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Regional differences in lectin binding patterns of vestibular hair cells

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Surface glycoconjugates of hair cells and supporting cells in the vestibular endorgans of the bullfrog were identified using biotinylated lectins with different carbohydrate specificities. Lectin binding in hair cells was consistent with the presence of glucose and mannose (CON A), galactose (RCA-I), *N*-acetylglucosamine (WGA), *N*-acetylgalactosamine (VVA), but not fucose (UEA-I) residues. Hair cells in the bullfrog sacculus, unlike those in the utriculus and semicircular canals, did not stain for *N*-acetylglucosamine (WGA) or *N*-acetylgalactosamine (VVA). By contrast, WGA and, to a lesser extent, VVA, differentially stained utricular and semicircular canal hair cells, labeling hair cells located in peripheral, but not central, regions. In mammals, WGA uniformly labeled Type I hair cells while labeling, as in the bullfrog, Type II hair cells only in peripheral regions. These regional variations were retained after enzymatic digestion. We conclude that vestibular hair cells differ in their surface glycoconjugates and that differences in lectin binding patterns can be used to identify hair cell types and to infer the epithelial origin of isolated vestibular hair cells.

Lectins; Glycoconjugates; Otolith organs; Semicircular canals; Bullfrog; Guinea pig

Introduction

The surface coat of eukaryotic cells consists of extracellular carbohydrate chains of membrane-bound or membrane-associated glycoconjugates (Luft, 1976; Hook et al., 1984). These cell surface glycoconjugates include the glycolipids, glycoproteins, and the proteoglycans with their glycosaminoglycan groups (Glick and Flowers, 1978). A common feature of the glycoconjugates is the presence of negatively-charged carbohydrates bound to either protein or lipid molecules. Although few of these functions have been directly demonstrated, cell surface glycoconjugates are suspected of subserving important functions in neural development, including cell differentiation, recognition, and adhesion (Edelman, 1984; Dodd and Jessell, 1986; Peinado et al., 1987).

The presence of a glycoconjugate-rich surface coat surrounding hair cells and supporting cells of the vertebrate inner ear has been well documented. Hirokawa and Tilney (1982), using frozen, unfixed tissue, described a network of fibrils associated with the plasma membrane of hair cell stereocilia in the chick vestibular organs. The presence of negatively-charged glycoconjugates associated with the plasma membrane of cochlear and vestibular hair cells has also been indirectly demonstrated by binding experiments with polycationic ferritin (Flock et al., 1977), ruthenium red (Slepecky and Chamberlain, 1985; Khan et al., 1990), alcian blue (Santi and Anderson, 1986; 1987) and tannic acid (Prieto and Merchan, 1986; Khan et al., 1990).

Lectins, non-immune proteins able to recognize specific carbohydrate structures, have been widely used as biochemical tools to identify and localize specific glycoconjugates in cell membranes (Sharon and Lis, 1982; Lis and Sharon, 1986). Lectins have previously been used to examine carbohydrates present in the tectorial (Gil-Loyzaga et al., 1985a; Khalkhali-Ellis et al., 1987; Sugiyama et al., 1991; Tachibana et al., 1987a,c) and cupular and otolith membranes (Gil-Loyzaga et al., 1985b; Tachibana et al., 1987b). They have also been used to study the expression of specific glycoconjugates in the inner ear endorgans during postnatal development (Rueda and Lim, 1988; Lim and Rueda, 1990; Prieto et al., 1990; Endo et al., 1991). More recently, investigators have used lectins to demonstrate the presence of a number of carbohydrates on the apical surface of hair cells in the cochlea and vestibular endorgans (Gil-Loyzaga and Brownell, 1988; Khan et al., 1991). They have not, however, attempted to examine regional variations in the glycoconjugate composition of hair cells within individual inner ear endorgans. This is of critical importance in the vestibular endorgans, where Type I and Type II hair cells with differing hair bundle and cell body morphology are located in close proximity to one another (Lindeman, 1969a; Wersall and Bagger-Sjoback, 1974). Variations in hair bundle morphology are particularly striking in the bullfrog

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otolith organs, where several types of Type II hair cells differing in hair bundle morphology (Lewis and Li, 1975; Baird and Lewis, 1986) and physiological response properties (Baird, 1992, 1993a,b) have been described.

In our laboratory, we are studying the development and differentiation of hair cell types in the vestibular endorgans. These studies have been hampered by a lack of cell specific markers that could be used to identify subpopulations of vestibular hair cells. Separation methods used to date have used differences in cell body and hair bundle morphology. It would be highly desirable to separate and characterize subpopulations of hair cells on the basis of their biochemical phenotype. We were also interested in determining whether enzymatically dissociated hair cells maintained their ability to bind specific lectins and, if so, whether differences in lectin binding patterns could be used to infer the epithelial origin of isolated vestibular hair cells. As recent patch-clamp studies have found, it is difficult to infer the epithelial origin of a hair cell once it has been isolated from the sensory epithelium. This problem is compounded in higher vertebrates, where Type I hair cells (Correia and Lang, 1989; Valat et al., 1989; Rennie and Ashmore, 1991) do not retain their cellular morphology after enzymatic dissociation.

In the present study, we used biotinylated lectin probes to identify and characterize the surface glycoconjugates of hair cells in the vestibular endorgans. The primary aim of these studies was to determine if hair cells in different epithelial regions could be distinguished by their surface glycoconjugates. Our results demonstrate that vestibular hair cells in central and peripheral epithelial regions differ in their lectin binding patterns. These differences are preserved after enzymatic dissociation, demonstrating that they can be used to separate Type I and Type II hair cells and to infer the epithelial origin of isolated vestibular hair cells.

Preliminary accounts of portions of this data have been presented in abstract form (Baird et al., 1991).

Methods

Removal of the vestibular endorgans

Adult bullfrogs (*Rana catesbeiana*) were anesthetized by immersion in 0.2% MS-222; guinea pigs (*Cavia porcellus*) received intraperitoneal injections of Nembutal (50 mg/kg body wt). Anesthetized animals were decapitated and the sacculus, utriculus, and horizontal and anterior vertical semicircular canals were dissected from the membranous labyrinth and maintained in cold, oxygenated physiological saline. The cupular and otolith membranes of vestibular endorgans were removed with gentle mechanical agitation following a 15–45 min proteolytic digestion in 50 μg/ml subtilopeptidase BPN′ (Sigma). Vestibular endorgans were then trimmed of excess nerve and connective tissue to improve the visibility of hair bundles and, in some experiments, sectioned with a teflon-coated double-edge razor to separate central and peripheral regions of the sensory epithelium.

Isolation of vestibular hair cells

Hair cells and supporting cells were isolated from the sacculus and horizontal and anterior vertical semicircular canals by enzymatically dissociating the appropriate endorgan for 20–45 min with 500 μg/ml papain (CalBiochem) and 300 μg/ml L-cysteine (Sigma) dissolved in Ca^{2+}-free, PIPES-buffered saline (pH = 7.25). The utricular macula was pre-treated for 5 min with 250 μg/ml collagenase (Sigma, Fraction IV) dissolved in the same buffer. After enzymatic dissociation, endorgans were washed for 10–20 min each in 500 μg/ml bovine serum albumin (BSA) and 2 μg/ml DNAse 1 (Sigma) dissolved in standard HEPES-buffered saline. They were then mechanically triturated with fire-polished pasteur pipettes, depositing isolated cells into small cover-slipped chambers. Isolated cells were allowed 15–20 min to settle to the chamber bottom. In early studies, chamber bottoms were coated with 1 mg/ml poly-L-lysine (Sigma, > 300,000 mol.wt.). In later experiments, isolated cells were embedded in a 1% solution of low-temperature agarose (BioRad). This change did not affect lectin binding patterns, but did enable larger numbers of isolated cells to survive histological processing.

Lectin histochemistry

Vestibular endorgans and isolated vestibular hair cells were fixed by immersion for 1–4 h in 4% PFA and rinsed with 0.02M phosphate buffered saline containing 0.5 mM $CaCl_2$ (PBS). If desired, vestibular endorgans were pre-treated for 1 h with 0.1 U/ml neuraminidase (Calbiochem, *Vibrio cholerae*) to remove sialic acid residues. Endorgans were then pre-treated for 1 h in 3% BSA to block non-specific binding sites, rinsed briefly in PBS, and incubated for 1 h in biotinylated lectins (E-Y Labs) diluted 50–100 μg/ml in PBS. Lectins from other suppliers produced weaker responses than those from E-Y Laboratories. The lectins studied, their carbohydrate specificities, and their inhibitory sugars are listed in Table I.

Biotinylated lectins were visualized by incubating vestibular endorgans for 1 h in 10 μg/ml streptavidin-Texas Red conjugate (Amersham). Agarose-embedded hair cells were reacted with biotinylated lectins as above except that all incubation times were halved

TABLE I

Lectins used for characterization of vestibular hair cells

Lectin (common name)	Abbreviation	Carbohydrate specificity	Inhibitory sugar
Canavalia ensiformis (jack bean)	CON A	α-Man ≫ α-Glc	α-methyl mannopyranoside
Galanthus nivalis (snowdrop)	GNA	α-Man	α-methyl mannopyranoside
Griffonia simplicifolia (no common name)	GS-II	α,β-GlcNAc	β-GlcNAc
Ricinus communis-I (castor bean)	RCA-I	β-Gal	β-Gal
Ulex europaeus (gorse seed)	UEA-I	α-Fuc	α-Fuc
Vicia villosa (hairy vetch)	VVA	α-GalNAc	α-GalNAc
Triticum vulgaris (wheat germ)	WGA	β-GlcNAc, Neu5Ac	βb-GlcNAc, Neu5Ac

Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosimine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Neu5Ac, neuraminic (sialic) acid.

from those used for vestibular endorgans. Controls for lectin staining included: (1) exposure of tissue to streptavidin-Texas Red conjugate without lectin and (2) pre-incubation of lectins with 0.1 M–1.0 M of the appropriate inhibitory sugar (Table I).

At the completion of histological procedures, vestibular endorgans and agarose-embedded hair cells were mounted in Fluoromount (Fischer Scientific) and examined with ×40 and ×63 oil-immersion objectives using Nomarski optics and epifluorescent illumination. Vestibular endorgans were also dehydrated in ethanol, embedded in glycol methacrylate (Polysciences, JB-4), and serially sectioned at 5–10 μm. In sectioned material, Type I hair cells were identified by the presence of a calyx ending (Lindeman, 1969a; Wersall and Bagger-Sjoback, 1974). Type II hair cell subtypes were identified, as in previous studies (Baird, 1992; Baird 1993a,b), from their macular location and hair bundle morphology (Fig. 1). Nomarski photomicrographs were taken with Tech-Pan film developed to ASA 50 with HC-110 developer. Fluorescent photomicrographs were photographed, whenever possible, at similar illumination and exposure times with T-Max 400 and 3200 film (Kodak).

TABLE II

Lectin binding patterns in the vestibular endorgans

Lectin	Sacculush		Utriculus			Semicircular Canals		
	HC	SC	HC		S	HC		SC
			S	ES		I	P	
CON A	+ + +	+ +	+ + +	+ + + +	+	+ + +	+ + + +	+
	+ +		+ +	+ +		+ +	+ +	
GNA	+ +	+	+	+ +	−	+	+ +	−
	+		+	+		+	+	
GS-II	−	−	−	−	−	−	−	−
	+		+	+		+	+	
RCA-I	+ + +	+ +	+ + + +	+ + + + +	+	+ + + +	+ + + + +	−
	−		−			−	−	
UEA-I	−	−	−	−	−	−	−	−
	+		+	+		+	+	
VVA	+ +	−	+	+ +	−	+	+	−
			+	+		+	+	
WGA	−	−	−	+ +	+ +	−	+ +	+ +
	−		−	−		−	−	
WGA & neuraminidase	+	+	−	+ + +	+ + +	−	+ + +	+ + +
			−	−		−	−	

Degree of lectin binding on apical surface of hair cells (HC) and supporting cells (SC) based on a visual assessment of relative staining intensity on a scale from +, least stained, to + + + + +, most stained, with − corresponding to no staining. Each value was obtained from examination of ten or more stained sections. Where applicable, HC staining is separated into hair bundle (top) and apical surface, (bottom) staining, respectively. S, striola; ES, extrastriola; I, Isthmus; P, Planum semilunatum.

Results

In agreement with earlier studies (Gil-Loyzaga et al., 1985b; Takumida et al., 1989c), significant lectin staining was observed in the cupular and otolithic membranes. For many lectins, this staining was so intense that it obscured the lighter staining of the sensory epithelium. This was particularly true for CON A, RCA-I, and WGA. By contrast, little or no staining of these structures was seen with UEA-I.

With the cupular or otolithic membrane removed, lectin binding was generally confined to the apical surfaces of hair cells and supporting cells. The lectin binding patterns of lectins in the sacculus, utriculus, and horizontal and anterior vertical semicircular canals are summarized in Table II. Endorgans incubated without lectins or lectins pre-incubated with their inhibitory sugars exhibited no specific staining (Fig. 3d).

Amphibian vestibular endorgans

Sacculus

The hair bundle morphology and macular distribution of hair cells in the bullfrog sacculus are illustrated in Fig. 1. The saccular macula is composed largely of hair cells with short stereocilia and bulbed kinocilia no longer than their longest stereocilia (Type D). These hair cells occupy the entire central region of the macula inside the first 2–4 rows of its perimeter. A second type of hair cell, located on the perimeter of the saccular macula, has very short stereocilia and kinocilia longer than its longest stereocilia (Type A).

Lectin binding patterns in the sacculus are illustrated in Fig. 2. Two lectins – CON A and RCA-I – strongly labeled peripheral remnants of the otolithic membrane and, less strongly, supporting cells and the hair bundles of hair cells (Figs. 2a,b). A similar, albeit weaker, response was seen for WGA (Fig. 2c). CON A, unlike RCA-I and WGA, also labeled the apical surfaces of saccular hair cells. The latter two lectins labeled only the apical surfaces of supporting cells, leaving unlabeled the apical surfaces of hair cells (Figs. 2b,c). A fourth lectin, VVA, weakly labeled the hair bundles of hair cells but not supporting cells (Fig. 2d). GS-II and UEA-I weakly labeled the apical surfaces, but not the hair bundles, of saccular hair cells.

The binding pattern of GNA, a lectin with a strong affinity for mannose but not glucose residues (Goldstein and Poretz, 1986), was significantly weaker than that of CON A, suggesting that CON A binding was due to the presence of both glucose and mannose residues. Lectin staining produced by WGA was stronger when preceded by neuraminidase treatment, suggesting that sialic residues blocked the access of this lectin to *N*-acetylglucosamine residues (Peters et al., 1979; Monsigny et al., 1980; Debray et al., 1981).

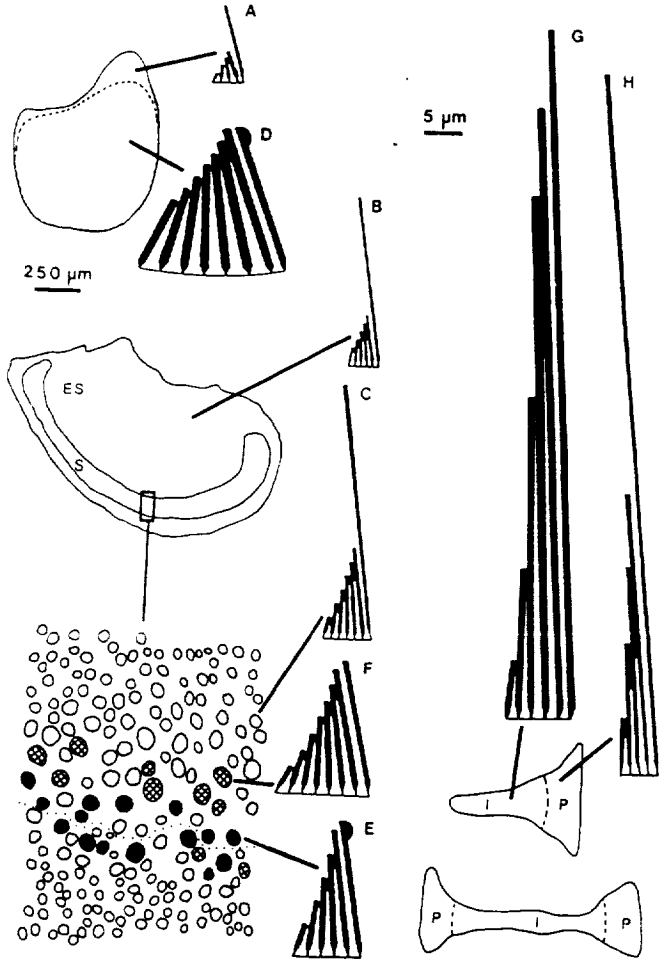

Fig. 1. Surface maps of the sensory surfaces of the bullfrog sacculus (top left), utriculus (lower left) and horizontal and anterior vertical semicircular canals (bottom right), showing the distributions of vestibular hair cell types. Idealized cross-sections (drawn to scale) of the hair bundles of vestibular hair cells are shown to the right of each endorgan. S, Striola; ES, Extrastriola; I, Isthmus; P, Planum semilunatum; A–H correspond to the hair type. Scale bar, 250 μm.

Utriculus

The utricular macula in the bullfrog is divided into medial and lateral parts by the striola, a narrow ribbon-shaped zone that runs for the length of the sensory epithelium near its lateral border (Wersall and Bagger-Sjoback, 1974; Lewis and Li, 1975). Hair cells in the bullfrog utriculus differ significantly in hair bundle morphology (Fig. 1). Type B hair cells, the predominant hair cell type in the utricular macula, have uniformly short stereocilia and kinocilia 2–6 × as long as their longest stereocilia. These cells are found throughout the medial and lateral extrastriola and, more rarely, in the striolar region. Three other hair cell types are confined to the striolar region. Type C hair cells, found throughout the striolar region, resemble an enlarged version of the predominant hair cell type. Moving inward, these cells are gradually replaced by

Type F hair cells, cells with shorter kinocilia and visibly larger hair bundles. Type E hair cells, restricted to the inner striolar rows, have short kinocilia with prominent kinociliary bulbs.

Lectin binding patterns in the bullfrog utriculus are illustrated in Fig. 3 and Fig. 4. CON A and RCA-I, as in the sacculus, strongly labeled the hair bundles of all hair cells, regardless of their macular location (Figs. 3a,b;4a,b). By contrast, WGA (Figs. 3c;4c) and, to a lesser extent, VVA (Fig. 4d), labeled the hair bundles of extrastriolar, but not striolar, hair cells. As in the sacculus, CON A strongly labeled the apical surface as well as the hair bundles of hair cells (Fig. 4b). A similar, weaker, response was seen for RCA-I and VVA (Figs. 4a,d). GS-II and UEA, as in the sacculus, weakly labeled the apical surfaces but not the hair bundles of hair cells. Basal staining was not observed with any lectin except for VVA and, with this lectin, was most pronounced in the striolar region (Figs. 4a–d).

The regional pattern of lectin binding associated with WGA was not correlated with differences in hair bundle morphology. Type B hair cells, for example, although found throughout the utricular macula (Fig. 1), were labeled only in the extrastriolar region (Fig. 4c). Furthermore, striolar hair cells with differing hair bundle morphology did not exhibit different lectin binding patterns (Fig. 3c,4c).

Semicircular canals

The semicircular canals in the bullfrog consist of a central isthmus and one (horizontal canal) or two (vertical canals) planar extensions (Fig. 1). Hair cells in the semicircular canals have significantly longer kinocilia than hair cells in the otolith organs. In the

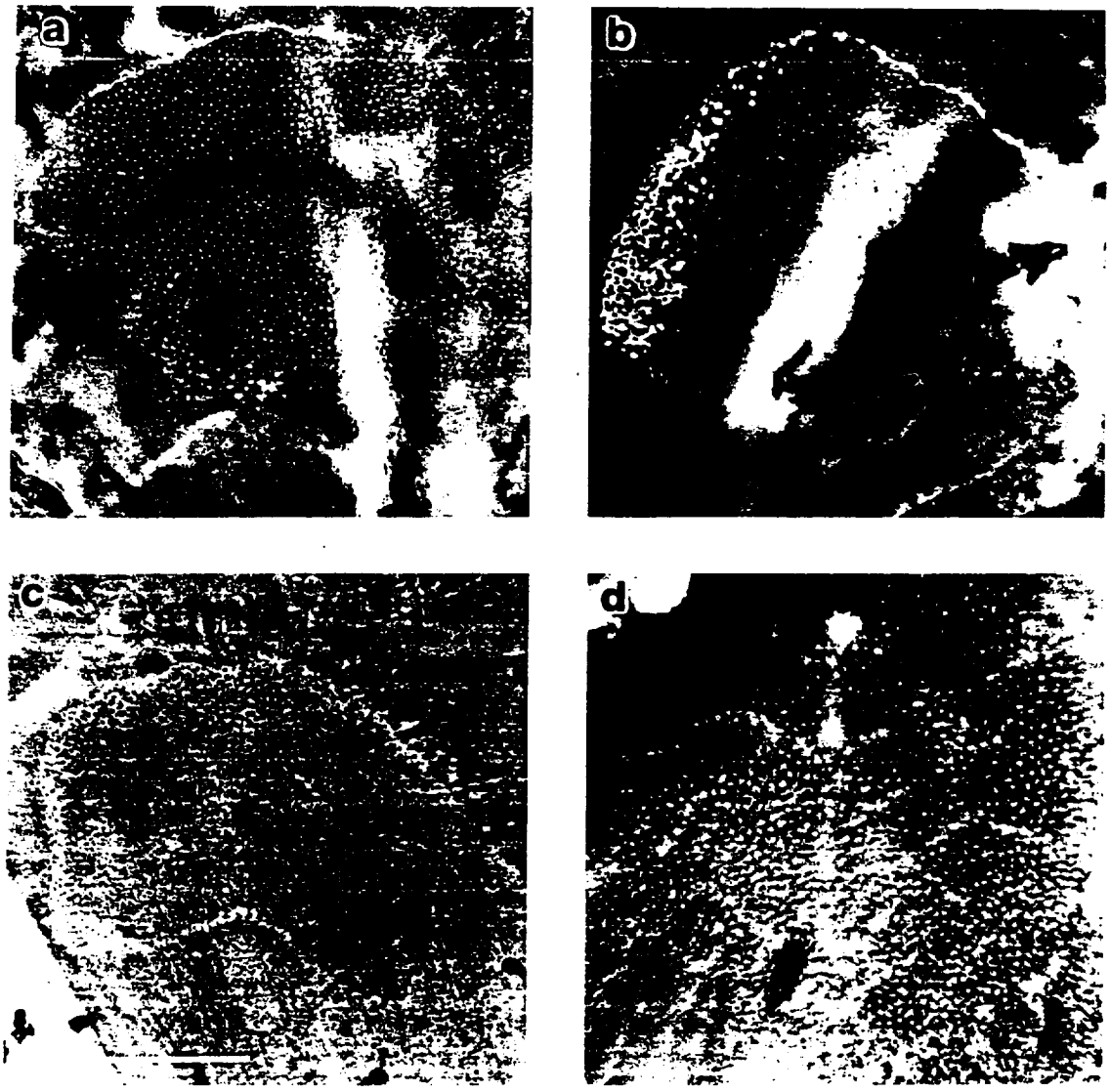

Fig. 2. Lectin binding patterns to CON A (a), RCA-I (b), WGA (c) and VVA (d) in the wholemount bullfrog sacculus. Photomicrographs exposed for 1 s at ASA 400. Scale bar, 250 μm.

planar extensions, hair cells have short stereocilia (Type H). Hair cells in the central isthmus, on the other hand, have stereocilia only slightly shorter than their kinocilia (Type G).

Lectin binding patterns in the semicircular canals, illustrated in Fig. 5, were similar to those in the utricular macula. RCA-I and CON A, as in the utriculus, labeled the hair bundles of all hair cells, regardless of their epithelial location (Fig. 5a). By contrast, WGA labeled the hair bundles of hair cells in the planar extensions but not the central isthmus (Fig. 5b).

Mammalian vestibular endorgans

Lectin binding in the utriculus and semicircular canals of the guinea pig was compared to their counterparts in the bullfrog. CON A and RCA-I labeled the hair bundles of both Type I and Type II hair cells, regardless of their epithelial location. WGA labeled the hair bundles of Type I hair cells in all regions. The hair bundles of Type II hair cells, as in the bullfrog, were labeled only in peripheral regions (Fig. 6).

Isolated vestibular hair cells

Lectins were also applied to hair cells isolated from the bullfrog otolith organs and semicircular canals. Basal staining of isolated hair cells (Fig. 7) was more pronounced than in sectioned material (Figs. 4,5). Regional differences in apical staining patterns, however, were preserved in isolated hair cells. CON A and RCA-I, for example, uniformly labeled the hair bundles of all utricular hair cells (Fig. 7, top). WGA, on the other hand, labeled the hair bundles of Type B hair cells isolated from the medial extrastriola (Fig. 7, bottom left) without labeling the hair bundles of hair cells isolated from the utricular striola (Fig. 7, bottom right). Similarly, WGA labeled hair cells isolated from the planar extensions but not the central isthmus of the semicircular canals (not shown).

Discussion

Our results indicate that a number of carbohydrate residues exist on the apical surfaces of hair cells and

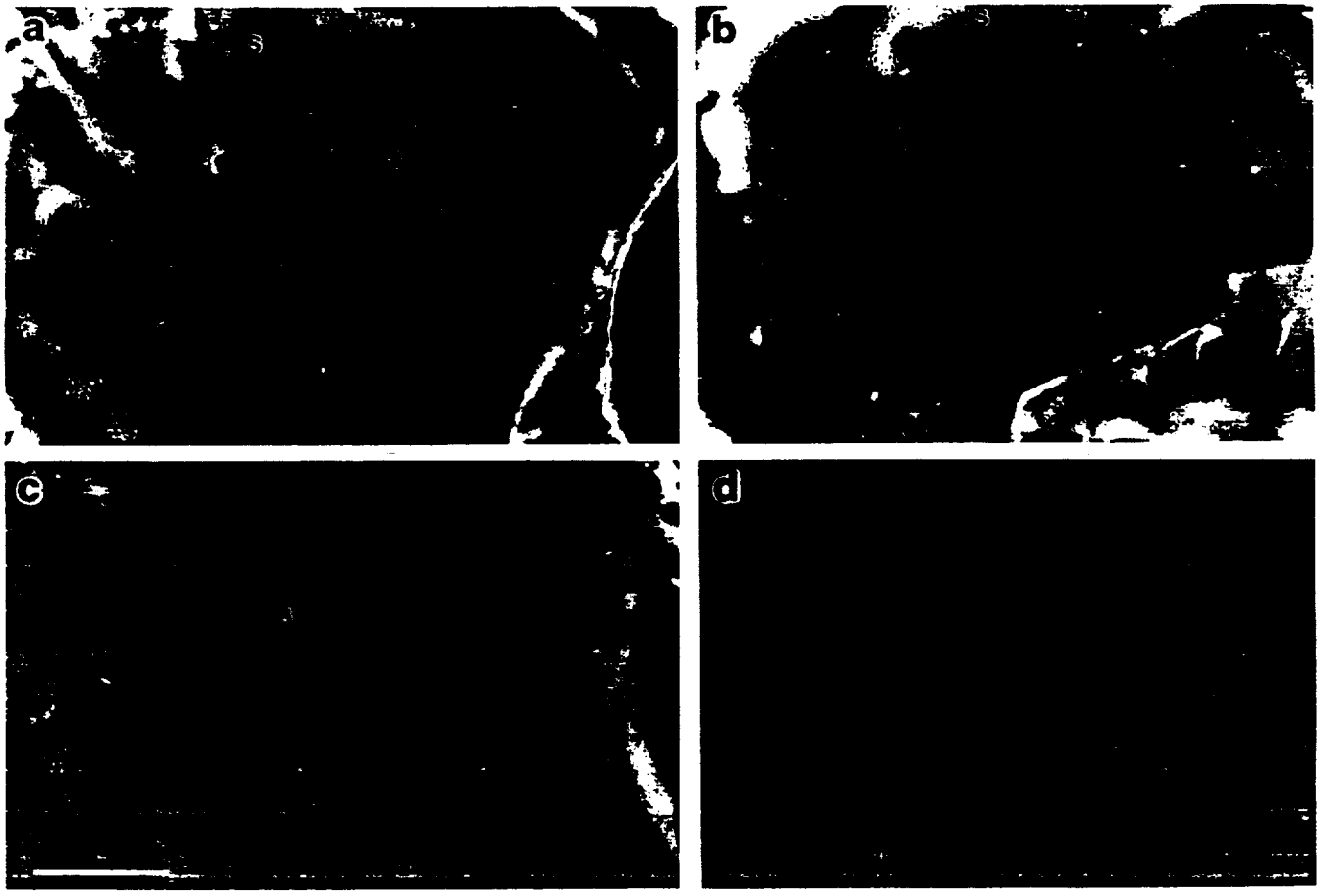

Fig. 3. Lectin binding patterns to CON A (a), RCA-I (b), WGA (c) and WGA pre-incubated with 1 M *N*-acetylglucosamine (d) in the wholemount bullfrog utriculus. Photomicrographs exposed for 1 s (a,b) and 30 s (c,d) at ASA 400. S, striola; MES, medial extrastriola; LES, lateral extrastriola. Scale bar, 250 μm.

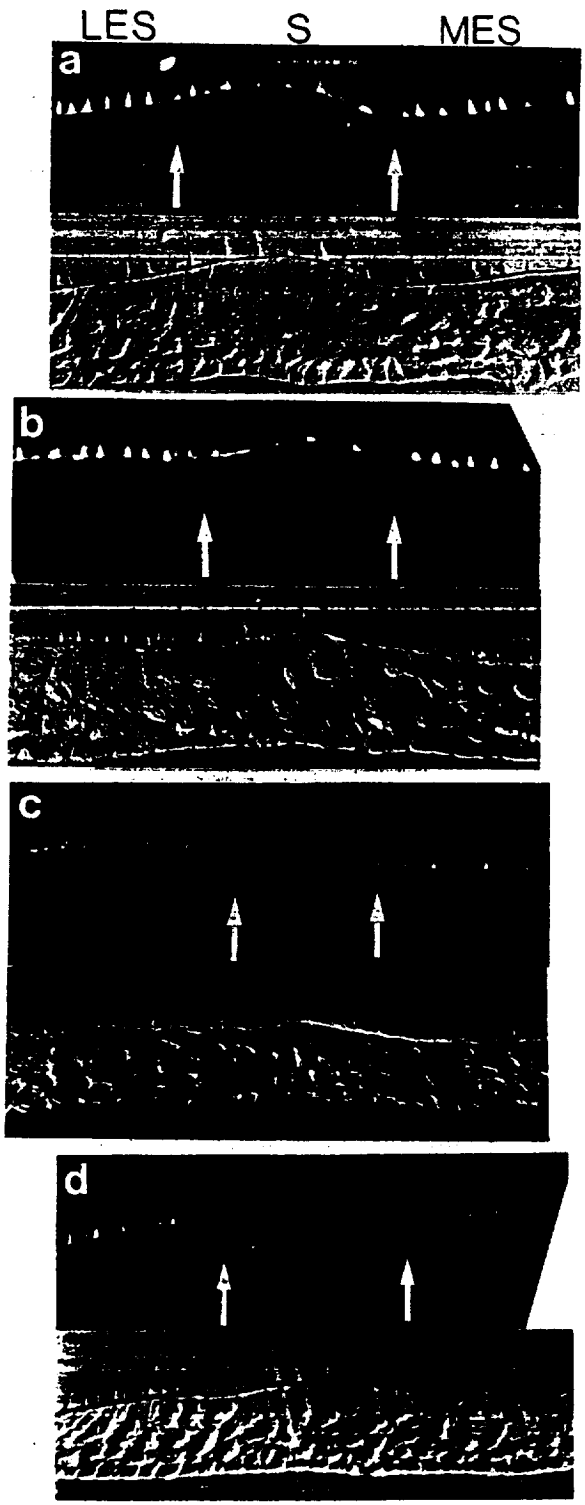

Fig. 4. Photomicrographs of utricular cross-sections viewed with epifluorescent illumination (top) and Nomarski optics (bottom) after incubation with CON A (a), RCA-I (b), WGA (c) and VVA (d). White arrows delineate limits of striolar region. In c, dark arrow indicates striolar Type B hair cell unlabeled by WGA incubation. Fluorescent photomicrographs exposed for 15 s at ASA 400 (a–c) and 4 s at ASA 3200 (d). S, striola; MES, medial extrastriola; LES, lateral extrastriola. Scale bar, 50 μm.

supporting cells in the vestibular endorgans. The amount of lectin binding to these residues could not be quantified with our techniques. The photographic exposure times required to observe fluorescently labeled lectins, however, is inversely correlated with and provides a qualitative measure of lectin binding. In particular, the intense staining produced by CON A is consistent with the presence of a high concentration of mannose and glucose residues on hair cells and supporting cells (Goldstein and Hayes, 1978; Debray et al., 1981). The lesser staining seen with GNA, a lectin with a specific affinity for mannose residues (Goldstein and Poretz, 1986), suggests that CON A staining was due to both glucose and mannose residues. Similarly, the heavy staining produced by RCA-I indicates an abundance of galactose residues on these cells (Hennigar et al., 1978; Baenzinger and Fiete, 1979). The relatively lighter staining seen in endorgans treated with WGA suggests a lower concentration of *N*-acetylglucosamine (GlcNAc) or sialic acid (Peters et al., 1979; Monsigny et al., 1980; Debray et al., 1981). The increase in staining to WGA seen in endorgans pre-treated with neuraminidase suggests that much of this staining was due to contiguous GlcNAc masked by sialic acid residues. This interpretation is supported by the poor staining seen with GS-II, a lectin which does not possess an extended binding site and recognizes only terminal GlcNAc residues (Ebisu et al., 1978; Goldstein et al., 1981). The staining density of VVA was even lower than that of WGA, suggesting that *N*-acetylgalactosamine (GalNAc) residues are relatively rare in the vestibular endorgans (Tollefsen and Kornfeld, 1983; Goldstein and Poretz, 1987). Other lectins, such as UEA-I, bound only weakly to hair cells, indicating a lack of fucose residues (Allen et al., 1977; Periera et al., 1978; Sugii et al., 1982).

Cell surface glycoconjugates include the glycolipids, glycoproteins, and the proteoglycans with their glycosaminoglycan groups (Glick and Flowers, 1978). The carbohydrate residues detected in our experiments are probably attached to glycoproteins since proteoglycans are generally not detected by lectin histochemistry and glycolipids are not well preserved by paraformaldehyde fixation (Spicer and Schulte, 1992). Glycoproteins consist of a protein backbone with oligosaccharide side chains and fall into two main categories according to the attachment of their oligosaccharides to the peptide chain (Kornfeld and Kornfeld, 1985). These include glycoproteins in which a terminal GalNAc is *O*-linked to serine or threonine and those in which a terminal GlcNAc is *N*-linked to asparagine. The intense staining by CON A suggests that most glycoproteins in the vestibular endorgans are of the *N*-linked type. This lectin has its strongest affinity for the core region of high mannose, *N*-linked glycoproteins (Debray et al., 1981) and does not detect *O*-linked glycoproteins, since

O-linked chains generally do not contain the mannose residues on which the lectin affinity depends (Loomis et al., 1987). The intense staining to RCA-I further suggests that these *N*-linked glycoproteins may be of the high *N*-acetyllactosamine (GalNAcb1,4GlcNAc) subtype (Kornfeld and Kornfeld, 1985). The weaker binding produced by WGA is consistent with this interpretation since this lectin does not bind strongly to lactosamine oligosaccharides (Debray et al., 1981). The presence of VVA staining, however, demonstrates that *O*-linked glycoproteins are also present. This lectin does not detect *N*-linked chains, since these glycoconjugates lack GalNAc (Tollefsen et al., 1983; Kornfeld and Kornfeld, 1985).

With the exception of the bullfrog sacculus (see below), our results are largely in agreement with the

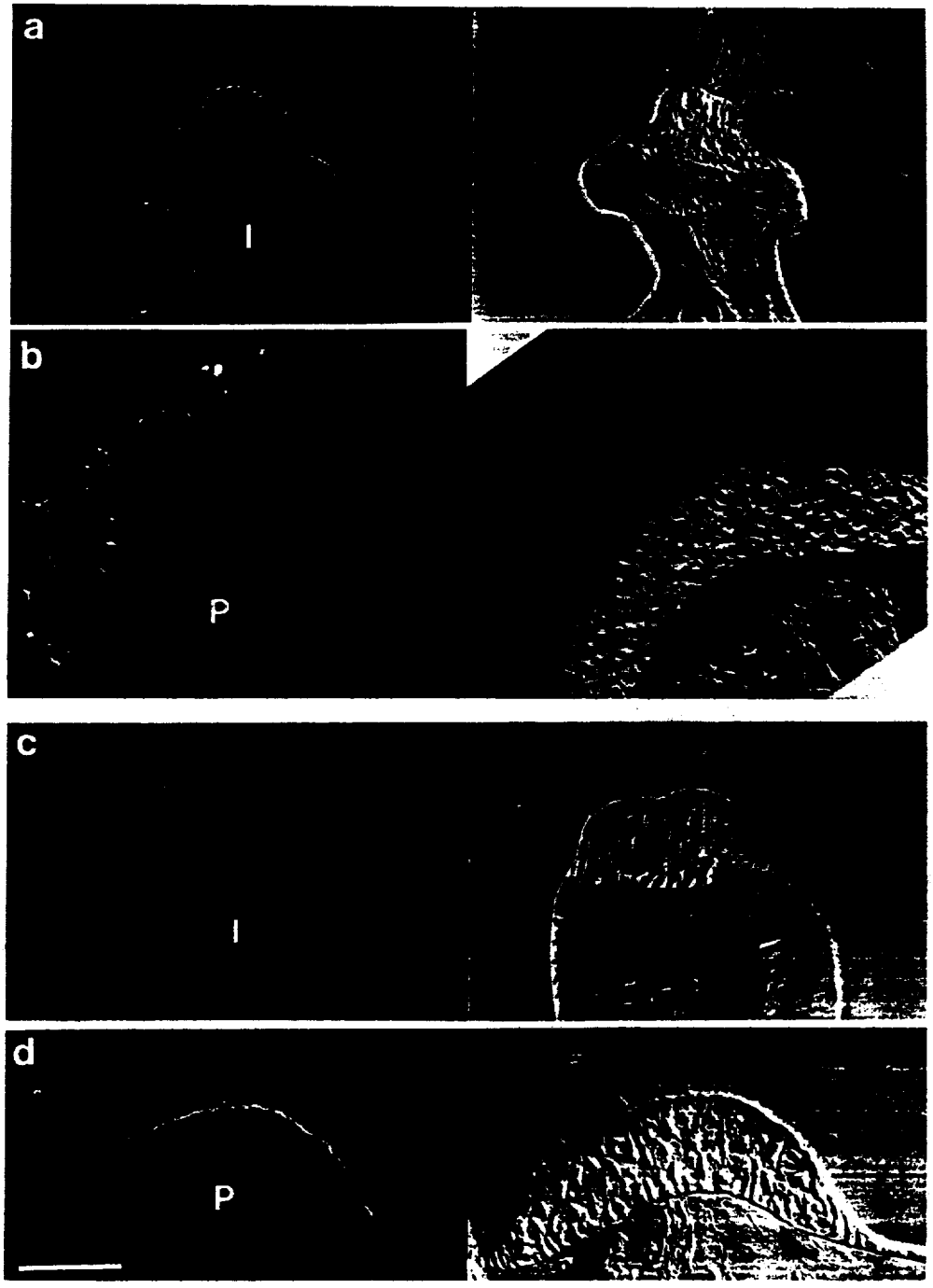

Fig. 5. Photomicrographs of cross-sectioned central isthmus (a,c) and planum semilunatum (b,d) of the bullfrog anterior vertical semicircular canal under epifluorescent illumination (left) and Nomarski optics (right) after incubation with CON A (a,b) and WGA (c,d). Fluorescent photomicrographs exposed for 15 s at ASA 400. I, Isthmus; P, Planum semilunatum. Scale bar, 100 μm.

results of previous lectin binding studies in fish (Khan et al., 1991), rats (Gil-Loygaza et al., 1985b), and guinea pigs (Tachibana et al., 1987b; Takumida et al., 1989b), suggesting that the glycocalyx of vestibular hair cells is strongly conserved in the lower and higher vertebrates. Similar results have also been obtained in inner hair cells (Gil-Loygaza et al., 1985a; Gil-Loygaza and Brownell, 1988; Khalkhali-Ellis et al., 1987; Tachibana et al., 1987a,c; Lim and Rueda, 1990; Prieto et al., 1990), suggesting that the glycocalyx of auditory and vestibular hair cells is not dissimilar. These studies, using lectin probes, demonstrated the presence of glucose and mannose (CON A), galactose (RCA-I), and GlcNAc (WGA) residues and the absence of GalNAc (VVA) and fucose (UEA-I) residues on hair cells. Unlike previous studies, the staining produced by WGA in our study was significantly weaker than that seen to CON A or RCA-I. The reason for this discrepancy is not clear, but may be age-related since WGA labeling declines progressively during postnatal development (Endo et al., 1991).

Our results also differ from previous histochemical observations (Gil-Loygaza et al., 1985b, Tachibana et al., 1987b; Takumida et al., 1989b) but confirm biochemical data (Khalaki-Ellis et al., 1987) in showing the presence of GalNAc residues on vestibular hair cells. Presumably, this sugar was not detected in earlier studies because these studies used HPA rather than VVA to detect GalNAc residues. Lectins which bind GalNAc differ in their affinity for different oligosaccharides with terminal GalNAc (Debray et al., 1981; Sugiyama et al., 1991). In particular, VVA is specific for terminal a-linked GalNAc and binds preferentially to the disaccharides GalNAc-a1,3Gal and GalNAc-a1,6Gal (Kaladas et al., 1981). Negligible staining with HPA in previous experiments suggests the absence of

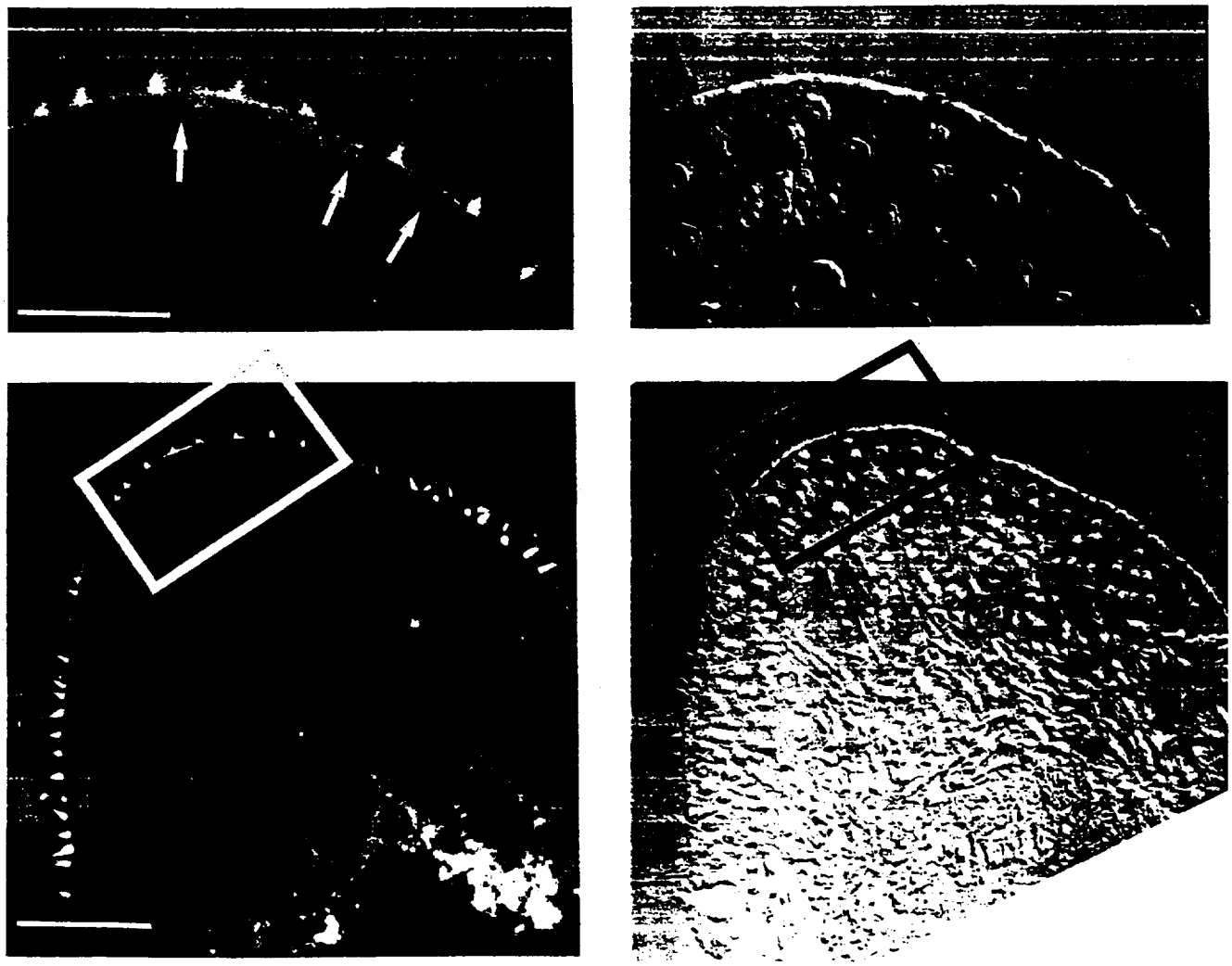

Fig. 6. Photomicrographs of cross-sectioned central isthmus of the guinea pig horizontal semicircular canal under epifluorescent illumination (left) and Nomarski optics (right) after incubation with WGA. Arrows indicate Type II hair cells unlabeled by WGA incubation. Insets of boxed region shown at top left and top right. Fluorescent photomicrographs exposed for 1/2 s at ASA 3200. I, Type I hair cell; II, Type II hair cell. Scale bar, 25 μm (top), 50 μm (bottom).

GalNAc-a1,3GalNAc for which this lectin has a high affinity (Baker et al., 1986). Outer hair cells, unlike inner hair cells and vestibular hair cells, are strongly labeled by HPA (Gil-Loygaza and Brownell, 1988).

Lectin binding patterns in the bullfrog sacculus were distinctly different from those in other vestibular endorgans. Saccular hair cells, unlike those in the utriculus or semicircular canals, were only weakly labeled by lectins and displayed a marked absence of staining to WGA and VVA. Unlike the latter endorgans, the bullfrog sacculus also did not exhibit regional variations in its binding patterns (see below). These differences may be correlated with the distinctive function of this endorgan which, unlike the sacculus of higher vertebrates, is a sensor of substrate-vibration (Koyama et al., 1982; Lewis et al., 1982).

In the utriculus and semicircular canals, WGA and, to a lesser extent, VVA, selectively labeled hair cells in different epithelial zones. In particular, these lectins labeled hair cells in peripheral regions but not those located in more central regions. In mammals, WGA uniformly labeled Type I but not Type II hair cells, demonstrating that this regional variation was confined to Type II hair cells. Among Type II hair cells, this regional variation was not correlated with hair bundle morphology. In the bullfrog utriculus, for example, WGA labeled Type B hair cells in the extrastriolar, but not the striolar, regions. Regional variations in WGA staining were also preserved after enzymatic digestion, demonstrating that this lectin can be a useful probe for separating Type I and Type II hair cells from central epithelial regions and for infering the epithelial origins of isolated Type II hair cells.

In retrospect, it is surprising that regional variations in lectin binding were not recognized in earlier studies. This might be because most previous investigations were made in mammalian tissue. Regional variations in lectin binding, while preserved in mammalian tissue, are more difficult to visualize because they are obscured by differences in the lectin binding of Type I

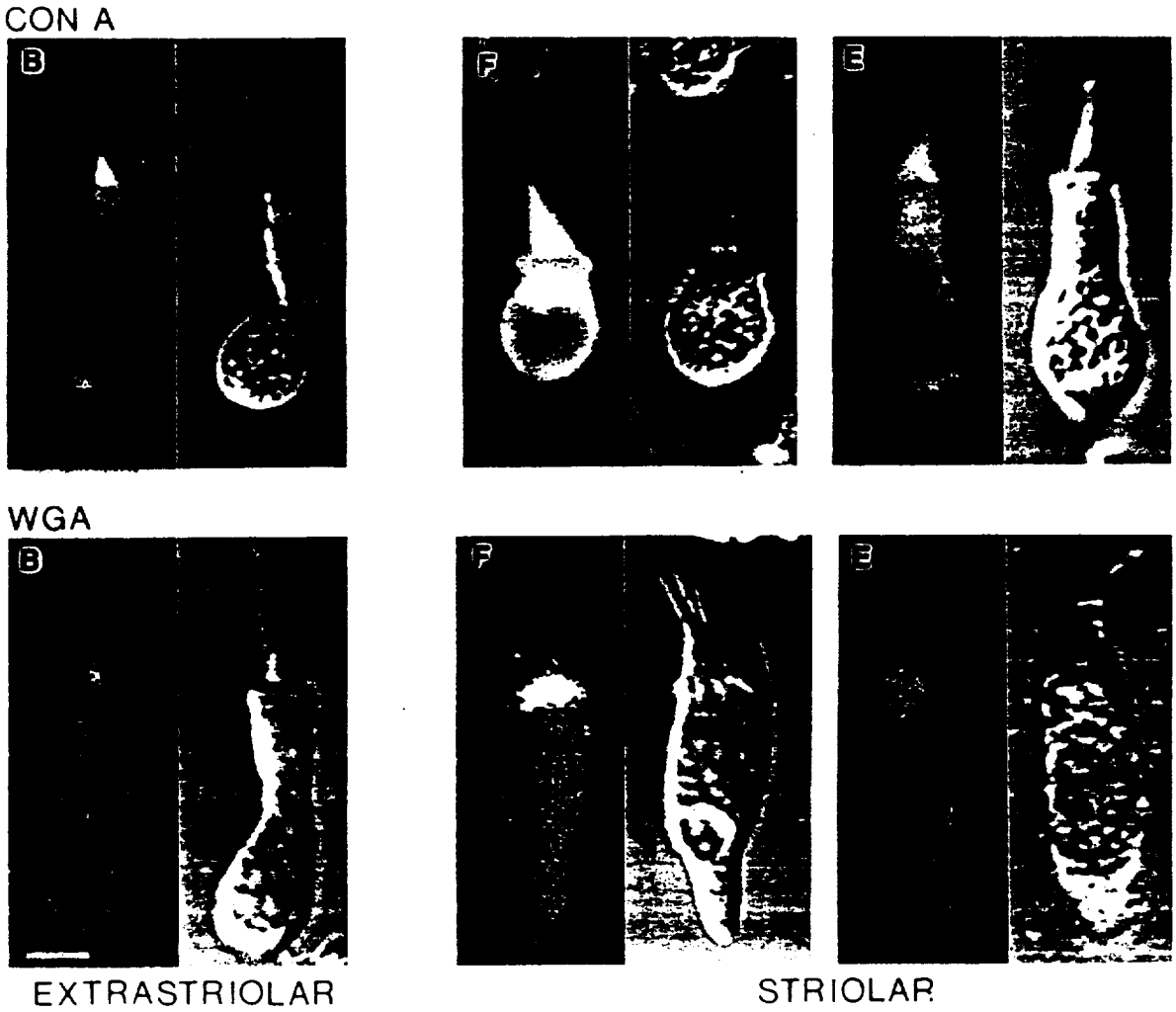

Fig. 7. Lectin binding patterns to CON A (top) and WGA (bottom) in utricular hair cells isolated from the medial extrastriola (left) and the striolar region (right). Fluorescent photomicrographs exposed for 1 s at ASA 3200. B, Type B; E, Type E; F, Type F. Scale bar, 10 μm.

and Type II hair cells. In addition, most previous studies of lectin binding were carried out in sectioned material rather than wholemount endorgans. Regional variations in lectin binding would be more difficult to observe at this level. Many previous investigations have also used FITC-labeled lectins, resulting in a weaker and less specific staining than that obtained with biotinylated lectins (R. Baird, unpublished observations).

The exact role(s) that glycoconjugates play in inner ear function have not yet been established. Membrane-bound glycoconjugates usually function locally in receptor activity, influencing ion transport across cell membranes (Spicer and Schulte, 1992). In hair cells, it has been suggested that the negative charges on these glycoconjugates, by influencing the surface charge of the plasma membrane, may play a role in sensory transduction by maintaining the integrity of the stereociliary array (Flock et al., 1977; Slepecky and Chamberlain, 1985; Neugebauer and Thurm, 1986; Santi and Anderson, 1987). It has also been suggested that the negative charge of these glycoconjugates may control the microenvironment of the transduction channel (Slepecky and Chamberlain, 1985), sequestering ions such as calcium that are important for maintaining mechanoelectric transduction (Assad et al., 1991; Crawford et al., 1991) and adaptation (Eatock et al., 1985; Crawford et al., 1989). Regional variations in lectin binding may therefore reflect differences in the transduction mechanisms of striolar and extrastriolar hair cells.

Glycoconjugates are also undoubtedly involved in drug-induced ototoxicity processes (Flock et al., 1977). It has been recently speculated (Schacht, 1986) that aminoglycoside antibiotics bind electrostatically with negatively charged components of the hair cell glycocalyx prior to being transported into the hair cell by an energy-dependent transport process (Takada et al., 1985). It has also been suggested that morphological changes observed in gentamicin-treated animals are due to a gradual decrease in the thickness of the glycocalyx (Takumida et al., 1989a) and that, once inside the cell, aminoglycoside antibiotics interfere with glycoconjugate biosynthesis (Schacht, 1986; DeGroot and Veldman, 1988; Takumida et al., 1989c). Thus, the resistance of hair cells to aminoglycoside antibiotics may be correlated with the size and integrity of their glycoconjugate coat. Vestibular hair cells are known to be differentially sensitive to aminoglycoside antibiotics. In the bullfrog, for example, gentamicin is known to damage saccular hair cells more than utricular hair cells (Baird et al., 1993). Moreover, these antibiotics have been shown to selectively affect hair cells in central epithelial regions (Lindeman, 1969b; Yan et al., 1992; Baird et al., 1993). This pattern of selective sensitivity is correlated with the lectin binding pattern of WGA to utricular and semicircular canal hair cells.

It is also possible that glycoconjugates play a role in inner ear development. In other systems, glycoconjugates are known to play important roles in cell recognition and adhesion, serving as recognition markers for specific subsets of sensory cells (Edelman, 1984; Dodd and Jessell, 1986; Peinado et al., 1987). In the inner ear, glycoconjugates are synthesized in different cells at different developmental times (Rueda and Lim, 1988; Lim and Rueda, 1990; Prieto et al., 1990). In a recent study, Endo et al. (1991) have also demonstrated that WGA labeling of the otolith membrane, otoconia, and the sensory epithelium is high in the early embryo and declines progressively during postnatal development. Because differences in WGA binding were correlated with epithelial location and not hair cell type per se, it seems unlikely that glycoconjugates control the differentiation of individual hair cell types in the vestibular endorgans. It is possible, however, that glycoconjugates are used to define the central epithelial regions of vestibular endorgans during development. Glycoconjugates might also be involved in the formation of the cupular or otolith membranes or in determining the relationship between hair cells and these accessory structures. It has previously been suggested that hair cells with differing hair bundle morphology may be coupled to the cupular or otolith membrane in different ways (Lim, 1976; Lim, 1979). Regional differences in staining density might reflect differences in the coupling of hair bundles to the cupular or otolith membranes in central and peripheral regions.

In summary, our results indicate that Type I and Type II vestibular hair cells and Type II hair cells in different epithelial regions differ in their complement of surface glycoconjugates. We conclude that lectin probes are useful for separating and identifying Type I and Type II hair cells and for infering the epithelial origins of Type II hair cells isolated from central and peripheral regions. Additional biochemical and physiological experiments are needed to identify the surface glycoconjugates of vestibular hair cells and to determine their role(s) in inner ear development and mechanoelectric transduction.

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