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Additional Evidence That Small Amounts of a Peptide Can Cross the Blood-Brain Barrier

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KASTIN, A. J., C. NISSEN, A. V. SCHALLY AND D. H. COY. *Additional evidence that small amounts of a peptide can cross the blood-brain barrier.* PHARMAC. BIOCHEM. BEHAV. 11(6) 717–719, 1979.—It was determined that an antiserum against delta-sleep inducing peptide (DSIP) required eight of the nine constituent amino acids for antigenic activity. Measurement by this radioimmunoassay (RIA) of DSIP-like material in the rat brain, therefore, would necessarily involve almost the entire molecule present in essentially intact form. Injection of 200 μg DSIP into the carotid artery of rats resulted in a doubling of brain levels of peptide as measured shortly afterwards by RIA. The brain tissue to plasma ratio of radioactivity in rats injected with labeled DSIP was much higher than that in rats injected with labeled inulin; this suggests that the increased amount of material measured by RIA was not merely trapped in the blood vessels. Thus, the results indicate that a small amount of essentially intact peptide can cross the blood-brain barrier. This could represent one of the mechanisms by which central effects of peripherally injected peptides can be exerted.

Blood-brain barrier Radioimmunoassay Peptide antigenic-determinants DSIP

IT HAS become increasingly evident that peripheral administration of brain peptides affects the central nervous system (CNS) [3]. Although it has not been established how a peptide injected peripherally can exert an action centrally, several possibilities have been discussed [3,4]. Among these possibilities is the penetration of the blood-brain barrier (BBB) by the peptide in its intact form.

Several experimental approaches have been used in attempts to determine whether systemically injected peptides enter the brain. Most of the early studies of this issue involved localization of the labeled peptide by radioautography [7] or radioactivity [1]. Additional techniques such as chromatography were then required to identify the intact molecule in the brain tissue and small amounts of apparently intact material have been found [1]. Continued attachment of the label to the peptide, however, remained difficult to prove completely [1]. Oldendorf's technique, involving the simultaneous injection of two different isotopes [6], offers another approach, but the sensitivity of this technique is partly limited by the lack of sufficiently high specific activity of the injected peptide [3,4]. Radioimmunoassay (RIA) in cerebrospinal fluid of a peripherally injected peptide has recently been reported [8] and similar measurements in brain tissue will undoubtedly be developed for most peptides. Until now, however, the antigenic determinants for existing RIAs constitute only a small portion of the peptide, so that additional identification of the measured compound is required, as is the case after injection of radioactively labeled peptides.

A partial solution to this dilemma would be offered by a radioimmunoassay with an antiserum which required essentially the whole molecule for antigenic activity. This paper reports evidence that an antiserum against delta-sleep inducing peptide (DSIP) [11] has been generated [2] which meets this requirement. The RIA which was developed with this antiserum [2] was used to determine penetration of the BBB by an almost intact brain peptide.

METHOD

Peptides

DSIP, seven fragments, and two analogues of it were synthesized and purified as previously described [2]. They were used to test for cross-reactivity with DSIP.

Animals

Male, albino, adult rats (Zivic Miller, Allison Park, PA) weighing about 300 g were anesthetized with sodium pentobarbital (5 mg/100 g) and the test material was injected in 100 μl saline in alternating design into the carotid artery as described previously [1]. A dose of 200 μg/rat of DSIP was used. The rats were then decapitated at various times afterwards, beginning at five seconds.

Procedure

Penetration of the BBB by DSIP was investigated in four experiments. In the first study, six animals were injected into the carotid artery (IC) with 200 mg DSIP and another six with the saline diluent. The rats were decapitated at various times after injection, beginning at five seconds. Whole brains, without pineal, pituitary or hypothalamus, were carefully rinsed, extracted, and the DSIP-like material measured by RIA. The method was the same as that described previously [2] except that chloramine-T was used for iodination and the percent of dextran in the mixture with charcoal was changed to 0.1%.

The second experiment was identical to the first except

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that the brains of four animals injected with DSIP and four with saline were divided into nine areas for assay. Data from the first two experiments were analyzed by analysis of variance with subsequent comparisons by Duncan's Multiple Range Test.

In the third experiment, radioactivity in the whole brain was compared to that in trunk blood five seconds after injection of 1.5 μCi of the iodinated DSIP. A similar ratio was measured after injection of tritiated inulin, the quench being equalized by addition of the sample of plasma to that of a digest of whole brain obtained from uninjected rats.

In the fourth experiment, a DSIP standard was compared by thin layer chromatography (TLC) to material present in the brain five seconds after IC injection of either 200 μg DSIP or saline. Samples of extracts from the whole brains used in the first experiment were extracted with 1 M acetic acid, defatted with ether, deproteinated with the addition of acetone, lyophilized, and then passed through a column of Sephadex G-25 fine. After TLC in a solvent system of butanol, acetic acid, water, ethyl acetate (1:1:1:1), the plates were sprayed with ninhydrin.

RESULTS

At five seconds after IC injection of 200 μg DSIP, the mean ± SEM content of DSIP-like material in the whole brain extracts of six rats was 50.1 ± 6.9 pg/mg wet brain tissue. This amount was more than double that found after injection of six other rats with the saline diluent (22.3 ± 1.4 pg/mg). There was no overlap of any value in one group with any value in the other group, the difference between groups being highly significant ($p<0.01$). No significant increase remained at 1, 5, 15, or 30 min after injection.

These results were confirmed in a second experiment in which the mean levels of DSIP measured in the brains of rats receiving the DSIP were much higher than those of the controls receiving saline when analyzed by parts. The main effect of treatment was highly significant ($p<0.01$). If DSIP receptors are localized in specific areas of the brain, the whole brain measurements of the first experiment could obscure the values for the amount of DSIP entering brain areas with high affinity for DSIP. In the second experiment, assay of nine brain parts in each of four rats receiving DSIP and four rats receiving saline showed that the pons-medulla, thalamus, and posterior cortex ranked high in content of DSIP-like material. Occipital cortex also ranked high in the rats injected with saline. The main statistical effect of brain area and the interaction of area with treatment were significant ($p<0.05$). Although those parts not entirely behind the BBB (pineal, pituitary, hypothalamus) were excluded, no special dissection was performed of the circumventricular areas. Nevertheless, comparison of individual brain parts showed all but striatum to be significantly higher in rats injected with DSIP than in those injected with saline.

The high concentration of DSIP in the pons-medulla (including fourth ventricle) in animals given DSIP was confirmed in the third experiment in which iodinated DSIP was injected and measured in the various brain areas. These results were consistent with penetration of peptide into the CSF first, a concept included in our general use of the term BBB. Radioactivity in the brain compared to that in blood after injection of iodinated DSIP or tritiated inulin showed that the tissue to plasma ratio for the labeled DSIP was 0.58 whereas that for the labeled inulin was only 0.08, with no

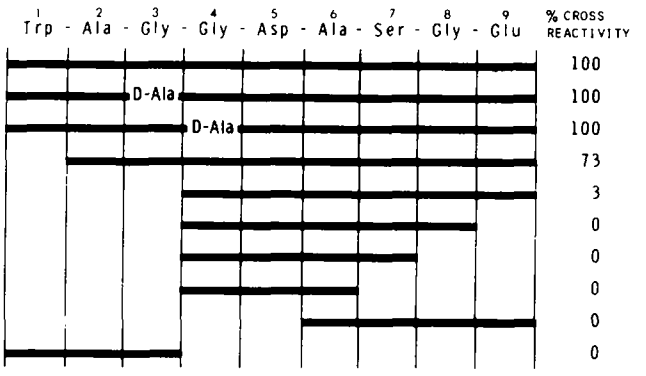

FIG. 1. Percent cross-reactivity of various fragments and analogues of DSIP with the antiserum used in the RIA. DSIP is shown on top.

overlap among the seven rats ($p<0.01$). No attempt was made to determine whether the label was still attached to DSIP.

In the fourth experiment, TLC showed some material from fraction 6 of brain extracts of rats injected with DSIP to have about the same Rf as the DSIP standard (0.53–0.54). This was not evident in the sample from the six rats injected with saline, although endogenous DSIP-like material is frequently found in this early fraction.

DISCUSSION

A major advantage in the use of DSIP to determine penetration of the BBB by a brain peptide is the demonstration that almost the entire molecule is required as an antigenic determinant by our antiserum. Of the nine amino acids in DSIP, less than 0.1% cross-reactivity was seen with fragments 4–6, 4–7, 4–8, and 6–9. This indicates that the small amount of immunological binding seen with DSIP 4–9 requires the presence of at least the last five amino acids. The lack of cross-reactivity of DSIP 1–3 and unaffected cross-reactivity when the third and fourth amino acids are substituted by D-Ala demonstrate that the antiserum requires more than the N-terminal amino acids for binding. DSIP 2–9 showed 73% cross-reactivity, indicating that the first amino acid is not required for complete binding, an expected finding because of the presumed site of conjugation. Although substitutions can be made in the molecule which do not affect its cross-reactivity, it is obvious that almost the entire sequence is necessary for full binding with the DSIP antiserum. Thus, measurement of increased DSIP-like immunoreactivity in the brain after peripheral administration of DSIP constitutes strong evidence that some DSIP can cross the BBB essentially unchanged. This evidence was strengthened by the use of the five second interval, a time at which little degradation would be expected.

By one minute after the rapid IC injection of 200 μg DSIP, the increased values were no longer significantly different from controls, and by five minutes basal levels had been reached. If penetration by the intact molecule is required for central effects, then these effects would seem to be dependent upon initial stimulation rather than continued presence of the intact peptide. Other explanations include actions primarily in the periphery, or a biologically active degradation product not detected by the RIA.

The ranking of brain parts in relation to their content of DSIP-like material tended to show some differences between the rats receiving DSIP and those receiving saline. Since the relative levels of DSIP in the various areas of the controls represent the endogenous distribution of the peptide, the slightly different pattern of concentrations after injection of DSIP could reflect an inverse profile of receptor occupancy by endogenous peptide, different binding properties, or the pattern of penetration of the BBB. A similar phenomenon may have been observed with α-MSH [1].

A major problem in measuring the presence of any compound in the brain after systemic injection is the possibility of residual material remaining in the blood vessels rather than in the tissue. This also applies to studies involving localization of radioactivity after injection of labeled material. Radioautographic localization can circumvent this problem [7], but involves other difficulties such as establishing that the radioactivity represents the intact molecule.

To determine whether the measured immunoreactivity was mainly trapped in the blood vessels, we compared the amounts of radioactivity in the brain after IC injection of radioactively labeled DSIP and radioactively labeled inulin. Inulin is thought to be relatively inert in terms of its penetration of the BBB [6]. Although persistence of label on the injected compound must also be considered, the much greater proportion of radioactivity in brain compared with blood after injection of labeled DSIP than after injection of labeled inulin indicated that the accumulated material measured by RIA was not confined to the blood vessels.

The presence of increased radioactivity in the brain tissue of rats injected with labeled DSIP strengthens the suggestion that DSIP crossed into the brain. Although some material in the brain of rats receiving DSIP had a similar Rf to that of DSIP by TLC, identity was not proven for this or the labeled peptide. It does not seem likely, however, that exogenous DSIP caused a secondary increase in endogenous levels of DSIP. It is possible that exogenous DSIP increased levels of other substances which cross-reacted with the antiserum. Although 19 other peptides are known not to react in our RIA [2], there are a large number of protein fragments which share as many as five amino acids with DSIP (National Biomedical Research Foundation, Washington, D.C.). Part of the IG α-chain, for example, is identical to the C-terminal tetrapeptide of DSIP, but this portion did not appreciably react with our antibody. A DSIP fragment consisting of the last five amino acids is identical to part of a 48 amino acid bovine posterior pituitary peptide [9] and showed 3% cross reactivity with DSIP. Even though no other known peptide contains the peptide sequence required to react with our antiserum, it is theoretically possible that a natural compound could exist which differed structurally but not immunologically from DSIP.

The evidence for penetration of the BBB by a peptide (in this case DSIP) agrees with the findings of a few other studies of this same question recently reviewed elsewhere [3,4]. All these investigations, including the present one, indicate that the actual amount of peptide entering the brain is relatively small. It remains a question of semantics whether the word "readily", which means "quickly" as well as "easily" [10], should be used to describe this small degree of penetration. It also remains unclear whether the CNS effects observed after the peripheral administration of peptides are exerted only by direct penetration into the brain (or CSF) or whether other mechanisms are also involved.

Preliminary studies with E. Fritschka showed that tritiated Met-enkephalin infused IV for 90 min in dogs and cats was not found in unchanged form by TLC in the CSF even though radioactivity increased with time. Although this may represent a methodological problem, it emphasizes the caution which should be used in generalizing from the data of these experiments with DSIP to other peptides.

Our results indicate that some DSIP is capable of crossing the BBB essentially intact. Whether this occurs by retrograde flow from the pituitary, entry into the CSF first or other means is not known. The percentage as well as absolute amount penetrating the brain is probably small, but may be sufficient to exert the CNS effects already described for DSIP [5]. Systemically injected DSIP may also affect the brain in other ways discussed previously [3,4]. The stringent binding requirements of our antiserum against DSIP has enabled DSIP to serve as a model for other brain peptides. It may be reasonable to assume that small amounts of other peptides can also cross the BBB essentially intact.

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