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Estimation of opioid receptor agonist dissociation constants with β-chlornaltrexamine, an irreversible ligand which also displays agonism

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1 The irreversible opioid receptor antagonist β-chlornaltrexamine (β-CNA) has been shown previously to have agonist activity in the guinea-pig ileum preparation. However, the receptor type or types mediating this effect have not been established.

2 In this study, the agonism of β-CNA was investigated by use of the competitive antagonist 16-methylcyprenorphine (RX8008M). Non-cumulative concentration-effect curves for β-CNA were displaced in a non-parallel fashion indicating that the agonism was mediated by both μ- and κ-receptors.

3 In principle, expression of agonism by an irreversible receptor antagonist could compromise its use in estimating agonist dissociation constants (pK_As) due to desensitization operating in addition to receptor inactivation. For κ-receptors, this possibility was checked by use of ethylketocyclazocine (EKC) to mimic the agonist effects of β-CNA and test whether subsequent EKC concentration-effect curves were displaced. For μ-receptors it was necessary to perform more involved experiments in which [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL) was used as a standard agonist and its pK_A was estimated under different conditions of β-CNA incubation.

4 These analyses indicated that neither the μ- nor the κ-receptor-mediated agonism of β-CNA was associated with appreciable receptor desensitization. In turn it was concluded that the usefulness of β-CNA as a pharmacological tool for the estimation of μ- and κ-opioid receptor agonist dissociation constants is not compromised by the agonist effects that the compound demonstrates at these receptors.

Introduction

In a previous study (Dougall & Leff, 1987) we used the irreversible opioid receptor antagonist β-chlornaltrexamine (β-CNA; Portoghese *et al.*, 1979) to estimate the affinity of the pentapeptide [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL) (Handa *et al.*, 1981) for μ-receptors in the guinea-pig ileum preparation. It was evident from these studies that β-CNA showed agonist activity, a phenomenon that has been reported previously in this tissue (Caruso *et al.*, 1979) as well as in the mouse vas deferens (Ward *et al.*, 1982). That this agonism is mediated by interaction with opioid receptors was suggested by its susceptibility to blockade by the reversible competitive antagonist, naloxone. However, the receptor type or types mediating the agonist activity of β-CNA have not been established. Since the guinea-pig ileum is generally accepted to possess both μ- and κ-opioid receptors (Lord *et al.*, 1977) either or both of these receptor systems may be involved. The ability of a compound such as β-CNA to stimulate as well as to alkylate opioid receptors irreversibly introduces potential problems to the process of agonist dissociation constant estimation. If the stimulus imparted to the system is large enough, receptor desensitization like other post-receptor interventions, may result in erroneous measurements of this parameter (Leff *et al.*, 1985; Eglen & Whiting, 1987).

The present paper describes an attempt to characterize the agonism of β-CNA in the guinea-pig ileum

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preparation and to assess if such agonism is associated with appreciable μ- and/or κ-receptor desensitization.

The results of this study are discussed with regard to their implications for the pharmacological estimation of opioid agonist dissociation constants.

Methods

Guinea-pig isolated coaxially-stimulated ileum

Male albino Dunkin-Hartley guinea-pigs (250–400 g) were killed by cervical dislocation. The terminal ileum was removed and the 10 cm section closest to the ileocaecal junction discarded. Approximately 2 cm portions of ileum were cleared of adherent tissue and the contents gently flushed out before being transferred to 20 ml organ baths containing modified Krebs solution of the following composition (mM): NaCl 118.41, NaHCO 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10, $CaCl_2$ 2.50. This was maintained at 37°C and continually gassed with 95% O_2 and 5% CO_2. The upper end of the tissue was attached by cotton thread to a Grass FT03C force-displacement transducer, the bottom end being tied to the tissue holder. Contractions of the tissue were elicited by coaxial supramaximal stimulation (0.5 ms duration, 0.1 Hz, 20 V). Changes in isometric force were recorded on Gould BS272 pen recorders.

Experimental protocols

General At the beginning of each experiment, a force of 1.0 g was applied to each tissue. This was followed by a 30 min stabilization period before electrical stimulation was started (unless indicated otherwise). At the end of the stabilization period the tension was re-instated. Reproducible responses to supramaximal electrical stimulation were established after about 45 min.

DAGOL and ethylketocyclazocine (EKC) concentration-effect, E/[A], curves were constructed by cumulative additions of agonist at $0.5 \log_{10}$ unit increments.

In experiments involving the above agonists only a single E/[A] curve was obtained in each tissue, therefore the number of replicates refers to the number of preparations.

β-CNA E/[A] curves were constructed by applying a single dose to each of six tissues from the same animal such that a single E/[A] curve consisted of six points increasing in $0.5 \log_{10}$ unit increments. Peak responses were measured. It was necessary to construct the curves in this manner to minimize the possibility of irreversible alkylation of opioid receptors attenuating the agonist response. Responses were recorded as percentage inhibitions of the stimulated twitch.

Competitive antagonist studies Tissues were exposed to 16-methylcyprenorphine (RX8008M) (Smith, 1987) for 40 min before the construction of E/[A] curves. DAGOL and EKC curves were constructed cumulatively, β-CNA curves were established as outlined above.

EKC desensitization studies Tissues were exposed to 3×10^{-9} M EKC for 30 min after which the agonist was removed by several changes of the organ bath Krebs solution. EKC E/[A] curves were then constructed cumulatively.

Irreversible receptor inactivation Two conditions of incubation with β-CNA were employed. In the first, tissues were incubated with either 20 nM or 100 nM β-CNA for 30 min, after which excess inhibitor was removed by several changes of the organ bath Krebs solution. DAGOL E/[A] curves were then constructed cumulatively. In the second, tissues were incubated with 2 nM β-CNA for 300 min. In these latter experiments electrical stimulation of the tissues was carried out for a comparable time period to that employed in the former experiments. After removal of excess β-CNA, DAGOL E/[A] curves were constructed cumulatively.

Analysis of data

Each individual set of E/[A] curve data, recorded as percentage inhibitions of twitch were fitted to a logistic function of the form:

$$E = \frac{\alpha[A]^m}{[A_{50}]^m + [A]^m} \quad (1)$$

In which E and [A] are the pharmacological effect and the concentration of the agonist, respectively; α, $[A_{50}]$ and m are the asymptote, location and slope parameters, respectively. Location parameters were actually estimated as logarithms. For the analysis of competitive interactions, this fitting procedure also performed one-way analyses of variance comparing computed slope and asymptote parameters between and within treatment groups. Further analysis of competitive antagonism was performed by fitting computed $\log_{10}[A_{50}]$ values to the following linear form of the Schild equation (Trist & Leff, 1985):

$$\log_{10}[A_{50}] = \log_{10}[A_{50}^c] + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

in which $[A_{50}^c]$ is a control $[A_{50}]$ value, [B] is the concentration of antagonist, K_B is its dissociation

constant and n is equivalent to the Schild plot slope parameter (unity for simple competition). When n was not significantly different from unity, it was constrained to this value in order to estimate $pK_B(-\log_{10} K_B)$.

Operational model fitting

Concentration-effect curve data obtained in experiments in which β-CNA was used to occlude μ-receptors irreversibly were fitted according to the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (3)$$

in which K_A is the agonist dissociation constant, τ is the efficacy of the agonist in a particular tissue, E_m is the maximum possible effect in the receptor system and n determines the sensitivity of the occupancy-effect relation.

Drugs

Ethylketocyclazocine methanesulphonate (Sterling Winthrop Research Institute, Rensselaer, New York); DAGOL acetate (prepared by Mr L. Lowe, Medicinal Chemistry Department, Wellcome Research Laboratories, Beckenham, Kent); β-CNA dihydrochloride and 16-methylcyprenorphine hydrochloride (both prepared by Dr S. Wilkinson, Medicinal Chemistry Department, Wellcome Research Laboratories, Beckenham, Kent) were used. Analysis of the sample of β-CNA revealed that it did not contain the epimer α-CNA, an important consideration since this compound has previously been shown to elicit profound agonism in the guinea-pig ileum (Sayre *et al.*, 1983) (see Discussion).

16-Methylcyprenorphine was dissolved in distilled water with the addition of a few drops of 1 M HCl. All other drugs were dissolved and diluted in distilled water.

Results

Comparison of the agonism of β-chlornaltrexamine with that of standard μ- and κ-receptor agonists

Figure 1 illustrates the agonist effects of a standard μ-agonist (DAGOL), a standard κ-agonist (EKC) and β-CNA on the guinea-pig ileum preparation. The degree of agonism produced by the three compounds is comparable. Since DAGOL and EKC are known to behave as full agonists in this tissue, β-CNA could be behaving as a full μ-agonist, a full κ-agonist or a mixed receptor agonist.

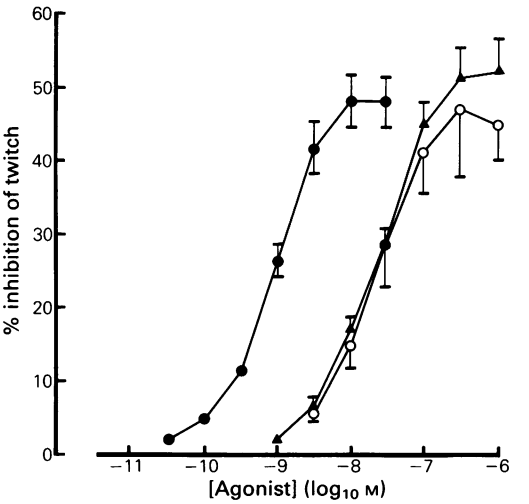

Figure 1 Percentage inhibitions of twitch produced by ethylketocyclazocine (●), [D,Ala²-MePhe⁴,Gly-ol⁵]enkephalin (▲) and β-chlornaltrexamine (○) in the isolated, coaxially stimulated guinea-pig ileum. The diagram shows averaged data points (4–5 replicates). Vertical lines show s.e.

Interactions between DAGOL and RX8008M and EKC and RX8008M

Average concentration-effect data for the interaction between DAGOL and RX8008M and between EKC and RX8008M are shown in Figures 2a and b respectively. In both cases, RX8008M produced displacements of agonist curves which, according to analyses of variance, did not significantly deviate from parallelism.

Analysis of the computed $[A_{50}]$ values by equation (2) for the two sets of data indicated that the interactions between RX8008M and the agonists conformed to simple competition in each case. Figure 2c and d show the $[A_{50}]$ data for DAGOL and EKC respectively in Clark Plot form. The Schild slope parameters were 1.02 ± 0.09 (s.e., 22 d.f.) and 0.96 ± 0.08 (s.e., 23 d.f.) respectively. Neither value was significantly different from unity and the resulting pK_B estimates were 8.67 ± 0.08 (s.e., 23 d.f.) and 7.06 ± 0.07 (s.e., 24 d.f.).

Interaction between β-CNA and RX8008M

Figure 3 illustrates the average concentration-effect data for the interaction between β-CNA and RX8008M. Although in the absence of RX8008M the β-CNA curves appeared monophasic, in the presence of the antagonist they were rendered biphasic.

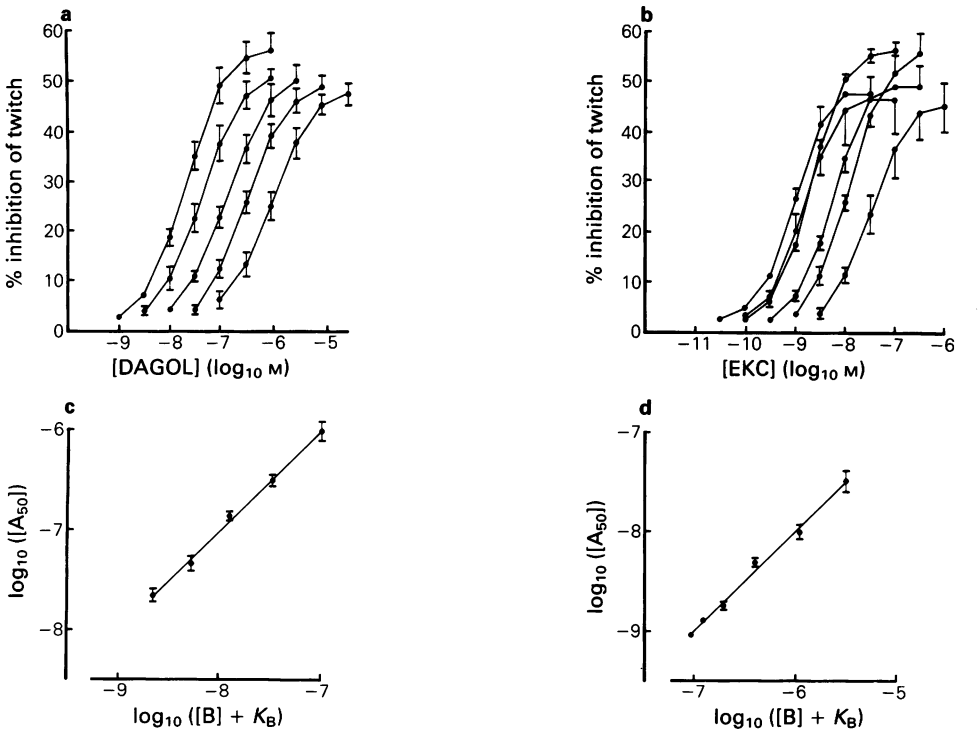

Figure 2 Panels (a) and (b) show the antagonistic effects of 16-methylcyprenorphine (RX8008M) on [D-Ala^2, MePhe^4,Gly-ol^5]enkephalin (DAGOL) E/[A] curves and ethylketocyclazocine (EKC) E/[A] curves respectively. The data are the averages of 4–5 replicate E/[A] curves with vertical lines indicating s.e. The concentrations of antagonist used were 3×10^{-9} M, 10^{-8} M, 3×10^{-8} M and 10^{-7} M in panel (a) and 3×10^{-8} M, 10^{-7} M, 3×10^{-7} M, 10^{-6} M and 3×10^{-6} M, in panel (b). Panels (c) and (d) illustrate the $[A_{50}]$ data in Clark Plot form for DAGOL and EKC respectively. The adherence of the data with the unit slope line drawn through them indicates consistency with simple competition. The pK_B values, estimated by fitting equation (2) to the $[A_{50}]$ values, were 8.67 ± 0.08 (s.e. 23 d.f.) and 7.06 ± 0.07 (s.e. 24 d.f.) respectively.

These changes are broadly consistent with expectation for a two-receptor system in which the two phases of agonism are superimposed initially, then separated by the action of an antagonist selective for one of them. No detailed analysis was carried out but as the antagonist in this case is evidently selective for μ-receptors over κ-receptors (see Figure 2 and analysis) it was deduced that the first phase of agonism revealed in the presence of RX8008M was κ-receptor-mediated and that the second phase was μ-receptor-mediated.

Effect of prior EKC incubation on subsequent EKC E/[A] curves

Thirty minutes prior exposure of tissues to a concentration of EKC (3×10^{-9} M) which elicited the same effect as the maximal response which β-CNA could achieve through κ-receptor activation (that is approx. 35% inhibition of twitch height) did not cause any significant change in EKC E/[A] curve asymptote or location (data not shown, $n = 3$).

Irreversible antagonism of DAGOL effects by β-CNA under two different incubation conditions

The irreversible antagonism of DAGOL effects by β-CNA were examined under two different incubation conditions. The average data shown in Figure 4a and b illustrates the effects of 20 nM and 100 nM β-CNA each for 30 min and 2 nM β-CNA for 300 min respectively on DAGOL E/[A] curves. The lines drawn through the data are the results of operational model-fitting. Unfortunately, incubation conditions which produced more pronounced DAGOL E/[A] curve asymptote depression than that shown in Figure 4b could not be established because of problems with tissue viability. Although this is less than ideal, it did not preclude analysis by operational model-fitting (see below and Discussion).

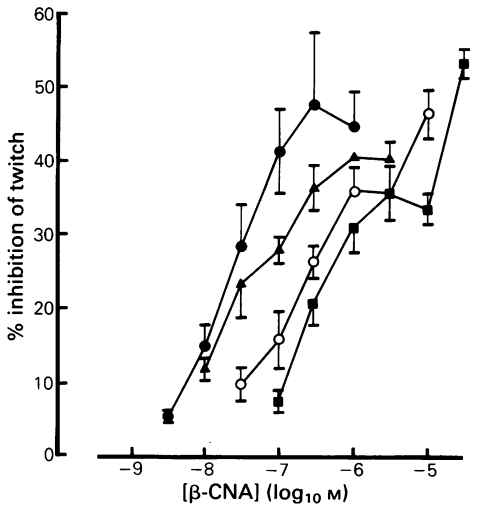

Figure 3 Antagonism of β-chlornaltrexamine (β-CNA) by 16-methylcyprenorphine (RX8008M). The data are the averages of 4–6 replicate E/[A] curves. Vertical lines show s.e. Control (●); 10^{-7} M RX8008M (▲); 5.6×10^{-7} M RX8008M (○) and 3×10^{-6} M RX8008M (■).

Operational model-fitting of the effects of β-CNA treatment on DAGOL E/[A] curves

The data illustrated in Figure 4 were fitted to Equation (3), different values of E_m, n, K_A and τ being estimated for each data set. These parameter values are shown in Table 1. The most important result from these analyses was the similarity of pK_A estimates obtained for DAGOL under the different conditions.

Discussion

In this study we have attempted to characterize the agonist activity of β-CNA in the guinea-pig ileum preparation and to assess if such agonism compromises agonist dissociation constant estimations made by employing this agent to inactivate opioid receptors irreversibly. Since it is generally accepted that the guinea-pig ileum possesses both μ- and κ-opioid receptors, β-CNA's agonism could potentially cause errors in affinity estimations for both μ- and κ-receptor agonists. The degree of agonism that β-CNA exhibits will determine its propensity to densitize the receptor systems. In the present study the degree of agonism that β-CNA expressed was greater than that observed by Ward *et al.* (1982) in the mouse vas deferens. However, no attempts have been made to quantify the agonism in this tissue or in the guinea-pig ileum and it is conceivable that differences in receptor reserve could account for the variable degree of agonism observed. Certainly the reserve for both μ- and κ-receptors is lower in the mouse vas deferens than in the guinea-pig ileum and

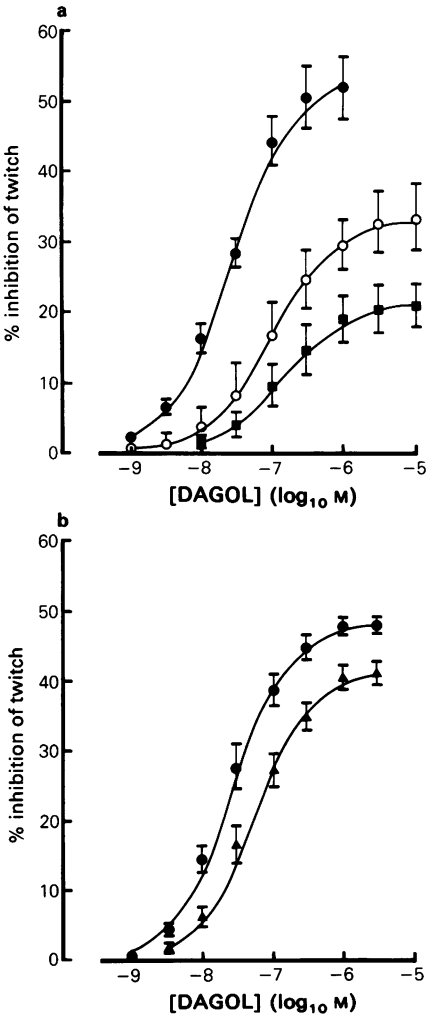

Figure 4 Effect of β-chlornaltrexamine (β-CNA) treatment on [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGOL) E/[A] curves. Panel (a) shows DAGOL E/[A] curves obtained in the absence of (●) and following 30 min exposure to 20 nM (○) or 100 nM (■) β-CNA. Panel (b) shows DAGOL E/[A] curves obtained in the absence of (●) and following 300 min exposure to 2 nM (▲) β-CNA. Each point is the mean of 5–8 replicates. Vertical lines show s.e. The lines drawn through the data are the results of operational model-fitting. For each data set only τ varied between curves, a single value of n, E_m and K_A being estimated. Under the different conditions represented by the separate panels, n, E_m and K_A were allowed to vary.

Table 1 Operational model-fitting of the effects of β-chlornaltrexamine (β-CNA) treatment on [D-Ala^2,MePhe^4, Gly-ol^5]enkephalin (DAGOL) E/[A] curves

Incubation condition	E_m	n	τ_1	τ_2	τ_3	pK_A	*RSS*
20 nM or 100 nM for 30 min	62.55	0.93	8.03	1.16	0.51	6.61	9.73
2 nM for 300 min	53.87	1.06	7.85	3.22	—	6.69	7.14

RSS = Residual sum-of-squares.
In both cases τ_1 denotes values estimated in the absence of β-CNA. For the short incubation period τ_2 and τ_3 denote respectively values estimated in the presence of 20 nM and 100 nM β-CNA. For the long incubation period, τ_2 denotes the value estimated in the presence of 2 nM β-CNA.

therefore the propensity of β-CNA to cause desensitization would be greater in the latter tissue. Another possible source of variation in the amount of agonism expressed is the purity of the sample of β-CNA employed. α-CNA, the epimer of β-CNA elicits profound agonism in the guinea-pig ileum (Sayre *et al.*, 1983) and it is therefore conceivable that variations in the relative amounts of the two epimers in the sample could result in varying degrees of agonism. However, this was not a problem in the present study as the sample did not contain α-CNA. Figure 1 illustrates that the maximum response to β-CNA in the guinea-pig ileum preparation is similar to that observed with full μ- and κ-agonists as represented by DAGOL and EKC respectively. Thus β-CNA may be behaving as a full μ-agonist, a full κ-agonist or a mixed receptor agonist.

The characterization of the agonism of β-CNA required the use of a competitive antagonist of suitable μ/κ-receptor selectivity. Recently 16-methylcyprenorphine (RX8008M) has been found to be considerably more selective than naloxone in this respect (Smith, 1987). The data shown in Figure 2 confirm this claim, indicating that RX8008M exhibits approximately 40 fold higher affinity for μ- than κ-receptors. Its subsequent use in studies designed to characterize the agonism of β-CNA rendered the monophasic control curve biphasic. This curve profile is predicted for an agonist that interacts with 2 different receptor types for which a competitive antagonist shows differential affinity. Although the data are qualitatively consistent with an interaction of β-CNA with both μ- and κ-receptors it did not lend itself to detailed quantitative analysis. However, it seemed reasonable to deduce that the μ- and κ-receptor-mediated effects were initially superimposed, that the higher potency phase exposed in the presence of the antagonist was κ-receptor-mediated and that the lower potency phase was μ-receptor-mediated. In turn, this implied that β-CNA acts as a partial κ-receptor agonist. The maximal effect which β-CNA appeared to be able to elicit through κ-receptors was, from the position of the inflection in the curves in Figure 3, of the order of 60% of the maximal effect of EKC in this tissue. Whether the μ-receptor-mediated phase of the action of β-CNA represents full or partial agonism cannot be deduced from these data, although the compound was evidently able to elicit the maximum possible effect when both populations were saturated. It is certainly possible that β-CNA acts as a full agonist through μ-receptors meaning that desensitization of μ-receptor-mediated effects by β-CNA was considered to be more likely than desensitization of κ-receptor-mediated effects. However, both eventualities needed to be checked since β-CNA has been used in the estimation of both μ- (Porreca & Burks, 1983; Dougall & Leff, 1987) and κ- (Leff & Dougall, unpublished work) agonist dissociation constants.

Theory predicts that reductions in the efficiency in the cellular apparatus which couples agonist-receptor occupancy to pharmacological effect may or may not resemble a simple reduction in $[R_0]$, the functional receptor population (Leff *et al.*, 1985). Whereas $[R_0]$ reduction provides the basis for estimation of agonist dissociation constants (Furchgott, 1966) post-receptor interventions cannot be relied upon to do so. Overestimation of agonist affinity is the predicted error (Leff *et al.*, 1985). Since desensitization could involve such changes in post-receptor coupling it is an unreliable method for attempting to estimate agonist affinities (Eglen & Whiting, 1987). When an irreversible antagonist: which ideally should cause only $[R_0]$ reduction, elicits agonism in its own right, it is possible for desensitization and, therefore, erroneous agonist affinity estimation to result.

In the present study, this question was readily addressed in the case of κ-receptors by use of EKC to mimic β-CNA. Thus, at a concentration of EKC that elicited the same effect as the maximal response which β-CNA could achieve through κ-receptors no change occurred in the location or size of a sub-

sequent EKC E/[A] curve. Thus desensitization of κ-receptor effects does not appear to be associated with the κ-receptor agonism of β-CNA.

In the case of μ-receptors it was not possible to address the question in the same way. As the μ-receptor agonism of β-CNA could not be analysed quantitatively it was not possible to choose a concentration of a μ-agonist like DAGOL to mimic the effects of β-CNA. Instead, the problem was approached by use of conditions under which β-CNA did not elicit agonism and, therefore could not produce desensitization, namely lower concentrations of the compound and an extended incubation time. The DAGOL pK_A estimate obtained under these conditions would, in principle, be more reliable than that obtained when β-CNA elicits agonism. The conditions chosen (a 10 fold lower β-CNA concentration but a 10 fold longer incubation period than originally) assumed a pseudo-first order reaction between β-CNA and the receptors. The results indicate that this assumption was not valid since there was significantly less reduction in $[R_0]$ and therefore τ, than under the original conditions (see Table 1). Although the degree of asymptote depression shown in Figure 4b is less than ideal for the purpose of pK_A estimation it did not preclude analysis by operational model-fitting. In fact, the fitting error associated with these data as measured by the residual sum of squares (see Table 1) was less than that for the data shown in Figure 4a. The resulting pK_A estimate was considered valid, therefore, and evidently it was indistinguishable from that obtained under the original conditions. Obviously such analysis of pK_As implicitly assumes that the agonist in question activates only a single receptor population in the concentration-range studied. In analysing DAGOL this assumption is justified by results of other workers (Handa *et al.*, 1981; Goldstein & James, 1984).

In conclusion, the present analysis indicates that the use of β-CNA as an irreversible antagonist in the estimation of μ- and κ-receptor agonist dissociation constants is not compromised by the agonist effects that the compound demonstrates at these receptors.

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