HAIR CELL TYPES AND DISTRIBUTIONS IN THE OTOLITHIC AND AUDITORY ORGANS OF THE BULLFROG

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SUMMARY

Based on surface morphology, 6 hair cell types have been identified on the sensory epithelia of the bullfrog otolithic and auditory organs. One of these types apparently is the morphogenetic precursor of vibrational and auditory hair cells. Another type, with a kinocilium several times as long as the longest stereocilia, apparently mediates gravistatic sensitivity. A third type, with a bulbed kinocilium no longer than the longest stereocilia, apparently mediates vibrational and auditory sensitivities. The other types may serve a variety of functions, depending on their location. One suggested function is that of an anchor for the gelatinous superstructure.

INTRODUCTION

In its early development, the membranous labyrinth of the bullfrog is divided into two cavities, the pars superior and the pars inferior, which are connected by the primitive utriculosaccular foramen[14]. Four sensory organs develop within each cavity, the three semicircular canals and the utriculus forming within the pars superior and the sacculus, lagena, amphibian papilla and basilar papilla forming within the pars inferior. The innervation of these organs does not correspond precisely with their cavities of origin, the two anterior canals along with the utriculus and the sacculus being innervated by the anterior branch of the VIIIth nerve (whose afferent cell bodies are in the anterior VIIIth nerve ganglion), and the lagena, the amphibian papilla, the basilar papilla and the posterior canal all being innervated by the posterior branch of the VIIIth nerve (whose afferent cell bodies are in the posterior VIIIth nerve ganglion)[7,9].

Although the specific sensitivities of the 8 organs remain somewhat open to question, the literature contains rather compelling evidence for the following relation-

ships. The utriculus and lagena apparently are gravistatic organs associated with static equilibrium of the bullfrog[24,25,31]. The sacculus apparently is sensitive to substrate-borne vibrations[2]. The amphibian papilla is sensitive to substrate-borne vibrations and to airborne vibrations, while the basilar papilla is sensitive to airborne vibrations alone (acoustical stimuli)[8,10]. The two presumedly gravistatic organs are otolithic, with the otolith of the lagena being much more massive than that of the utriculus. The most massive otolith of all occurs in the sacculus, however. The amphibian and basilar papillae possess tectorial masses, but no otoliths[11].

Based on cell-body and synapse morphology, two types of hair cells have been identified in mammals and birds; the bottle-shaped type I cells and the cylindrical type II cells[32]. Only type II cells have been found in frogs[33]. Based on surface morphology, two hair cell types have been identified in the inner ear of the mudpuppy (*Necturus maculosus*), one type being associated with the sacculus and amphibian papilla, the other being associated with the utriculus[19], and similar distinctions were noted between the hair cells of the sacculus and utriculus in the bullfrog[18]. In addition, a third type of hair cell has been found at the growing edge of the bullfrog saccular macula and the undifferentiated edge of the bullfrog amphibian papilla, and strong evidence exists indicating that in the saccular macula this is an immature type of hair cell that eventually undergoes transformation into the dominant type of that organ[17]. The distinction between these hair cell types is sharpened by the occurrence of a kinociliary bulb on the dominant hair cell of the sacculus[13] and on the dominant hair cell types of the amphibian papilla[16,17]. This bulb is not present in the dominant hair cell of the utriculus or the immature hair cell of the growing edges of saccular macula and amphibian papilla.

Since the sensory modalities have been established reasonably well for the various organs of the bullfrog inner ear (whereas they have not been established for the mudpuppy), and since the distinctions between hair cell types are so sharp in the bullfrog, we set out to determine the distributions of the various types in the hope of finding correlations between those distributions and sensory modalities. Since we were interested primarily in surface structure, we undertook this study with the aid of the scanning electron microscope, which already has proved extremely useful in such work[5,21,26,28,30].

MATERIALS AND METHODS

Well over 100 frogs have been used in this study to date. In each case, the animal was pithed and decapitated to prevent movement during dissection and the early stages of fixation. Occasionally all 8 sensory surfaces were taken from each inner ear of a specimen. However, it was often necessary to damage or destroy some of the surfaces in order to preserve others intact. This was particularly true when entire, intact maculae or papillae were desired. Much of the effort necessarily was devoted to the improvement of preparation techniques; nonetheless, virtually every specimen provided new information or corroborated previous observations.

Basically, the preparation techniques employed in this study followed the general

outline that has been well established for scanning electron microscopy of soft tissues[3, 4,15]. The tissue was fixed, dissected to expose the surfaces of interest, carried through a sequence of solvent exchanges to a transitional fluid with low critical temperature and pressure, critical-point dried, coated with a very thin film of gold, and observed in the scanning electron microscope. Precise details varied from specimen to specimen, but two of the variations proved most consistently successful.

Generally, the cleanest macular and papillar surfaces were obtained when the specimen was fixed in osmium alone. However, the surfaces of the sensory cells are extremely sensitive to the osmolarity of the fixative in this case, and considerable effort is required to determine the optimum osmolarity[12]. Furthermore, this method inevitably failed to provide adequate fixation of the large sensory cells along striolae or the large sensory cells in the center of the amphibian papillae. After fixation in osmium alone, removal of the gelatinous superstructure (gelatinous membrane or tectorial membrane) was quite easy over most of the sensory structures, but extremely difficult and damaging along the striolae of the lagenar macula and utricular macula.

Fixation with 2% glutaraldehyde in 400 mos$M$ sodium cacodylate buffer, pH 7.4, followed by postfixation in 1% osmium tetroxide in the same buffer proved to be especially good for the large sensory cells. However, remnants of the fibrous mat underlying the gelatinous membrane, especially in the three otolithic organs, often obscured the smaller hair cells. These remnants were not present in preparations fixed with osmium alone. In addition to providing better fixation for the large cells of the utricular and lagenar striolae, the glutaraldehyde/osmium sequence also made removal of the gelatinous superstructure from the striolae much easier and less damaging than it was after fixation in osmium alone. Interestingly, the opposite appears to be true in the bullfrog tadpole, where the membranes are extremely difficult to remove after fixation with glutaraldehyde.

After pithing and decapitation, very fine rongeurs were used to open a small hole in the cartilaginous wall of the otic capsule, usually from the roof of the mouth, but occasionally at the point of contact with the columella (*i.e.* the round window). The inner ear was flushed periodically with fixative solution passed through the hole by means of a syringe with a fine, blunt hypodermic needle, a method employed previously by Hillman[12]. After fixation, the membranous labyrinth was removed from the otic capsule and dissected while immersed in buffer. Gelatinous superstructures of otolithic organs were removed mechanically with a fine jet of buffer solution or with very fine forceps. The exposed tissues then were placed in 60% ethanol and taken rather briskly through a graded sequence to absolute ethanol, then on through a graded sequence of ethanol–Freon TF mixtures to 100% Freon TF (a commercially available solvent, $CCl_2F \cdot CClF_2$, also sold under the name 'Freon 113'), and, finally, on to either liquid Freon 13 ($CClF_3$) or liquid carbon dioxide, from which they were dried by the critical-point method[1,6,18]. Both transitional fluids provided excellent drying, but the liquid carbon dioxide is considerably less expensive and therefore has become the preferred fluid in our laboratory. The exchange of carbon dioxide for Freon TF can be made just as rapidly as the exchange of Freon 13 for Freon TF (*i.e.* in a matter of a few minutes), whereas the usual substitution sequence (amyl acetate to carbon di-
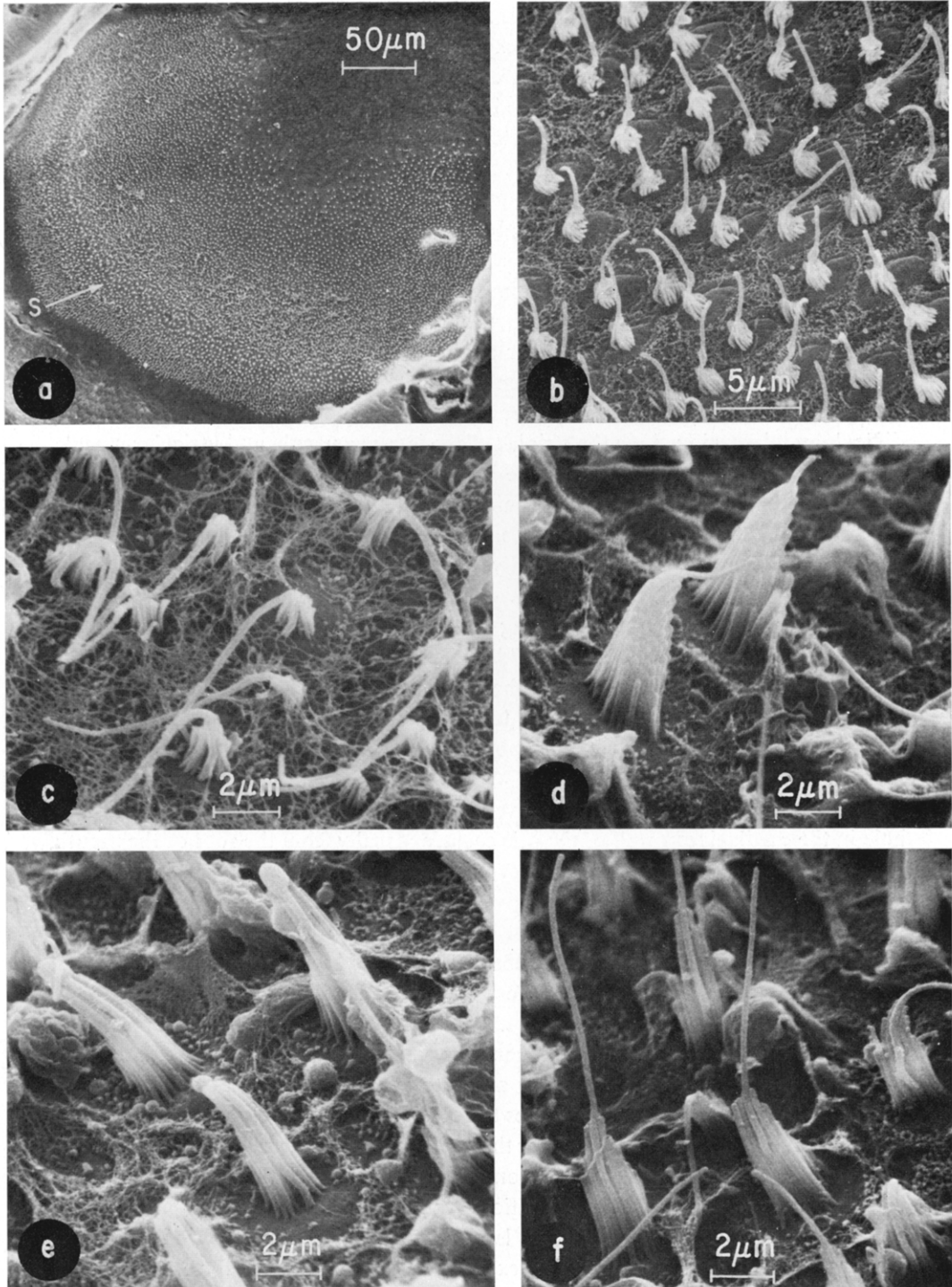
Fig. 1. Bullfrog utricular macula. a: whole macula, note 'striola' (S). b: dominant hair cell type (type B). c: close-up of type B hair cells. d: (type F) hair cell from striola. e: (type E) hair cell from striola. f: (type C) hair cell from striola.
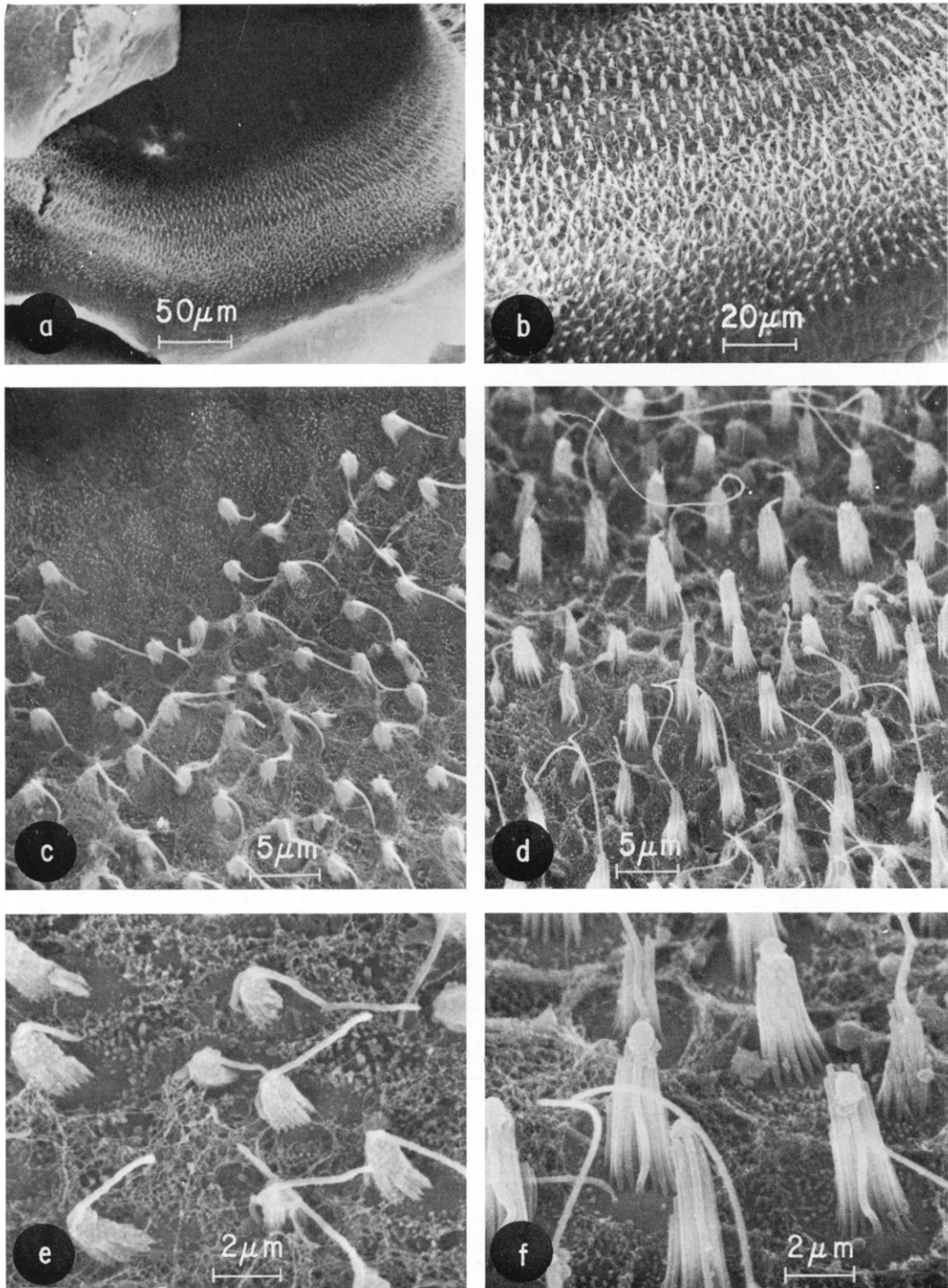
Fig. 2. Bullfrog lagenar macula. a: nearly entire macula. b: close-up, showing 'striola'. c and e: dominant hair cell type (type B). d and f: striola hair cells (types C and E) note reversal of hair cell orientation at mid-striola.
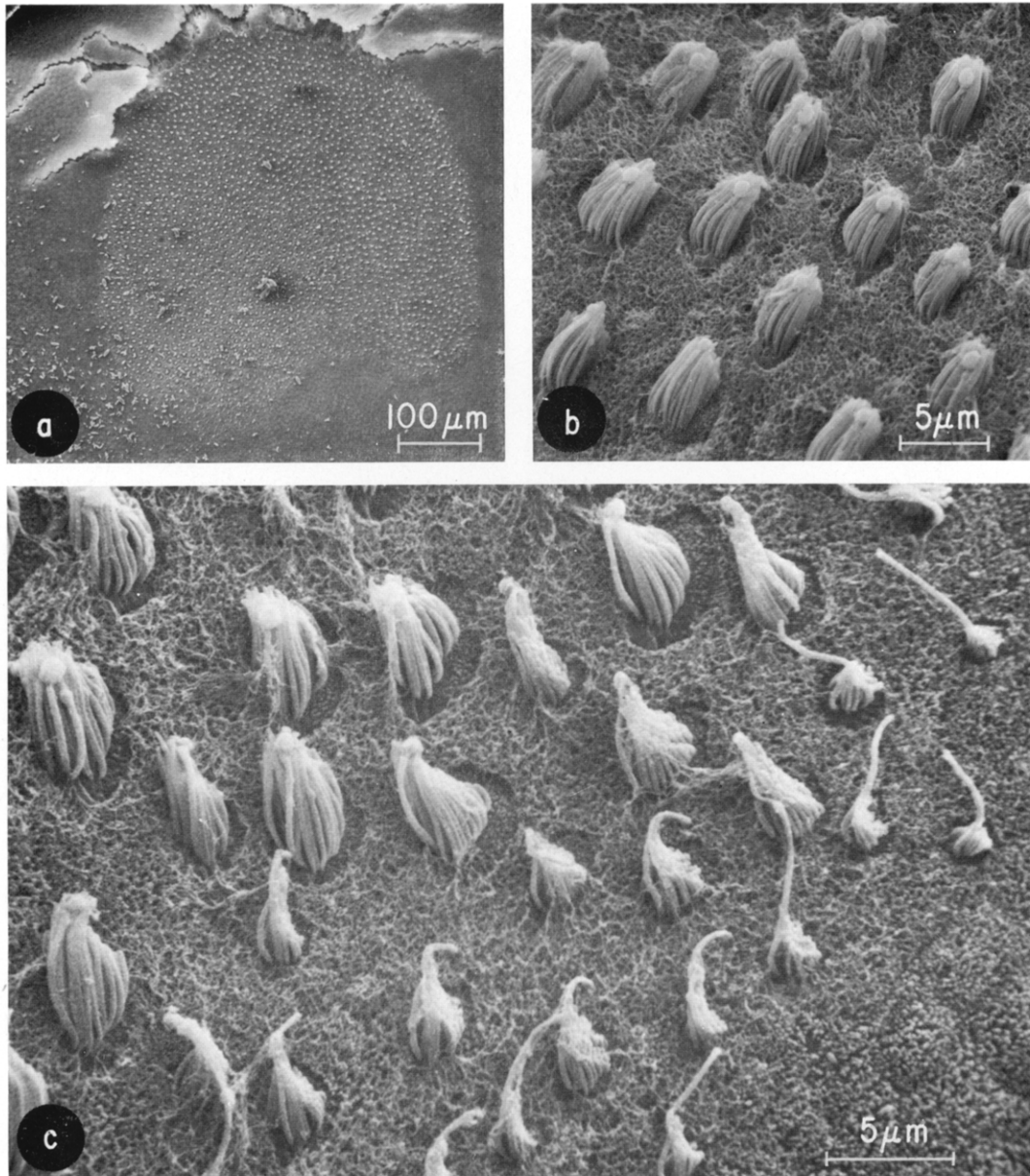
Fig. 3. Bullfrog saccular macula. a: entire macula, note absence of striola. b: dominant hair-cell type (type D). c: hair cells near periphery of macula, note the gradation of hair cell types (type A to type D).

oxide) reported in the literature requires several hours[1]. Immediately after critical-point drying, the tissues were mounted on stubs with double-coated tape (Scotch brand, type 400) and coated with a very thin layer of gold in a vacuum evaporator approximately 5×10^{-6} Torr.
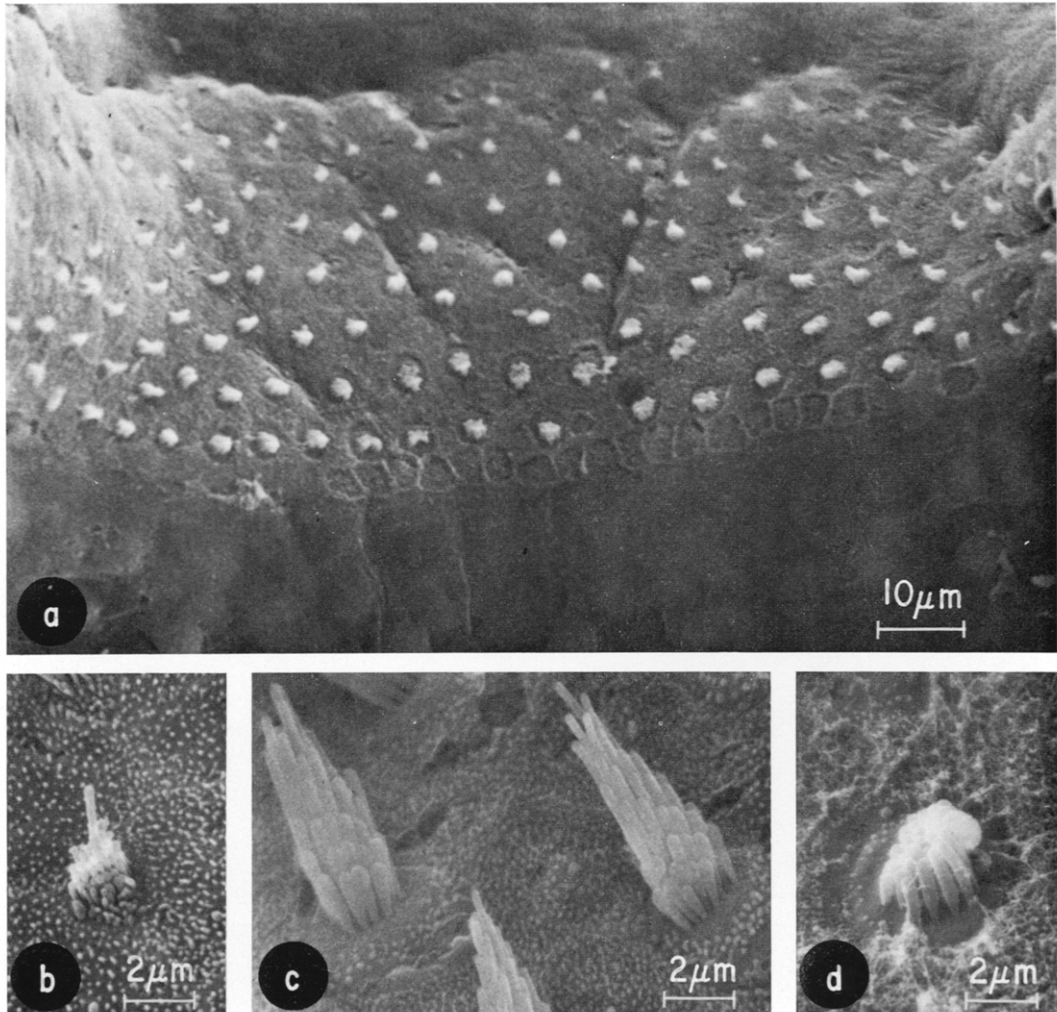
Fig. 4. Bullfrog basilar papilla. a: entire papilla. b: hair cell type of the lateral periphery (type A). c: hair cell type of the mid-papilla (type F). d: hair cell type of the medial periphery (type D).

RESULTS

Both gravistatic maculae (utricular and lagenar) exhibit central bands in which receptors are more sparsely distributed and have markedly larger luminal surfaces (Figs. 1 and 2). The hair cells outside this band are quite similar in the two organs, exhibiting relatively short stereocilia, with kinocilia approximately 3–4 times as long as the longest stereocilia (Figs. 1b, c and 2c, e). This is by far the most predominant hair cell type in these organs. In the central band itself, the hair cells exhibit a variety of surface topographies, with kinocilia and stereocilia that are markedly longer than those of the hair cells outside the band. Some of these cells have the appearance of being simply enlarged versions of the predominant type (Figs. 1f and 2d). Others
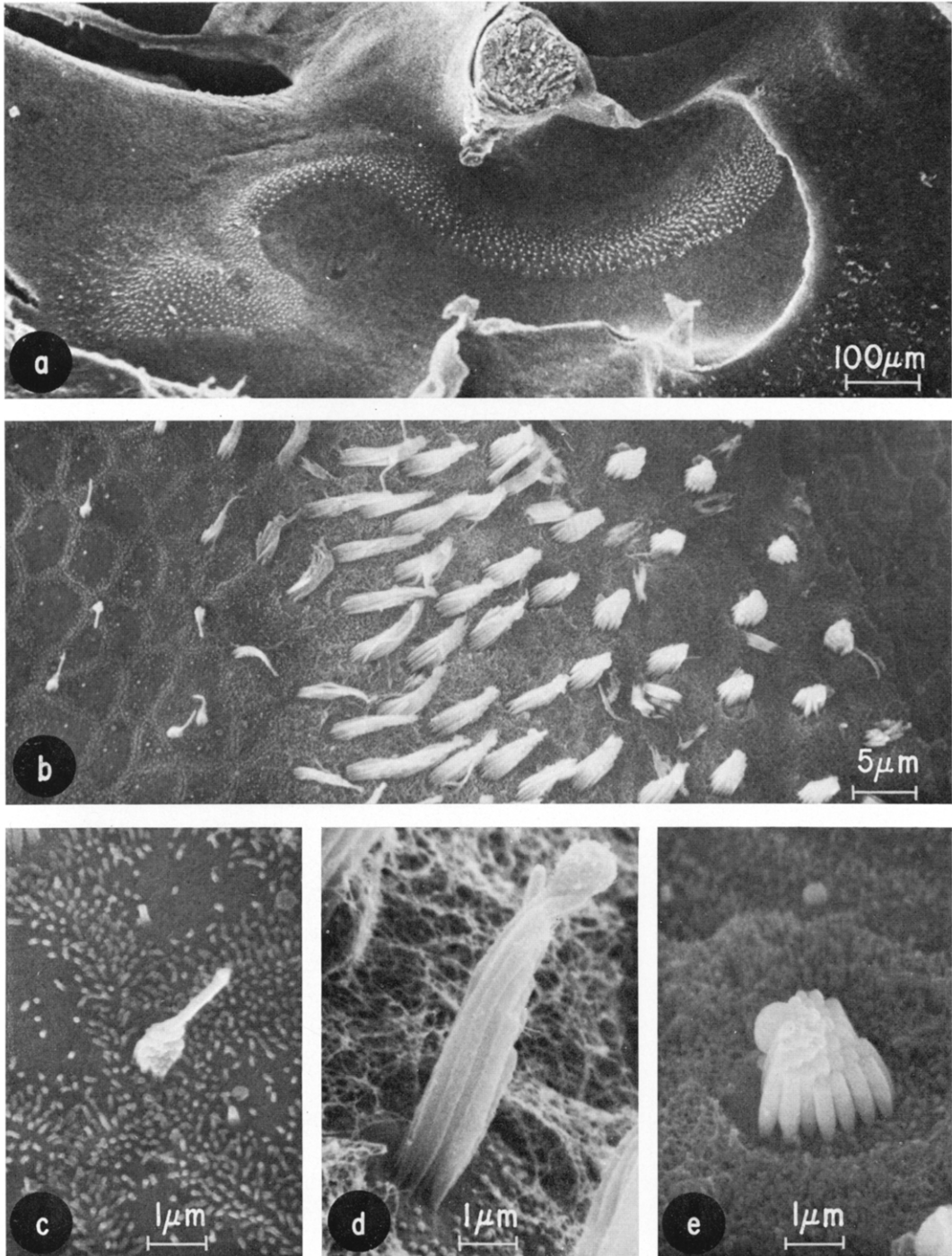
Fig. 5. Bullfrog amphibian papilla. a: entire papilla and cross-section of papillar branch of VIIIth nerve. b: entire papillar width, showing the 3 hair cell types (medial edge is on the right). c: lateral hair cell type (type A). d: mid-papillar hair cell type (type E). e: medial hair cell type (type D).
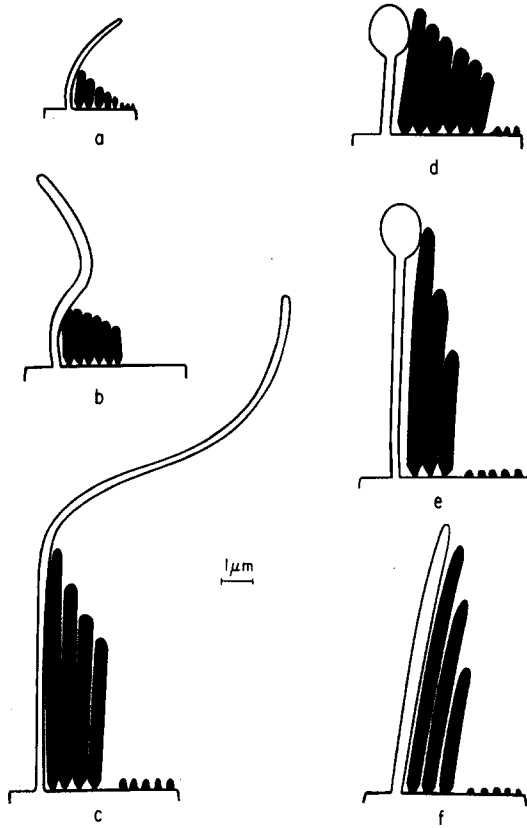
Fig. 6. Idealized cross-sections of the cilia and microvilli of the 6 hair cell types. a: type A. b: type B. c: type C. d: type D. e: type E. f: type F. (All drawn to same scale.)

are quite distinct, exhibiting markedly shorter kinocilia with bulbs (Figs. 1e and 2f). These are abundant in the lagena and sparse in the utriculus. Sparsely distributed in the central band of the utriculus, one also finds hair cells with short kinocilia, but no kinociliary bulbs (Fig. 1d). On either side of the central band in both maculae, the orientation of hair cells is quite uniform; the orientation reverses in the middle of the band, the large cells of the band itself being oriented in the same direction as the adjacent cells outside the band. In the utriculus, the hair cell orientation generally is such that the kinocilium is on the same side of the cell as the central band; in the lagena it is on the side opposite the central band. In other words, by the standard convention, the polarization of the utricular hair cell is directed toward the central band, while that of the lagenar hair cells is directed away from the band.

The vibrational macula (saccular), the vibrational and auditory papilla (amphibian), and the auditory papilla (basilar) all exhibit hair cells with short stereocilia and bulbed kinocilia no longer than the longest stereocilia (Figs. 3b, 4d and 5e). This is by far the predominant hair cell type of the sacculus, occupying the entire central region of the macula inside the first 2–4 rows of the perimeter. It also occupies
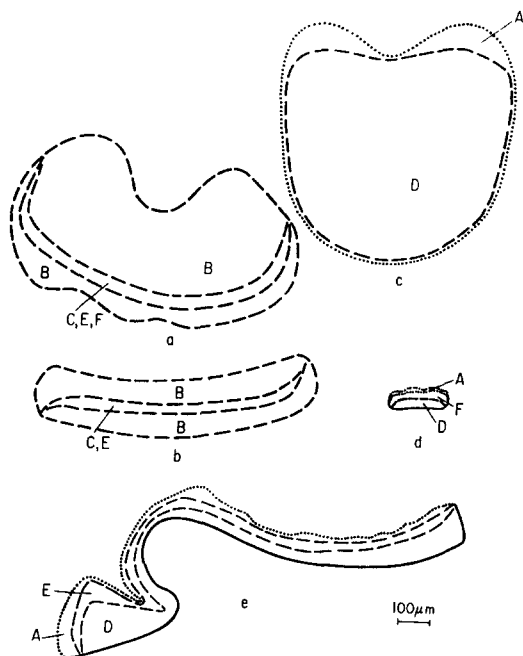
Fig. 7. Outlines of the 5 sensory surfaces, showing the distributions of hair cell types. a: utricular macula. b: lagenar macula. c: saccular macula. d: basilar papilla. e: amphibian papilla. (All drawn to same scale.)

the first 4 or 5 rows on the medial side of the amphibian papilla and the first 2 or 3 rows on the medial side of the basilar papilla (Figs. 3a, 4a and 5a). All three organs exhibit a second, variable type of hair cell, which typically has the appearance of a miniature version of the predominant hair cell of the utriculus and lagena (Figs. 3c, 4b and 5c). The stereocilia of this hair cell type often exhibit very small diameters, quite comparable to those of the surrounding microvilli. However, one usually finds a gradation of size and shape in this hair cell type, progressing from an extremely small version to a version that closely resembles the hair cells of the neighboring region (Figs. 3c and 5b). This variable type of hair cell occurs around the entire perimeter of the saccular macula and on the lateral edges of the amphibian and basilar papillae. The central 3 or 4 rows of the basilar papilla are occupied by a hair cell type very similar to one found sparsely on the central band of the utricular macula (Fig. 4c). The central 4 or 5 rows of the amphibian papilla are occupied by hair cells with bulbed kinocilia and relatively long stereocilia, very similar to those of the bulbed hair cells in the central bands of lagenar and utricular maculae (Fig. 5d).

The results are summarized in Figs. 6 and 7. Based on surface morphology, we can identify 6 hair cell types, as depicted in Fig. 6. Type A occurs along the perimeter of the saccular macula and the lateral edges of the amphibian and basilar papillae (Fig. 7c, d and e). Type B is the predominant hair cell of the utriculus and lagena (Fig. 7a and b). Type C occurs in the central bands of the utricular and lagenar maculae, along with type E, which also occupies the central region of the amphibian papilla

(Fig. 7a, b and e). Type D is the predominant hair cell of the sacculus and also occupies the medial regions of the amphibian and basilar papillae (Fig. 7c, d and e). Type F occurs in the central band of the utricular macula and occupies the central region of the basilar papilla (Figs. 7a and d).

The polarization of hair cells in the sacculus is very similar to that in the lagena, being directed away from a central line of polarization reversal; the type A cells are polarized in the same direction as neighboring type D cells[17]. In the basilar papilla, the polarization of all hair cells is approximately the same, being directed toward the medial (type D) edge of the sensory surface. The polarization patterns in the amphibian papilla are rather complicated, apparently stemming partially from the fact that two separate sensory surfaces in the tadpole join to form the single papilla in the adult[20]. The details of polarization in the adult and tadpole papilla are presented elsewhere[20]. The 3 hair cell types (A, E, and D) in a given region of the papilla all share a common polarization.

DISCUSSION

Although our classification of hair cells of the frog otolithic and auditory organs is somewhat arbitrary, we believe that if we have erred it is on the side of lumping rather than splitting. For example, if one looks closely at the central band of the lagenar macula one finds hair cells with a wide variety of kinociliary lengths, all of which we have lumped into type C (see Fig. 2d). Similarly, type A also represents a wide variety of sizes and shapes, as mentioned in the results. Furthermore, at the boundary between the medial and central regions of the amphibian papilla, one finds hair cells intermediate between type D and type E. Therefore, we believe that our divisions are minimal and that we should attempt to find explanations for the presence of *at least* 6 hair cell types in these organs.

As we see it, the presence of differences in hair cell surface topographies could be dictated by morphogenesis (*i.e.* different types might represent necessary steps in a morphogenetic sequence in which case one still faces the problem of explaining the necessity of the ultimate type in the sequence). It could be dictated by geometric relationship with the gelatinous superstructure (*e.g.* the superstructure topography might be specified independently and the hair cells required to conform to its variations), or it could be dictated by specialization of sensitivity (*i.e.* different hair cell surfaces might be specific adaptations with respect to stimulus modality). With the present evidence, one cannot eliminate any of these possibilities, but some interesting points can be made.

Type A hair cells, for example, occur along the edge of the amphibian papilla identified as being undifferentiated (*i.e.*, the growing edge of the macula)[11]. This same type of cell is found along the entire perimeter of the saccular macula, a region identified as being a growing edge of that organ[17]. Furthermore, in the basilar papilla, the amphibian papilla and the saccular macula, one finds a continuous gradation from the typical 'type A' size and shape to the size and shape of the hair cell type in the neighboring region (Figs. 3c and 5b), which is different in each of the three sensory

TABLE

SUMMARY OF MORPHOLOGICAL AND PHYSIOLOGICAL RELATIONSHIPS

Organ	Morphogenetic origin	Dominant hair cell types	Other hair cell types	Modality	Frequency response	Accessory structure	Innervation (VIIIth nerve branch)
Utricular macula	Pars superior	B	C,E,F	Gravistatic	Low	Otolithic	Anterior
Lagenar macula	Pars inferior	B	C,E	Gravistatic	Low	Otolithic	Posterior
Saccular macula	Pars inferior	D	A	Vibrational	Moderate	Otolithic	Anterior
Amphibian papilla	Pars inferior	D,E	A	Vibrational/ auditory	Moderate to high	Tectorial	Posterior
Basilar papilla	Pars inferior	D,F	A	Auditory	High	Tectorial	Posterior
Canal cristae (anterior)	Pars superior	Similar to C	Similar to A	Angular acceleration	Low	Cupula	Anterior
Canal crista (posterior)	Pars superior	Similar to C	Similar to A	Angular acceleration	Low	Cupula	Posterior

surfaces. This evidence tends to favor the possibility that type A is a morphogenetic precursor of other hair cell types and that its presence may be dictated by morphogenesis. The gelatinous superstructures apparently are bound to their respective sensory surfaces through a dense mat of fibers emanating from the microvilli of the supporting cells[12,13]. The absence of remnants of this mat from regions containing type A cells (Figs. 3c, 4b and 5c) suggests that the superstructure may be weakly attached, or not attached at all in those regions; which in turn might be interpreted to imply that type A cells are marginally operational or not operational at all with respect to transduction. Finally, Table I shows a correlation of 1.0 between the presence of type A cells and the presence of higher-frequency sensitivity (auditory or vibrational). This might imply that type A cells are themselves higher-frequency receptors, or that they are morphogenetic precursors of higher-frequency receptors.

No obvious morphogenetic relationships can be ascribed to type B hair cells. Their distributions seem to begin abruptly at the edges of the lagenar and utricular macula and to extend all the way to the central bands, where they cease abruptly. Both organs in which they occur are otolithic, but they do not occur in the third otolithic organ (the sacculus), so the correlation is not perfect. In fact, in Table I, the only perfect correlation involving type B cells is that with low-frequency sensitivity; type B cells are by far the most numerous hair cells on the two gravistatic maculae, and they occur only on those maculae.

Type C cells also lack obvious morphogenetic relationships with other hair cells, except that they appear to be exaggerated versions of the type B cell. The central bands of the lagenar and utricular maculae, in which type C cells occur, appear to correspond to the striolae of mammalian utricular and saccular maculae[22]. Like these central bands, the striolae exhibit hair cells with larger luminal surfaces and more sparsely distributed than those of the surrounding regions, and they contain the loci of reversal of hair cell orientation. In mammals, the striolae also exhibit distinctive superstructure geometries[22,34]. The same sorts of geometric distinctions were not seen in the bullfrog lagena and utriculus, but the gelatinous membrane in those organs did tend to adhere very strongly and specifically in the 'striolae' after fixation in osmium alone, so much so that it could not be removed from those regions without severe damage to the underlying epithelium. Thus it is possible that the striolae serve as anchors for the gelatinous membrane and that the striolar hair cells are modified accordingly, either to help with the anchoring function or to carry out specific sensory function in the face of it. It also is possible that the hair cells of the lagenar and utricular striolae respond to different stimuli than do those of the rest of the maculae. Lowenstein and Roberts[23] found functional differences between regions of the utricular and saccular maculae of the ray, but the divisions of function apparently did not involve the striolae. If receptors other than gravistatic hair cells occur in the lagena and utriculus, they went unnoticed by McNally and Tait[25] and by Ross[29], but one certainly cannot exclude that possibility. On the other hand, type C hair cells are quite similar in general shape to the predominant type B cells of the lagena and utriculus, which very likely are gravistatic, and also to the hair cells of the cristae, which in the

bullfrog apparently respond to static deformation of the cupulae[27] and thus are low-frequency receptors.

The morphogenesis of type D cells is not altogether clear. In the sacculus, they seem to be derived directly from type A cells. In the basilar papilla and amphibian papilla, however, their distributions border on those of type F and type E cells, which may be morphogenetic intermediates between types A and D. Type D cells occur in regions that exhibit remnants of dense fibrous mats, indicating strong attachment of the gelatinous superstructure. Furthermore, strong connections appear to exist between the gelatinous membrane and the kinociliary bulbs of type D cells in the sacculus[13]. We have not yet looked for similar attachments in the two papillae. In Table I, the only perfect correlation involving type D cells is that with higher-frequency sensitivity, auditory and vibrational.

Type E cells are enigmatic. They have the appearance of exaggerated type D cells, suggesting possible vibratory or auditory sensitivity, but they occur not only in the vibrational/auditory amphibian papilla, but also in the striolae of the presumedly gravistatic lagenar and utricular maculae. Perhaps they serve an anchoring function in the striolae. The observation that the gelatinous membrane is rather strongly connected directly to the kinociliary bulbs of saccular hair cells, which in turn are connected to the longest stereocilia[12,13], coupled with the observation that hair cells apparently are compliant in response to shear directed down the gradient of stereocilia lengths and resistant to shear in the opposite direction (i.e. directed toward the kinocilium)[19], suggests that oppositely oriented, bulbed hair cells in the middle of the striola (such as those shown in Fig. 2f) could serve as a very effective anchor against shearing motion normal to the midline of the striola. This leaves unanswered the question of why this same type of hair cell is so numerous in the amphibian papilla, which has a large number of bulbed hair cells of another type. Perhaps, in the latter case, their presence is dictated by the geometry of the tectorial membrane. On the other hand, Frishkopf et al.[8] observed both vibrational and auditory units which they attributed to the amphibian papilla. It is possible that this dichotomy of sensitivities corresponds to the predominance of two distinct hair cell types in the amphibian papilla.

Type F hair cells, which occur in the basilar papilla and the utricular striola, are even more difficult to explain. Perhaps in the utricular striola these rather sparse hair cells are transitory morphogenetic intermediates that may become either type C or type E. On the other hand, they make up at least 3 of the approximately 7 rows of hair cells in the basilar papilla and clearly are not transitory intermediates there. In fact it seems extremely likely that these relatively populous hair cells participate in the observed auditory function of the papilla.

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