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Nutrient Storage Cells Isolation from Mantle Tissue of *Mytilus galloprovincialis*: Glucose Release and Glycogen Content

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A method for obtaining isolated mantle nutrient storage cells and purifying vesicular (VC) and adipogranular (ADG) cells from mantle tissue of *Mytilus galloprovincialis* is reported. Tissue digestion is partly mechanical (stirring) and partly enzymatic (collagenase + dispase). Purification is carried out through continuous and discontinuous Percoll gradients. VC appears in fraction 3 (d = 1.05-1.08 g/ml) and ADG in fraction 2 (d = 1.09 g/ml). Intracellular glycogen and free-glucose content in September-April period is studied. When glycogen is detectable it is always accompanied by intracellular free-glucose pool in a concentration relationship glycogen/glucose 10:1. Furthermore, a glucose releasing activity elicited by the Ca^{2+}-ionophore A23187 was found in isolated cells, which reproduce the former behaviour found with mantle tissue fragments in our laboratory.

Key words: Mytilus, Mantle cells, Cell purification.

In bivalves glycogen is an important energy material. It is found particularly in special storage cells, mainly in vesicular cells (VC) and also in adipogranular cells (ADG), which are particularly abundant in the mantle tissue (4, 10, 12). These cells altogether are named mantle storage cells (MSC), which underline the predominant functional features of these types of cells. VC are characterized by the presence of a large mass of glycogen which occupies almost the entire volume of the cell; and ADG by the presence of lipid droplets, protein granules and glycogen particles (4, 8, 10).

In the Mytilidae family the seasonal cycle of storage and utilization of glycogen reserves is closely linked to the annual reproductive cycle. Glycogen accumulates in summer and then declines in autumn and winter (7). Little is known about the factors which control glycogen metabolism in MSC. Recently we found evidences for the existence of a glucose release stim-

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ulation in the mantle tissue of *Mytilus galloprovincialis* by Ca^{2+} (6). It would clearly be advantageous if isolated MSC were available for detailed studies of the various aspects of glycogen metabolism in these cells.

In this paper a method for obtaining isolated MSC and purified VC and ADG from mantle tissue is described. The isolated cells were used to investigate the intracellular glycogen and free-glucose content and to carry out experiments in absence of Ca^{2+} ions to compare with similar experiments previously performed with pieces of mantle (6).

Materials and Methods

Animals.— All experiments were performed on adult specimens of *Mytilus galloprovincialis* from Ría de Arosa (Spain) (shell height 8-10 cm). They were kept at 14 ± 1 °C, 12h/12h light/dark cycle and fed with a diet of a microalgal mixed. The sea water was changed weekly.

Chemicals.— All chemicals used were of the highest grade. Thus, amyloglucosidase (from *Rhyzopus mold*), collagenase (from *Clostridium hystoliticum*, type V) and ethylenediaminetetraacetic acid (EDTA) were from Sigma; neutral protease (dispase) from Böehringer Mannheim. Percoll (d = 1.130 g/ml) and density marker beads (DMB) were from Pharmacia Ibérica (Spain). For the glucose test a kit (Glucinet) from Sclavo (Siena, Italy) was used. The trypan Blue reagent was obtained from Flow Laboratories (U.S.A.). Ultra-pure water (18 MΩ · cm) was used throughout.

The osmolality of the solutions was measured by the freezing point depression technique, using a digital osmometer (Osmomat 030, Gonotec). The density of the solutions was determined by gravimetry, using a picnometer. All solutions for density gradient centrifugation were adjusted to 900 mOsm/kg H_2O.

Cellular isolation and purification.— Eight to ten hemimantles (dissected from specimens) were collected in artificial sea water (ASW) pH 7.0, with the following ionic composition in mEq/l: Na^+, 512; K^+, 10; Cl^-, 500; Ca^{2+}, 5 and 20 mM HEPES. This solution was used as basic medium throughout the preparation and the incubation of the isolated cells. The pooled mantles were chopped, washed in ice-cold ASW (ICASW) for 2 min and filtered through a nylon cloth (200 µm mesh); after that, the fragments were transferred to Petri dishes and washed for 30 min with ICASW under stirring; finally the fragments were filtered and washed twice (5).

Cell isolation was carried out with the pooled fragments (2-3 g) in a PVC beaker with collagenase (700 UI/ml), dispase (6 UI/ml) and 1 mM EDTA in Ca^{2+}-free ASW, pH 7.0 (20 ml, final digestion volume). The beaker was placed in a water bath (37 °C) and the fragments were in-

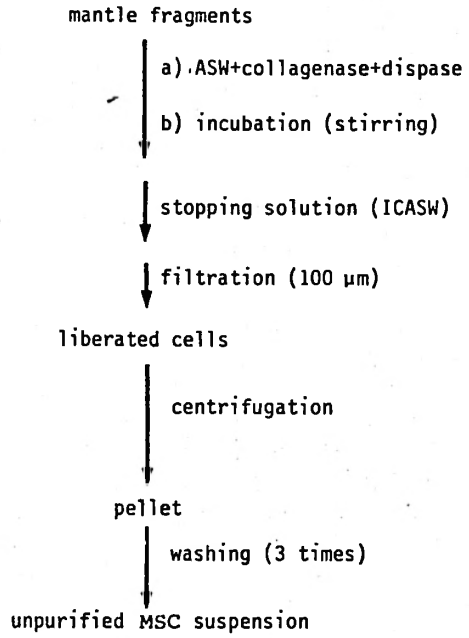

Fig. 1. *Isolation of MSC by mechanical and enzymatic treatment.*
For further details of the method see text.

cubated during 30 min with vigorously internal stirring (magnetic bar). The incubation was stopped by addition of 5 ml of ICASW. The liberated cells were concentrated by centrifuging the filtrate for 5 min at 100 × *g* (4 °C) and were washed three times in the same conditions of centrifugation with ASW (final volume, 10 ml) (fig. 1).

Purification of the MSC was effected by continuous and discontinuous density gradients of Percoll. A stock solution of Percoll was prepared by mixing 4 parts of Percoll with 1 part of ASW (5X). To obtain the desired density this stock solution of Percoll was diluted with ASW. Continuous density gradients were performed in a gradient-maker as described by HAMES (11) (density steps, top: 1.04 g/ml; bottom: 1.10 g/ml). Discontinuous density gradients were performed with density steps: 1.04, 1.06, 1.08 and 1.10 g/ml). The final pellet obtained above was resuspended in ASW and approximately 10^7 cells/ml were layered on both gradient and centrifuged at 390 × g, 20 °C for 30 min. For both kinds of gradients parallel tubes containing a DMB profile were simultaneously centrifuged (fig. 2).

Cell viability was tested by the trypan blue exclusion (14).

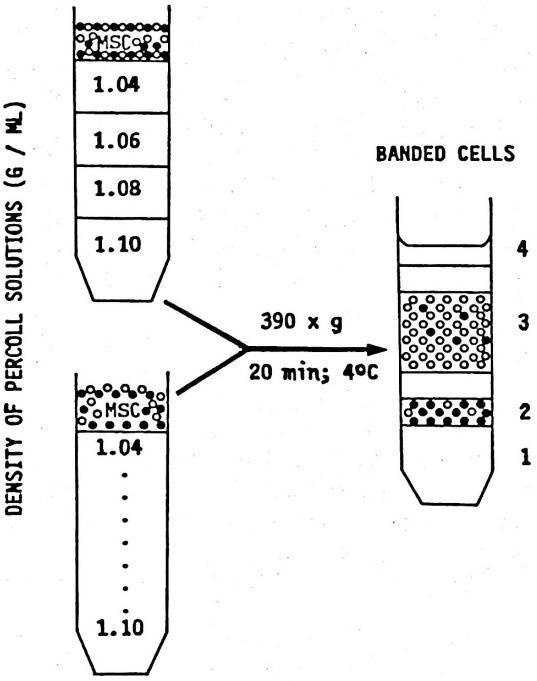

Fig. 2. *Purification of VC and ADG.* 1.5-3.0 × 10^7 isolated cells (mixed VC:ADG) in 1 ml of ASW were purified by centrifugation through continuous and discontinuous Percoll density gradients. In both cases, unpurified MSC were layered on the density column as shown in the figure.

Intracellular glycogen and free-glucose content.— Glycogen and free-glucose content in purified cells was determined by the micromethod previously described (5). The glucose yield was tested by the glucose oxidase peroxidase method (3).

Glucose release experiments.— Aliquots of 10^6 purified cells were incubated in ASW pH 7.0 (1 ml final volume). When required Ca^{2+} ions were replaced with the osmotically equivalent amount of NaCl as well as other treatments were added. Incubations were carried out at 20 °C for 60 min in a giratory shaker (150 cycles/minute) and were stopped at 4 °C by centrifugation (1,060 × g, 10 min). Glucose was determined in the supernatant.

Statistical analysis.— When required results were analysed using the Student's t test for unpaired data (15). Values of $p < 0.05$ were considered significant. Generally results are expressed as the mean ± SEM specifying the number of individual determinations (n).

Results

Cellular isolation.— When mantle tissue fragments are incubated with collagenase and dispase a great number of cells are released. Tissue digestion being partly

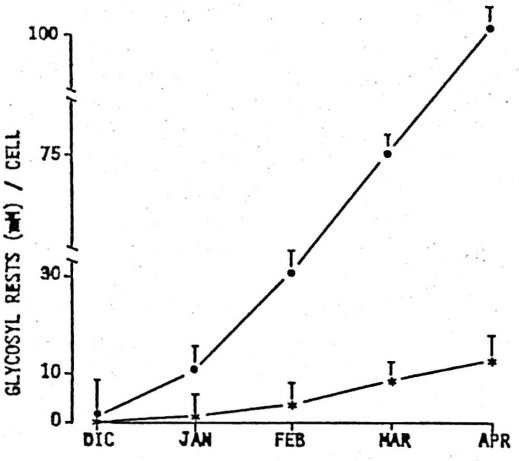

Fig. 3. *Monthly profiles of intracellular glycogen and free-glucose pools.*
For comparative reasons, glycogen (●) and glucose (⁎) values are expressed as glycosyl rests. Each point represents the mean ± SEM (n = 8).

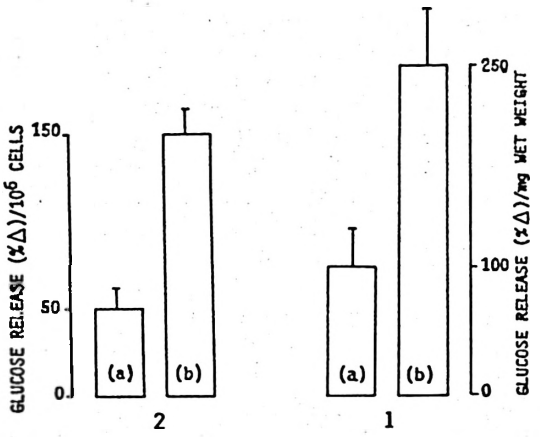

Fig. 4. *Effects of A23187 (10 μM) on glucose release by mantle tissue fragments n = 6 (1) and isolated MSC, n = 8 (2).*
Experiments were carried out with (a) and without (b) Ca^{2+} ions. Each bar represents mean ± SEM for each experimental group.

mechanical (stirring) and partly enzymatic. The material released from the mantle consists of large spherical cells (vesicular and adipogranular cells), together with numerous other cells and particles. However, if the tissue is not washed before digestion, the dispersed suspension of cells is largely contaminated with spermatozoa.

Continuous and discontinuous Percoll gradients yielded homogenous VC population in fraction 3 (d = 1.05-1.08 g/ml) for both cases and purified ADG in fraction 2 (d = 1.09 g/ml) for both cases.

After an overall purification flow diagram has been achieved, the cells obtained have shown a viability better than 90 %. The purification degree was in the ranges of 70-80 and 80-90 % for VC and ADG respectively.

The number of VC/ADG isolated per 2-3 g of mantle was ca $3 \times 10^7/10^7$.

Intracellular pools of glycogen and free-glucose.— Intracellular glycogen and free-glucose pools can be precisely determined in isolated MSC. When intracellular glycogen is detectable it is always accompanied by an intracellular free-glucose pool in a concentration relantionship glycogen/glucose 10:1. Thus, both pools correlated positively during the seasonal changes in glycogen cell content (fig. 3).

Glucose releasing activity.— When isolated MSCs were incubated with A23187 treatment (with and without Ca^{2+}), the glucose releasing activity elicited reproduced the former behaviour found with mantle tissue fragments (fig. 4).

Discussion

Whenever a function operating at tissue level can be reduced to some cellular type(s) of the tissue, it is of great value to obtain the responsible cells in isolated form. The method suggested here can significantly achieve it by obtaining MSC and purifying VC and ADG from mantle

tissue of Mytilus, while keeping the cell functional viability.

It has been stated that the main component of glycogen metabolism in the mantle tissue of Mytilus resides in these two kinds of cells (8, 12, 13). After both of them are obtained, in an isolated mode or as mixed populations (VC + ADG) the way is opened to study directly not only glycogen metabolism, but the specialized functions of these cells, which are needed for the animal physiology as well.

The glycogen content obtained in the isolated MSC, mainly stored in VC, represents 25 % of the total mantle glycogen content from female specimens in comparison with results obtained by Lubet *et al.* (12) in stages I-II of gametogenesis (study period). This occurs because the other glycogen fraction remains mainly in previtellogenic eggs, as it has been reported (1, 9).

To gain an understanding of how these cells work, it is important to know whether they need to be partially or totally lysed in order to make the glycogen to be broken available, as has been claimed (7, 8, 12). The first was performed on pieces of mantle tissue of *Mytilus galloprovincialis*, which showed a glucose releasing activity elicited by A23187 (4, 6). This A23187-activated glucose releasing activity has been used here as a marker for the functional viability of the isolated MSC. The data obtained reproduce the former behaviour found with mantle tissue fragments and suggest that a rise in cytosolyc Ca^{2+} in MSC may be a sufficient condition for a possible glycogenolysis, to be «turned on». If so, the suggestion that a rise in cytosolyc Ca^{2+} activates glycogenolysis in MSC remains to be confirmed.

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Resumen

Se describe un método para obtener células vesiculares (VC) y adipogranulares (ADG) aisladas y purificadas del tejido del manto de *Mytilus galloprovincialis*. La digestión tisular es parcialmente mecánica (agitación) y parcialmente enzimática (colagenasa + dispasa). La purificación se lleva a cabo mediante gradientes continuos y discontinuos de Percoll. Las VC aparecen en la fracción 3 (d = 1,05-1,08 g/ml) y las ADG en la fracción 2 (d = 1,09 g/ml). Se estudia el contenido intracelular de glucógeno y glucosa libre durante el período septiembre-abril. Cuando el glucógeno es detectable siempre está acompañado de un fondo de glucosa libre en una relación de concentraciones glucógeno/glucosa 10:1. Además, existe una actividad liberadora de glucosa, estimulada por el ionóforo de Ca^{2+} A23187, que coincide con el comportamiento experimentado por fragmentos del tejido del manto.

Palabras clave: Mytilus, Células del manto, Purificación celular.

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