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REGULAR ARTICLE

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Intracellular vesicular trafficking in the gill epithelium of urea-excreting fish

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Abstract Most teleost fish are ammoniotelic, and relatively few are ureotelic, in which the majority of nitrogenous waste is excreted as urea. This study aimed to determine whether the gill ultrastructure of ureotelic fish might have specific, unique characteristics compared with ammoniotelic fish. The gill morphology was studied in three closely related species of the family Batrachoididae: *Opsanus beta*, the gulf toadfish; *Opsanus tau*, the oyster toadfish; and *Porichthys notatus*, the plainfin midshipman, because prior studies have demonstrated that the two former species are ureotelic and excrete urea in unique, short daily pulses, whereas the latter is ammoniotelic. Ultrastructural studies demonstrated significant trafficking of dense-cored vesicles (50–200 nm) between the Golgi apparatus and the apical membrane of epithelial cells surrounding gill filaments and lamellae in these two *Opsanus* spp. The material constituting the core of these vesicles was intensely stained by lead salt and was unloaded externally when vesicles contacted the apical membrane. Another characteristic of these urea-secreting fish was the presence of numerous large, black-stained lysosomes, which contained cored vesicles, suggesting a second destination for the dense-cored vesicles. As a working hypothesis, the present data suggest that the urea-transporter protein, recently found in toadfish gills, is inserted in the vesicle. Subsequently, it could serve to either sequester cytosolic urea that ultimately is secreted into the water after contact of these vesicles with the pavement cell apical membrane, or it could allow facilitated diffusion of urea across the plasma membrane following insertion into the membrane. As further comparative evidence, the ammoniotelic *P. notatus* exhibited neither the vesicular trafficking nor the population of lysosomes both found in *Opsanus* spp.

Keywords Gill epithelium · Ultrastructure · Pavement cell · Lysosome vesicle trafficking · *Opsanus beta* · *Opsanus tau* · *Porichthys notatus* (Teleostei, Batrachoididae)

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Introduction

The nitrogen excretion physiology of the marine toadfish has received intense attention recently (reviewed by Walsh 1997). The two species that have been most extensively studied are the gulf toadfish (*Opsanus beta*), which inhabits the coastal waters of Florida and the Gulf of Mexico, and the oyster toadfish (*Opsanus tau*), which inhabits the north-east coastal waters of the USA. Unlike almost all other teleosts which are ammoniotelic (and thus excrete the majority of nitrogenous waste, "N-waste," as ammonia), these two toadfish species express a complete ornithine-urea cycle in their liver and are capable of complete ureotelism in some circumstanc-

es. At such times, the majority of nitrogenous waste is excreted as urea. Conditions known to induce ureotelism in toadfish include high ammonia exposure, crowding, and confinement in small but flowing volumes of water (reviewed by Walsh, 1997; see also Wang and Walsh 2000). In the wild it is thought that *O. beta* excretes more than 50% of N-waste as urea (Hopkins et al. 1997). For the purposes of the present study, the most interesting characteristic of ureotelism in *Opsanus* spp. is that 90% of excreted urea occurs in pulses of short duration (30 min to 3 h), typically about once a day (Wood et al. 1995). This pulsing behavior reflects the expression of a pulsatile excretion mechanism which quickly lowers body urea stores, rather than a pulsatile production mechanism as the rate of urea production by the liver stays constant (Wood et al. 1997). Recent evidence suggests that the gills are the site of pulsatile urea excretion in toadfish (summarized by Gilmour et al., 1998). However, the gill cell type(s) responsible for urea excretion have not yet been identified.

The fish gill epithelium is composed of several cell types, of which chloride cell (CCs; reviewed by Jurss and Bastrop, 1995; Perry 1997) and pavement cells (PVCs) are generally the most prevalent (Laurent et al. 1994; Goss et al. 1994, 1998). PVCs constitute the outermost layer of an epithelium, which is generally bilayered over the lamellae and multilayered over the filaments. PVCs are flat, large, thin cells on the lamellae, but more cuboidal on the filaments. Their apical membrane displays various extensions, including folds, ridges, and villi that may impart a variety of functional processes. Based on substructural features (endoplasmic reticulum, Golgi apparatus, varied vesicles), these cells probably represent several subtypes that have varied functions.

In freshwater fish there is evidence that PVCs, in addition to operating as an osmotic and protective barrier, also contribute to ionic exchanges between the fish and its external medium. In particular, recent work suggests that CCs are the site of Cl^-/HCO_3^- exchange, whereas PVCs are concerned with Na^+ and H^+ movements (Morgan et al. 1994; Sullivan et al. 1995, 1996). Thus, both cell types contribute to acid-base and ionic regulation in freshwater fish (Perry and Laurent 1993; Laurent and Perry 1995). In seawater fish, the function of the CC is well established (reviewed by Marshall and Bryson, 1998), whereas the role of the PVC is unknown and indeed ignored in most models. Thus, PVCs have not, so far, been implicated in the literature in any specific function in seawater fish, including urea excretion. This may reflect the general paucity of details concerning the ultrastructure of PVCs in seawater fish (Laurent 1984).

Therefore, the aim of the present study was to examine the ultrastructure of the batrachoidid fish gill epithelia (CCs and PVCs) in relation to their particular physiology. Specifically, our goal was to determine whether excretory pulses of urea were associated with any unique gill cell ultrastructure or changes in this ultrastructure. A comparison was made with a relevant species, the plainfin midshipman *(Porichthys notatus*), a close relative of the toadfish (i.e., another member of the family Batrachoididae) living on the west coast of North America. Unlike the ureotelic toadfish, *P. notatus* is ammoniotelic, excreting less than 10% of its N-waste as urea, and importantly it does not exhibit pulsatile urea excretion (Wang and Walsh 2000).

Materials and methods

Sexually mature specimens of gulf toadfish (90–203 g) were captured from Biscayne Bay (Florida), in May 1996 and November to December 1998, and treated as previously described (Wood et al. 1995). Mature oyster toadfish (64–100 g) were obtained from the Marine Biological Laboratory (Woods Hole, Mass.) and again treated as previously described (Wang and Walsh 2000). Both species were first held in large tanks of running, aerated seawater and then subjected to the confinement protocol of Wood et al. (1995) to induce pulsatile ureotelism. Water samples in confinement were taken every hour by a fraction collector/pump system and analyzed chemically for urea and ammonia as previously described (Wood et al. 1995). In some experiments where it was necessary to detect and kill fish that were in "midpulse", 1.5–3.7 kBq of [^{14}C]urea were injected through an indwelling catheter inserted prior to experimentation into the caudal artery (see Wood et al. 1995). In these fish urea excretion could be monitored at 30- to 60-min intervals by rapid liquid scintillation counting of water samples, with a delay time of only about 15 min between sampling and detection. By using one of either method, fish could be identified as either pulsing or being at a particular time since the previous pulse. After the pulsing pattern was determined, fish were killed using an overdose of tricaine (MS-222) in water (0.5 g/l; fish typically succumbed within 2 min with minimal struggle). Fish were kept on ice all the time during further tissue processing. Immediately following death, gill arches (right side), were excised from each fish and quickly rinsed in ice-cold 0.15 mol l^{-1} sodium cacodylate. The individual gill filaments were then carefully dissected away from each gill arch in the buffer. Only the anterior and posterior rows of filaments remained attached to the septum of the arch. Each piece was then fixed in 5% iced glutaraldehyde in cacodylate buffer for 1 h and processed for transmission electron microscopy (TEM; Siemens Elmiskop 101 and Jeol 200TM) or scanning electron microscopy (SEM; Cambridge Stereoscan 100) and ESEM (Philips XL30) as outlined by Laurent et al. (Laurent and Hebibi 1990; Laurent et al. 1994). Morphometric analysis was performed using a lattice test system (Weibel et al. 1966) or morphometry software on silver photos (Sigma Scan, Jandel) or on digitized photos (Scanpro, Jandel) according to protocols described previously (Laurent and Hebibi 1990; Laurent et al. 1994, 2000).

Pacific midshipman (*P. notatus*) were caught by otter-trawl close to Bamfield Marine Station (British Columbia, Canada) in August 1999. At the Station, they were held in large groups in sand-covered tanks served with flowing seawater for approximately 1 week prior to being killed. They were then transferred to flux chambers, experimentally confirmed to be excreting predominantly ammonia (see Results), and then killed and prepared for morphological examination of the gills in the same manner as above.

These studies examined 50 specimens of *O. beta*, 10 specimens of *O. tau*, and 6 specimens of *P. notatus*. Differences between pulsing and nonpulsing fish were analyzed for statistical significance, when appropriate, by means of a two-sample Student's *t*-test. The fiducial limit of significance was 5%.

Results

The gross organization of toadfish gills did not differ from that of other marine teleosts. A noteworthy feature,

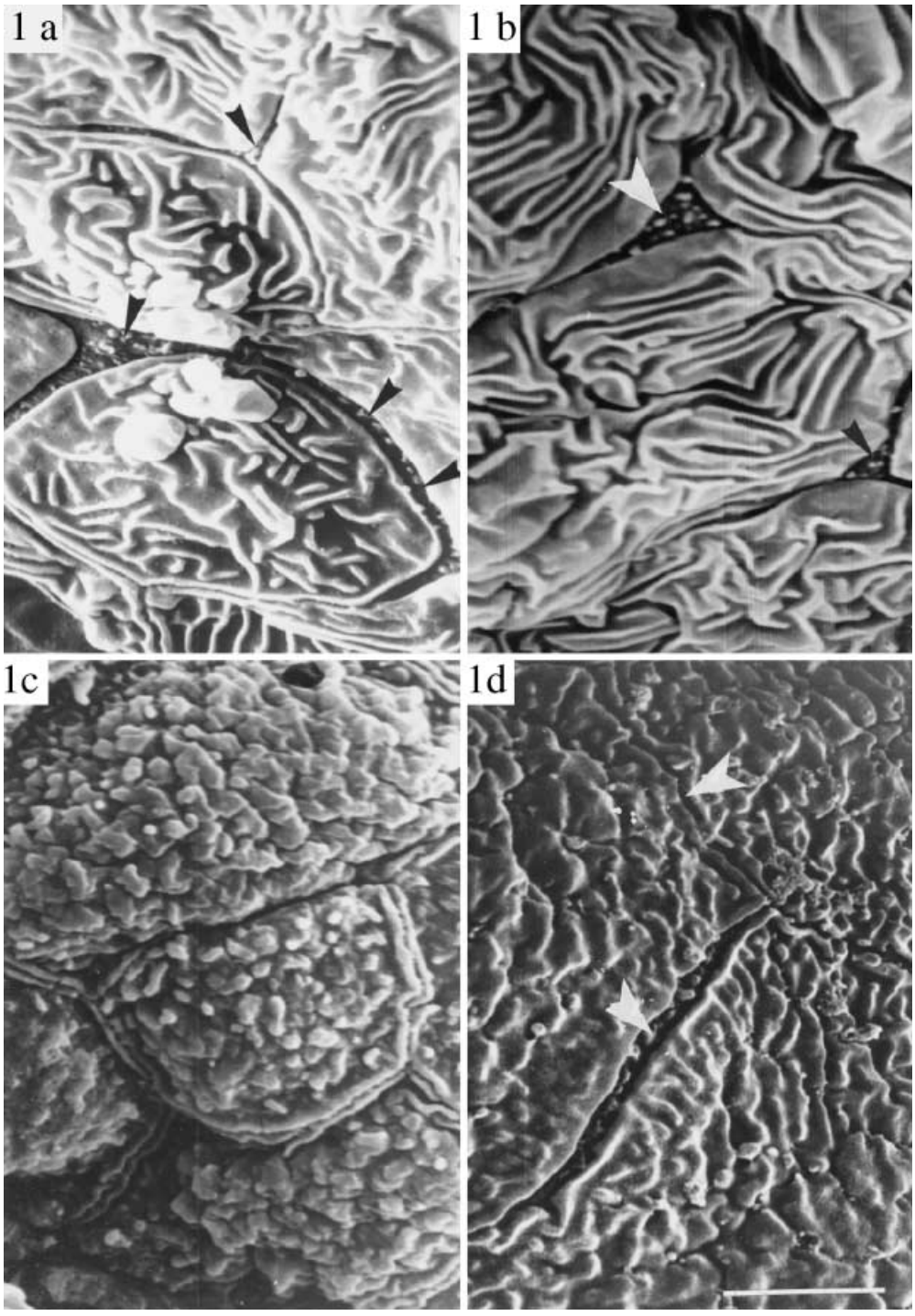


Fig. 1a–d Pavement cell apical surface ornamentation of the lamella. Ridges, foldings, and villi cause a huge amplification of the contact area between the fish and its environment. Comparisons between ureotelic non-pulsing (**a**) and pulsing (**b**) *Opsanus beta* did not reveal any significant difference in ornamentation density. *O. tau* (**c**) and *Porichthys notatus* (**d**), respectively less ureotelic and more ammoniotelic, had more ornamentations. Note the location of chloride cells (*arrowheads*) between and beneath the pavement cells. *Bar* 10 µm

however, was the high density of CCs on the lamellae. CCs in the three species studied conformed to the seawater type, being flanked by an accessory cell having cytoplasmic processes inlaying the CC apical surface. The interposition of CCs and PVCs as depicted in Fig. 1 was somewhat unusual.

The PVC organelles

In this study, our attention was focused on the PVCs, and the descriptions given hereafter concern both *O. beta* and *O. tau*, which appeared to be similar. (*P. notatus* will be considered separately.) These flat cells were morphologically variable according to their location. On the filamental epithelium, the PVCs' individual mean apical surface area was approximately 100 µm^2, whereas on the

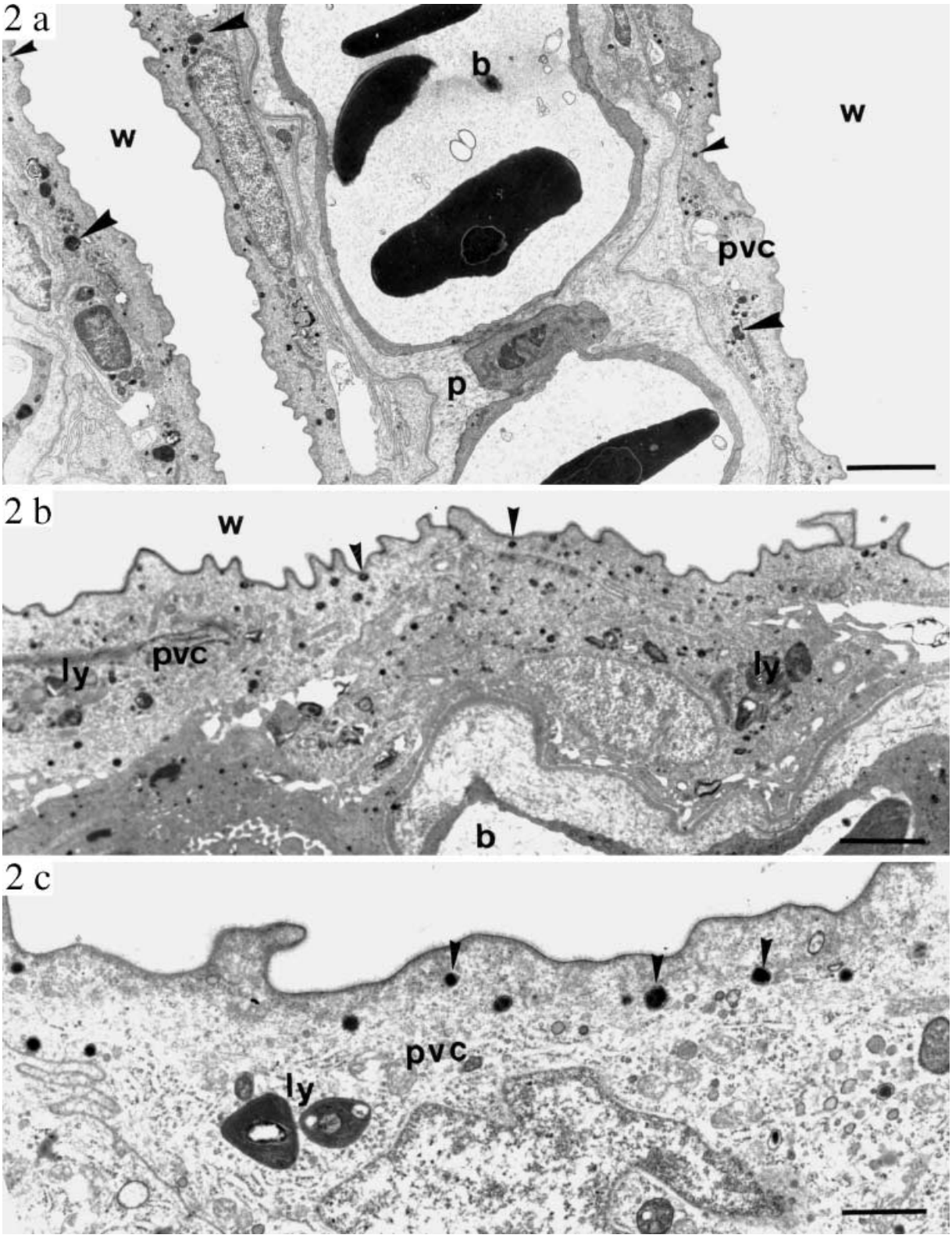


Fig. 2a–c Representative transmission electron-microscopic gill lamella sections of pavement cell in nonpulsing (**a**) and pulsing *Opsanus beta* (**b**) and pulsing *O. tau* (**c**). Note the dense-cored vesicles and lysosomes (*large arrowhead*; *ly*) *Bars* **a** 4 µm; **b** 1 µm; **c** 1 µm

lamellar epithelium it was close to 200 µm^2. This apical surface was ornamented with concentric ridges on the filament, but with microvilli or short ridges on the lamella. The density of this ornamentation was greater in *O. tau* than in *O. beta* (Fig. 1a–c). The apical membrane was strongly osmiophilic and bore a dense coat of fuzzy

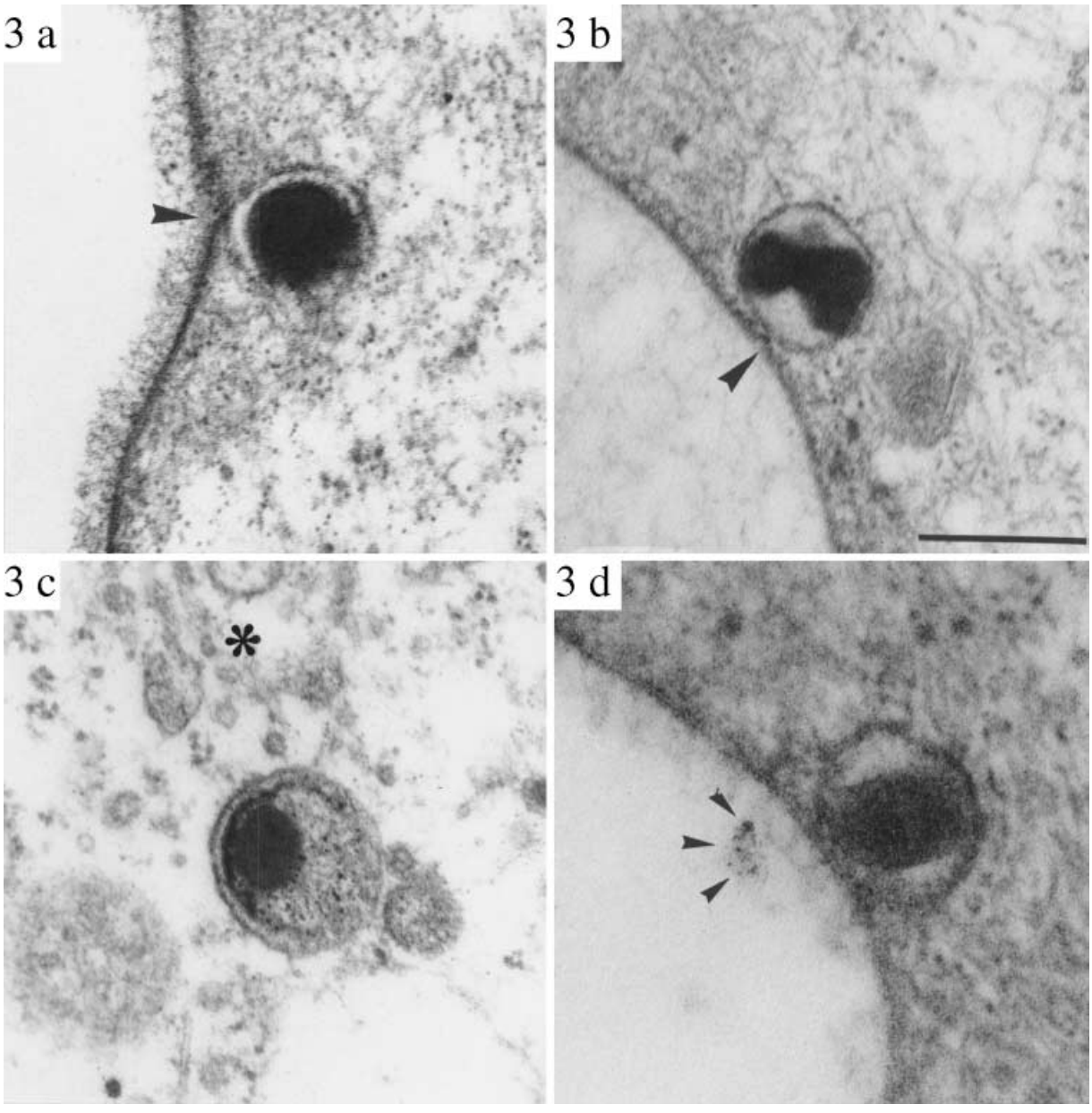
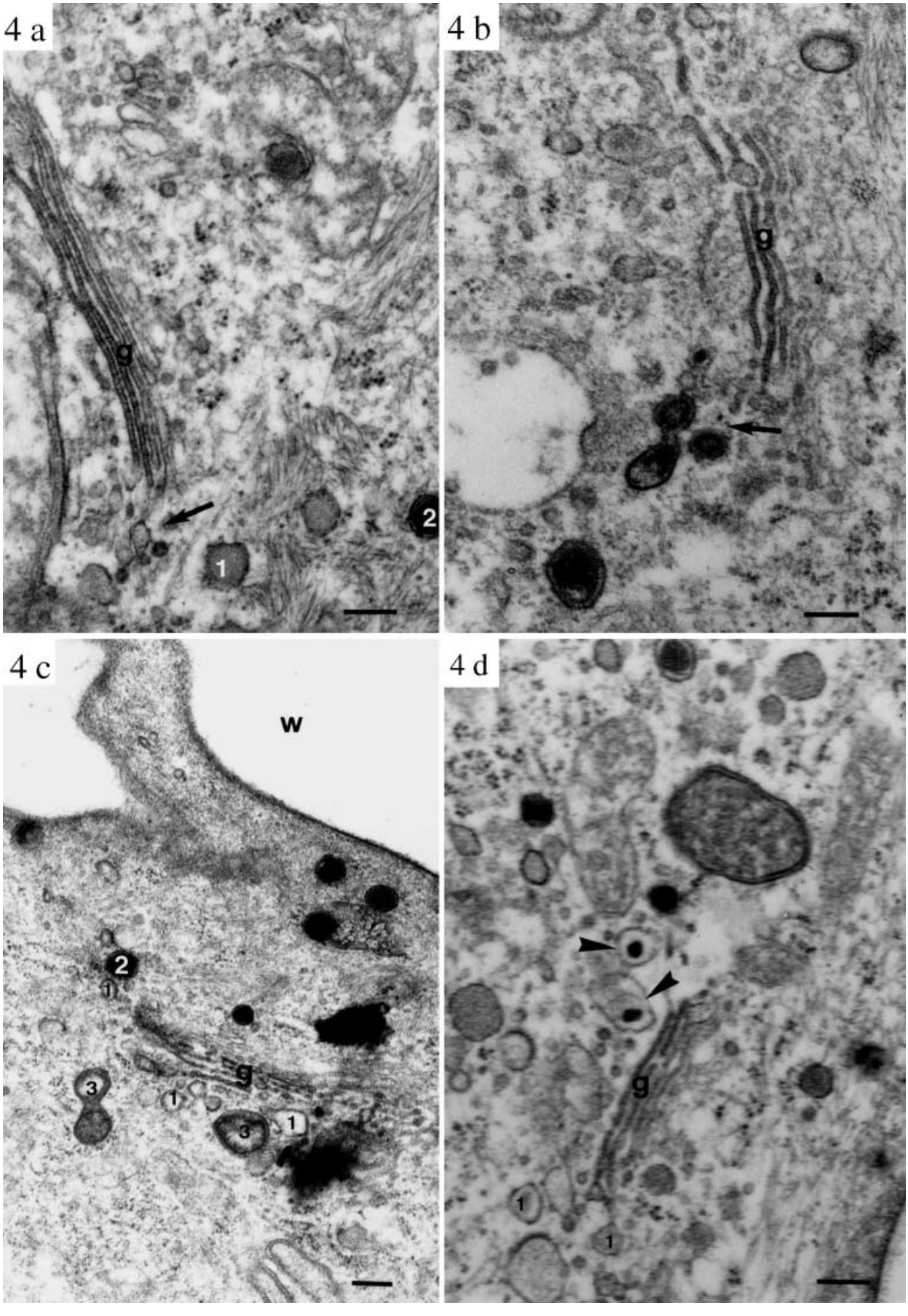


Fig. 3a–d Dense-cored vesicles in gill pavement cell of *Opsanus beta* (**a**) and *O. tau* (**a–d**). In **a** and **b**, the apical membrane of the cell is slightly depressed facing the vesicle (*arrowhead*), suggesting the existence of a pore. In **a** the vesicle was still full of black material. In **b** the vesicle was partly empty. In **c** this vesicle close to Golgi cisternae (*asterisk*) appears to have been scalloped by the diamond knife, showing its structure. Note the lucent space beneath the vesicle membrane. On its right, a small, clear, empty vesicle. Further to the right, a small vesicle displays a dense core. In **d**, just a thin operculum separates the inner side of the vesicle from the external medium, and some black material, presumably secreted, is fixed and stained externally. *Bar* 200 nm

material (glycocalix); however, due to the sample processing this coat was not always preserved. After lead-uranyl staining, a conspicuous feature of the toadfish pavement cell was the presence, in variable density, of cored vesicles of 100–200 nm (Fig. 2), and these vesicles were not present in the CCs. These vesicles (which we designate "type II") were very black after staining and were already visible on unstained sections of osmicated samples, indicating a strongly osmiophilic content. They displayed three layers from the outside: (1) a thick (10 nm) bilayered membrane, supporting discrete units, and (2) a lucent area of variable thickness, surrounding (3) a black core (Fig. 3). The shape of this core was variable: sometimes spherical with a thin lucent area, or lobular when the core was small, suggesting variable vesicle filling (Fig. 3b). The type II vesicles (often seen as dense-cored vesicles throughout this study) were mostly located in an area bounded by the Golgi zones and the apical membrane of PVCs (Fig. 3). Often, some of them were seen closely contacting the apical membrane.

Vesicles of another type, from 50 to 200 nm in diameter, were intermingled with the type II vesicles (Fig. 4a–c). These vesicles (which we designate as "type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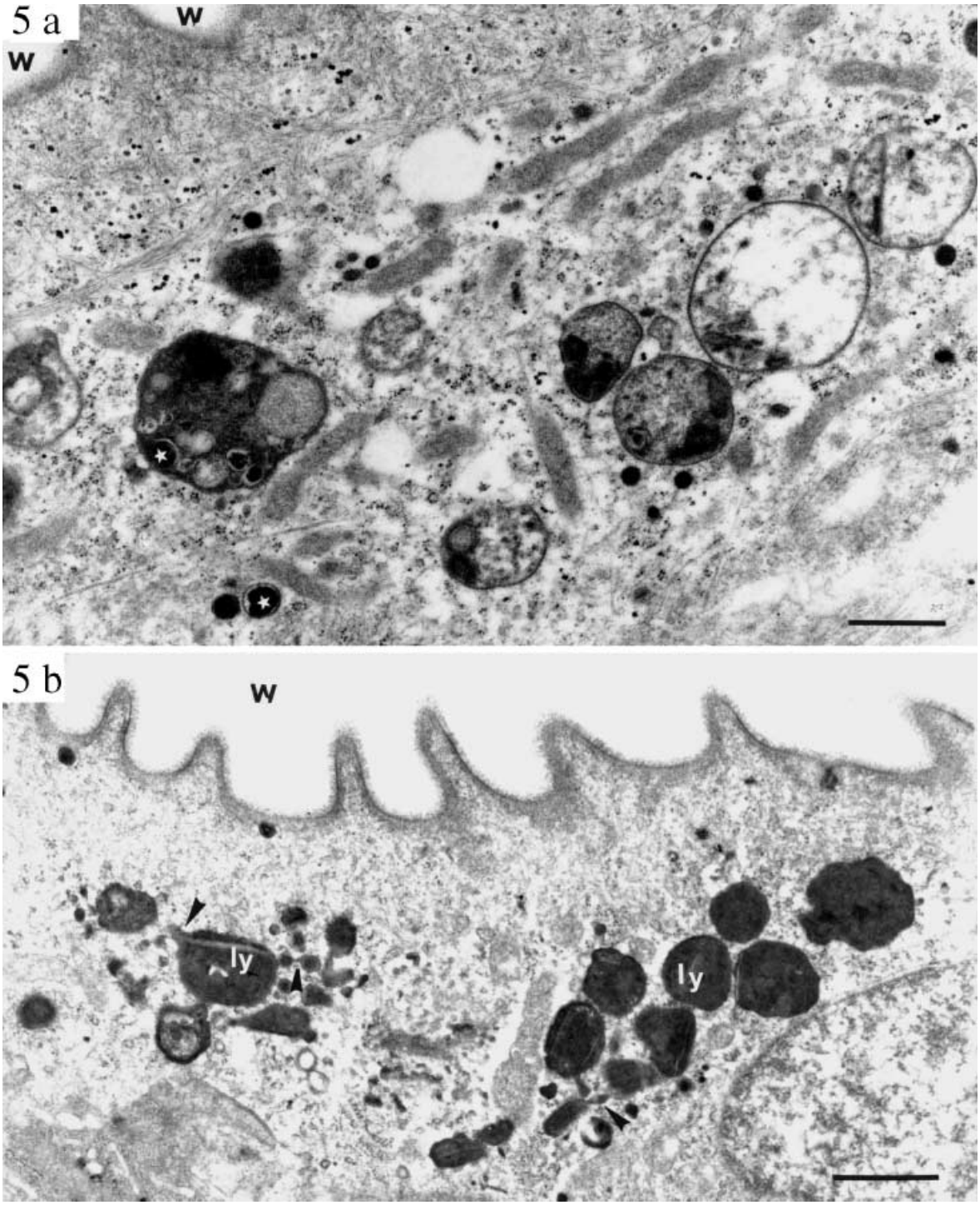


◀ **Fig. 4a–d** Representative transmission electron micrographs of the relationships between the Golgi apparatus (*g*) and the cored (type II) and noncored (type I) vesicles in pulsing *Opsanus tau* pavement cells. The budding was very active (*arrow*), giving rise to type I vesicles (*1* in **a**, **c**) and a *chaplet* of rapidly growing type II vesicles (**b**). **c** Presumed developing secondary lysosomes (*3*) and collections of black material. **d** The starting steps of nucleation of type II vesicles (*arrowhead*). Note the presence of discrete units on the membrane of the type I vesicle. *Bar* 200 nm

Fig. 5a, b Lysosomes were extremely numerous in pavement cells of *Opsanus beta* and *O. tau*, but, as shown in Fig. 7c, d, were scanty in *Porichthys notatus*. We found a spatial association between lysosomes and vesicles in *O. beta* and *O. tau*. In **a** (*O. beta*, nonpulsing), dense-cored vesicles are trapped inside a large lysosome on the *left*, suggesting that when the fish is not pulsing, cored vesicles are stored in lysosomes. Conversely, **b** (*O. beta*, pulsing) could be interpreted as illustrating that dense-cored vesicles are released by budding from the lysosome (*arrowhead*) and finally reach the apical membrane; *arrowhead* points to *chaplet* of vesicles budding from the lysosome (on the *left*). *Bar* 500 nm

Table 1 Density of type II vesicles in pavement cells (*PVC*)

Opsanus beta pavement cell	Number of PVC	Vesicle II density/ 100 µm² of PVC profile	Mean vesicles number within 0.5 µm of PVC apical membrane	Percentage of vesicles II within 0.5 µm of PVC apical membrane
Not pulsing	29	37.09±5.37	4.25±0.57	11%
Pulsing	37	63.77±5.60*	11.00±0.91*	17%
Porichthys notatus	15	<1	<1	<1

*P<0.001, two-sample Student t-test

I") exhibited small dense granules on their membrane (Fig. 4a, b). In contrast with type II, type I vesicles were apparently empty or filled with a clear (less opaque) material and often were seen budding from the Golgi cisternae (Fig. 4b, d). In some instances, some clear vesicles displayed a dumbbell shape, suggesting a process of partition (Fig. 4c). A third vesicle type (which we designate "type III"), consisting of large (often more than 1 µm) polymorphic membraned vacuoles, was concentrated in the most internal regions of the cell. By shape and internal structure, these vacuoles were identified, according to Alberts et al. (1983), as secondary lysosomes, containing heterogeneous patchy material of variable opacity, and membraned vesicles (Fig. 5). It is worth noting that dense-cored vesicles were readily recognizable inside the secondary lysosomes (Fig. 5a, white star). This observation suggests that type II vesicles (dense cored) might be considered as organelles determined under the classic name of primary lysosomes (Alberts et al. 1983). Another observation worth noting is that vesicles are filled with a black core of variable size (Fig. 4d, arrowheads).

Another significant feature of the toadfish pavement cell was an extreme abundance of Golgi stacks (Fig. 4). Several Golgi profiles were often visible on the same PVC ultrathin section. Golgi cisternae were generally surrounded by a mixed population of vesicles of variable size, both clear and dense, which we consider as a mix of type I and II vesicles (Fig. 4d). As usual, rough and smooth endoplasmic reticulum were seen close to the nucleus.

Relationships with urea excretion pulse

In the above description of type II vesicles, we pointed out their varied numerical density. Indeed, it was apparent clearly that the number of cored vesicles, and particularly the number located near the PVCs' apical membrane, varied amongst fish. Moreover, we suspected that the occurrence of a pulse of urea excretion was coincident with an increased density of type II vesicles. Therefore, by continuously monitoring urea excretion, we took separate sets of samples to compare pulsing and nonpulsing fish. Fish examined in the present study clearly conformed to previously published patterns of nitrogen excretion, in that *O. beta* and *O. tau* were predominantly ureotelic, whereas *P. notatus* was not (values are means ± SE: *O. beta* 181±20 µmol urea-N kg^{-1} h^{-1}, 22 µmol ammonia-N kg^{-1} h^{-1}; *O. tau* 90±22 µmol urea-N kg^{-1} h^{-1}, 28±10 µmol ammonia-N kg^{-1} h^{-1}; *P. notatus*, 10.1±2.9 µmol urea-N kg^{-1} h^{-1}, 208.6±51.8 µmol ammonia-N kg^{-1} h^{-1}). To more precisely establish relationships between vesicle traffic and time elapsed after a urea pulse, we also sampled at 6 h, 8 h, and 12 h postpulse. Some uncertainty was unavoidable with respect to the coincidence of fixation and the peak of urea pulsing; a time lag of unknown duration (but probably not greater than 15 min) separated urea detection in the surrounding water from the collection of gill tissue.

The most striking difference between nonpulsing and pulsing fish concerned the numerical density and location inside the pavement cell of the dense-cored type II vesicles (Fig. 2) and therefore numbers of type II vesicles per unit of cell profile area were determined (Table 1). In *O. beta*, the dense-cored vesicles were significantly more numerous during urea pulses (Table 1). The number of dense-cored vesicles located close to the apical membrane (within 500 nm) was also significantly greater (Table 1), suggesting that when the fish were excreting urea there was an increased trafficking of vesicles from the Golgi area to the apical membrane. Close "apposition" of the dense-cored vesicles to the plasma membrane was frequently observed during urea pulses. Note that we have used *apposition* instead of *fusion*, because we did not observe the "omega-shape" image of membrane fusion characteristic of exocytosis as it was originally described by Palade and coworkers (Palade 1975). A narrow pore was observed at the point of contact between the vesicle and the PVC apical membrane (Fig. 3a–b). The fate of the vesicle membrane after voiding should also be considered. Classically, a true fusion process involves insertion of new material in the plasma membrane, causing an amplification of the apical plasma membrane. As representatively shown by the scanning electron micrographs of Fig. 1, the present study revealed a high density of various indentations on the PVC apical membrane, but no difference between pulsing and nonpulsing *O. beta* (Fig. 1a, b).

Since the vesicle membrane does not appear to be inserted in the apical membrane (i.e., no fusion), we hypothesized potential mechanisms for retrieving this vesicular membrane material and found ultrastructural evidence to support two mechanisms. Firstly, empty vesicles, appeared to be recycled, suggesting that they might function as a back-and-forth shuttle between the cytosol and the apical membrane. This process involved the rela-

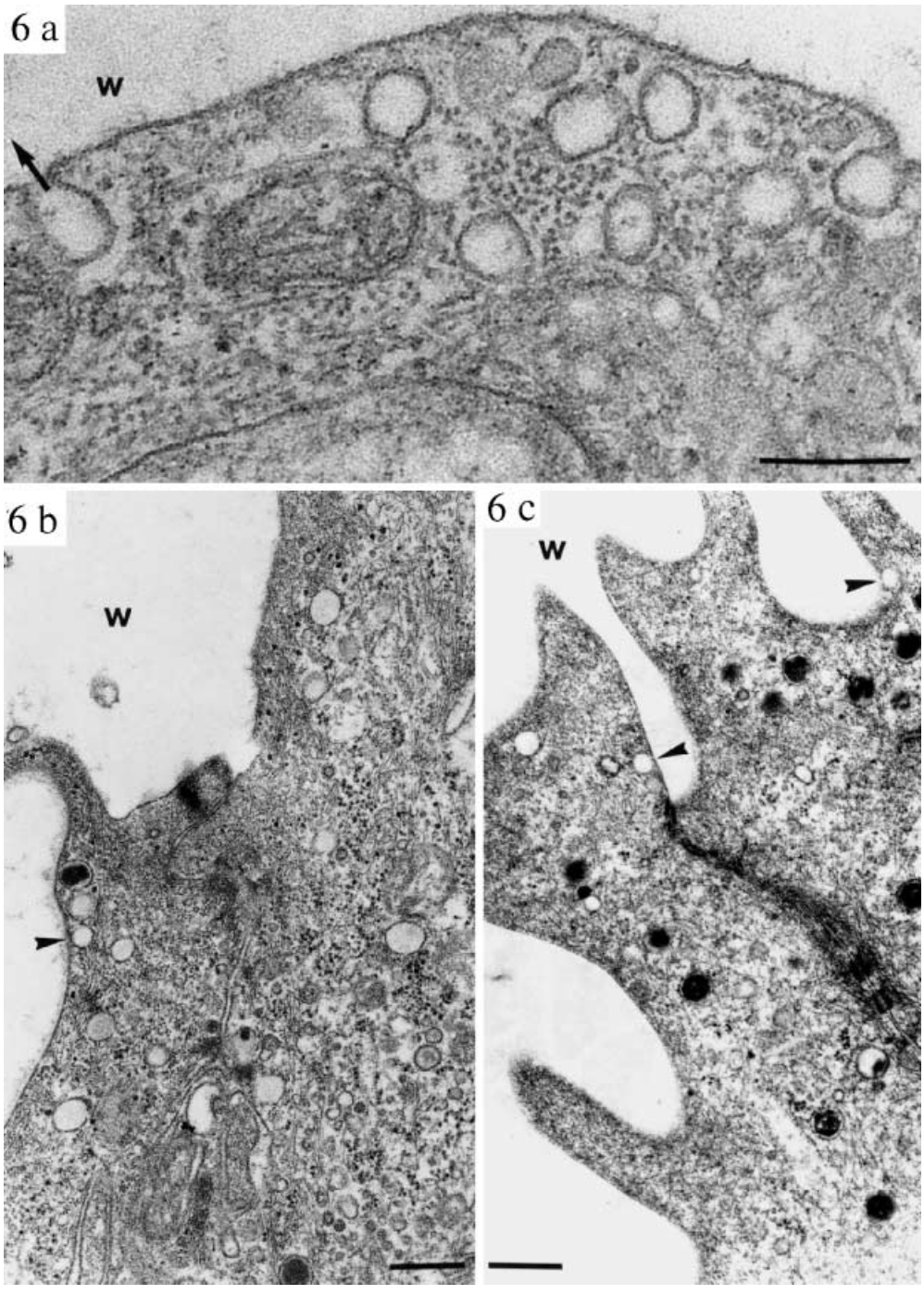
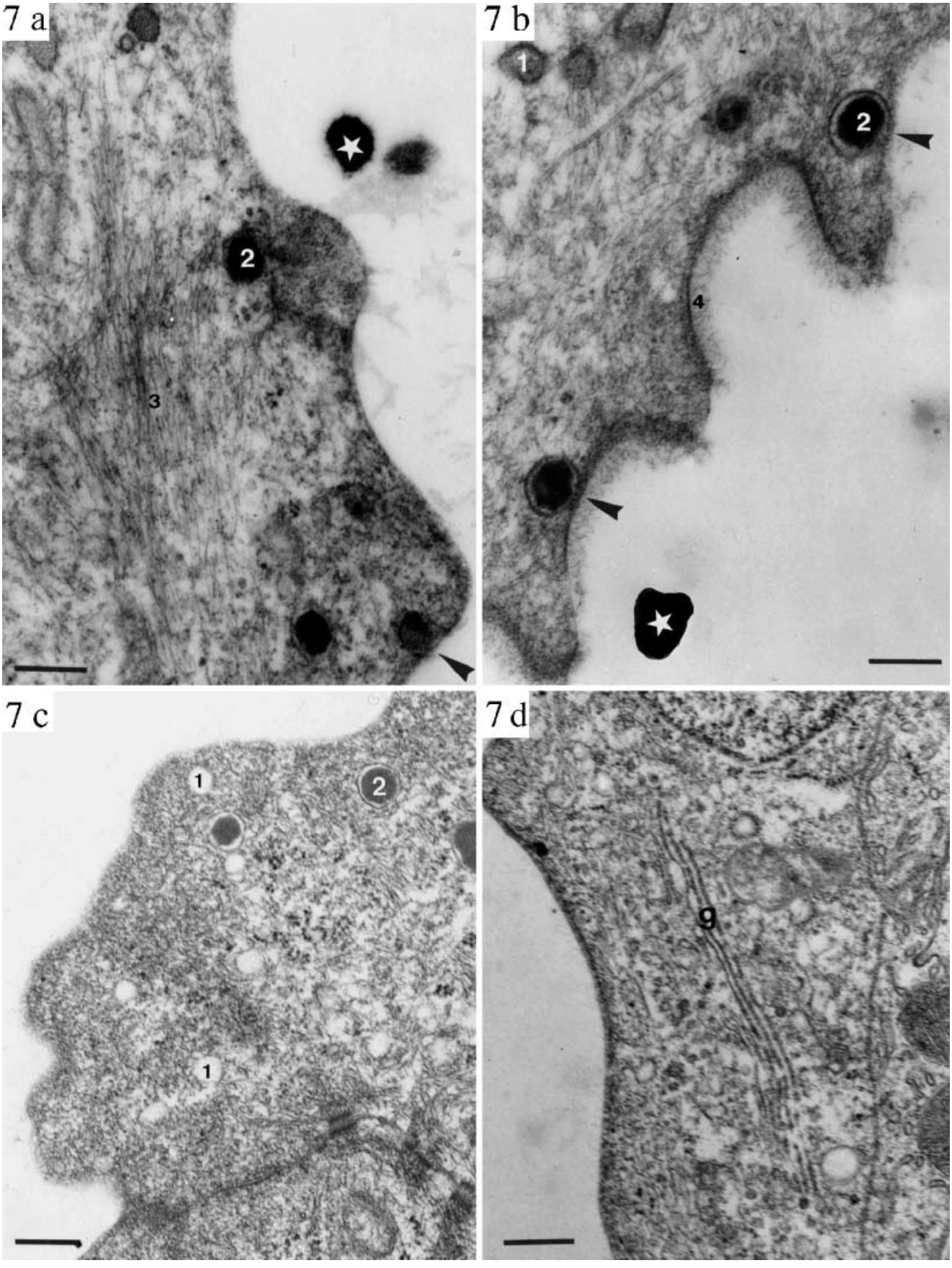


Fig. 6a–c Like dense-cored vesicles (type II), clear vesicles (type I) were found in varied locations: close to or even apposed on the pavement cell apical membrane, or deeper inside the cell surrounding the Golgi apparatus and lysosomes. Thus, the main question which arises is whether these clear vesicles are part of the dense-cored vesicle trafficking (empty or not yet refilled) or form by themselves a distinct trafficking. **a** A population of clear vesicles within the range of 70 nm in diameter, close to the apical membrane, some of these clearly opening externally. **b** A large population of clear vesicles intermingled with dense-cored ones. Both pictures came from a specimen (*O. tau*) that had last pulsed 6 h earlier; **c** was obtained from *O. tau* during pulsing. Note that the dense-cored vesicle density is higher than in **b**, whereas the density of clear vesicles is much lower. Some of the vesicles apposed to the pavement cell membrane are incompletely filled with dense material. *Bar* 200 nm

tionship between the cored vesicles (type II) and the clear vesicles (type I). When a type II vesicle was attached to the apical membrane, its dark core was variably lysed, disorganized, or absent (Fig. 6). In some instances, black material located outside of, but close to, the membrane as it exited the vesicle was fixed and stained (Fig. 7a, b). Type II vesicles observed at this time were very similar to the type I vesicles which were frequently observed beneath the apical membrane (Fig. 6b, c). The question of whether type II and type I vesicles are different stages of the same type of vesicle (i.e., are type I vesicles simply empty type II vesicles?) has yet to be elucidated. In this regard, an interesting set of observations was made on *O. tau*: after the pulsing period, a high density of type I vesicles was observed (Fig. 6a). In Fig. 6a, the process of vesicle opening was particularly well visible, as was a population of empty vesicles located not only beneath the apical membrane but also disseminated more centrally in the cytosol. Secondly, we found vesicles more or less disorganized in situ still attached to the apical membrane or close to it. (Fig. 3b, arrow). Presumably, the material constituting the vesicle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was somehow reused in new synthesis. The present study suggests that both processes for recycling vesicle membrane material coexist.

Is vesiculation from the Golgi modified by pulsing? This point was not addressed statistically by the present study. However, the Golgi stacks appeared to be less well developed and less visible in fish that had not pulsed for 6 h. Shortly after a pulse, we found a high number of small (less than 50 nm diameter) empty vesicles (type I). The size of the cored vesicles (100–200 nm) did not seem to depend upon the pulse cycle, but their density close to the PVC apical membrane was significantly increased in *O. beta* during pulsing (Table 1; not tested in *O. tau*).

The plainfin midshipman

The gross gill morphology of *P. notatus* was similar to that of *O. beta* and *O. tau*. Filamental and lamellar PVCs exhibited different ornamentations from the *Opsanus* spp. Ornamentations of the apical membrane were mainly ridges on the filamental PVCs, whereas the surface of lamellar PVCs was abundantly covered with villi (Fig. 1d). The most striking difference concerned the PVC ultrastructure, which was poorly differentiated in the midshipman (Fig. 7c, d); cellular organelles were scarce and the Golgi apparatus much less well developed than in *Opsanus* spp. Type I and type II vesicles, similar to those found in *Opsanus*, were present, but in a much lower density (about one type I vesicle for every ten PVCs profiles; Fig. 7c).

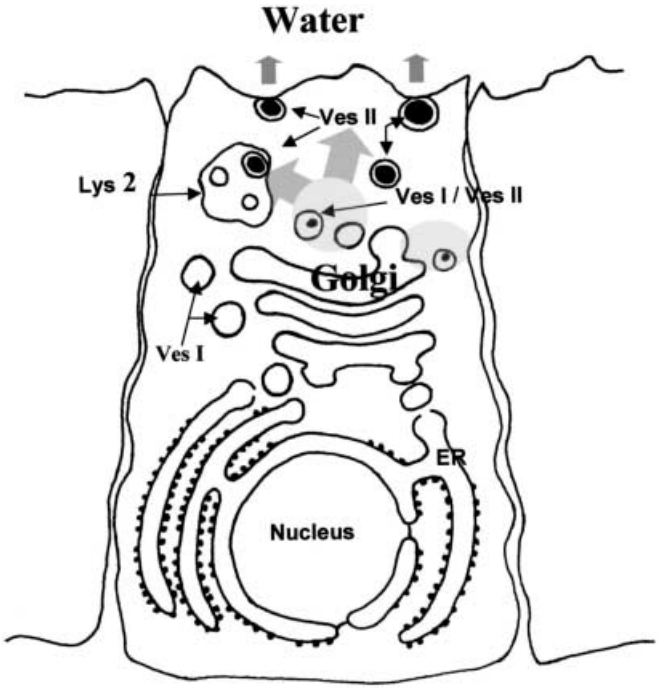

Fig. 8 Schematic of vesicle trafficking in ureotelic toadfish gill epithelium pavement cells. Type I (*Ves I*) vesicles are budding from the Golgi apparatus. Owing to the presence in their membrane of a urea transporter, they accumulate urea and progressively acquire a black core, forming the dense-cored vesicles (*Ves II*). Vesicles II are trapped in the secondary lysosomes (*Lys 2*) or contact the apical membrane and excrete their content in the water (*ER* endoplasmic reticulum). (Modified from Alberts et al. 1983, Fig. 7-39)

Discussion

In the present study, we have collected strong morphological evidence indicating that a significant trafficking of vesicles in the gill PVCs of *Opsanus* spp. coincides with the transfer of material from the gills to the external medium, which is further coincident with the pulsatile urea excretion event. The gill (and the kidney) have previously been identified as sites of urea excretion in eel (Smith 1929; Masoni and Payan 1974). In particular, the gill chloride cells were implicated in urea excretion, based on the clearance of injected [^{14}C]urea and autoradiography experiments (Masoni and Garcia-Romeu 1972).

The observations reported here, while circumstantial, allow us to reconstruct a testable hypothesis regarding the dynamics of vesicular trafficking. We have summarized the ultrastructural aspects of this tentative model in Fig. 8 for visual reference. The key piece of the trafficking mechanism appears to reside with the Golgi system, where the buildup of vesicles presumably begins. The Golgi apparatus is particularly abundant in the toadfish PVCs, where several Golgi profiles often could be counted in the same ultrathin section. The present study also points to a probable relationship between the Golgi apparatus and the magnitude of vesicle trafficking in *Opsanus* spp. The second key observation is the coincidence between the cyclic excretion of urea as a pulse and increased vesicular trafficking. During a pulse, the Golgi machinery was more highly developed in size and density, and consequently more visible than in the postpulse period; both the number and size of Golgi cisternae increased. During the pulsing period, small (less than 50 nm diameter) membraned, clear vesicles budded from the edge of the Golgi cisternae. These vesicles apparent-

◀ **Fig. 7a–d** Comparison of the pavement cell apical region of the ureotelic *Opsanus beta* (**a, b**) and its ammoniotelic relative *Porichthys notatus* (**c, d**). In *Opsanus* spp., the core of the type II vesicles is stained intensely black. Apparently, material which constitutes the vesicle core (*white star*, **a, b**) was expelled outside, caught within the mucus layer, fixed, embedded, and finally stained in situ. The cytoskeleton appeared to be closely associated with the vesicles. *Arrowheads* are pointing to type II vesicles (compare vesicles *1* and *2*). The pavement cells of *P. notatus*, an ammoniotelic batrachoidid, are poor in organelles in comparison with *Opsanus* spp. (**c, d**). The Golgi apparatus is present but not very active; the cisternae are shrunken and a limited number of vesicles are budding. However, a few typical dense-cored vesicles were occasionally observed within the cytosol (*2* in **c**), indicating that the same basic organization as in *Opsanus* spp. is present but is probably inefficient. In addition, clear type I vesicles are also observed (*1*). *Bar* 200 nm

ly grew in size to 200 nm, and we conclude this growth must be rapid because not many intermediate sizes were visible. The vesicles then progressively acquired a dense black core, presumably by loading material from the cytosol. The exact coincidence between this budding and the pulsatile event has not yet been established due to the difficulty in obtaining specimens for a nonpredictable phenomenon. However, once a specific in vivo triggering molecule (hormone) has been identified, it could presumably be used in more precisely timed observations.

Subsequent to the growth and formation of type II cored vesicles, two alternative vesicle pathways appear to exist. In the first, cored vesicles, considered to be primary lysosomes, were directed into and engulfed by secondary lysosomes (the type III vesicle). This is the classic fate of a primary lysosome; indeed secondary lysosomes typically result from the repeated fusion of primary lysosomes with a variety of membrane-bound substrates (Alberts et al. 1983). In the present study, secondary lysosomes exhibited the morphology of multivesicular bodies, in which cored vesicles are incorporated into the secondary lysosomes but remained clearly identifiable (Fig. 5b). In a second pathway, possibly preponderant, vesicles approached the PVC apical membrane. Figure 7a shows close relationships between vesicles and the cytoskeleton. A small pore developed where the vesicle membrane contacted the apical membrane, presumably to allow the vesicle to void its contents to the outside medium. Although an open aperture was rarely observed, the small size of the pore meant that the probability of a section passing directly through it was minimal. The mechanism of vesicle opening is not completely understood, but it appears to contrast with the classical description of exocytosis, which is characterized by the omega shape aspect of membrane fusion (Palade 1975).

Nevertheless, we collected structural evidence for vesicle voiding. First, cored vesicles close to the PVC apical membrane more often displayed a spherical core, leaving a thin translucent area beneath the vesicle membrane. Once apposed to the PVC apical membrane, many cored vesicles exhibited a smaller, irregular core, suggesting progressive emptying (Fig. 3b–d). Second, we occasionally observed extruded black material that was extracellular but still close to the membrane. This observation could be explained by either artifactual disruption of the vesicle during fixation or extracellular fixation of material freshly extruded (Figs. 3d, 7a). Finally, a large population of clear vesicles was present beneath the apical membrane and deeper within the cytosol (Fig. 6b, c). This population could represent cored vesicles after voiding, now empty and translucent. Whether these vesicles are able to be refilled when disseminated within the cytosol from the apical membrane up to the Golgi area is a hypothesis worth testing. This hypothesis implies that vesicles move back inside the PVC after discharging their contents as suggested by Fig. 6.

The plainfin midshipman (*P. notatus*) is a close relative of the genus *Opsanus* and is considered to be almost strictly ammoniotelic, excreting urea at a very low rate, in a nonpulsatile fashion (Wang and Walsh 2000). We believe that this species served as a negative control for purposes of comparison with the *Opsanus* spp., and that the poorly developed Golgi apparatus and the very limited vesicle trafficking observed is consistent with the relative absence of urea excretion. Notably, secondary lysosomes were also much less abundant or absent in *Porichthys* sp. than in *Opsanus* spp. and, above all, devoid of black material.

Although the presence and timing of the vesicular traffic in the *Opsanus* spp. PVCs was strongly suggestive of its involvement in urea excretion, we cannot yet conclude this, nor can we distinguish between at least two potential mechanisms of vesicle action in urea excretion. One hypothesis suggested by the present morphological data is that urea is concentrated in the vesicles as they are formed, by means of a urea transport protein inserted in the vesicular membranes, and that urea would subsequently be expelled directly to the environment (along with any other vesicular contents). In this scenario, the transporter would need to be synthesized in the endoplasmic reticulum and incorporated as it passed through the Golgi apparatus, into the type I vesicle membrane according to the classic scheme for primary lysosomes (Fig. 8; Hasilik 1980). Also in this model, the transporter(s) inserted in the vesicle membrane would start transporting urea from the cytosol into the vesicle, as soon as vesicles are liberated from the Golgi system. [This hypothesis does not, however, provide an explanation for why the vesicle contents become so intensely electron-opaque. One possibility is that acid phosphatase, a marker enzyme for lysosomes (Holzman 1976), might release phosphate which could then react with lead when the grids were stained. On the other hand, it is well known that urea reacts with aldehyde to form resins (Windholtz 1983]. Stoichiometric X-ray electron-microscopic analysis is now in process to determine the nature of the compounds located within the vesicle core.) Relative to the above hypothesis, there is a key requirement for an *active* urea transporter to obtain vesicular urea concentrations high enough, relative to the volume of the vesicles, to account for the size of the urea pulse (Wood et al. 1995). To date, no *active* urea transporter has been cloned from a vertebrate, but physiological evidence exists for active urea transport in several systems (reviewed by Walsh and Smith, 2000).

An alternative hypothesis for the mechanism of urea excretion is similar to that proposed for mammalian kidney urea transport (Nielsen et al. 1996), namely that the vesicular traffic allows the insertion of the urea transport protein into the apical PVC membrane; the inserted transporter then allows urea to exit the PVC down its concentration gradient. In this second hypothesis, the vesicles would not necessarily contain urea, but could have a different substance that is either directly or indirectly related to the urea pulse event. Consistent with this hypothesis is the precedent in the mammalian kidney (Nielsen et al. 1996), as well as the lack of a need for an

active urea transporter. Furthermore, it should be noted that the physiological data available for toadfish at present suggest that the transport mechanism is a freely reversible, bidirectional facilitated diffusion transport system which normally operates in only the efflux direction, because the urea gradient is normally outwardly directed (Walsh 1997; Wood et al. 1998). Also consistent with this hypothesis is the recent cloning of a gene from toadfish gill cDNA (tUT) which is homologous to the UT-A family of facilitated diffusion urea transporters (GenBank AF 165893; Walsh et al. 2000). However, a key finding from the current study may be inconsistent with this second hypothesis. Notably, a true fusion process between vesicles and the PVC apical membrane was not observed, at least for the dense-cored vesicles. In addition, effective insertion of vesicular membrane material should cause an enlargement of the PVC apical membrane and therefore the appearance of increased folding. Although the question was not statistically addressed, the ornamentation density of the PVC surface did not appear to increase during pulsing (Figs. 1, 2).

With regard to both hypotheses, it is not known whether regulation of the transport mechanism ultimately takes place at the apical membrane, the basolateral membrane, or at both locales in the pavement cell. Presumably, based on permeability constants (reviewed by Walsh and Smith 2000), the lipid bilayer of either the basolateral or the apical membrane should serve as an effective barrier to urea permeation during the nonpulsing state, such that urea "leak" is not an issue. However, this raises the question of how urea enters the gill cells at the basolateral membrane. Presumably a UT-type transporter is present there. Nor is it known whether a single facilitated diffusion transporter protein is involved (at both membranes) or some combination of different types of transporter proteins (e.g., active and facilitated diffusion) that give the summed (physiological) appearance of reversible facilitated diffusion in vivo. An important characteristic of the urea flux was its reversibility (Wood et al. 1998). This reversibility was not the result of a simple, unmediated diffusive process, in that the membrane permeabilities for urea and water were not found to increase in parallel (Pärt et al. 1999). In the experiments of Wood et al. (1998), the creation of an inward urea concentration gradient by raising the external urea concentration to threefold higher than the internal concentration resulted in each efflux pulse event (as detected by [^{14}C]urea) being accompanied by a net urea uptake rather than a net efflux.

To conclude, an intense traffic of dense-cored vesicles was observed in the pavement cells of ureotelic teleosts, but not in their nonureotelic relatives. The pattern in the seawater *Opsanus* spp. contrasts with the traffic of ornamented vesicles described in the pavement cell of ammoniotelic freshwater teleosts, where small, coated vesicles that bud from the Golgi cisternae fuse with the PVC apical membrane, giving rise to the classic exocytotic, omega-shaped image (Laurent et al. 1994). In the present study, it appeared that dense-cored vesicles arising from the Golgi were apposed to the apical membrane and apparently discharged their contents to the outside. Thereafter, vesicle membranes were inwardly retrieved. The pronounced development of the lysosomal compartment appeared to be a specific morphofunctional characteristic of these, and probably other, ureotelic teleosts; lysosomes and cored vesicles are apparently intimately implicated in the specific physiology of these animals. Whether this mechanism is totally or partly responsible for urea excretion is a question which clearly warrants further study at the molecular level, and probes based on the recently cloned tUT facilitated diffusion transporter (Walsh et al. 2000) will be useful in this regard.

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