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Research Paper

Characteristics of Substance P Transport Across the Blood–Brain Barrier

Arvind K. Chappa,[1] Kenneth L. Audus,[1] and Susan M. Lunte[1,2]

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Purpose. Substance P (SP; NH_3^+-Arg$^+$-Pro-Lys$^+$-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) belongs to a group of neurokinins that are widely distributed in the central nervous system and peripheral nervous system. The biological effects mediated by SP in the central nervous system include regulation of affective behavior, emesis, and nociception. Many of these actions are believed to be the result of the binding of SP to the neurokinin-1 (NK-1) receptor and subsequent transport across the blood–brain barrier (BBB). The objective of the study was to investigate the involvement of the NK-1 receptor in the permeation of SP across the BBB.
Methods. Transport of ^{3}H SP (1–13 nM) was investigated using BBMEC monolayers grown on polycarbonate membranes mounted on a Side-bi-Side™ diffusion apparatus. ^{3}H SP samples were analyzed by scintillation spectrometry. Liquid chromatography-tandem mass spectrometry was used to monitor the transport at higher concentrations (micromolar).
Results. SP transport across BBMEC monolayers was found to be saturable (K_m = 8.57 ± 1.59 nM, V_{max} = 0.017 ± 0.005 pmol min^{-1} mg^{-1} protein) in the concentration range of 0–13 nM. Significant ($p < 0.05$) decline in ^{3}H SP permeation was observed in the presence of unlabeled SP and at 4°C, indicating that the transport process is carrier-mediated. High-performance liquid chromatography analysis showed no significant metabolism of ^{3}H SP in either the donor or receiver chambers. ^{3}H SP transport was inhibited by 2–11 SP ($p < 0.05$) but not by any other fragments, indicating that both the C- and N-terminal regions are essential for molecular recognition by the receptor. Endocytic inhibitors (chloroquine, phenylarsine oxide, monensin, and brefeldin) did not inhibit SP transport, suggesting the involvement of a nonendocytic mechanism in SP permeation. Pro^9 SP, a high-affinity substrate for the NK-1 major subtype receptor, significantly ($p < 0.05$) inhibited the transport of SP. However, Sar^{9}Met(O$_2$)^{11} SP, a high-affinity substrate for the NK-1 minor subtype receptor, septide, and neurokinin A, inhibitors of NK-1 and neurokinin-2 (NK-2) receptors, respectively, did not produce any inhibition of SP transport. Western blot analysis confirmed the presence of the NK-1 receptor in BBMEC monolayers.
Conclusions. The above results provide functional and molecular evidence for the existence of a carrier-mediated mechanism in the transport of SP across the BBB. The effects of specific inhibitors and the results of Western blot analyses demonstrate the involvement of the NK-1 receptor in the transport of SP across the BBB.

KEY WORDS: blood–brain barrier; neurokinin receptor; substance P.

INTRODUCTION

Substance P (SP) belongs to the class of small peptides called tachykinins that are widely distributed in the central nervous system (CNS) and peripheral nervous system. To date, three mammalian tachykinins [SP, neurokinin A (NK-A), and neurokinin B (NK-B)] have been identified. The prepro tachykinin gene *PPT-A* encodes for SP and NK-A whereas, the *PPT-B* gene encodes for NK-B. SP is the natural ligand for the tachykinin receptor neurokinin-1 (NK-1) (1).

Since the discovery of SP in the early 1930s, its pharmacological actions have been extensively studied. SP has been identified as a neurotransmitter and neuromodulator in the CNS (1). It has also been shown to be involved in the generation or progression of various physiological and pathophysiological conditions such as pain and depression and a variety of neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's, and schizophrenia (2–7). Changes in SP concentrations and *PPT-A* gene expression have been observed in brains of patients suffering from these neurodegenerative diseases. SP has been shown to improve memory and counter age-related performance deficits in animals (8,9). It works in concert with several hormones and other endogenous substances such as lutenizing hormone, angiotensin II, and serotonin, thus modulating their actions (10–13). Many of these effects require the

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66047, USA.
[2] To whom correspondence should be addressed. (e-mail: slunte@ku.edu)

selective passage of SP across the blood–brain barrier (BBB) following its release in the periphery.

The BBB serves as an obstacle to the movement of molecules between the blood and the CNS. The BBB is made of a complex vasculature lined by endothelial cells and supported by astrocytic end-feet and extracellular matrix. The capillary endothelial lining of the BBB consists of tightly adjoined cells with no intercellular junctions. These tight junctions limit the passive diffusion of many hydrophilic compounds. Specialized transport mechanisms such as receptor- and adsorptive-mediated endocytosis exist at this interface for the permeation of large molecular weight compounds such as peptides and proteins (14). Along with synaptic transmission, these specialized transport systems serve as another mode of communication between the CNS and the periphery (15,16). The long-term effects exhibited by blood-borne peptides and their metabolites in the CNS have been shown to be the result of their selective passage across the BBB (15,16).

SP serves as a model compound for the study of the transport of tachykinins and other neuropeptides across the BBB. Modulation of the permeation of neuropeptides across biological barriers and their subsequent receptor binding is an attractive approach for the treatment of depression and anxiety disorders (17,18). Also, a better understanding of the mechanisms of transport of SP and other blood-borne peptides will provide information that can be used in the rational design of peptidergic drugs and neuropeptide conjugates for the treatment of neurodegenerative disorders.

Recently, cytotoxic proteins conjugated to SP (SP–saporin, SP–diphtheria toxin, and SP–pseudomonas exotoxin) have been shown to selectively destroy nociceptive neurons expressing NK-1 receptors (19–21). These results demonstrate the exciting potential of delivering large molecular weight protein drugs selectively to their target sites by conjugating them to peptides such as SP. An effective application of this strategy to brain drug delivery requires a better understanding of the factors and mechanisms involved in the permeation of SP across the BBB. Based on the physicochemical properties of SP (high molecular weight, hydrophilic nature, and presence of ionic sites in its structure) and the above evidence for its role in the CNS, we hypothesize the existence of a carrier-mediated mechanism for the transport of SP across the BBB. Because SP is a natural ligand for the NK-1 receptor and NK-1 receptors are widely expressed in neuronal cells and their adjacent tissues, this paper investigated the role of the NK-1 receptor in SP permeation across the BBB.

MATERIALS AND METHODS

Materials

^{3}H SP was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA); SP 1–11, NK-A, and other SP fragments were obtained from Bachem (King of Prussia, PA, USA). Sar^{9}Met(O$_2$)^{11} SP was purchased from Sigma (St. Louis, MO, USA). Minimum essential medium (MEM) and Ham's-F12 solutions were from Invitrogen (Carlsbad, CA, USA). All other cell culture materials were obtained from Sigma.

Methods

Cell Culture

BBMEC cells were isolated as described by Audus *et al.* (22). Cells were seeded at a density of 50,000 cells/cm^2 on a sterile petri dish containing collagen- and fibronectin-coated polycarbonate membranes (Nuclepore Track-etch, PC MB, 13 mm, Fisher Scientific, St. Louis, MO, USA) using plating medium (50% Ham's F-12 nutrient mixture, 50% MEM), 10 mM HEPES, 13 mM sodium bicarbonate, penicillin G (100 μg/mL), streptomycin (100 μg/mL), and 10% platelet-poor horse serum. Medium was changed every 48 h using changing medium (plating medium + endothelial cell growth factor and heparin) until the cells attained confluency (10–14 days).

Transport Studies

Transport studies were performed using polycarbonate membranes mounted on silanized Side-bi-Side™ diffusion cells (Crown Glass Co., Somerfield, NJ, USA). Transport of SP (^{3}H-labeled or unlabeled) in the blood-brain direction was conducted in a buffer made of 50% Ham's F-12 and 50% MEM adjusted to pH 7.4. The donor and the receiver chambers were sampled at various time intervals for a period of 2 h after adding either unlabeled or ^{3}H SP at time 0. Unlabeled SP samples were then spiked with a known concentration of the internal standard (Tyr^{8} SP) and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The samples were monitored for the appearance of parent peptide as well as metabolites. ^{3}H SP samples were analyzed by scintillation counting. Total cell protein content was determined by Pierce BCA protein assay kit (Fisher Scientific) as per the manufacturer's protocol.

For studying the effects of competitive (Pro^{9} SP, septide, NK-A, and Sar^{9}Met(O$_2$)^{11} SP) and endocytic inhibitors (phenylarsine oxide, chloroquine, monensin, and brefeldin), cells were exposed to these compounds for 30 min before the addition of SP.

LC-MS/MS

Optimal separation was achieved on a Vydac Microbore C-18 MS column (5 μm, 1 mm ID × 15 mm OD) at a flow rate

Table I. Permeation of Various Substance P Fragments Across BBMEC Monolayers

SP Fragment (1 μM)	Permeation (cm/s) × 10^5
1–11	5.57 + 1.48
3–11	1.92 + 0.42
5–11	2.49 + 0.49
6–11	3.62 + 0.20
1–9*	0.53 + 0.04
^{14}C sucrose	3.82 + 0.47

*$p < 0.05$ and 10 μM.

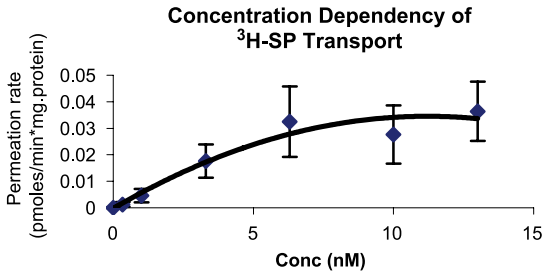

Fig. 1. Concentration dependency of substance P (SP) permeation across BBMEC monolayers. Transport of ^{3}H SP was studied at increasing amounts of unlabeled SP for 2 h using Side-bi-Side™ diffusion chambers. Permeability was determined from the slope of the plot of cumulative amount transported *vs.* time. The apparent Michaelis-Menten parameters (V_{max} and K_m) were estimated using Sigma Plot software as described under "Materials and methods." Data points are mean ± SD ($n \geq 3$).

of 0.2 mL/min using a gradient of 0–25% acetonitrile in 0.1% formic acid. MS/MS was performed in positive electrospray ionization (ESI+) mode on a Micromass QuattroMicro™ instrument coupled with a Waters 2690 solvent delivery system. Samples were analyzed by diluting with an equal volume of mobile phase A (0.1% formic acid) containing internal standard (Tyr^{8} SP).

Western Blot

Cell lysate (25 μg of protein) was separated on 4–20% Tris–glycine gel (Invitrogen) at 110 V for 2 h and then transferred onto a nitrocellulose membrane at 25 V for 2 h on ice. After blocking overnight with Tris-buffered saline (TBS) + 5% milk, the membrane was treated with primary NK-1 receptor antibody (EMD Biosciences, La Jolla, CA, USA) at 1:1000 dilution for 1 h. The membrane was then washed with TBS–Tween (TBST) + 1.5% bovine serum albumin (BSA) for 1 h and incubated with a secondary antibody, goat anti-rabbit IgG conjugated with peroxidase (Jackson Immuno Research Labs, West Grove, PA, USA), at 1:5000 dilution for 1 h. After washing the membrane with TBST + 1.5 % milk and TBST for 1 h to remove secondary antibody, the bound immunoreactive proteins were detected with an ECL system (Amersham-Pharmacia Biotec, Buckinghamshire, UK).

Data Analysis

The permeability of SP was calculated using the following equation:

$$P_{app} = (\Delta Q/\Delta t)/A \times C_o$$

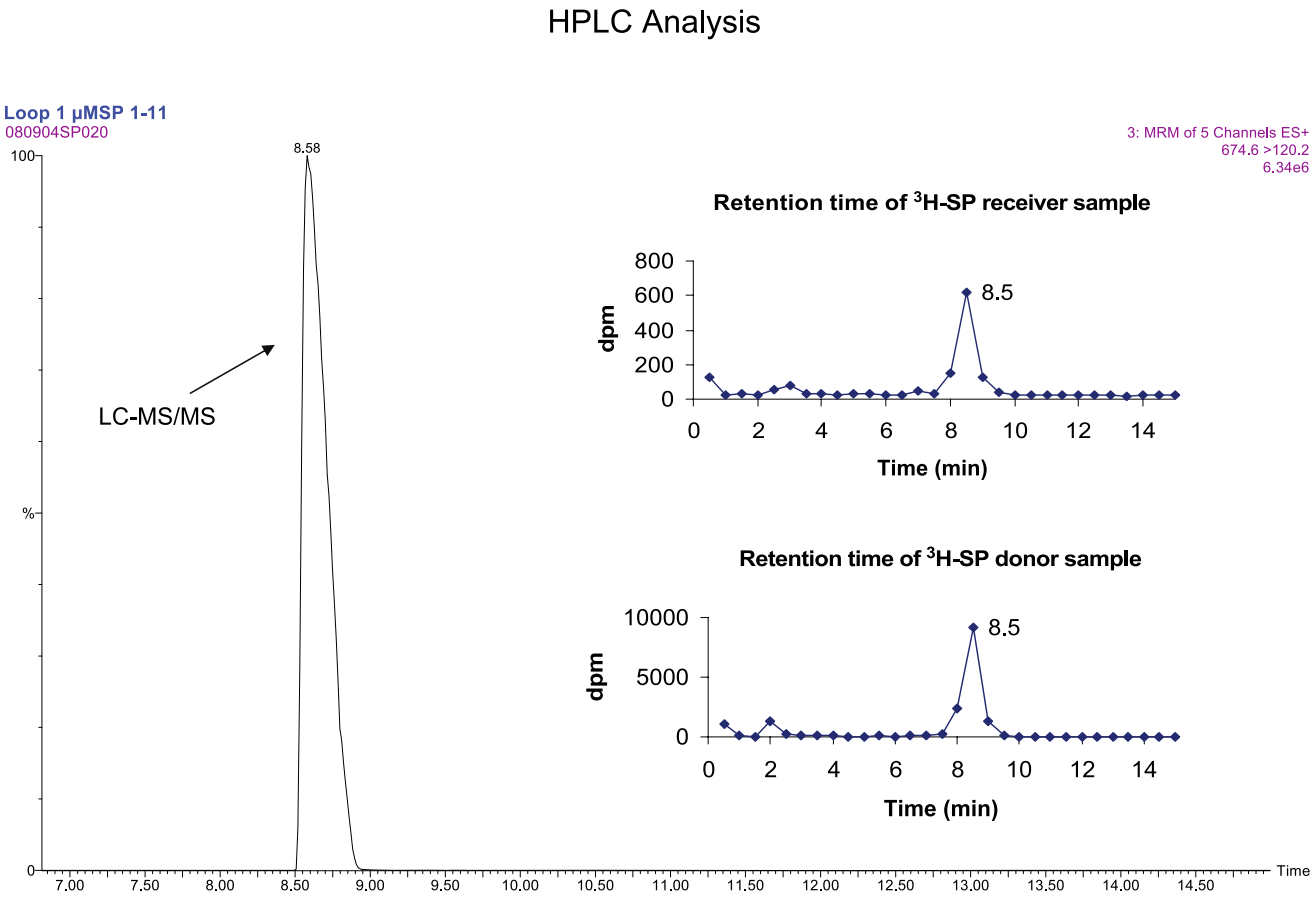

Fig. 2. High-performance liquid chromatography analysis of the retention times of unlabeled SP and ^{3}H SP from the donor and receiver chambers. Unlabeled SP was analyzed by liquid chromatography-tandem mass spectrometry as described in "Materials and methods." ^{3}H SP samples were separated under chromatographic conditions similar to those for unlabeled SP except that the column eluents were collected at 30-s intervals for 15 min and analyzed by scintillation spectrometry. The data were plotted as dpm *vs.* time.

where $\Delta Q/\Delta t$ is the linear appearance rate of the SP in the receiver chamber, A is the cross-sectional area (0.636 cm^2), and C_o is the initial concentration of the test compound in the donor chamber at $t = 0$. The values obtained for the saturable permeation of SP were fitted to the Michaelis-Menten equation by nonlinear regression using Sigma Plot software (SPSS Inc., Chicago, IL, USA):

$$V = V_{max} * C/(K_m + C)$$

where V is the permeation rate (in picomoles per minute per milligram of protein), C is the concentration (nanomolar), V_{max} is the saturable permeation rate, and K_m is the SP concentration at half-saturation. The data are reported as mean ± SD. The criteria for model selection included the coefficient of determination (r^2), the standard error of parameter estimates, and visual inspection of the residuals.

RESULTS

Concentration Dependency of SP Transport

Transport studies (Table I) employing unlabeled SP and its metabolites at high concentrations (micromolar) displayed a marked decrease (10-fold) in the permeation of the SP 1–9 fragment compared with the fragments with intact C terminus. The permeation of the paracellular marker, ^{14}C sucrose, was in agreement with the values reported previously from our laboratories (23).

SP permeation of BBMEC monolayers was saturable with increasing concentrations (2–13 nM) of SP in the donor chamber (Fig. 1). The data when plotted and fitted by nonlinear regression displayed a typical Michaelis-Menten curve with the kinetic parameters V_{max} = 0.017 ± 0.005 pmol min^{-1} mg^{-1} protein and K_m = 8.57 ± 1.59 nM.

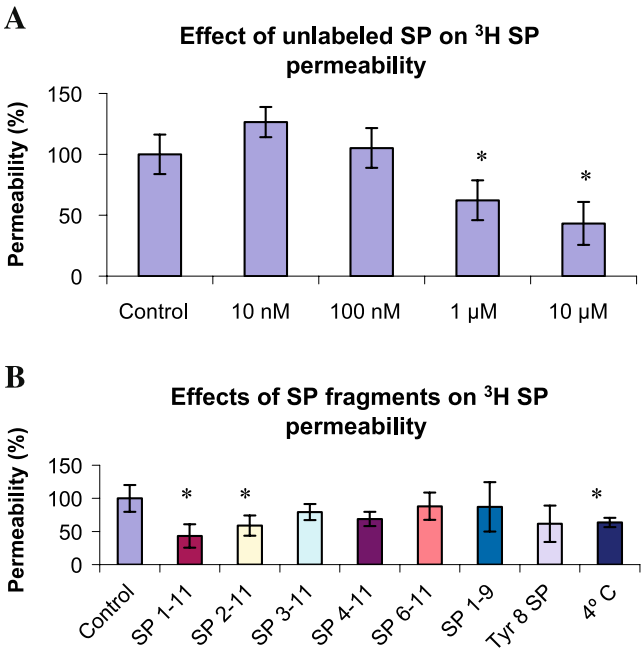

Fig. 3. (A, B) Effects of unlabeled SP, SP fragments, and temperature (4°C) on the permeation of ^3H SP. The cells were incubated with unlabeled SP or SP fragments for 30 min before the addition of ^3H SP. For determining the effect of temperature, the buffer surrounding the cell monolayers was maintained at 4°C throughout the experiment. Each column represents the mean ± SD ($n \geq 3$). *$p < 0.05$.

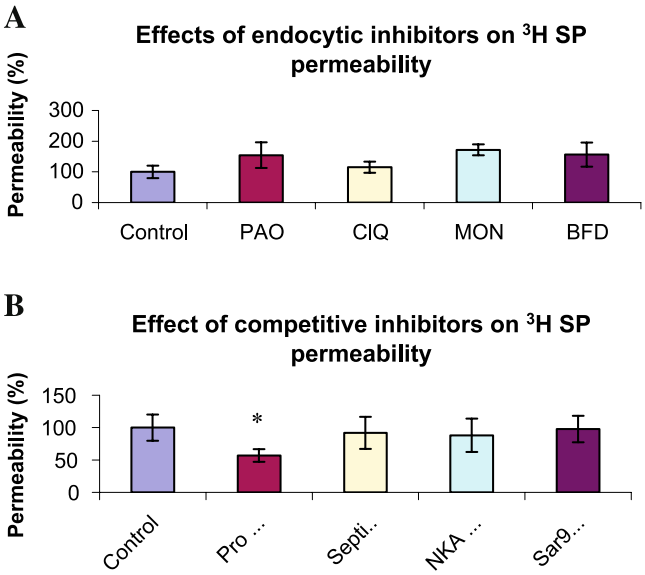

Fig. 4. (A) Effect of endocytic inhibitors on the permeation of ^3H SP across BBMEC monolayers. PAO = phenylarsine oxide, CLQ = chloroquine, MON = monensin, and BFD = brefeldin. (B) Effect of competitive inhibitors on the permeation of ^3H SP across BBMEC monolayers. The cells were exposed to endocytic or competitive inhibitors for 30 min before the addition of ^3H SP. Each column represents the mean ± SD ($n \geq 3$). *$p < 0.05$. Pro^9 SP = neurokinin-1 major receptor substrate, septide = NK-1 receptor substrate, neurokinin-2 = NK-2 receptor substrate, and $Sar^9Met(O)_2^{11}$ SP = neurokinin-1 minor receptor substrate.

Retention Time Analysis of ^3H SP

High-performance liquid chromatography (HPLC) analysis performed on ^3H SP samples from both the receiver and donor chambers at the end of the experiment displayed a single peak accounting for more than 90% of the radioactive counts (Fig. 2). The retention time of this radiolabeled compound (8.5 min) matched that of the unlabeled compound (8.5 min), indicating little or no metabolism of SP under the experimental conditions employed.

Effect of Temperature, Unlabeled SP, and SP Fragments on the Permeation of ^3H SP

SP transport across BBMEC monolayers decreased significantly ($p < 0.05$) at 4°C compared with that at 37°C (Fig. 3B), signifying the involvement of a carrier-mediated mechanism in its permeation. Higher concentrations (micromolar) of unlabeled SP significantly ($p < 0.05$) inhibited the permeation of ^3H SP in a dose-dependent fashion. Maximum inhibition (more than 60%) of the permeation of ^3H SP was observed at a concentration of 10 µM of unlabeled compound. The extent of the drop in ^3H SP permeability in the presence of 10 µM SP is similar to that obtained at 4°C, indicating the maximum inhibition of the carrier. Thus, studies involving the determination of the effect of various

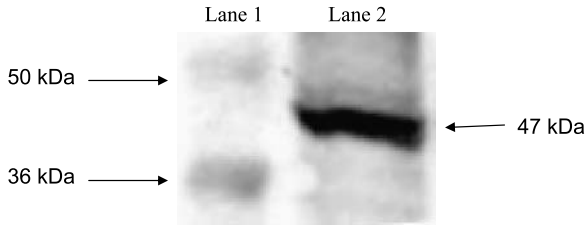

Fig. 5. Western blot analysis for the expression of the NK-1 receptor in BBMEC monolayers. Lane 1 represents the molecular weight marker, and lane 2, the cell lysate.

SP fragments on the permeation of SP were performed at this concentration (Fig. 3A).

The effect of different SP fragments on the permeation of ^{3}H SP was investigated to determine the minimum SP sequence essential for receptor recognition and concomitant transport. Of the various SP fragments analyzed, only the 2–11 SP fragment produced significant ($p < 0.05$) inhibition of SP permeation (Fig. 3B).

Effect of Endocytic and Competitive Inhibitors on the Permeation of SP

Various endocytic inhibitors such as phenylarsine oxide, chloroquine, monensin, and brefeldin were investigated for their effect on the permeation of SP through BBMEC monolayers. No significant inhibition was observed in the permeation of SP in the presence of endocytic inhibitors (Fig 4A); this suggested the involvement of a nonendocytic transport mechanism.

The permeation of SP through BBMEC monolayers was further investigated in the presence of various high-affinity ligands of the tachykinin receptors (Fig 4B). Septide, a substrate for the NK-1 receptor; NK-A, a substrate for the NK-2 receptor; Pro^9 SP, a substrate for the NK-1 major (NK-1M) type receptor; and Sar^{9}Met(O$_2$)^{11}, a substrate for the NK-1 minor (NK-1m) type receptor were chosen to determine their effect on SP permeation across BBMEC monolayers. Of the above inhibitors studied, significant inhibition ($p < 0.05$) in SP permeation was observed only in the presence of Pro^9 SP. SP permeation decreased by more than 50% in the presence of Pro^9 SP.

Western Blot Analysis of the NK-1 Receptor

BBMEC cell lysate was probed by Western blot analysis with the NK-1 receptor antibody to determine the expression of the NK-1 receptor. Figure 5 demonstrates the expression of the NK-1 receptor in BBMEC cells. A dense clear band was observed in the region between 50 and 36 kDa in agreement with the reported molecular weight (47 kDa) for the NK-1 receptor (24).

DISCUSSION

SP and its analogs show promise as therapeutic agents for a variety of conditions such as pain, cancer, and CNS diseases and, more recently, as vehicles for delivery of potent drug substances (19–21,25–29). Also, understanding the mechanism of transport of SP across the BBB is essential for regulating its activity in the brain and the design of drug candidates for depression and anxiety disorders (17,18). SP conjugates have been successfully utilized for drug targeting to tumors and nociceptive neurons (30,31). The specificity of targeting in these cases can be attributed to the presence of high-density NK-1 receptors. Studies by Mantyh *et al.* (31) have demonstrated that chronic pain-producing spinal neurons can be selectively destroyed *in vivo* by using an SP–toxin (SP–saporin) conjugate. This significant observation generates great interest in the utilization of SP and its analogs as vectors for the delivery of highly potent drugs to their target sites in brain.

The present study focuses on identifying the characteristics of SP transport across the BBB. Results from an earlier report from our laboratory provided indirect evidence for the involvement of a carrier-mediated mechanism in the transport of SP across the BBB (32). To further determine the structural requirements of the peptide for its permeation of the BBB, transport studies were performed with unlabeled SP fragments. Interestingly, results from these studies showed a marked drop in the permeation of SP 1–9 fragment in comparison to the parent peptide. Taking into consideration the requirement of C-terminal residues of SP in binding to its receptor (1), the above outcome strongly suggests a possible carrier-mediated mechanism operating in the permeation of SP across BBMEC monolayers. Thus, further studies were conducted using ^{3}H SP to characterize the transport of SP at physiologically relevant concentrations (nanomolar).

Permeation of ^{3}H SP across BBMEC monolayers at physiological concentrations was determined to be saturable with a K_m value of 8.57 ± 1.59 nM. This K_m value is in good agreement with the reported affinity values for the SP receptor (NK-1 receptor) in various tissues (33–35). The permeation of ^{3}H SP across BBMEC monolayers as an intact molecule was confirmed by HPLC analysis with MS and radiochemical detection. The observed dose-dependent inhibition of ^{3}H SP in the presence of increasing concentrations of unlabeled SP indicates that both the labeled and the unlabeled compounds compete for the same carrier during their transport across BBMEC monolayers. Further, the significant drop in SP permeation at 4°C supports the involvement of a carrier-mediated mechanism. Evidence for the lack of paracellular transport in the permeation of SP across BBMEC comes from a comparison of permeation rates of different SP fragments (Table I). Although paracellular transport cannot completely be ruled out in a model system like this, it seems to be negligible for SP permeation. If paracellular pathway were to be the determinant factor in the permeation of SP, the permeation rates of smaller fragments of SP should be greater than the parent peptide. However, this work shows quite the opposite, permeation of SP decreases with reduction in chain length and molecular size, indicating the sequence requirement for carrier recognition. Further, permeation changes in SP were independent of any changes in the permeation of sucrose (paracellular marker).

The importance of the C-terminal residues in the selective receptor binding of tachykinins is well documented (1,36). In the present study, we investigated the minimum

sequence of SP that is essential for receptor recognition. Of the various fragments of SP that were studied for their potential to inhibit SP permeation across the BBB, only the SP 2–11 fragment produced significant ($p < 0.05$) inhibition. This result indicates that, in addition to the C-terminal residues, the N-terminal residues of SP are essential for adopting the necessary conformation of the peptide in binding to its receptor.

Endocytic processes are widely known to be involved in the permeation of high molecular weight compounds such as peptides and proteins across endothelial barriers such as the BBB (37). Whereas adsorptive-mediated endocytosis involves the attachment of cationic compounds to the negatively charged cell surface components and subsequent formation of cell membrane vesicles, receptor-mediated endocytosis is associated with the internalization of the receptor protein along with its bound ligand into the membrane vesicles. The internalized vesicles then undergo lysis in the cytoplasm and release their contents. The released receptors are recycled back to the cell surface. Receptor-mediated endocytosis differs from adsorptive-mediated endocytosis in its higher affinity, higher specificity, and lower capacity for the substrates. Also, multiple ligands for the same receptor can competitively inhibit the internalization of one another (38).

To elucidate the involvement of endocytic processes in the permeation of SP across the BBB, various endocytic inhibitors (phenylarsine oxide, chloroquine, monensin, and brefeldin A) were investigated for their effect on SP transport. These inhibitors are known to effectively impair the various steps in the endocytic pathway. Phenylarsine oxide inhibits receptor internalization, monensin blocks receptor recycling, chloroquine inhibits lysosomal breakdown, and brefeldin A causes uncoating of membrane tubules that fail to transport molecules (39–42). SP permeation was found to be unaffected by the presence of any of these endocytic inhibitors. Also, the absence of a lag time (data not shown) in the permeation of SP across BBMEC suggests the involvement of a possible nonendocytic mechanism operating in the transport of SP. The lack of effect of endocytic inhibitors on SP permeation across BBB at lower (nanomolar) concentrations is in contrast to our earlier studies with SP at higher (micromolar) concentrations (32). A possible explanation for this discrepancy is that at saturating concentrations of NK-1 receptor, SP could still be transported via an adsorptive-mediated endocytic pathway. Interestingly, at higher SP concentrations, a lag time was observed in its permeation across BBMEC, suggestive of adsorptive-mediated endocytosis.

In addition to the NK-1 receptor, SP is also known to exhibit an affinity for other neurokinin receptors, such as the NK-2 receptor, although it is much lower (16). Also, the NK-1 receptor itself is reported to possess at least two distinct binding sites for SP, NK-1M and NK-1m, which bind SP with differing affinities. The NK-1M binding site is shown to be the most abundant of the NK-1 receptor binding sites and has an affinity in the nanomolar range for SP, whereas the NK-1m binding site is less abundant and its affinity for SP ranges from picomolar to sub nanomolar. Further, these multiple binding sites have been proposed to correspond to different conformations of the NK-1 receptor that exhibit different functional properties. The NK-1M conformer has been shown to be temperature dependent and resistant to the effects of endocytic inhibitors, whereas the internalization of the NK-1m conformer is temperature independent (43–45).

Further studies were thus designed to elucidate the receptor involved in the permeation of SP by exploiting the differences in the functional and affinity properties of tachykinin receptors (specifically NK-1). Various ligands were identified as competitive substrates for SP at the neurokinin receptors. Pro^9 SP is a known substrate for the NK-1M receptor, whereas $Sar^9Met(O_2)^{11}$ SP, NK-A, and septide are preferred substrates for NK-1m, NK-2, and NK-1 receptors, respectively (16,44,45). Of the various competitive substrates studied, significant inhibition of SP permeation was observed only with Pro^9 SP, indicating the involvement of the NK-1M receptor. Also, the lack of inhibitory effect of septide on SP permeation suggests that this SP binding site on the NK-1 receptor is distinct from the septide-sensitive binding site (46,47). Further, the K_m value obtained in our present study agrees well with the affinity values reported for the NK-1M binding site. In addition to the above results, the temperature dependency of SP permeation and the lack of effect of endocytic inhibitors support the conclusion that SP permeation across the BBB is mediated via the NK-1M conformation of the receptor (44). The expression of the NK-1 receptor in BBMEC was confirmed by Western blot analysis.

In summary, the present study constitutes the first report of functional as well as molecular evidence for the involvement of a carrier-mediated mechanism in the permeation of SP across the BBB. Results obtained from this study demonstrate that the carrier involved in SP permeation across the BBB is NK-1. This newly identified carrier could serve as a target for drug delivery of SP conjugates to the brain.

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