, if the transition of the state of the opiate receptor from an inactive to an active state were restricted by the occupation of other receptors by buprenorphine. Recent evidence that opiate receptors cluster (Hazum, Chang & Cuatrecasas, 1979) makes the possibility of such a receptor-receptor interaction feasible. The extremely slow receptor kinetics of buprenorphine, mentioned earlier might also result from allosteric changes accompanying receptor occupation. Another explanation for the shape of the dose-response curve of buprenorphine is that it might be due to a slow, but high affinity binding to an inactive state of the opiate receptor. This would also fit with the slow kinetics of the drug. However, it would be inconsistent with the appearance of agonist effect at high doses, several hours after the time when lower doses reach their peak activity. The question remains as to whether or not cooperativity of opiate binding can be found by means of *in vitro* binding

assay methods, since such methods generally disturb the natural state of the receptor and it is difficult to distinguish active and inactive states, further pharmacological investigations with other partial opiate agonists may be more fruitful.

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