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RETINAL PIGMENT EPITHELIUM CONTAINS A DISTINCTIVE STRYCHNINE-BINDING SITE

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Membranes prepared from the retinal pigment epithelium of several species possess a specific site which binds [^{3}H]strychnine. This binding has a somewhat lower affinity and a much greater density than the corresponding interaction in the hindbrain or neural retina. Binding is not greatly altered in the presence of 10^{-3} M glycine, L-alanine, β-alanine, taurine, or serine. Thus, the receptor does not resemble the classical glycine receptor of the hindbrain and spinal cord. This new type of binding site appears to be confined to the pigment epithelial layer of the retina.

INTRODUCTION

The retina is an accessible, relatively simple part of the central nervous system. It contains significant amounts of neurotransmitter-related high-affinity binding sites resembling those found in the brain. These include dopaminergic (1), glutamate (2), and muscarinic cholinergic (3) receptors as well as binding sites for benzodiazepine (4, 5) and muscimol (6). These

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receptors are generally reported to have characteristics very similar to those in the brain.

The retina also exhibits a high glycine concentration (7, 8) and a high affinity uptake system for the amino acid (9, 10). Light-stimulated release of glycine has been reported (11). We, as well as others (12, 13) have thus examined retinal tissue for the existence of a high-affinity binding site toward strychnine, a potent glycine antagonist. We also have examined the retinal pigment epithelium (PE) a single, neuroepithelial cell layer in close apposition to the retinal photoreceptor out segments. This tissue functions together with the photoreceptors in several critical aspects of the visual process (14). We now report that the PE contains an exceptionally high number of strychnine binding sites that, are quite distinct from those observed in retina and brain.

EXPERIMENTAL PROCEDURE

Fresh Tissue Preparation. Bovine eyes were obtained on ice from a local slaughter house and light adapted before use. After removal of the anterior segment and vitreous body, the eye cup was filled with 67 mM potassium phosphate buffer, pH 7.6, swirled gently, and the neural retina removed after cutting at the optic nerve head and frozen on dry ice. After removal of the retina as described above, a small amount of the phosphate buffer was added to the eye cup and PE cells were gently brushed from the underlying chorodial layer using a fine camel hair brush. Cells were removed by aspiration, the cellular membrane preparation concentrated by centrifugation at 5,000*g* for 10 min and frozen for use. This preparation consists largely of intact and broken pigment epithelial cells with a small proportion of outer rod segments and erythrocytes present as contaminants (15). Brains and eyes were removved from freshly slaughtered pigs and immediately put on ice. Hindbrain (pons and medulla) and rostral spinal cord were removed from the brain; neural retina, pigment epithelium, and iris were dissected from the eye. Porcine retina and PE are tenaciously adherent, thus the retinal preparation in this case was somewhat contaminated with PE, unlike the corresponding retinal tissue from cow or chick.

For preparation of purified bovine photoreceptor outer segments, retinas were removed from light-adapted fresh bovine eyes using the 67 mM phosphate buffer with 5% sucrose added. Eighteen retinas were used for a normal preparation. Retinas were gently homogenized and rod outer segments prepared by differential sucrose gradient density centrifugation as previously described (16).

Chick Tissues and Cultured PE Cells. Neural retinas of chick embryo (13 days of embryonation) were dissected under a stereomicroscope using Dulbecco's PBS medium (Gibco, Grand Island, New York). The PE-choroid unit was then dissected from the posterior portion of the remaining eye cup using the same medium and frozen on dry ice. A usual preparation consisted of tissue from 48 eyes.

For culture purposes, pure PE tissue from chick embryo eyes (7 days of embryonation, Truslow Farms, Chestertown, Maryland) was dissected cleanly from underlying choroidal tissue under a stereomicroscope as previously described (17). PE sheets were dissociated into individual cells with collagenase-trypsin-chick serum-EDTA) solution (6 U/ml collagenase, 0.01% trypsin, 2% chick serum, 4 mM EDTA). Cells were plated in 60 mm tissue

culture plates and maintained in 3 ml of Eagle's MEM medium containing 5% heat-inactivated fetal calf serum (Gibco). Cells were grown for about 1 month in culture. For harvesting, medium was drained from 18 culture dishes, rinsed with Dulbecco's phosphate buffered saline solution, cells removed from substratum by scraping with a rubber policeman and frozen for use.

Receptor Assay. All tissues were frozen in dry ice and stored at −40°C. Membrane fractions were prepared by homogenization and repeated resuspension of centrifugal precipitates, using 40 mM Tris-HCl pH 7.4 (18, 19). Briefly, a crude membrane fraction was prepared by homogenization of tissues in 19 volumes of 0.32 M sucrose followed by centrifugation (50,000 *g*, 10 min). The pellet from this step was then homogenized in distilled water pH 7.4 and recentrifuged. The final pellet was suspended in 40 mM Tris-HCl pH 7.4 buffer at a concentration corresponding to 50 mg original tissue/ml. Binding assays were carried out by the glass fiber filtration method (20). Membranes (125–400 μg protein) were incubated (37°C, 15 min) in 40 mM Tris-HCl pH 7.4 together with 3.6×10^{-9} M [G-^{3}H]strychnine (17 Ci/mmol). The receptor-ligand complex was separated by filtration through 0.3 μ pore size glass fiber discs (Gelman Inc., Ann Arbor, Michigan), washed twice rapidly with 5 ml Tris, dried and counted in order to determine the amount of bound strychnine. The extent of nonspecific binding was determined by a series of parallel incubations in the presence of 10^{-5} M unlabeled strychnine. Specific binding was routinely 60–77% of the total bound radioactivity. Previous studies established the validity of our procedure as evaluated by regional binding, saturability, reversibility, and attainment of equilibrium. Protein was determined by the method of Lowry et al. (21). Each data point presented is the mean of 3–8 separate preparations. Scatchard analysis of tissues were carried out between 1 and 70×10^{-9} M strychnine and plots were computed by the Least Squares method.

RESULTS AND DISCUSSION

Specific binding of [^{3}H]strychnine was roughly comparable in spinal cord, brain, and retina (Table I). The higher value for porcine retina than that observed in cow or chick is possibly due to species variation but more probably due to contamination of porcine retinal membranes with tenaciously adherent PE cell processes. In contrast, bovine and chick retina can be prepared essentially free of PE contamination and demonstrate lower total binding. Purified bovine photoreceptor outer segment membranes had binding comparable to that of membranes prepared from the entire retina. This indicates that photoreceptor organelles are not especially rich in [^{3}H]strychnine binding sites and that much of the binding occurs to membranes of the inner retinal layers (10). Photoreceptor outer segments in the 13-day old chick embryo have not yet developed. Inner layers of the retina are quite well developed by this time however, and [^{3}H]strychnine binding is similar to that seen in bovine retina.

Binding to membranes prepared from the retinal pigment epithelium of cow, pig, and chick embryo was much higher than that observed in spinal cord, hindbrain, or neural retina. In the cow, binding to PE was about 30-fold higher than that to retina. Melanin granules have been reported

TABLE I

SPECIFIC BINDING OF [^{3}H]STRYCHNINE TO MEMBRANES PREPARED FROM RETINAL AND CEREBRAL TISSUES

Tissue		pmol strychnine bound per gm protein
Porcine		
	Spinal cord	183 ± 27
	Hindbrain	119 ± 11
	Neural Retina	560 ± 39
	Pigment epithelium	4319 ± 461
Bovine		
	Neural retina	54 ± 3
	Rod outer segments	38 ± 2
	Pigment epithelium	1545 ± 84
Chick		
	Neural retina	54 ± 2
	Pigment epithelium-choroid	1734 ± 223
	Cultured pigment epithelial cells	1845 ± 108

Membrane samples in 40 mM Tris-HCl buffer (pH 7.4) were incubated with 3.6×10^{-9}M [^{3}H]strychnine for 15 min at 37°C. Binding was assayed by glass fiber filtration (20). Values given are mean values ± SE from 3–8 separate preparations.

to interact with several drugs (22) and their role in the binding described here cannot be excluded. However, no strychnine binding sites could be detected in the membrane fraction of iris and ciliary body which is rich in melanin and thus the binding to PE was quite specific.

Confirmation of the high level of binding of [^{3}H]strychnine in pigment epithelium was obtained using cultured PE cells derived from the chick embryo. These cells show excellent differentiation both morphologically and biochemically (17, 23) and were a rich source of [^{3}H]strychnine binding sites.

Further kinetic analysis (24) of the specific strychnine binding site was carried out comparing hindbrain membranes with those prepared from bovine PE cells. The K_D values for the two tissues respectively, were $(27 \pm 2) \times 10^{-9}$ M and $(81 \pm 8) \times 10^{-9}$ M, values close to those in the brain of several species (25, 26) and recently reported for bovine (13) and rat (12) retina. The K_D range determined for bovine PE ($67\text{-}141 \times 10^{-9}$ M), was similar to that of retina. However, the binding site density in the PE membranes was very great, 6025 ± 45 pmol/100 mg protein as compared to 40 ± 4 pmol/100 mg protein in hindbrain and 20-150 pmol/100 mg protein in neural retina (12, 13). Our data also show that this distinctive pigment epithelial receptor class is widely distributed among animal spe-

cies. The dissociation of bound strychnine from retinal membranes was determined by addition of unlabeled 10^{-5} strychnine to a membrane preparation in equilibrium with 3.6×10^{-9} M [^{3}H]strychnine. The rate of displacement of the labeled strychnine was fairly slow and appeared to be first order with a $t_{1/2}$ of 85 seconds (mean of two separate determinations within 10% of each other). This result was in contrast to the corresponding value for hindbrain membranes where the $t_{1/2}$ was less than 10 seconds and could not be accurately determined in view of the total filtration time of 5–7 seconds. The rate of association of labeled strychnine to hindbrain membranes was also much more rapid than that found with pigment epithelium. In view of the difficulty of measuring association rates in the presence of simultaneous dissociation, this was not quantitated. This is further evidence of the unusual nature of the strychnine binding site of pigment epithelium.

In contrast to hindbrain, 10^{-3} M glycine was not effective in competing with retinal binding of [^{3}H]strychnine (Table II). This result raises the possibility of glycine-insensitive strychnine binding sites (26, 27). However, the antagonistic effects of glycine and strychnine (28) support the idea of the glycinergic nature of some strychnine binding sites within the retina. Glycinergic mechanisms have been associated with the amacrine cells of the rat retina (29) and are thus probably unrelated to the binding sites described here. Taurine, alanine, and serine have been reported as being able to displace bound strychnine in cerebral and spinal cord membranes (30). For this reason we examined the effect of several of these

TABLE II

COMPETITION OF VARIOUS AMINO ACIDS FOR SPECIFIC [^{3}H]STRYCHNINE BINDING

Amino Acid	Concentration (mM)	Displacement (%)	
		Pigment Epithelium	Hindbrain
strychnine	0.01	100	100
glycine	1.0	12.1 ± 2.9	100.0 ± 5.5
β-alanine	1.0	14.8 ± 4.3	52.6 ± 14.8
L-α-alanine	1.0	17.9 ± 6.5	14.8 ± 7.2
taurine	1.0	5.7 ± 1.4	5.8 ± 0.7
taurine	50.0	19.3 ± 3.4	30.2 ± 17.9
serine	1.0	7.6 ± 4.5	—

Specific binding was measured in membrane preparations from a standard porcine hindbrain preparation and from isolated bovine pigment epithelium as described in Table I. Values are given as percentage displacement of specific binding and represent the means of three determinations. Standard errors are given.

compounds on the binding of [^{3}H]strychnine to total retinal membranes and purified pigment epithelium (Table 2). The specificity of the hindbrain strychnine receptor was very similar to that previously decribed for rat spinal cord (30). However, in no case was major inhibition of retinal binding of the labeled ligand observed using relatively high concentrations of amino acids. The photoreceptors may contain as much as 0.05 M taurine (31) and a high affinity uptake system for taurine has been described in both pigment epithelium and photoreceptors (32), but 0.05 M taurine did not compete with the strychnine binding (Table II). The spectrum of inhibition caused by various chemicals was very similar in neural retina and in purified pigment epithelium.

The identity of the endogenous ligand for this binding site is not known at present. This receptor seems to be distinctive in its properties and not hitherto described. It clearly does not resemble the classical glycine receptors that have recently been characterized in the CNS and in bovine neural retina dissected free of pigment epithelium (13). The morphological, biochemical, and electrophysiological interdependence of the neural retina and pigment epithelium has been demonstrated (33). Substances are known to be released from the retinal photoreceptors as has recently been shown in the case of a light-induced flux of calcium from the outer segment layer (34). The pigment epithelium is also known to respond to light-induced changes in the metabolism of the retina (35). The receptors described here may play a role in mediating PE cell function by interacting with an as yet unidentified chemical released from the neural retina in response to light or other stimulus.

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