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Evidence for the ϵ-Type of Opioid Receptor in the Rat Vas Deferens

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SUMMARY

The ϵ-opioid receptor type has been postulated to be specific for β-endorphin on the basis of structure-activity studies in the isolated rat vas deferens (RVD). Since β-endorphin also displays high affinity for other opioid receptors, the present investigations were conducted to better define the ϵ-receptor in the RVD. Therefore, the interaction of concomitantly acting pairs of opioid agonists was examined and analyzed according to the predictions of the law of mass action and the receptor theory. Our data provide evidence for a specific receptor for β-endorphin in this organ, a receptor not recognized by either the δ-agonist D-Ala^2-D-Leu^5-enkephalin, or the μ-agonists FK-33824, fentanyl, and etorphine in the range of concentrations necessary for them to exert maximal inhibition of electrically induced twitch tension. The activity displayed by β-endorphin in the RVD is in agreement with the existence of the proposed ϵ-type of opioid receptor therein.

INTRODUCTION

The opioid peptide β-EP[1] is a potent inhibitor of electrically evoked twitching of the isolated RVD (1–3). The fact that only β-EP was a potent agonist in this organ led to the proposal of a novel type of opioid receptor, the ϵ receptor (1, 4, 5). In subsequent studies Schulz *et al.* (6), using fragments and derivatives of β-EP as well as preparations rendered tolerant to opioid agonists, concluded that in the RVD β-EP acts through an opioid receptor type different from μ, δ, and κ. In the RVD shorter sequences than the first 21 aminoacids are considerably weaker in inhibiting electrically evoked twitches than β-EP itself and fragments consisting of less than 17 aminoacids are practically inactive. Similar conclusions with respect to these requirements in the RVD were reported by Huidobro-Toro *et al.* (7). These data contrast with findings in the guinea-pig ileum and mouse vas deferens, where shorter fragments are strong opioid agonists.

The present study attempts to obtain further evidence for the existence of the ϵ receptor. We have analyzed the effects of pairs of opioid agonists acting simultaneously to inhibit the twitching of the RVD. Conceptually, this approach is based on the principles of the law of mass action and the receptor theory initially developed by Ariëns *et al.* (8–11). The concentration effect curves for one agonist are constructed after initial effects are induced by fixed concentrations of a second agonist. When both agonists act on the same receptor (simple competition) the curves are different from those seen when each one acts on a different one (functional synergism). The experimental results were compared with the theoretical predictions describing each one of these two situations. By making that comparison we could ascertain the number of opioid receptors involved in the over-all effect of the agonists.

THEORETICAL CONSIDERATIONS

When two agonists displaying qualitatively identical effects are both present in a biological system, they will behave either as competitors for the same receptor or as functional synergists, thus binding to different ones. Ariëns *et al.* (9, 11) introduced the concept of functional interaction of two drugs that may bind to their own specific receptor but produce their effect by means of a common effector. In 1973, a revised model of functional interaction was proposed by F. G. Van den Brink (12), which considered the concepts of receptor reserve and nonlinear stimulus-effect relationships.

It must be stressed that when two substances displaying an identical effect on a particular system are introduced together, their action will coincide at some level along the chain of events triggered by them and thus lead to the final effect observed. They may compete for the receptor (simple competition), they may activate the same effector but through different receptors (functional synergism with common effector), or they may just share the function, the effectors and the receptors being distinct (pure functional synergism). For two agonists producing the same maximal effect in the system, when the effector is common to both synergists, the maximum effect of both together is never larger than that of either

[1] The abbreviations used are: β-EP, β-endorphin; RVD, rat vas deferens; βh-EP, human β-EP; DADLE, D-Ala^2-D-Leu^5-enkephalin; β-FNA, β-funaltrexamine.

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one acting alone, and in their interaction sigmoid-like curves are always obtained (9, 11). On the other hand, when the effector system is not shared, the maximum effect might be larger than that of either substance at the time the sigmoidal shape of the curve may be lost (12).

Competition at the binding site. The agonists A and B bind to the same site of the receptor to induce the biological effect. The total effect when acting together is linearly proportional to the binding of A plus B in the presence of each other. The effect of A plus B is never larger than the maximum obtainable by either A or B alone. Their interaction in equilibrium is represented by

$$E_{AB} = E_{A(B)} + E_{B(A)} = \frac{1}{1 + \frac{K_A}{[A]} \cdot \left[1 + \frac{[B]}{K_B}\right]} + \frac{1}{1 + \frac{K_B}{[B]} \cdot \left[1 + \frac{[A]}{K_A}\right]}$$

where $E_{A(B)}$ and $E_{B(A)}$ are the effects induced through the binding of A and B, respectively, to the receptor in the presence of the other agonist. K_A and K_B are the dissociation constants of A and B from the site. $[A]$ and $[B]$ are the concentrations of A and B in the biophase. Assuming the total bound insignificant, then $[A]$ and $[B]$ are equal to the concentrations introduced in the medium.

For $[B] = n \cdot K_B$

$$E_{AB} = \frac{[A] + n \cdot K_A}{[A] + (n + 1) \cdot K_A} \quad (1)$$

and the fraction of effect induced by a fixed concentration of the agonist B before the concentration-response curve for A is constructed is given by

$$E_B = \frac{n}{n + 1} \quad (2)$$

Half of the remaining effect will be

$$E_B + \frac{1 - E_B}{2} = \frac{2 \cdot n + 1}{2 \cdot (n + 1)} \quad (3)$$

Combining Eqs. 1 and 3, it follows that

$$[A] = (n + 1) \cdot K_A \quad (4)$$

Once $[B]$ had produced the effect E_B, Eq. 4 gives the concentration of the agonist A needed to induce half of the remaining effect.

From Eqs. 2 and 4 we get

$$[A] = \frac{1}{1 - E_B} \cdot K_A$$

and also

$$[A] = \frac{1}{1 - E_B} \cdot \mathrm{EC}_{50}$$

EC_{50} is the concentration of agonist A inducing half of the maximum effect when it acts alone in the system.

In Fig. 1, *I*, the competitive interaction of two agonists, is simulated. It is shown how the concentration of the agonist A to reach $E_B + (1 - E_B)/2$ is clearly dependent on the initial E_B. The larger the E_B, the greater is $[A]$.

Functional synergism through a common effector system. The effect of agonist A binding to receptor I plus the effect of agonist B binding to receptor II is represented by

$$E_{AI,BII} = E_{BII} + (1 - E_{BII}) \cdot E_{AI}$$

$(1 - E_{BII})$ is the interference term, which expresses the coincidence of both actions at the same effector. For two full agonists, this interference term prevents the overall effect of both agonists together from being larger than the effect of either one acting alone in the system. If E_{BII} is the effect induced by $[B]$ acting on receptor II, then the concentration of A required to reach an effect $E_{BII} + E'$ is

$$E_{BII} + E' = E_{BII} + (1 - E_{BII}) \cdot E_{AI}$$

$$E' = (1 - E_{BII}) \cdot \frac{[A]}{[A] + K_A}$$

then it follows

$$[A] = \frac{E' \cdot K_A}{1 - (E_{BII} + E')}$$

For $E' = (1 - E_{BII})/2$, that is half of the remaining effect $[A] = K_A$.

Thus, for two agonists binding to different receptors after an initial effect is induced by one of them, the concentration of the other needed to reach half of the remaining effect is always its EC_{50} when acting alone in the system. In Fig. 1, *II*, the functional synergism between two agonists, is simulated.

Spare receptors in the interaction between agonists. The historical concept refers to the presence of a larger number of receptors than necessary to induce a full effect (13–15). Then the stimulus (receptor occupation)-effect relation is assumed to be nonlinear after the maximum effect is reached. However, in such situations, sigmoid-like curves are still observed in the action of the agonists (12). This fact has been explained in a number of ways. Levitzki *et al.* (16) and Boeynaems and Dumont (17) developed a model of receptor that accounted for the apparent presence of spare receptors. Other authors explained the presence of hyperbolic concentration-effect curves for the agonist in systems with spare receptors by considering the saturability of some postreceptor events in the transduction steps leading to the final response (18–20). The existence of a large number of spare receptors might reduce the probability of competition between two agonists known to bind to the same class of receptor. This phenomenon is better understood if we consider that one agonist is only required to bind to a very small fraction of receptors to practically saturate the response mediated by the effector. Thus, another agonist binding to the same class of receptor requiring a similar fraction of occupancy as the former to practically induce a maximal effect will compete mostly for the postreceptor steps in the expression of the response rather than for the binding sites. Therefore, this agonistic interaction will be close to the functional synergism. Obviously, the use

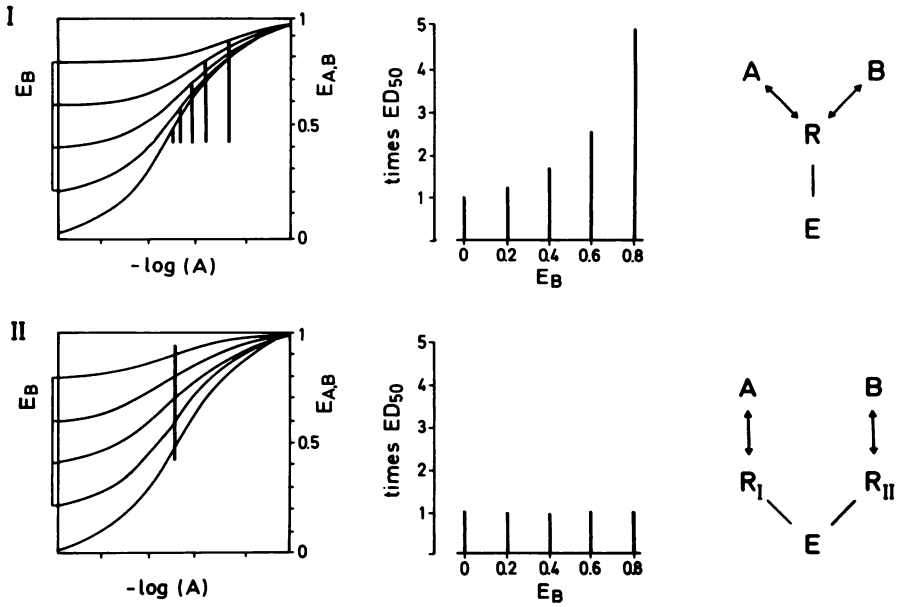

FIG. 1. *Computer-simulated log concentration-effect curves for agonist A combined with several concentrations of agonist B*
I, both agonists bind to the *same* receptor. *II*, each one binds to a *different* receptor.

of irreversible blockers to reduce the number of sites will help in distinguishing the nature of the interaction between the two agonists, particularly in situations in which the expected pattern of competition is not revealed.

Partial agonism. Of particular interest is the situation in which one agonist only produces a partial effect; then, depending on the kind of interaction that takes place between the two agonists, the resulting curves will be different (10, 11). In the competition for the same receptor, after the initial effect is induced by the full agonist, the partial agonist will bring the effect to its maximum in the system (Fig. 2, *I*) but if the initial effect is produced by the partial agonist, afterwards the full agonist will reach maximum effect, although its EC_{50} will be shifted beyond the value expected when two full agonists were interacting. The situation appears especially dramatic when the initial effect of the partial agonist is close to its ceiling effect (Fig. 2, *II*).

In contrast, functional synergism shows a different picture. After the initial effect is induced by the partial agonist, the remaining effect is reached by the full agonist with the same EC_{50} rather than without the partial agonist in the system, and if the initial effect is generated by the full agonist, the total effect will be greater than expected for the partial agonist alone (Fig. 2, *III*).

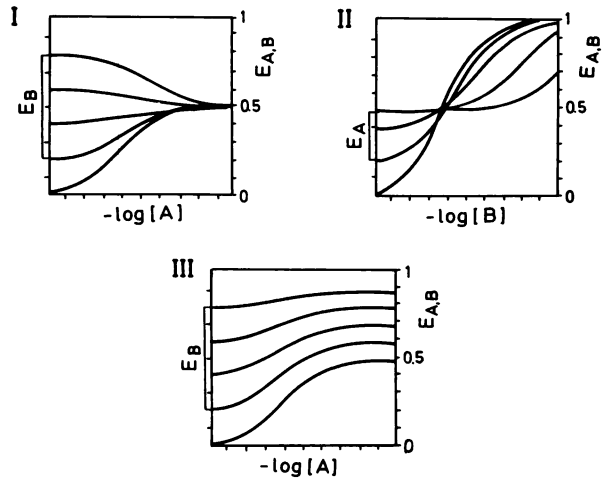

FIG. 2. *Computer-simulated log concentration-effect curves for partial agonist A (intrinsic activity = 0.5) interacting with the full agonist B*
I and *II*, both bind to the *same* receptor. *III*, each one binds to a *different* receptor.

MATERIALS AND METHODS

General procedures. Vasa deferentia from Wistar rats (200–300 g) were prepared and set up in 5-ml organ baths for electrical stimulation as described by Schulz *et al.* (1). The parameters for the electrical stimulation were 60 V, 0.1 Hz, 0.5 msec. The preparations were allowed to equilibrate for 90 min, receiving electrical stimulation from the first moment in the bath. The medium was changed every 10 min.

Stability of the agonist's effect. The stability of the inhibitory action of the agonist on the electrically induced twitch was monitored for 3 to 5 min; each agonist was added to the medium at a concentration able to inhibit the contracture between 30 and 70%. Preparations showing recovery from the inhibition were discarded. Not more than 15% presented this phenomenon.

Concentration-response curves. Concentration-response curves were constructed by increasing bath concentrations cumulatively (21, 22). The concentration was increased only when the previous one produced its maximum effect and remained constant. After completion of a concentration-response curve, the agonist was washed from the preparation. In this particular organ (RVD), the effect of accumulated concentrations was indistinguishable from that obtained using the single dose procedure. The same has been reported for other isolated organ preparations (23, 24).

Sensitivity to the agonist. In order to detect possible changes in the sensitivity of the preparation to the agonist during the course of the experiment, the "bracketing" procedure was utilized (25); therefore, the concentration-effect curve for the main agonist was always done both before and after studying its effects combined with the second agonist. Usually no more than two sets of combinations were carried out in

between the bracketing. Changes in sensitivity of the preparation in responding to the agonist were rarely detected during the interval required for our study. Although some figures depict experimental curves constructed over initial effects of smaller than 40%, to better differentiate between the two patterns of interaction described in Fig. 1, only initial effects from 40 to 80% were considered for our calculations. In general, the larger the initial effect, the better the information obtained, although it is important to compromise between the magnitude of the initial inhibition and the reliability of the remaining twitch to be inhibited afterwards by the other agent. In the RVD, we found that up to 90% of the twitch tension can be abolished and the remaining is still reliable. But as already mentioned, no initial inhibition greater than 80% was considered here.

Analysis of data. Plots of log concentration versus the efficiency to inhibit the twitch tension were constructed with 7 to 10 concentrations of the agonist. The results were analyzed transforming the ordinate (fraction of inhibition) into a probit scale. IC_{50} values were then obtained from linear regression analysis. Student's t test was utilized to evaluate statistically the differences between theoretical (predicted) and experimentally obtained IC_{50} values. The level of significance was set at $p < 0.05$.

Drugs. The following substances were used: βh-EP, human β-endorphin(1–27), DADLE (Peninsula Labs, San Carlos, CA); FK-33824 (Sandoz, Basle, Switzerland); etorphine (Reckitt and Colman, Kingston-upon-Hull, England); fentanyl (Janssen Pharmaceutica, Beerse, Belgium); and β-FNA (National Institute of Drug Abuse).

RESULTS

Interaction between human β-endorphin and other opioid agonists in the rat vas deferens. The interaction of βh-EP with other opioid agonists of defined receptor selectivity was studied by examining their inhibition of the electrically induced twitch of the RVD. An initial inhibition was induced by a fixed concentration of one opioid and when a stable effect had been reached, then the concentration-effect curve for βh-EP was constructed. In other experiments, βh-EP induced the initial effect and the curves for the other opioids were prepared. In Fig. 3, representative concentration-effect curves corresponding to the interaction of βh-EP with DADLE, FK-33824, etorphine, and fentanyl are shown. All these opioids gave identical patterns in interacting with βh-EP. A comparison between these curves and the models of Fig. 1 provides evidence for the similarity of the experimental set of curves with those predicted when the agonists are functional synergists through a common effector system. The curves obtained for βh-EP in the presence of an initial effect induced by one of the other opioids run apart from the curve for βh-EP when it was acting alone in the system. Only on reaching the maximum effect do the curves converge as expected from a common effector system (9, 11). They do not reach the maximum effect at lower concentrations than βh-EP alone, as would happen if the agonists were using different effectors (12).

When both agonists compete for the same receptor, the IC_{50} values displayed by the second agonist will increase as the initial inhibitions induced by the first agonist increase. However, in the case where the agonists do not share the same receptor but bind to different ones, then the IC_{50} values for the second agonist will be completely independent of the magnitude of the initial inhibition induced by the first agonist. On these bases, the IC_{50} values are predicted for each situation, simple competition and functional synergism, and are compared afterwards with those experimentally obtained for the opioids in inhibiting the remaining twitch tension. The procedure is also explained in Table 1. Our results yielded IC_{50} values in good agreement with those predicted for the case of different receptors for the two agonists (Table 2). Therefore, βh-EP binds to a receptor that is in a separate class from that of DADLE, FK-33824, etorphine, and fentanyl. On the other hand, βh-EP in interacting with itself gave ratios for one and two receptors of 1.02 ± 0.05 and 2.72 ± 0.26 ($n = 10$), respectively.

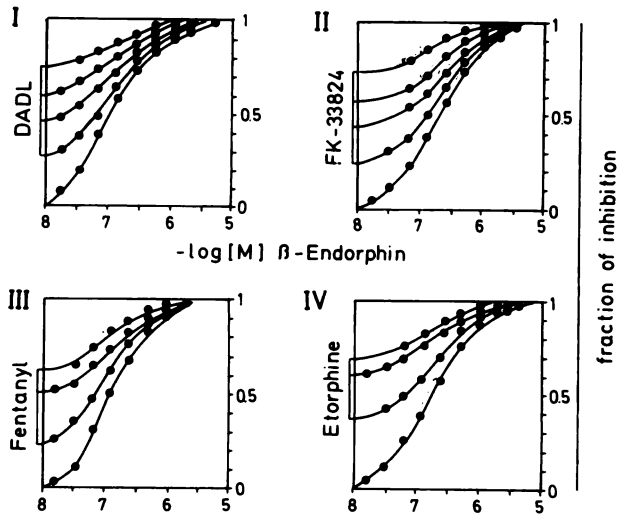

FIG. 3. *Experimental log concentration-effect curves for human β-endorphin combined with several concentrations of different agonists in the inhibition of the electrically induced twitch of the isolated RVD*

For each organ, the procedure used was as follows. A concentration-response curve for β-EP was cumulatively constructed and the IC_{50} was determined. After removing the peptide by repeated washing, one of the other agonists, used at a fixed concentration, induced an initial inhibition of the twitch amplitude. At this stage, a concentration-effect curve for βh-EP was again constructed and the IC_{50} for βh-EP in reducing to half the remaining twitch amplitude was evaluated as described in Materials and Methods. *I*, DADLE was used at 300, 750, 1400, and 2700 nM to induce fractional inhibitions (E_i) of 0.25, 0.45, 0.58, and 0.75, respectively. The IC_{50} of βh-EP was 127 nM in the absence of, and 115, 120, 106, and 127 nM, respectively, in the presence of the successively higher concentrations of DADLE. *II*, FK-33824 was used at 90, 250, 380, and 800 nM to induce E_i values of 0.25, 0.5, 0.6, and 0.75. The IC_{50} of βh-EP was 185 nM in the absence of, and 173, 223, 202, and 228 nM, respectively, in the presence of the successively higher concentrations of FK-33824. *III*, fentanyl (50, 200, and 300 nM) induced E_i values of 0.2, 0.5, and 0.6. The βh-EP IC_{50} was 114 nM in the absence of, and 107, 101, and 115 nM, respectively, in the presence of the increasing concentrations of fentanyl. *IV*, etorphine (5, 6.5, and 9.5 nM) induced E_i values of 0.6, 0.7, and 0.75. The βh-EP IC_{50} was 120 nM in the absence of 115, 100, and 127 nM, respectively, in the presence of the increasing concentrations of etorphine.

Interaction among opioid agonists other than βh-EP in the RVD. The previous results indicate a specific receptor for βh-EP that is not shared with the other opioids. Now the question is whether the other opioids share just another receptor or, on the contrary, whether more than two receptor types are present in the organ. An interesting interaction was the one between the μ-agonist FK-33824 and the δ-agonist DADLE. The results are summarized in Fig. 4 (*I*) and Table 3. The curves resemble

TABLE 1

Interaction between agonists in the rat vas deferens

Agonist	E_i[a]	IC_{50}[b]	Expected IC_{50}[c]		Ratio	
			1	2 receptors	1	2 receptors[d]
	nM		*nM*			
βh-EP	0	185				
+FK-33824						
350	0.5	223	370	185	0.60	1.20
700	0.6	202	462	185	0.43	1.09
FK-33824	0	142				
+DADLE						
1200	0.44	280	253	142	1.10	1.97
4200	0.75	547	568	142	0.96	3.85

[a] E_i is the effect induced by an agonist (FK-33824 or DADLE) before the concentration-effect curve for the other agonist is constructed (βh-EP or FK-33824). E_i values are computed from the recordings.

[b] IC_{50} is the concentration of agonist (β-EP in its interaction with FK-33824 and FK-33824 interacting with DADLE) that inhibits 50% of the remaining twitch.

[c] In the interaction between two agonists, these are the predicted IC_{50} values for the main agonist when they compete for the same receptor (1-receptor system) or each agonist binds to a different one (2-receptor system, functional synergism). For one receptor, the expected IC_{50} is $1/(1-E_i)$ times the IC_{50} obtained in absence of E_i, and for two receptors it is always the IC_{50} without E_i. For further details, see Theoretical Considerations.

[d] Ratio between experimental and predicted IC_{50} values. A value of 1 indicates which of the two possibilities (1 or 2 receptors) is mediating the agonistic interaction.

TABLE 2

Interaction of human β-endorphin with other opioid agonists in the RVD

The interaction of βh-EP with other opioid agonists was studied in the inhibition of electrically induced twitching of the RVD. The experimental procedure is described in Materials and Methods and Table 1. E_i values were all between 0.4 and 0.8 of fractional inhibition of the twitching. Mean ± standard error ratios (experimental versus predicted IC_{50} values for 1- and 2-receptor interaction) from n independent determinations are shown. n is the number of times the agonistic interaction was carried out for a particular pair of agonists. At least four different organs were used for each pair of agonists. IC_{50} values found for these agonists when acting along were (nM): βh-EP, 132.3 ± 14.9; FK-33824, 230.7 ± 27.0; DADLE, 2602.3 ± 205.0; etorphine, 15.3 ± 1.52, and fentanyl, 230.2 ± 25.2 (n = 10). For details see Table 1.

Agonist	n	IC_{50} ratio	
		1 receptor	2 receptors
FK-33824	8	0.452 ± 0.051[a]	1.075 ± 0.042
DADLE	7	0.465 ± 0.066[a]	0.930 ± 0.057
Etorphine	7	0.424 ± 0.058[a]	0.968 ± 0.050
Fentanyl[b]	7	0.465 ± 0.048[a]	0.952 ± 0.047

[a] Statistically different from 1. Student's t test; $p < 0.05$.

[b] In some preparations, fentanyl behaved as a partial agonist.

those simulated in Fig. 1 (*I*) for the competition at the receptor level. Here, the FK-33824 concentration-response curves, combined with different concentrations of DADLE, converge with the main curve for FK-33824 in absence of DADLE before the total inhibition is reached. The interaction between FK-33824 and etorphine (Fig. 4, *II*; Table 3) also showed a pattern coincident with competition at the receptor. Pilot experiments

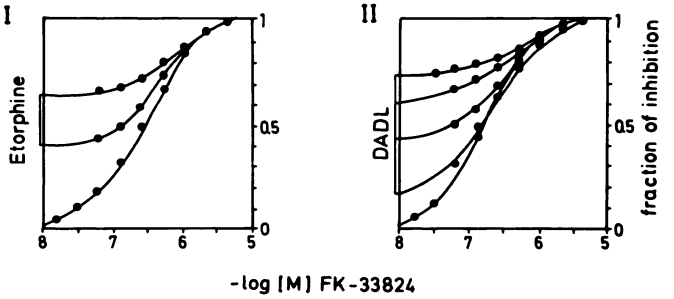

FIG. 4. *Experimental log concentration-effect curves for FK-33824 combined with several concentrations of DADLE and etorphine in the inhibition of electrically induced twitch of the isolated RVD*

The experimental procedure was as described in Fig. 3. FK-33824 was used instead of βh-EP. *I*, DADLE was used at 300, 1200, 3200, and 4500 nM to induce fractional inhibitions of the twitch amplitude (E_i) of 0.17, 0.44, 0.6, and 0.75, respectively. The IC_{50} of FK-33824 in this organ was 142 nM in the absence of and 210, 254, 380, and 547 nM, respectively, in the presence of increasing concentrations of DADLE. *II*, etorphine at 6 and 15 nM induced E_i values of 0.4 and 0.65, respectively. The FK-33824 IC_{50} was 235 nM in the absence and presence of the successively higher concentrations of etorphine.

TABLE 3

Interaction of FK-33824 with DADLE and etorphine in the RVD

For details, see Tables 1 and 2.

Agonist	n	IC_{50} ratio	
		1 receptor	2 receptors
DADLE	9	1.020 ± 0.048	2.311 ± 0.283[a]
Etorphine	6	0.961 ± 0.059	2.865 ± 0.295[a]

[a] Statistically different from 1. Student's t test; $p < 0.05$.

confirmed the identity of etorphine- and DADLE-binding sites. Therefore, at least another type of opioid receptor different from that selective for βh-EP is present in the organ and is involved in the effect of FK-33824, DADLE, and etorphine.

Fentanyl as partial agonist: interaction with other opioids in the RVD. A partial agonist in its interaction with full agonist gives different pictures depending on whether the same or different receptors are acted upon (8–11; see theoretical considerations). In several organs, mainly obtained from younger animals (200 g), fentanyl did not reach total inhibition of the twitch tension. In these organs, higher concentrations than those necessary to get a maximum effect induced an enhancement of the twitch amplitude. This excitatory effect has been described for morphine and its derivatives in the RVD (3, 26, 27).

Fig. 5A shows a representative concentration-effect curve for fentanyl behaving as partial agonist. In other organs where the opioid displayed similar behavior, the presence of an initial effect induced by fentanyl shifted the subsequent IC_{50} values for DADLE (Fig. 5B), etorphine, and FK-33824 (Fig. 5C) beyond the point expected for the competition of two full agonists. The crossing under the main curve of the full agonist after the initial effect of fentanyl introduced into the system agrees with the dualistic action of partial agonists. These curves compare satisfactorily with the computer-simulated graphs in Fig. 2, *II*. In Fig. 5D, the interaction of fentanyl

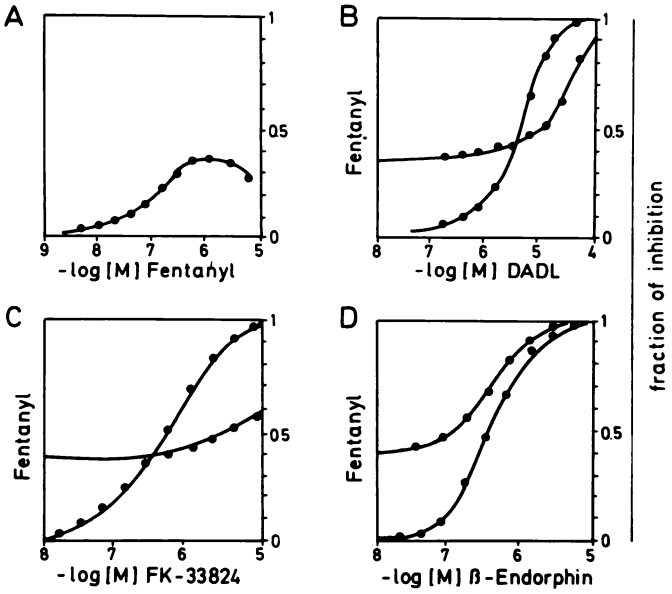

FIG. 5. *Experimental concentration-effect curves for fentanyl and its interaction with other opioids in the RVD*

Experimental procedure was as described in Figs. 1 and 2. A, fentanyl acting alone in inhibiting the electrically induced twitch of the isolated RVD. This curve is representative of fentanyl behaving as a partial agonist. Maximum effect was about 0.4. B, DADLE effect was reduced by the presence of 500 nM fentanyl. This concentration induced an E_i of 0.35. Fentanyl acted as a partial agonist in this organ. Maximum effect was 0.5. IC_{50} for DADLE acting alone was 3,467 nM. In the presence of fentanyl, it was 11,111 nM. C, FK-33824 concentration-effect curve was shifted to the right in the presence of 1,100 nM fentanyl. This concentration induced an E_i of 0.4. Fentanyl was a partial agonist in this organ. Maximum effect of 0.45. IC_{50} for FK-33824 acting alone was 400 nM. In the presence of fentanyl, it was 63,840 nM. D, βh-EP in interacting with fentanyl showed a pattern of synergism. The organ is the same as in C. The concentration and E_i induced by fentanyl are the same as above. IC_{50} for βh-EP acting alone was 180 nM. In the presence of fentanyl, it was 170 nM.

with βh-EP is shown and this particular organ is the one used for the interaction between fentanyl and FK-33824 depicted in Fig. 5C. Here, as predicted in Fig. 1, *II*, fentanyl was not different from a full agonist; the IC_{50} for βh-EP alone was 180 nM and in the presence of fentanyl 170 nM. Fentanyl at higher concentrations also reverses the inhibitory action of βh-EP, most likely through a complex mechanism involving its direct access to the βh-EP receptor plus the concurrence of the twitch enhancement. In Table 4, results from the interaction of fentanyl with FK-33824, DADLE, and etorphine are summarized. Fentanyl acted as a partial agonist in most of the organs. To provide a better understanding, the results are presented as in Table 1, and the E_i induced by fentanyl is normalized with respect to the maximum effect produced by the opioid in each organ. When the initial effect was close to the maximum attainable, i.e., FK-33824-fentanyl third interaction, then the shift for the FK-33824 curve was very pronounced (see Fig. 5C). The ratio of 11.9 found for one receptor interaction is likely to be due to the difficulty in evaluating the magnitude of the E_i induced by fentanyl when the effect of this opioid is reaching its asymptotic maximum effect. The results from Fig. 5 and Table 4 provide evidence to

TABLE 4

Interaction of FK-33824, DADLE, and etorphine with fentanyl in the RVD

Numbers in parentheses refer to the approximate maximum obtainable inhibition of twitching reached by fentanyl in a particular organ. Values smaller than 1 indicate the partial agonistic behavior of fentanyl in the organ. E_i values are referenced to maximal inhibitory effect of fentanyl, i.e., if fentanyl only reached 0.5 of the maximum effect (1.0), a submaximal fixed concentration of the opioid may induce 0.33 inhibition of the total twitch amplitude. This value is now referenced to the initial 0.5, giving 0.66. On the basis of that last number, the expected IC_{50} for the interaction at one receptor is computed. The concentrations of fentanyl inducing E_i values in these experiments were, from top to bottom, 300, 500, 1100, 500, 400, 500, and 500 nM.

Agonist	E_i[a]	IC_{50}[b]	Expected IC_{50}		Ratio	
			1	2 receptors	1	2 receptors
		nM		nM		
FK-33824	0	574				
+Fentanyl (1.0)	0.51	1,230	1,171	574	1.05	2.14
FK-33824	0	190				
+Fentanyl (1.0)	0.78	1,057	863	190	1.22	5.56
FK-33824	0	400				
+Fentanyl (0.4)	0.92	63,840	5,333	400	11.9	159.6
DADLE	0	3,467				
+Fentanyl (0.5)	0.66	11,111	10,197	3,467	1.08	3.20
DADLE	0	1,178				
+Fentanyl (0.7)	0.74	6,309	6,914	1,778	0.91	3.54
Etorphine	0	12				
+Fentanyl (0.7)	0.81	69	64	12	1.07	5.75
Etorphine	0	21				
+Fentanyl (0.7)	0.71	90	73	21	1.22	4.28

[a] E_i is the effect induced by fentanyl before the concentration-response curve for the other agonist is constructed.

[b] IC_{50} is the agonist concentration that inhibits 50% of the total remaining twitch after fentanyl initial inhibition. Expected values and ratio are as in Table 1.

TABLE 5

Interaction between βh-EP(1–27) and βh-EP or FK-33824 in the RVD

IC_{50} for βh-EP(1–27) was 55.5 ± 5.8 nM (n = 4). For details see Tables 1 and 2.

Agonist	n	IC_{50} ratio	
		1 receptor	2 receptors
Human β-endorphin(1–31)	3	1.01 ± 0.063	2.40 ± 0.352[a]
FK-33824	3	0.32 ± 0.052[a]	1.05 ± 0.125

[a] Statistically different from 1. Student's t test; $p < 0.05$.

conclude that FK-33824, DADLE, etorphine, and fentanyl bind to the same receptor in the RVD.

Interaction of βh-EP (1–27) with βh-EP and Sandoz FK-33824 in the RVD. βh-EP(1–27) was included in this study because this fragment has been reported to be the most active among several tested in the RVD (6). The results are shown in Table 5. It is evident that βh-EP(1–27) and βh-EP bind to the same receptor but one that is distinct from that binding FK-33824.

Pretreatment with β-funaltrexamine on the effect of agonists in the RVD. The RVD was incubated at 37° in the presence of the irreversible antagonist β-FNA (28) during electrical stimulation. Free antagonist was re-

moved by washing the preparations every 10 min for 90 min. Organs preincubated with β-FNA (300 nM) for 30 min were less sensitive to the inhibitory action of all the opioids in the study. In the same organ, FK-33824, DADLE, and etorphine displayed similar maximum effects but did not completely inhibit the twitch tension (27.7 ± 4.7, 25.2 ± 6.1, and 20.0 ± 4.3%, respectively; n = 4). The maximum effect of fentanyl was the lowest (about 8%), thus confirming the preliminary observation on its lower efficacy in its interaction with the receptor in the RVD. In contrast, βh-EP always reached about 2-fold higher levels of inhibition than the other opioids 52.5 ± 5.7%, n = 4; same organs as above).

The exposure of the organs to β-FNA (100 nM) for 30 min resulted in a decrease of β-EP and FK-33824 potencies (from 113.3 ± 10.8 to 329.3 ± 28.9 nM, ratio, 2.91, n = 3; and from 127.3 ± 28.1 to 563.0 ± 62.5 nM, ratio, 4.43, n = 3, respectively). In a series of pilot experiments, we found that 300 nM β-EP added to the incubation media 5 min prior to β-FNA (100 nM) prevented the β-EP efficacy from becoming deteriorated (ratio IC_{50} β-EP before/after β-FNA exposure of 1.27). However, the FK-33824 inhibitory effect on the twitching was diminished 3.21-fold. A higher concentration of β-EP (1.3 μM) not only protected β-EP effect on the RVD (IC_{50} ratio of 1.1) but also the FK-33824 action (IC_{50} ratio of 2.17). When 1.3 μM FK-33824 was utilized instead of β-EP in a similar protocol, the potency of FK-33824 after β-FNA (100 nM) only shifted 2.05 and 2.39 times the one of β-EP. In using 5.4 μM FK-33824 for protecting the receptors from β-FNA blocking action, FK-33824 IC_{50} only increased 1.57 times and β-EP 1.43-fold, confirming by this the possibility of having each of the agonists bound to both receptors, although at higher concentrations than those required to induce their practically maximum effect in the system through the occupation of their most specific opioid receptor in the RVD.

After these preliminary experiments, we selected 300 nM β-EP in an attempt to selectively protect the opioid receptors to which β-EP binds preferentially in the RVD from the inactivating action of β-FNA. After this procedure, the IC_{50} for βh-EP was changed very little. However, the IC_{50} values of FK-33824 and DADLE shifted 3- to 4-fold. In these preparations, fentanyl initially acted as a full agonist; after incubation in the presence of β-FNA plus βh-EP, fentanyl did not inhibit more than 40% of the twitch tension (Table 6). After the blockade of receptors by β-FNA (100 nM for 30 min), the agonistic interaction of β-EP and FK-33824 was studied. The ratios between experimental and predicted IC_{50} values for the interaction at one or two receptors were 0.401 ± 0.037 and 1.00 ± 0.056 (n = 6). Therefore, the existence of two different receptors for these two agonists is again indicated.

TABLE 6

βh-EP protection from β-FNA-blocking effect of the opioid receptors in the RVD

βh-EP (300 nM) was incubated with 100 nM β-FNA for 30 min. IC_{50} and IC_{50}' are the concentrations reducing the twitch tension to half before and after the preincubation with the irreversible agent. n is the number of experiments performed on different organs. After the incubation of the organs with β-FNA, β-EP still produced 100% of inhibition; FK-33824, 84.3 ± 1.66%; DADLE, 82.3 ± 2.1%. Fentanyl only attained 29.0 ± 5.6% with a half-concentration of 265.5 ± 52.5 nM.

Agonist	n	IC_{50}	IC_{50}'	Ratio
		nM	*nM*	
Human β-endorphin	4	121.2 ± 9.1	154.0 ± 14.6	1.27
FK-33824	4	152.6 ± 13.6	491.0 ± 41.4	3.21
DADLE	4	645.0 ± 82.1	2607.6 ± 460.3	4.04
Fentanyl	4	195.2 ± 19.1		

DISCUSSION

The present study has shown that βh-EP inhibits the electrically evoked twitch of this organ after binding to a receptor distinct from that for DADLE, FK-33824, etorphine, and fentanyl. Our results have been evaluated considering the two simplest possible interactions between the pairs of agonists, that is, both binding to the same receptor or alternatively to a different receptor with no noticeable cross-binding. In our case, there was no need to assume more complex models. The experimental results fit the predictions quite satisfactorily. On the other hand, the possibility that the presence of a large number of spare receptors would make the probability of competition at the receptor smaller than at the effector level was disregarded. One agonist "interacting with itself" would give a pattern of functional synergism in this case, but in our study, we did not find such a situation. βh-EP interacting with itself gave a competition image; moreover, βh-EP(1–27) also presented this pattern in interacting with βh-EP. It was also found that after the blockade of receptors by the irreversible agent β-FNA, the interaction between βh-EP and FK-33824 continued to indicate two different receptors. β-FNA has been reported to distinguish between μ and other opioid receptors (29); in the RVD, β-FNA diminished all the agonists' effects, although βh-EP always remained more potent than the other opioids. This result, together with the smaller shift found for βh-EP IC_{50} after the protection experiments against the effect of β-FNA, also supports the idea of a distinct receptor for βh-EP in the RVD. The other four opioids studied do not bind to this receptor over the range of concentrations required to induce their maximum effect in the test. It is likely that at higher concentrations they will also bind to the βh-EP site and vice versa, as we already mentioned in Results when dealing with protection experiments.

The multiplicity of opioid receptors in the RVD has been suggested before (6, 30, 31) on the basis of agonist activities, pA_2 determinations, and cross-tolerance studies. It has also been reported for this organ that the concentration-effect curves for agonists in the presence of increasing concentrations of antagonists lack parallelism (32). This phenomenon was also described for the mouse vas deferens (24), a system recognized for the heterogeneity of its opioid receptors (5), and was interpreted as the result of changes in the agonist affinity ratio for the different receptors in the presence of the antagonist. Thus, a similar finding in the RVD agrees with the plurality of opioid receptors in this organ.

Since etorphine, fentanyl, and FK-33824 are μ-ago-

nists and DADLE is a δ-agonist, it does not seem likely that the ε-receptor is constituted even partially by μ- and/or δ-sites, since a partial coincidence or overlapping in the binding site would give a pattern of competition between the agonists mentioned and βh-EP. On the other hand, all the types of opioid receptor so far described, μ, δ, κ, and ε, must have a common part of the binding site involving enkephalin recognition or, more critically, the tyrosine structure. Most of the endogenous opioid peptides have similar affinities for the δ-receptor and are not very much affected by elongation of the enkephalin pentapeptide (33). In contrast, the μ- and κ-affinities of these peptides are highly dependent on their length and sequence. Thus, for μ, κ (33), and ε (6, 7), activities, the presence of additional attachments is required. Evidently, unless an antagonist binds to different areas of these recognition sites unique to a particular receptor type, it will not distinguish among the receptors. Therefore, the agonistic interaction is a practical tool for revealing one or more receptors when selective antagonist are lacking. Moreover, this approach does not have the negative implications of changes in affinity ratios for the agonist's receptors that the presence of an antagonist may induce (24, 32), which would interfere with the correct interpretation of the results, for instance, establishing different receptors on the basis of pA_2 values.

The character of the ε-receptor is being ascertained by different studies, Schulz *et al.* (6) found no cross-tolerance between etorphine and βh-EP in the RVD, and studies carried out with iodinated (34) and tritiated β-EP (35–37) showed the complexity of its binding to brain membranes. At this initial stage, only μ- and δ-receptors were considered and, consequently, the existence of a specific receptor for β-EP was not revealed. Later on, additional studies provided some support for the binding of tritiated β-EP to the ε-receptor in the central nervous system (38, 39). All of these studies agree upon the variety of opioid receptors involved in β-EP binding. The fact that β-EP binds with similar affinities to some of these receptors complicates the issue of characterizing the ε-receptor. It is likely that cross-protection techniques as well as the specific blockade of the different opioid receptors binding β-EP will help in ascertaining the presence of this ε-receptor in the central nervous system. A similar approach has been successful in revealing the presence of the κ-type of opioid receptor in the binding of benzomorphans to brain membranes (40, 41). More recently, autoradiographic studies of [^{3}H]βh-EP in the rat brain have shown a pattern of binding for this peptide partially different from that of [^{3}H]dihydromorphine or [^{3}H]DADLE and therefore consistent with the presence of ε-receptors in the central nervous system (42). In conclusion, this evidence together with our finding in the RVD strongly suggests the existence of the ε-type of opioid receptor in the RVD as well as in the mammalian central nervous system.

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