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NEUROTRANSMITTER-RELATED FEATURES OF THE RETINAL PIGMENT EPITHELIUM

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Abstract—Various neurotransmitter-related biochemical features of the separated pigment epithelium and neural retina of the cow have been examined. The pigment epithelium contains high affinity binding sites for several pharmacological agents thought to attach to neurotransmitter receptor sites with a high degree of specificity. Thus, serotonergic, adrenergic and opiate receptors appear to be present in the pigment epithelium. Serotonin has also been detected in this region.

Several neuropeptides were found in the pigment epithelium. Relatively large amounts of neurotensin and met-enkephalin were present, but substance P was not detected.

The pigment epithelium of the retina contains no neuronal elements but interdigitates with and is intimately connected with the neural components within the retina. Some functions of the pigment epithelium have been recognized for many years. These include phagocytosis of shed photoreceptor elements (Hollyfield and Raybourne, 1979) and storage of vitamin A, a precursor of retinaldehyde (Berman *et al.*, 1979). The relation of pigment epithelium with neural retina has also been shown by demonstration that it participates in the electroretinogram (Nilsson, 1980). The pigment epithelium is involved in many visual disorders (Foulds, 1979).

In view of the evidence of interactions between the neural retina and pigment epithelium, we have surveyed the two separated tissues for their content of various receptor species, peptides and neurotransmitters. Evidence for the presence of several neurotransmitter-related molecular species within the pigment epithelium has been found.

EXPERIMENTAL PROCEDURES

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 pigment epithelial cells were gently brushed from the underlying choroidal layer using a fine camel hair brush. Cells were removed by aspiration, the cellular membrane preparation concentrated by centrifugation at 5000 ***g*** for 10 min and frozen.

Receptor assay

A crude membrane fraction was prepared from frozen tissues by homogenization of tissue in 19 vol of 0.32 M sucrose followed by centrifugation (50,000 ***g***, 10 min). The precipitate from this step was then homogenized in 40 mM Tris pH 7.4 and recentrifuged. The final pellet was suspended in the Tris–HCl buffer at a concentration representing 50 mg original tissue/ml.

Binding incubations were carried out in triplicate in a final volume of 1 ml containing 40 mM Tris–HCl (pH 7.4) together with appropriate labeled and unlabeled pharmacological agents. The incubation mixture used in the assay of serotonin also included 10^{-5} M pargyline, 4×10^{-3} M $CaCl_2$, and $5–7 \times 10^{-3}$ M ascorbic acid. The amount of tissue used per tube corresponded to 2–5 mg original wet weight and contained 100–200 μg protein as determined by the method of Lowry *et al.* (1951). At the end of a 15 min incubation at 37°C samples were filtered on glass fiber discs (25 mm diameter, 0.3 μm pore size, Gelman Inc., Ann Arbor, MI) and washed rapidly two times with 5 ml Tris buffer. In the case of the strychnine binding assay, only one wash was used. The naloxone binding method included a 30 min tissue preincubation at 37°C before addition of naloxone. Filter discs were then dried

Table 1. Pharmacological agents used in high affinity binding studies

Labeled ligand	Specific activity (Ci/mmol)	Conc. (nM)	Unlabeled competitor	Conc. (μm)	Putative receptor species assayed
[1-phenyl-4, ^{3}H] spiroperidol	29.6	1.0 Haloperidol	Haloperidol	1.0	Dopamine
DL-[benzilic-4,4-^{13}H] quinuclidinyl benzilate	40.2	1.0	Atropine	1.0	Acetylcholine
(methyl-^{3}H)-diazepam	87.6	0.75	Diazepam	1.0	Benzodiazepine
[methylene-^{3}H (N)] muscimol	9.3	1.0	GABA	1.0	GABA
[G-^{3}H]strychnine sulfate	15	4.0	Strychnine	1.0	Glycine
[1,2^{3}H (N)]Serotonin	32.2	3.0	Serotonin	1.0	Serotonin
9,10-[9,10-^{3}H(N)]-Dihydro-α-Ergocryptine	23.0	1.3	Ergocryptine	1.0	α-adrenergic
Levo-[propyl-1,2,3-^{3}H] dihydroalprenolol	47.5	0.7	Alprenolol	1.0	β-adrenergic
[*N*-allyl-2,3-^{3}H] naloxone	50.2	1.0	Levalorphan	1.0	Opiate

and counted in 5 ml of a scintillation mixture using a scintillation counter at an efficiency of 38–43%. Control incubations were carried out in order to determine the extent of non-specific binding simultaneously with the experimental series. Details of isotopes and competing compounds used are in Table 1. The method used was essentially similar to other filtration binding methods (Yamamura *et al.*, 1978). It was felt necessary to establish basic binding characteristics prior to this study. This included delineation of saturability, specificity and reversibility of the binding interaction (Bondy, 1981).

Estimation of neuropeptides

The regional content of met-enkephalin (ME), substance P (SP), neurotensin (NT) and β-endorphin (βE) was determined by radioimmunoassay. Tissue was homogenized in 2 N acetic acid, then immersed in boiling water for 5 min, and centrifuged at 25,000 g for 20 min. The supernatant was lyophilized and the residue was reconstituted with H_2O and radioimmunoassayed using [tyrosyl-3,5-^{3}H]met-enkephalin (36 Ci/mmol), ^{125}I-substance P, [tyrosyl-3,5-^{3}H]neurotensin (61 Ci/mmol) and ^{125}I-β-endorphin (original specific activity of iodine, 65 μCi/μg). Antisera were raised in rabbits using polylysine conjugates. Six injections were given at 2 week intervals before animals were bled. Nonlabeled retinal or brain extract was incubated with antiserum and isotopically-labeled peptides in 0.5 ml of 0.2 M Tris buffer, pH 7.4, containing 0.1% albumin and 0.06% dextran. The incubation was carried out at 4°C for 15–24 h. The labeled peptide bound to antibody was separated from the unbound peptide by adding 0.2 ml of 1.5% charcoal slurry containing 0.15% dextran (suspended in 0.2 M Tris buffer, pH 7.4) and a sample of supernatant fluid was counted in a liquid scintillation spectrometer. The validation and specificity of this method have been described in detail (Hong, Costa and Yang, 1976; Hong *et al.*, 1978). Fractionation of extracts by column chromatography followed by radioimmunoassay using antiserum against met-enkephalin revealed that over 90% of the immunoreactivity appeared in the fraction where authentic met-enkephalin was eluted (Hong *et al.*, 1980). This result indicates that this antiserum does not cross-react with enkephalin precursors or related peptides unless these larger molecules are first trypsinized (Yang *et al.*, 1978). Using the same method we found that over 90% of substance P-immunoreactivity of tissue extract represented authentic substance P (unpublished observation).

Estimation of dopamine and serotonin and their metabolites

The biogenic amine content was assayed by high performance liquid chromatography (HPLC) using the method of Wilson *et al.* (1982). In brief, tissue was homogenized in 19.3 vol. of chilled 0.1 M $HClO_4$ containing 0.002 M sodium bisulfite. The homogenate was centrifuged (40,000 *g* for 20 min) and the supernatant filtered through 0.2 μm pore size regenerated cellulose filter (Bio-Analytical Systems, Inc., W. Lafayette, IN) prior to chromatography. The filtrate was used for automated analysis of serotonin, dopamine, and their acid metabolites by reversed phase HPLC using an electrochemical detection system.

RESULTS

The extent of binding of various labeled ligands possessing a relatively high and selective affinity

Table 2. High affinity binding of various labeled pharmacological agents to membranes of pigment epithelium and neural retina

Labeled ligand	Pigment epithelium	Neural retina	Pigment epithelium/ neural retina
Spiroperidol	828 ± 26	125 ± 12	6.6
Serotonin	61 ± 5	1.6 ± 0.2	38
Dihydroalprenolol	156 ± 3	32 ± 13	4.9
Dihydroergocryptine	64 ± 4	169 ± 6	0.4
QNB	16 ± 3	75 ± 5	0.2
Muscimol	12 ± 5	30 ± 4	0.4
Strychnine	2426 ± 45	65 ± 4	39
Benzodiazepine	3.7 ± 0.8	18.7 ± 2.5	0.2
Naloxone	25 ± 6	<2.0	—

Each value represents a mean derived from 5 to 8 samples.
Data are expressed as *p* mol bound/100 mg protein ± S.E.
Experimental details are given in the text.

toward specific receptor species was determined in bovine pigment epithelial and neural retinal membranes (Table 2). No significant binding of [^{3}H]muscimol to pigment epithelium was found and values for [^{3}H]dihydroergocryptine, [^{3}H]QNB, and [^{3}H]benzodiazepine binding were all very low. Binding of [^{3}H]strychnine to pigment epithelium was many times greater than to neural retina. Similar relatively high values were obtained for the binding of tritiated spiroperidol, serotonin, and dihydroalprenolol. [^{3}H]naloxone binding to neural retina was not detectable and was low in pigment epithelial membranes. In order to determine whether serotonin and spiroperidol were binding to the same site in the pigment epithelium, the extent to which serotonin could compete with spiroperidol binding was measured. Haloperidol and serotonin at 10^{-6} M competed in an additive manner with the binding of 10^{-9} M [^{3}H]spiroperidol to pigment epithelial membranes. The percentage of total counts competed out was 35 + 4% in the case of haloperidol and 39 ± 7% for serotonin. The simultaneous presence of these two unlabeled competitors displaced 88 ± 3% of the total binding. Thus, each agent appeared to act on a different receptor population at the concentrations used. It is possible that these are both serotonergic (Peroutka and Snyder, 1979).

The data supported the concept that the major pigment epithelial monoamine receptor possessed serotonergic properties. This idea was strengthened by analysis of monoamine content by HPLC (Table 3) which revealed serotonin to be a significant component and of both the pigment epithelium and the neural retina. The absolute values for serotonin and dopamine in the neural retina agreed well with those reported for chick retina (Parkinson and Rando, 1981) and were also similar to values reported by Ehinger *et al.* (1981). The concentrations of monoamine metabolites such as 5-hydroxyindole acetic acid and dihydroxyphenyl acetic acid were too low to permit quantitation in these tissues. This may reflect a relatively slow rate of turnover of retinal monoamines or a rapid efflux of metabolites by way of the vascular system, and is similar to the results obtained by Parkinson and Rando (1981). The content of several neuropeptides in the two tissues was compared (Table 4). While substance P-like immunoreactivity was present in considerable quantity in the neural retina, this peptide could not be detected in pigment epithelium. A greater concentration of met-enkephalin-like immunoreactivity was found in the neural retina than in the pigment epithelium while the neurotensin-like immunoreactivity of each tissue was similar.

DISCUSSION

Retinal amacrine cells have been reported to contain several neuropeptides by immunohistochemical localization. By this means specific cell types have been found to contain enkephalins (Brecha *et al.*, 1979; Brecha *et al.*, 1981), neurotensin (Buckerfield *et*

Table 3. Levels of biogenic amines in bovine neural retina and pigment epithelium

	Dopamine	Serotonin
Neural retina	307 ± 91	290 ± 57
Pigment epithelium	13 ± 7	57 ± 17

Results expressed as pmol/g wet tissue. Each value represents a mean derived from 5 to 7 animals ± S.E.

Table 4. Neuropeptide content of bovine pigment epithelium and neural retina

Peptide	Pigment epithelium	Neural retina	Pigment epithelium/ neural retina
Met-enkephalin	35 ± 10	77 ± 17	0.45
Substance P	0	478 ± 20	0
Neurotensin	30 ± 3	39 ± 7	0.78

Each value represents a mean ± S.E. from 5 to 8 samples.
Data expressed as pg/10 mg wet weight of tissue.
Experimental details given in the text.

al., 1981; Brecha *et al.*, 1981) and substance P (Karten and Brecha, 1980). Retinal membranes have also been reported to contain opioid binding sites (Howells *et al.*, 1980; Osborne and Herz, 1981). The role of such peptides is uncertain but each appears to be largely confined to a distinctive cell population (Stell *et al.*, 1980). Neuropeptides have been found capable of altering the excitability of retinal ganglion cells (Djamgoz *et al.*, 1981, Dick and Miller, 1981).

The neural retina has recently been found to contain several neurotransmitter and related receptor sites which closely resemble those found in the brain. These include receptors for benzodiazepine (Altstein *et al.*, 1981; Osborne, 1980; Paul *et al.*, 1980), strychnine (Borbe, Muller and Wollert, 1981; Schaeffer and Anderson, 1981), muscarinic acetylcholine (Hruska *et al.*, 1978; Vogel and Nirenberg, 1976), dopamine (Magistretti and Schordoret, 1979; Redburn and Kyles, 1980) and GABA (Osborne, 1981, 1981a; Schaeffer, 1980; Yazulla and Brecha, 1981; Pourcho, 1981). These findings imply the presence of a variety of neurotransmitter species within the neural retina and several of these have been described including GABA, glycine, acetylcholine and dopamine (Watling, 1981; Yazulla and Schmidt, 1976), norepinephrine (Osborne, 1981b), glutamate and aspartate (Berger *et al.*, 1977; Altschuler *et al.*, 1982), and serotonin (Suzuki *et al.*, 1977; Osborne *et al.*, 1981; Parkinson and Rando, 1981). However, no comparison with pigment epithelium has been attempted.

Since the pigment epithelial preparation is almost totally devoid of vascular elements, the binding sites described here do not appear to be of capillary origin. Furthermore, cerebral microvessels contain no serotonin, opiate, or benzodiazepine binding sites (Peroutka *et al.*, 1980) while these sites were present in our pigment epithelial preparation. Deproteinized ocular melanin has been reported to bind several drugs (Atlasik *et al.*, 1980). Such interactions appear to be of relatively low affinity, however, taking place at around 10^{-4}–10^{-5} M. The binding of strychnine to pigment epithelium was not a non-specific binding to melanin since no strychnine binding sites could be detected in the membrane fraction of iris and ciliary body which is rich in melanin (Bondy *et al.*, 1982). Strychnine antagonizes taurine action in the retina (Miller *et al.*, 1977) and taurine is also known to be accumulated in the pigment epithelium (Lake *et al.*, 1977). However, the strychnine binding reported here is not significantly displaced in the presence of 10^{-3} M taurine and is thus unlikely to be attached to a taurine receptor (Bondy *et al.*, 1982). The presence of β-adrenergic receptors in cultures of pigment epithelium has been previously deduced by demonstration of stimulation of adenyl cyclase by isoproterenol in a tissue culture preparation (Chader and Koh, 1982). It may be relevant that amphibian melanocytes possess β-adrenergic receptors and also respond to two neuropeptides (MSH and ACTH) by increased levels of cyclic AMP (Garcia *et al.*, 1979).

The presence of serotonin in the pigment epithelium may be related to the fact that melanin is an indole polymer or to the phagocytosis of outer rod segments which are of neural origin. Evidence for a neurotransmitter role for serotonin in the retina is growing but this transmitter seems largely confined to the amacrine cells (Ehinger and Floren, 1980; Thomas and Redburn, 1979).

An analogy can be drawn between the anterior pituitary and the pigment epithelium. Both are devoid of neuronal elements but interact closely with a specific neuronal population. The anterior pituitary also contains receptors for several neurotransmitters including dopamine, acetylcholine and opiate binding sites (Creese *et al.*, 1977; Schaeffer and Hsueh, 1980) and neuropeptides (Blackwell and Guillemin, 1973; Salih *et al.*, 1979). Tissues with such intimate connections to nerve tissue may possess some similar biochemical features related to neural communication. Since both anterior pituitary and retinal pigment epithelium are of ectodermal origin, they also are ontogenically related to nerve tissue.

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