

# Role of Opioid Receptors in Bombesin-induced Grooming<sup>a</sup>

DEBRA E. GMERER<sup>b</sup> AND A. COWAN<sup>c</sup>

<sup>b</sup>*Department of Pharmacology  
University of Michigan Medical School  
Ann Arbor, Michigan 48109-0010*

<sup>c</sup>*Department of Pharmacology  
Temple University School of Medicine  
Philadelphia, Pennsylvania 19140*

## INTRODUCTION

Bombesin, a tetradecapeptide originally isolated from the skin of the frog *Bombina bombina*,<sup>1</sup> elicits dose-related excessive grooming when injected centrally in rats<sup>2-4</sup> as well as other species.<sup>5</sup> The grooming induced by intracerebroventricular (i.c.v.) bombesin in rats consists of hindlimb scratching directed primarily at the head and neck, although facial grooming, body washing, nail licking and biting, forepaw tremors, wet-dog shakes, and stretching also occur. Bombesin-induced grooming behavior can be observed within a few minutes of i.c.v. injection and continues for 30-45 minutes.

In contrast to the grooming induced by a variety of other peptides such as ACTH<sub>1-24}</sub>, thyrotropin-releasing hormone, substance P,  $\beta$ -endorphin, and nonpeptides such as RX 336-M (7,8-dihydro-5',6'-dimethylcyclohex-5'-eno-1',2',8',14 codeinone), bombesin-induced grooming is not inhibited by nonsedative doses of morphine.<sup>6-11</sup>

In keeping with multiple types of cholinergic, adrenergic, histaminic, and dopaminergic receptors, it is also well established that there are multiple types of opioid receptors. Based on observations in dogs, the existence of three types of opioid receptors was postulated: mu (for which morphine was the prototype), kappa (for which ketocyclazocine was the prototype) and sigma (for which *N*-allyl-normetazocine was the prototype).<sup>12,13</sup> Since then, mu and kappa types of opioid receptors have been differentiated by biochemical studies using receptor binding and visualization techniques (see, for example, refs. 14 and 15) and by pharmacological studies in which differences in acute effects, differences in the ease of antagonism by the opioid-specific antagonist naloxone, and the selective development of tolerance and dependence were identified (see refs. 16-18). Whereas sigma receptors are no longer considered to be opioid (*i.e.*, naloxone is not an antagonist of effects mediated by the "sigma" receptor), a third type of opioid receptor has been identified primarily through *in vitro* studies, the delta receptor.<sup>19</sup> The endogenous enkephalins have been proposed as natural ligands for the delta receptor.

In light of the multiple classes of opioids, we decided to examine opioids other than morphine for their ability to inhibit bombesin-induced grooming in rats. In our

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preliminary studies,<sup>8,9,20</sup> it became evident that ethylketazocine (a compound structurally and pharmacologically related to ketazocine) could antagonize bombesin-induced grooming. It was therefore decided to study further the role of opioid receptors in the modulation of bombesin-induced grooming.

Ethylketocyclazocine, and more recently tfluadom and U-50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(pyrrolidinyl)cyclohexyl-1]benzeneacetamide), have been used as prototypical kappa agonists. Ethylketazocine is a benzomorphan showing selectivity for kappa receptors in binding assays as well as in most tests *in vitro* and *in vivo*. It can act through mu receptors, however, to produce hot water tail-withdrawal analgesia and inhibit gastrointestinal transit in rats.<sup>21</sup> Tfluadom is a very interesting compound in that it has the structure of a benzodiazepine but little or no activity at benzodiazepine receptors; it has been found to be very selective for the kappa type of opioid receptor, however.<sup>22,23</sup> U-50,488 is a compound of novel structure for opioids.<sup>24</sup> In binding studies, U-50,488 has a selectivity of approximately 250-fold for kappa over mu or delta binding sites in quinea pig brain.<sup>25</sup> It has a strictly kappa profile of activity both *in vitro* and *in vivo* (see, for example, refs. 18, 24, 26, and 27).

In this work, the role of opioid receptors in bombesin-induced grooming was investigated by means of classical methods in opioid pharmacology: by classification of opioid-like compounds on the basis of their ability to inhibit the grooming elicited by a standard submaximal dose of bombesin; by examination of the stereospecificity of the interaction between the opioid and bombesin; by examination of the sensitivity of the opioids to naloxone; and through tolerance and cross tolerance studies.

## METHODS

### *Animals and Surgery*

Male Sprague-Dawley albino rats weighing 180–200 g were each implanted with a stainless steel cannula (Plastic Products Co., Roanoke, VA) directed towards the lateral cerebral ventricle as described previously.<sup>4</sup> The rats were housed individually at 23 ± 1°C with free access to food and water for at least 4–7 days before testing. They were exposed to a 12-h timer-regulated light period from 7 A.M. to 7 P.M.

### *General Procedure*

Experiments were carried out in the afternoons. At least 15 min before testing, the rats were transferred from their home cages and placed individually in Plexiglas observation boxes (22 cm long; 18 cm wide; 25 cm high). Four rats were monitored at a time with the aid of a portable microcomputer as described previously.<sup>11,28</sup> Test compounds, with the exception of dynorphin A, were injected 15 min prior to a standard submaximal groom-inducing dose of bombesin<sup>4</sup> (0.1 µg, i.c.v.). Dynorphin A and [D-Pen-2,D-Pen-5]enkephalin were given i.c.v. 5 min before bombesin (0.1 µg, i.c.v.).

### *Quantification of Behavior*

Beginning immediately after the last bombesin or i.c.v. saline control injection, each rat was observed for 5 s out of every 20 s for a total of 30 min. A positive grooming score was given if the rat demonstrated any type of grooming behavior (*e.g.*, scratching,

washing, licking, biting, etc.) during the 5-s observation period. Consequently, there was a total of 90 grooming episodes scored.

Results are presented as the %MGE (maximum number of grooming episodes scored as positive)  $\pm$  SEM. The %MGE was calculated for each rat (*i.e.*, number of positive grooming scores times 100, divided by 90). Groups of at least five rats were used to determine each data point. Intracerebroventricular saline control animals had %MGE scores of  $6 \pm 1$ . If, following the pretreatment with a test compound and bombesin administration, the majority of rats had %MGE of less than 6, their grooming behavior was said to be suppressed. Compounds were said to have the ability to antagonize bombesin-induced grooming only if they inhibited the scratching behavior in a dose-related manner at doses that did not suppress grooming behavior below *i.c.v.* saline controls.

Absolute grooming scores were analyzed statistically by means of ANOVA followed by Dunnett's test or the Mann-Whitney U-test, as appropriate.<sup>29</sup> Percent inhibition of bombesin-induced grooming was calculated as follows:

$$\frac{1 - (\text{bombesin grooming score in opioid-pretreated rats} - \text{saline controls})}{(\text{bombesin grooming score in saline-pretreated rats} - \text{saline controls})} \times 100.$$

$A_{50}$  values (*i.e.*, doses at which test compounds antagonize an effect by 50%) were determined by linear regression analysis<sup>30</sup> of absolute data from which saline control values had been subtracted. Confidence limits (95%) of  $A_{50}$  values were also determined.

### Compounds and Injections

The following compounds were used: azidomorphine bitartrate (J. Knoll, Semmelweis University of Medicine, Budapest, Hungary), bombesin (Sigma and Boehringer Mannheim, Indianapolis, IN), bremazocine HCl (D. Romer, Sandoz, Basel, Switzerland), buprenorphine HCl (National Institute on Drug Abuse, Rockville, MD), codeine phosphate (Merck Sharp & Dohme, West Point, PA), cyclazocine (Sterling-Winthrop, Rensselaer, NY), dextrorphan (NIDA), dynorphin A (Penninsula Laboratories, Belmont, Calif.),  $\beta$ -endorphin (A. A. Manian, National Institute of Mental Health, Bethesda, MD), [D-Pen-2,D-Pen-5]enkephalin (DPDPE, Penninsula), ethylketazocine methanesulfonate (EKC, Sterling-Winthrop), *d*-EKC and *l*-EKC (Sterling-Winthrop), ethylmorphine HCl (Merck Sharp & Dohme), heroin HCl (NIDA), ketocyclazocine methanesulfonate (Sterling-Winthrop), levorphanol tartrate (Hoffmann-La Roche, Nutley, NJ), meperidine HCl (Sterling-Winthrop), methadone HCl (NIDA), metkephamid (R. C. A. Frederickson, Eli Lilly Co., Indianapolis, IN), morphine sulfate (Mallinckrodt Inc., St. Louis, MO), nalbuphine HCl (Endo Laboratories, Inc., Garden City, NY), nalorphine HCl (Sigma), *d*-naloxone HCl and *l*-naloxone HCl (A. Jacobson, National Institutes of Health, Bethesda, MD), naloxone HCl (Endo), *l*-pentazocine (Sterling-Winthrop), phenazocine HBr (Smith Kline & French, Philadelphia, PA), quadazocine methanesulfonate (also known as Win 44,441-3; Sterling-Winthrop), thebaine (NIDA), racemic tipluadom, (+) and (-)-tipluadom (referring to the optical rotation as measured in ethanol; Kali-Chemi AG, Hannover, FRG), U-50,488 (*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(pyrrolidinyl)cyclohexyl-1]benzeneacetamide) (J. Collins, Upjohn Co., Kalamazoo, MI), xorphanol mesylate (H. G. Pars, Pharmaceutical Laboratories, Inc., Cambridge, MA), and zomepirac (McNeil Laboratories, Fort Washington, PA).

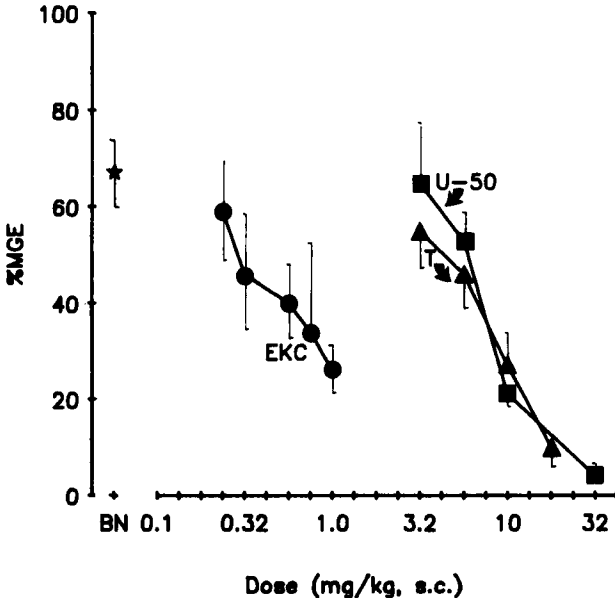
The alkaloids were dissolved in a minimal volume of glacial acetic acid, if necessary, and diluted with saline. Tipluadom (racemic and optical isomers) were dissolved

in a solution of 40% propylene glycol, 10% ethanol, 5% sodium benzoate and benzoic acid, and 15% benzyl alcohol by sonication and heat. Injections were given s.c. in volumes of 1 ml/kg with doses calculated as the base or salt as indicated above; the dose of *d*- and *l*-naloxone was calculated as the free base. Aliquots of bombesin, DPDPE, dynorphin A, and  $\beta$ -endorphin were each dissolved in saline daily as needed. Bombesin, DPDPE, dynorphin A and  $\beta$ -endorphin were injected i.c.v. to hand-held, conscious rats in volumes of 3–4  $\mu$ l followed by 0.5–1  $\mu$ l saline wash.

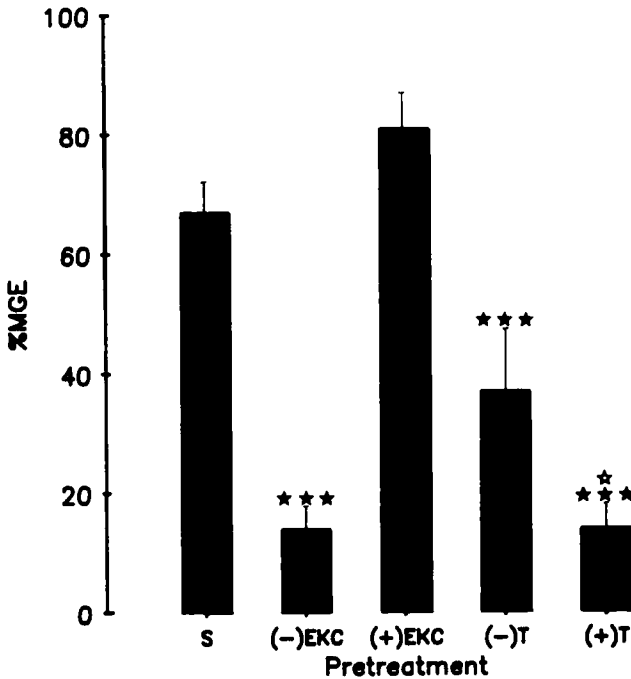
## RESULTS

### Opioid Classification

The following compounds given as 15 min s.c. pretreatments to a standard 0.1  $\mu$ g dose of i.c.v. bombesin did not attenuate bombesin-induced grooming at nonsedative doses, *i.e.*, doses that did not suppress grooming behavior below i.c.v. saline controls (highest nonsedative dose used in mg/kg, s.c., in parentheses): azidomorphine (0.05), buprenorphine (0.5), codeine (40), dextrorphan (30), ethylmorphine (100), heroin (0.5), levorphanol (1), meperidine (25), methadone (1), metkephamid (30), morphine (10), nalbuphine (10), naloxone (10), and thebaine (25).  $\beta$ -Endorphin (10  $\mu$ g, i.c.v.) given



**FIGURE 1.** The effect of 15-min pretreatment with ethylketazocine methanesulfonate (EKC), U-50,488 (U-50), or tifluadom (T) on the grooming induced by bombesin (0.1  $\mu$ g, i.c.v.). %MGE is the percentage of the maximum number of grooming episodes scored as positive during the 30-min observation session. Data points indicate the mean  $\pm$  SE for 5–8 rats. BN: %MGE following saline (1 ml/kg, s.c.) pretreatment.



**FIGURE 2.** The effect of 15-min pretreatment with saline (1 ml/kg, s.c.), the stereoisomers of EKC (both 0.5 mg/kg, s.c.), or the optical isomers of tifluadom (*T*, both 10 mg/kg, s.c.) on the grooming induced by bombesin (0.1  $\mu$ g, i.c.v.). %MGE is the percentage of the maximum number of grooming episodes scored as positive over the 30-min observation session. Bars indicate the mean  $\pm$  SE for 5-8 rats; \*\*\* $p$  < 0.001, ANOVA and Mann-Whitney U-test compared to saline + bombesin controls; ☆ $p$  = 0.02, ANOVA and Mann-Whitney U-test compared to (-)-tifluadom + bombesin.

15 min before bombesin or DPDPE (1 and 5  $\mu$ g, i.c.v.) given 5 min before bombesin also did not attenuate bombesin-induced grooming at doses that did not produce competing behaviors (catalepsy and rearing, respectively). Zomepirac (5 mg/kg, s.c.) had no marked effect on bombesin-induced grooming.

Bremazocine (10 mg/kg), cyclazocine (5 mg/kg), ketazocine (1 mg/kg), *l*-pentazocine (20 mg/kg), and phenazocine (0.5 mg/kg) attenuated bombesin-induced grooming significantly compared to saline + bombesin controls ( $p$  < 0.001; ANOVA followed by Dunnett's test for multiple comparisons) at non-sedative doses (%MGE scores remained greater than i.c.v. saline controls). Xorphanol attenuated bombesin-induced grooming in a dose-related manner with an  $A_{50}$  of 0.71 (0.41-1.21) mg/kg, s.c. Dynorphin A also antagonized bombesin-induced grooming in a dose-related manner with an  $A_{50}$  of 4.1 (2.8-5.9)  $\mu$ g, i.c.v.

FIGURE 1 shows the dose-related decrease in bombesin-induced grooming produced by EKC, U-50,488, and tifluadom. The  $A_{50}$ 's for EKC, tifluadom, and U-50,488 to antagonize bombesin-induced grooming were 0.36 (0.33-0.40), 6.61 (5.37-8.13), and 6.80 (5.52-8.36) mg/kg, respectively.

**TABLE 1.** Naloxone Antagonism of the Effect of s.c. EKC (0.5 mg/kg), U-50,488 (10 mg/kg), and Tifluadom (10 mg/kg) on Bombesin-induced Grooming (0.1  $\mu$ g, i.c.v.).

Opioid Agonist	Naloxone A <sub>50</sub> (95% C.L. <sup>a</sup> ), mg/kg, s.c.
EKC	0.076 (0.068–0.086)
Tifluadom	0.12 (0.08–0.20)
U-50,488	0.43 (0.30–0.60)

<sup>a</sup> Confidence limit.

### *Stereoselectivity of EKC and Tifluadom*

FIGURE 2 shows the stereoselectivity of EKC and tifluadom in their ability to antagonize bombesin-induced grooming. The levorotatory enantiomer, but not the dextrorotatory enantiomer, of EKC inhibited bombesin-induced grooming. Whereas both isomers of tifluadom antagonized bombesin-induced grooming, the (+)-optical isomer of tifluadom was significantly more effective than the (–)-isomer.

### *Sensitivity to Naloxone*

Naloxone itself, up to 10 mg/kg, had no effect on bombesin-induced grooming. Naloxone, however, reversed the effects of EKC, tifluadom, and U-50,488 (doses that inhibited bombesin-induced grooming by approximately 80%) in a dose-related manner. TABLE 1 shows the potency of naloxone against the effects of the three kappa agonists on bombesin-induced grooming. This action of naloxone was stereospecific in that *l*-naloxone (0.08 mg/kg) prevented the action of 0.5 mg/kg EKC on bombesin-induced grooming significantly ( $p < 0.01$ ), whereas *d*-naloxone (0.1 mg/kg) had no effect on the ability of the same dose of EKC to attenuate bombesin-induced grooming (data not shown).

**TABLE 2.** Effect of Multiple Injections of EKC or Morphine on the Ability of EKC to Attenuate Bombesin-induced Grooming

Treatment <sup>a</sup> (8 ×)	Challenge <sup>b</sup>	Bombesin-induced Grooming (%MGE ± SEM)
saline	saline	68 ± 3
saline	EKC	20 ± 4 <sup>c</sup>
EKC	saline	73 ± 6
EKC	EKC	85 ± 4 <sup>d</sup>
morphine	saline	62 ± 6
morphine	EKC	23 ± 4

<sup>a</sup> Eight injections over consecutive days as described in RESULTS.

<sup>b</sup> Challenge with saline (1 ml/kg, s.c.) or EKC (0.5 mg/kg, s.c.) was given 19–20 h after the eighth injection of saline, EKC, or morphine, and 15 min before bombesin (0.1  $\mu$ g, i.c.v.).

<sup>c</sup>  $p < 0.001$ , ANOVA followed by the Mann-Whitney U-test compared to saline + saline controls.

<sup>d</sup>  $p < 0.001$ , ANOVA followed by the Mann-Whitney U-test compared to saline + EKC controls.

### Tolerance Studies

Morphine was given at 5 P.M. and 8 A.M. at doses of 10, 10, 30, 30, 100, 100, 100, and 100 mg per kg per injection for a total of eight injections over consecutive days to two groups of eight rats. EKC was given eight times over consecutive days at the doses of 5, 5, 10, 10, 20, 20, 20, and 20 mg per kg per injection to two groups of rats. Another two groups of rats received twice-daily injections of saline (1 ml/kg, s.c.) for four consecutive days. Beginning 19–20 h after the last of the multiple morphine, EKC, or saline injections, the rats were challenged with saline (1 ml/kg, s.c.) or EKC (0.5 mg/kg, s.c.) and followed 15 min later with the standard dose of bombesin (0.1  $\mu$ g, i.c.v.) as indicated in TABLE 2. Multiple treatments with saline, morphine, or EKC had no effect on bombesin-induced grooming. Multiple injections of morphine also did not affect the ability of EKC to attenuate bombesin-induced grooming. However, multiple injections of EKC decreased significantly ( $p < 0.001$ ) the ability of EKC (0.5 mg/kg) to inhibit bombesin-induced grooming; *i.e.*, tolerance developed to EKC.

## DISCUSSION

The excessive grooming (scratching) behavior elicited by a standard submaximal dose of bombesin was attenuated in a dose-related and stereoselective manner by opioids showing activity at the kappa type of receptor. These included benzomorphan analgesics; the endogenous kappa-selective opioid peptide, dynorphin A; a morphinan mixed agonist-antagonist analgesic of the kappa type, xorphanol; and the most kappa-receptor-selective opioid available, U-50,488. It is important to note that the inhibition of bombesin-induced grooming by these opioids occurred in a dose-related fashion and at doses that did not obliterate natural grooming behavior.

In contrast, compounds whose agonist effects are thought to be mediated by the mu or delta types of opioid receptors were unable to affect bombesin-induced scratching at doses that did not completely suppress grooming behavior. Most of these compounds eventually prevented bombesin-induced grooming, but it was not a dose-related effect, and grooming behavior was prevented altogether: %MGE scores were below those of i.c.v. saline controls. These included the prototype mu-receptor agonist, morphine; the prototype delta-receptor agonist, DPDPE; and a mixed agonist-antagonist analgesic of the mu-type, nalbuphine. In addition, the nonopioid analgesic, zomepirac, had no marked effect on bombesin-induced grooming.

Ethylketazocine, tifluadom, and U-50,488 were examined more thoroughly as prototype kappa opioid agonists in their ability to affect bombesin-induced grooming. All three compounds inhibited bombesin-induced grooming in a dose-related and parallel manner. Ethylketazocine was approximately 18 times as potent as U-50,488 and tifluadom, which were equipotent. A similar potency relationship has been observed among EKC and U-50,488 and tifluadom in the rat tail-immersion test.<sup>24,31</sup> U-50,488 was found to be 10 times less potent than EKC in the rat tail-flick test.<sup>24</sup>

Ethylketazocine was stereospecific in its ability to affect bombesin-induced grooming in that the (+)-enantiomer was ineffective at doses at which the (–)-enantiomer had a significant effect. Tifluadom showed stereoselectivity, but not to as great an extent. Thus, 10 mg/kg of both the (+)- and (–)-isomers did suppress bombesin-induced grooming. However, (–)-tifluadom was significantly less effective than the (+)-isomer. The stereoselectivity of the (+)-optical isomer of tifluadom (determined in ethanol) for opioid receptors has been observed previously in mice<sup>32</sup> and monkeys.<sup>33</sup> The (–)-isomer of tifluadom has some affinity for the benzodiazepine receptor<sup>32,34</sup> and may

inhibit bombesin through this receptor; diazepam also inhibits bombesin-induced grooming at nonsedative doses (unpublished observation). This could be tested easily by examining the ability of naloxone to antagonize the actions of (-)-tifluadom on bombesin-induced grooming.

The ease at which naloxone reversed the actions of EKC, U-50,488, and tifluadom on bombesin-induced grooming and the stereospecificity of naloxone in having this effect verifies that opioid receptors mediate their action. Naloxone is less potent at kappa than mu receptors. Its decreasing potency in antagonizing EKC, tifluadom, and U-50,488 may therefore correspond to the increasing relative affinity of the three agonists for kappa over mu receptors.<sup>26</sup>

The development of tolerance to the ability of EKC to inhibit bombesin-induced grooming, taken together with the stereoselectivity, reversibility by naloxone, and the lack of effect of the nonopioid analgesic zomepirac indicate that opioid receptors mediate the attenuation of bombesin-induced scratching by analgesics. It becomes clear, however, based on tests of 35 opioid-like compounds with a large variety of chemical and pharmacological properties, that the kappa type of receptor is the only known opioid receptor linked to bombesin-induced grooming. Thus, mu opioid receptor agonists (morphine, codeine, levorphanol, heroin), mixed agonist-antagonists of the mu type (buprenorphine, nalbuphine), opioid antagonists (naloxone), peptides with mu- and delta-receptor activity ( $\beta$ -endorphin, metkephamid, DPDPE), and chemically related nonopioids (dextrophan, thebaine) had no effect at nonsedative doses in this test. However, all the compounds tested that act at the kappa-type of opioid receptor were effective. Furthermore, the lack of tolerance to EKC in rats that received multiple injections of morphine is in agreement with the kappa-receptor specificity of opioid link to bombesin-induced scratching.

Dynorphin A was proposed as an endogenous ligand for the kappa receptor.<sup>35</sup> However, its kappa receptor activity *in vivo* has been questioned. The dose-related antagonism of bombesin-induced grooming may be used as new evidence of a kappa-receptor-mediated effect of supraspinally administered dynorphin A. The inhibition of bombesin-induced grooming occurred at doses similar to those at which dynorphin A increased feeding in rats<sup>36</sup> (another effect thought to be mediated by kappa receptors). Similarly, the attenuation of bombesin-induced grooming by xorphanol may be used as further evidence of the kappa agonist nature of this compound.<sup>36</sup>

Having established that opioids act through kappa receptors to affect bombesin-induced scratching, we can speculate on the role of bombesin and opioids in scratching behavior. It is known that low doses of opioids acting at mu receptors (*e.g.*, morphine and levorphanol) often cause scratching behavior in animals, and itching in humans. Bombesin given *i.c.v.* to morphine-dependent rhesus monkeys produces scratching behavior that is similar to, but more intense, than that observed following low doses of morphine in drug-naive monkeys (ref. 5 and unpublished observations). The itching produced by low doses of morphine is thought to be due to the release of histamine; however, it is not clear that histamine is the sole cause of this effect. In contrast, the acute administration of kappa opioids does not elicit scratching behavior. However, abstinence or naloxone-induced withdrawal behavior following the chronic administration of kappa opioids includes scratching as a prominent response.<sup>18,38</sup> This behavior, and the possible role of bombesin in its production, needs to be studied further. Similarly, the role of bombesin in different types of pruritus and a possible link to kappa opioids should be investigated further. For example, opioids with kappa-receptor selectivity might be a drug class of choice for the relief of the pain and itching caused by small cell carcinoma of the lung, which is known to produce large amounts of bombesin-like immunoreactivity.<sup>39</sup>



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