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Delta Sleep-Inducing Peptide Crosses the Blood-Brain-Barrier in Dogs: Some Correlations with Protein Binding

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BANKS, W. A., A. J. KASTIN AND D. H. COY. *Delta sleep-inducing peptide crosses the blood-brain-barrier in dogs: Some correlations with protein binding.* PHARMAC. BIOCHEM. BEHAV. 17(5) 1009–1014, 1982.—To determine if the nonapeptide delta sleep-inducing peptide (DSIP) and some of its analogs can enter the cerebrospinal fluid (CSF) from the peripheral circulation, dogs anesthetized with sodium pentobarbital were injected with a 100 μg/kg bolus of DSIP, desTrp^1-DSIP, or D-Ala^3-DSIP. Using an antibody that is highly specific for these nonapeptides, we found significant increases in CSF after IV injection of each. Column chromatography of CSF withdrawn after an IV bolus of DSIP showed the increase in immunoreactivity to be due to DSIP and desTrp^1-DSIP. Chromatography of radioactivity appearing in the CSF after an IV bolus of ^{125}I-N-Tyr-DSIP showed co-elution with the intact labeled peptide. The CSF/plasma ratios for DSIP and ^{125}I-N-Tyr-DSIP were higher than that for tritiated insulin injected IV. It was also shown that DSIP is reversibly bound to a large molecule in dog plasma and CSF, and that the binding is greater in blood than in CSF. The binding protein demonstrates some specificity for DSIP, binding a smaller percentage of D-Ala^3-DSIP, and it appears that it is probably free DSIP that crosses the blood-brain-barrier. Although non-specific crossing cannot be ruled out, the results presented here are also consistent with a specific saturable process for DSIP.

Sleep DSIP Peptide BBB Brain CSF RIA Plasma binding Protein binding Column chromatography Protein(s) Canine

NUMEROUS studies have described changes in behavior after the peripheral administration of peptides [7]. Alterations in the EEG occur with the peripheral administration of many peptides [7], including delta sleep-inducing peptide (DSIP) [3, 6, 12, 13], and these may correlate with endogenous blood levels [2]. Correlation between CSF and blood levels have been noted for a few other peptides [1,14].

Several studies have shown increased immunoreactivity in the CSF or brain after peripheral administration of a peptide or measured radioactivity after administration of labeled peptide [5]. These have been interpreted as evidence of peptides crossing the blood-CSF or blood-brain-barrier (BBB). These investigations, however, have not excluded the possibilities of stimulation of endogenous levels of peptide in the CNS, passage of immunoreactive or radioactive fragments of the peptide, or passage of unattached radioactive label. Our study attempted to clarify these points and to determine if exogenous DSIP can enter the CSF after peripheral administration. Since DSIP has been shown to be bound to serum proteins in human blood [4], we also attempted to determine if protein binding occurs in the plasma and the CSF of the dog, and to determine the possible role of this binding in BBB transport.

METHOD

Validation of DSIP Assay for CSF

Dilution curves for endogenous DSIP were determined with dog and rabbit CSF. The effects of thawing, lyophilization, and addition of synthetic DSIP on parallelism of dog CSF were determined, and recovery measured. Column chromatography (Sephadex G-15) was performed on endogenous immunoreactivity of pooled dog CSF.

Bolus Injections

Mongrel dogs (10–25 kg) were anesthetized with sodium pentobarbital (30–35 mg/kg). Animals were placed on their right side and CSF was obtained by percutaneous puncture of the cisterna magna using a 22 g 3.8 cm needle. Two intermittent intravenous (IV) infusion sets kept patent with heparin sulfate were used, a set in the hindlimb for bolus injection and a set in the forelimb for withdrawal of blood. Two ml of blood were collected in tubes containing 50 U of heparin. CSF was also collected, and all samples used were visually clear. Both CSF and blood were immediately centrifuged at 2300 g for 10 minutes and the supernatants collected and frozen at −20°C until time of assay. CSF was collected at −10 min, 0 min, 20 sec, 40 sec, 60 sec, 90 sec, 2 min, 3 min, 5 min, 10 min, 15 min, and 30 min and plasma at −10 min, 0 min, 2.5 min, 5.5 min, 15 min, and 30 min.

Animals were injected with 100 μg/kg of DSIP, desTrp^1-DSIP, D-Ala^3-DSIP, or the vehicle of normal saline (0.9% NaCl (NS)). From three of the dogs injected with DSIP, plasma and CSF samples were also taken at 60 min, 90 min, and 120 min. In two more dogs injected with DSIP, samples

were taken only at 0 min and 30 min. Additional dogs were injected with ^{125}I-N-Tyr-DSIP, tritiated inulin, or a tritiated and unlabeled inulin mixture.

Determination of Protein Levels

Protein levels were measured in the pooled samples (basal CSF, basal plasma, post-injection CSF, post-injection plasma). This was done on a Dupont Instruments Automatic Clinical Analyzer using the biuret color reaction method.

Column Chromatography

DSIP and desTrp^1-DSIP were used to calibrate a column of Sephadex G-15 (fine) (1×120 cm) in 0.02 M acetic acid. One ml of pooled basal samples (−10 and 0 min) or post-injection samples (10–30 min) of CSF or plasma were separated on the G-15 column. DSIP and D-Ala^3-DSIP were also incubated in vitro for 1 hr at 4°C in 1 ml of pooled CSF or plasma and then separated. Two ml fractions were collected, lyophilized, reconstituted in 0.5 ml Trizma (pH 8.0), and measured in the radioimmunoassay (RIA).

A column of Sephadex G-25 was calibrated using ^{125}I-N-Tyr-DSIP. One ml of pooled basal CSF or plasma was incubated at room temperature for 10 min with ^{125}I-N-Tyr-DSIP and separated on this column. CSF collected after IV injection of ^{125}I-N-Tyr-DSIP was also chromatographed.

Determination of Immunoreactivity and Radioactivity

An RIA for detecting DSIP in human plasma and rat brain including techniques of antibody production, cross reactivity, iodination, purification of labeled material, incubation time, and statistical analysis is described elsewhere [4, 8, 9]. Immunoreactive levels of DSIP-like material were measured by RIA using 100 μl samples of CSF or reconstituted column fraction or 10 μl of plasma. Samples with a $B/B_0 < 0.20$ were diluted and reassayed. Radioactivity was measured in a Hewlett Packard 4/200 automatic gamma counter.

Statistics

Data were evaluated for statistical significance with a one-way analysis of variance program on a DEC 2060 Computer. Means are reported with the standard error. A Texas Instruments 58C calculator was used for the linear regression analysis. The coefficient of variation was calculated both within and between assays. Half-time disappearance was calculated using linear regression for $\log_{10}$ of plasma concentration vs time.

RESULTS

Validation of Assay for CSF

Dilution curves of dog and rabbit CSF were parallel to the standard curve (Fig. 1), and this parallelism was unaffected by either freezing or lyophilization. CSF (100 μl) from dogs added to all tubes in the standard curve shifted it to the right, but parallelism was unaffected, and CSF with exogenous DSIP was also parallel to the standard curve (Fig. 1). Immunoreactivity increased linearly with increasing amounts of CSF (10–200 μl/tube). The sensitivity of the assay was 13.5±1.2 (80% B/B_0). Recovery of immunoreactive material added to CSF was 100% by RIA.

Exogenous DSIP added to human CSF was stable for 4 cycles of freezing and thawing (91±9% decrease in levels of immunoreactivity). DSIP incubated in CSF was stable at 4°C

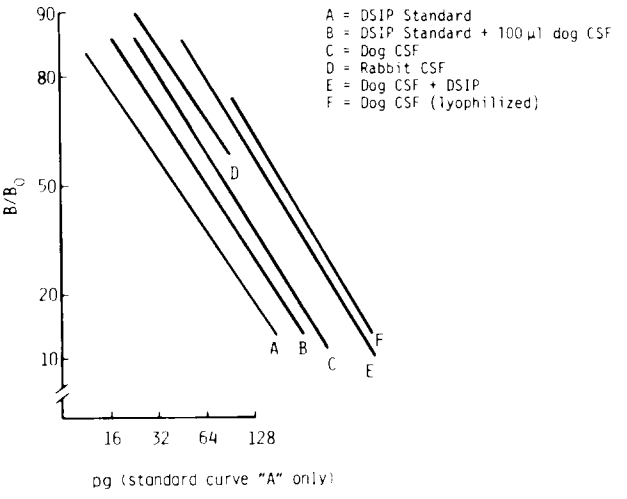

FIG. 1. Parallelism of DSIP-like immunoreactivity in CSF of the dog and rabbit. Numbers on the abscissa refer to line A, the standard curve for synthetic DSIP in buffer; the other curves are shifted to the right for clarity.

with a 2–3% decrease in levels of immunoreactivity after 100 hours, while at room temperature, 33–36% immunoreactivity was lost after 100 hours.

Column chromatography of basal samples of CSF on either Sephadex G-15 (fine) or G-25 (fine) revealed two peaks of endogenous immunoreactivity (Fig. 2, panel A), one that coeluted with the synthetic DSIP nonapeptide, and one that immediately followed the void volume. Five ng of exogenous DSIP added to either buffer or CSF, immediately lyophilized, and subsequently chromatographed were found to co-elute with the intact molecule.

The coefficient of variation at 32 pg/100 μl and 128 pg/100 μl was about 11% for both within and between assays. Validation of the DSIP assay for plasma is described elsewhere [4].

Bolus Injections

All CSF samples were visually clear. Dilution of CSF with added blood showed that the unaided eye can readily detect a concentration of 1 part in 2000.

Immunoreactive levels in basal (−10 and 0 min) samples of CSF correlated well with levels in the simultaneously drawn plasma samples ($y=0.085x + 22.4$, $n=75$, $r=0.686$, $p<0.001$). The r^2 value of 0.47 indicates that about half of the variability in the CSF is related to the variability in the plasma.

After bolus injection of 100 μg/kg DSIP (N=25), the CSF level increased from a baseline 0.56±0.03 ng/ml to 1.10±0.08 ng/ml at 30 min (Fig. 3) achieving statistical significance at 120 sec and 3 min ($p<0.05$) and 5 min, 10 min, 15 min and 30 min ($p<0.0001$). For the three dogs from which samples were taken up to 120 min, the 30 min value was the peak. No correlation existed between plasma levels at 2.5 min and CSF levels (either peak or 30 min) in the post-injection state. For the two dogs from which samples were taken only at 0 min and 30 min, CSF levels increased from 0.49 ng/ml to 2.34 ng/ml, suggesting that our repeated sampling of the other

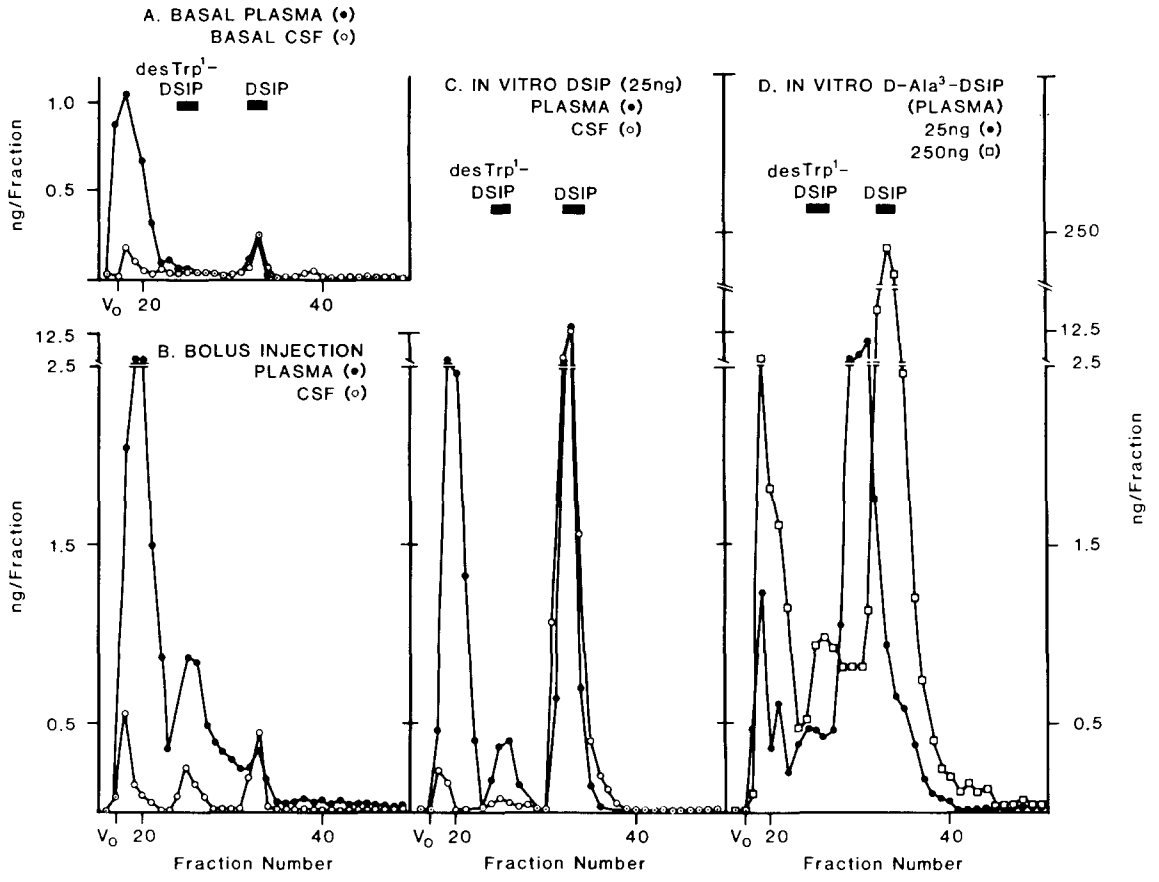

FIG. 2. Column chromatography of immunoreactivity in dog CSF and plasma. Panel A shows pooled basal plasma (●) and CSF (○). Panel B shows pooled plasma (●) and CSF (○) after an IV bolus of DSIP. Panel C shows pooled basal plasma (●) and CSF (○) incubated in vitro with 25 ng DSIP. Panel D shows pooled basal plasma incubated in vitro with 25 ng (●) or 250 ng (□) D-Ala³-DSIP.

dogs did not artifically elevate CSF levels of peptide. CSF values after injections of desTrp¹-DSIP (n=4) were similar to those for DSIP, showing statistically significant ($p<0.01$) increases at 3 min, 5 min, 10 min, 15 min and 30 min. After injection of D-Ala³-DSIP (n=6), however, CSF levels peaked earlier (at 10 min) and rose much higher to a value of 1.95±0.59 ng/ml although statistical significance occurred only at 15 min and 30 min ($p<0.01$). CSF and plasma values were unchanged after injection of the vehicle (0.9% NS) alone (Fig. 3).

The half-time disappearance and plasma levels at 2.5 min after bolus injection of DSIP, desTrp¹-DSIP, and D-Ala³-DSIP were 7.9 min and 282±30.6 ng/ml, 8.2 min and 319±15.7 ng/ml, and 10.9 min and 388±47 ng/ml respectively. At an average weight for the dogs of 18 kg, the volume of distribution for DSIP is 6.23 l, which is approximately equal to the extracellular volume. The CSF/plasma ratio at 30 min was 0.025, 0.020, and 0.016 for DSIP, desTrp¹-DSIP, and D-Ala³-DSIP. Although the CSF value peaked at 30 min for the three dogs studied to 120 min, their highest CSF/plasma ratio (0.145) occurred at 90 min.

In dogs injected with ^{125}I-N-Tyr-DSIP the peak in CSF levels also occurred at 30 min as did the peak CSF/plasma ratio (0.037). A good correlation existed between the levels of radioactivity at the CSF peak and plasma (2.5 min) ($y=0.0142\times-0.62$, n=5, r=0.993, $p<0.001$). The CSF/plasma ratio after injection of tritiated inulin (n=1) was 0.019 at 30 min and 0.045 at 90 min. The appearance rate of tritiated inulin in the CSF was reduced by half with the simultaneous injection of 1 g of unlabeled inulin (n=1).

Determination of Protein Levels

The protein levels in the pooled samples were 28 mg/dl (basal CSF), 6.45 g/dl (basal plasma), 33 mg/dl (post-injection CSF), and 6.08 g/dl (post-injection plasma). This gave a ratio of plasma protein to CSF protein of 230:1 for basal samples and 184:1 for post-injection samples, or an average of about 205:1.

Column Chromatography

Gel filtration of pooled basal CSF showed that 44% of the DSIP immunoreactivity eluted immediately after the void volume ("bound" fraction), 51% co-eluted with DSIP ("free" DSIP), and a small percent co-eluted with desTrp¹-DSIP ("free" desTrp¹-DSIP) (Fig. 2, panel A). Pooled basal plasma was 89% "bound" with 9% co-eluting as free DSIP. Pooled basal CSF and plasma had essentially equal amounts of free DSIP (355 pg/ml vs 370 pg/ml), although total levels were six times higher in plasma.

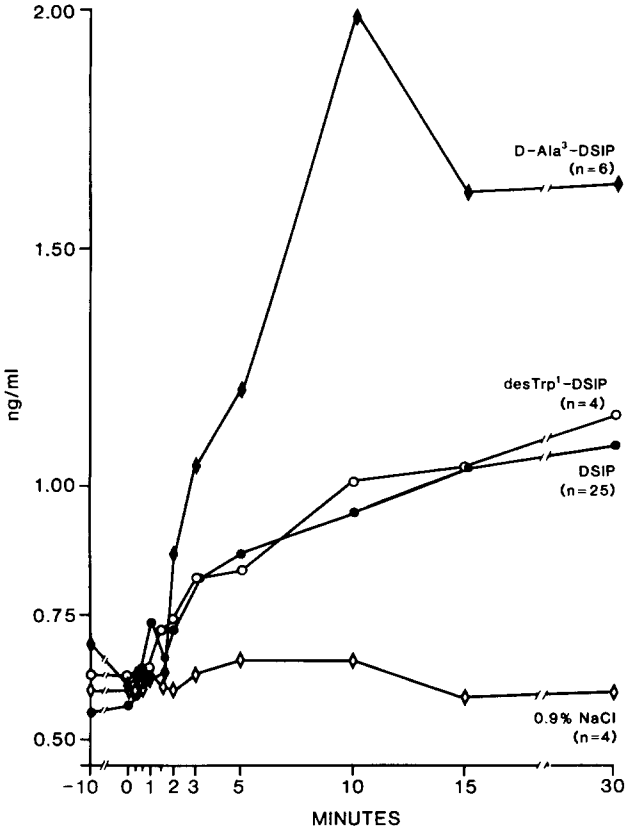

FIG. 3. Immunoreactive levels of DSIP-like material in CSF after IV bolus of DSIP peptides. See text for statistically significant points.

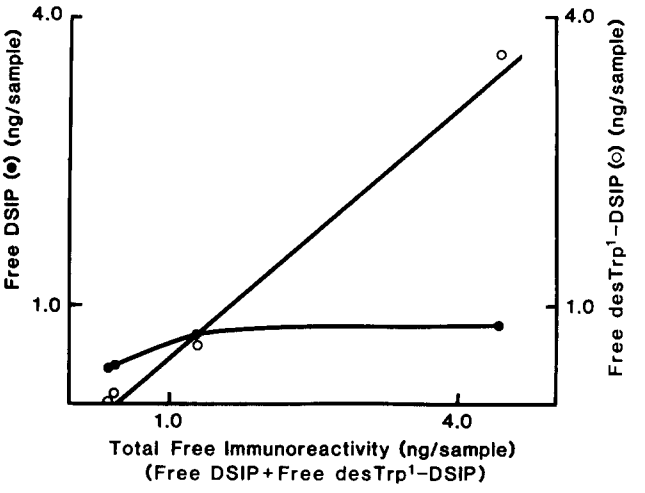

FIG. 4. Relationship of free DSIP, free desTrp¹-DSIP, and total free immunoreactivity. Results are derived from immunoreactive values obtained after column chromatography of basal and post injection samples.

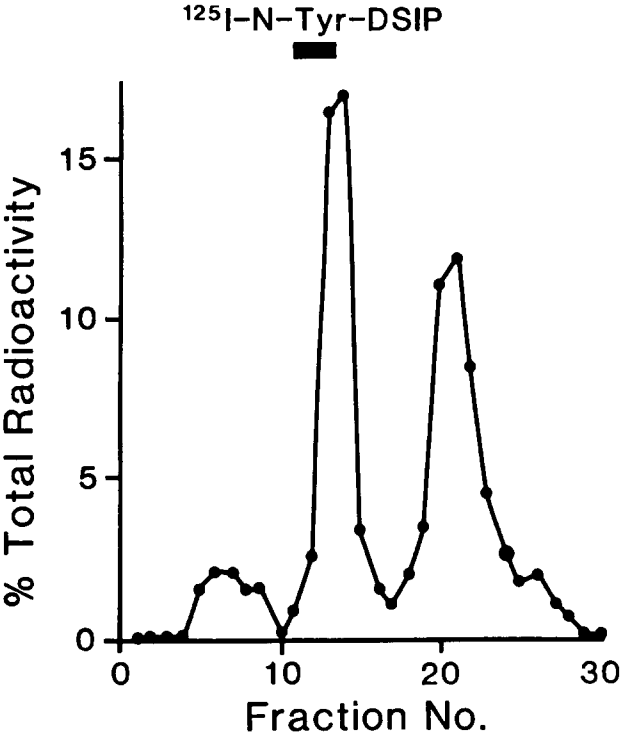

FIG. 5. Column chromatography of radioactivity appearing in CSF after IV bolus of ¹²⁵I-N-Tyr-DSIP.

After bolus injection of DSIP, all fractions (bound, free DSIP, and free desTrp¹-DSIP) were increased in both CSF and plasma (Fig. 2, panel B). Total immunoreactivity increased 3.5 times in plasma, but only 4% of the increase in immunoreactivity co-eluted with free DSIP, most (64%) having been bound. The increase in total immunoreactivity in CSF was proportionate to that in plasma (3.1 times), but the majority of the increased immunoreactivity co-eluted with the free DSIP, while the percent bound remained stable (44% basal, 42% post injection). Once again, the concentration of free DSIP was nearly equal in CSF and plasma. The percent free desTrp¹-DSIP in CSF and plasma was 28% and 24% respectively.

Figure 4 shows the relationship between free desTrp¹-DSIP, free DSIP, and total free immunoreactivity found after gel filtration in the basal and post injection states in CSF and plasma. As the total free immunoreactivity increased, free DSIP appeared to increase asymptotically to a relatively stable level whereas free desTrp¹-DSIP continued to increase linearly. "Bound" fraction 18 obtained by chromatography of post-injection plasma was acidified with 1 N hydrochloric acid and rechromatographed. The acidification apparently liberated some free immunoreactivity (mostly desTrp¹-DSIP), although the majority still co-eluted in the "bound" region.

Addition of 25 ng DSIP to one ml pooled basal plasma incubated for 1 hour at 4°C (Fig. 2, panel C) resulted in a doubling of the "bound" fraction and an increase in free desTrp¹-DSIP and DSIP as compared to the basal state (Fig. 2, panel A). Addition of the same amount of DSIP to CSF resulted in a 32% increase in the "bound" fraction and an increase in free desTrp¹-DSIP. Most of the added immunoreactivity remained as free DSIP. D-Ala³-DSIP was bound much less in plasma, and only after addition of 250 ng did the increase in the "bound" fraction approach that ob-

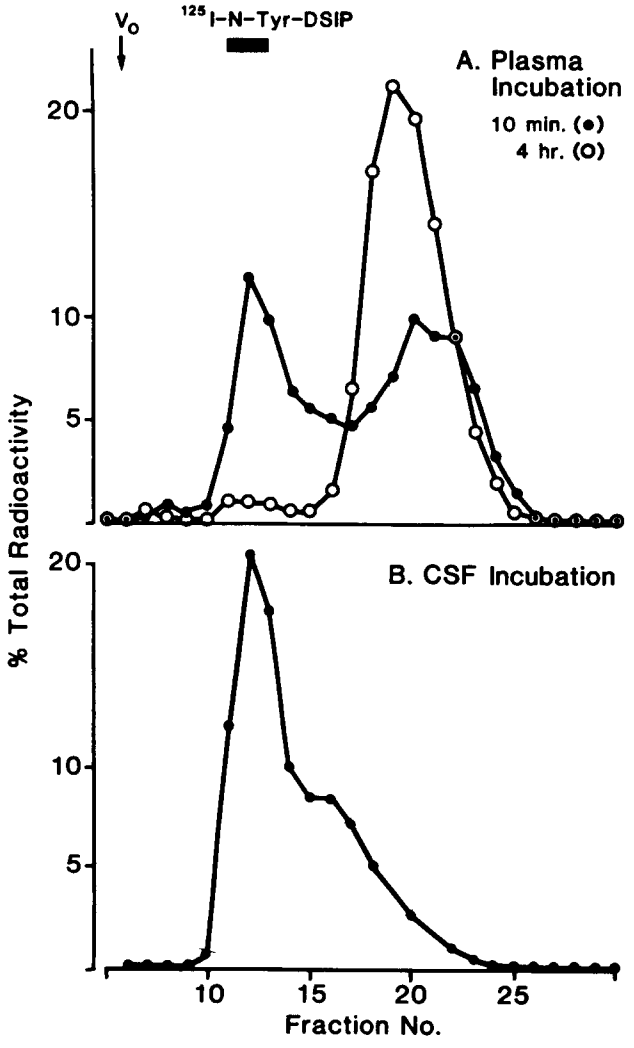

FIG. 6. Incubation of ^{125}I-N-Tyr-DSIP in pooled basal CSF and plasma. Panel A shows incubation in plasma for 10 min (●) and 4 hr (○). Panel B shows incubation in CSF for 10 min.

tained with 25 ng of DSIP (Fig. 2, panel D). Recovery from these columns was essentially 100%.

Chromatography of CSF obtained after IV injection of ^{125}I-N-Tyr-DSIP showed 10% of the radioactivity to elute in the "bound" fraction (Fig. 5). About half of the remaining radioactivity co-eluted with ^{125}I-N-Tyr-DSIP which, after neutralization to pH 8.0, was shown to bind with our antibody.

Incubation of ^{125}I-N-Tyr-DSIP in vitro for 10 min with subsequent chromatography showed that in plasma 2% of the radioactivity was "bound" and in CSF 0.04% (Fig. 6). This gives a ratio for the binding protein found in plasma to that in CSF of 50:1 (a similar ratio calculated for immunoreactivity was 42:1), and contrasts to the ratio for total protein measured in these samples of 205:1. An increase in the time of incubation in plasma to 4 hours resulted in a decrease in the "bound" fraction to 1% and an increase in the portion eluting as a radioactive fragment from 47% to 95%. Acidification and chromatography of the fraction "bound" in plasma resulted in liberation of free ^{125}I-N-Tyr-DSIP and a radioactive fragment, with 19% of the radioactivity remaining in the "bound" position.

DISCUSSION

The results show the presence of endogenous DSIP-like immunoreactivity in the CSF of dogs. This immunoreactivity diluted parallel to synthetic DSIP, co-eluted with synthetic DSIP, was unaffected by lyophilization, and was reasonably stable with incubation at 4°C or 37°C and with repeated freezing.

Plasma and CSF levels of DSIP immunoreactivity were correlated in the basal state. This correlation, noted for a few other peptides [1,14], suggests a similarity of control or distribution between CSF and blood for these peptides.

Protein binding of DSIP apparently occurred in both dog CSF and plasma. This was demonstrated with labeled and unlabeled DSIP by measurement of both radioactivity and immunoreactivity, in vivo and in vitro in the basal state and after injection as a bolus. The binding was much greater in plasma than CSF, although the amounts of free DSIP were equivalent both before and after injection. Acidification reversed most of the binding of radioactivity and immunoreactivity. The presence of an immunoreactive precursor to DSIP could not account for the increase in the "bound" fraction after addition of synthetic DSIP, nor the appearance of radioactivity in this fraction and its subsequent release by acidification. Such a precursor, however, might account for some of the residual immunoreactivity, though not radioactivity, noted in the "bound" fraction even after acidification.

Most (64%) of the DSIP injected IV was bound in the plasma with only 4% remaining as free DSIP. A tight control of the level of free DSIP is further suggested in Fig. 4, where it is noted that the increase of free DSIP, in relation to increasing amounts of total free immunoreactivity, appears asymptotic. The plasma to CSF "bound" ratio of 50:1 in comparison to the total protein ratio of 205:1 suggests that the DSIP binding protein is relatively more concentrated in CSF in comparison to the total serum proteins. Degradation, as determined by the increase in desTrp^1-DSIP, and binding both occur to a greater extent in plasma than in CSF and in vivo than in vitro. The binding protein appears to have a high specificity for DSIP, since much less D-Ala^3-DSIP was bound in vitro. Our antibody detects bound as well as free DSIP and almost all added DSIP can be recovered. The presence of specific reversible binding of DSIP in CSF and plasma with tight regulation of free DSIP levels should be taken into account when assessing the physiological roles of DSIP.

Passage of intact DSIP from the plasma to the CSF was demonstrated with the use of immunoreactive and radioactive DSIP. Immunoreactive levels in the CSF doubled after IV injection of DSIP or desTrp^1-DSIP and tripled after injection of D-Ala^3-DSIP. Column chromatography demonstrated increased amounts of intact peptide and a level of free DSIP in CSF essentially equal to that in plasma both before and after injection of DSIP. This suggests that free DSIP may be very important in crossing. A key role for free DSIP is further suggested by the observation that the percent of free DSIP in basal plasma (9%) is equivalent to the slope of the line (0.085=8.5%) relating basal CSF and plasma levels. Finally, the appearance in CSF of radioactivity that co-elutes with free ^{125}I-N-Tyr-DSIP shows that these observations are due to crossing rather than stimulation of endogenous levels.

Higher levels of free peptide as a result of less binding may explain why more D-Ala^3-DSIP and ^{125}I-N-Tyr-DSIP

than DSIP appear to enter the CSF, although their longer half lives or a selective transport may also be contributory. The order of increasing passage of the DSIP peptides tested, as assessed by their CSF/plasma ratios at 30 min, is in the same order of their half-time disappearance from blood. Although the longer half-time of D-Ala^3-DSIP may account for higher CSF levels after peripheral injection, it does not explain its earlier peak in CSF. The amount of peptide that crosses the BBB, therefore, appears to be a function of several factors, including unbound peptide, half-life, and structural conformation.

The mechanism by which DSIP enters the CSF was not investigated, but some indirect evidence raises the possibility that it might be relatively specific. If crossing were by a nonspecific mechanism such as pinocytosis, one would expect free and bound DSIP to cross equally well, with similar bound/free ratios in CSF and plasma. The different CSF/plasma ratios for DSIP, desTrp^1-DSIP, D-Ala^3-DSIP, and ^{125}I-N-Tyr-DSIP could reflect specificity, although factors such as plasma protein binding and half-life complicate this consideration. The lack of correlation between plasma levels and CSF levels after injection of immunoreactive DSIP is also compatible with a saturable transport mechanism, especially since similar analysis for ^{125}I-N-Tyr-DSIP, which involved injection of only 0.1% of the amount of peptide, showed a strong statistical correlation between plasma and CSF levels.

Inulin, a substance known to be poorly permeable to the BBB, had a CSF/plasma ratio at 30 min of 0.019, only slightly lower than that of DSIP (0.025) and desTrp^1-DSIP (0.020) but half that of ^{125}I-N-Tyr-DSIP (0.037); at 90 min, the ratio was 0.045, one third that of DSIP (0.145). The exact way in which inulin enters the CSF and brain tissue is unknown [10], but a nonspecific mechanism such as bulk transport has been postulated [11]. Our finding of reduction of the entry rate by cold inulin raises the possibility that a saturable mechanism may be involved. It appears, then, that DSIP and some of its analogues cross the BBB to a greater extent than does labeled inulin.

Although the exact mechanism by which injected DSIP enters the CSF from plasma remains unknown, even nonspecific penetration could have practical implications. Regardless, the basal levels of DSIP-like material in CSF and plasma are interrelated and some passage of intact DSIP does occur after peripheral administration.

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