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Ca^{2+} Activates Glycogenolysis in Isolated Mantle Storage Cells of *Mytilus galloprovincialis Lmk*

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Glycogenolytic activity (GA) in isolated mantle storage cells (MSC) from *Mytilus galloprovincialis* was studied, while glycogen and free-glucose content, as well as glucose released from cells were tested. In the period studied (November-December), the glucose releasing activity measured can be considered as an output of GA. In both, whole cells system (WCS) and crude cell-free system (CFS), a non-stimulated GA was detected. In WCS, dopamine and 5-hydroxytryptamine (5-HT) stimulated glycogenolysis, while epinephrine, norepinephrine and isoproterenol did not show any effect. Furthermore, mellitin and the Ca^{2+}-ionophore, A23187, had a stimulating effect on the GA. In CFS, the absence of Ca^{2+} ions was a sufficient condition to depress GA. These and other findings suggest that: 1) GA in MSC may be stimulated by dopamine and 5-HT and not by adrenergic agonists; 2) cytosolyc Ca^{2+} signalling may have become an absolute requirement for activation of the glycogenolytic cascade in MSC; 3) a rapid high-affinity glucose transport may occur in these cells.

Key words: Mytilus, Mantle cells, Glycogenolysis.

The bioenergetic metabolism of *Mytilidae* family has been reported as carbohydrate based (8, 11, 12). The mantle tissue of *Mytilus* genus is the main site of glycogen storage (9, 23). Glycogen in the mantle is stored in the vesicular cells (VC), also named glycogen cells (GC), as well as in the adipogranular cells (ADG) but to a lower extent (4, 18, 19). These cells altogether are named mantle storage cells (MSC).

Little is known about the factors which control the glycogen mobilization from the MSC; nothing is known about the type of intracellular messenger if there is any, which could have a definitive role in that mobilization. It is well established that the glycogen breakdown in muscle and liver cells of mammals is controlled by

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several hormones each binding to a specific cell surface receptor and acting through one or more intracellular signalling pathways: α-adrenergic receptors, which release inositol trisphosphate and diacylglycerol, or β-adrenergic receptors, which act via cAMP (2, 3, 15). In several invertebrates the hormonal regulation of glycogen breakdown follows a similar pattern, as indicated by the role of hyperglycemic factors in crustaceans, insects and gasteropods (13, 14, 17, 21).

Recently evidences were found by us for the existence of a glucose release stimulation in the mantle tissue of *Mytilus galloprovincialis* by Ca^{2+} ions (6). Furthermore, a procedure was developed to obtain isolated MSC (7), which allows a more direct study of glycogen breakdown in these cells; and the exploration of such control in the mantle tissue. The present paper investigates the possible role of factors that could act in promoting receptor-coupled signals and intracellular messengers that could cause a glucose release by MSC. Two different approaches have been used: 1) whole cell system (WCS): dose-response experiments with factors with a possible glycogenolytic activity; 2) cell-free system (CFS): dose-response experiments in which the intracellular rise of cAMP was simulated.

Materials and Methods

Animals. — Sea mussels, *Mytilus galloprovincialis*, from Ría de Arosa (Galicia, Spain) (shell length 8-10 cm) were used. They were maintained in the laboratory with discontinuous feeding of mixed microalgae, and kept in tanks with discontinuous sea water at 15 ± 1 °C and a light/dark cycle of 12 L/12 D.

MSC isolation. — Mantle fragments from 8-10 specimens of both sexes were used to obtain cellular suspensions with a mixture of VC:ADG (70:30 %) as previously described (7). Briefly, MSC were isolated by enzymatic digestion at 37 °C with a mixture of collagenase and dispase for 30 min. Purification of the MSC was carried out by density gradients of Percoll. Cell viability, tested in each experiment by the trypan blue exclusion, was superior to 95 % (20).

Cytotoxicity. — The cytotoxicity of the used compounds was determined up to 60 min in parallel incubations of the same cellular preparation used in each experiment, the cellular viability being tested afterwards with a resulting 5-10 % cytotoxicity.

Dose-response experiments. — In WCS-experiments, aliquots of 10^6 cells/ml were incubated in glass tubes containing: 0.5 ml ASW (artificial sea water) pH 7.0 with the following ionic composition (mEq/l): Na^+, 512; K^+, 10; Cl^-, 550; Ca^{2+}, 5; and 20 mM HEPES. When required, Ca^{2+} was omitted and replaced with an osmotically equivalent amount of NaCl. The compounds were added in 100 µl of ASW pH 7.0 and the final incubation volume was adjusted to 1 ml with ASW. Unless otherwise stated, the incubations were carried out for 60 min at 20 °C with orbital shaking (150 cycles/min) and stopped at 4 °C by centrifugation (1,060 × g, 5 min).

In CFS-experiments, aliquots of 10^6 cells were sonicated in the presence of 20 mM HEPES pH 7.0 (0.5 ml) and incubated at 37 °C with orbital shaking (150 cycles/min) for 60 min. The compounds were added in 20 mM HEPES (100 µl) and the final incubation volume was adjusted to 1 ml with the same medium. The reaction was stopped by TCA precipitation (5 % in tube) and further centrifugation (1,060 × g, 5 min, 4 °C).

In the experiments with isobutylmethylxantine (IBMX), a preincubation for 20 min was performed.

In all experiments aliquots were taken from the supernatant for glucose assay.

Transmission electron microscopy. — For ultrastructural analysis mantle fragments were processed as previously described (4). Briefly, pieces were fixed by immersion in 2.5 % glutaraldehyde in ASW, pH 7.0. Postfixation was performed in 2 % osmium tetroxide in the same medium. Tissue blocks were then dehydrated and embedded in Spurr's epoxy resin (25). Ultra thin sections were stained using aqueous uranyl acetate and lead citrate.

Analytical procedures. — Glucose in the supernatant was determined by the glucose oxidase peroxidase method (1). Glycogen content in cells was tested as glucose after enzymatic hydrolysis with amyloglucosidase (5). Statistical analysis was carried out using unpaired Student's t-test (24). A probability < 0.05 was accepted as significant.

Chemicals. — All enzymes for cell isolation and glycogen determination, as well as catecholamines, 5-hydroxytryptamine, mellitin, isobutylmethylxantine (IBMX), A23187 and dibutyryl-cAMP were obtained from Sigma; NaF was supplied by Merck (Darmstadt, FRG), the glucose test kit, from Sclavo (Siena, Italy). All other chemicals were of analytical grade and glass-distilled water was used throughout.

Results

Basal glucose release. — An intracellular free-glucose pool was not detected in the MSC used. However a non-stimulated glucose releasing activity was observed after a 60 min incubation time. These basal values were in the range of 9-21 nmoles/60 min · 10^6 cells. Moreover, in the CFS-experiments a basal glycogenolysis was also observed with similar values to those obtained in WCS-experiments.

Appropriate controls for the basal glucose release were included in all experiments as required and used to express the results as the increment percent (%Δ) in the basal value.

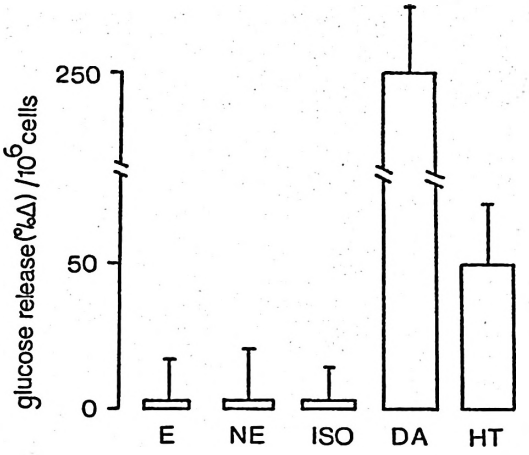

Fig. 1. *Effects of epinephrine (E), norepinephrine (NE), isoproterenol (ISO), dopamine (DA) and 5-hydroxitriptamine (5-HT) at 250 μM on glucose release by isolated mantle storage cells (MSC).* Incubation time 60 min. Values are mean ± SEM for five determinations and are expressed as the increment percent (% Δ) over basal glucose release.

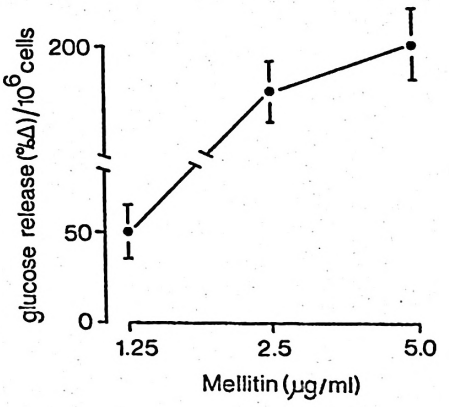

Fig. 2. *Dose-response relationship of the effects of mellitin concentrations on glucose release by isolated mantle storage cells (MSC).* Data are expressed as the increment percent (%Δ) over basal glucose release. Values as mean ± SEM (n = 6) are given.

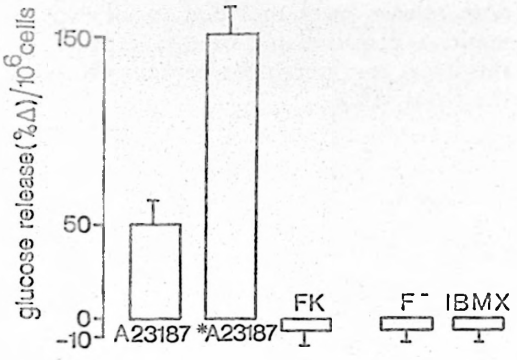

Fig. 3. *Effects of Forskolin (FK) (100 μM), NaF (F^- (10 mM), isobutylmethylxantine (IBMX) (50 μM) and A23187 (10 μM) on glucose release by isolated mantle storage cells (MSC).*
All experiments were carried out in the presence of Ca^{2+} (5 mEq/l), except *A23187 where no Ca^{2+} were added. Values are mean ± SEM for five determinations and are expressed as the increment percent (%Δ) over basal glucose release.

. *Glucose release in WCS.* — Glucose releasing activity above basal levels, 50 % and 250 % respectively, was shown by 5-HT and DA at 250 μM, while no significant increase over basal activity (fig. 1) was shown by E, NE and ISO at 250 μM.

A dose-dependent response, was shown by Mellitin (1.25, 2.5, 5 μg/ml), reaching values above 200 % of the basal activity with 5 μg/ml (fig. 2).

Glucose release was caused by 10 μM A23187 in the presence or absence of external Ca^{2+}. In the presence of Ca^{2+} an increase of 50 % on basal activity was observed and in its absence the basal levels were increased (fig. 3).

A non-significant effect on glucose release was shown by 100 μM Forskolin (FK), 10 mM NaF and 50 μM IBMX (figure 3).

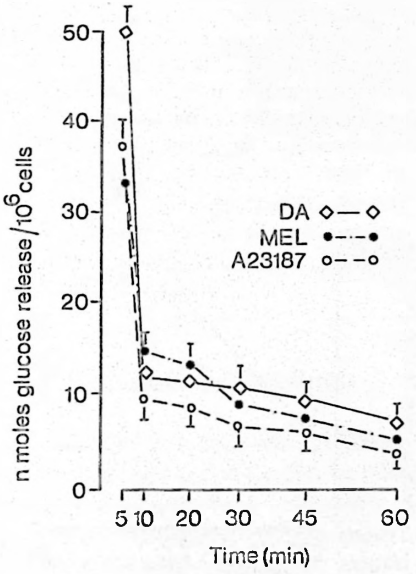

Fig. 4. *Time-dependence of glucose release stimulation by dopamine (DA) (250 μM), mellitin (MEL) (5 μg/ml) and A23187 (10 μM) in isolated mantle storage cells (MSC).*
Values, corrected for the basal release, are given as mean ± SEM (n = 5). The experiments with A23187 were carried out in external Ca^{2+} absence.

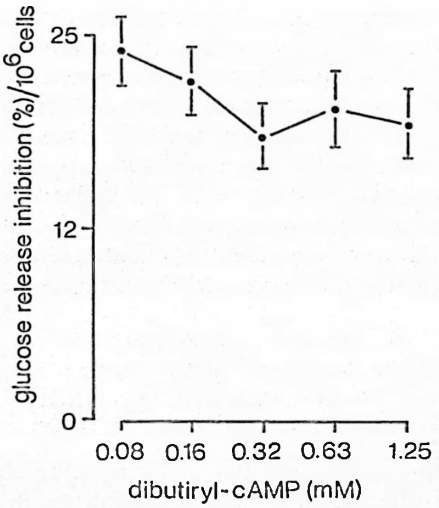

Fig. 5. *Effect of dibutyryl-cAMP on glucose release by mantle storage cells (MSC).*
Experiments were done in the presence of isobutylmethylxantine (50 μM), ATP (3 mM), Mg^{2+} (3 mM) and NaF (10 mM) in 20 mM HEPES, pH 7.0. Incubation time 60 min. Values are mean ± SEM (n = 5) and are expressed as the inhibition percentage of the basal glucose release.

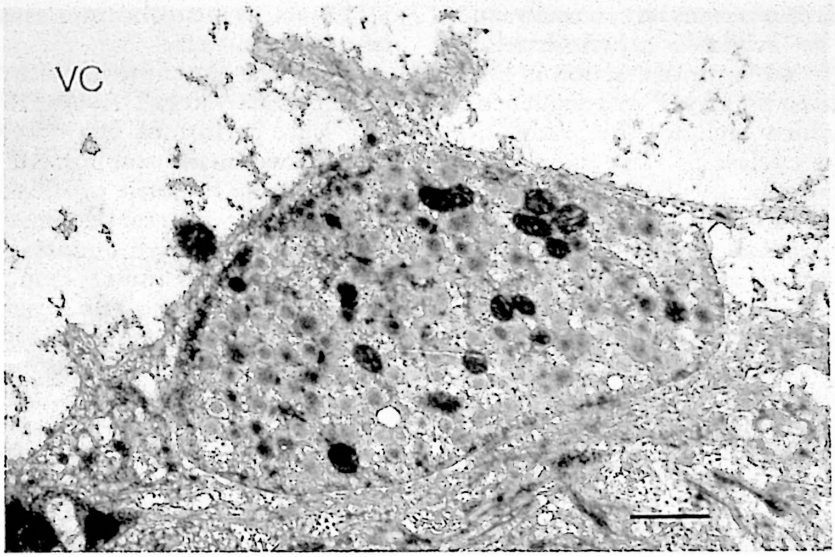

Fig. 6. *Electronmicrograph of a secretory-like cell in contact with the surface of a vesicular cell (VC).* In the cytoplasm an outstanding amount of low-density core membrane-surrounded granules can be observed. The opposite side of the cell is in contact with the stroma of the mantle connective tissue. Bar: 0.6 μm.

Time course of glucose release stimulation. — Three compounds which showed a stimulatory effect on glucose release (DA, mellitin, A23187) were chosen in order to test the possible time-dependence of that output. A maximun in glucose release was obtained for all compounds at 5 min of incubation, with a sudden decrease at 10 min, reaching a constant value from 10-60 min (fig. 4).

Glucose release in CFS. — A non-specific inhibition of the basal glucose releasing activity was shown by 0.08, 0.16, 0.32 and 1.25 mM of dibutyryl-cAMP along with 50 μM IBMX, 5 mM NaF, 3 mM ATP, 3 mM Mg^{2+} intended to simulate a rise in cAMP level (fig. 5).

Ultrastructural analysis. — Secretory-like anchored cells making contact with the surface of the VC were frequently found. The non-adjacent surface to the VC is in contact with the stroma of the mantle connective tissue. In the cytoplasm of these cells a considerable amount of membrane-surrounded granules of low electron-density core were visualized (figure 6).

Discussion

The present experiments were performed in the November-December period when the intracellular free-glucose pool was not detected, so that the glucose releasing activity measured in MSC may be considered as due to its GA. Two components for the GA of MSC have been observed: 1) a basal one, and 2) an activated one. The basal component has been measured, both in the WCS and in the crude CFS, the values being similar for both systems.

Two intracellular pathways to activate the glycogenolysis cascade in mammals,

insects and crustaceans are actually substantiated by available experimental results. Firstly the hormone action is likely to be mediated by cAMP in a sequence of reactions which includes the stimulation of adenylate cyclase to raise the intracellular concentration of cAMP, while a second mechanism, making use of Ca^{2+} as an intracellular messenger, leads to the phosphorylase kinase activation. In this context it should be noted that Ca^{2+} is absolutely required for the activation of phosphorylase kinase (3, 21, 26).

According to the present data a rise in cytosolyc Ca^{2+} in MSC may be a sufficient condition for the glycogenolysis to be «turned on» (experiments with A23187 and mellitin), which parallels the results previously obtained at the tissue level (4). The role of cAMP as second messenger for glycogenolysis in MSC cannot be clearly explained, as the effects from forskolin, F^- and IBMX in WCS, and from the rise of intracellular cAMP simulated with dibutyryl-cAMP in CFS, showed a non significant inhibition of the basal glycogenolytic component in the first case. Furthermore, the effect of cAMP in the second case, can be explained by the absence of Ca^{2+} ions in the medium, in accordance with the results reported by STEELE (26). Whether cAMP therefore, plays a role as intracellular messenger via adenylate cyclase system on glycogenolysis in MSC requires further investigation.

Catecholamines and 5-HT are present in ganglia of *Mytilus* (22). The data from these amines suggest that α and β-adrenergic agonists are not effective on GA, no effects are produced by E, NE and ISO. However, a GA as well as DA but to a lesser extent was manifested by 5-HT. These facts suggest that the glycogenolytic cascade in MSC is probably activated by dopaminergic and serotoninergic receptors and not by α or β-adrenergic receptors as it generally happens in mammals (10, 15). The absence of nerve terminals in the mantle tissue (4) suggests that DA and 5-HT act as neurohormones and not as neurotransmitters.

When the experiments with the glycogenolytic activators DA, A23187 and mellitin were performed in a time-dependent mode, two main components were observed in the response profile: 1) an early strong glycogenolytic response which occurs in the first five minutes; 2) a rapid decline from ten minutes on. The non-sustained glycogenolytic response is an unexpected result, since the glucose released from the cells at least might remain in the incubation medium. The loss in glucose can be explained if its uptake is subsequently taking place. After five minutes the released glucose concentration from the cells amounts to 30-50 μM in the incubation medium. If this concentration is sufficient to stimulate the glucose uptake by the cells then a rapid and high-affinity glucose-transport system should occur in these cells. Our recent experiments have just confirmed it (manuscript in preparation).

The glycogen content of the MSC, mainly stored in VC, is a reservoir of glucose for other cells. To understand how these cells work, it is important to know whether they need to be partially or totally lysed in order to supply the glycogen to be broken, as it has been claimed (12, 19). Results show that MSC are able to have GA by themselves as well as interpret extracellular factors intended to activate the glycogenolytic cascade. In such a case the VC might become target cells for the ones liberating the glycogenolytic factors as reported for *Mytilus edulis* and *Crassostrea gigas* (16, 19) and also for *Lymnaea stagnalis* (13, 14). The possible anatomical loci where these factors may arise are: 1) ganglia; 2) a possible mantle scattered paracrine system, whose cells have been found close to the VC. The light-density cored granules of these cells suggest that they may store polypeptides. Their existence and whether they activate glycogenolysis in VC remains to be proved.

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Resumen

Se estudia la actividad glucogenolítica en las células de reserva aisladas del manto de *Mytilus galloprovincialis* y se determina el contenido en glucógeno y glucosa libre, y glucosa liberada por las mismas. En el período estudiado (noviembre-diciembre) se detecta una actividad liberadora de glucosa que puede considerarse como actividad glucogenolítica. Tanto en los experimentos con células enteras, como en sistema libre de células, se observa una actividad glucogenolítica no estimulada. Con células enteras, la dopamina y la 5-hidroxitriptamina (5-HT) estimulan la glucogenolisis, mientras que la epinefrina, la norepinefrina y el isoproterenol no lo alteran. También la melitina y el ionóforo de Ca^{2+}, A23187, estimulan la actividad glucogenolítica. Con el sistema libre de células, la ausencia de iones Ca^{2+} detiene la actividad glucogenolítica. Estos y otros resultados sugieren: 1) la actividad glucogenolítica en las células de reserva del manto puede estimularse por dopamina y 5-HT y no por agonistas adrenérgicos; 2) la señal de Ca^{2+} citosólico puede ser un requerimiento absoluto para la activación de la cascada glucogenolítica; 3) las células de reserva del manto posiblemente presenten un transporte de glucosa rápido y de alta afinidad.

Palabras clave: Mytilus, Células de manto, glucogenolisis.

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