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Pharmacological analysis of the calcium-dependence of μ-receptor agonism

I.G. Dougall & P. Leff

Analytical Pharmacological Group, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 A number of studies in isolated tissues have shown that μ-opioid-receptor-mediated agonism can be augmented or potentiated with reduction in extracellular calcium concentration ($[Ca^{2+}]_o$). These effects have been ascribed to alterations in post-receptor coupling but the nature of the changes involved have not been quantitatively elucidated.

2 In this paper, logistic curve-fitting and operational model-fitting (Black & Leff, 1983) were used to analyse the effects of variations in $[Ca^{2+}]_o$ on the μ-receptor-mediated effects of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL) in the isolated, coaxially-stimulated ileum of the guinea-pig. At each value of $[Ca^{2+}]_o$, the effects of irreversible receptor alkylation by β-chlornaltrexamine (β-CNA) were also investigated.

3 From these analyses it is concluded that with reduction in $[Ca^{2+}]_o$ the efficacy of DAGOL in this system is increased and the sensitivity of the transducer relation is also enhanced, the latter indicating a trend towards positive co-operativity. Reduction of $[Ca^{2+}]_o$ also appeared to produce a reduction in agonist affinity.

4 Experimental manipulation of $[Ca^{2+}]_o$ may provide a useful means of enhancing μ-agonist efficacy, allowing detection of agonism in compounds with low intrinsic efficacies. However, the accompanying change in co-operativity of the transducer relation must be considered when quantifying agonism under these conditions.

Introduction

A number of studies have described the dependence of opioid-receptor-mediated agonism on extracellular calcium concentration ($[Ca^{2+}]_o$) in isolated tissue experiments (Opmeer & Van Ree, 1979; 1980; Kamikawa & Shimo, 1983; Hayes & Sheehan, 1986; Johnson *et al.*, 1986). Earlier (Opmeer & Van Ree, 1979), the inhibitory effects of morphine in the guinea-pig ileum preparation were demonstrated to be antagonized competitively by raising $[Ca^{2+}]_o$ above its normal value (2.50 mM). More recently, lowering $[Ca^{2+}]_o$ below this value was shown to potentiate opioid agonist effects in the guinea-pig ileum (Johnson *et al.*, 1986) and rat vas deferens (Hayes & Sheehan, 1986) preparations. Both μ-receptor (Hayes & Sheehan, 1986; Johnson *et al.*, 1986) and κ-receptor mediated (Johnson *et al.*, 1986) agonist effects were susceptible to these changes and they were attributed to alterations in receptor-effector coupling (Hayes & Sheehan, 1986). This interpretation seems more plausible than the previous one (Opmeer & Van Ree, 1979) particularly in the light of evidence that opioid receptor ligands, which demonstrate little or no significant agonism under normal conditions exhibit agonism when the $[Ca^{2+}]_o$ is reduced (Hayes & Sheehan, 1986), implying a change in efficacy. However, accepting that a post-receptor alteration is involved, the nature of the change has not been quantitatively elucidated.

According to theoretical modelling (Black & Leff, 1983; Black *et al.*, 1985b) agonist action can be described by four parameters: K_A, the agonist-receptor dissociation constant; τ, the operational efficacy; E_m, the maximal effect in a particular receptor system; n, the slope parameter for the function relating receptor occupancy to pharmacological effect. In theory, an experimental intervention could affect any of these parameters.

Experimental interventions which affect τ (Black *et al.*, 1985a; Barrett *et al.*, 1986) and both τ and E_m (Leff *et al.*, 1985; Eglen & Whiting, 1986) have been exemplified although no examples of an intervention affecting n are evident in the literature to these authors' knowledge.

The present paper describes an attempt to define, in operational terms, the effect of $[Ca^{2+}]_o$ variation on μ-

receptor-mediated agonism in the guinea-pig ileum preparation. As shown previously (Black *et al.*, 1985a; Leff *et al.*, 1985; Barrett *et al.*, 1986; Eglen & Whiting, 1986) elucidation of undefined interventions depends partly on a comparison of their effects with those of a well-characterized irreversible antagonist at the receptor in question. β-Chlornaltrexamine (β-CNA) has been shown to be an irreversible opioid-receptor ligand (Portoghese *et al.*, 1979) albeit non-selective. Its utility as a classification tool for μ-receptors was confirmed in the present study. [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL) (Handa *et al.*, 1981) was used as the standard μ-receptor agonist.

The results of this study are discussed with regard to their implications for the pharmacological quantification of μ-receptor agonism.

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 ileo-caecal 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, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10, $CaCl_2$ (2.50, 1.25 or 0.63). 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 FTO3C force-displacement transducer, the bottom end being tied to the tissue holder. Contractions of the tissue were elicited by coaxial stimulation (0.5 ms duration, 0.1 Hz, 20 V) the stimuli being about 30% greater than that required to elicit a maximum contraction. 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. At the end of the stabilization period the tension was re-instated. Reproducible responses to supramaximal electrical stimulation were established after about 45 min.

Agonist-concentration-effect, E/[A], curves were constructed by cumulative additions of DAGOL at 0.5 $\log_{10}$ unit increments. Only a single curve was generated in each tissue. A multiple curve design could not be used due to a gradual gain of spontaneous activity in tissues, a phenomenon which was particularly marked at low $[Ca^{2+}]_o$.

Responses were recorded as fractional inhibitions of the stimulated twitch.

Irreversible receptor inactivation In each extracellular calcium concentration (2.50, 1.25 and 0.63 mM) tissues were incubated with β-CNA (20 nM or 100 nM) for 30 min, after which excess inhibitor was removed by several changes of the organ bath Krebs solution. DAGOL E/[A] curves were then established as outlined above.

Competitive antagonist studies In both low (0.63 mM) and high (2.50 mM) $[Ca^{2+}]_o$ tissues were incubated with naloxone for 40 min before construction of DAGOL E/[A] curves.

Protection against irreversible receptor inactivation In high (2.50 mM) $[Ca^{2+}]_o$, tissues were incubated with naloxone (300 nM) for 40 min. Ten minutes after the addition of naloxone, β-CNA (20 nM) was added, both drugs were removed 30 min later by several washes with fresh Krebs solution. DAGOL E/[A] curves were then constructed. To determine whether any naloxone antagonism remained after washing, the above experiment was repeated except that the irreversible antagonist was omitted.

Drugs

DAGOL (Cambridge Research Biochemicals Ltd, Harston, Cambridge); β-CNA hydrochloride and naloxone hydrochloride (both prepared by Dr S. Wilkinson, Wellcome Research Laboratories, Beckenham, Kent). All drugs were dissolved in distilled water.

Analysis of data

Each individual set of E/[A] curve data, recorded as fractional 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 ($-\log_{10}K_B$).

E/[A] data obtained in experiments using variable $[Ca^{2+}]_o$ and β-CNA treatment were fitted to the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985b):

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

in which E_m is the maximum possible effect; K_A is the dissociation constant of the agonist from the receptor (this was estimated as the negative logarithm, that is, pK_A); τ is the ratio $[R_o]/K_E$, where $[R_o]$ is the total, functional receptor concentration and K_E defines the value of occupancy, [AR], for half E_m; n is the slope parameter for the assumed logistic relation linking [AR] to effect, E. Operationally, τ defines the efficacy of an agonist in a system.

In data analysis, the results of logistic curve fitting will be considered in terms of the operational model. To do this it is necessary to define the logistic curve parameters in terms of their operational model counterparts:

$$\alpha = \frac{E_m \tau^n}{1 + \tau^n} \quad (4)$$

$$[A_{50}] = \frac{K_A}{[(2 + \tau^n)^{1/n} - 1)]} \quad (5)$$

$$m = \frac{n(2 + \tau^n)((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}(1 + \tau^n)} \quad (6)$$

Results

Effect of varying $[Ca^{2+}]_o$ on DAGOL E/[A] curves

Figure 1 shows the dependence of DAGOL E/[A] curves on $[Ca^{2+}]_o$. Individual curves were fitted to Equation (1) providing estimates of α, $[A_{50}]$ and m in each case. Average estimates of these parameters from replicate curves obtained at each value of $[Ca^{2+}]_o$ are shown together with standard errors in Table 1. One-way analyses of variance showed that each parameter was significantly affected by variation in $[Ca^{2+}]_o$: α and m were both enhanced by decreasing $[Ca^{2+}]_o$; $[A_{50}]$ was reduced. Figure 1 illustrates the average E/[A] curve data together with logistic fits corresponding to the average parameter estimates given in Table 1.

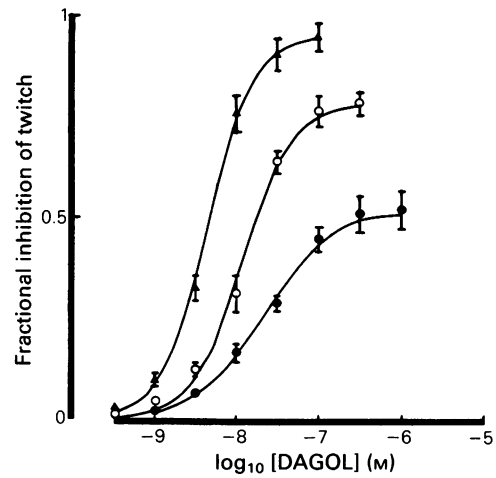

Figure 1 Dependence of [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAGOL) E/[A] curves on $[Ca^{2+}]_o$. DAGOL E/[A] curves were obtained at 0.63 mM (▲), 1.25 mM (○) and 2.50 mM (●) $[Ca^{2+}]_o$. The diagram shows averaged data points (5–7 replicates) as fractional inhibition of the twitch; vertical lines indicate s.e.mean. The lines drawn through the data are the results of logistic curve fitting.

Table 1 Logistic fitting of [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin E/[A] curves with varying $[Ca^{2+}]_o$

$[Ca^{2+}]_o$ (mM)	*Number of replicate curves*	$p[A_{50}]$	α	*m*
2.50	5	7.64 ± 0.09	0.54 ± 0.04	1.07 ± 0.07
1.25	5	7.91 ± 0.05	0.80 ± 0.02	1.50 ± 0.15
0.63	7	8.35 ± 0.03	0.96 ± 0.03	1.60 ± 0.07
One-way analysis of variance:		$P<0.01$	$P<0.01$	$P<0.01$

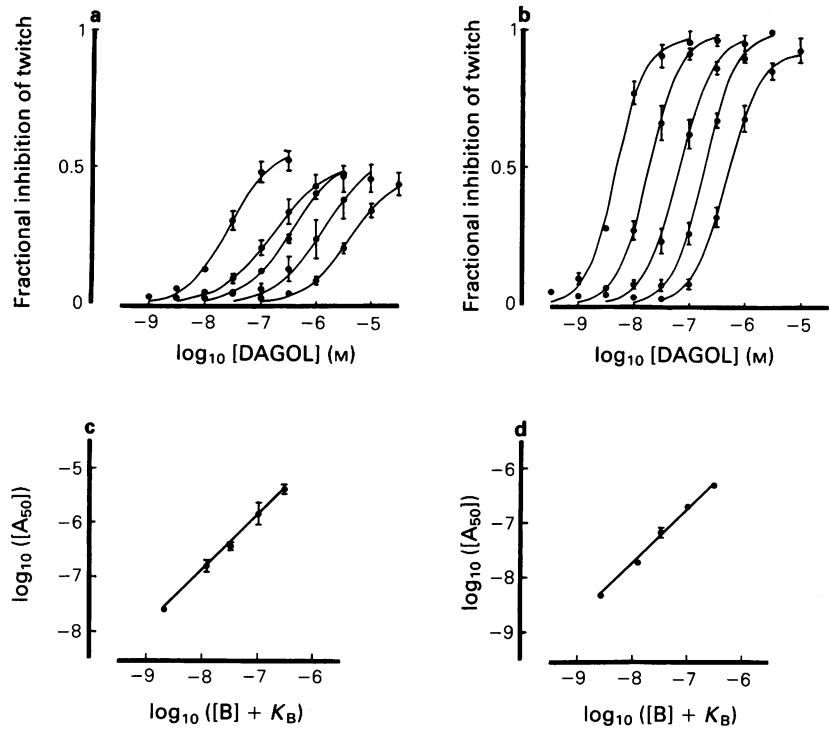

Figure 2 Antagonism of [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin (DAGOL) by naloxone. (a) and (b) The effects of naloxone on DAGOL E/[A] curves at 2.50 mM and 0.63 mM $[Ca^{2+}]_o$, respectively. The lines drawn through the data are the results of logistic curve fitting. In each case, analyses of variance on slope and asymptote parameters revealed no significant differences; (c) and (d) illustrate the $[A_{50}]$ data in Clark Plot form at 2.50 mM and 0.63 mM $[Ca^{2+}]_o$, respectively.

At 2.50 mM $[Ca^{2+}]_o$, the Schild slope parameter (n) was 1.01 ± 0.12 (s.e., 17 d.f.); at 0.63 mM $[Ca^{2+}]_o$, it was 1.00 ± 0.06 (s.e., 17 d.f.). Neither value was significantly different from unity and the resulting pK_B estimates obtained with n constrained to unity were: 8.68 ± 0.12 (s.e., 18 d.f.) and 8.58 ± 0.07 (s.e., 18 d.f.), respectively. These estimates were not significantly different from one another.

Interaction between DAGOL and naloxone at different $[Ca^{2+}]_o$

The antagonism of DAGOL effects by naloxone was studied at 2.50 mM and 0.63 mM $[Ca^{2+}]_o$. Average E/[A] data are shown in Figure 2a and b. The lines drawn through the data correspond to the average logistic fit in each case. At 2.50 mM $[Ca^{2+}]_o$, naloxone produced displacement of DAGOL E/[A] curves which according to analyses of variance, did not significantly deviate from parallelism (Figure 2a).

At 0.63 mM $[Ca^{2+}]_o$, naloxone demonstrated apparent agonism in the concentration range used. This effect was variable although broadly concentration-dependent. Moreover, the agonism was transient and when DAGOL E/[A] curves were obtained it had completely faded. The resulting curve displacements elicited by naloxone did not deviate significantly from parallelism (Figure 2b).

Analysis of the computed $[A_{50}]$ values by Equation (2) for the two sets of data indicated that the interaction between naloxone and DAGOL conformed to simple competition in each case. Figure 2c and d shows the $[A_{50}]$ data in Clark Plot form. At 2.50 mM $[Ca^{2+}]_o$, the Schild slope parameter was 1.01 ± 0.12 (s.e.mean; 17 d.f.); at 0.63 mM $[Ca^{2+}]_o$, it was 1.00 ± 0.06 (s.e.mean; 18 d.f.), respectively. These estimates were not significantly different from one another or from unity.

The data illustrated in Figure 2a and b also confirm the results shown in Figure 1 and Table 1 regarding the differences between E/[A] curves at 2.50 mM and 0.63 mM $[Ca^{2+}]_o$. In the present case the estimated curve parameters were: $\alpha = 0.56 \pm 0.02$,

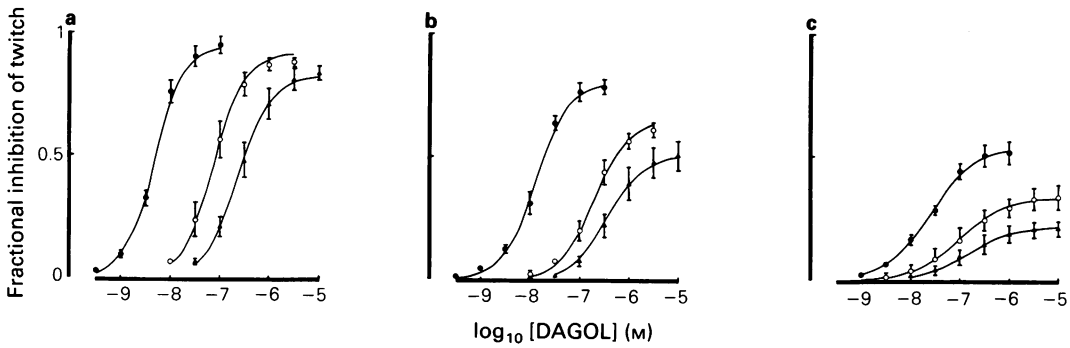

Figure 3 Effect of β-chlornaltrexamine (β-CNA) treatment on [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAGOL) E/[A] curves at 0.63 mM (a), 1.25 mM (b) and 2.50 mM (c) $[Ca^{2+}]_o$. DAGOL E/[A] curves were obtained in the absence of (●) and following 30 min exposure to 20 nM (○) or 100 nM (▲) β-CNA. Each point is the mean of 4–7 replicates. Vertical lines show s.e.mean. The lines drawn through the data are the results of operational model-fitting. At each value of $[Ca^{2+}]_o$, only τ varied between curves, a single value of n, E_m and K_A being estimated. Under the different conditions of $[Ca^{2+}]_o$, n, E_m and K_A were allowed to vary.

$m = 1.22 \pm 0.13$, $p[A_{50}] = 7.58 \pm 0.04$ (s.e.mean; 4 d.f.) at 2.50 mM $[Ca^{2+}]_o$ and $\alpha = 0.97 \pm 0.02$, $m = 1.71 \pm 0.17$, $p[A_{50}] = 8.32 \pm 0.02$ (s.e.mean; = 3 d.f.) at 0.63 mM $[Ca^{2+}]_o$.

Irreversible antagonism of DAGOL effects by β-CNA

The effects of β-CNA (20 nM and 100 nM each for 30 min) on DAGOL E/[A] curves were examined at 0.63 mM, 1.25 mM and 2.50 mM $[Ca^{2+}]_o$. The average data are illustrated in Figure 3a, b and c. At each value of $[Ca^{2+}]_o$, the results accord with the expectation of the effects of irreversible antagonism, although the curves obtained at 0.63 mM $[Ca^{2+}]_o$ were clearly more resistant to asymptote depression than those obtained at the higher values, implying that lowering $[Ca^{2+}]_o$ had enhanced the operational efficacy of DAGOL. Quantitative evaluation of these effects of β-CNA at varying $[Ca^{2+}]_o$ was carried out using operational model-fitting (see below). The lines drawn through the data in Figure 3 are the results of such model-fitting.

Table 2 Unconstrained operational model-fitting of [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin E/[A] curves with varying $[Ca^{2+}]_o$ and β-chlornaltrexamine (β-CNA) treatment

$[Ca^{2+}]_o$ (mM)	E_m	n	τ_1	τ_2	τ_3	pK_A
2.50	0.62	0.94	7.98	1.17	0.51	6.62
1.25	0.81	1.37	46.73	3.02	1.49	6.23
0.63	0.94	1.48	187.47	12.00	3.89	6.08

τ_1, τ_2, τ_3 denote respectively τ values estimated in the absence and the presence (20 nM and 100 nM) of β-CNA.

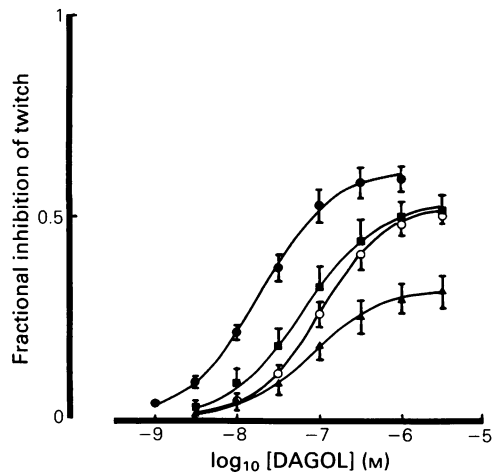

Figure 4 Effect of concomitant naloxone incubation on β-chlornaltrexamine (β-CNA)-induced irreversible antagonism of responses to [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin (DAGOL) at 2.50 mM $[Ca^{2+}]_o$. Average data points (4–5 replicates) are shown with vertical lines indicating s.e.mean. The lines drawn through the data are the results of logistic curve fitting: control (●); naloxone (300 nM) then washout (■); β-CNA (20 nM) then washout (▲); naloxone (300 nM) and β-CNA (20 nM) then washout (○).

Protection of β-CNA effects by naloxone

Figure 4 shows the results of concomitant naloxone (300 nM) and β-CNA (20 nM) treatment, followed by subsequent wash-out, on DAGOL E/[A] curves. The data obtained with naloxone alone under these condi-

tions indicated that the competitive antagonist was not completely dissociated from the receptors when DAGOL E/[A] curves were constructed. However, these data virtually superimpose with those obtained when β-CNA was also present, indicating effective protection by naloxone of the irreversible effect of β-CNA, confirming previous results (Caruso *et al.*, 1979), and indicating that the effects of β-CNA were confined to the receptor.

Effect of $[Ca^{2+}]_o$ variation on basal twitch size

During the course of these experiments it was observed that twitch size in the absence of drug additions was reduced by lowering $[Ca^{2+}]_o$. In the experiments which produced the data shown in Figure 3 the average twitch sizes were; at 2.50 mM $[Ca^{2+}]_o$, 3.59 ± 0.13 g (s.e.mean; 15 d.f.); at 1.25 mM $[Ca^{2+}]_o$, 2.96 ± 0.15 g (s.e.mean; 13 d.f.) and at 0.63 mM $[Ca^{2+}]_o$, 2.15 ± 0.12 g (s.e.mean; 17 d.f). These changes had repercussions in the interpretation of the effects of $[Ca^{2+}]_o$ variation on μ-receptor-mediated agonism (see Discussion).

Operational model-fitting of the effects of $[Ca^{2+}]_o$ variation and β-CNA treatment on DAGOL E/[A] curves

The data illustrated in Figure 3 were fitted to Equation (3). Average data points were fitted, rather than all the data points and goodness-of-fit was assessed on the basis of the residuals between these average points and fitted values. A series of fits was performed in each of which the three curves obtained in the absence and presence of β-CNA were assumed to differ only by τ. In the first, unconstrained fit, different values of E_m, n, K_A and a set of three τ values were estimated at each $[Ca^{2+}]_o$.

In subsequent fits, E_m, n, K_A and the set of three τ values, were individually constrained to common estimates for all the data. The constrained and unconstrained fits were compared by F-ratio analysis (see Table 3) which indicated that all constraints resulted in significant worsening of fit.

The above results indicated that $[Ca^{2+}]_o$ reduction produced a significant increase in n, E_m and each τ value and a significant decrease in pK_A.

Discussion

In this study we have attempted to elucidate the changes in μ-receptor-mediated agonism brought about by $[Ca^{2+}]_o$ variation.

DAGOL was used as a standard μ-receptor agonist and its inhibitory effects in the stimulated guinea-pig ileum preparation were studied at 2.50 mM, 1.25 mM and 0.63 mM $[Ca^{2+}]_o$ with and without prior exposure to the irreversible antagonist, β-CNA.

Analysis of DAGOL E/[A] curves by logistic fitting indicated that curve asymptote, α, location, $p[A_{50}]$, and slope, m, depended on $[Ca^{2+}]_o$. Reducing $[Ca^{2+}]_o$ from 2.50 mM produced significant increases in α, m and $p[A_{50}]$ (see Table 1). The purpose of the experiments using β-CNA and their analysis by operational model-fitting was to assist in the interpretation of these changes. An important consideration in these analyses was the extent to which averaging of response data affects interpretation of curve shape, as in principle, this process can cause distortions (Ariëns *et al.*, 1964). In the present experiments, for a particular treatment, the average logistic curve, (obtained by fitting each individual curve then averaging the logistic curve parameter estimates) and the average data points invariably superimposed (see Figures 1, 2 and 4). This demonstrates that the average data convey the genuine curve shape characteristics, a result which is expected for experimental data where the source of variation is essentially vertical, that is, on the effect scale, rather than on the horizontal, concentration axis. This result justifies the use of average data in the operational model-fitting and in turn allows a more stringent goodness-of-fit analysis than would have been possible if it had been necessary to use all the data points.

In order to use the operational model of agonism to interpret the changes in DAGOL E/[A] curves caused by $[Ca^{2+}]_o$ variation, we use the definitions of α, $[A_{50}]$ and m in terms of the model parameters, K_A, τ, n and

Table 3 Goodness-of-fit analyses of operational model fitting of [D-Ala², MePhe⁴, Gly-ol⁵]enkephalin E/[A] curves with varying $[Ca^{2+}]_o$ and β-chlornaltrexamine treatment

Constraint	*Residual sum of squares*	*Degrees of freedom*	*F ratio*	P *value*
None	116.42	51	—	—
K_A	161.40	53	9.85	<0.001
n	156.98	53	8.89	<0.001
E_m	271.62	53	34.00	<0.001
τ	692.86	57	42.09	<0.001

E_m (see Methods section). According to the operational model, the asymptote, α, depends on τ (the operational efficacy), E_m (the maximal effect possible through the particular transducer system coupled to the receptors involved) and n (the slope index of the transducer system, that is, the sensitivity); $[A_{50}]$ depends on K_A (the dissociation constant for the agonist-receptor complex), τ and n; and m depends on τ and n.

In the case of α, its dependence on τ and n can be assessed by estimating this parameter as a fraction of E_m at each $[Ca^{2+}]_o$, in the absence of β-CNA. According to Equation (4) using the estimates of τ and n given in Table 2 for the unconstrained fit, α/E_m was 0.875 at 2.50 mM $[Ca^{2+}]_o$, 0.995 at 1.25 mM $[Ca^{2+}]_o$ and 1.000 at 0.63 mM $[Ca^{2+}]_o$. Although increases in τ and n in principle can lead to increases in intrinsic activity, in this case these effects were minimal due to the almost full agonism that DAGOL exhibited at normal $[Ca^{2+}]_o$. It was logical to deduce, therefore, that E_m was responsible for the increase in α. Certainly, constraining E_m to a single value for all the data resulted in a drastic loss of goodness-of-fit of the operational model (see Table 3). However, in considering this the response scale upon which the data were expressed is important. It is conventional when using the stimulated guinea-pig ileum preparation to record inhibitory responses such as those induced by μ-opioid agonists as fractional inhibitions of the twitch. In the present series of experiments this convention was employed but it was noted, as stated in the Results section, that the twitch size itself was reduced by lowering $[Ca^{2+}]_o$ meaning that the accompanying increase in α could have been the result of an altered response measurement scale. Indeed, when the factors by which $[Ca^{2+}]_o$ variation reduced twitch size were used to re-express DAGOL E/[A] data in g tension, it was found that the curves obtained in the absence of β-CNA had very similar asymptotes (1.94 g (2.50 mM $[Ca^{2+}]_o$); 2.37 g (1.25 mM $[Ca^{2+}]_o$); 2.05 g (0.63 mM $[Ca^{2+}]_o$). Therefore, it would not be justified to conclude that E_m is enhanced by lowering $[Ca^{2+}]_o$.

The increase in m accompanying reduction in $[Ca^{2+}]_o$ could in theory have been the result of the concomitant change in τ. As shown previously (Black *et al.*, 1985b; Leff, 1987), if n in the operational model is not unity then the gradients of E/[A] curves depend on τ. For example, curves which are steep, that is, n (and therefore m) is greater than unity, can become flatter by reducing τ without a change in n. In the present case, if the DAGOL E/[A] curve at 0.63 mM $[Ca^{2+}]_o$ is considered as the control, then, because it exhibited a slope value greater than unity (m = 1.60, see Table 1), flattening may have been predicted due to the reduction in τ that accompanied increasing $[Ca^{2+}]_o$. However, according to the operational model definition of m (Equation (6)) the factor by which τ changed is not sufficient to account for the observed flattening. Assuming that the value of n is 1.48 (see Table 2) the value of m at 0.63 mM $[Ca^{2+}]_o$, for a τ value of 187.47 is predicted by Equation (6) to be 1.48 which is close to the experimentally obtained value (1.60); the value of m at 2.50 mM $[Ca^{2+}]_o$ for a τ value of 7.98 would be predicted to be 1.36, which is significantly higher than the experimental value (1.07). Therefore, a change in n is necessary to account for the alteration in m. This is confirmed by the significant worsening of fit that results from constraining the model to estimate a single value of n for all the data (see Table 3).

The increase in $p[A_{50}]$, that is, the leftward shifting of E/[A] curves accompanying reduction in $[Ca^{2+}]_o$ appeared to be dominated by the enhancement in τ (see Table 2). The previous analysis showed that n is enlarged; this change by itself produces rightward displacement of curves (Black *et al.*, 1985b) and therefore cannot have accounted for the potentiation. Similarly, the effect of lowering $[Ca^{2+}]_o$ is to have reduced pK_A rather than enhancing it (see Table 2). Inspection of the effects of β-CNA on DAGOL E/[A] curves (Figure 3) indicates quite clearly that there is more resistance to asymptote depression as $[Ca^{2+}]_o$ is lowered, implying an enhancement of τ. This is confirmed by the drastic worsening of fit that results from constraining the three τ values (in the absence and presence of β-CNA) to be the same at each $[Ca^{2+}]_o$ (see Table 3). The apparent reduction in agonist affinity as $[Ca^{2+}]_o$ is lowered was also significant as judged by F-ratio analysis (see Table 3). Rather than enhancing agonist potency, this change would have offset the effect of τ enhancement.

Summarizing this analysis, significant changes in the asymptote, location and slope of DAGOL E/[A] curves were the result of significant changes in K_A, τ and n, although probably not E_m. Thus, by lowering $[Ca^{2+}]_o$, the affinity of DAGOL appears to have been reduced, its efficacy in this system enhanced and the transduction of its receptor occupancy into effect increased in sensitivity. In principle, the change in affinity and efficacy could have resulted from Ca^{2+}-dependent alterations in the state of the μ-receptor in this tissue. It is noteworthy in this context that, if such a change of state occurred, it did not affect the affinity of the antagonist, naloxone.

These findings can be interpreted in two ways. It is quite conceivable that alkaloid-like structures, such as naloxone, and peptides, such as DAGOL utilize different attachment sites in the μ-receptor – the changes in state may not involve those sites to which naloxone binds. Alternatively, the change in agonist affinity, though statistically significant, should not be considered as meaningful in the context of the naloxone result. A change in the state of the receptor does not have to be invoked in order to explain the change in τ. This could have resulted from an enhancement of

the efficiency of the transducer relation linking μ-receptor occupancy to effect. This corresponds to a decrease in K_E, in the operational model. It is possible to devise hypothetical mechanisms for this change which also explain the gain in sensitivity in transducer relation based on the suggested coupling of μ-receptor occupancy to K^+-conductance (and, therefore inhibition of acetylcholine release) via elevation of intracellular calcium concentration (Tokimasa *et al.*, 1981; North, 1986). Any hypothesis of this kind must remain speculative in the absence of electrophysiological experiments designed to elucidate the mechanism of the operational changes, and, therefore, will not be considered further here. The point is that most of the $[Ca^{2+}]_o$-dependent change in the expression of agonism by DAGOL can be attributed to alterations in transduction. A change in state of the receptor is required only in order to account for the change in agonist affinity.

Regardless of the mechanistic bases for these results, they have implications for the analyses of μ-receptor agonism. Firstly, variation in $[Ca^{2+}]_o$ may be a useful experimental manipulation for converting weakly-effective agonists into more strongly-effective ones, but it should be recognized that the conversion involves a change in the shape of the transducer relation as well as an increase in its efficiency. In fact, the increase in co-operativity detected here actually acts against the desired increase in agonist expression. The effects of varying n in the operational model have been analysed previously (Black *et al.*, 1985b) showing that for partial agonists of efficacy, τ, less than unity, the effect of increasing n is actually to lower, rather than to raise effectiveness as indicated by E/[A] curve asymptotes. In the present example, the increase in τ would oppose this depressive effect, but it is interesting to speculate that there may be systems in which the change in co-operativity dominates to the extent that the actions of partial agonists are depressed rather than enhanced by lowering $[Ca^{2+}]_o$. The converse prediction applies for agonists which demonstrate efficacies greater than unity (Black *et al.*, 1985b), meaning that observations made on high efficacy ($\tau > 1$) agonists are not representative, indeed they are totally misleading of expectations for weakly-effective ($\tau < 1$) agonists. Also, with increases in n there is increased resistance of full agonist E/[A] curves to asymptote depression by irreversible antagonism (Black *et al.*, 1985b). Whether the changes in n are experimentally induced or unavoidable due to differences between tissues, they are likely to influence calculations of so-called receptor reserve. An agonist demonstrating equal efficacies in two systems characterized by different values of n will exhibit different receptor reserves, as estimated by the proportion of occupied receptors required to achieve a certain fraction of E_m. Neglect of such co-operativity changes could lead to errors in estimation of the relative intrinsic efficacies of agonists at different values of $[Ca^{2+}]_o$.

More generally, the present analysis illustrates another situation in which E/[A] curve shape information is critical in the interpretation of data (for other examples, see Leff, 1987). Methods of analysis which, unlike the direct model-fitting approach, avoid curve shape considerations, such as the traditional, null equation approach, are not appropriate to elucidating problems of this kind.

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