Estimation of affinities and efficacies for κ -receptor agonists in guinea-pig ileum

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1 Ethylketocyclazocine (EKC) and U50,488H have been employed widely as κ -receptor agonists in the study of opioid receptor systems. However, the quantification of their agonism in terms of affinities and relative efficacies has not been investigated.

2 In this study, operational model-fitting was used to analyse the effects of irreversible receptor alkylation by β -chlornaltrexamine (β -CNA) on the κ -receptor mediated effects of EKC and U50,488H in the isolated, coaxially stimulated ileum of the guinea-pig.

3 EKC produced monophasic inhibitory concentration-effect curves which were readily amenable to analysis. In contrast U50,488H produced biphasic curves characterized by a higher potency phase of agonism that was susceptible to antagonism by 16-methylcyprenorphine (RX8008M) and a lower potency phase that was apparently non-opioid in nature.

4 Analysis of the κ -receptor-mediated effects of both agonists indicated that EKC has fifteen fold higher affinity than U50,488H and that the two agonists possess similar intrinsic efficacies.

Introduction

Opioid receptor classification studies in isolated tissues have for the most part relied on competitive antagonist affinity estimates. The estimation of opioid agonist affinities and relative efficacies has received little attention despite the contribution such information makes to receptor classification. A few investigators have reported functional estimates of μ -agonist affinities (Porreca & Burks, 1983; Williams & North, 1984; Dougall & Leff, 1987) but no attempts similarly to estimate κ -agonist affinities are evident in the literature to these authors' knowledge.

With the objective of providing more quantitative information than currently available on two established κ -receptor agonists we have analysed the action of ethylketocyclazocine (EKC) and U50,488H in the guinea-pig ileum using the irreversible receptor antagonist β -chlornaltrexamine (β -CNA).

The results of this study are discussed with regard to their implications, for the classification of opioid receptors. A preliminary account of this work was

presented to the British Pharmacological Society (Leff & Dougall, 1988b).

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, NaHCO₃ 25.00, KCl 4.75, KH₂PO₄ 1.19, MgSO₄ 1.19, glucose 11.10, CaCl₂ 2.50. This was maintained at 37°C and continually gassed with 95% O₂ and 5% CO₂. The upper end of the tissue was attached by cotton thread to a Grass FTO3C force-displacement transducer, the bottom end being tied to the tissue holder. Contractions of the tissue were elicited by coaxial supramaximal

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stimulation (0.5 ms duration, 0.1 Hz, 20 V). Changes in isometric force were recorded on Gould BS-272 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. At the end of the stabilization period the tension was reinstated. Reproducible responses to supramaximal electrical stimulation were established after about 45 min.

Agonist concentration-effect, $E/[A]$, curves were constructed by cumulative additions of either EKC or U50,488H at 0.5 $\log_{10}$ unit increments. Only a single curve was generated in each tissue. Responses were recorded as percentage inhibitions of the stimulated twitch.

Competitive antagonist studies Tissues from the same animal were incubated with 1 of 4 concentrations (10^{-7} M, 3×10^{-7} M, 10^{-6} M or 3×10^{-6} M) of 16-methylcyprenorphine (RX8008M) (Smith, 1987) or vehicle for 40 min before construction of U50,488H $E/[A]$ curves. This procedure was repeated until 5 replicates were obtained.

Irreversible receptor inactivation Tissues from the same animal were either treated as controls or were incubated with β -CNA (10 nM or 30 nM) for 30 min after which excess alkylating agent was removed by 20 changes of the organ bath Krebs solution over a period of 60 min. EKC or U50,488H curves were then constructed as outlined above. This procedure was repeated until at least 4 replicates were obtained.

Analysis of data

$E/[A]$ curve data obtained in competitive antagonist studies was 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. This fitting procedure also performed one-way analyses of variance comparing computed slope and asymptote parameters between and within treatment groups. If the treatments did not significantly ($P < 0.05$) modify these parameter estimates, then computed $\log_{10} [A_{50}]$ values were fitted to the following linear form

of the Schild equation (Trist & Leff, 1985):

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

where $[A_{50}^c]$ is the estimated control $[A_{50}]$, $[B]$ is the concentration of antagonist, K_B is its equilibrium dissociation constant and n is equivalent to the Schild plot slope parameter (unity for simple competition). If 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

$E/[A]$ curve data obtained in experiments with β -CNA treatment were fitted to the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985) in order to estimate agonist dissociation constants and efficacies:

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

in which τ defines the efficacy of an agonist in a system; E_m is the maximum possible effect; K_A is the agonist dissociation constant and n is the slope parameter for the transducer relation linking occupancy to effect.

Drugs

Ethylketocyclazocine methane sulphonate (Sterling-Winthrop); U50,488H (*trans*-($\pm$)-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide methane sulphonate (Upjohn); β -CNA dihydrochloride and 16-methylcyprenorphine hydrochloride (both prepared by Dr S. Wilkinson, Medicinal Chemistry Department, Wellcome Research Laboratories).

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

Interaction between U50,488H and RX8008M

Average concentration-effect data for the interaction between U50,488H and RX8008M are shown in Figure 1a. Both in the absence and presence of the antagonist U50,488H $E/[A]$ curves were clearly biphasic. RX8008M produced displacements of the higher potency phase of U50,488H's agonism which according to analyses of variance did not signifi-

Figure 1 (a) Antagonist effects of RX8008M on U50,488H $E/[A]$ curves. The data are the averages of 5 replicate $E/[A]$ curves with vertical lines indicating s.e. The concentrations of the antagonist were zero ($\circ$); 10^{-7} M ($\bullet$); 3×10^{-7} M ($\blacktriangle$); 10^{-6} M ($\blacksquare$); 3×10^{-6} M ($\blacktriangledown$). The dotted lines illustrate the presence of a RX8008M-resistant phase of agonism. (b) The $[A_{50}]$ data in Clark Plot form. The adherence of the data with the unit slope line drawn through them indicates consistency with simple competition. The pK_B value, estimated by fitting equation (2) to the $[A_{50}]$ values was 7.26 ± 0.06 (s.e., 23 d.f.).

cantly deviate from parallelism (Figure 1a). Analysis of the computed $[A_{50}]$ values by Equation (2) indicated that the interaction between RX8008M and U50,488H conformed to simple competition. Figure 1b shows the $[A_{50}]$ data in Clark Plot form. The Schild slope parameter (n) was 1.11 ± 0.06 (s.e.mean, 22 d.f.) and the resulting pK_B estimate with n constrained to unity was 7.26 ± 0.06 (s.e.mean, 23 d.f.). This is consistent with U50,488H activating κ -receptors in this concentration-range since we have previously demonstrated that RX8008M competi-

tively antagonizes EKC in this tissue yielding a pK_B of 7.06 (Leff & Dougall, 1988a). Figure 1a also illustrates the resistance of the lower potency phase (dotted lines) of U50,488H's agonism to antagonism by RX8008M. This agonism was also resistant to naloxone antagonism at a concentration of 300 nM.

Irreversible antagonism of ethylketocyclazocine effects by β -chlornaltrexamine

The average data shown in Figure 2a illustrate the effects of β -CNA (10 nM or 30 nM each for 30 min) on EKC $E/[A]$ curves. The lines drawn through the data are the results of operational model-fitting. The parameter values corresponding to these lines were as follows: $E_m = 63.32\%$; $n = 1.05$; $pK_A = 8.33$; τ_1 (control) = 9.76; τ_2 (10 nM β -CNA) = 0.97; τ_3 (30 nM β -CNA) = 0.45.

Irreversible antagonism of U50,488H effects by β -chlornaltrexamine

The average data shown in Figure 2b illustrate the effects of β -CNA (10 nM for 30 min) on U50,488H $E/[A]$ curves. The solid lines drawn through the data are the results of operational model-fitting. The parameter values corresponding to these lines were as follows: $E_m = 43.07\%$; $n = 1.20$; $pK_A = 7.16$; τ_1 (control) = 12.10; τ_2 (10 nM β -CNA) = 1.15. The dotted lines merely indicate the presence of the resistant phase of agonism (see above) and the data points connected by these lines were not used in the model-fitting analysis.

It is evident from Figure 2 that the E_m values for the two sets of data were different. In fact, the two sets of experiments corresponding to the two panels were conducted at different times. However when EKC and U50,488H were applied to portions of ileum from the same guinea-pig, maximum responses observed were not significantly different (data not shown, $n = 3$) implying that the observed differences in E_m were the result of animal variation.

Discussion

The quantitative classification of opioid receptors has for the most part relied upon estimates of affinity for competitive antagonists. Few attempts have been made to complement these with estimates of affinity and efficacy for agonists. When using agonists, most investigators have been content to quote potency orders despite the fact that these offer unreliable information for receptor classification. Agonist potency depends on both affinity and efficacy (Stephenson, 1956; Black & Leff, 1983) and it is possible for agonists to demonstrate the same potency

Figure 2 Effect of β -chlornaltrexamine (β -CNA) treatment on ethylketocyclazocine (EKC) and U50,488 E/[A] curves: (a) shows EKC E/[A] curves obtained in the absence of ($\bullet$) and following 30 min exposure to 10 nM ($\blacktriangle$) or 30 nM ($\blacksquare$) β -CNA. (b) Shows U50,488H E/[A] curves in the absence of ($\bullet$) and following 30 min exposure to 10 nM β -CNA ($\blacktriangle$). Each point is the mean of 4–7 replicates. Vertical lines show s.e. The solid lines drawn through the data are the results of operational model-fitting. The dotted lines in (b) illustrate the presence of a non-opioid phase of agonism.

orders in two tissues containing different receptors. Also, changes in agonist potency order can occur between different tissues containing the same receptors (Kenakin, 1984). Therefore, in order for results with agonists to contribute reliably to receptor classification, attempts should be made to estimate their affinities and efficacies. The absence of such information on κ -agonists instigated the present study.

Here, we have attempted to quantify the action of two commonly used κ -agonists, EKC and U50,488H. Initially, the agonist effects of the two compounds were confirmed as being κ -receptor mediated using the competitive antagonist RX8008M. This antagonist is reported to have similar affinities for μ - and δ -receptors and less, though still substantial affinity for κ -receptors (Smith, 1987); in this study, it demonstrated competitive dynamics against U50,488H. We have previously reported similar behaviour when EKC is employed as the agonist (Leff & Dougall, 1988a) and in both cases the pK_B values were consistent with the antagonist's reported affinity for κ -receptors (Smith, 1987). Attempts were made to support these results by use of a putative κ -receptor selective antagonist nor-binaltorphimine (Portoghese *et al.*, 1987; Birch *et al.*, 1987). However, although it blocked the responses to both agonists in the concentration-range expected on the basis of its reported κ -receptor affinity, it did not demonstrate simple competition and pK_B estimates could not be made (data not shown).

Assuming that EKC and U50,488H were κ -receptor agonists in this tissue they were subjected to analysis using the operational model. According to this analysis these agonists possess similar efficacies (10 and 12 respectively in this tissue), but different affinities, EKC having some fifteen fold higher affinity than U50,488H. It is important to emphasise that the difference in the curve maxima between the two agonists was due to E_m differences, reflecting a tissue difference rather than intrinsic efficacy differences between the agonists.

Unlike EKC, U50,488H clearly demonstrated a biphasic E/[A] curve in the presence and absence of β -CNA treatment. It is known that the guinea-pig ileum possesses both μ - and κ -receptors and, in principle, the lower affinity phase of agonism could have been due to activation of μ -receptors.

Sheehan *et al.* (1986) have suggested that U50,488H is able to activate μ -receptors in this tissue. Those authors used β -CNA in the presence of the selective μ -receptor agonist [D-Ala², MePhe⁴, Gly-ol⁵] enkephalin (DAGOL) to produce a preponderantly μ -receptor-enriched tissue. Under those conditions U50,488H produced agonist effects in a concentration range 150 fold higher than under control conditions. These effects were attributed to μ -receptor activation.

However, the present study indicates that the lower potency phase of U50,488H's agonism was not mediated by opioid receptors, due to its resistance to both RX8008M and naloxone. Although the study of Sheehan *et al.* (1986) did not reveal a clearly-defined biphasic curve for U50,488H it is notable that the resistant phase described here occupies the same

concentration-range as the apparently μ -receptor phase of agonism described in their study. As those authors did not apply naloxone or any other antagonist in their study it would seem reasonable to adopt the present explanation. However, although the lower potency phase of U50,488H's agonism was not μ -receptor mediated, the present study did not rule out μ -receptor properties of EKC and U50,488H. Until any such properties associated with these com-

pounds are adequately quantified their classification will remain incomplete and interpretation of *in vivo* data limited.

In conclusion, the present analysis represents the first attempt to quantify the action of κ -opioid receptor agonists in terms of their affinities and efficacies. Similar attempts to provide such information would do much to further the classification of opioid receptors.

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