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BEHAVIORAL AND NEUROCHEMICAL EFFECTS OF THE NEW OPIOID PEPTIDE DYNORPHIN-(1-13): COMPARISON WITH OTHER NEUROPEPTIDES

H. Zwiers, V.J. Aloyo, and W.H. Gispen

Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology and Laboratory of Physiological Chemistry, Medical Faculty, Institute of Molecular Biology, University of Utrecht, Padualaan 8, Utrecht, The Netherlands

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Summary

This study was undertaken to determine whether the recently discovered opioid-like peptide dynorphin-(1-13) could influence both excessive grooming in the rat and also the activity of the ACTH-sensitive B-50 protein kinase in vitro. Dynorphin-(1-13), when injected intracerebroventricularly at a dose of 1 to 10 μg, resulted in excessive grooming behavior similar to that observed after administration of ACTH-(1-24). In contrast, leu-enkephalin was not effective in the same dose-range. The grooming behavior elicited by both ACTH-(1-24) and dynorphin-(1-13) was blocked by pre-treatment of the rats with naloxone. Furthermore we observed that dynorphin-(1-13) and ACTH-(1-24) were potent inhibitors of B-50 protein kinase. Leu-enkephalin was not effective whereas β-endorphin was a relatively weak inhibitor. Naloxone did not block these in vitro effects. The relationship of these phenomena to the opioid receptor is discussed.

Phosphorylation of membrane components [proteins and (poly)phosphoinositides] appears to play an important role in membrane function (1,2). In brain, this phosphorylation may modulate membrane permeability and synaptic efficacy (3). Recently, a rat brain ACTH-sensitive protein kinase and its corresponding substrate protein (B-50) were isolated, purified and characterized (4,5). The structural requirement of various ACTH-analogs necessary to inhibit B-50 phosphorylation in vitro and those necessary to induce excessive grooming in the rat after intracerebroventricular (icv) administration, showed a striking similarity (6,7,8). Knowledge of these structural requirements led to the deduction that the presence of basic amino acids in behaviorally active neuropeptides are important elements for the induction of grooming in the rat and the inhibition of the B-50 protein phosphorylation as studied in vitro. In line with this, is the previously reported discovery of a new polar peptide (PIP), released from rat SPM, with these two properties (9). In the present communication we report that the recently isolated potent opioid peptide dynorphin-(1-13) (10), a small basic peptide, is also capable of inhibiting B-50 kinase and inducing excessive grooming. The properties of dynorphin-(1-13) in these two assays are compared and contrasted with those of ACTH-(1-24), β-endorphin and leu-enkephalin.

Methods

B-50 kinase assay: B-50 protein kinase was isolated and partially purified from rat brain synaptosomal plasma membranes (SPM) as detailed elsewhere (4,5). The

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total procedure took place at 0-4°C. In short, a crude SPM fraction was prepared from rat brain cortex cerebri (30 g, wet weight). The endogenous B-50 phosphorylating activity was solubilized from the membranes using 0.5% Triton X-100 in 75 mM KCl and was then subjected to column chromatography over DEAE cellulose. The B-50 protein kinase activity was eluted with a NaCl gradient and the eluate fractions containing that activity were pooled and subjected to ammonium-sulfate fractionation. The protein precipitating between 55 and 80% saturation (ASP 55-80) were collected by centrifugation, redissolved in 200 µl buffer A (10 mM Tris-HCl, 0.1 mM dithiothreitol, 1 mM $CaCl_2$, pH 7.4) and dialysed for 5 h against one liter of buffer A. The volume of the dialysed ASP 55-80 fraction was then adjusted to 600 µl with buffer A. This ASP 55-80 fraction contains only one protein kinase (B-50 protein kinase) and one major acceptor protein (B-50; 5 and Fig. 1A). In order to prevent proteolytic breakdown of B-50 (9) only freshly prepared ASP 55-80 was used. The B-50 protein kinase activity was assayed under the following conditions: 15 µl ASP 55-80, 50 mM Na-acetate, 10 mM Mg-acetate, 1 mM Ca-acetate, 7.5 µM ATP, 1 µCi $|\gamma\text{-}^{32}P|$ATP (approx. 3000 Ci/mmol, Amersham, UK) pH 6.5, final volume 25 µl. After the ASP 55-80 proteins were preincubated for 5 min at 30°C, the reaction was initiated by the addition of 5 µl $|\gamma\text{-}^{32}P|$ATP. After 1 min at 30°C the reaction was terminated by addition of 12.5 µl of a denaturing solution, containing 6% SDS (1). Previously, it was demonstrated that the phosphorylation of B-50 under these conditions was linear over time for at least 15-20 min (5). The effect of the following synthetic peptides was studied: ACTH-(1-24), leu-enkephalin and β-endorphin (Organon Int. BV, Oss, The Netherlands) and dynorphin-(1-13) (Peninsula Labs, CA, gift of Dr. A. Goldstein). Known quantities of peptides were transferred to plastic Eppendorf vials, dissolved in buffer A and without delay added to the preincubated samples. This procedure circumvented the hazard of peptide degradation and absorption to the wall of the peptide-containing vial (see also 12). The peptide (5 µl) was added 10 sec prior to the $|\gamma\text{-}^{32}P|$ATP aliquot. The peptides were tested in triplicate over a concentration range of 10^{-4} to 10^{-6} M. At the end of the phosphorylation assay, the ASP 55-80 proteins were separated on SDS-polyacrylamide slab gels (11% acrylamide). The gels were fixed and stained with Fast Green, dried and subsequently subjected to autoradiography as described previously (11). Incorporation of radioactive phosphate into B-50 protein was quantitatively measured, using liquid scintillation counting of excised gel bands (13).

Excessive grooming test: The behavioral assay was essentially that described by Gispen and coworkers (7). In short, one week prior to the test, a polyethylene cannula was implanted into the interventricular foramen of the brain ventricular system by the method of Brakkee et al. (14). Naive male rats of an inbred Wistar strain weighing 150 g were used. The peptides were dissolved in normal saline immediately prior to use. To initiate the grooming test, the conscious rats received an intraventricular injection of 3 µl and were then placed individually into a novel glass box (24 x 12.5 x 14 cm) in a soundproof observation room. After 5 min, the grooming behavior was scored every 15 sec for a period of 50 min. Thus, every 15 sec an observer scored whether or not a given rat displayed vibration, washing, grooming, scratching, paw licking, etc. The data are expressed either as the percentage of maximal possible grooming score (200) or as the number of bouts observed during the 50 min test period (15). The behavioral responses observed after treatment with dynorphin-(1-13), ACTH-(1-24), β-endorphin or leu-enkephalin was compared to similarly treated rats receiving only normal saline.

Results

B-50 kinase assay: In confirmation of the recent findings of our laboratory (5), the protein staining pattern of the ASP 55-80 protein fraction of rat brain SPM contained a number of bands of which the B-50 protein and its kinase were the

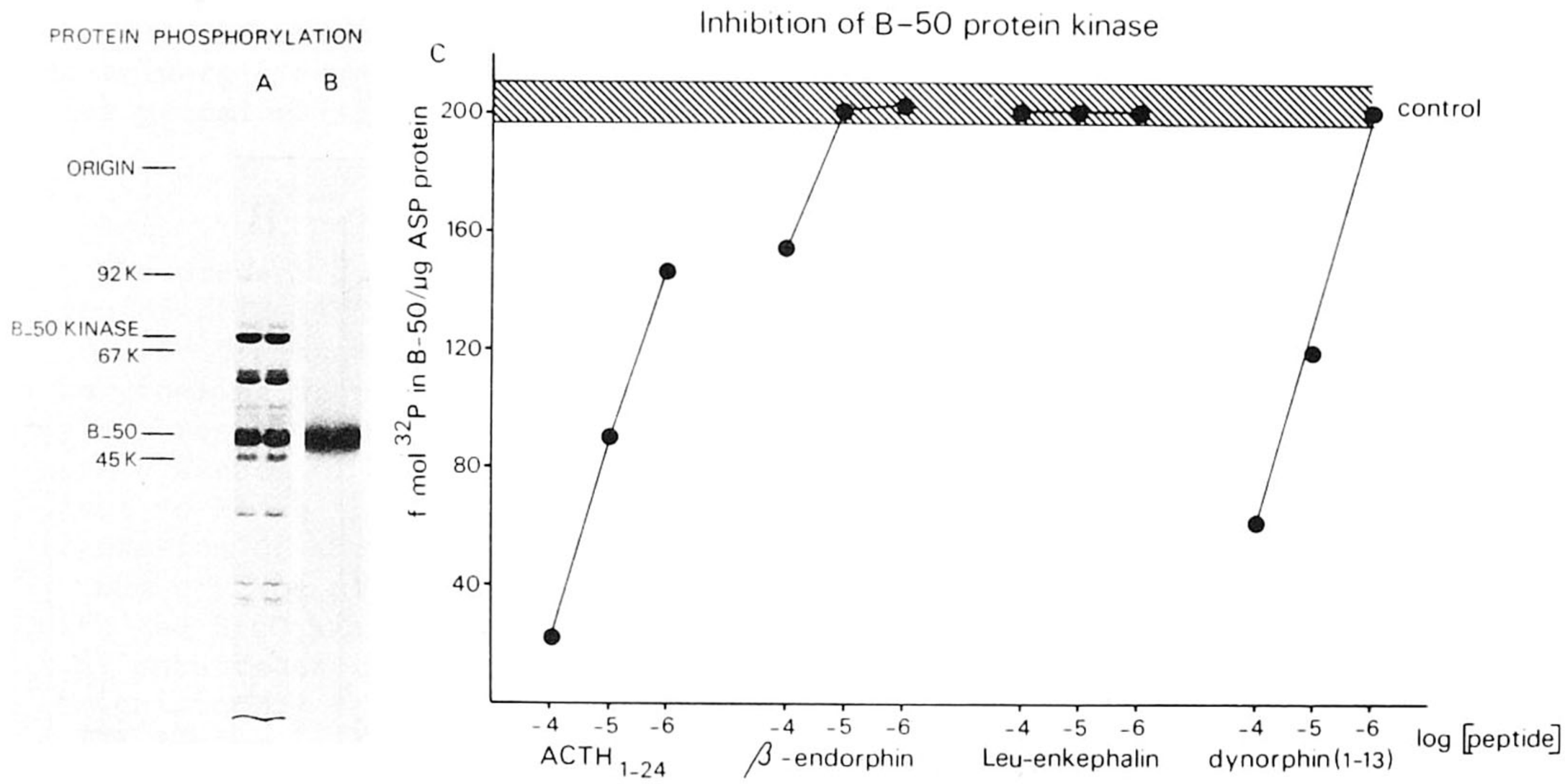

FIG. 1

Part A shows protein staining pattern of ASP 55-80 separated by SDS-polyacrylamide gel electrophoresis. The B-50 kinase (70K) and the B-50 protein (48K) are indicated.
Part B shows the autoradiograph after phosphorylation (1 h exposure).
Part C shows the incorporation of ^{32}P-phosphate into B-50 after incubation of ASP 55-80 with different concentrations of ACTH-(1-24), β-endorphin, leu-enkephalin and dynorphin-(1-13). Incorporation into B-50 is expressed as fmol ^{32}P-phosphate (1 cpm = 0.05 fmol) per µg total incubated protein. Incubations were performed in triplicate (SEM < 5%). Control value (± SEM) is indicated as a shaded area.

most pronounced (Fig. 1A). Autoradiography of previously phosphorylated ASP 55-80 proteins revealed that B-50 was the only phosphoprotein labelled under the present conditions (Fig. 1B). The effects of the four neuropeptides on the B-50 protein phosphorylation is shown in Fig. 1C; as previously reported, 10^{-5} M β-endorphin did not inhibit B-50 kinase (5) and 10^{-4} M resulted in only a slight (20%) inhibition. Leu-enkephalin failed to inhibit B-50 kinase at all concentrations tested. In contrast, dynorphin-(1-13), which is a C-terminally elongated analog of leu-enkephalin, resulted in a marked inhibition of this kinase (IC50, 3 x 10^{-5} M). This enzyme proved to be most sensitive to inhibition by ACTH-(1-24) (IC50, 6 x 10^{-6} M). Preincubation of ASP 55-80 with 10^{-4} M naloxone did not block the inhibition of B-50 kinase caused by either 10^{-5} M ACTH-(1-24) or by 10^{-5} M dynorphin-(1-13). In view of the substantially purified nature of the B-50 kinase preparation used, it is unlikely that the absence of inhibitory effects by leu-enkephalin and β-endorphin is the result of proteolytic degradation in this rapid assay. However, minor differences in effectiveness may be attributable to possible differences in absorption to the test tubes used (12). Notwithstanding this possibility, it is concluded that the rank order of potency in this kinase assay is ACTH-(1-24) > dynorphin-(1-13) > β-endorphin > leu-enkephalin.

Excessive grooming: ACTH-(1-24), at all doses studied, resulted predominantly in grooming behavior (Fig. 2A) interrupted by short episodes of stretching and yawning syndrome (7,16). ACTH-(1-24) extended grooming bout duration but not the

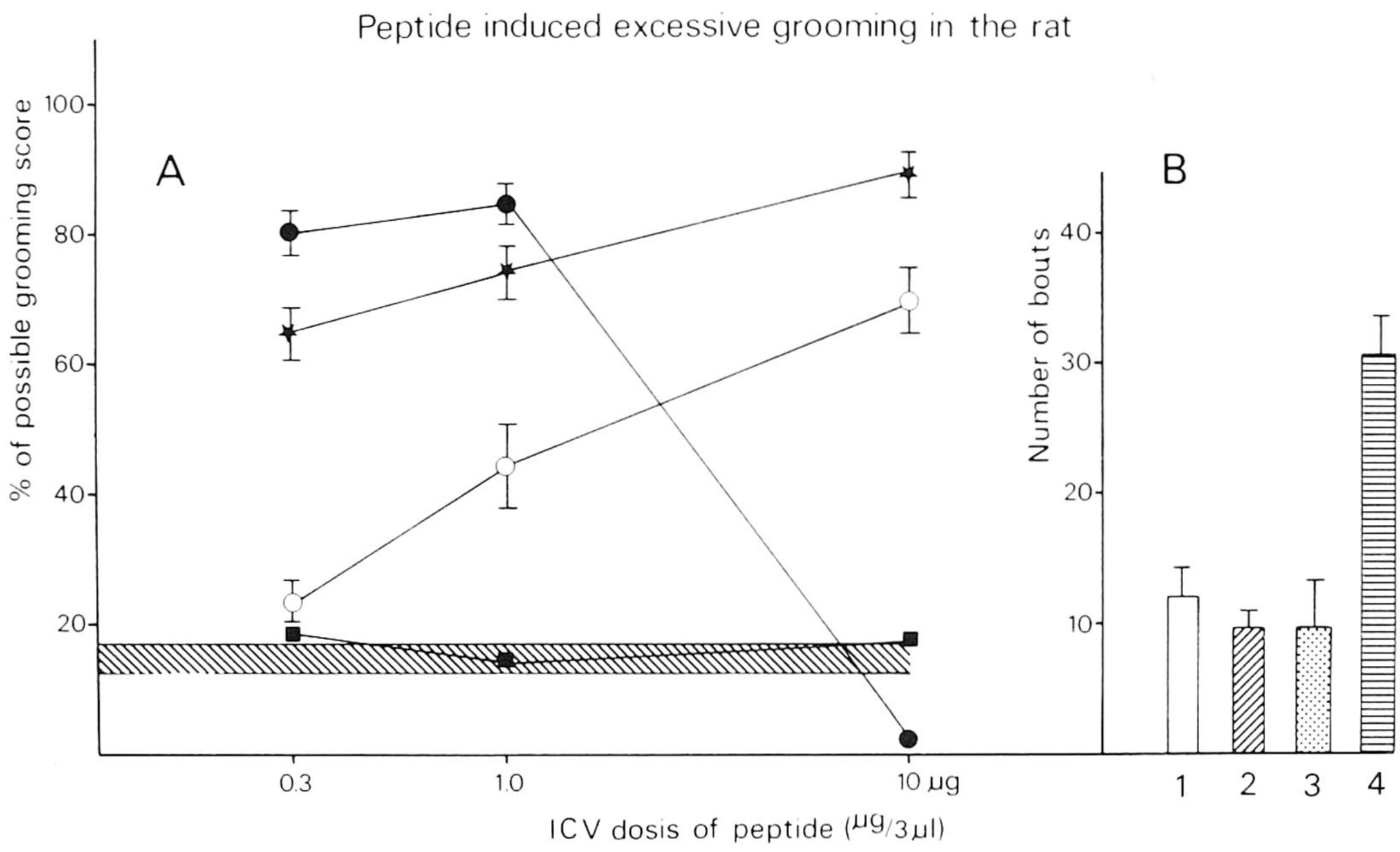

FIG. 2

Effect of intracerebroventricular administered peptides on excessive grooming in the rat.
Part A shows the percentage of maximal possible grooming score ± SEM (n = 5) after doses of ACTH-(1-24) (✱), β-endorphin (●), leu-enkephalin (■) and dynorphin-(1-13) (○). The score of control rats (± SEM) treated with normal saline is indicated as a shaded area.
Part B shows the number of grooming bouts ± SEM during the observation of rats that received 1 µg/3 µl dynorphin-(1-13) (2), ACTH-(1-24) (3), β-endorphin (4) or 3 µl of normal saline (1).

number of bouts, when compared with saline-treated rats (Fig. 2B; 15). In contrast to this type of grooming is the behavior elicited by β-endorphin. At either 0.3 or 1 µg of β-endorphin, excessive grooming was observed. In this case the grooming was typified by short bouts and, as previously reported (15,17), was often interrupted by quick movements of head and body, jumping, gnawing and body shakes (Fig. 2B). At the highest dose (10 µg), β-endorphin induced a cataleptic state (18,19,20). As reported before, leu-enkephalin failed to induce excessive grooming behavior at all doses tested (Fig. 2A; 17). Interestingly, dynorphin-(1-13), which has the leu-enkephalin sequence at its N-terminus, resulted in a dose-dependent increase in grooming behavior (Fig. 2A). The grooming appeared to be of the ACTH-type in that the number of bouts did not increase when compared to saline treated rats. In addition, like rats treated with ACTH-(1-24), but in contrast to those treated with β-endorphin, the grooming observed in dynorphin-(1-13) treated rats was occasionally interrupted by stretching and yawning. However, at the 10 µg dose, the dynorphin-(1-13) treated rats displayed barrel rotation which started immediately after the icv injection but lasted for only a few minutes. In addition, such rats often stood in an upright position but without obvious catalepsia. The excessive grooming induced by 1 µg of ACTH-(1-24), β-endorphin or dynorphin-(1-13) could be blocked by systemic administration of haloperidol (0.5 mg/kg, i.p.) or naloxone (1 mg/kg, s.c.; data not

shown). These data support previous findings on the involvement of opiate and dopaminergic components in the neural pathway underlying peptide-induced excessive grooming (15).

Discussion

The present data suggest similarities in the neurochemical mechanisms of action of ACTH-(1-24) and dynorphin-(1-13). Both of these peptides not only inhibit B-50 kinase activity but also stimulate excessive grooming by increasing the grooming score without changing the number of grooming bouts. This similarity may result from the basic nature of these two peptides (6,9). However, the mere presence of basic amino acid residues in short peptides per se in insufficient to block the enzyme or induce excessive grooming and thus the stereo configuration of the peptide is essential for activity (6).

One can speculate on the mechanism by which such basic peptides interact with the B-50 kinase. Kemp and coworkers (21) have shown, using synthetic peptide substrates that basic residues are important in determining substrate site recognition by rabbit muscle protein kinase. Substitution of the phosphorylatable serine in a peptide by alanine produced a competitive inhibitor of phosphorylation of the original peptide. Thus, it may well be that ACTH-(1-24) and dynorphin-(1-13) act as competitors with B-50 for the active site on the B-50 kinase.

In contrast to the report by Raese et al. (22) we were unable to detect phosphate incorporation into the peptides tested (9). Phosphorylation of ACTH-(1-24) and dynorphin-(1-13) seems unlikely because of the amino acid composition and sequence (see Fig. 3).

β-Endorphin	Tyr Gly Gly Phe Met Thr Ser Glu Lys Ser Gln Thr Pro Leu Val Thr Leu Phe Lys Asn Ala Ile Val Lys Asn Ala His Lys Lys Gly Glu
Leu-Enkephalin	Tyr Gly Gly Phe Leu
Dynorphin(1-13)	Tyr Gly Gly Phe Leu Arg Arg Ile Arg Pro Lys Leu Lys
ACTH (1-24)	Ser Tyr Ser Met Glu His Phe Arg Try Gly Lys Pro Val Gly Lys Lys Arg Arg Pro Val Lys Val Tyr Pro

FIG. 3

Primary structure of the peptides tested. Note the homology of leu-enkephalin and the N-terminal of dynorphin-(1-13). The basic sequences which are thought to be important in both the induction of grooming and the inhibition of B-50 kinase are underlined.

Recently, evidence was obtained for an endorphin/enkephalin-sensitive brain membrane protein phosphorylation (23,24,25). It is unlikely that the B-50 protein/B-50 kinase complex is part of the above mentioned membrane system. There are several discrepancies between these systems with respect to membrane preparation, substrate proteins, effect of naloxone, effects of opioid peptides and ACTH-(1-24).

The inhibition of B-50 protein kinase in vitro most likely reflects a non-opiate effect on ACTH-(1-24) and dynorphin-(1-13) since their effect is not blocked by naloxone. However, at present one should reserve judgment with respect to such a statement. Although, in vitro ACTH-(1-24) displaces labelled dihydromorphine, naloxone and β-endorphin from their respective CNS binding sites (26,27,28), there is evidence for non-naloxone reversible effects of both ACTH-(1-24) (27) and dynorphin-(1-13) (30) on rat behavior, presumably involving

opioid-sensitive structures.

Certainly, peptide-induced excessive grooming behavior involves an opioid-sensitive neural substrate since it is blocked by naloxone and naltrexone (31). It remains to be elucidated whether this behavioral response is elicited by direct opioid receptor activation or by peptide activation of brain structures containing opiate receptors. For example, peptide-induced excessive grooming could also be blocked by the dopamine receptor blocker haloperidol, thus supporting previous findings on the involvement of dopaminergic components in the neural pathways underlying this behavior (32). Therefore it is possible that the presently reported effects of dynorphin-(1-13) reflect a non-opioid influence of this peptide on central nervous structures reminescent of the modulatory role ascribed to ACTH.

In conclusion, the present data fit the previously observed correlation between the ability of several neuropeptides to induce excessive grooming in the rat after intraventricular administration and the inhibitory effect of these peptides on the B-50 protein kinase.

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