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An *Alpha*$_1$-Adrenergic Receptor-Mediated Phosphatidylinositol Effect in Canine Cerebral Microvessels

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SUMMARY

In microvessels isolated from canine cerebral cortex, $^{32}P_i$ is incorporated into phospholipids when incubated in physiological buffer containing [$^{32}P_i$]orthophosphate. Norepinephrine (NE) selectively increases $^{32}P_i$ incorporation into phosphatidylinositol (PI) and phosphatidic acid (PA) 60–200% over control levels. Half-maximal stimulation of PI labeling is observed with 1 μM NE, whereas maximal stimulation occurs at approximately 100 μM. *Alpha*$_1$-adrenergic agonists, phenylephrine and methoxamine, mimic the effects of NE, whereas isoproterenol, a *beta*-adrenergic receptor agonist, is ineffective. A wide variety of other agents tested had no specific effect on $^{32}P_i$ incorporation into PI or PA. Prazosin, a selective *alpha*$_1$-receptor antagonist, at a concentration of 0.05 μM inhibits 50% of the stimulation due to NE (100 μM), whereas 1 μM yohimbine, an *alpha*$_2$-selective antagonist, is required to achieve the same effect. These results demonstrate the existence of an *alpha*$_1$-adrenergic receptor-mediated PI effect in isolated canine cerebral microvessels.

INTRODUCTION

Increased $^{32}P_i$ labeling of PI[2] and PA in response to external chemical stimuli has been demonstrated in a variety of tissues and cell types. This phenomenon, although not completely understood, has been suggested to be an intermediary link between certain cell surface receptors and their physiological responses. Although all intermediate events in the receptor-response coupling pathway have not yet been characterized, several reports have suggested that receptor-stimulated PI metabolism may be related to prostaglandin synthesis, calcium mobilization, and/or protein secretion (1–3).

The association of adrenergic neurons with the cerebral microvasculature is well documented. Anatomical evidence indicates that axonal projections are in direct contact with capillary endothelial cell basal lamina and form synapse-like structures (4–6). Various neurotransmitters have been identified in these neuronal projections to the cerebral capillaries, including NE, serotonin, vasoactive intestinal polypeptide, and substance P. Changes in blood flow and capillary surface area and/or permeability have also been demonstrated in response to electrical or pharmacological stimulation of the locus ceruleus (7, 8). Cerebral metabolism is dependent on local regional blood flow and on microvascular permeability for providing nutrient supplies adequate to meet the metabolic demands of brain cells. The regulation and coupling of cerebral blood flow and capillary permeability to cerebral metabolic activity have been the focus of several lines of investigation in recent years.

Short segments of the cerebral microvasculature consisting predominantly of capillaries have been isolated, and the receptor binding specificity for a variety of neurotransmitters and drugs has been examined (9–11). The presence of adrenergic and histaminergic receptors has been demonstrated by these studies. The function of these receptors is unknown, but they may be involved in eliciting changes in vessel caliber or endothelial membrane permeability.

This paper reports the presence of an *alpha*-adrenergic receptor-stimulated incorporation of $^{32}P_i$ into PI and PA in microvessels isolated from dog brain. Characteristics of this response were further examined using various pharmacological agents, and this effect appears to be mediated through *alpha*$_1$-adrenergic receptors.

EXPERIMENTAL PROCEDURES

Materials. Nylon screening was obtained from Tetko (Rolling Meadows, Il.). Glass beads (Glasperlen) were from Sargent-Welch (Skokie, Il.). Precoated Silica Gel 60 thin-layer chromatography plates were purchased from Merck (Darmstadt, Germany). Silica gel HL plates were from Analtech (Newark, Dl.). Film (X-Omat R) for autoradiography was obtained from Eastman Kodak (Rochester N. Y.). Prazosin was a gift from Pfizer (New York, N. Y.). Methoxamine was a gift from Burroughs Wellcome (Research Triangle Park, N. C.). [$^{32}P_i$]

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[2] The abbreviations used are: PI, phosphatidylinositol; PA, phosphatidic acid; NE, norepinephrine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Orthophosphoric acid (carrier-free) was from New England Nuclear Corporation (Boston, Mass.). NE, epinephrine, dopamine, isoproterenol, yohimbine, phospholipid standards, and all other reagents were purchased from Sigma Chemical Company (St. Louis, Mo.). All solvents were high-pressure liquid chromatography grade.

Microvessel isolation. Microvessels were isolated by a modification of a procedure previously reported (12). A healthy adult mongrel dog (14–22 kg) was anesthetized with halothane, and the brain vasculature was flushed via carotid artery cannulas with cold phosphate-buffered saline (pH 7.4) to remove erythrocytes and other blood components. The brain was removed and placed in cold Hepes buffer solution containing 25 mM Hepes, 120 mM NaCl, 1.4 mM $CaCl_2$, 5.2 mM KCl, 1.4 mM $MgSO_4$, 10 mM glucose, and 1.2 mM KH_2PO_4, adjusted to pH 7.4. Pial membrane and blood vessels were removed with forceps, and the cortical gray matter was dissected with scissors and placed in a beaker containing 200 ml of the Hepes buffer solution. Razor blades were used to mince the gray matter into small pieces. The tissue was homogenized with a Tekmar Tissumizer at 5000 rpm for 25 sec, and the homogenate was passed through a 110-μm nylon screen. The filtrate was then centrifuged at 1,000 × *g* for 10 min. The supernatant fluid was discarded, and the pellet was resuspended in two volumes of 20% dextran (average molecular weight 60,400) and centrifuged at 2,500 × *g* for 10 min in a swinging bucket rotor. The pellets were combined and resuspended in cold buffer solution, then layered on a 2 × 1.6 cm diameter column of glass beads (0.25–0.30 mm diameter) and washed with an additional 200 ml of cold buffer. The microvessels were trapped on the glass beads and collected by gentle agitation in buffer solution. Approximately 50 mg of microvessel protein were obtained from 40–50 g of cortical gray matter.

Capillary incubation. The purified microvessel preparation was suspended in medium containing 25 mM Hepes (pH 7.4), 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgCl_2$, 0.75 mM $CaCl_2$, 1.6 mM cytidine, 1.6 mM myoinositol, and 10 mM glucose. Aliquots of the microvessel preparation were added to tubes containing [$^{32}P_i$]K_2HPO_4 (10–15 μCi), along with the agents to be tested and medium to bring the final incubation volume to 0.25 ml. Incubations were begun by the addition of the microvessels and were continued for 30 min at 37° in a shaking water bath. Incubation was stopped by the addition of 2.0 ml of $CHCl_3$/CH_3OH/HCl (20:40:1), and the tubes were placed on ice.

Phospholipid extraction. Phospholipids were extracted by a modification of the method of Bligh and Dyer (13). After the addition of the $CHCl_3$/CH_3OH/HCl to stop the reaction, the tubes were covered and allowed to stand for 30 min. Chloroform (0.8 ml) and water (0.7 ml) were then added, and the tubes were vortexed and centrifuged for 10 min at 1,000 × *g* at 4°. The aqueous phase was removed, and the chloroform phase was washed twice with 2.0 ml of 0.1 N HCl by centrifugation for 10 min at 1,000 × *g* at 4°. A 1.0-ml aliquot of the chloroform phase was dried under nitrogen and stored overnight at −20°.

Thin-layer chromatography and autoradiography. The dried lipid extracts were dissolved in 20 μl of $CHCl_3$/CH_3OH (2:1), and 10-μl aliquots were spotted on Silica Gel 60 plates (20 × 20 cm, 0.2 mm thick). The phospholipids were usually separated by the two-solvent system described by Schacht and Agranoff (14). In some experiments the identity of the phospholipids was confirmed by 2-dimensional chromatography using silica gel HL plates (15). Radioactive phospholipids were detected by autoradiography. The $^{32}P_i$-labeled phospholipids were scraped from the plate, and the radioactivity was determined by liquid scintillation detection. The phospholipid content was estimated by phosphate analysis as described by Bartlett (16).

RESULTS

Microvessels. The microvessel preparations consisted almost exclusively of capillary segments (150–200 μm in length) as viewed by phase-contrast microscopy (Fig. 1). Vessels with diameters larger than capillaries, either arterioles or venules, were observed as minor contaminants.

Incorporation of $^{32}P_i$ into phospholipids. Incubation of isolated canine brain microvessels in the presence of $^{32}P_i$-labeled orthophosphate resulted in the rapid incorporation of $^{32}P_i$ into phospholipids. $^{32}P_i$ incorporation into total phospholipids (counts per minute per microgram of phospholipid) was linear with time for at least 90 min. PI-4-phosphate, PI-4,5-bis-phosphate, PI, phosphatidylcholine, and PA accounted for nearly all $^{32}P_i$ labeling (Table 1). Labeling of phosphatidylserine, phosphatidylethanolamine, and sphingomyelin was negligible. Following incubation with $^{32}P_i$ (60 μCi/ml) for 30 min, the specific radioactivities of PI and PA were 14,500 ± 2,200 cpm/μg P_i and 40,600 ± 6,100 cpm/μg P_i, respectively. These results are the average ± standard error of the mean of three separate experiments.

Effects of various agents on $^{32}P_i$ labeling of phospholipids. A wide variety of agents was tested to determine their effects on $^{32}P_i$ labeling of microvessel phospholipids. Prostaglandins E_1, E_2, and A_1 (0.01–1 μM), as well as lysine vasopressin (0.1 IU), carbachol (1–100 μM), histamine (10 μM), adenosine (10 μM), indomethacin (10–100 μM), and serotonin (10 μM) had no effect on $^{32}P_i$ labeling of phospholipids.

Alpha-adrenergic receptor agonists (NE, phenylephrine, methoxamine) stimulated $^{32}P_i$ labeling of PI and PA. Dopamine also stimulated PI and PA labeling, presumably because of its *alpha*-agonist activity. Labeling of phospholipids other than PI and PA were not affected by NE (Table 2) or the other *alpha*-adrenergic agonists. NE was found to be more effective than either phenylephrine or methoxamine at stimulating PI and PA labeling at concentrations of 100 μM. There were no detectable changes in the absolute amounts of PI or PA in response to 100 μM NE as determined by phosphate analysis. The dose-response curves for stimulation of $^{32}P_i$ incorporation into PI and PA by NE were sigmoidal in shape (Fig. 2). Half-maximal stimulation of labeling occurred at approximately 1 μM for PI and 8 μM for PA. Maximal stimulation of PI and PA labeling occurred after 30 min of incubation in the presence of 100 μM NE. In repeated experiments with microvessel preparations, maximal stimulation averaged 120% above control values. In every case, an increase in labeling was observed; however, the values ranged from 60% to 200%. Stimulation of PA labeling was usually 15–25% less than that of PI. Labeling of phospholipids continued for up to 120 min, but there was no significant increase in the level of stimulation after 30 min. The concentration of NE in the incubation medium after 30 min was examined by high-pressure liquid chromatography and electrochemical detection of catecholamines. No significant change in NE was detected in the concentrations used in this study.

Determination of adrenergic receptor type. To characterize the type of receptor mediating the PI effect in microvessels, the influence of a number of adrenergic receptor agonists and antagonists on $^{32}P_i$ labeling was examined.

Isoproterenol had no stimulatory effect on $^{32}P_i$ labeling of PI or PA, nor did propanolol block the stimulation by NE, thus indicating that NE does not mediate the effect on PI through *beta*-adrenergic receptors. Propanolol alone increased labeling of PI and PA; however, this was most likely due to inhibition of PA-phosphohydrolase

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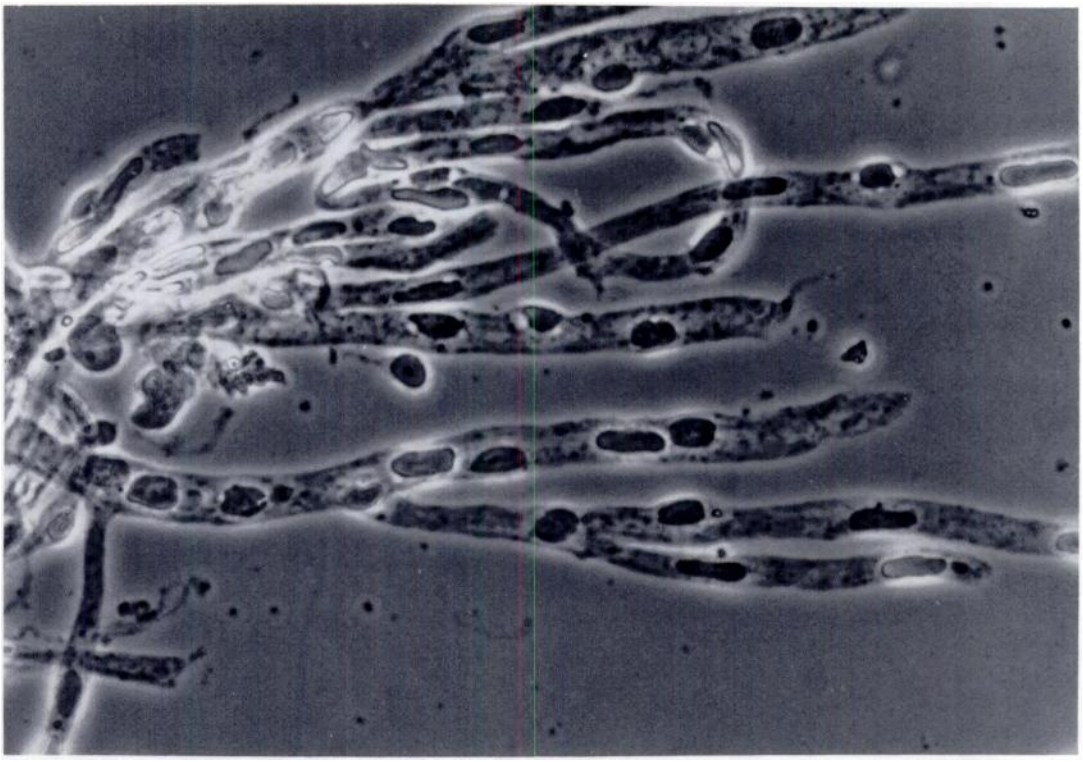

FIG. 1. *Phase-contrast micrograph of microvessel segments from canine cerebral cortex*
Microvessels were isolated by the procedure outlined under Experimental Procedures. The preparations consisted virtually entirely of capillaries and were essentially free of nonvascular material (×1280).

(17). Dibutyryl cyclic AMP (10 μM) had no effect on phospholipid labeling, further suggesting that NE is not acting through the adenylate cyclase-linked receptors reportedly present on cerebral microvessels (9–11).

The *alpha*$_1$-adrenergic receptor-selective agonists, methoxamine and phenylephrine, but not clonidine (*alpha*$_2$-agonist), selectively increased $^{32}P_i$ labeling of PI and PA (Table 3). The selective adrenergic antagonists, prazosin (*alpha*$_1$) and yohimbine (*alpha*$_2$), were found to block the stimulatory effect of 100 μM NE. However, prazosin was much more effective at blocking NE stimulation than was yohimbine. Prazosin at a concentration of 0.05 μM inhibited 50% of the NE-stimulated $^{32}P_i$ labeling of PI, whereas 1 μM yohimbine was required to achieve the same degree of inhibition (Fig. 3). Neither prazosin nor yohimbine alone had an effect on labeling of PI or PA at the concentrations used to block the NE response.

DISCUSSION

The microvessel isolation technique employed in these studies produces short, sometimes branching, segments

TABLE 1

Phospholipid composition and $^{32}P_i$ labeling of phospholipids from isolated canine brain microvessels

Approximately 2.75 mg of microvessel protein were incubated at 37° for 30 min in incubation medium containing $^{32}P_i$ (60 μCi/ml). The lipids were extracted, approximately 26 μg of phospholipid were chromatographed, and the phosphate content and $^{32}P_i$ radioactivity were determined as described under Experimental Procedures. The results are expressed as averages ± standard error of the mean of three or more experiments with duplicate determinations.

Phospholipid	Phosphate	Incorporation of $^{32}P_i$
	% of total	*% of total*
Phosphatidylcholine	32 ± 1	10.0 ± 0.5
PA	1 ± 0.1	8.7 ± 0.3
Phosphatidylserine	10 ± 1	0.5 ± 0.1
PI	4 ± 0.5	15.2 ± 1.0
PI-4-phosphate	Trace	18.0 ± 2.0
PI-4,5-bisphosphate	Trace	41.4 ± 2.0
Phosphatidylethanolamine	23 ± 1	Trace
Sphingomyelin	19 ± 2	Trace

TABLE 2

Effect of norepinephrine on $^{32}P_i$ labeling of microvessel phospholipids

Microvessels were incubated at 37° for 30 min in incubation medium containing $^{32}P_i$ (60 μCi/ml) in the presence or absence of 100 μM NE. Approximately 26 μg of phospholipid were separated by thin-layer chromatography, and the phosphate content and $^{32}P_i$ radioactivity were determined as described under Experimental Procedures. The results are expressed as averages ± standard error of the mean of three or more experiments with duplicate determinations.

Phospholipid	Control	100 μM NE
	cpm/μg P_i	*cpm/μg P_i*
PI	12,900 ± 340	22,300 ± 660[a]
PA	42,200 ± 1,000	70,300 ± 2,100[a]
Phosphatidylcholine	1,530 ± 85	1,570 ± 79
PI-4-phosphate	881 ± 29[b]	902 ± 33[b]
PI-4,5-bisphosphate	2,250 ± 80[b]	2,210 ± 93[b]

[a] Significantly different from control ($p < 0.01$).
[b] Phosphate levels below sensitivity of assay; values expressed in counts per minute.

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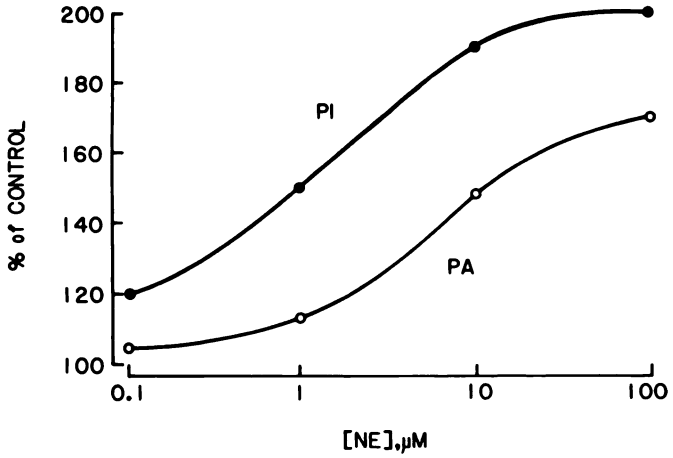

FIG. 2. *Dose-response curve for NE-stimulated labeling of PI (●) and PA (○)*

Microvessels were incubated for 30 min at 37° in medium containing $^{32}P_i$ (60 μCi/ml) and various concentrations of NE. $^{32}P_i$ labeling of PI and PA was determined as described under Experimental Procedures. The data shown are averages of eight determinations from four microvessel preparations.

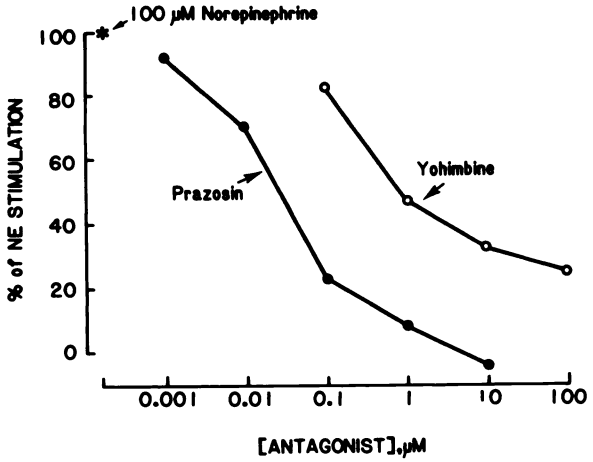

FIG. 3. *Effect of alpha-adrenergic antagonists on NE-stimulated $^{32}P_i$ labeling of PI*

Microvessels were incubated under standard conditions with $^{32}P_i$ (60 μCi/ml) and various concentrations of prazosin (●) or yohimbine (○) and the percentage inhibition of stimulation due to 100 μM NE was determined. The data shown are averages of eight determinations from four microvessel preparations.

of the microvasculature. They are free of erythrocytes and other blood elements and consist primarily of capillary endothelial cells connected by a surrounding wall of basement membrane. The purity of each microvessel preparation was monitored by light or phase-contrast microscopy. Preparations were used for experimentation only when nonvascular elements were absent and when the preparations consisted virtually entirely of segments of the microvasculature. Minor contaminants were vessels with larger caliber, presumably arterioles and venules. No differences in appearance were noted between these microvessels and those described previously (18–20). Some residual membrane elements from complex intercellular contacts with astrocyte processes and pericytes reportedly are also present on brain microvessels following isolation (21).

TABLE 3

Effect of various agents on $^{32}P_i$ incorporation into PI

Microvessels were incubated at 37° for 30 min in incubation medium containing $^{32}P_i$ (60 μCi/ml) and the agents listed. Approximately 26 μg of phospholipid were separated by thin-layer chromatography, and the $^{32}P_i$ radioactivity was determined by liquid scintillation detection. The results are expressed as averages ± standard error of the mean of three or more experiments with duplicate determinations.

Additions	$^{32}P_i$ Incorporation into PI
	cpm
None	754 ± 19
NE, 100 μM	1750 ± 38[a]
Dopamine, 100 μM	1130 ± 18[a]
Phenylephrine, 100 μM	1330 ± 37[a]
Methoxamine, 100 μM	1280 ± 40[a]
Clonidine, 100 μM	730 ± 40
Isoproterenol, 100 μM	797 ± 68
Propranolol, 10 μM	1620 ± 200[a]
Propranolol, 10 μM, + NE, 100 μM	1560 ± 93[a]

[a] Significantly different from control ($p < 0.01$).

The results of phospholipid composition are similar to those previously published for bovine cerebral endothelial cells (20). The major phospholipids present in dog brain microvessels are phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, PI, and PA (Table 1). The concentrations of PI-4-phosphate and PI-4,5-bisphosphate were present in microvessels below the level of sensitivity of the phosphate assay used; however, substantial incorporation of $^{32}P_i$ into these phospholipids occurred.

Approximately 75% of the total $^{32}P_i$ incorporated into phospholipids was present in the phosphoinositides, with the major amount in PI-4,5-bisphosphate. This is in contrast to other cells, such as hepatocytes, in which the labeling of the phosphoinositides is about 20% of the total $^{32}P_i$ incorporated into phospholipids (22). This observation with brain microvessels may reflect rapid turnover or net synthesis of the phosphoinositides in cerebral endothelial cells. Enzymes of phosphoinositide metabolism reportedly are elevated in neural tissues (23).

The viability and metabolic activity of the cells associated with dog brain microvessels remain high following isolation. This is illustrated by the linear rate of $^{32}P_i$ incorporation into phospholipids for up to 90 min and by the successful culture of endothelial cells derived from this microvessel preparation.[3] Metabolic viability of microvessel-associated cells for several hours also has been demonstrated by others (24, 25).

In canine cerebral microvessels, stimulation of $^{32}P_i$ labeling of PI and PA by NE is the result of activation of *alpha*$_1$-adrenergic receptors. Only agents reported to have *alpha*-adrenergic agonist activity were found to stimulate $^{32}P_i$ labeling of PI and PA. The *alpha*$_1$-selective antagonist prazosin was found to block NE stimulation at a much lower concentration than was yohimbine, an *alpha*$_2$-selective antagonist. This indicates that *alpha*$_1$-

[3] R. J. Zeleznikar, Jr., and L. R. Drewes, unpublished results.

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adrenergic receptors are involved in mediating this PI effect. An *alpha*$_1$-receptor-linked PI response has been demonstrated by similar criteria in rat brown fat cells, hepatocytes, parotid glands, and pineal glands; in rabbit iris smooth muscle; and in hamster adipocytes (2, 22, 26, 27). The agonist concentrations required to elicit the PI effect in microvessels are similar to those reported for the above tissues.

Herbst *et al.* (9) and Palmer *et al.* (28) observed a stimulation of adenylate cyclase activity in rat brain microvessels in response to 100 μM NE. This response via *beta*-adrenergic receptors is independent of the NE response reported here. The PI effect in canine brain microvessels was not induced by dibutyryl cyclic AMP or the *beta*-agonist isoproterenol. In addition, the *beta*-adrenergic antagonist propanolol did not block the NE-stimulated PI effect.

Radioligand binding has been used to detect various types of receptor binding sites on cerebral microvessels (10, 11). Harik *et al.* (11) reported negligible binding of the *alpha*$_1$-selective ligand [^{3}H]WB-4101 to rat and pig microvessels. However, Peroutka *et al.* (10) detected substantial binding of [^{3}H]WB-4101 to bovine cerebral microvessels. The results reported here indicate that microvessels from canine brain also contain *alpha*$_1$ receptor sites. These differences in receptor populations may reflect a species variability.

The physiological significance of *alpha*$_1$-adrenergic receptors on cerebral microvessels is unknown, although a role in the regulation of blood-brain barrier permeability or cerebral blood flow is possible. Evidence has been presented which, indeed, suggests that this may be the case. Blood-brain barrier permeability to water is increased by pharmacological concentrations of the tricyclic antidepressant amitriptyline, which acts by blocking the neuronal reuptake of NE and potentiating its action (29, 30). This increase in permeability can be blocked by treatment with the *alpha*-adrenergic antagonist phentolamine or by ablation of central adrenergic neurons (7). This evidence supports the theory that *alpha*-adrenergic receptors are involved in regulating the blood-brain barrier.

The presence of *alpha*$_1$-adrenergic receptors linked to the PI effect on canine cerebral microvessels and the association of adrenergic neurons with the cerebral microvasculature suggest that PI metabolism may be important to the proper functioning and regulation of the brain microvasculature.

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