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ISOLATION OF PIGMENTED GRANULES INVOLVED IN EXTRA-RETINAL PHOTORECEPTION IN *APLYSIA CALIFORNICA* NEURONS

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Summary

Many neurons in the ganglia of *Aplysia californica* contain pigmented, membrane-bound granules (lipochondria), which are thought to mediate the light response of some of the neurons, including the giant cell of the abdominal ganglion. A method of isolating the lipochondria by centrifugation of ganglia homogenates has now been developed. Electron microscopy was used to demonstrate that most of the lipochondria remain morphologically intact. As shown by X-ray microanalysis, isolated lipochondria contain the same elements, including calcium, as do lipochondria in intact giant cells. The calcium can be released into the medium by treatment of the organelles with the Ca^{2+} ionophore A23187. It appears that the lipochondria of *Aplysia* ganglia are similar in their morphology, elemental content and susceptibility to the ionophore. Two pigments were isolated from the lipochondria, and chromatography and spectrophotometric studies indicated that they are β-carotene and a "retinol-like" compound.

Introduction

Blue-green light increases a calcium-activated membrane potassium conductance in identifiable neurons of the marine mollusc *Aplysia californica* [1,2]. The cells which have been investigated most thoroughly are the giant cell R2 [3] and the ventral photoresponsive neuron (Andresen, M.C. and Brown, A.M., unpublished) of the abdominal ganglion.

The response is, in some ways, similar to that of retinal photoreceptors, but its slowness, high threshold, and lack of amplification are more often associated

Abbreviation: Arsenazo III, 2,2'-[1,8-dihydroxy-3, 6-bisulfo-2,7-naphthalene-bis (azo)]-dibenzenearsonic acid.

with extra-retinal photoreception. The latter is a poorly understood phenomenon that is widespread throughout the animal kingdom. The cytoplasm of light-sensitive and many other *Aplysia* neurons abounds with masses of membrane-bound yellow-orange granules [4] containing calcium which is depleted following illumination [5,6]. These pigmented granules (lipochondria), therefore, probably mediate the light response. The first major event after photon capture is believed to be calcium release within the cell. In order to facilitate study of the initial light-induced processes, we have successfully isolated the lipochondria from *Aplysia* neurons.

Chalazonitis and Arvanitaki [4] prepared pigmented granules from optic ganglia of *Sepia* and visceral ganglia of *Aplysia depilans*. These fractions, however, included other cell components besides the pigmented bodies. The isolation procedure reported here gives excellent purity and reasonable yields of intact lipochondria containing pigments and calcium which can be released by the Ca^{2+} ionophore, A23187. A preliminary report of this research has been published [7]. Lipochondria isolated from the combined ganglia are shown to be similar in several respects to those found in the intact giant cell R2.

Methods

The experimental animals, *A. californica* (Pacific Bio-Marine Supply Co., Venice, Calif.), are kept in an aerated aquarium and fed dried seaweed. They are dark-adapted overnight prior to removal of the ganglia. Under dim red light, the abdominal and circumesophageal ganglia are removed and placed in artificial sea water. As much extraneous nervous and connective tissue as possible is trimmed from the ganglia and they are placed in fresh, chilled artificial sea water.

The isolation method is summarized in Fig. 1. All glassware and solutions are chilled in an ice bath. The ganglia, which weigh about 0.2 g (3 animals), are rinsed in Solution 1 (see Fig. 1). They are then placed on a Plexiglas block over ice and minced thoroughly with a razor blade. Minced ganglia are rinsed into a Dounce homogenizer with 2 ml of homogenizing medium. When a larger quan-

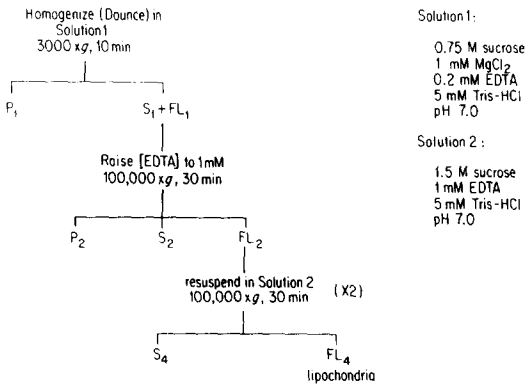

Fig. 1. Isolation of lipochondria from *Aplysia* ganglia. P, pellet; S, supernatant; FL, floating layer.

tity of tissue is used, it is best to homogenize it in small batches to prevent breakage of the organelles. The mixture is homogenized gently by hand until most of the yellow-orange material has been suspended (about 3—5 strokes). The homogenate is centrifuged (Fig. 1) in a Sorvall RC2-B centrifuge (SS-34 rotor) to produce an initial pellet (P_1), supernatant (S_1) and "floating layer" (FL_1). This step removes heavier cellular elements (P_1).

The orange-colored lipochondria (FL_1), which float or stick to the side of the tube, are carefully brought into suspension with a glass rod, and this suspension is aspirated off and diluted with Solution 1. EDTA is added to a final concentration of 1 mM (if this step is omitted, other organelles are found in the lipochondrial pellet). The mixture is poured into a centrifuge tube and centrifuged on an SW 27.1 swinging bucket rotor in a Spinco L2-65B ultracentrifuge.

The resulting pellet (P_2) contains yellow and sometimes brownish material, including mitochondria, microsomes and a few small lipochondria (unpublished electron microscope observations). Most of the lipochondria (FL_2) float at the top of the supernatant. They are easily removed by gently touching a rounded ground glass homogenizer pestle, which is slightly smaller in diameter than the tube, to the floating layer and immediately dispersing the lipochondria into Solution 2. If lipochondria stick to the side of the tube, they can be carefully brought into suspension with a glass rod and aspirated out. The resuspended floating layer is centrifuged again to produce FL_3, with which the centrifugation is repeated to yield FL_4. These steps remove vesicular material, including synaptosome-like bodies.

FL_4 is resuspended in a medium of the same osmotic strength as Solution 2. When the lipochondria are to be fixed for electron microscopy, they are dispersed in a minimum amount of Solution 2 to which is then added an equal amount of the following fixative: 12% glutaraldehyde (Taab Laboratories, Reading, U.K.) [5], 2% OsO_4, in Solution 2 (based on the fixative of Trump and Bulger [8]). This fixative must be made immediately before use, as it darkens rapidly. It is necessary to fix the lipochondria with OsO_4 in order to precipitate them, and better fixation is obtained when glutaraldehyde is included.

The fixed lipochondria are allowed to stand at room temperature for about 15 min and are then centrifuged in 400 μl plastic tubes in a Beckman 152 Microfuge for 2 min. Usually it is necessary to remove the supernatant and add more fixed lipochondria to the pellet in order to obtain a pellet large enough to be embedded. The pellet may be stored overnight in the cold in 1% OsO_4; it may become resuspended if left with supernatant. The pellet is cut out of the plastic tube with a razor blade, and if desired, cut into pieces. The specimen is rinsed in distilled water, dehydrated in ethanol series, and embedded in Epon or Spurr resin. Thin sections are cut on a DuPont MT2-B ultramicrotome and stained with uranyl acetate and lead citrate. A Philips 300 electron microscope was used to examine the sections.

For elemental analysis of lipochondria, they were isolated as described above. The last floating layer was dispersed in a minimum amount of Solution 2, and drops of the suspension were frozen rapidly in liquid nitrogen on stubs for cryosectioning at $-70°C$ on a DuPont MT 2-B ultramicrotome with low temperature controller and frozen thin sectioner accessories. Sections 1 μm thick were cut, placed on celloidin-coated Ni folding grids, and lyophilized. The

specimens were examined in a Cambridge S4-10 scanning electron microscope in the scanning-transmission mode. An energy dispersive X-ray analyzer (EDAX International, Inc., 707A) attached to the microscope was used for qualitative determination of the elements present in the lipochondria.

Ca^{2+} discharge from lipochondria was monitored in a dual-beam spectrophotometer (fabricated by Johnson Research Foundation, University of Pennsylvania), using the metallochromic dye arsenazo III at the wavelength pair 675–685 nm. Under red light a 0.2 ml aliquot of lipochondria was suspended in 1.5 M sucrose, 5 mM Tris · HCl, pH 7.0 and 150 μM arsenazo III at a final volume of 1.3 ml. After establishment of a baseline trace, 10 μg of the Ca^{2+} ionophore A23187 were added to induce Ca^{2+} discharge from the lipochondria. The appearance of Ca^{2+} in the medium was indicated by a change in the absorbance of the arsenazo III dye. The A23187 was the kind gift of Dr. Robert L. Hamill of Eli Lilly and Co., Indianapolis, Ind., and a stock solution of 1.0 mg/ml in 70% ethanol was used. The arsenazo III was obtained from Sigma Chemical Co., St. Louis, Mo. and was purified by the procedure of DiPolo et al. [9].

Results

Fig. 2A is an electron micrograph of an intact dark-adapted R2 cell from the *Aplysia* abdominal ganglion. The lipochondria (arrows) contain moderately and

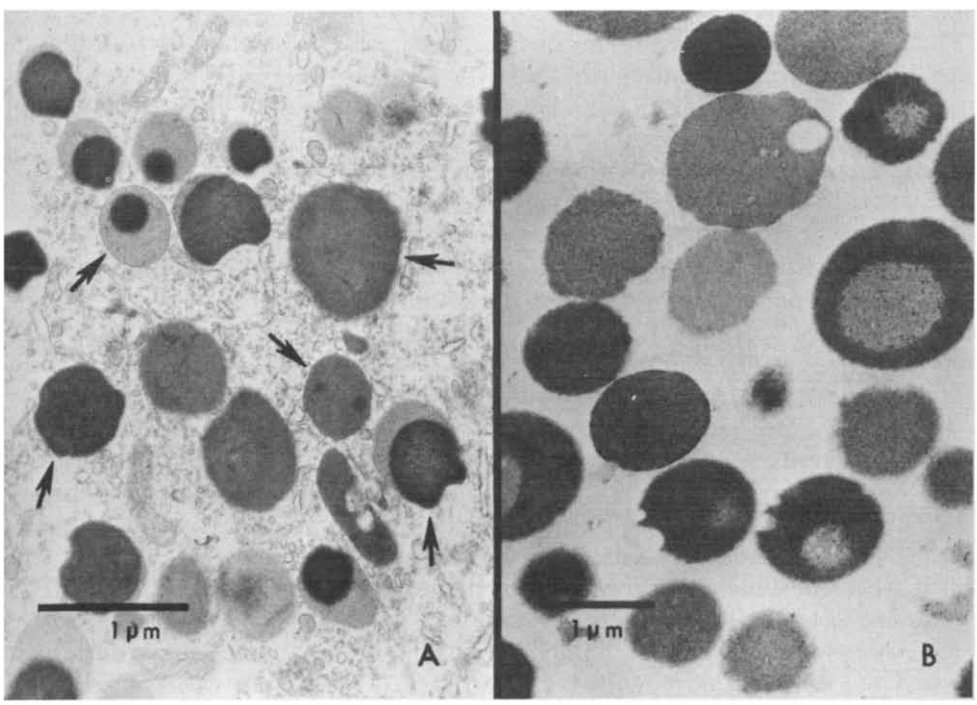

Fig. 2. Electron micrographs of lipochondria in an intact cell and an isolated suspension. A. Lipochondria (arrows) in an intact, dark-adapted R2 cell. The specimen was fixed in 5% glutaraldehyde in 0.1 M piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], pH 7.6. It was postfixed in 1% OsO_4 and stained with uranyl acetate and lead citrate. B. Isolated lipochondria, fixed as described in the text and stained with uranyl acetate and lead citrate.

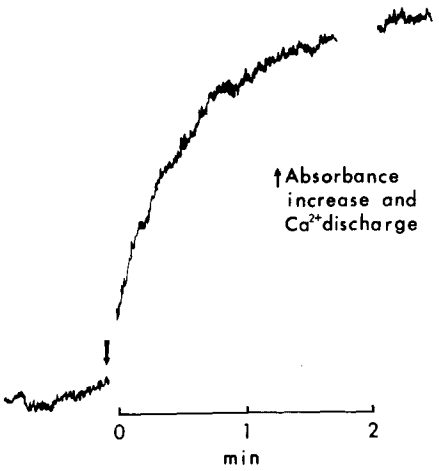

Fig. 3. Representative dual-beam spectrophotometric trace of A23187-induced discharge of Ca^{2+} from isolated lipochondria. The arrow indicates addition of A23187 (10 μg) to the medium inducing Ca^{2+} discharge from the lipochondria (upward deflection of trace). The calcium-sensitive dye arsenazo III (150 μM) at the wavelength pair 675—685 nm was used to detect the presence of Ca^{2+} in the medium. Blank regions on the trace are deletions of recorder artifacts made during reproduction. See Methods for details.

highly electron-dense material and are bounded by a membrane. Fig. 2B illustrates the appearance of a pellet of isolated lipochondria. This pellet was quite small (about 0.5 mm^3), so that it could be examined thoroughly. All sections of it appeared uniform. The pellets contained only lipochondria, all of which were in the dark-adapted form. Most had a boundary membrane. They were essentially indistinguishable from the lipochondria in the intact cell (Fig. 2A). Scanning electron microscopy of isolated, fixed lipochondria also showed that they were oblate spheroids, and no other organelles were seen (data not shown).

X-ray spectra of isolated lipochondria showed that their elemental composition was similar to that of intact R2 cell lipochondria [6] and included Na, Mg, Br, Si, P, S, Cl, K, and Ca.

The Ca ionophore A23187 was observed to release Ca^{2+}, which was then complexed by arsenazo III, from the isolated lipochondria. Fig. 3 is a dual-beam spectrophotometric tracing showing the A23187-induced release of Ca^{2+} (upward deflection) from a suspension of isolated lipochondria. The volume of the lipochondria used in this experiment was estimated, from experiments in which lipochondria were pelleted, to be about 0.5 mm^3. The total increase in absorbance corresponded to a final concentration of 38.5 μM Ca^{2+}, as determined by calibration of the instrument with known concentrations of Ca^{2+}. The concentration of Ca in the lipochondria was calculated to be approx. 10^{-1}—10^{-2} M.

Discussion

Morphological integrity and extent of contamination of the preparation of Chalazonitis and Arvanitaki [4] is not known. Our lipochondrial preparations have been thoroughly examined with the electron microscope (Fig. 2) and essentially contain only lipochondria which are intact and comparable morphologically and in elemental content to lipochondria of intact R2 cells [6].

The lipochondria are easily disrupted, and this factor appears to vary with the physiological state of the animals. Some (if not most) granules are likely to burst if they are transferred from high to low osmotic strength, and once most of them were lost even when they remained in the same medium and had been homogenized very gently.

It is extremely unlikely that a significant number of the isolated lipochondria are from the giant R2 cell or the ventral photoresponsive neuron of the abdominal ganglion. The pigmented portion of this ganglion comprises no more than 20% of the total weight of the ganglia, and all of the ganglia have the same orange color. The R2 and ventral photoresponsive neuron cells are only two of many pigmented cells in the abdominal ganglion [3]. Therefore, many if not all of the lipochondria of *Aplysia* ganglia, as observed in our lipochondrial pellets, are morphologically similar to those of the R2 cell.

The similarity of X-ray spectra of isolated lipochondria and those of intact R2 cells indicates that the isolation procedure does not remove significant amounts of material from the lipochondria, and lipochondria from other cells besides R2 have an elemental content comparable to that of R2 lipochondria.

Exposure of intact R2 cells to the Ca^{2+} ionophore A23187 has been shown to produce morphological changes in the lipochondria similar to those seen upon exposure to light for 20 min [6]. Illumination also reduces the amount of Ca in R2 lipochondria [5,6]. It was therefore of interest to determine whether or not Ca, which was detected by X-ray microanalysis in isolated lipochondria, is released from them by A23187. This was found to occur. Although A23187 does release some Mg^{2+} as well as Ca^{2+} [10], interference from Mg^{2+} is negligible when the absorbance difference at 675–685 nm of arsenazo III is used [9]. Ionophore-induced release of Ca^{2+} from isolated lipochondria demonstrated that, although the X-ray peak for Ca is small, the lipochondria do contain Ca^{2+} which can be released into the medium. There is evidence [11] that the lipchondrial Ca^{2+} is in a precipitated form, so that our estimate of 10^{-1}–10^{-2} M Ca^{2+} in the lipochondria does not seem unreasonable. Lipochondria from many other cells besides R2 must have contributed Ca^{2+}, so that susceptibility to release of Ca^{2+} by A23187 must also be a common characteristic of lipochondria.

Many neurons of *Aplysia* ganglia are pigmented, but only a few have been found to exhibit a change in potential in response to illumination. The factor(s) responsible for this difference is (are) unknown. The experiments reported here indicate that the pigmented granules of all *A. californica* ganglia are similar in morphology, elemental content, and susceptibility to Ca^{2+} release by ionophore A23187. It appears, then, that none of these characteristics has a role in determining which neurons will respond electrophysiologically to illumination.

We have used lipochondria isolated by the method described here to attempt to identify their pigment(s) by thin-layer chromatography and spectrophotometry. The organelles contain at least two pigments, β-carotene and a compound that appears to be retinol. These were extracted from the lipochondria by homogenization in $CHCl_3$/methanol (2 : 1, v/v). On thin-layer chromatography plates developed in 100% acetone, extracts of ganglia or isolated lipochondria were separated into two major pigments. Both of these were stained blue by $SbCl_3$ and by phosphomolybdic acid. The spot migrating farthest was

yellow-orange and appeared dark under long-wave ultraviolet light, while the other major spot was colorless but brightly fluorescent (yellow-green). It was necessary to add 0.25% sodium dodecyl sulfate to the extract in order to remove most of the fluorescent material from the origin; therefore it seems likely that it is attached to protein. The yellow-orange spot had the same R_F (0.78) as β-carotene, which is also dark under ultraviolet light. The spot was eluted with $CHCl_3$ and its spectrum was measured in the visible region. The spectrum (peaks at 433 and 460 and shoulders at 410 and 492 nm) was quite similar to that of β-carotene (peaks at 437, 465 and 492 nm), although the shoulder at 410 nm probably indicates the presence of an impurity. Evidence from resonance Raman spectroscopy [12] also indicates that this pigment is β-carotene. The fluorescent spot had the same R_F (0.70) as retinol, which is also fluorescent. The absorption spectrum of this spot, which had been irradiated with ultraviolet light in order to locate it, had its λ_{max} at 277—278 nm. This is in the range (275—280 nm) of the λ_{max} of ultraviolet-irradiated retinol [13]. The lipochondria pigments also migrated with β-carotene and retinol in a 2-dimensional thin-layer chromatography system, with light petroleum (30—60°C)/acetone (7 : 3, v/v) used for the second development. The "retinol-like" pigment appeared to be much more abundant than the β-carotene in isolated lipochondria. We could not detect any hemeprotein, which was reported by Arvanitaki and Chalazonitis [15] to be present in neurons of *Aplysia depilans* and *punctata*. The same authors also reported the presence of two carotenoids conjugated with protein in these neurons; these pigments might be comparable to the ones we extracted from *A. californica*. Retinaldehyde and 3-dehydroretinaldehyde are the only compounds known to act as chromophores in light-sensitive nerve cells [14]. Our studies indicate that if either of these is present in lipochondria, it occurs in very small amounts or in only a few cells. It is possible that the presence of one of these compounds in only light-sensitive cells could be a factor determining which cells respond to light.

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