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Permeability of Blood-Brain Barrier to DSIP Peptides

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KASTIN, A. J., C. NISSEN AND D. H. COY. *Permeability of blood-brain barrier to DSIP peptides.* PHARMAC. BIOCHEM. BEHAV. 15(6)955–959, 1981.—An improved radioimmunoassay (RIA) for delta sleep-inducing peptide (DSIP) was used to study the permeability of the blood-brain barrier (BBB) to DSIP peptides. Although the synthetic analog [D-Ala^3]-DSIP reacts fully in the assay, at least 8 of the 9 amino acids of the naturally occurring DSIP sequence are required for recognition by the antibody. A significant increase in DSIP-like immunoreactivity found in rat brain in one part of the study after peripheral injection of DSIP indicated, therefore, that some peptide crossed the BBB. The much higher levels found in brain tissue after intracarotid administration of [D-Ala^3]-DSIP as compared with DSIP made it unlikely that non-specific leakage of peptide across the BBB was the only explanation. Furthermore, the findings after infusion with saline for one minute tended to rule out contamination of brain tissue by peptide remaining in blood. Thus, the results provide additional evidence that one of the ways peptides administered peripherally can influence the brain is by transport across the BBB essentially intact.

Sleep peptide Blood-brain barrier Radioimmunoassay

THE initially controversial concept that peptides injected peripherally can act on the brain [6] is now widely accepted. How this action is exerted, however, still remains in contention. Recently, papers have appeared supporting [4,12] as well as not supporting [1, 10, 11] passage of intact peptides through the blood-brain barrier (BBB). Earlier studies in this area have been reviewed elsewhere [5,8] and recent evidence [14] supports the existence of additional mechanisms [6].

Most methods used to determine permeability of the BBB to peptides involve measurement of radioactivity or immunoreactivity. The difficulty inherent in these methods is the assumption that the labeled or immunoreactive compound being measured remains intact. Further procedures are required to determine if only a fragment or degradation product of the peptide is being assayed.

The generation of a very specific antibody [3] to the nonapeptide delta-sleep inducing peptide (DSIP) [15] circumvents the problem. This antibody is unusual in that it requires almost the entire molecule for cross-reactivity [4]. Detection of increased immunoreactivity in the brain after its peripheral administration, therefore, indicates that DSIP penetrates the BBB. Contamination of the sample of brain with blood containing exogenous DSIP, although unlikely [4], remains as a possible explanation of the results as does non-specific leakage of peptides across the BBB. In the present study, an improved radioimmunoassay (RIA) was used to help rule out these possibilities.

EXPERIMENT 1

METHOD

DSIP and the more stable [D-Ala^3]-DSIP were synthesized by solid-phase methods, and highly purified. The peptides were diluted immediately before use with 0.9% NaCl and injected into the carotid artery as described previously [4]. All statistical comparisons were made by analysis of variance followed by Duncan's Multiple Range Test.

Thirty male, Sprague-Dawley derived albino rats were purchased from Zivic Miller, Allison Park, PA. Half of the rats weighed about 100 g (99.4±2.6 g) and half about 275 g (275.9±7.2 g). At each range of weight, 5 rats were injected with 10 mg/kg DSIP, 5 rats with 200 μg DSIP/rat, and 5 rats with saline. Five sec after intracarotid injection, the rats were decapitated; the pituitary, pineal, hypothalamus, and all visible superficial blood vessels were carefully removed from each brain. All tissue was frozen. After being thawed, the brain was minced and divided into 2 portions. Part was extracted by the method described previously [3] and part by a revised method. For both extraction procedures, the assay was conducted in a tube containing 2 mg in 100 μl of buffer as the initial dilution (0.4 mg/100 μl final dilution) and 5 mg in 100 μl of buffer as the initial dilution (1 mg/100 μl final dilution). The design, involving samples from 5 rats in each group, is shown in Table 1.

In the revised method, tissue was put in polypropylene tubes containing cold 0.1 M acetic acid and 4% Trasylol. The tubes were placed in a cooled, multi-sample cup horn and sonicated (Heat Systems Ultrasonic, Plainview, NY) for 5 min. Sonication with added solution was repeated 2 more times, centrifuged at 2500 g for 30 min, and the supernatants were lyophilized. The previous method involved a single homogenization in 0.1 M acetic acid after heating and rapid cooling [3].

The remainder of the RIA for DSIP was the same as that used by us previously [3,4]. Although antiserum from a later

TABLE 1

COMPARISON OF VARIABLES AFFECTING MEASUREMENT OF DSIP-LIKE IMMUNOREACTIVITY (pg/mg) IN RAT BRAIN AFTER RAPID INTRACAROTID INJECTION OF DSIP OR NaCl

Extraction procedure:	Revised				Previous			
Body weight:	100 g		275 g		100 g		275 g	
Tissue concentration (mg/tube):	2	5	2	5	2	5	2	5
Injectate: 10 mg/kg DSIP	69.8	39.2	82.4	43.0	54.3	35.5	71.5	37.2
±SEM	9.4	1.1	7.3	2.2	5.4	1.5	8.8	1.0
0.2 mg/rat DSIP	31.4	23.2	30.7	25.9	27.8	15.2	18.5	14.4
±SEM	3.4	2.0	2.6	1.9	1.4	0.9	2.7	1.3
NaCl	23.6	19.4	27.9	23.3	20.6	12.7	20.9	11.9
±SEM	1.6	1.2	4.8	3.3	1.8	1.4	1.8	1.2

bleeding was used, the specificity was identical to the earlier one except that it was perhaps slightly less reactive with [des-Trp[1]]-DSIP (65% vs 73%). The dose-response curves of DSIP and [D-Ala[3]]-DSIP remained parallel. Recovery of DSIP added to brain tissue and then run through the revised extraction and RIA procedures was 92%. We also checked for cross-reactivity with up to 100 ng of the α-human globulin fraction IV-4 to which DSIP was conjugated; there was none. Since the variation between assays continued to be greater than that within an assay, all samples in each experiment were measured in the same assay.

RESULTS

This experiment examined several variables in the procedure in an attempt to improve the RIA. The results are summarized in Table 1. The revised extraction procedure, the lower (2 mg/tube) tissue concentration, and the higher dose (10 mg/kg) of injected DSIP all produced higher levels of DSIP-like immunoreactivity in the brain. These differences were supported by a significant effect of dose, $F(2,96)=195.19$, $p<0.001$, extraction technique, $F(1,96)=28.43$, $p<0.001$, and tissue concentration, $F(1,96)=91.82$, $p<0.001$. Separate analyses showed that the levels of immunoreactive peptide were significantly higher after injection of 10 mg/kg DSIP ($p<0.001$) or 200 μg/rat ($p<0.05$) than after NaCl; the dose of 10 mg/kg produced a greater effect ($p<0.001$) than that of 200 μg/rat. Although the higher level of peptide in the heavier (275 g) animals was not significantly different from that observed in 100 g animals, the differences produced by varying the dose of DSIP injected were more evident in the heavier animals. This tendency was supported by a significant weight by dose interaction, $F(2,96)=4.14$, $p<0.02$. The effect of dose was also more evident with the lower concentration of tissue, as shown by the significant interaction between dose and concentration, $F(2,96)=26.23$, $p<0.001$. In summary, as shown by Table 1, the highest concentrations of DSIP-like immunoreactivity occurred in brain samples of the 275 g rats injected with 10 mg/kg and extracted at 2 mg/tube by the revised method.

EXPERIMENT 2

METHOD

This experiment compared penetration of the BBB by [D-Ala[3]]-DSIP and DSIP in 30 male, Sprague-Dawley derived albino rats obtained from 2 suppliers (Blue Spruce and Zivic Miller). The animals weighed 79.8±1.4 g at the time of the experiment; the low weight was chosen on the basis of preliminary experiments and Experiment 1 to reduce possible passage across the BBB of a minimal dose of DSIP fixed by body weight and to exaggerate the differences from [D-Ala[3]]-DSIP. The 5 rats in each group were decapitated 5 sec after injection of 10 mg/kg DSIP, 10 mg/kg [D-Ala[3]]-DSIP, or an equal volume (0.1 ml) of 0.9% NaCl. Since aliquots were taken after sonication of the brain, the relative amounts of each part were consistent.

TABLE 2

PENETRATION OF BLOOD-BRAIN BARRIER BY [D-Ala[3]]-DSIP AS COMPARED WITH DSIP AND NaCl IN 2 STRAINS OF 80 g RATS (pg/mg ± SEM DSIP-LIKE IMMUNOREACTIVITY)

	[D-Ala[3]]-DSIP	DSIP	NaCl
Blue Spruce	72.0 ± 18.7	27.5 ± 4.4	23.4 ± 3.8
Zivic Miller	58.3 ± 12.1	22.8 ± 3.6	20.9 ± 4.7

RESULTS

Analysis of variance revealed a highly significant effect of peptide, $F(2,28)=9.12$, $p<0.001$, but not supplier. There was no reliable interaction between these two factors. Duncan's Multiple Range Test showed significantly ($p<0.05$) higher levels of DSIP-like immunoreactivity in brain after intracarotid injection of [D-Ala[3]]-DSIP than after 0.9% NaCl or DSIP (Table 2). The difference in values after injection of DSIP and saline was not statistically significant.

EXPERIMENT 3

METHOD

Rats weighing about 100 g were obtained from Blue Spruce, Altamont, NY and divided into 7 groups, each consisting of about 6 rats. Three groups were decapitated at 5 sec and 4 groups at 65 sec after the rapid injection of peptide (10 mg/kg) or diluent (0.9% NaCl). Three groups receiving DSIP, [D-Ala[3]]-DSIP, and 0.9% NaCl were decapitated at 5

TABLE 3

EFFECT OF WASHOUT ON DSIP-LIKE IMMUNOREACTIVITY (MEAN pg/mg ± SEM) IN RAT BRAIN AFTER THE RAPID INTRACAROTID INJECTION OF [D-Ala^3]-DSIP

Injectate	5 sec	65 sec	Washout (65 sec)
[D-Ala^3]-DSIP	126.1 ± 17.0	33.9 ± 6.1	60.5 ± 9.5
NaCl	19.0 ± 1.6	16.0 ± 1.4	15.7 ± 1.4

sec to compare the permeability of the BBB to these substances. Two groups of rats were decapitated 65 sec after injection of a bolus of [D-Ala^3]-DSIP or NaCl; these served as additional controls for the 2 groups being washed out (Table 3).

Rats to be washed out were additionally prepared at the time the carotid was cannulated by exposure of both jugular veins. A 15 gauge needle was placed in the left jugular vein, immobilized with tape, and connected by a closed valve to a reservoir of 0.9% NaCl situated about 1 meter above the rat. Five sec after injection of [D-Ala^3]-DSIP or diluent, the IV flow of at least 14 ml of saline was begun into the left jugular vein while a 15 gauge needle was rapidly inserted into the right jugular to collect the venous outflow. The effluent collected between 50 and 60 sec after the start of the washout with saline (10 sec before decapitation) was saved for determination of the relative volume of red cells to fluid. Cessation of respiration and heart beat appeared to occur a little more than halfway through the 1 min infusion.

RESULTS

In this experiment (Table 3), results obtained from rats injected with [D-Ala^3]-DSIP or NaCl 5 sec before decapitation and 65 sec (with and without washout) earlier were compared by analysis of variance. A significant main effect was found for the injected substance, $F(1,28)=19.67$, $p<0.001$. Duncan's Multiple Range Test revealed that the levels of DSIP-like immunoreactivity in the brains of rats injected with [D-Ala^3]-DSIP and killed 5 sec later were significantly ($p<0.001$) higher than in the brains of any of the 3 groups of rats injected with 0.9% NaCl (5 sec, 65 sec, 65 sec with washout) or in the 2 other groups of rats injected with the same peptide but decapitated 65 sec later (with and without washout). In a separate analysis, rats receiving DSIP 5 sec before decapitation had significantly ($p<0.001$) less DSIP-like immunoreactivity than rats injected with the same dose of [D-Ala^3]-DSIP, and there was no significant difference from the saline controls. The levels of DSIP-like immunoreactivity among the groups injected only with saline were not significantly different.

Significantly ($p<0.05$) higher immunoreactive levels of peptide were found in rats infused with 0.9% NaCl immediately after injection of [D-Ala^3]-DSIP and decapitated 65 sec later than in those decapitated at the same time but not infused. The increased immunoreactivity in the brain of rats receiving [D-Ala^3]-DSIP 65 sec before decapitation was not significantly different from that in the brains of rats receiving saline. Rats injected with the same peptide and killed after the 1 min infusion of saline, however, showed significantly ($p<0.01$) higher levels of DSIP-like immunoreactivity than those in each of the groups of rats receiving saline.

The ratio of blood cells to fluid ("hematocrit") in the venous effluent obtained from rats during the last 10 sec of washout was 0.047 for rats receiving [D-Ala^3]-DSIP and 0.046 for rats receiving 0.9% NaCl. The mean (±SEM) weights of the brains of the rats receiving the DSIP analog and infused for 1 min with saline was 1.645±0.034 g as compared with 1.661±0.028 g for rats receiving the same peptide and decapitated at the same time (65 sec) but not infused with saline.

GENERAL DISCUSSION

On the basis of preliminary experiments, several improvements made in the method for measuring DSIP in tissue by RIA were verified in the present study. The revised procedure resulted in significantly greater extraction of DSIP from brain as well as reduced time for processing as compared with the previous method (Table 1). By either process, relatively more DSIP was extracted with a concentration of 2 mg of tissue in a tube than from 5 mg/tube. This reflected the gradual flattening recently detected in the slope of the dose-response curve for brain tissue at concentrations greater than 2.5 mg/tube; up to this level, the curve remains strictly parallel to that of the DSIP standard.

Preliminary experiments also indicated that penetration of the BBB by DSIP was not consistent in rats receiving the same dose (200 μg) used previously [4] regardless of body weight, even after a 72 hr fast. This was confirmed in Experiment 1 where the effect of 200 μg/rat was very small although it was statistically significant. Injection of a dose 15–20 times greater (10 mg/kg) resulted in significantly higher levels in brain than injection of 200 μg/rat (Table 1). Yet even this higher dose did not always cause increased levels in the brain (Table 2), perhaps due to undetermined experimental variables.

Passage of DSIP across the BBB seemed less in smaller rats. At 10 mg/kg in a pilot study, 2.4 times higher levels were found after injection of [D-Ala^3]-DSIP into 2 rats weighing 500 g as compared with the levels in 2 rats weighing 100 g, both groups being substantially higher than the 2 controls. Administered as a function of weight, larger rats obviously receive more peptide. Whether there is a crucial, though variable, total amount of peptide necessary for rapid penetration of the BBB isn't known, but such a threshold could help explain some of the results with DSIP in small rats. It might even contribute to the variability frequently seen in other experiments involving peripheral administration of peptides.

The levels of DSIP-like immunoreactivity after injection of [D-Ala^3]-DSIP in the pilot study just mentioned were higher than we had seen after injection of DSIP itself. The 6 rats used in that preliminary experiment had been obtained from Blue Spruce. It could not be determined whether the high levels were due to the altered structure of the DSIP peptide, the type of rat used, or chance alone. Experiment 2 showed that the results could be fully accounted for by [D-Ala^3]-DSIP (Table 2).

The increased amount of DSIP-like immunoreactivity consistently found in brain after peripheral administration of [D-Ala^3]-DSIP (Tables 2 and 3) represents essentially intact peptide. Small substitutions in the middle of the DSIP nonapeptide do not alter immunoreactivity, but no fragments of DSIP can be detected by our antibody with the exception of the 2–9 segment that shows about 65% cross-reactivity [4].

Even if DSIP were completely degraded in the rat to [des-Trp^1]-DSIP within 5 sec after injection, an unlikely occurrence, the results still demonstrate a large part (8 amino acids) of DSIP to be present in brain.

In both experiments in which [D-Ala^3]-DSIP and DSIP were injected peripherally, significantly higher levels of DSIP-like immunoreactivity were found in brain after administration of the analog than after the parent compound. This implies a differential penetration of the BBB. It is also possible that both peptides entered the brain to the same extent but that DSIP was removed from brain at a faster rate than the DSIP analog. Interactions could occur in both entry and removal from brain. Regardless, these results would clearly not be expected if the increased levels of DSIP-like immunoreactivity observed after peripheral injection were due only to non-specific leakage of peptide.

Although the brain was rinsed after dissection, it was possible that some of the injected peptide was present in blood trapped in the tissue being assayed. Increased immunoreactivity, therefore, might reflect the exogenous peptide remaining outside the BBB in the vascular lumen. The results of this study provide 2 pieces of evidence that this did not occur.

First, the levels of immunoreactivity were several times higher after injection of the analog than after injection of the parent compound (Tables 2 and 3). Since equal amounts of each peptide were injected, equal amounts should have remained in the blood. It is conceivable, of course, that [D-Ala^3]-DSIP binds better to blood proteins or to the walls of blood vessels than does DSIP; differential binding to vessel endothelium could also affect the next piece of evidence as well as penetration of the BBB in general.

Second, the levels of immunoreactive DSIP in the brain of rats injected with peptide were still significantly higher than those in the saline controls after removal of the blood by infusion of large amounts of saline (Table 3). The 14 ml of saline used in each approximately 100 g rat was sufficient to dilute the red cells to less than 5% of the volume of the effluent. The use of rats initially injected with NaCl instead of peptide before the washout ruled out the additional possibility that the procedure itself changed endogenous peptide levels in the brain.

It was expected that the levels of DSIP-like material would be significantly higher after the 1 min washout in rats receiving [D-Ala^3]-DSIP than in those receiving NaCl, but it remains puzzling that they were higher than the levels found in rats killed at the same time after receiving the same amount of the same peptide but not washed out (Table 3). This could be explained by maximal extraction of peptide by tissue within the first few passages of blood through the brain followed by active removal of peptide from brain tissue or the surrounding extracellular fluid (ECF). The active removal would be expected to be greater in the rats kept alive for 65 sec than in those killed during that time by the infusion. Passive diffusion with differential binding could also be involved. It is also possible that the infused saline could have expanded the ECF surrounding the brain so that the diffusion gradient for the diluted peptide was smaller; such a process was not detectable at a gross level since the brain weights of the infused group were the same as the group not washed out after the DSIP analog.

The evidence for DSIP exerting CNS effects has been reviewed elsewhere together with the suggestion of the possibility that sleep may not be the main function of the nonapeptide [7]. Whatever the role of a DSIP peptide, our results with the quickly decreasing levels between 5 and 65 sec (Table 3) suggest that its continued presence in the brain in the intact form probably is not essential for its actions. The central actions of many other peptides persist much longer than their presence in blood [6]. It is unlikely that the peripherally injected DSIP peptides acted only by stimulating endogenous DSIP [4], and 5 sec seems too short a time for transmission of a signal to the brain that could affect synthesis.

Substitution of D-alanine for L-glycine as the third amino acid of the DSIP decapeptide apparently resulted in a compound better able to penetrate the BBB. It might, therefore, exert greater effects on the brain. Since glycine and alanine are the simplest monoamino, monocarboxylic neutral amino acids, it is unlikely that the presence of an additional carbon atom in alanine is sufficient by itself to explain fully the better entry of the analog into the brain.

Although [D-Ala^3]-DSIP is more resistant than DSIP to enzymatic degradation [9], not much degradation would have occurred during the 5 sec after arterial injection. Differential degradation, however, could have occurred during extraction and assay, but preliminary results showed that recovery of immunoreactivity after addition of [D-Ala^3]-DSIP to brain tissue was not greater than that found after addition of DSIP. One of the main cleavage points by brain enzymes occurs at the N-terminal, releasing Trp [9]. Degradation by enzymes extracted from the brain is not necessarily the same as that occurring in the functioning tissue or at another site like the capillary endothelium, but our antibody cross-reacts well enough with [des-Trp^1]-DSIP (DSIP 2–9) that it should be detected in the RIA. Nevertheless, the spatial configuration or lipophilicity of [D-Ala^3]-DSIP is probably more important for its increased transport across the BBB as compared with the parent compound.

A very large increase in DSIP-like immunoreactivity was found after administration of DSIP and its analog in the few pineals examined. The accumulation of intact peptides like MIF-1 (Pro-Leu-Gly-NH_2) has been reported in the highly vascular pineal gland since 1973 [13] and a related but novel peptide was recently found there [2]. Increased immunoreactive levels were also found in the pituitary and hypothalamus after injection of the DSIP peptides. These parts, like the pineal, were not included in the brain tissue assayed for DSIP-like immunoreactivity, but several of the circumventricular organs, through which penetration of the BBB may occur, were included.

The results clearly demonstrate that intracarotid injection of [D-Ala^3]-DSIP causes an increase in DSIP-like immunoreactivity in rat brain. The increase is greater than that found after similar injection of the same dose of the parent DSIP and cannot be fully explained by trapping of blood in the extracted tissue or by non-specific leakage of peptide. Although this indicates that small amounts of a peptide can penetrate the BBB in essentially intact form, it remains to be determined what part this mechanism plays in mediating the CNS effects of peripherally administered peptides, particularly DSIP.

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