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Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) Can Cross the Vascular Component of the Blood-Testis Barrier in the Mouse

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ABSTRACT: Pituitary adenylate cyclase activating polypeptide (PACAP), present in highest concentrations in the hypothalamus and testes, affects the release of LH, FSH, and prolactin, as well as Sertoli cell function. We examined the ability of the 38-amino acid form of PACAP labeled with ^{125}I (I-P38) to cross the vascular component of the blood–testis barrier. The unidirectional influx constant (K_i) was 4.23 × 10^{-3} ml/g-minute, which is about 5 times faster than the entry of LH and about 17 times faster than that of serum albumin. Entry occurred in part by a saturable transport system, with 20 μg/mouse of unlabeled P38 inhibiting transport by 40%. An analog of peptide T, which like PACAP is related to vasoactive intestinal polypeptide and has been found to have its own saturable transport system into the brain, did not alter the uptake of I-P38 by the testes. A dose of 10 μg/mouse, but not of 20 μg/mouse, was associated with a contraction of the vascular space of the testes. HPLC confirmed that a small but persistent percentage of the radioactivity recovered from the testes represented intact I-P38. These results suggest that circulating P38 may contribute to the testicular pool of PACAP, which may play an active role in the function of the testes.

Key words: Influx constants, transport, peptide T, vasoactive intestinal polypeptide (VIP).

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Pituitary adenylate cyclase activating polypeptide (PACAP) is a newly discovered member of the vasoactive intestinal polypeptide (VIP) family. It occurs primarily in two forms composed of 27 and 38 (P38) amino acid residues. It has been found in numerous tissues and exerts various effects on brain, gastrointestinal, and respiratory function (Arimura, 1992). However, its highest concentrations occur in the hypothalamus and testes (Arimura et al, 1991), and the cDNA coding P38 has been cloned from the human testis λgt11 cDNA library (Kimura et al, 1990). Several studies have linked PACAP to aspects of reproductive function. For example, it releases prolactin and LH from the pituitary (Miyata et al, 1989) and has a synergistic effect with LH-RH on the release of LH and FSH (Culler and Paschall, 1991). PACAP has binding sites in the testis, epididymis, and seminal vesicles (Schivers et al, 1991) and stimulates cAMP accumulation and secretion in Sertoli cells (Heindel et al, 1992).

Although PACAP is probably synthesized by the testes (Arimura et al, 1991), it is not known whether PACAP circulating in the blood can affect testicular function. To do so, PACAP would have to cross the blood–testis barrier (BTB). The vascular part of the barrier is comprised of the capillary endothelial cells. This retards the entry into the testes of albumin and other circulating substances (Setchell and Sharpe, 1981; Setchell et al, 1988). In this study, we determined whether P38 could cross the vascular component of the BTB.

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Methods

Measurement of Blood-to-Testis Influx

Adult male ICR mice (Charles River Labs Inc., Wilmington, MA) weighing about 25 g and anesthetized with intraperitoneal (i.p.) urethane (2 g/kg of body weight) received an injection into the jugular vein of 0.2 ml of lactated Ringer's solution containing 1% bovine serum albumin (BSA), 2 × 10^6 cpm of ^{125}I-PACAP38 (I-P38), and 2 × 10^5 cpm of ^{131}I-albumin (I-Alb). Some mice also had 10 μg or 20 μg of unlabeled PACAP38 or 20 μg of D-Ala^1-peptide T-amide included in their injections. Arterial blood from the carotid artery was collected 1, 1.5, 2, 2.5, 3, 4, and 5 minutes after the intravenous (i.v.) injection. Immediately after the collection of arterial blood, the mice were decapitated and the testes removed, freed of the major external blood vessels, the epididymis, and the efferent ducts, and weighed to the nearest mg. Serum and the testes were then counted in a gamma counter, and the unidirectional influx constant (K_i in ml/g-minute) from the blood into the testes was determined by the multiple time regression analysis method of Patlak et al (1983) as applied to the testicular influx of cytokines (Banks and Kastin, 1992). Brief-

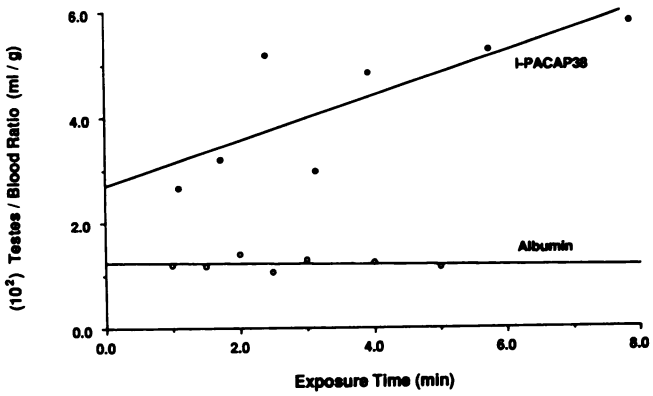

FIG. 1. Testis/blood ratios for I-P38 and I-Alb over time. The slope measures K_i in ml/g-minute. There was a statistically significant correlation between testis/blood ratios and exposure time for I-P38, but not for I-Alb.

ly, this was determined by plotting the testis/blood ratio (in ml/g) against exposure time (Exp t) defined as:

$$\text{Exp t} = \int_0^t \text{Cp}[(\tau)\, d\tau]/\text{Cpt} \qquad (1)$$

where t is time and Cp is the concentration of radioactivity in blood.

The percentage of the i.v. injection entering each gram of testis (%Ct) was determined with the equation (Banks and Kastin, 1992):

$$\%\text{Ct} = 100(\text{Rp} - \text{Ra})\text{Sp}/\text{I} \qquad (2)$$

where Rp and Ra are the testis/serum ratios for I-P38 and I-Alb, respectively, Sp is the cpm/ml of serum for I-P38, and I is the total cpm of I-P38 injected i.v.

Radioactive Labeling

P38 was synthesized with solid-phase methodology, labeled by the lactoperoxidase method with ^{125}I, and purified with a Vydac C18 column on reverse-phase HPLC (Tatsuno et al, 1990). Specific activity was 450–550 μCi/μg. BSA was labeled with ^{131}I by exposure to chloramine-T for 60 seconds. The I-Alb was separated from free I on a column of Sephadex G-15.

HPLC

To determine whether the ^{125}I entering the testes was still attached to P38, radioactivity was extracted from the testes and analyzed by HPLC. Testes harvested 2, 5, or 10 minutes after the i.v. injection of I-P38 were placed in 5 ml of 30% trifluoroacetic acid (TFA) and, 10 minutes later, homogenized with a polytron (Brinkmann Instruments, Westbury, NY). The mixture was centrifuged at 4°C for 10 minutes at 5,000 × *g* and the supernatant lyophilized. Later, the material was reconstituted in 90% water with 10% acetonitrile, 0.1% TFA, and 0.1% Triton X-100. The radioactivity was then eluted HPLC with a gradient that progressed from 90% solution A (0.1% TFA in water) to 100% solution B (0.1% TFA in acetonitrile) over 70 minutes. To determine the amount of degradation resulting from the processing, testes removed from mice that had not received an i.v. injection were mixed with TFA and I-P38 and processed as above. Results were corrected for degradation that occurred during processing.

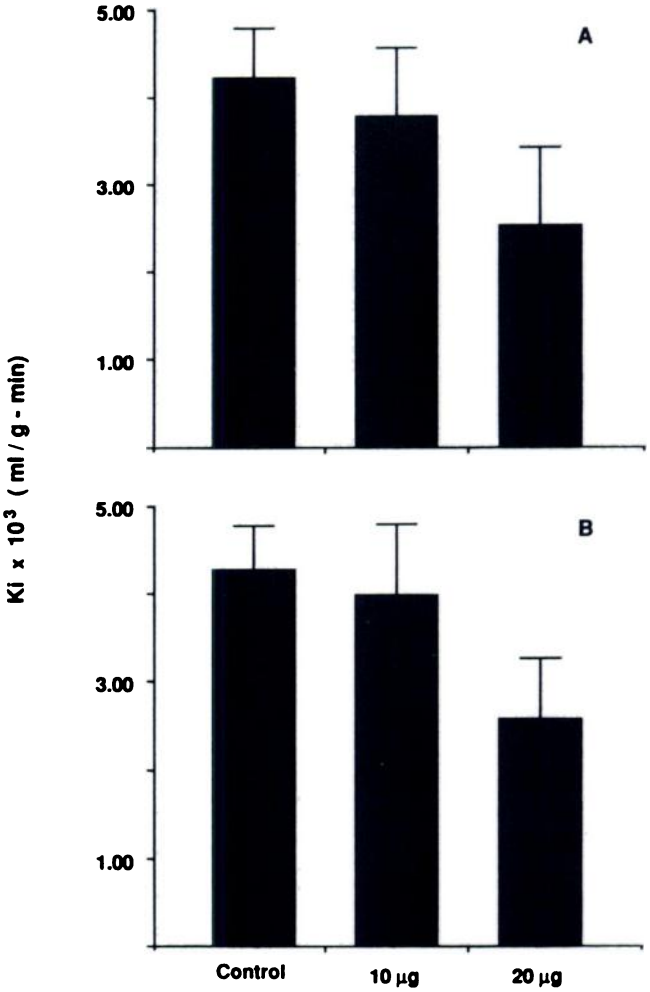

FIG. 2. Effect of unlabeled P38 (10 or 20 μg/mouse) on the K_i of I-P38. **(A)** Not corrected for vascular space of testes. **(B)** Corrected for vascular space. Unlabeled P38 produced statistically significant inhibition in the K_i for I-P38. The mean of six/group is shown.

Statistics

Means were compared by analysis of variance (ANOVA), which was followed by Duncan's multiple range test. Regression lines were computed by the least-squares method and compared for statistical differences with the BMDP P1R program (BMDP Statistical Software, Los Angeles, CA).

Results

Influx of I-P38 into Testes

A statistically significant entry into the testes could be measured for I-P38: $r = 0.787$, $n = 7$, $P < 0.05$ (Fig. 1). The K_i was determined to be $(4.23 \pm 1.5) \times 10^{-3}$ ml/g-minute. At 5 minutes, 0.034% of the i.v. injection had entered each gram of testis. Unlabeled P38 significantly

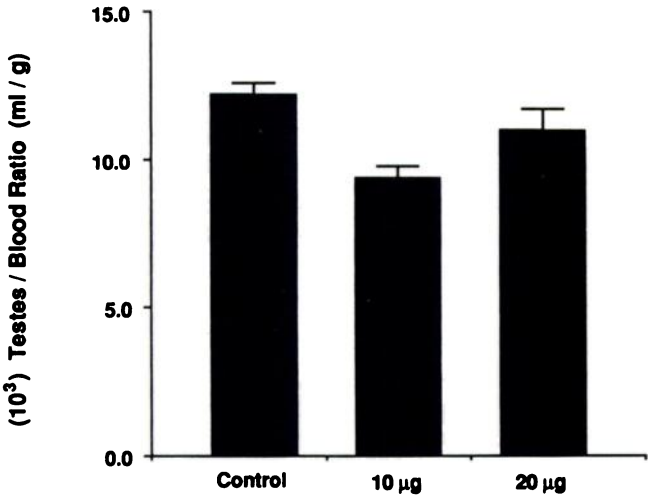

FIG. 3. Effect of unlabeled P38 (10 or 20 µg/mouse) on vascular space of testes. The 10-, but not the 20-, µg/mouse dose caused statistically significant contraction in vascular space of testes. The mean of six/group is shown.

inhibited entry: $F(4,13) = 3.42$, $P < 0.05$, with 10 µg/mouse inhibiting influx by 11% and 20 µg inhibiting by 40% (Fig. 2A). Similar findings were obtained when the results were corrected for the vascular space of the testes as measured with I-Alb (Fig. 2B). The peptide T analog had no effect on the influx of I-P38.

No significant entry of I-Alb into the testes occurred during this time period, so the testis/blood ratios were measures of the vascular space of the testes. ANOVA showed a statistically significant effect of P38 $F(2,16) = 6.72$; $P < 0.01$, with the multiple range test showing that the low ($P < 0.005$), but not the high, dose of P38 decreased the vascular space of the testes (Fig. 3).

In the processing controls, 75% of the radioactivity eluted as intact I-P38. After correction, between 4.9 and 20.5% of the radioactivity recovered from the testes eluted as intact I-P38 (Table 1).

Table 1. *Percent of radioactivity recovered from testes eluting as intact I-P38*

Time (minutes)	Percent eluting as	
	I-P38	Peptide fragments
2	14.6	0.0
5	4.9	8.8
10	20.5	33.0

Discussion

The results show that I-P38 enters the testes by a saturable transport system. The uptake of I-P38 exceeds that due to the vascular space of the testes, indicating that I-P38 can cross the vascular component of the BTB. This means that circulating P38 is available to the components of the basal compartment of the testes, which include the Leydig cells, the spermatogonia, and the basal aspect of the Sertoli cells.

The unidirectional rate of influx is relatively high in comparison with that of other substances also found to cross the mouse BTB. The K_i of I-P38 is about 2 times greater than that of human interleukin (IL)-1α, about 5 times greater than that of LH, about 16 times greater than that of FSH, and about 17 times greater than that of albumin (Banks and Kastin, 1992, 1993). This relatively high rate of entry is due at least in part to a saturable, carrier-mediated component to passage. Based on the doses tested, about 25 µg/mouse would be required to inhibit passage by 50%. By contrast, the peptide T analog had no effect on entry, although it is even more closely related to PACAP than VIP and has been found to cross the blood–brain barrier by a saturable system (Barrera et al, 1987).

Albumin did not show a statistically significant degree of entry into the testes over the short period of time measured here. Therefore, its testis/blood ratio reflects the vascular space of the testes. The low, but not the high, dose of P38 produced a statistically significant contraction in the vascular space of the testes. This biphasic effect is consistent with the known actions of PACAP on other vascular beds. PACAP produces vasodilation through a direct effect and vasoconstriction through the release of catecholamines from the adrenal, with the dominant effect being dose and time dependent (Minkes et al, 1992a,b). The contraction of the vascular space, however, did not have a significant effect on the K_i, as can be seen by a comparison of panels A and B in Figure 2.

Although I-P38 was largely degraded after entry into the testes, a small but consistent level of radioactivity eluted in the position of intact I-P38. Much of the degraded material may not have been produced in the testis but may have entered from the circulation. P38 is rapidly degraded in the circulation with only 6.9% being intact 10 minutes after i.v. injection (unpublished data), compared with 20.5% in the testis. Therefore, the testis, compared with serum, may represent a relatively protected area for P38 with regard to enzymatic degradation. This suggests that circulating P38 is able to enter the testes and contribute to the intratesticular pool of P38, which is thought to play an active role in the functions of the testes.

Two functions that P38 would be likely to affect are production of testosterone and spermatogenesis. Other substances shown to cross the BTB, such as IL-1α (Banks and Kastin, 1992), can regulate gonadal steroid secretion (Rivier and Vale, 1989). The presence of PACAP Type I receptors on germ cells, especially spermatozoa, suggests a role in spermatogenesis (Arimura, 1992). In addition to acting at its own receptors, P38 might also potentiate the actions of other hormones upon the testes, just as it po-

tentiates the actions of LH-RH on LH release at the pituitary (Culler and Paschall, 1991).

The ability of substances such as P38, LH, and IL-1α to cross the BTB suggests that the BTB may function as a regulatory as well as a restrictive membrane. A similar role has recently been suggested for the blood–brain barrier (Banks and Kastin, 1990), which also has transport systems for LH, P38, and IL-1α (Banks et al, 1991; Banks and Kastin, 1993). Taken together, these findings are consistent with previous evidence indicating that P38 and other PACAP-like peptides exert an effect on testicular function.

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