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OPIOID ACTIVITIES OF ß-CASOMORPHINS

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Summary

ß-Casomorphin-7 (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) and its analogues: ß-casomorphin-6, (-5) and (-4) (derived by sequential removal of respectively one, two or three amino acid residues from the C-terminus), were tested for their opioid activities in a variety of assay systems. Each of the four peptides displayed opioid activity in an opiate receptor binding assay, the isolated mouse vas deferens (MVD), the guinea-pig ileum longitudinal muscle myenteric plexus preparation (GPI) and produced naloxone-reversible analgesia after intracerebroventricular injection into rats. In contrast, none of the peptides displayed opioid activity in the isolated rat vas deferens preparation (RVD). ß-Casomorphin-5 was the most potent compound in all the assays employed. Each ß-casomorphin was more potent on the GPI than on the MVD. In view of the fact that the GPI, MVD and RVD are populated predominantly by μ-, δ- and ε-receptors, respectively, the ß-casomorphins probably represent μ-type opiate receptor agonists.

In the last few years, the existence of a number of endogenous opioid peptides (endorphins) has been demonstrated in various mammalian tissues (for review see (1)). Although these peptides vary considerably in size, ranging from 5 to 31 amino acid residues, their N-terminal sequences invariably consist of the following residues: Tyr-Gly-Gly-Phe. This sequence is, thus, presumably responsible for the opioid properties of these endorphins. Recently, a novel type of natural opioid peptide was discovered. These peptides, named ß-casomorphins, represent fragments of ß-casein, a constituent of milk, and their N-terminal amino acid sequence Tyr-Pro-Phe-Pro (2,3,4,5) differs from that of other endogenous opioid peptides (e.g. enkephalins, ß-endorphin and dynorphin).

In this report, details are presented concerning the opioid activities of ß-casomorphin-7 (H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH) and its analogues, ß-casomorphin-6 (Tyr-Pro-Phe-Pro-Gly-Pro),

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ß-casomorphin-5(Tyr-Pro-Phe-Pro-Gly) and ß-casomorphin-4 (Tyr-Pro-Phe-Pro) in opiate receptor binding assays, in isolated organ preparations and in the intact animal after intracerebroventricular injection. Part of the present results have been published previously in abstract form (5).

Methods

Determination of opioid activity: radioreceptor assay

Opioid activities were determined by an opiate receptor binding assay as described by Pert and Snyder (6). Inhibition of ^{3}H-naloxone binding to opiate receptors in rat brain homogenate (washed 4-times by centrifugation) at 25°C using ß-casomorphins was evaluated and IC_{50} values were determined.

Determination of opioid activity in isolated organ preparation

The ß-casomorphins, normorphine and met-enkephalin were tested for opioid activity in three isolated organ preparations:
Guinea-pig ileum longitudinal muscle myenteric plexus preparation:
Preparation, mounting and electrical stimulation (frequency: 0.1 Hz, pulses: 60 V, 0.5 ms) were carried out as described by Kosterlitz et al. (7) and Schulz and Goldstein (8).
Mouse vas deferens and rat vas deferens:
The method employed was as originally described by Henderson et al. (9) and the experimental details may be found in Schulz et al. (10).

In these isolated organ preparations, opioid activities were determined in terms of an inhibition of electrically induced contractions, in so far as these proved antagonizable by the opiate antagonist (-)-naloxone, but not by its optical isomer (+)-naloxone. From these effects IC_{50} values were calculated for quantitative evaluation.

Measurement of analgesic effects

Following intracerebroventricular (i.c.v.) injection of ß-casomorphins, analgesic measurements were made as previously described by Herz and Bläsig (11). Briefly, stainless steel guide cannulae were implanted under pentobarbitone anaesthesia into the right lateral ventricle of Sprague Dawley rats one week prior to experimentation. For testing of analgesic effects, the compounds were dissolved in physiological saline and up to 10 µl of solution (or saline as a control) injected by use of a Hamilton gastight syringe. The degree of analgesia produced was evaluated by by application of the "vocalization test", whereby a sequence of incremental current pulses (rectangular pulses, frequency 50 Hz, duration 10 ms for 2 sec) is delivered to the tail via a bipolar electrode. The minimum current strength (measured in mA) evoking vocalization was taken as a measure of the analgesic threshold. After completion of testing, methylene blue was injected through the cannulate in order to locate cannulae positions. Only results obtained with rats possessing a correctly positioned cannula were used for evaluation of data. (-)-Naloxone was injected intraperitoneally (3-10 mg/kg) in order to establish the opioid nature of the analgesic effects.

Substances

ß-Casomorphin-3, (-4), (-5) and (-6) were obtained by synthesis (Lottspeich et al., Hoppe-Seyler's Z. Physiol. Chem., in press) or purchased from Peninsula Laboratories, San Carlos, USA;

ß-casomorphin-7 was obtained by isolation from casein hydrolysate by Brantl et al. (3) or purchased from Peninsula. Met-enkephalin was purchased from SERVA, Heidelberg, FRG, and normorphine from BIOS, Brussels, Belgium; (-)-naloxone was obtained from ENDO Laboratories, Garden City, USA. (+)-Naloxone was a generous gift of Dr. A.E. Jacobson, Bethesda, USA.

Results

The opioid activities of ß-casomorphin-4, (-5), (-6) and (-7) in an opiate receptor binding assay (BA), in the guinea-pig ileum longitudinal muscle myenteric plexus preparation (GPI), in the mouse vas deferens (MVD) and in the rat vas deferens (RVD) may be seen from table I.

TABLE I

	B A	G P I	M V D	GPI/MVD
Normorphine	0.035	0.23±0.022	1.1 ± 0.14	0.21
Met-enkephalin	not tested	0.19±0.018	0.019± 0.002	10.00
ß-Casomorphin-7	14.0	57.00±7.5	>200	<0.29
ß-Casomorphin-6	3.2	27.40±1.7	>150	<0.18
ß-Casomorphin-5	1.1	6.50±0.6	42.1 ± 5.9	0.15
ß-Casomorphin-4	2.7	21.90±2.6	84.3 ±12.8	0.26

Opioid activities of ß-casomorphins in an opiate receptor binding assay (BA). IC_{50} values (µM) are given for peptide concentrations inhibiting ^{3}H-naloxone (10^{-9} M) binding by 50% (values derived from the intercepts of regression line with 8 separate concentrations). Opioid activities of ß-casomorphins in the guinea-pig ileum longitudinal muscle myenteric plexus preparation (GPI) and in the mouse vas deferens (MVD). IC_{50} (µM) values are given for peptide contractions causing a 50% inhibition of the contractions of the organ preparations induced by electrical stimulation. Mean values ± standard deviations from at least 3 determinations. The value GPI/MVD expresses the potency ratio in both bioassays.

The affinities of the ß-casomorphins for opiate receptors from rat brain, as indicated by their IC_{50} values, are lower than the affinity of normorphine by a factor of 30- to 400-fold and lower than morphine (not shown), which is 10-times more potent than normorphine in the binding assay, by ca. 300-4000-fold. The affinities of morphine and normorphine in the GPI and MVD bioassays do not, however, differ: thus, the relative affinities of the ß-casomorphins in these bioassays are similar when compared to either normorphine or morphine. The highest affinity, i.e. the lowest IC_{50} value, was found for ß-casomorphin-5. The degree of binding of the ß-casomorphins was not significantly decreased during a one hour incubation period under our experimental conditions. Inhibition of ^{3}H-naloxone binding by 60-80% was observed with the highest concentrations of ß-casomorphins used in the binding assay (2×10^{-5} M).

Opioid activities, as evaluated in the bioassays, clearly

parallel the relative affinities of the ß-casomorphins for the opiate receptor as determined in the binding assay. It is noteworthy that the ß-casomorphins displayed a maximal potency in the GPI, whereas their potencies in the MVD were much lower: in the RVD they were found to be inactive. The tripeptide ß-casomorphin-3 proved to be practically inactive in the GPI and in the MVD. The potency ratio (GPI/MVD) of their opioid activities demonstrates that, in contrast to met-enkephalin, the ß-casomorphins behave similarly to normorphine in these assays.

After i.c.v. injection into rats (0.06 to 2.0 µmoles), all four ß-casomorphins elicited opioid effects, such as analgesia, which could be completely antagonized by naloxone (3 to 10 mg/kg, i.p.). The maximum analgetic effect was reached about 5 minutes post-injection in the case of ß-casomorphin-4, 15 minutes for ß-casomorphins-5 and (-6), and about 30 minutes with ß-casomorphin-7. Analgesia persisted for up to 45 minutes post administration with ß-casomorphins-4, (-5) and (-6) and in excess of 90 minutes with ß-casomorphin-7.

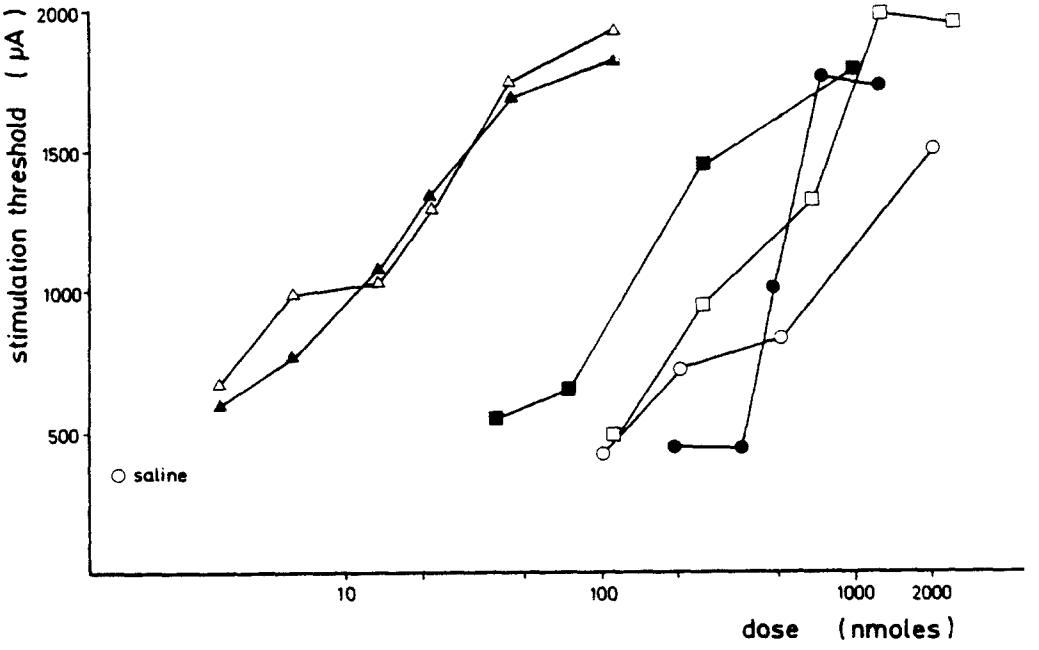

FIG. 1
Dose-response relationship for analgetic effects elicited by morphine (▲), [D-Ala^2,D-Leu^5]-enkephalin (DADL) (△), ß-casomorphin-4 (□), ß-casomorphin-5 (■), ß-casomorphin-6 (○) and ß-casomorphin-7 (●) after intracerebroventricular injection in rats. Mean values from 3 to 8 animals, deviation of single values from mean value <15%.

In figure 1, the dose-response relationships for the analgetic effects of the ß-casomorphins after intracerebroventricular injection (i.c.v.) and for comparison, morphine and [D-Ala^2,D-Leu^5]-enkephalin are presented. ß-Casomorphin-5 was again the

most potent of the ß-casomorphins. In this test, its potency is lower than that of morphine by a factor of 10 only.

Discussion

Four natural opioid peptides of the ß-casomorphin type have been identified (3,4,5) and tested for their opioid activities in a variety of assay systems, as described in the present paper. All four peptides displayed activity in the opiate receptor binding assay, i.e. an affinity for specific opiate binding sites, indicating that their opioid effects are not elicited via a release of endogenous opioids but by a direct interaction with opiate receptors. The relative affinity of the peptides to opiate receptor binding sites was very similar to their relative potencies in the in-vitro assay systems employed. Thus, as previously established for both alkaloid and peptide opiates (12), the pharmacological potency of the ß-casomorphins is dependent upon their affinities to opiate receptors rather than on differences in "intrinsic activities". The relative affinities of the ß-casomorphins to the opiate receptor are evidently not correlated in a simple fashion with their chain length. NMR studies may assist in the elucidation of the problem of the relationship between the steric configurations of the ß-casomorphins and their opioid potencies (Sonnenbichler et al., in preparation). The ß-casomorphins and their D-Ala^2-derivatives are the first natural opioid peptides discovered to possess the amino acid phenylalanine at position 3 from the N-terminus. (The enkephalins, ß-endorphin and dynorphin, in contrast, possess phenylalanine at position 4.) The recently discovered dermorphin (13), which also possesses a phenyl alanine moiety at position 3, in addition to possessing other common residues is, thus, structurally very similar to the ß-casomorphins.

Each of the ß-casomorphins displayed a much higher potency in the guinea-pig ileum (populated predominantly by µ-type opiate receptors (14)) than in the mouse vas deferens (which contains primarily δ-type opiate receptors (15)). A complete absence of activity was observed in the rat vas deferens which possesses mainly ε-type opiate receptors (10). Thus, in contrast to met-enkephalin, each of the ß-casomorphins shows a potency ratio for the guinea-pig ileum as compared to the mouse vas deferens very similar to that of normorphine, a typical µ-type opiate receptor agonist. Therefore, it is appropriate to consider the ß-casomorphins to be µ-type opiate receptor agonists. Since the ß-casomorphins can be derived from cleavage of ß-casein by proteolytic microorganisms (16) or possibly proteolytic enzymes in the gastro-intestinal tract after ingestion of milk, they may represent natural agonists for the µ-type opiate receptor detected in the gut.

It is of interest that substitution of L-Pro^2 in ß-casomorphin-5 by D-Ala^2 (Tyr-D-Ala-Phe-Pro-Gly) led to a 12-fold increase in potency of this peptide in the mouse vas deferens in comparison to a less accentuated increase in the guinea-pig ileum (Brantl et al., Lottspeich et al., in preparation). Amidation of the C-terminus of ß-casomorphins led to a pronounced increase in opioid potencies in all assay systems used.

In pilot experiments, ß-casomorphin-5 and ß-casomorphin-7 were found to be degraded in rat plasma, whereas ß-casomorphin-4

and the ß-casomorphin-4-amide (Tyr-Pro-Phe-Pro-NH_2, Bachem, Bubendorf, Switzerland) proved to be comparatively stable under these conditions. The synthetic D-Ala^2-ß-casomorphin-5 similarly proved to be stable in these conditions. In addition, this analogue had an analgetic action after intravenous injection in rats or subcutaneous application to mice (Brantl et al., in preparation). After intravenous injection of the ß-casomorphin-4-amide, a naloxone-antagonizable fall in heart rate of vagal origin was observed (17).

In addition, after i.c.v. injection of ß-casomorphins, a cataleptic state was observed. This state was not accompanied by a muscular rigidity which typifies the cataleptic condition produced by opiates. The catalepsy produced was completely blocked by pretreatment with naloxone in the cases of ß-casomorphin-5, (-6) and (-7), but only partially blocked with ß-casomorphin-4, an observation which may be explicable by an occupation of non-opiate, e.g. neuroleptic receptors, in addition to opiate receptors by ß-casomorphin-4.

It should be noted that ß-casomorphins are the first short opioid peptides which elicit effects after i.c.v. injection for periods of 45 to 90 minutes. In other opioid peptides (e.g. enkephalins), it has been necessary to replace one of the amino acids residues, usually the one next to the N-terminal tyrosine by D-amino acids in order to obtain long lasting effects. The finding is in complete agreement with the striking stability of ß-casomorphins towards proteolytic enzymes (3,4). This stability can be explained by the fact that the ß-casomorphins have a proline residue in every second position and that most proteases are unable to cleave peptide-bonds involving proline residues: degradation is therefore, less rapid.

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References

1. H. TESCHEMACHER, Developments in Opiate Research, A. Herz, Ed. Marcel Dekker, Inc., New York, pp. 67-151 (1978).
2. V. BRANTL and H. TESCHEMACHER, Naunyn-Schmiedeberg's Arch. Pharmacol. 306 301-304 (1979).
3. V. BRANTL, H. TESCHEMACHER, A. HENSCHEN and F. LOTTSPEICH, Hoppe-Seyler's Z. Physiol. Chem. 360 1211-1216 (1979).
4. A. HENSCHEN, F. LOTTSPEICH, V. BRANTL and H. TESCHEMACHER, Hoppe-Seyler's Z. Physiol. Chem. 260 1217-1224 (1979).
5. V. BRANTL, A. HENSCHEN and F. LOTTSPEICH, Naunyn-Schmiedeberg's Arch. Pharmacol. 311 R67 (1980).
6. C.B. PERT and L.H. SNYDER, Science 179 1011-1014 (1973).
7. H.W. KOSTERLITZ, R.J. LYDON and A.J. WATT, Brit. J. Pharmacol. 39 398-413 (1970).
8. R. SCHULZ and A. GOLDSTEIN, J. Pharmacol. exp. Ther. 183 404-410 (1972).
9. G. HENDERSON, J. HUGHES and H.W. KOSTERLITZ, Brit. J. Pharmacol. 46 764-766 (1972).

10. R. SCHULZ, E. FAASE, M. WÜSTER and A. HERZ, Life Sci. 24 843-850 (1979).
11. A. HERZ and J. BLÄSIG, Endorphins in Mental Health Research, E. Usdin, Ed. Macmillan Press, London, pp. 269-278 (1979).
12. V. HÖLLT and M. WÜSTER, Developments in Opiate Research, A. Herz, Ed. Marcel Dekker, Inc., New York, pp. 1-65 (1978).
13. V. ERSPARMER and P. MELCHIORRI, Trends in Pharmacological Sciences, Elsevier/North-Holland Biomedical Press, Amsterdam, 1, pp. 391-395 (1980).
14. J.A.H. LORD, A.A. WATERFIELD, J. HUGHES and H.W. KOSTERLITZ, Nature 267 495-499 (1977).
15. F.M. LESLIE and H.W. KOSTERLITZ, Europ. J. Pharmacol. 56 379-383 (1979).
16. H. TESCHEMACHER, G. AHNERT, M. UMBACH, G. KIELWEIN and S. LEIB, Naunyn-Schmiedeberg's Arch. Pharmacol. 311 R 67 (1980).
17. E.T. WEI, A. LEE and J.K. CHANG, Life Sci. 26 1517-1522 (1980).