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Evidence for a Dual Mechanism in the Anesthetic Action of an Opioid Peptide

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Loss of righting reflex (LRR) produced by various concentrations of the leucine–enkephalin analog BW831c (TYR.D-ALA.GLY.PHE.D-LEU.NHEt.HCl) was determined in amphibia at 1 atm and 120 atm of helium. EC_{50} for LRR was 22.1 ± 1.6 μM and 44 ± 6.9 μM, respectively. The octanol/water partition coefficient (P) was 26 ± 3.6, suggesting that this peptide is sufficiently lipid soluble for a classic Meyer-Overton type of anesthetic action. The ratio (EC_{50} at 120 atm)/(EC_{50} at 1 atm) for the peptide (2.0 ± 0.31) was essentially the same as that for the long-chain alcohol, octanol (1.8 ± 0.08), and similar to those reported for phenobarbital and the gaseous anesthetics. Thus, peptide-induced LRR was reversible by pressure. Peptide-induced LRR also was completely reversible by naloxone, whereas octanol-induced LRR was unaffected by up to 100 μM naloxone. These findings are consistent with a dual mechanism of anesthetic action for this peptide: one, an opiate receptor-specific mechanism, reversible with the specific opiate antagonist, naloxone; the other, a nonspecific mechanism, related to lipid solubility and reversible with the application of the physical agent, pressure. (Key words: Analgesics: narcotic, mechanisms of action. Antagonists, narcotic: naloxone. Polypeptides: enkephalins; mechanism of action. Theories of anesthesia: lipid solubility; pressure reversal.)

MECHANISMS FOR ANESTHETIC ACTION have been debated for over a century. Many articles have been written either supporting or contradicting a unitary hypothesis.[1,2] A nonspecific mechanism is implied by the chemical heterogeneity of the compounds that induce anesthesia, and, on this basis, theories of anesthesia have emphasized the physicochemical properties of these drugs.[3–5] To date, the most consistent physicochemical correlation is the relationship between anesthetic potency and lipid solubility, the so-called Meyer-Overton Hypothesis.[6]

In contrast, the pharmacologic potency of the opiates and their antagonists are determined by the affinity and efficacy of these compounds for specific opiate receptor sites. The opiate antagonist naloxane reverses all opiate effects, consistent with the site-specific mechanism of action.[7] The identification of the enkephalins with their weak, short-lived analgesic properties spurred the synthesis of more potent, stable synthetic enkephalin analogs.[8] Miller *et al.*[9] developed one such experimental agent, the leucine–enkephalin analog BW831c (TYR.D-ALA.GLY.PHE.D-LEU.NHEt.HCl), a naloxone-reversible antinociceptive compound with a potency three times that of morphine. They also reported that the analog induced anesthesia, defined as the loss of righting reflex (LRR), when injected intraventricularly (ivc) or intravenously (iv) in rodents.[9]

Although, by definition, LRR is induced by all general anesthetics, it has not been a consistent finding for all opiates. In both rodents[10,11] and tadpoles,[12] some opiates did while others did not induce LRR. This observation led us to question whether, in addition to its opiate properties, BW831c also might exert a second pharmacologic action, fitting the physicochemical criteria of a general anesthetic. Two criteria were selected to test this hypothesis: 1) Was the peptide sufficiently lipid soluble to induce anesthesia as predicted by the Meyer-Overton Hypothesis? and 2) Was the peptide-induced anesthesia reversible by pressure? In this article we present data for this peptide consistent with both a specific and a nonspecific mechanism of pharmacologic action, and with these findings then postulate a possible dual mechanism for the anesthetic action of this opioid.

Materials and Methods

Experiments were performed at 23 ± 1° C on prelimb bud tadpoles, approximately 1 cm in length (*Rana pipiens,* Connecticut Valley Biological Supply Co., Southamton, Massachusetts). To determine dose–response curves at 1 atm,‡ the animals were placed in neutral (*p*H 7.0) oxygenated solutions of BW831c, octanol (the control anesthetic), or dextrorphan, and LRR was determined as previously described.[13] The octanol concentrations were verified by gas liquid chromatography (Beckman GC 72-5® with a 6 × ¼″ Porapak® P packed column at 215° C).

Dose–response curves at pressure were determined in a 0.3 l stainless-steel high-pressure chamber with a

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‡ One standard physical atmosphere = 760 mmHg = 0.101MPa(SI).

TABLE 1. Anesthetic Potency Expressed as the EC_{50} for LRR at 1 atm and 120 atm

Agent	n	EC_{50} 1 atm	n	EC_{50} 120 atm	EC_{50} 120 atm/EC_{50} 1 atm
Octanol	39	63 ± 6.0 μM	40	112 ± 4.8 μM	1.8 ± 0.08
BW831c	40	22 ± 1.6 μM	14	44 ± 6.9 μM	2.0 ± 0.31

Plexiglass® viewing port. Pressure was raised with helium (Yankee Oxygen, Boston, 99.999% pure) and measured by a Master Test® gauge (Type 200, Marsh Instrument Company, Skokie, Illinois), measuring to 5,000 psi. The animals were tipped by rolling the chamber, and their ability to right themselves (rolling response) was determined as previously described.[14] The rolling response has been shown to be equivalent to the right response.[15]

The partition coefficient of the peptide was determined by measuring the depletion of 5-ml aliquots of a 0.1 mM stock aqueous solution of BW831c (*p*H 7.0) by increasing volumes of octanol. This depletion was quantified by the decrease in the absorption spectrum of tyrosine in the aqueous phase. Spectra of known concentrations of the peptide were used as controls. The samples were measured in quartz cuvettes in a Varian DMS 90 ultraviolet (UV) spectrophotometer at wavelengths of 220 through 400 nm, with a 2-nm slit length and a 10 nm/min scanning rate.

The ability of opiate antagonists to reverse both peptide- and octanol-induced LRR was studied using naloxone. Antagonism of peptide-induced LRR also was studied using the stereospecific, pure opiate antagonist, WIN 44441-3[16] (2α, 6α 11S*)-(−)-1-cyclopentyl-5-(1,2,3,4,5,6-hexahydro-8-hydroxy-3,6,11-trimethyl-2,6-methano-3-benzazocin-11-yl)-3-pentanone methanesulfonate) as well as its opiate inactive (+) enantiomer, WIN 44441-2.§ The effects of the antagonists alone also were examined to determine if they possessed any intrinstic anesthetic or toxic properties.

Dose–response curves were analyzed by the method of Waud for quantal responses.[17] Values are expressed as the mean ± standard deviation unless otherwise designated. Significant differences were determined by Student's t-test, Fisher's Exact Probability Test, or χ^2 test as appropriate.[18] A *P* value of ≥0.05 was considered insignificant.

Results

The EC_{50} for octanol at 1 atm was 63 ± 6.0 μM (table 1), comparable to values previously published.[4,¶] At 120 atm it increased to 112 ± 4.8 μM. The EC_{50} of BW831c at 1 atm was 22 ± 1.6 μM and increased to 44 ± 6.9 μM at 120 atm (fig. 1). The ratios of (EC_{50} at 120 atm)/(EC_{50} at 1 atm) for octanol and BW831c were 1.8 ± 0.08 and 2.0 ± 0.31, respectively. These ratios are not significantly different and are essentially the same as those reported for other classes of anesthetics studied under similar conditions.[19] The EC_{50} for dextrorphan at 1 atm was 2.4 ± 0.38 mM.

If the effect of two drugs are additive, then a solution containing an ($EC_{50}/2$) concentration of drug A and an ($EC_{50}/2$) concentration of drug B should induce LRR in 50% of the test animals. That is:

$$\frac{EC_{50(A)}}{2} + \frac{EC_{50(B)}}{2} = EC_{50(A\&B)}$$

A mean LRR of 12.0 (n = 20) was induced by 60 μM octanol. Likewise, 25 μM BW831c induced a mean LRR of 7.7 (n = 20). A solution containing 30 μM octanol and 12.5 μM BW831c induced a mean LRR of 11.3 (n = 20). None of these values are significantly different from the expected EC_{50} response of 10 for n = 20. Therefore, the effects of octanol and the peptide appear to be at least additive.

The opioid peptide and octanol displayed different time courses for onset of action. Octanol-induced LRR plateaued after 30 min and remained constant for the remaining 120 min of observation. Seventy-five minutes were required to reach a plateau in peptide-induced LRR. This plateau also remained constant for the remainder of the observation period.

The peptide has a partition coefficient, P, of 26 ± 3.6 as measured by the depletion assay. This value was confirmed by measuring the concentration of the peptide in both the octanol and aqueous phases in samples that contained equal volumes of octanol and water.

At 100 μM, but not 10 μM, both naloxone and WIN 44441-3 reversed the LRR induced by 33.5 μM BW831c (table 2). Concentrations up to and including 100 μM of the pharmacologically inactive enantiomer, WIN 44441-2, did not reverse the peptide-induced LRR. The LRR induced by either dextrorphan or octanol was not reversed by up to and including 100 μM naloxone. Animals placed in a 10-mM aqueous naloxone solution, or in a 0.1 mM aqueous solution of either WIN compound, demonstrated no behavioral changes during a 3-h observation period. An aqueous 1 mM solution of either WIN compound produced a toxicity that was not

§ Personal communication, Dr. W. F. Michne, Sterling-Winthrop Research Institute, Rensselier, New York.

¶ Meyer KH, Hemmi H: Beitrage Zur Theorie der Narkose III. Biochem Zeit 277:39–71, 1935.

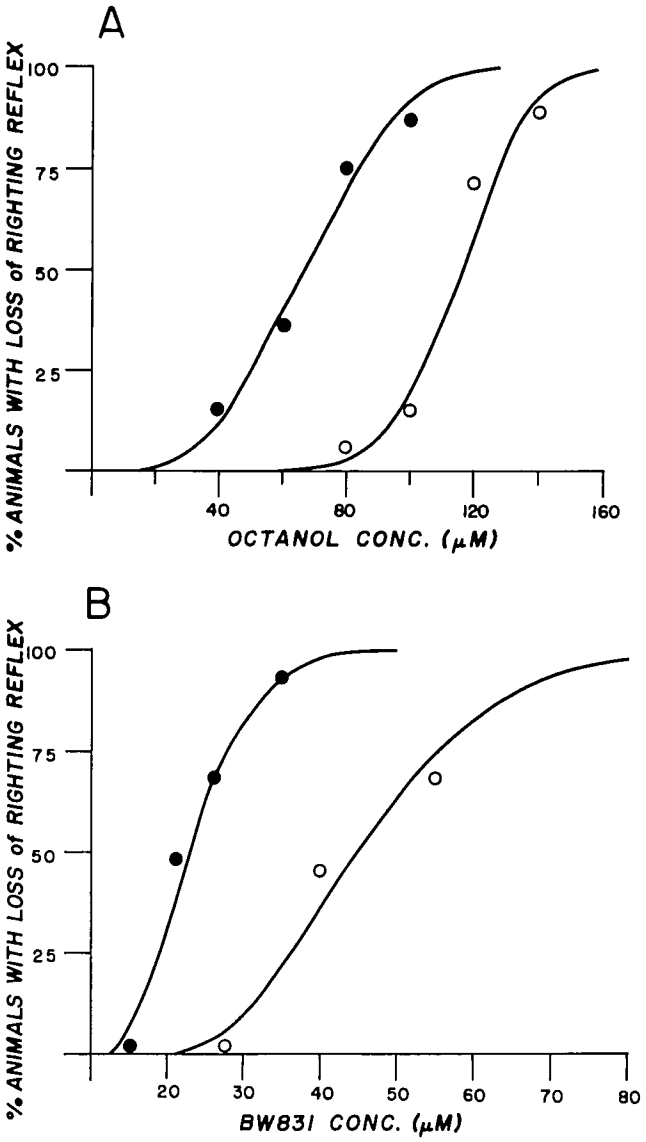
naloxone reversible. At 50 mM naloxone, the animals exhibited an extremely sluggish behavior, although a strictly defined[14] LRR could not be obtained consistently. Irreversible toxicity was induced rapidly (<15 min) with 100 mM of naloxone.

Discussion

LRR is an endpoint commonly used to assess anesthetic potency. Its advantage over other endpoints, such as the minimum alveolar anesthetic concentration (MAC), is that it does not measure a response to a painful stimulus, and therefore should allow a clearer delineation between anesthetic and antinociceptive effects. BW831c induces LRR in rodents[9] and, as shown in this article, in amphibia. Tadpoles, a traditional model in pressure studies, also provide an ideal practical model for quantifying the effective concentration (EC) of a drug. Because they are aquatic and quite small, they equilibrate rapidly with the drug in aqueous solution. Therefore, the drug concentration of the anesthetic solution may be assumed to be the concentration of the drug in equilibrium with its site(s) of action *in vivo*, thus bypassing the problems of protein binding and drug metabolism that normally affect the calculation of dose–response curves for intravenous agents. Similarly, we found that by simple diffusion the oxygen tension present in the anesthetic solutions was sufficient to maintain, without sequelae, completely curarized tadpoles (50 μM *d*-tubocurarine) for a minimum of 210 min. Therefore, respiratory depression was not considered to be a significant factor in this model.

No exceptions have been found yet to the hypothesis that a compound must be lipid soluble to possess general anesthetic potency. Little information exists on peptides in lipid bilayers, but BW831c, with its five neutral amino acid side chains, should have considerable hydrophobicity.[20] Furthermore, with each of its constituent amino acids able to cross lipid bilayers,[21] BW831c should likewise be sufficiently lipid soluble to cross the blood–brain barrier, as also inferred from its ability to induce anesthesia when injected iv in rodents. The *P* value of the peptide is much higher than for many neuropeptides,[22] but is comparable to the anesthetic phenobarbital.[23] Solubility in octanol is likely to provide only a semiquantitative prediction of the general anesthetic potency of the peptide when comparison is made with such dissimilar compounds as volatile anesthetics and alcohols. In fact, a bulk solvent should underestimate the actual interaction of an amphiphilic peptide with a bilayer. Indeed, opiates do interact with lipids, even in their charged form.[24] The essential point is that BW831c is, by several criteria, a highly lipophilic peptide.

The second physicochemical criterion assessed was



FIGS. 1*A* and *B*. Figures demonstrate (no. of animals with LRR/total no. of animals) × 100 as a function of the concentration of octanol (*A*) or BW831c (*B*) at 1 atm or 120 atm (● = values determined at 1 atm, O = values determined at 120 atm). The dose–response curves were computer generated using a program based on the method of Waud for quantal responses.[17]

TABLE 2. Reversal of Peptide-induced LRR by Opiate Antagonists

Drug	Control (33.5 μM BW831c)	Control + Drug (33.5 μM BW831c + 0.1 mM drug)	*P*
Naloxone	9/14*	1/14	†
WIN 44441-3	9/10	3/10	‡
WIN 44441-2	7/10	7/10	NS

NS = not significant.
* (number of animals with LRR)/(total number of animals).
† Significant at $P = 0.005$.
‡ Significant at $P = 0.01$.

whether pressure would reverse peptide-induced LRR. Although reported for virtually every other class of general anesthetic,[12] pressure reversal of anesthesia has never been examined in an enkephalin-like compound. The rightward shift in the peptide dose–response curve (fig. 1*B*) illustrates the pressure reversibility of peptide-induced LRR. This is the first demonstration, of which we are aware, that the action of a peptide can be reversed with pressure.

It is possible that the pressure reversal of the peptide-induced LRR is secondary to a direct effect on the opiate receptor mechanism rather than a nonspecific lipid interaction. There are, however, several arguments against this. In our study we found no significant difference in the magnitude of the pressure effect on the EC_{50} of either compound as expressed as the ratios in table 1. This implies a common mechanism of action for pressure on anesthetic effect of both BW831c and the nonopiate octanol. This argument does not entirely rule out the possibility that pressure acts on an opiate activated pathway (*e.g.*, on an opiate stimulated adenylate cyclase) by a mechanism similar to that employed at the anesthetic site.

A further argument against pressure reversal of opiate-specific action can be found in the results of studies on the effect of pressure on opiate analgesia. No significant differences have been reported in morphine pharmacokinetics,[25] microsomal metabolism,[26] or analgesia[27] in rodents exposed to pressures above those capable of inducing significant pressure reversal of anesthesia.[12] Therefore, although a receptor-mediated pressure effect cannot be ruled out, it is more likely that we are observing pressure reversal of the general anesthetic effect of the peptide.

Thus, by the two criteria tested—1) sufficient lipid solubility, and 2) pressure reversibility—BW831c may be considered to be a general anesthetic. The peptide differed from octanol in two respects. The first was a slower onset of action. A similar difference in onset has been reported in a study comparing BW831c and β-endorphin with pentobarbital injected iv in rats.[9] This increase in time necessary to reach an effect may be explained, in part, by the lower permeability of the blood–brain barrier to peptides.[22]

The second and more significant difference was the ability to naloxone to reverse peptide-induced LRR but not octanol-induced LRR. Reversal of LRR by naloxone in tadpoles and rodents is consistent with the peptide possessing opiate properties, as are the naloxone-induced parallel shifts of the BW831c dose–response curves in antinociceptive studies.[9]

The dose of naloxone required to reverse peptide-induced LRR was between 10 μM and 100 μM, considerably higher than the dissociation constant (K_d) for naloxone in brain tissue (10 nM).[7] This raises the possibility of a nonspecific naloxone effect.[28] There are also several arguments against this possibility. First, if the reversal were completely nonspecific, naloxone should have affected the octanol-induced LRR. Second, the results with the active opiate antagonist WIN 44441-3 and its inactive enantiomer strongly suggest a stereospecific opiate mechanism. Third, there are subpopulations of opiate receptors in nervous tissue that appear to have different affinities for naloxone. The enkephalin site, the so-called δ receptors, with (D-ala,[2] D-leu[5]) enkephalin the prototypical ligand, appears to have at least a tenfold lower affinity for naloxone than the other subpopulations.[29,30] Lower affinity partially could explain, although not completely, the high concentration of naloxone required for reversal. (The specificity of naloxone as an opiate antagonist also has been reviewed recently.[28]) Finally, although the K_d of BW831c has not been determined, the K_ds of similar peptides are in the range of 1–3 nM.[30,31] The EC_{50} for peptide-induced LRR is 10^4-fold higher than this, and a comparable excess of naloxone over its K_d likewise should be required for effective competitive antagonism.[32] This provides the most straightforward explanation for the high naloxone concentrations required to overcome anesthesia.

The results suggest that BW831c is an opioid analgesic with general anesthetic properties. Other opiates have been reported with this combination of properties. Murphy and Hug[33,34] found a dose-dependent lowering in the MAC requirements of enflurane by both fentanyl and morphine. There was, however, a "ceiling" in the reduction of enflurane requirements by both of these compounds. One explanation of this ceiling could be found in the painful stimulus (tail clamp) used to estimate MAC.[33,34] An opiate should mute the perception of this stimulus proportional to the number of opiate receptors occupied. Once sufficient receptors are occupied to elicit a maximum response, no further increase in drug concentration should be effective.

Therefore, the ability of a drug to induce analgesia does not appear to be sufficient to ensure the ability to induce anesthesia. We propose that an opiate also must be sufficiently lipid soluble in order to function as a general anesthetic. One test of the proposal would be to antagonize the opiate action and then raise the concentration of the opiate to that required for lipid-mediated action alone. Assuming octanol to be a fair model of the anesthetic site and the Meyer-Overton rule to apply equally to alcohols and peptides, BW831c should have an EC_{50} (in the aqueous phase) of 3 mM. This high concentration is toxic (and would require a correspondingly high concentration of naloxone to pre-

vent BW831c from occupying opiate receptors.) Therefore, as an alternative test we used dextrorphan, the inactive stereoisomer of the opioid levorphanol. The above rule predicts dextrorphan (P = 1.76 at pH 7.0)[35] to have an EC_{50} of 50 mM. The observed EC_{50} of 2.4 mM was in reasonable agreement with the predicted value, considering the simplicity of the model. This enhanced potency in the observed EC_{50} also could have arisen from other nonopiate mechanisms, such as those reported by Carney and Sirochman,[36] from a weak intrinsic opiate activity[37] or from contamination with the opioid (−) isomer. The last two possibilities seem unlikely, as naloxone did not reverse dextrorphan-induced LRR. We also attempted to induce anesthesia using naloxone as another example of a lipid-soluble (P = 6.1 at pH 7.0)[35] inactive opiate analog. Naloxone has a predicted EC_{50} of 15 mM. At three times this concentration the animals became sluggish, and toxicity was induced rapidly at six times the predicted EC_{50}. Therefore, it appears that high-dose naloxone possesses too small a therapeutic safety margin to enable the unequivocal demonstration of naloxone-induced anesthesia.

In conclusion, we have shown a leucine–enkephalin analog to satisfy the criteria of both an opiate and a general anesthetic. One possible explanation for these findings is that the peptide has two separate mechanisms, one an opiate receptor-specific mechanism, reversible with opiate antagonists; the second, a nonspecific mechanism, related to lipid solubility and reversible with the application of the physical agent, pressure. Finally, we believe these findings reaffirm the role of hydrophobicity as a fundamental requirement in the mechanism of anesthetic action.

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