Carbonic anhydrase is required for statoconia homeostasis in organ cultures of statocysts from Aplysia californica

Abstract A novel organ culture system has been developed to study the regulation of statoconia production in the gravity sensing organ in Aplysia californica. Statocysts were cultured in Leibovitz (L15) medium supplemented with salts and Aplysia haemolymph for four days at 17°C. The viability of the system was evaluated by examining four parameters: statocyst morphology, the activity of the mechanosensory cilia in the statocyst, production of new statoconia during culture and change in statoconia volume after culture. There were no morphological differences in statocysts before and after culture when ciliary beating was maintained. There was a 29% increase in the number of statoconia after four days in culture. Mean statocyst, statolith and statoconia volumes were not affected by culture conditions. The presence of carbonic anhydrase in the statocysts was shown using immunohistochemistry. When statocysts were cultured in the presence of 4.0 x 10^{-4} M acetazolamide to inhibit the enzyme activity, there was a decrease in statoconia production and statoconia volume, indicating a role for this enzyme in statoconia homeostasis, potentially via pH regulation. These studies are the first to report a novel system for the culture of statocysts and show that carbonic anhydrase is involved in the regulation of statoconia volume and production.

Key words Aplysia · Carbonic anhydrase · Statoconia · Organ culture

Introduction

The gravity sensing organ of the gastropod mollusk Aplysia californica consists of bilaterally paired statocysts which are responsible for spatial orientation of the animal and crucial for swimming and locomotion. Each statocyst is contained in a connective tissue sheath attached to the antero-lateral portion of the corresponding pedal ganglion, which in turn forms part of the circumesophageal ring of ganglia. This ring of ganglia constitutes the central nervous system of Aplysia, and is located distal to the buccal mass and surrounds the esophagus (Fig. 1A).

The statocyst (Fig. 1B) is a fluid-filled sac composed of supporting cells and 13 receptor cells (Coggeshall 1969; Wiederhold 1974). In embryonic and early metamorphosed animals, the statocysts contain a single calcium carbonate stone, the statolith, found in the lumen. As the animals age, the statocyst is found to contain, in addition to the statolith, up to 1,000 multiple stones called statoconia (Wiederhold et al. 1990).
Ciliary beating is essential for sensory transduction in the statocyst (Wiederhold 1977). The statocyst's receptor cells are hair-cell-like neurons whose cilia are motile and mechanosensory. In the intact statocyst, statoconia are seen in constant motion due to the continuous beating of these cilia. Gravity pulls the statoconia down which obstructs the beating of the cilia on the bottom one-third of the statocyst. This causes an increase in membrane conductance to Na⁺, leading to a large depolarizing receptor potential, which in turn leads to the formation of an action potential (Gallin and Wiederhold 1974; Wiederhold 1977).

Both the statolith and the statoconia are composed of an organic and an inorganic component. Fourier transform infrared spectroscopy indicates that *Aplysia* statoconia are composed of calcium carbonate of the aragonite form whereas the statolith appears to be an amorphous form of calcium carbonate (unpublished data). The statoconia are produced by the supporting cells (Wiederhold et al. 1990); however, the site of production of the statolith and the mechanism of statoconia and statolith production are unknown.

Carbonic anhydrase catalyzes the hydration of carbon dioxide to form carbonic acid, which then dissociates to form hydrogen and bicarbonate ions. It is known that this enzyme is found in the inner ear of higher animals (Takumida et al. 1989; Wanatabe and Ogawa 1984; Lim et al. 1983) and is thought to play an important role in cochlear, vestibular and endolymphatic sac electrolyte and fluid balance (Erulkar and Maren 1961; Johnson and Spoendlin 1966). Inhibition of carbonic anhydrase has been shown to cause changes in cochlear epithelial tissue in guinea pigs (Johnson and Spoendlin 1966) as well as changes in calcium carbonate deposition in the inner ear. It has been shown that the presence of carbonic anhydrase inhibitors prevents otolith formation in chicks in a dose-dependent manner when administered prior to the initiation of otolith morphogenesis (Vicentiis and Marmo 1967). Carbonic anhydrase inhibition also caused otolith defects in embryonic mice (Portencinis and Erway 1972). At the electron microscopic level, the same treatment resulted in remarkable morphological changes in the otoconia still present (Kido et al. 1991). Acetazolamide, a carbonic anhydrase inhibitor, is known to reduce the number of otoconia in adult guinea pigs as well as in mice (Harada 1984). Taken together, these results suggest that carbonic anhydrase may be important in the regulation of statoconia formation in *Aplysia*.

In previous studies, investigators have used in vivo models to study the formation and development of otoconic organs (Erulkar and Maren 1961; Vicentiis and Marmo 1967). However, to study the underlying mechanism of statoconia formation and its regulation, it was necessary to develop an in vitro organ culture model. In this paper, we present the first description of such a system. Statocysts, isolated from developmental stage 10 *Aplysia*, were cultured in vitro and the role of carbonic anhydrase in statoconia production, including number and size, was examined.

**Materials and methods**

**Statocyst isolation**

*Aplysia californica* were obtained from the *Aplysia* Resource Facility at the University of Miami, Florida, and maintained in artificial sea water (Instant Ocean®; Aquarium Systems, Mentor, Ohio) at room temperature (22°C) until the time of dissection.

Individual *Aplysia* were weighed and placed in a small amount of artificial sea water on a dissecting pad, making sure the animal was totally immersed. The animal was then pinned to the dissecting pad.
A. Observation Slide

B. Specimen Holder

Fig. 2 Diagram of the observation slide (A) and specimen holder (B) used in this study. The observation slide (A) is a laboratory-made slide used for observing live statocysts under the light microscope. It consists of a standard microscope slide coated with a thin layer of Sylgard (Dow Corning Corp., Midland, MI). A 2.5 mm well is made by piercing through the Sylgard in the center of the slide, where the specimen is placed for observation. The specimen holder (B) is used to hold the statocyst during culture. It is also made in the laboratory using glass and Sylgard.

Optimization of the culture system

Pedal ganglia, containing the statocysts, were dissected out of preweighed animals and placed in culture for four days in a 24-well culture plate. Each well contained 850 μl complete media (SL15 plus haemolymph). Humidity was maintained at 100%, at atmospheric CO₂, and unless otherwise stated the temperature was maintained at 17°C. The effect of changing the following culture parameters was examined:

Haemolymph donor age

The effect of haemolymph donor age on both ciliary beating and statocoria production was evaluated by comparing the number of statocoria before and after culture, as described below. Haemolymph was obtained from adult (150 g) and juvenile (40 g) Aplysia.

Incubation temperature

The effect of incubation temperature was evaluated. Organs were incubated at 22-26°C (room temperature) or 16-18°C.

Antibiotic used

The effect of streptomycin plus penicillin or penicillin alone on ciliary beating was assessed. 0.2 mg streptomycin and/or 61.36 mg penicillin G (Sigma Cell Culture™, St. Louis, MO) were added to 1 l of media.

Media storage

The effect of culture medium age was examined. Statocysts were incubated in fresh (1-day old) and aged (14-day old) media stored at 4°C.

Organ culture

Isolated statocysts were cultured in Leibovitz (L15) medium (Sigma, St. Louis, MO) (Leibovitz 1963) supplemented to reach a final concentration of the following: 1.14 M NaCl and 10 mM KCl, 29 mM MgCl₂·6H₂O, 25.3 mM MgSO₄·7H₂O, 2.3 mM NaHCO₃, 34.6 mM D-glucose (dextrose), and 14.4 mM HEPES·Na (Sigma, St. Louis, MO) as described by Goldberg (1991). The salts were added sequentially, in the order indicated above, while stirring the solution, and each solute was allowed to completely dissolve before addition of the next solute. After adjusting the pH to 7.4, 1.488 g/l of CaCl₂·2H₂O and 0.4 ml/l of 250,000 U/ml of penicillin stock solution were added. The supplemented medium (SL15) was then filter sterilized through a 0.2 μm pore size filter and stored at 4°C.

Prior to use, the media (SL15) were diluted 1:1, v/v, with haemolymph obtained from either adult (around 150 g) or juvenile (around 40 g) animals. Haemolymph was removed from the coxcom of the animal with a sterile 25 gauge needle. Special care was taken not to contaminate the haemolymph with digestive contents. The animals were then allowed to recuperate for at least 24 h before obtaining additional haemolymph. The haemolymph was centrifuged for 15 min at 10,000 × g. The supernatant was filter sterilized before storage at −70°C.

Microscopic techniques

Tissue was fixed overnight in 8% paraformaldehyde in 0.1 M phosphate buffer that had been adjusted to the osmolarity of sea water.
of haemolymph, since prior studies by Weiss and Wilbur (1978) have shown that DMSO had no effect on the calcareous alga, *Cryophyraea carterae*, including the appearance of calcium carbonate coccoliths. DMSO was added to control cultures at the same concentration used in acetazolamide treated cultures (1% by volume of full media). The cultures were wrapped in aluminium foil due to the light sensitivity of acetazolamide. In two experiments (data not shown), acetazolamide stock solutions were prepared in 100% ethanol and tested at a 1% concentration to assess the effect of a different diluent.

Immunohistochemistry was used to localize carbonic anhydrase in the statocyst. The tissue was fixed in phosphate buffered 8% paraformaldehyde for 2 h and washed twice (15 min wash) with sodium cacodylate buffer. It was then dehydrated, processed, and infiltrated as described above. Tissue sections (1 μm) were placed on chrome alum coated slides and heated at 60 °C for at least 1 h. The resin was etched from the sections by immersing the slides in sodium ethanolate (4 g NaNH in 100 ml of 100% ethanol) for 30 min and washing with PBS (3 × 5 min each). Endogenous peroxidase activity was quenched by incubating the sections for 30 min in 0.3% hydrogen peroxide in methanol at room temperature and washed in PBS. The tissue sections were incubated in 1.5% normal rabbit serum (Vector Laboratories Inc., Burlingame, CA) for 20 min to reduce nonspecific staining. Sections were incubated overnight at 4 °C with a 1:50, 1:100, 1:500 or 1:1000 dilution of the primary antibody [affinity purified, polyclonal sheep anti-human carbonic anhydrase I or II (The Binding Site, San Diego, CA)] and washed with PBS. Sections were prepared for immunohistochemistry using the sheep and mouse IgG Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Specimens were mounted and photographed with an Olympus Vanox II photomicroscope using Kodachrome 64 film. A negative control was run by incubating the specimens in the absence of primary antibody.

**Statistical analysis**

Differences among experiments were determined by using analysis of variance. Student’s *t*-tests were used to assess differences in statocyst number and volume between control and experimental groups. *P* < 0.05 was considered statistically significant. All statistical tests were performed using Statview 4.0 and Excel for the Macintosh. Statistically significant differences are indicated by * or # in the graphs and tables.

**Results**

Under optimal culture conditions, there was an increase in the number of statocyst, statoconia, and statolith volumes, and statoconia production. The length of time the culture
Table 1: Statoconia number before and after culture. Statoctysts were cultured under the optimal conditions described in Materials and methods and the number of statoconia before and after culture determined. For all 7 experiments the mean change in statoconia number was 2.3 ± 0.1. The mean treatment/control (after vs. before) ratio for the 7 experiments was 1.29 ± 0.03. Data are presented as mean ± SEM of the N indicated. *P < 0.05, time 0 vs. 4 days in culture, unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>N (Statocysts)</th>
<th>Number of Statoconia Before culture</th>
<th>After culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>12.4 ± 2.1</td>
<td>16.1 ± 1.9*</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>8.0 ± 0.9</td>
<td>9.9 ± 0.8*</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>9.7 ± 1.1</td>
<td>12.3 ± 1.2*</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>11.5 ± 1.4</td>
<td>18.8 ± 1.4*</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>7.1 ± 1.1</td>
<td>9.6 ± 1.2*</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>8.6 ± 1.4</td>
<td>10.7 ± 1.7*</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>6.9 ± 0.5</td>
<td>8.8 ± 0.7*</td>
</tr>
</tbody>
</table>

Fig. 3 The ratio of the number of statoconia after culture to that before culture as a function of experiment number. The values represent the mean ± SEM. The sample sizes for experiments 1–7 were 10, 12, 6, 11, 12, 19 and 24, respectively. *P < 0.05, before vs. after, paired Student’s t-test.

media was stored affected ciliary beating; 83.3% of the cultures in fresh media maintained vigorous ciliary beating, while none of the cultures in stored media showed beating (Table 3). Although ciliary beating was inhibited in statoctysts cultured in stored media, there

Table 2: Effect of culture on statoctyst, statoconia and statolith volumes. Statoctysts were cultured under the optimal conditions described in Materials and methods and statoctyst, statolith and statoconia volume determined. The volumes from specimens from 5 individual experiments are shown. The values are the mean of each experiment’s mean. The N’s for the individual experiments were 6, 8, 4, 9 and 5. Within each experiment the single statoconia volumes from each statoctyst were pooled together into one group. The number of statoconia per statoctyst varies considerably (between 1 and 15). Data are presented as mean ± SEM. No statistically significant differences were found.

<table>
<thead>
<tr>
<th>Volumes (μm³)</th>
<th>Statocyst</th>
<th>Statoconia</th>
<th>Statolith</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before culture</td>
<td>7,623 ± 591</td>
<td>16.9 ± 3.4</td>
<td>208.0 ± 36.6</td>
</tr>
<tr>
<td>After culture</td>
<td>8,769 ± 457</td>
<td>14.1 ± 1.6</td>
<td>258.4 ± 47.4</td>
</tr>
</tbody>
</table>

Fig. 4A, B The effect of temperature on statoconia number and ciliary beating. The values represent the mean ± SEM of 7 experiments for the 16–18°C group and 8 experiments for the 22–26°C group, respectively. A shows that the number of statoconia is always greater after culture, irrespective of temperature; however, the fold increase is less at the higher temperature. B shows that temperature dramatically affects ciliary beating. *P < 0.05 after vs. before culture, unpaired Student’s t-test, and #P < 0.05 16–18°C vs. 22–26°C, paired Student’s t-test.

Table 3: Effect of media storage on statoconia number and ciliary beating. Culture media were prepared and used immediately (Fresh) or after two weeks at 4°C (Stored). The change in statoconia number and the ratios of statoconia number before and after culture were determined. Data represent the mean ± SEM; N = 24 organs per media type.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Number of statoconia</th>
<th>Change in number</th>
<th>After/Before</th>
<th>% Ciliary beating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stored</td>
<td>Before culture: 7.0 ± 0.9</td>
<td>After culture: 8.3 ± 1.0*</td>
<td>1.3 ± 0.3*</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Fresh</td>
<td>Before culture: 6.9 ± 0.5</td>
<td>After culture: 8.8 ± 0.7*</td>
<td>1.8 ± 0.3*</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05, time 0 vs. four days in culture, unpaired Student’s t-test.
was no effect on the production of statoconia when compared to statocysts cultured in fresh media.

Both the number of statoconia produced in culture and maintenance of ciliary beating were sensitive to temperature. When temperature was constantly maintained at 16–18 °C there was a 29.0 ± 2.6% increase in statoconia number (mean ± SEM, N = 7 experiments). In contrast, when statocysts were incubated at 22–26 °C, fewer statoconia were produced; there was an 18.3 ± 3.0% increase in statoconia number over the 4-day incubation period (mean ± SEM, N = 8 experiments) (Fig. 4A). While 74.0 ± 8.4% of the statocysts cultured at 16–18 °C showed vigorous ciliary beating, there was no ciliary beating in the statocysts incubated at room temperature (Fig. 4B).

The statocysts retained their structural integrity throughout the culture period as long as ciliary beating was maintained (Fig. 5). Disruption of ciliary beating led to ciliary demise and structural changes in the statocyst. Prior to culture, the prominent histologic features of the organ included cilia, supporting and receptor cells, as well as the statolith and several statoconia (Fig. 5A). When statocysts were cultured at 16–18 °C, the same features were observed (Fig. 5B). However, when the statocysts were cultured at 22–26 °C, the receptor cells appeared to remain intact but absence of mechanosensory cilia and invasion of the intraluminal side of the statocyst were observed (Fig. 5C).

The ultrastructural morphology of the statocyst was unaffected by culture at 16–18 °C (Fig. 6). Nuclei, mitochondria, and endoplasmic reticulum in the freshly isolated organs (Fig. 6A) were comparable to those of the cultured statocysts (Fig. 6B). The 9 + 2 cilia structure was also apparent in both as well as the microvilli lining the surface of the supporting cells.

The presence of carbonic anhydrase I and II in statocysts of *Aplysia californica* was demonstrated by immunohistochemistry (Fig. 7). There was no reaction product when statocysts were incubated in the absence of the primary antibody (Fig. 7A). However, immunohistochemical staining was found when the organs were incubated with the primary antibody. Staining was greater in the supporting cells, while the statoconia were produced, than in the receptor cells (Fig. 7B and C). There were no differences in immunohistochemical staining between carbonic anhydrase I (Fig. 7B) and II (Fig. 7C).

Inhibition of carbonic anhydrase activity altered the in vitro production of statoconia. When statocysts were cultured in the presence of acetazolamide, a specific inhibitor of carbonic anhydrase, there was a significant reduction in statoconia number (Fig. 8A). However, exposure to acetazolamide had no effect on ciliary beating (Fig. 8B). Acetazolamide treatment also had no effect on the mean statocyst volume (Fig. 9A). In contrast, it caused a decrease in both statoconia and statolith volumes (Fig. 9B and C, respectively). No differences were noted as a function of acetazolamide vehicle, i.e., DMSO or ethanol (data not shown), nor were there differences between untreated cultures and cultures treated with vehicle alone.

**Discussion**

The main goal of this study was to devise a system in which *Aplysia* statocysts could be maintained in culture so that the direct effects of treatment on statoconia formation could be separated from systemic effects on the animal. We chose *Aplysia* as an experimental animal to examine the formation and development of statoconia because of our previous experience with this system in vivo (Pedrozo and Wiederhold 1994; Wiederhold et al. 1990). We have successfully isolated and cultured statocysts from adult, juvenile, early juvenile and developmental stage 10 animals, but only studies using developmental stage 10 animals are presented here. The simplicity of *Aplysia*’s gravity sensing organ and the limited number of statoconia during these early stages of development make it possible to accurately measure their number and volumes.

There was a 29% increase in the number of statoconia during the culture period, indicating that the statocysts were able to remain viable and produce new statoconia. This was not affected by storage of the media or by age of the animal from which the haemolymph was obtained. However, some aspects of statocyst physiology were affected by culture conditions. Ciliary beating was maintained throughout the culture period when fresh media were used, when the statocysts were incubated at 16–18 °C, and when penicillin alone was used as antibiotic. Culture media stored for 2 weeks, or maintaining cultures at ambient temperature or in the presence of streptomycin, reduced or totally inhibited ciliary beating. The age of the haemolymph donor had no effect on ciliary beating.

There were no morphological differences at the light microscopic or transmission electron microscopic levels between cultured and freshly dissected statocysts unless ciliary beating was absent. In the latter case, no cilia were found in the statocyst; however, the receptor cells and other neurons in the pedal ganglia showed nucleation, and proper general cellular integrity under light microscopy. Whether or not ciliary beating was present, there was no statistically significant difference in mean statocyst, statolith, or statoconia volumes between freshly isolated and cultured statocysts.

While the volume of the organ and individual statoconia was not affected by culture conditions, the number of statoconia produced in culture was sensitive to temperature. This may have been a consequence of the loss of ciliary beating seen at ambient room
Fig. 5 Photomicrographs of 1 μm sections through Aplysia statocysts before culture (A), after 4 days in culture at 16–18°C (B), and after 4 days in culture at 22–26°C (C). Note the lack of cilia in C. The greater number of statoconia present in C is because this is a statocyst from an early juvenile animal (1.5 g in weight). St, statocyst; Sc, statoconia; Sl, statolith; C, cilia; RC, receptor cell; SC, supporting cell; I, intraluminal invasion; and N, nucleus. Bar = 10 μm. Magnification: 120×
temperature. It is also possible that the loss of ciliary beating and the decrease in statoconia number were both consequences of some other effect of temperature.

Using immunohistochemistry, carbonic anhydrase I and II were found to be present in the *Aplysia* statocysts, as in the case of the inner ear in higher animals (Erulkar and Maren 1961; Lim et al. 1983; Takumida et al. 1989; Wanatabe and Ogawa 1984). The fact that antibody binding was greater in the supporting than in the receptor cells of the statocyst is
Fig. 7 Photomicrographs of 1 μm sections through adult *Aplysia* statocysts after staining with anti-carbonic anhydrase I or II antibody. A Control (i.e., no primary antibody used); B after incubation with anti-carbonic anhydrase I antibody; C after incubation with anti-carbonic anhydrase II antibody. Sc, statoconia; C, cilia; RC, receptor cell; SC, supporting cell; and N, nucleus. *Bar* = 20 μm. Magnification: 85 ×
consistent with previous studies in guinea pigs and chinchillas showing localization of carbonic anhydrase in the supporting cells of the sensory epithelium and the dark cells lining the walls of the utricular and saccular maculae as well as the crista ampullaris (Lim et al. 1983; Takumida et al. 1989). These cells are thought to play an important role in the homeostasis of the otoliths and the endolymphatic fluid.

Histologic localization of carbonic anhydrase in supporting cells suggests a possible role of carbonic anhydrase in statoconia formation, since statoconia are made by the supporting cells (Wiederhold et al. 1990). This hypothesis is supported by the observation that inhibition of this enzyme’s activity led to a decrease in statoconia number in vitro. In earlier studies inhibition of carbonic anhydrase has been shown to cause otolith abnormalities (Kido et al. 1991) and, in some instances, to inhibit otolith development (Vincenti and Marmo 1967) in chick embryos. Similar treatment also caused a reduction in the number of otoconia in adult guinea pigs and mice (Harada 1984).

In our study, acetazolamide caused a decrease in existing statoconia and statolith volumes without a change in statocyst volume. This indicates that the enzyme is involved in the mechanism of statoconia biogenesis since the changes are apparently not due to toxicity of the inhibitor, the DMSO vehicle, or damage to the statocyst itself. The results also indicate that inhibition of carbonic anhydrase causes a decrease in statoconia formation as well as a decrease in the volume of statoconia and statoliths already present in the statocyst lumen.

Erulkar and Maren (1961) found that cats treated with acetazolamide showed a significant decrease in endolymphatic K⁺ levels. On the other hand, Ross et al. (1980) demonstrated that a decrease in the K⁺/Na⁺ ratio in endolymph and perilymph leads to a decrease in ⁴²Ca²⁺ uptake and/or exchange in in vitro saccular and utricular preparations. Therefore, carbonic anhydrase may play a role in statoconia homeostasis by regulating the ionic composition of the statolymph. More specifically, it can be envisioned that a reduction in enzyme activity causes a decrease in endolymphatic K⁺ concentration which leads to a de-
crease in Ca$^{2+}$ incorporation. The reduction in statoconia volume with acetazolamide treatment seen in our results certainly supports this contention.

Our results are also supported by previous research indicating that carbonic anhydrase plays a role in bone mineral homeostasis through pH regulation. Treatment of cultured chick osteoclasts with PTH and calcitonin altered the intracellular localization of carbonic anhydrase (Cao and Gay 1985). Also, treatment with calcitonin and PTH, and inhibition of this enzyme has been shown to cause changes in pH in cultured chick osteoclasts (Hunter et al. 1988). Furthermore, PTH-induced bone resorption in vitro was inhibited by carbonic anhydrase inhibition (Minkin and Jennings 1972). The presence and activity of carbonic anhydrase has also been demonstrated in matrix vesicles from rat epiphyseal growth plates (Stechschulte et al. 1992), implicating this enzyme in calcification, since matrix vesicles are associated with initial mineral formation in cartilage, bone, and dentine.

In summary, a novel organ culture system has been successfully established. Under appropriate conditions, as discussed above, the statocysts show vigorous ciliary beating at the end of the culture period. This is the most obvious indication of proper physiological function. Second, there are no morphological differences between control and cultured statocysts at the light microscopic or transmission electron microscopic levels. And third, statoconia are produced in vitro. Furthermore, inhibition of carbonic anhydrase causes a reduction in statoconia production as well as a reduction in mean statolith and statoconia volumes, indicating that carbonic anhydrase plays an important role in statoconia homeostasis as observed previously in otoliths.

Acknowledgement Supported by: USPHS Grant DE-05937, NSF Grant 1FC-9209162, NASA Grant NAG730, and Veterans Administration Medical Research Funds.

References


Leibovitz A (1963) The growth and maintenance of tissue cell cultures in free gas exchange with the atmosphere. Am J Hyg 78: 173-180


Ross M, Potz KG, Cloke PL, Corson C (1980) In vitro $^{40}$Ca$^{2+}$ uptake and exchange by otoconial complexes in high and low K$^+$ Na$^+$ fluids. The Physiologist 23: S129-S130


Vicentis M, Marano F (1967) Inhibition of the morphogenesis of the otoliths in the chick embryo in the presence of carbonic anhydrase inhibitors. Experimenta 24: 8: 818-820


Wiederhold ML (1977) Rectification in *Aplysia* statocyst receptor cells. J Physiol (Lond) 266: 139-156
