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ORIGINAL PAPER

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

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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 4.0×10^{-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

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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).

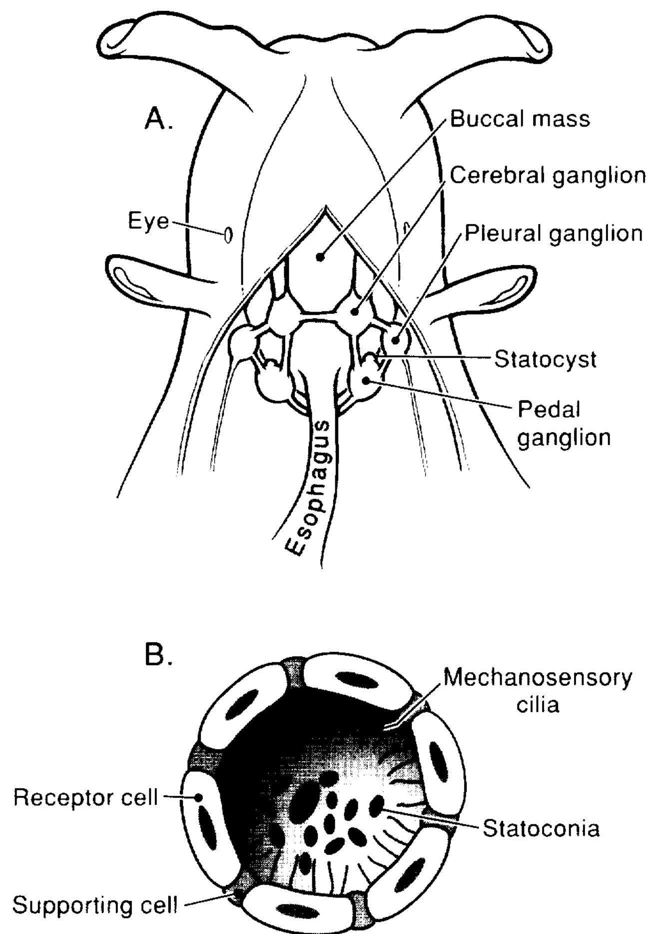


Fig. 1A. Artist's rendering of *Aplysia californica* (**A**) and a statocyst (**B**). **A** depicts a longitudinal section through the anterior portion of the animal showing the circumesophageal ring of ganglia. The statocysts are located on both pedal ganglia. **B** depicts a longitudinal section through a statocyst showing the ciliated receptor cells and the supporting cells, as well as the statoconia

The production of statoconia begins shortly after metamorphosis, when the animal reaches stage 10 of development, at about 1.3 mg of body weight.

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 (Purichia 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*.

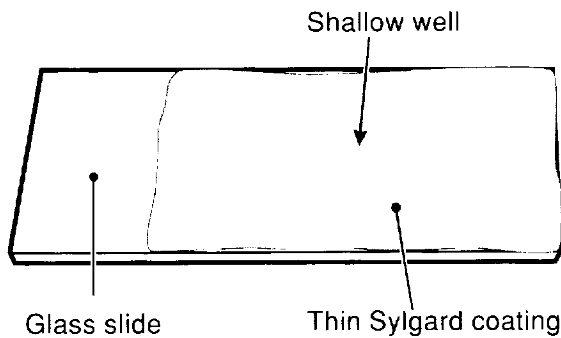
In previous studies, investigators have used in vivo models to study the formation and development of otolithic organs (Erulkar and Maren 1961; Vicentiis and Marmo 1967). However, to study the underlying mechanism of statoconia formation and its regulation, it was necessary to develop an in vitro organ 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.



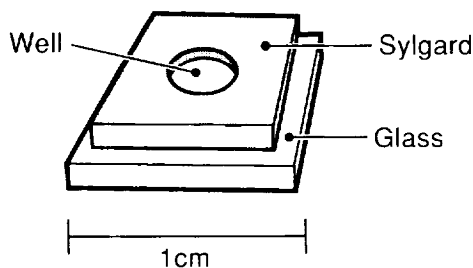


Fig. 2 Diagram of the observation slide (**A**) and specimen holder (**B**) used in this study. The observation slide (**A**) is a laboratory-made slide used for observing live statocysts under the light microscope. It consists of a standard microscope slide coated with a thin layer of Sylgard (Dow Corning 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

An incision was made just rostral to the parapodia, and the major nerves and the digestive tract cut. The statocysts, located on the antero-lateral pedal ganglia near the ventral part of the animal, were excised and immediately placed on the same dissecting pad in a drop of culture media (described below). A hook-shaped, ultra fine pin at the end of a wooden pick was used to aid in the dissection and manipulation of the ganglia. Both pedal ganglia were placed on a laboratory-made, Sylgard-well slide (Dow Corning Corp., Midland, MI) (Fig. 2A) and positioned so that the statocysts could be clearly seen. The specimen was covered with a 10 × 35 mm coverslip and the number of statoconia and the presence or absence of ciliary beating was visually determined under 400x. The slide was then transferred to the hood where each ganglion was placed in a separate laboratory-made organ holder (Fig. 2B) which allowed rapid identification of the ganglion and protected it during medium changes. The organ holder, containing the pedal ganglion, was then placed in the well of a 24-well culture plate. All of the steps above were carried out under a dissecting microscope.

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 $MgCl_2 \cdot 6H_2O$, 25.3 mM $MgSO_4 \cdot 7H_2O$, 2.3 mM $NaHCO_3$, 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 $CaCl_2 \cdot 2H_2O$ 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 coecum 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.

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 CO_2, 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 statoconia production was evaluated by comparing the number of statoconia 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.

Microscopic techniques

Tissue was fixed overnight in 8% paraformaldahyde in 0.1 *M* phosphate buffer that had been adjusted to the osmolarity of sea water

(about 1020 mOsm) and pH 7.5. Using a dissecting microscope, tissue was placed in 2% agar for processing because of the size of the specimen, roughly 400 μm in diameter (the statocyst is about 50 μm in diameter). Specimens were washed twice (15 min each) with 0.15 M sodium cacodylate buffer in Instant Ocean®, pH 7.5, and post-fixed for 1 h with 1% osmium tetroxide in 3 *M* sodium cacodylate buffer. After two more washes with buffer, the tissue was processed through an alcohol series (35, 50, 70, 80, 90, and 3 × 100%, 15 min each) into propylene oxide and then embedded in Medcast resin (Ted Pella, Inc., Redding, CA) and polymerized in a 60 C oven.

Various aspects of statocyst physiology were examined at the time of harvest from the animal and after 4 days in organ culture.

Statoconia production and ciliary beating

Statoconia production was determined by comparing the number of statoconia before and after culture. At the time of dissection and on the day of harvest, the ganglia were placed in Sylgard-well slides, positioned and cover slipped. Statoconia were counted and the status of ciliary beating noted. Ciliary beating was evaluated by observing the movement of the statoconia. In the present study, medium to vigorous statoconia movement was considered healthy ciliary beating, whereas low to almost unnoticeable ciliary beating was considered absent ciliary beating.

Statolith and statoconia volume

To assess the effect of culture on statolith and statoconia volume, one statocyst was fixed before culture (time 0) and the second statocyst from the same animal was placed in culture for four days and then fixed at the end of the culture period. To ensure there were no differences between the right and left statocysts, organs were assigned to time 0 and culture groups alternatively (i.e., left for time 0 and right for culture and vice versa).

Tissue was fixed, embedded and processed, and serial sections (1 μm) were stained with toluidine blue, mounted with Entellan (Electron Microscopy Sciences, Fort Washington, PA), and examined by light microscopy. Three dimensional reconstructions were made using a Bioquant™ System IV image analysis system (R&M Biometrics, Inc., Nashville, TN).

Statocyst morphology

Morphological differences were assessed by light and transmission electron microscopy. In both instances, tissues were processed as described above. Sections for light microscopy were treated as explained above under statolith and statoconia volume. Sections for transmission electron microscopy were 100 nm thick, placed on cellulose copper grids, stained with lead citrate, and uranyl acetate and examined using a Philips 301 transmission electron microscope (Philips, Holland).

Role of carbonic anhydrase

To determine the role of carbonic anhydrase in statoconia homeostasis, statocysts were incubated in the presence and absence of 0.4 m*M* acetazolamide (Sigma, St. Louis, MO), a specific inhibitor of this enzyme (Erulkar and Maren 1961; Kido et al. 1991; Minkin and Jennings 1972; Vicentiis and Marmo 1967). At the end of culture, the number of statoconia present was determined and specimens were fixed, processed and embedded for volume determination as described above. Acetazolamide was dissolved in dimethyl sulfoxide (DMSO) and added to the media (2% by volume) prior to addition of haemolymph, since prior studies by Weiss and Wilbur (1978) have shown that DMSO had no effect on the calcareous alga, *Cricosphaerea 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 aluminum 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% paraformaldahyde 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 allum 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 NaOH 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 statoconia 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 statoconia after 4 days in culture, indicating that new statoconia were produced in vitro (Table 1). At time 0, statocysts contained a mean of 9.2 ± 1.5 statoconia. The mean increase in statoconia number for all 7 experiments was 2.3 ± 0.1. This corresponds to a 29.0 ± 2.6% increase in the number of statoconia after culture, based on treatment/control ratios for each experiment (Fig. 3). However, there was a considerable range in the number of new statoconia produced among the experiments. Mean statocyst, statoconia and statolith volumes did not change over the culture period (Table 2).

Optimal culture conditions for maintaining statocyst function was defined with respect to ciliary beating, statocyst, statoconia, and statolith volumes, and statoconia production. The length of time the culture

Table 1 Statoconia number before and after culture. Statocysts 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.

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

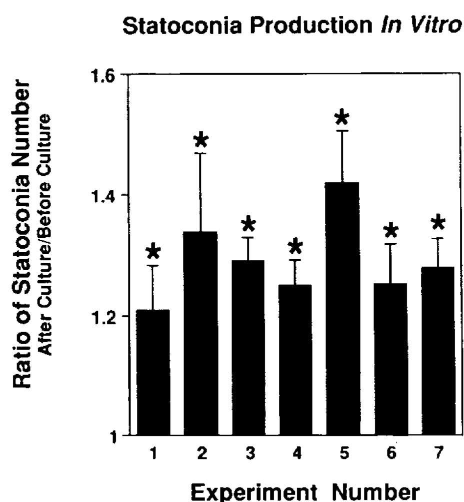


Fig. 3 The ratio of the number of statoconia after culture to that before culture as a function of experiment number. The values represent the mean ± SEM. The sample sizes for experiments 1–7 were 10, 12, 6, 11, 12, 19 and 24, respectively. $*P < 0.05$, before vs. after, paired Student's t-test

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 statocysts cultured in stored media, there

Table 2 Effect of culture on statocyst, statoconia and statolith volumes. Statocysts were cultured under the optimal conditions described in Materials and methods and statocyst, 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 statocyst were pooled together into one group. The number of statoconia per statocyst varies considerably (between 1 and 15). Data are presented as mean ± SEM. No statistically significant differences were found.

	Volumes (μm^3)		
	Statocyst	Statoconia	Statolith
Before culture	7,623.0 ± 591.0	16.9 ± 3.4	208.0 ± 36.6
After culture	8,769.0 ± 457.0	14.1 ± 1.6	258.4 ± 47.4

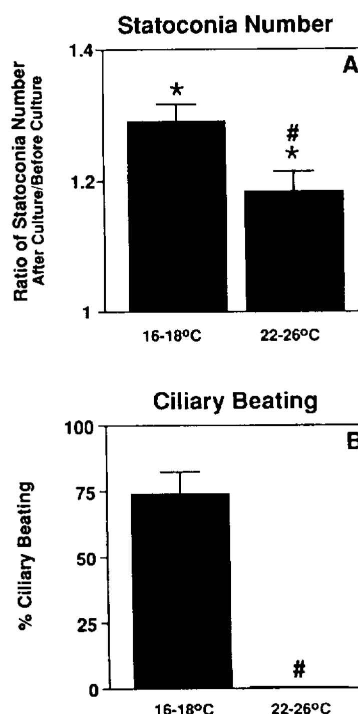


Fig. 4A, B The effect of temperature on statoconia number and ciliary beating. The values represent the mean ± SEM of 7 experiments for the 16–18°C group and 8 experiments for the 22–26°C group, respectively. **A** shows that the number of statoconia is always greater after culture, irrespective of temperature; 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.

	Number of statoconia		Change in number	After/Before	% Ciliary beating
	Before culture	After culture			
Stored Media	7.0 ± 0.9	8.3 ± 1.0*	1.3 ± 0.3*	1.3 ± 0.1	0
Fresh Media	6.9 ± 0.5	8.8 ± 0.7*	1.8 ± 0.3*	1.3 ± 0.1	83.3

$*P < 0.05$, 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, where the statoconia are 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.

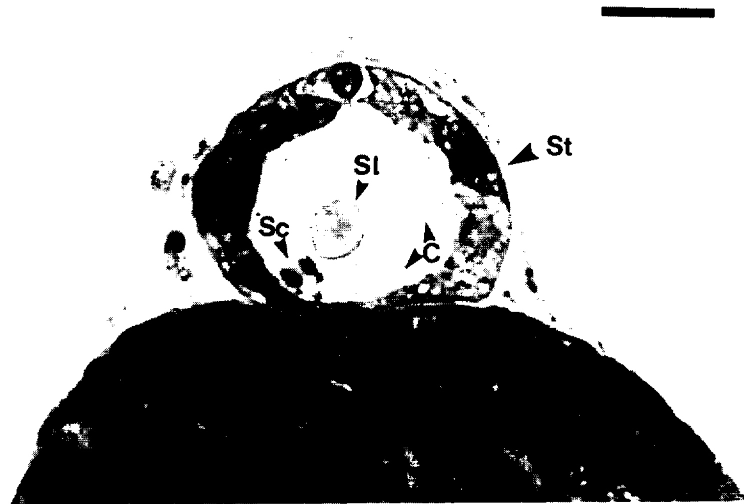
Discussion

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



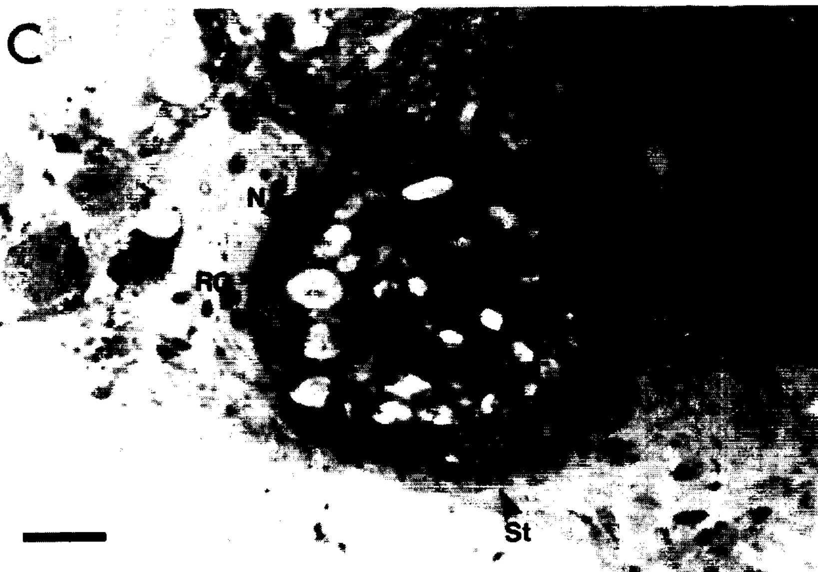
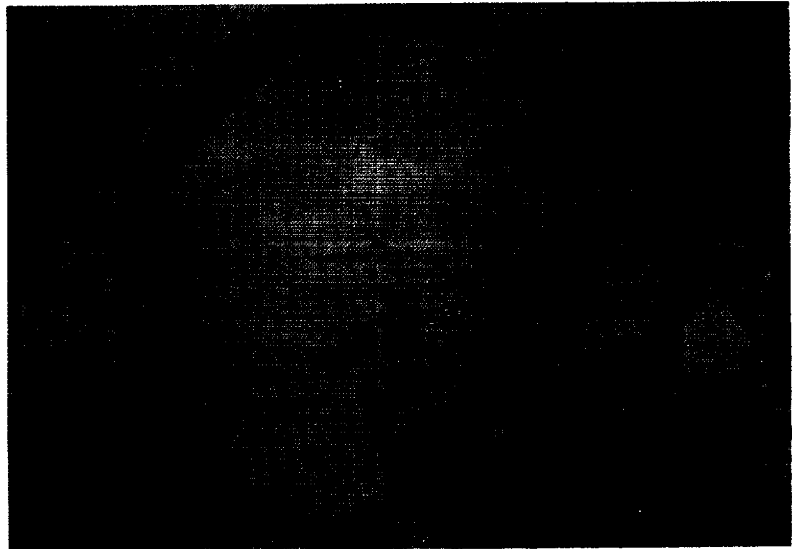


Fig. 5 Photomicrographs of 1 μm sections through *Aplysia* statocysts before culture (**A**), after 4 days in culture at 16–18 C (**B**), and after 4 days in culture at 22–26 C (**C**). Note the lack of cilia in **C**. The greater number of statoconia present in **C** is because this is a statocyst from an early juvenile animal (1.5 gm 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 ×

Fig. 6 Transmission electron micrographs of statocysts before (**A**) and after culture for four days (**B**). *C*, cilia; *RC*, receptor cell; *SC*, supporting cell; *MV*, microvilli; and *N*, nucleus. *Bar* = 2 μm. Magnification: 13,500 ×

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



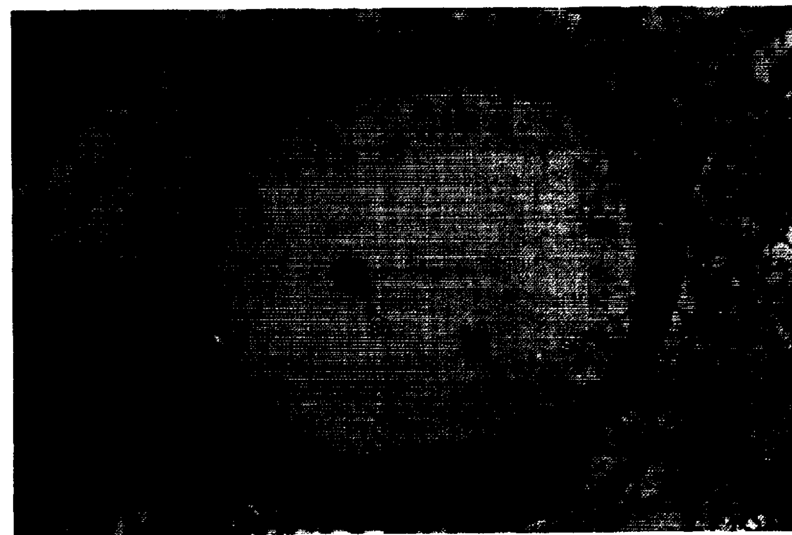


Fig. 7 Photomicrographs of 1 μm sections through adult *Aplysia* statocysts after staining with anti-carbonic anhydrase I or II antibody. **A** Control (i.e., no primary antibody used); **B** after incubation with anti-carbonic anhydrase I antibody; **C** after incubation with anti-carbonic anhydrase II antibody. *Sc*, statoconia; *C*, cilia; *RC*, receptor cell; *SC*, supporting cell; and *N*, nucleus. *Bar* = 20 μm. Magnification: 85 ×

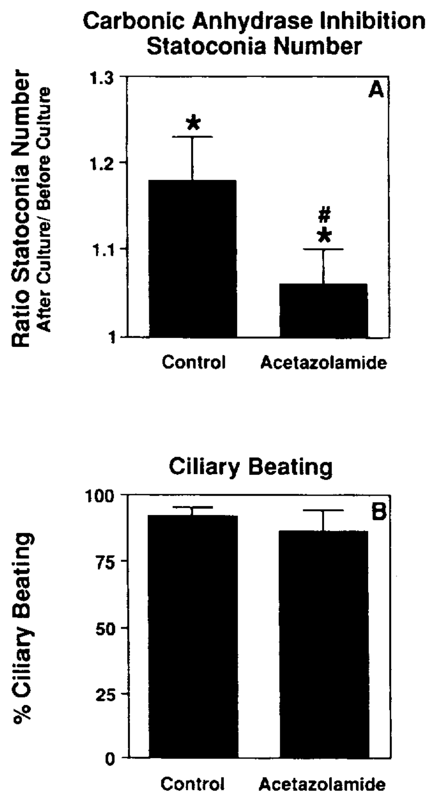


Fig. 8 Effect of the carbonic anhydrase inhibitor, acetazolamide, on in vitro statoconia production (**A**) and ciliary beating (**B**). Values represent the mean ± SEM of a single experiment. This experiment was performed 3 times. $^{*}P < 0.05$, before culture vs. after culture, paired Student's *t*-test and $^{\#}P < 0.05$, acetazolamide vs. control, unpaired Student's *t*-test

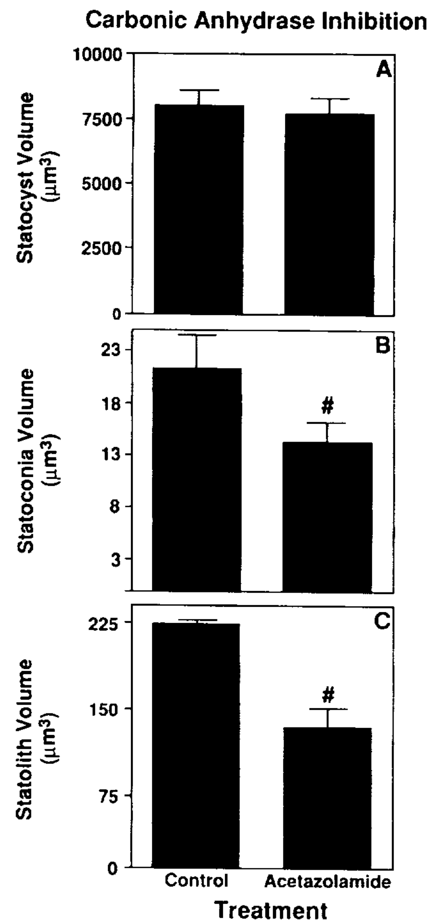


Fig. 9 Effect of the carbonic anhydrase inhibitor, acetazolamide, on statocyst (**A**), statoconia (**B**), and statolith (**C**) volumes in μm^3. This experiment was performed 3 times. Values represent the mean ± SEM of a single experiment. $^{*}P < 0.05$, unpaired Student's *t*-test

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 supprted 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 (Vicentiis 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 $^{45}Ca^{2+}$ 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 location 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.

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References

Cao H, Gay CV (1985) Effects of parathyroid hormone and calcitonin on carbonic anhydrase location in osteoclasts of cultured embryonic chick bone. Experientia 41: 1472–1474

Coggeshall RE (1969) A fine structural analysis of the statocyst in *Aplysia californica*. J Morphol 127: 113–131

Erulkar SD, Maren TH (1961) Carbonic anhydrase and the inner ear. Nature 189: 459–460

Gallin EK, Wiederhold ML (1974) Response of *Aplysia* statocyst receptor cells to physiologic stimulation. J Physiol (Lond) 266: 123–137

Goldberg DJ (1991) Culturing the large neurons of *Aplysia*. In: Banker G, Goslin K (eds) Culturing nerve Cells. The MIT Press, Cambridge, Mass., pp 155–175

Harada Y (1984) Effects of carbonic anhydrase inhibitor to the otolithic organs. In: Nishimura N (ed) The vestibular organs morphology, physiology and pathology, Tokyo pp 183–188

Hunter SJ, Schraer H, Gay CV (1988) Characterization of isolated and cultured chick osteoclasts: the effects of acetazolamide, calcitonin, and parathyroid hormone on acid production. J Bone Min Res 3(3): 297–303

Johnson RL, Spoendlin HH (1966) Structural evidence of secretion in the stria vascularis. Ann Otol Rhinol Laryngol 75: 127–138

Kido T, Sekitani T, Yamashita H, Endo S, Masumitshu Y, Shigomori H (1991) Effects of carbonic anhydrase inhibitor on the otolith organ in developing chick embryos. Am J Otolaryngol 12: 191–195

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

Lim DJ, Karabinas C, Trune D (1983) Histochemical localization of carbonic anhydrase in the inner ear. Am J Otolaryngol 4: 33–42

Minkin C, Jennings JM (1972) Carbonic anhydrase and bone remodeling: sulfonamide inhibition of bone resorption in organ culture. Science 2: 1031–1033

Pedrozo HA, Wiederhold ML (1994) Effects of hypergravity on statocyst development in Embryonic *Aplysia californica*. Hearing Res 79: 137–146

Purichia N, Erway LC (1972) Effects of dichlorophenamide, zinc, and manganese on otolith development in mice. Dev Biol 27: 395–405

Ross M, Pote KG, Cloke PL, Corson C (1980) In vitro $^{45}Ca^{2+}$ uptake and exchange by otoconial complexes in high and low K^+/Na^+ fluids. The Physiologist 23: S129–S130

Stechschulte DJJr, Morris DC, Silverton SF, Anderson HC, Vaananen HK (1992) Presence and specific concentration of carbonic anhydrase II in matrix vesicles. Bone and Mineral 17: 187–191

Takumida M, Bagger-Sjoback D, Wersall J, Harada Y (1989) Ultrastructural localization of carbonic anhydrase in the vestibular end organs of the guinea pig. Arch Otolaryngol 246: 56–60

Vicentiis M, Marmo F (1967) Inhibition of the morphogenesis of the otoliths in the chick embryo in the presence of carbonic anhydrase inhibitors. Experientia 24/8: 818–820

Watanabe K, Ogawa A (1984) Carbonic anhydrase activity in stria vascularis and dark cells in vestibular labyrinth. Ann Otol Rhinol Laryngol 93: 262–266

Weiss RE, Wilbur KM (1978) Effects of cytochalasin B on division and calcium carbonate extrusion in a calcareous alga. Exp Cell Res 112: 47–54

Wiederhold ML (1974) *Aplysia* statocyst receptor cells: intracellular responses to physiological stimuli. Brain Res 78: 490–494

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

Wiederhold ML, Sharma JS, Driscoll BP, Harrison JL (1990) Development of the statocyst in *Aplysia californica* I. Observations on statoconial development. Hearing Res 49: 63–78