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# Opioid receptor reserve in normal and morphine-tolerant guinea pig ileum myenteric plexus

(tolerance)

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**ABSTRACT** **We have measured the opioid receptor reserve in the guinea pig ileum myenteric plexus by means of the site-directed alkylating agent, β-chlornaltrexamine. Treatment of the tissue with low (<10 nM) concentrations of β-chlornaltrexamine caused a parallel shift of the log concentration–response curves for both normorphine and dynorphin A-(1–13). Analysis of the resulting curves indicated that the $K_d$ values were $1.5 \pm 0.5 \times 10^{-6}$ and $10 \pm 4 \times 10^{-9}$, respectively. Using the naloxone $K_e$ to distinguish between the μ and κ receptors in this tissue, we found that the receptor selectivities of normorphine and dynorphin A-(1–13) were unchanged after a maximum parallel shift, thus demonstrating that there are both spare μ and spare κ receptors present. The spare-receptor fraction for both receptor types was about 90%. In morphine-tolerant preparations (chronic pellet implantation), there was an apparent reduction in the fraction of spare μ receptors without any change in the apparent affinity of normorphine. Reduction in the spare receptor fraction does not necessarily imply reduction in the number of binding sites. We suggest that this reduction in receptor reserve is the basis of opioid tolerance, since the agonist concentration needed to produce a given effect is expected to increase as the receptor reserve decreases.**

The irreversible opioid receptor antagonist, β-chlornaltrexamine (β-CNA) is a site-directed alkylating agent synthesized and characterized by Portoghese and coworkers (1, 2). We showed previously (3) that *in vitro* treatment of the myenteric plexus-longitudinal muscle preparation from guinea pig ileum with low concentrations of β-CNA results in a parallel shift to the right of the log concentration–response curves for [Leu]enkephalin, normorphine, and dynorphin A-(1–13). Higher concentrations of β-CNA result in a reduced maximum response with concomitant decrease in slope. These findings provide classical evidence that there are spare opioid receptors (4, 5) in the myenteric plexus.

With spare receptors present, the $EC_{50}$ in the pharmacologic preparation should be lower than the $K_d$ measured by radioreceptor binding techniques. Yet when Creese and Snyder (6) and later Cox *et al.* (7) compared the $K_d$ and $EC_{50}$ values of various opioid agonists in the guinea pig ileum preparation, they found good agreement between these two parameters. Thus, our result was in sharp conflict with theirs. To rationalize the discrepancy, we hypothesized that the spare receptors we had observed with μ agonists were of the κ type and those observed with κ agonists were of the μ type. Thus, with each type of agonist, $EC_{50}$ and $K_d$ values might still agree. On this model, if the preferred receptor for an agonist were inactivated, that agonist would then exert its effects (at higher concentration) through another type of opioid receptor. Cox and Chavkin (8) showed that this phenomenon occurred after the mouse vas deferens was made selectively tolerant to the δ agonist [D-Ala$^2$,-D-Leu$^5$]enkephalin; then [Leu]enkephalin, which previously had acted on the δ receptor, now exerted its full effect (with potency reduced by a factor of 100) through the μ receptor, as indicated by a change in its naloxone sensitivity (see below). The first goal of the present study was therefore to determine what types of spare receptors are present in the guinea-pig myenteric plexus.

Our initial demonstration of the presence of spare opioid receptors in an intact tissue preparation (3) had two important implications. The first was that the sensitivity of a neuron to an opioid could be controlled by the magnitude of the opioid receptor reserve. This follows from the classical work on spare receptors by Stephenson (4), Ariens *et al.* (5), and Furchgott (9) showing that the greater the excess of functional receptors present, the lower the concentration of agonist required for effect. The second was the suggestion that tolerance to an opioid could be the consequence of a reduced opioid receptor reserve (10). In binding studies neither affinity nor site number in the guinea pig ileum myenteric plexus is affected by chronic morphine administration (11). The observed reduction in sensitivity of the tolerant tissue to opioids therefore implies that a higher fractional receptor occupancy is required for a given effect, as would be the direct consequence of a reduced spare-receptor fraction. The hypothesis that opioid tolerance is associated with a reduction of receptor reserve can be tested by the β-CNA technique. A preliminary account of our results was presented at the 1982 International Narcotic Research Conference (10); Porreca and Burks (12) later provided additional experimental support for this idea.

## MATERIALS AND METHODS

Guinea pig ileum longitudinal muscle strips were prepared from male Hartley guinea pigs (300–450 g) (Simonsen Laboratories, Gilroy, CA) as described (13). Strips (5 cm) in oxygenated Krebs bicarbonate buffer [composition (mM); NaCl, 118; KCl, 4.75; $CaCl_2$, 2.54; $KH_2PO_4$, 1.19; $MgSO_4$, 1.20; $NaHCO_3$, 25; glucose, 11; choline chloride, 0.02; pyrilamine maleate, $1.25 \times 10^{-4}$] under 1-g tension were stimulated maximally (field stimulation, 90 V, 0.1 Hz, 0.5 msec) with platinum electrodes, and the isometric contractions were recorded by a force-displacement transducer (Grass FT 0.03C) and a Grass polygraph. Before testing, the muscle strip was stimulated for 1–2 hr, with repeated changes of the bath solution. Opioid inhibition of the stimulated muscle twitch was measured as the ratio of minimum twitch amplitude in the presence of drug to that immediately prior to drug addition. Muscle contractions were allowed to return to pre-drug amplitude before the next dose was tested. Preparations not inhibited at least 95% by 1 μM normorphine were discarded.

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Abbreviation: β-CNA, β-chlornaltrexamine.

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Drugs and peptides were diluted in methanol/0.1 M HCl, 1:1 (vol/vol), which, in amounts <25 μl added to the 5-ml tissue bath, had no effect on the amplitude of contraction.

To measure the apparent naloxone equilibrium dissociation constant ($K_e$), the agonist $EC_{50}$ was first determined with at least two concentrations bracketing 50% inhibition of the twitch amplitude. Then, naloxone (50 nM final concentration) was added to the organ bath and, after a 20-min equilibration period, the agonist $EC_{50}$ was determined. The naloxone $K_e$ is given by $c/(dr - 1)$, where $c$ is the concentration of naloxone (here 50 nM) and $dr$, the *dose ratio*, is the ratio of agonist $EC_{50}$ in the presence of naloxone to that in its absence (14). Schild plots (15) showed that the naloxone $K_e$ is independent of the naloxone concentration used (unpublished observations).

β-CNA was dissolved in 0.1 M HCl in ethanol and stored at −70°C. Muscle strips were treated in the organ bath at 37°C for 20 min with freshly prepared dilutions of β-CNA. For the selective protection experiments, 3 nM β-CNA was used for 20 min in the presence of either 10 μM DADLE or 100 nM dynorphin A-(1–13) added 1 min prior to addition of β-CNA. These treatment conditions have been shown to confer selective protection on the μ and κ receptors, respectively, while allowing β-CNA to inactivate the unprotected receptor type (3, 16).

To prepare morphine-tolerant ilea, guinea pigs were implanted subcutaneously under ether anesthesia with morphine pellets (75 mg of base each), four on day 1 and six on day 3. This method was previously shown to induce morphine tolerance (11, 13). Animals were killed on day 6, and ileum longitudinal muscle strips were set up in the organ bath, then allowed to equilibrate for 2 hr before testing. The effect of β-CNA treatment on the agonist dose–response relationship was evaluated by the method of Furchgott (9) using the equation $1/[A] = [(1 - q)/qK_d] + 1/q[A']$. Here [A] and [A'] are equieffective concentrations of agonist before and after receptor alkylation. The fraction of receptors remaining after β-CNA treatment is defined as $q$; and $K_d$ is the equilibrium dissociation constant of the agonist.

[D-Ala$^2$,D-Leu$^5$]enkephalin, dynorphin A (heptadecapeptide), and dynorphin A-(1–13) were purchased from Peninsula Laboratories, San Carlos, CA; normorphine hemihydrate, from Applied Science Laboratories, State College, PA. Naloxone hydrochloride was a gift from Endo Laboratories, New York. Morphine pellets were prepared as described (13), and β-CNA was a gift from P. S. Portoghese and A. E. Takemori.

## RESULTS

The effects of treatment with various concentrations of β-CNA on the log concentration–response curve of normorphine are shown in Fig. 1*a*. Low (≤10 nM) concentrations of β-CNA resulted in parallel shifts without reduction in the maximum response. A higher (30 nM) concentration caused both reduction in the maximum response and concomitant depression of the slope. Comparison of normorphine potency before and after β-CNA treatment of the guinea pig ilium using the Furchgott method of analysis is shown in Fig. 1*b*. The apparent $K_d$ of normorphine calculated by this method was $1.5 \pm 0.5 \times 10^{-6}$ M. The $EC_{50}$ of normorphine in the normal preparation was 74 ± 9 nM ($N = 31$), a value 1/20th the $K_d$. The difference between the $EC_{50}$ and $K_d$ values indicates that only about 10% of the available functional opioid receptors present need to be occupied by normorphine to achieve maximal effect.

A similar result was obtained with dynorphin A-(1–13), except that a reduced maximum and shallow slope were seen after 10 nM β-CNA treatment, as shown in Fig. 2*a*. These effects of the higher β-CNA concentrations on slope were

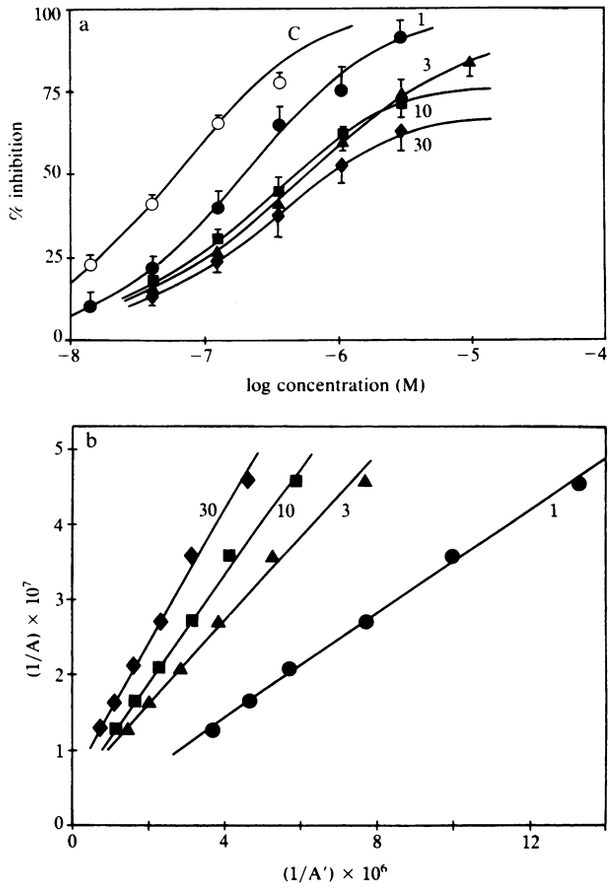


FIG. 1. Effects of β-CNA on normorphine log concentration–response curves. (*a*) Effects of different concentrations of β-CNA on normorphine potency in guinea pig ileum. Each point represents a mean, with the SEM shown as vertical bars. The following numbers of independent determinations were made on separate muscle strips: untreated (C), 31; β-CNA at 1 nM, 6; at 3 nM, 6; at 10 nM, 9; at 30 nM, 8. (*b*) Furchgott analysis of the equieffective normorphine concentrations before (1/A) and after (1/A') β-CNA treatment. Linear regression of the data points yields the slope ($m$) and $y$ intercept ($b$) that are used to calculate the $K_d$ by the equation $K_d = (m-1)/b$.

significant ($P < 0.01$) by least-squares analysis of data in the central nearly linear portions of the curves. As shown in Fig. 2*b*, Furchgott analysis of the equieffective concentrations before and after β-CNA treatment yielded a $K_d$ value for dynorphin A-(1–13) of $10 \pm 4 \times 10^{-9}$ M. The 22-fold difference between the estimated $K_d$ and the $EC_{50}$ of 0.47 ± 0.08 nM prior to β-CNA treatment indicates that about 90% of the dynorphin (κ) receptors are spare.

The receptor selectivities of normorphine and dynorphin A-(1–13) after maximum parallel shifts of the log concentration–response curves were investigated by measuring the naloxone $K_e$ (14). To increase the likelihood of being able to detect a change in the receptor type responding to a given agonist, the nonpreferred type was protected selectively during β-CNA treatment. In the guinea pig ileum, dynorphin A and dynorphin A-(1–13) act through the κ receptor (16–18), which is distinguished from the μ receptor by its lower (a factor of 10–15) affinity for naloxone. As shown in Table 1, the naloxone $K_e$ for dynorphin A-(1–13) was not significantly changed after β-CNA treatment caused a 19-fold shift in $EC_{50}$, under conditions previously shown to protect the μ receptor selectively. Similarly, the naloxone $K_e$ for antagonism of normorphine, which acts through the μ receptor in this preparation (19), was not altered after a 20-fold shift in $EC_{50}$ caused by β-CNA under conditions previously shown

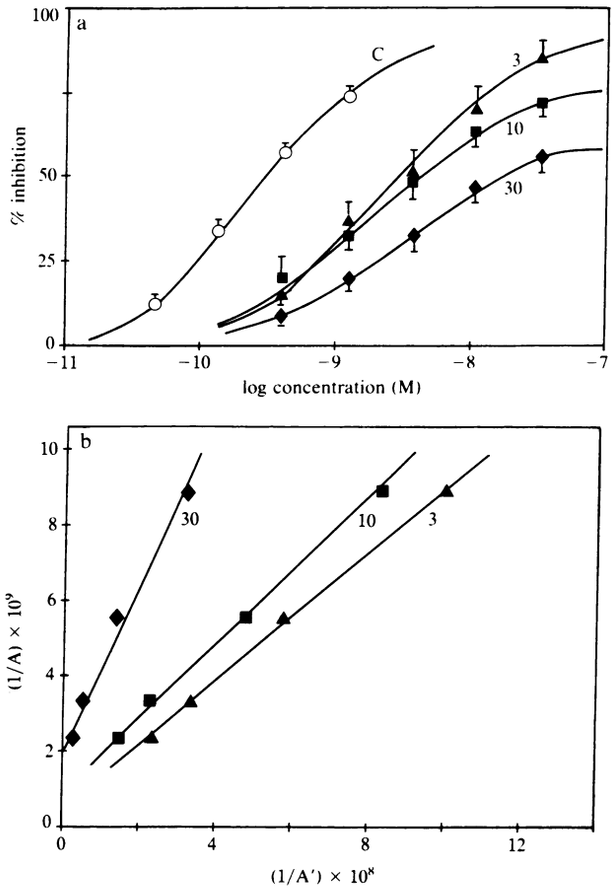


FIG. 2. Effects of β-CNA on dynorphin A-(1–13) log concentration–response curves. (*a*) Effects of different concentrations of β-CNA on dynorphin A-(1–13) potency in guinea pig ileum. Each point represents a mean, with the SEM shown as vertical bars. The following numbers of independent determinations were made on separate muscle strips: untreated (C), 22; β-CNA at 3 nM, 9; at 10 nM, 8; at 30 nM, 7. (*b*) Furchgott analysis of the equieffective concentrations before and after β-CNA treatment as described in Fig. 1.

to protect the κ receptor selectively. These findings indicate that there are spare receptors of both types in the guinea pig ileum myenteric plexus.

To ascertain whether morphine tolerance is associated with a reduction in the spare μ-receptor fraction, we used ileum muscle strips from guinea pigs implanted with morphine pellets. As shown in Fig. 3*a* and Table 2, chronic morphine exposure reduced the potency of normorphine by a factor of 3 on strips washed free of morphine for 2 hr. Such morphine-tolerant strips were distinctly more sensitive to the effects of β-CNA than were nontolerant preparations. For example, treatment with 3 nM β-CNA significantly reduced the slope and maximum here, whereas similar treatment of nontolerant preparations did not (Fig. 1*a*). Furchgott analysis (Fig. 3*b*) yields a $K_d$ value of $2.5 \pm 0.9 \times 10^{-6}$ for normorphine on morphine tolerant guinea pig ilea. This $K_d$ value is not significantly different from that obtained on nontolerant strips (Fig. 1*b*) ($P > 0.05$). Thus, in morphine-tolerant tissue, the spare receptor fraction is reduced, and 26% μ receptor occupancy is required for maximal effect, as compared with 10% in nontolerant tissue. This result is consistent with the observed factor of 3 reduction of potency in the tolerant strips. Table 2 also shows that, without β-CNA treatment, the slope of the normorphine log concentration–response curve for morphine-tolerant preparations was significantly steeper than that for nontolerant controls. This confirms an earlier finding by Cox and Padhya (11).

## DISCUSSION

Our conclusion that there are spare μ and κ opioid receptors in the guinea pig ileum myenteric plexus depends on the prior demonstration that β-CNA binds covalently to opioid receptors at the agonist binding site. It was shown that, when one receptor type is protected selectively during exposure to β-CNA, the number of binding sites is reduced without change in affinity of a type-selective ligand for the protected sites (20). Similar results were reported by Fantozzi *et al.* (21), who studied binding of [D-Ala$^2$-Met$^5$]enkephalinamide to NG108-15 cells. Moreover, the covalent binding of β-CNA can be blocked by opioid antagonists (1, 22) as well as by agonists (3, 16, 22). β-CNA is an opioid receptor-specific ligand demonstrated to have no effect on a wide range of other receptors (21, 22). Furthermore, the effects of β-CNA are not mimicked by nonopioid nitrogen mustard alkylating agents at similar concentrations (2, 22).

Our finding of a substantial receptor reserve in the guinea pig ileum (3) is at variance with the conclusions of Creese and Snyder (6) and of Cox *et al.* (7), who found good agreement between $K_d$ values (estimated by binding experiments on homogenates) and potencies in the bioassay. However, since ligand–receptor interactions in homogenates are studied under completely different conditions than obtain in intact preparations, such agreement could be fortuitous. Spare

Table 1. Characterization of spare receptors by type

| Treatment | Receptor type protected | Agonist | $EC_{50}$ shift (*N*) | Naloxone $K_e$, nM (*N*) |
|---|---|---|---|---|
| None | None | Dyn A-(1–13) | None | 20 ± 2 (25) |
| DADLE + β-CNA | μ | Dyn A-(1–13) | 19 ± 5 (5) | 24 ± 6 (5) |
| None | None | Normorphine | None | 3.3 ± 0.4 (24) |
| Dyn A-(1–13) + β-CNA | κ | Normorphine | 20 ± 5 (12) | 5.5 ± 0.8 (12) |

Guinea pig ileum longitudinal muscle strips were treated with 3 nM β-CNA in the organ bath at 37°C for 20 min in the presence of either 10 μM [D-Ala$^2$, D-Leu$^5$]enkephalin (DADLE) or 100 nM dynorphin A-(1–13) (Dyn A-(1–13)). Although DADLE is a partially selective δ agonist, in this tissue preparation it exerts its effect through μ receptors (16, 19). $EC_{50}$ shift is the ratio of the agonist $EC_{50}$ 90 min after β-CNA treatment to the $EC_{50}$ immediately before β-CNA treatment. Excess β-CNA and protecting ligand were removed by replacing the bath fluid every 10 min during the 90-min period after β-CNA treatment. The adequacy of this washing procedure has been demonstrated (3, 16). Treatment of muscle strips with 10 μM DADLE alone resulted in an $EC_{50}$ shift for Dyn A-(1–13) of 0.6 ± 0.04 ($n$ = 5), and treatment with 100 nM Dyn A-(1–13) alone resulted in an $EC_{50}$ shift of 0.9 ± 0.1 ($n$ = 8). Thus, no residual tachyphylaxis was evident after exposure to protecting ligand. Results represent mean ± SEM. Numbers in parentheses (*N*) are numbers of independent determinations on separate bioassay preparations. Receptor type (μ or κ) was characterized by sensitivity to naloxone.

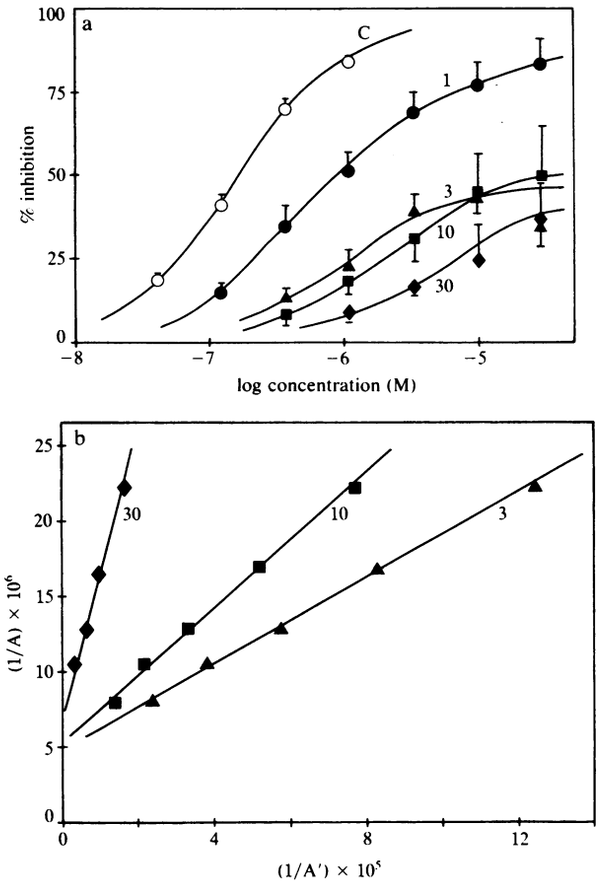


FIG. 3. Effects of β-CNA on nontolerant and morphine-tolerant preparations. (*a*) Effects of different concentrations of β-CNA on the log concentration–response curve of normorphine with ileum muscle strips from morphine-tolerant guinea pigs. The tissue bath did not contain an opioid except as added to generate points on the response curve. Each point represents the mean of the number of independent determinations listed as *N* in Table 2; the SEM is shown by vertical bars. The curves are labeled with the concentration (nM) of β-CNA used for treatment; C = untreated. (*b*) Furchgott analysis as described in Fig. 1.

opioid receptors have also been detected *in vivo* by Perry *et al.* (23), who concluded that only 2% receptor occupancy was required for full etorphine analgesia in rats. Similar evidence for spare opioid receptors was published by Tallarida and Cowan (24), who used an analgesia assay.

The pharmacologic potency of an agonist at its receptor is determined by binding site affinity and intrinsic activity (5) [or efficacy (4)] and also by the receptor density (8). Clearly, the potency of a given agonist could be different in various tissues and regions of the nervous system, depending on the local receptor density. As an example, Cox and Chavkin (8) showed that the mouse vas deferens contains fewer spare μ and κ receptors than does the guinea pig ileum, in agreement with the observed lower potencies of μ and κ agonists in the vas.

The spare-receptor fraction represents the excess of receptor binding sites over effector molecules (e.g., of an intracellular enzyme or an ion channel) to which the receptors can become coupled and which mediate the biologic effect. Thus, the spare-receptor fraction could be under physiologic control to regulate the sensitivity of the system by altering the number or activity of effector molecules or by altering the number or properties of the receptors. According to this view, tolerance to an exogenous opioid reflects a regulatory mechanism that is normally operative with respect to endogenous opioids. In the present study, the myenteric plexus exposed chronically to morphine was evidently able to reduce its sensitivity to opioids by decreasing its receptor reserve.

The explanation for reduced potency with reduction in receptor reserve is to be found in the stoichiometric interaction between receptors and effectors. A given biologic effect requires the switching on (or off) of a certain *number* of effector molecules. To accomplish this requires a smaller *fraction* of receptors to be occupied, the greater the excess of receptors over effectors. Since the law of mass action relates *fractional* occupancy to ligand concentration, it follows that a lower agonist concentration will suffice (higher potency), the greater the receptor reserve.

Adaptation to chronic opioid exposure has been studied extensively (25). The adaptive process has been localized to changes within the opioid-sensitive neuron. Given that the number of receptor binding sites does not change in the tolerant state in this tissue (11), our present findings indicate that opioid tolerance may result from a reduction in receptor reserve that comes about through altered activity of the coupled effector system. The molecular correlates of this change in receptor reserve are difficult to specify until the details of the opioid receptor transduction mechanisms are better understood. The effects of opioids on adenylate cyclase activity (26, 27) and on transmembrane ion conduc-

Table 2. Slopes and potencies for the log concentration–response curves in nontolerant and morphine-tolerant preparations of guinea pig ileum (GPI)

| | Normorphine on nontolerant GPI | | | Dynorphin A-(1–13) on nontolerant GPI | | | Normorphine on morphine-tolerant GPI | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | $EC_{50}$, nM | Slope | *N* | $EC_{50}$, nM | Slope | *N* | $EC_{50}$, nM | Slope | *N* |
| None | 74 ± 9 | 42 ± 2 | 31 | 0.47 ± 0.08 | 46 ± 4 | 26 | 230 ± 24 | 66 ± 3* | 53 |
| 1 nM β-CNA | 410 ± 140 | 46 ± 5 | 6 | ND | ND | ND | 980 ± 210 | 49 ± 5 | 10 |
| 3 nM β-CNA | 650 ± 250 | 39 ± 4 | 6 | 11 ± 4 | 35 ± 4 | 9 | >30,000 | 32 ± 5* | 16 |
| 10 nM β-CNA | 800 ± 330 | 32 ± 3* | 9 | 11 ± 4 | 32 ± 6† | 8 | >30,000 | 22 ± 8* | 5 |
| 30 nM β-CNA | 1200 ± 440 | 27 ± 1* | 8 | 28 ± 11 | 27 ± 3* | 7 | >30,000 | 15 ± 7* | 3 |

Effects of 20-min treatment with various concentrations of β-CNA on the slope of the normorphine log concentration–response curve. Potency is expressed as $EC_{50}$, the agonist concentration that exerts half-maximal effect. Slope is change in percentage inhibition per logarithmic unit of concentration, estimated by least-squares analysis of data in the nearly linear portion of the curve between 20% and 80% inhibition. Morphine-tolerant ileum longitudinal muscle strips were from morphine pellet-implanted animals; the tissue bath contained normal Krebs bicarbonate buffer. Values for normal ilea are from Fig. 1*a*, dynorphin A-(1–13) values are from Fig. 2*a*, and values for morphine-tolerant ilea are from Fig. 3*a*. ND, not determined. Results represent mean ± SEM for *N* independent determinations.

*$P < 0.01$ compared with control (untreated) values.

†$P < 0.05$ compared with control (untreated) values.

tance (28, 29), are two suggested effector systems. Within each of these systems, a change in receptor–effector coupling efficiency, in relative concentration of the molecular components, or in activity of the effector could result in the observed decrease in receptor reserve.

In view of the now well-established phenomenon of selective tolerance to the several types of opioid agonist (30), one has to suppose that, in tolerance to a $\mu$ agonist, as demonstrated here, the activity of a $\mu$ effector system is increased but not that of a $\kappa$ effector system; and presumably a corresponding selectivity applies in the case of tolerance to a $\kappa$ agonist. This implies that the different types of opioid receptor are coupled to different effector systems.

Morphine tolerance has been described by Cox as a two-component process (31). One component requires protein synthesis for its expression and reverses only slowly after removal of agonist from the system. The second component resembles receptor desensitization and reverses rapidly after drug withdrawal. Ileum muscle strips from morphine-tolerant guinea pigs, set up in the absence of morphine, have predominantly the first component; it was this preparation that was studied here and found to have a reduced spare-receptor fraction. Possible alteration of receptor reserve in the case of receptor desensitization could not be studied, because continuous presence of an opioid in the tissue bath would protect receptors against $\beta$-CNA and thus confound interpretation.

A limitation of our experiments on morphine-tolerant preparations is that we cannot rigorously exclude the unlikely possibility that some residual morphine continues to occupy some $\mu$ receptors. Although the exhaustive washing was sufficient to abolish morphine-induced inhibition in normal muscle strips, a tighter binding in tolerant ileum tissue is a possibility. In that case, some part of the reduction in receptor reserve could be due to such occupied sites.

In this paper, we have presented unambiguous evidence for separate spare $\mu$ and spare $\kappa$ receptors in the guinea pig ileum. We suggest that alteration of this receptor reserve could play a physiologic role in regulating the sensitivity to opioids of the neurons that bear opioid receptors. We also suggest that tolerance to an opioid agonist after chronic exposure may be due to an altered amount or activity of a post-receptor system, effectively reducing the spare fraction of receptors of the type with which the agonist interacts.

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