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The physiological relevance of low agonist affinity binding at opioid μ-receptors

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1 Inhibition constants (K_i) were determined for a range of opioid standards using two binding assays; [^{3}H]-[D-Ala^2, MePhe^4, Gly-ol^5]enkephalin ([^{3}H]-GLYOL) binding to guinea-pig brain membranes in HEPES buffer and [^{3}H]-naloxone binding to rat whole brain membranes in Krebs/HEPES buffer.

2 These values were compared with affinity measurements determined by antagonism of GLYOL on the rat isolated vas deferens preparation and by the receptor occlusion technique of Furchgott on the guinea-pig ileum longitudinal muscle, myenteric plexus preparation.

3 Agonists demonstrated markedly reduced binding affinity in the [^{3}H]-naloxone binding assay where binding was conducted in the presence of sodium.

4 A strong correlation was obtained between K_i values from the [^{3}H]-naloxone binding assay and affinity values determined in both isolated tissue preparations. K_i values obtained from [^{3}H]-GLYOL binding did not correlate well with affinity data determined by isolated tissue techniques.

5 These findings suggest that functionally relevant receptors exhibit low agonist affinity.

Introduction

It is now well established that opioid receptor binding is subject to regulation by both ions and nucleotides. As early as 1974 Pert & Snyder showed that the presence of sodium in the binding medium reduced the affinity of opiate agonists whilst having no effect on the binding of opiate antagonists. In 1978, Blume demonstrated that nucleotides, as well as ions, were capable of regulating opioid receptor binding, guanosine triphosphate (GTP) being shown to increase the rate of agonist dissociation from the opioid receptor. These effects have been reproduced by Carroll *et al.* (1984) in a μ-selective binding assay.

Ions and nucleotides are thought to induce a conformational change in the μ-opioid receptor (Simon *et al.*, 1975) converting between a 'high agonist affinity' conformation in the absence of sodium and GTP to a 'low agonist affinity' conformation in the presence of sodium and GTP. However, although it is now widely accepted that there are two conformations of the μ-opioid receptor there has been a lack of data concerning their physiological relevance.

Many early workers drew comparisons between binding affinities and pharmacological potency in order to verify the relevance of their binding assays. A highly significant correlation was found between the affinity of a range of narcotic analgesics, as determined by binding in the absence of sodium, and their analgesic potency (Stahl *et al.*, 1977). Similar findings have also been obtained by Pert & Snyder (1973a,b) although Pert *et al.* (1973) found that this correlation was strongest when binding was conducted in the presence of sodium. In addition, Stahl *et al.* (1977) found that, for antidiarrhoeal potency, a significant correlation was observed with binding to brain opioid receptors when the binding was carried out in sodium-containing medium. Binding in the presence of sodium was also found to correlate with the potency for inhibition of electrically induced contraction of the guinea-pig ileum preparation (Creese & Snyder, 1975). Thus the majority of early data, all based on comparisons with estimates of pharmacological potency, seem to implicate the 'low agonist affinity' conformation, as defined by binding in the presence of sodium, as the physiologically relevant conformation.

Unfortunately, potency is not affinity. There are

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many determinants of biological potency in addition to the drug-receptor affinity constant, and therefore ED_{50} and IC_{50} values do not necessarily reflect affinities. Bearing this in mind it is difficult to draw any firm conclusions about the relevance of μ-receptor conformations from the kind of experiments described above. In the absence of true affinity data it is not possible to discount binding in non-ionic buffers as physiologically irrelevant.

Carroll *et al.* (1984) described a series of experiments which compared binding in the presence and absence of sodium and the stable GTP analogue 5'-guanylylimidodiphosphate (GppNHp) with actual affinity values calculated from isolated tissue data. They reported that, for a range of μ-partial agonists, affinities calculated from binding experiments run in standard sodium-free buffer correlated poorly with antagonist equilibrium constants (K_e) determined by antagonism of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (GLYOL) on the rat vas deferens preparation. When these same K_e values were compared with affinities determined from binding in Krebs/HEPES buffer an excellent correlation was seen. These results would suggest that, for partial agonists at least, it is indeed the 'low agonist affinity' conformation that represents the relevant state of the μ-receptor.

However, according to the two state model proposed by Pert & Snyder (1974), the conformation to which antagonists bind is not necessarily the same as that to which agonists bind. On the basis that agonist and antagonist binding is differentially affected by sodium, they proposed that in the absence of sodium the opioid receptor takes on a conformation to which agonists bind preferentially. In the presence of sodium the formation of an antagonist-selective conformation is promoted. It would thus be of considerable interest to compare binding data with agonist affinities.

The purpose of the present study therefore, was to compare affinities determined from binding experiments, run both in the presence and absence of sodium and GppNHp, with affinity values calculated from both agonist and antagonist actions in functional assays. Affinities based on antagonism are easily determined on insensitive preparations such as the rat vas deferens (Smith & Rance, 1983) where μ-partial agonists behave as antagonists. Affinities can then be calculated by antagonism of a full agonist such as GLYOL. Affinities based on agonism are more difficult to measure. Nevertheless, with the availability of the irreversible μ-antagonist β-funaltrexamine (β-FNA) (Ward *et al.*, 1982), agonist affinities can be calculated (Porreca & Burks, 1983) by the method of Furchgott (1966). Such experiments should then allow firm conclusions as to the relevance of the two receptor conformations.

Methods

Rat vas deferens preparation

Vasa-deferentia were removed from Alderley Park rats (200–250 g) and suspended in 5 ml tissue baths containing Krebs solution of the following composition (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.18, $CaCl_2$ 2.52, and glucose 11.1, and bubbled with 95% O_2/5% CO_2. The upper end of the tissue was attached to an isotonic transducer under a tension of 0.4 g. Contractions were elicited by passing pulses (1 ms duration, 0.1 Hz at supramaximal voltage) between ring electrodes positioned above and below the tissue.

Guinea-pig ileum preparation

Guinea-pig ileum myenteric plexus longitudinal muscle strips were prepared according to the method of Paton & Zar (1968). These tissues were mounted and stimulated as described for the rat vas deferens, except that the tissues were suspended under a tension of 0.2 g.

Calculation of agonist affinities by the method of Furchgott

Agonist affinities were determined on the guinea-pig ileum myenteric plexus longitudinal muscle preparation. Agonist dose-response curves were constructed before and after addition of β-FNA (60 min incubation followed by 60 min continual washout). The concentration of β-FNA varied between 75 and 300 nM depending on the sensitivity of the tissue. Equi-effective agonist concentrations were determined. Agonist affinities were calculated according to the following theory:

$$\text{Response} = \text{f.e.} * [A]/\{[A] + \text{affinity}\}$$

where [A] = agonist conc, f = a function, e = efficacy, and for the same level of response in the presence of irreversible antagonist, provided f remains unchanged by the treatment:

$$\text{Response} = \text{f.e.q.} * [A']/\{[A'] + \text{affinity}\}$$

where [A'] = equi-effective agonist concentration and q = fraction of receptors remaining after partial receptor occlusion.

Therefore, for equi-effective agonist concentrations:

$$\{[A]/[A] + \text{affinity}\} = q \,.\, [A']/\{[A'] + \text{affinity}\}$$

which, rearranged gives:

$$1/[A] = 1/q[A'] + (1 - q)/q \,.\, \text{affinity}$$

Thus, a plot of 1/[A] against 1/[A'] yields a straight line where:

$$\text{slope} = 1/q$$

$$\text{intercept} = (1 - q)/q \cdot \text{affinity}$$

and affinity = (slope − 1)/intercept

The Furchgott technique is based on the assumption that the agonist has a single site of action. Thus, the method only gives a valid measure of affinity provided that the agonists used are sufficiently selective such that, even at the high concentrations needed to elicit a response in the presence of the irreversible antagonist, they continue to interact with a single receptor type. Thus, although the agonists used in this study are generally regarded as μ-receptor agonists it is questionable whether they continue to act solely at the μ-receptor following β-FNA pretreatment. It could be predicted however, that non-selectivity arising as a result of pretreatment with the irreversible antagonist would be recognisable since the double reciprocal plot of equi-active concentrations would not be a straight line. Errors arising from non-selectivity were minimized therefore, since affinity values were only determined from double reciprocal plots which showed no obvious deviation from linearity.

Calculation of antagonist equilibrium constants

For the calculation of antagonist equilibrium constants (K_e) both the rat vas deferens and the guinea-pig ileum preparations were used. K_e values were determined from their ability to antagonize the effects of GLYOL on these preparations.

Radioligand binding

Guinea-pig and rat whole brain membranes were prepared in 20 mM HEPES buffer (pH 7.4) and a modified Krebs HEPES buffer (pH 7.4) respectively according to the method of Magnan *et al.* (1982).

[^{3}H]-GLYOL binding assays were run for 40 min at 25°C, in HEPES buffer containing guinea-pig whole brain membranes and 1 nM [^{3}H]-GLYOL.

[^{3}H]-naloxone binding assays were run for 40 min at 25°C in Krebs/HEPES buffer containing rat whole brain membranes, 0.25 nM [^{3}H]-naloxone and 10 μM GppNHp.

Non-specific binding in both assays was defined using 10 μM naloxone.

Assays were terminated by filtration under vacuum through Whatman GFC filters followed by 2 × 5 ml washes with cold buffer.

IC_{50} values were determined from Hill plot analysis, and the data were expressed as K_i values according to Cheng & Prusoff (1973).

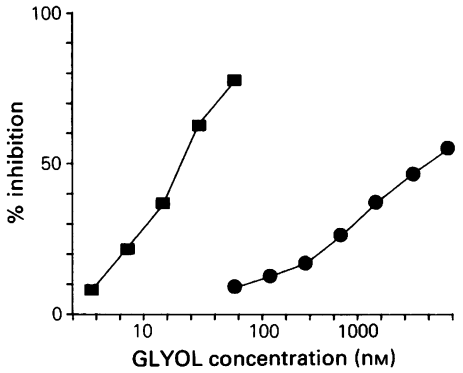

Figure 1 GLYOL dose-response curves on the guinea-pig ileum longitudinal muscle myenteric plexus preparation before (■) and after (●) incubation with 200 nM β-funaltrexamine.

Materials

Drugs were obtained from the following sources: ethylketocyclazocine, cyclazocine and pentazocine from Sterling Winthrop; bremazocine and tifluadom from Sandoz; naloxone, naltrexone and nalbuphine, Dupont; butorphanol, Bristol Laboratories; nalorphine, Wellcome; Mr2034 ((−)-α-(1R, 5R, 9R)-5,9-dimethyl-2-(L-tetrahydrofurfuryl)-2'-hydroxy-6,7-benzomorphan) and Mr1353 ((±)-α-5,9-dimethyl-2-(3-methylfurfuryl)-2'-hydroxy-6,7-benzomorphan), Boehringer Ingelheim; SKF10047 (N-allyl-normetazocine) National Institute for Drug Abuse; morphine and methadone, MacFarlane Smith; levallorphan, Roche; fentanyl, Janssen; etorphine, Reckitt and Colman; and [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin, [D-Met^2, Pro^5]enkephalinamide and β-funaltrexamine from Chemistry Department II, ICI Pharmaceuticals. Drugs were dissolved in distilled water with the addition of a few drops of 0.1 N HCl where necessary. Subsequent dilutions were made in Krebs solution.

Results

Figure 1 shows typical dose-response curves for GLYOL on the guinea-pig ileum. In the presence of β-FNA at 200 nM the curve was shifted to the right and showed a depressed maximum. These observations are consistent with the irreversible status of β-FNA. Equi-active concentrations were determined over the range of the dose-response curve and were expressed as a double reciprocal plot shown in Figure 2. As theory would predict, this shows a linear plot ($r = 0.99$) where (slope − 1)/intercept gives an affinity value for GLYOL of 1.11 μM. Agonist affinities were calculated in this way for a

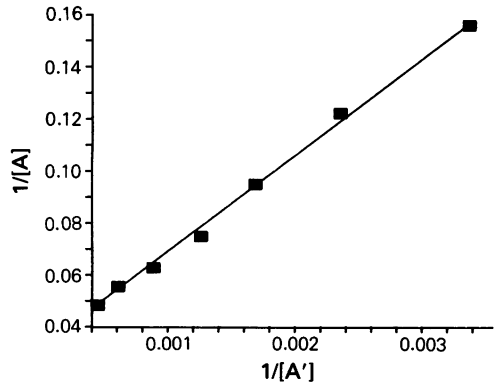

Figure 2 Double reciprocal plot of equi-effective GLYOL concentrations to inhibit electrically induced contraction in the guinea-pig ileum longitudinal muscle myenteric plexus preparation. [A] and [A'] represent agonist concentrations before and after incubation with 200 nM β-funaltrexamine respectively. Straight line fitted by least squares analysis.

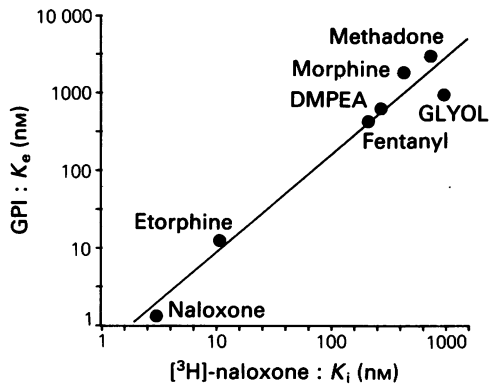

Figure 3 Relationship between the affinity of a range of opioid standards as determined on the guinea-pig ileum (GPI) preparation and by [^{3}H]-naloxone binding on rat whole brain membranes. Regression line by least squares analysis. $r = 0.98$; slope = 1.25. DMPEA = [D-Met^2, Pro^5]enkephalinamide.

range of opioid standards and Table 1 gives the mean affinity calculated from multiple determinations for each compound. Also shown in Table 1 is the affinity of naloxone calculated by antagonism of GLYOL on the guinea-pig ileum and the affinities of the same range of compounds determined by both [^{3}H]-GLYOL binding in HEPES buffer and [^{3}H]-naloxone binding in Krebs/HEPES buffer.

Values given in Table 1 are compared in Figures 3 and 4. Figure 3 shows a high degree of correlation ($r = 0.98$) between affinities determined on the guinea-pig ileum and those obtained from [^{3}H]-naloxone binding in Krebs/HEPES. This correlation was supported by a good agreement between values in absolute terms. For example, the agonist affinity of fentanyl measured on the guinea-pig ileum of 435 nM compared favourably with a K_i for the displacement of [^{3}H]-naloxone from rat brain whole membranes of 214 nM. On the other hand, Figure 4 shows a poor correlation between affinities determined on the guinea-pig ileum and those determined from [^{3}H]-GLYOL binding in HEPES ($r = 0.49$). No agreement exists between the absolute values. For example, the K_i for displacement of [^{3}H]-GLYOL from guinea-pig brain membranes by fentanyl was 1.67 nM compared to a value of 435 nM on the guinea-pig ileum.

Table 2 shows mean affinities determined from antagonism of GLYOL on the rat vas deferens, from

Table 1 A comparison of affinity values determined on the guinea-pig ileum (GPI) with [^{3}H]-naloxone binding in Krebs/HEPES and with [^{3}H]-GLYOL binding in HEPES

	Affinity (nM)		
Compound	K_e GPI	K_i [^{3}H]-*naloxone*	K_i [^{3}H]-*GLYOL*
GLYOL	936 ± 285	966 ± 93.4	2.84 ± 1.0
Morphine	1788 ± 617	416 ± 30.4	3.11 ± 0.56
Fentanyl	435 ± 124	214 ± 13.9	1.67 ± 0.22
Etorphine	12.9 ± 6.8	10.8 ± 0.74	0.55 ± 0.013
DMPEA[1]	645 ± 107	276 ± 25.4	0.27 ± 0.07
Methadone	2949 ± 1404	751 ± 76.5	7.14 ± 1.38
Naloxone	1.33 ± 0.2	3.0 ± 0.08	1.07 ± 0.19

All values are mean ± s.e. mean of multiple determinations ($n > 3$) and are expressed as nanomolar concentrations.
[1] DMPEA = [D-Met^2, Pro^5]enkephalinamide.

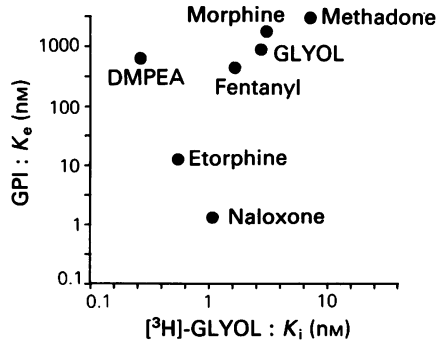

Figure 4 Relationship between the affinity of a range of opioid standards as determined on the guinea-pig ileum (GPI) preparation and by [^{3}H]-GLYOL binding on guinea-pig brain membranes. $r = 0.49$. DMPEA = [D-Met^2, Pro^5]enkephalinamide.

Table 2 A comparison of affinity values determined on the rat vas deferens (RVD) with [^{3}H]-naloxone binding in Krebs/HEPES and with [^{3}H]GLYOL binding in HEPES.

Compound	K_e RVD	K_i [^3H]-*naloxone*	K_i [^3H]-*GLYOL*
Naltrexone	3.1 ± 0.9	0.77 ± 0.03	—
Naloxone	3.9 ± 1.4	3.0 ± 0.08	1.07 ± 0.19
Bremazocine	4.5 ± 0.6	2.36 ± 0.19	1.13 ± 0.24
Levallorphan	10.9 ± 3.4	1.68 ± 0.14	1.05 ± 0.25
Cyclazocine	16.9 ± 6.1	2.5 ± 0.11	0.5 ± 0.04
Butorphanol	26.2 ± 4.9	4.96 ± 0.19	1.45 ± 0.57
Nalorphine	27 ± 6.9	19.5 ± 1.05	3.26 ± 0.54
Mr2034	27.4 ± 3.2	7.25 ± 0.74	0.58 ± 0.02
SKF10047	29.4 ± 7.3	9.82 ± 1.61	—
Nalbuphine	57 ± 5.1	16.7 ± 0.63	8.15 ± 2.9
EKC[1]	278 ± 49	224 ± 17.8	2.35 ± 0.2
Mr1353	349 ± 80	—	13.3 ± 0.6
Pentazocine	628 ± 233	270 ± 13	23.6 ± 4.4
Tifluadom	1100 ± 116	1960 ± 246	2.52 ± 0.08
Morphine	1500 ± 230	416 ± 30.4	3.11 ± 0.6

All values are mean ± s.e. mean of multiple determinations ($n > 3$) and are expressed as nanomolar concentrations.
[1] Ethylketocyclazocine.

[^{3}H]-GLYOL binding in HEPES and from [^{3}H]-naloxone binding in Krebs/HEPES. These data are compared in Figures 5 and 6. Figure 5 shows a high degree of correlation ($r = 0.96$) between affinities determined from [^{3}H]-naloxone binding in Krebs/HEPES and those determined from the rat vas deferens preparation. Again, this correlation is supported by excellent agreement between the absolute values. For example, the antagonist equilibrium constant for tifluadom on the rat vas deferens of

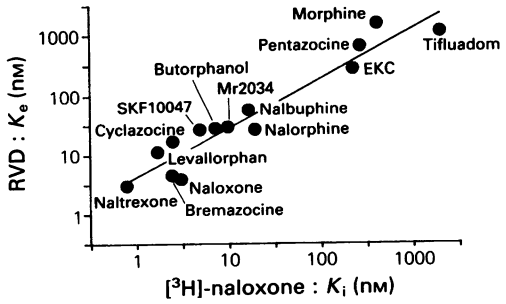

Figure 5 Relationship between the affinity of a range of opioid standards as determined on the rat vas deferens (RVD) preparation and by [^{3}H]-naloxone binding on rat whole brain membranes. Regression line by least squares analysis. $r = 0.96$; slope = 0.82. EKC = ethylketocyclazocine.

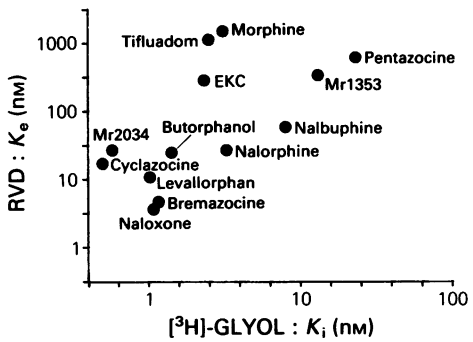

Figure 6 Relationship between the affinity of a range of opioid standards as determined on the rat vas deferens (RVD) preparation and by [^{3}H]GLYOL binding on guinea-pig brain membranes. $r = 0.66$.

1.1 μM compared favourably with the K_i for the displacement of [^{3}H]-naloxone from rat whole brain membranes by tifluadom of 1.9 μM. Figure 6 shows a poor correlation ($r = 0.66$) between the affinities determined from antagonism of GLYOL on the rat vas deferens and from [^{3}H]-GLYOL binding in HEPES. There is no agreement between the absolute values. The K_i for displacement of [^{3}H]-GLYOL from guinea-pig brain membranes by tifluadom of 2.5 nM differs from the antagonist equilibrium constant of tifluadom of 1.1 μM by several orders of magnitude.

Discussion

The main findings of this study are that affinities calculated from binding experiments run in the presence of sodium and GppNHp correlate significantly with affinities calculated on the basis of both agonism and antagonism on isolated tissues. These correlations are supported by good agreement between values in absolute terms. Such a close agreement in the actual values achieved by these different techniques indicates the correlation described is not simply fortuitous. No such similarities exist between data from functional assays and [^{3}H]-GLYOL binding in HEPES. These large discrepancies in absolute terms are reflected in Figures 4 and 5 where poor correlations are seen to exist between affinities determined in the absence of sodium and GppNHp and isolated tissue data. These findings have several important implications.

Firstly, it would appear that it is the low agonist affinity conformation of the μ-opioid receptor, as defined by binding in the presence of sodium and GppNHp, that represents the functionally relevant

receptor conformation. Consequently, if affinities calculated from binding experiments are to reflect physiological receptor interactions, then such experiments must be run in the presence of sodium and guanine nucleotides. The results of binding experiments run in the absence of sodium and GTP (Chang *et al.*, 1979; Kosterlitz *et al.*, 1980; Leslie *et al.*, 1980; Traynor *et al.*, 1982) should therefore be interpreted with caution. Furthermore, it is interesting to note that affinity values calculated from isolated tissue data are marginally but consistently larger than those calculated from binding in the presence of sodium and GppNHp (Tables 1 and 2). The most likely explanation for this is that the conditions under which the binding was carried out are unlikely to be truly physiological. The possible importance of other constituents to binding is yet to be established and their addition to the binding medium could well improve the correlations seen in Figures 3 and 5.

Secondly, the findings of this study are inconsistent with previous models proposed for the μ-opioid receptor. Based on the observation that sodium has no effect on the receptor binding of opiate antagonists but decreases the binding of opiate agonists (Pert *et al.*, 1973; Pert & Snyder 1974) Pert & Snyder postulated that the opiate receptor could exist in two interconvertible conformations, an agonist conformation which predominates in the absence of sodium and exhibits high agonist affinity, and an antagonist conformation, the formation of which is promoted by sodium and which exhibits low agonist affinity. In this model antagonists would bind preferentially to the antagonist or low agonist affinity conformation, thereby shifting the equilibrium of the two interconvertible receptor conformations and opposing the action of agonists. The present findings however, suggest that under physiological conditions both agonists and antagonists bind to the same receptor conformation, namely that of low agonist affinity. Such an arrangement is far more attractive in that antagonism can be described in terms of simple competition without the need to envisage shifts in the equilibrium between two receptor conformations.

What therefore does the high agonist affinity conformation of the μ-opioid receptor represent, since it does not appear to have a functional correlate? It is possible to suggest two explanations which are consistent with the results of this study.

As already discussed it would seem that the low agonist affinity conformation represents the functionally coupled form of the receptor and thus the conformation which is likely to be present under physiological conditions. However, under the unphysiological assay conditions in which the high affinity conformation has been demonstrated, it is not unreasonable to suggest that the receptor becomes uncoupled from its effector mechanism. It is possible that the high agonist affinity conformation of the receptor represents such an uncoupled form of the receptor.

However, from studies on the β-adrenoceptor, it is possible to suggest an alternative explanation. Kent *et al.* (1979) and De Lean *et al.* (1980) have observed that β-adrenoceptor antagonists bind to a homogeneous state of uniform affinity whereas β-adrenoceptor agonists manifest heterogeneous binding whereby two distinct binding sites are apparent, one of high affinity and one of low affinity. These workers found that the formation of the high affinity site was promoted by the presence of agonists, but unaffected by antagonists, whereas the formation of the low affinity site was promoted by guanine nucleotides. Thus, for the β-adrenoceptor at least, it is possible that a functional receptor interaction initiates a change which results in the formation of high affinity intermediate conformation of the receptor. Presumably, under physiological conditions, the existence of this high affinity intermediate would be short lived, guanine nucleotides promoting the regeneration of the low agonist affinity conformation and consequently agonist dissociation. However, under non-physiological conditions the absence of guanine nucleotides may stabilize this intermediate in some way.

Fundamental to both explanations is the fact that a functional interaction with the physiological conformation of the receptor is associated with the need for some energy input to the system in order to render the receptor capable of activating second messenger systems. It is likely therefore that this energy requirement will result in a change in apparent affinity. Thus, under physiological conditions, agonists, in inducing a functional response would bind with low affinity. On the other hand, under non-physiological conditions, where agonist binding is no longer associated with the same energy requiring processes they would bind with higher affinity. Antagonists, lacking the ability to induce functional responses, bind with high affinity irrespective of conditions.

Thus, it would seem that only compounds which possess intrinsic activity show reduced binding affinity under physiological conditions (i.e. show a 'sodium-shift') and therefore it seems likely that the magnitude of this sodium-shift is closely related to intrinsic activity. Indeed, Kent *et al.* (1979) have demonstrated that the ratio of dissociation constants of agonists for the high and low affinity states of the β-adrenoceptor correlates with an estimate of intrinsic activity based on ability to activate adenylate cyclase. Thus, comparisons of binding affinity data from assays run under both physiological and non-physiological conditions may provide a conve-

nient method for determining intrinsic activity without the inherent problems of isolated tissue or *in vivo* techniques.

In summary, therefore, it appears that it is the low agonist affinity conformation of the μ-opioid receptor which is functionally relevant and, under physiological conditions it is this conformation to which both agonists and antagonists bind. The nature of the high agonist affinity conformation is not clear. It may simply represent an uncoupled form of the receptor present under the non-physiological conditions used in many binding assays or alternatively, as evidence from other receptor systems would suggest, it may represent some short-lived intermediate conformation of the receptor formed as a consequence of a functional receptor interaction.

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