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A flow cytometric approach to study intracellular-free Ca2+ in Crassostrea gigas haemocytes

E. Atona[b], T. Renault[b], B. Gagnaire[b], H. Thomas-Guyon[a], C. Cognard[c] and N. Imbert[a*]

[a]Université de La Rochelle, Laboratoire de Biologie et Environnement Marin (LBEM) FRE 2727, avenue Michel Crépeau, 17042 La Rochelle, France
[b]IFREMER La Tremblade, Laboratoire de Génétique et Pathologie (LGP), Ronce-les-Bains, 17390 La Tremblade, France
[c]U.F.R. Sciences fondamentales et appliquées, Institut de Physiologie et Biologie Cellulaire (IPBC), 40, avenue du Recteur Pineau, 86022 Poitiers, France

*: Corresponding author : Tel.: +33 5 46 50 02 91. nathalie.imbert@univ-lr.fr

Abstract: Bivalve haemocytes are essential in defence mechanisms including phagocytosis. They also produce molecules including hydrolytic enzymes and antimicrobial peptides that contribute to pathogen destruction. Although haemocyte activities have been extensively studied, relatively little is known about the intracellular signalling pathways that are evoked during haemocyte activation and especially the role of calcium.

Flow cytometry has been used for the first time to define the effect of cell incubation in haemolymph and artificial sea water (ASW) on Pacific oyster, Crassostrea gigas, haemocytes. Cell viability, enzymatic activities (esterases and aminopeptidases), phagocytosis and granulocyte percentage were analysed. Viability and some activities were different in haemolymph and ASW. Cytoplasmic-free calcium in circulating haemocytes was then investigated by flow cytometry in both media using a calcium probe (Fluo-3/AM). To explore calcium homeostasis, different calcium modulators were tested. The calcium chelator Bapta/AM (10 µM) reduced significantly the percentage of Fluo-3-positive cells in ASW. In addition, ryanodine (5 µM) induced a significant enhancement of the percentage of Fluo-3 positive cells in haemolymph and in ASW. Flow cytometry may be used to study calcium movements in C. gigas haemocytes, but several haemocyte incubation media need to be tested in order to confirm results. The objective of the study should be considered before selecting a particular experimental medium.

Keywords: *Crassostrea gigas*; Haemocytes; Flow cytometry; Calcium; Fluo-3/AM; Cellular activities

I. Introduction

Bivalve defence mechanisms are supported by haemocytes that are considered to be the counterpart of vertebrate inflammatory cells and participate directly in eliminating pathogens by phagocytosis. In addition, they produce substances including lysosomal enzymes (esterases and aminopeptidases) and antimicrobial molecules that contribute to destruction of pathogenic organisms [1, 2].

Calcium acts as a second messenger in a variety of cell processes in different organisms. The increase of cytosolic free calcium in mammalian cells is due to both release from intracellular stores and influx through plasma membrane channels. The subsequent decrease is ensured by calcium pumps located in the plasma membrane and intracellular membrane [3, 4]. Transient variations in cytoplasmic calcium are necessary for cell activities and ensure calcium homeostasis [5]. Calcium is also involved in various processes including cardiac or skeletal muscle contraction [6, 7], reproduction [8], and immune processes [9, 10, 11] in marine invertebrates. However, the role of calcium has been little investigated in bivalve haemocytes and calcium channels remain unknown in these species. Nevertheless, Burlando et al. [12] demonstrated that cytosolic calcium induces activation of phospholipase A2 in mussel haemocytes. An *in vitro* approach to define calcium flux and stores in bivalve haemocytes may be suitable to better understand the relationship between environmental factors and immunity. Bivalves are sensitive to environmental modifications or stress which may affect immune parameters [13, 14, 15]. Heavy metals induce alteration of calcium homeostasis in mussel haemocytes [16, 17]. In most electrophysiological studies, physiological saline solutions are currently used. However, cell functions in such media are rarely checked before assays.

The first aim of this study was to determine whether artificial seawater (ASW) may modify Pacific oyster, *Crassostrea gigas* haemocyte functions. For this purpose, cellular activities were monitored and compared in ASW and in haemolymph (H). Esterase, aminopeptidase and phagocytic activities were investigated by flow cytometry using specific fluorescent markers. Haemocyte cytoplasmic-free calcium (Ca^{2+}) was also investigated by flow cytometry using a calcium probe (Fluo-3/AM), in both ASW and H. Finally, different calcium modulators known to affect cytosolic free calcium were tested in order to compare calcium responses in *C. gigas* haemocytes in both media.

II. Materials and methods

2.1. Oysters

One year old Pacific oysters, *C. gigas*, were purchased from January to May 2002 from IFREMER station (La Tremblade, Charente Maritime) located on the French Atlantic coast.

2.2. Haemocyte collection

Haemolymph was withdrawn from the pericardial cavity by puncture with a sterile 1 mL syringe equipped with a needle (0.6 x 25 mm) [18]. Haemolymph obtained from different individuals (15 individuals for each assay) were pooled and kept on ice to avoid aggregation. Haemocytes were collected by centrifugation (100g, 4°C, 10 min) and re-suspended (10^6 cells/mL) in 200 µl haemolymph or artificial seawater (ASW: 458 mM NaCl ; 10.5 mM KCl, 10.5 mM $CaCl_2$ ($2H_20$), 28 mM $MgCl_2$ ($6H_20$), 22 mM anhydrous $MgSO_4$; 5 mM Hepes ; pH 7.4 ; osmolarity 1100 mOsmol/L) [19].

2.3. Cellular parameter analysis by flow cytometry

Haemocytes were analysed using an EPICS XL 4 flow cytometer (Beckman Coulter). For each haemocyte sample, 3000 events were counted. Results were expressed as cell cytograms indicating size (FSC value), complexity (SSC values) and fluorescent values corresponding to the marker used. Enzymatic activities and phagocytosis were measured using FL1 (green fluorescence) and mortality using FL3 (red fluorescence).

2.3.1. Cell viability

Percentages of dead cells were assessed using propidium iodide (IP, Interchim) which only permeates through the membrane of dead cells and stains nucleic acids [18]. Two hundred µL of haemolymph was incubated in the dark for 30 min at 4 °C with 10 µL of IP (1.0 mg. mL^{-1}).

2.3.2. Phagocytosis

Phagocytosis was measured by ingestion of Fluorospheres® carboxylate-modified beads (Ø=1 µm, Interchim). Two hundred µL of cell suspension were incubated for 1h 30 min in the dark at 18°C with 10 µL of a 1/10 dilution of fluorescent beads [18]. Phagocytic activity was expressed as the percentage of haemocytes that had ingested at least three beads [20].

2.3.3. Enzymatic activities

Esterase and aminopeptidase activities were evaluated using commercial kits (Cell Probe ™ Reagents, Beckman Coulter) as previously described [20]. Each analysis required 200 µL of cell suspension in haemolymph or ASW and 20 µL of the corresponding kit (FDA Esterase and Aminopeptidase M). Haemocytes were incubated for 15 min for esterases and 30 min for aminopeptidase in the dark at 18°C. Enzymatic activities were expressed as percentages of fluorescent cells.

2.4. Cytoplasmic free-Ca^{2+} detection by flow cytometry

Fluo-3/acetoxymethyl ester (Fluo-3/AM, 10 µM, Interchim) dissolved in DMSO was used to detect calcium activity [17]. Fluo-3/AM binds cytoplasmic free calcium and emits a green fluorescence (peak at 526 nm) detected using FL1. Cells were incubated with 10 µM Fluo-3/AM for 30 min at 18°C in the dark in both H and ASW. During incubation with Fluo-3/AM, calcium modulators were added to individual tubes. Bapta/AM (Sigma), a calcium chelator [21], was added for 30 min at 18°C at a final concentration of 10 µM. To induce calcium release from intracellular stores, e.g. endoplasmic reticulum, ryanodine (Sigma), an activator of an intracellular calcium channel (ryanodine receptor) [22], was added at 5 µM final concentration, for 10 min at 18°C. Haemocytes were then analysed by flow cytometry to measure calcium activity in H and ASW. Where required, DMSO was added to controls. Mortality due to DMSO was checked as non significant.

2.5. Statistical analysis

Data were expressed as mean values ± SD. Each value was derived from two or three replicates. Flow cytometry data were statistically analysed using Statgraphics version 5.1 software and two-way ANOVA analysis. In the case of rejection of H_0, an *a posteriori* test was used. Significance was set at $P \leq 0.05$.

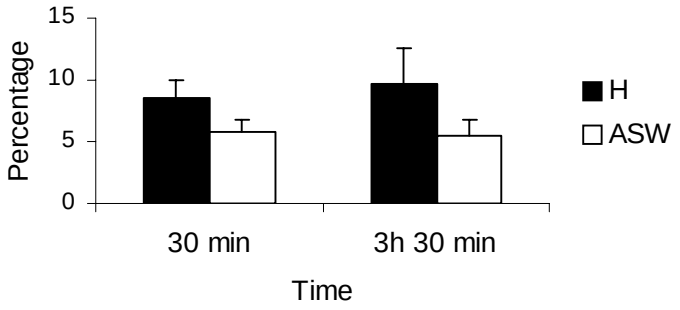
III. Results

3.1. Haemocyte viability

Haemocyte mortality rates were low in both H (10 %) and ASW (6 %) (Fig. 1). In both media, haemocyte viability was not affected by a 30 min or a 3h 30 min incubation period.

The percentage of dead cells was significantly lower in ASW than in haemolymph both at 30 minutes ($P<0.001$) and 3h 30 min ($P<0.01$).

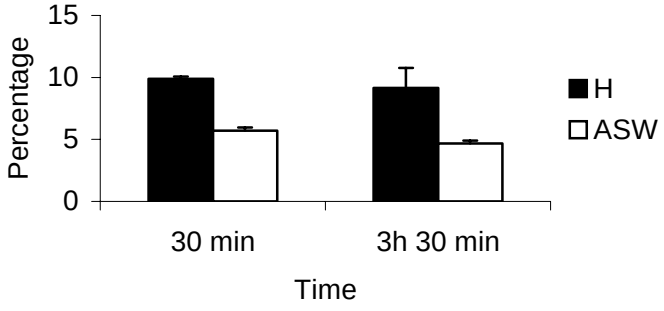
Fig. 1. Haemocyte mortality monitored by flow cytometry in haemolymph (H) and artificial sea water (ASW) at 30 min and 3h 30 min. Values are means of three replicates ± SD.
ANOVA indicated significant differences between H and ASW at 30 min and 3h 30 min (aaa, $P<0.001$; aa, $P<0.01$).



3.2. Haemocyte types

Granulocyte percentages were low (8 % to 9 %) in haemolymph (Fig. 2). However, granulocyte percentages in ASW were significantly lower at 30 min and 3h 30 min (around 5 %, $P<0.001$). No change was observed for either incubation media between 30 min and 3h 30 min incubation periods.

Fig. 2. Granulocyte percentages monitored by flow cytometry in haemolymph (H) and artificial sea water (ASW) at 30 min and 3h 30 min. Values are means of four replicates ± SD.
ANOVA indicated significant differences between H and ASW (aaa, $P<0.001$).



3.3. Cellular activities

No significant difference was observed in haemocyte activities (esterases, aminopeptidases and phagocytosis) over the incubation period (30 min and 3h 30 min) in both media (Figs. 3, 4 and 5). However, percentages of aminopeptidase positive cells and phagocytic cells in ASW were significantly lower than those observed in haemolymph ($P<0.001$, Figs. 3 and 4). In contrast, the percentage of esterase positive cells was significantly higher in ASW than in haemolymph ($P<0.001$), both at 30 min and 3h 30 min (Fig 5).

Fig. 3. Aminopeptidase activity monitored by flow cytometry in haemolymph (H) and artificial sea water (ASW) at 30 min and 3h 30 min. Values are means of two replicates ± SD.
ANOVA indicated significant differences between H and ASW (aaa, $P<0.001$).

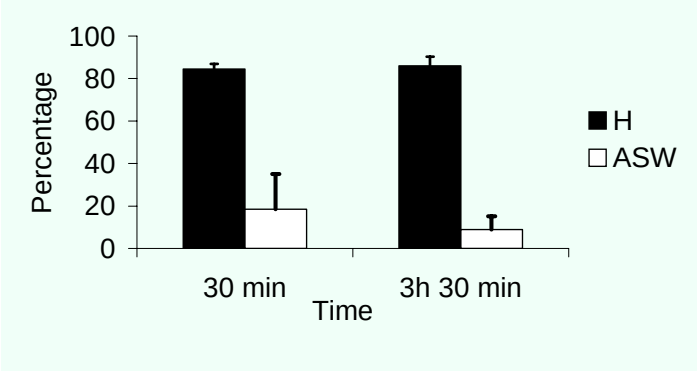


Fig. 4. Phagocytosis activity monitored by flow cytometry in haemolymph (H) and artificial sea water (ASW) at 30 min and 3h 30 min. Values are means of three replicates ± SD.
ANOVA indicated significant differences between H and ASW (aaa, $P<0.001$).

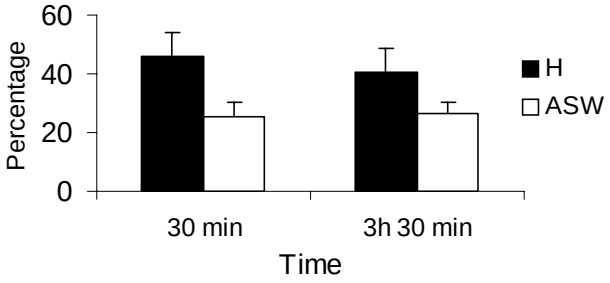
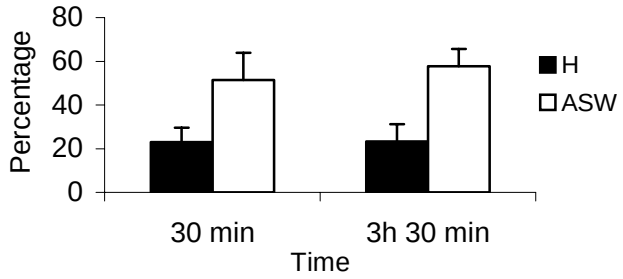


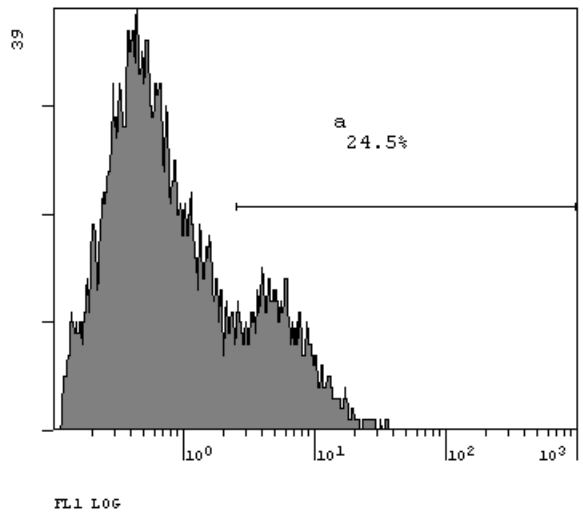
Fig. 5. Esterase activity monitored by flow cytometry in haemolymph (H) and artifcial sea water (ASW) at 30 min and 3h30. Values are means of three replicates ± SD.
ANOVA indicated significant differences between H and ASW (aaa, P<0.001).



3.4. Intracellular calcium in haemocytes

After Fluo 3-AM loading (10 µM), 19±7.1 % (H) and 16.5 ±6.5 % (ASW) of total haemocytes were detected as labelled haemocytes (Figs. 6 and 7). There was no statistical difference between labelled cells detection in H and ASW.

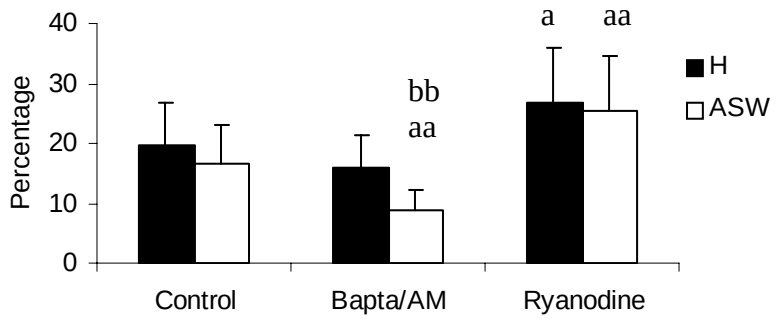
Fig. 6. Cytogram showing Fluo-3/AM labelled haemocytes monitored by flow cytometry. Percentage "a" correspond to cells stained by the probe (positive). X-axis: intensity of fluorescence (log scale); Y-axis: number of events.



3.5. Effect of calcium modulators on Fluo-3-AM labelled cells

The calcium chelator Bapta/AM (10 µM) induced a marked decrease ($P<0.01$) in the percentage of positive cells in ASW (Fig. 7). However, the decrease detected in H was not significant. In addition, 5 µM of ryanodine induced a significant enhancement of fluorescent cells in H ($P<0.05$) and in ASW ($P<0.01$).

Fig. 7. Fluo-3/AM loaded haemocytes monitored by flow cytometry. Values are obtained following the addition of calcium modulators : BAPTA/AM (10 µM) and Ryanodine (5 µM) in haemolymph (H) and artificial sea water (ASW). Values are means of four replicates ± SD. ANOVA indicated significant differences from control values respectively in H and ASW (a, $P<0.05$; aa, $P<0.01$) and indicated statistical difference between H and ASW for some modulators (bb, $P<0.01$).



IV Dicusssion

Marine invertebrates including bivalves, are isoosmotic: their extracellular medium (haemolymph) presents the same osmolarity as seawater (around 1000 mOsmoles). The haemolymph contains carbohydrates, glycosaminoglycans and proteins [23, 24]. Certain components are secreted by haemocytes e.g. esterases, aminopeptidases and antimicrobial peptides that participate in humoral immune defense [2, 25, 26]. ASW contains the same

inorganic compounds as haemolymph but it is nutrient free. Such artificial medium is commonly used for electrophysiological studies on marine invertebrate cells e.g. photoreceptors, oocytes, gill, cardiac and neuronal cells [6, 8, 19, 27]. Moreover, physiological saline solutions have been currently used to investigate calcium homeostasis in vertebrates and invertebrates [16, 17]. In simple media, the ionic composition can be adjusted However, haemocyte functions could be modified in such media. In haemolymph, various components could interact with the calcium probe and/or calcium modulators. The first objective of this work was to compare two incubation media : haemolymph and artificial seawater, a physiological saline solution, to determine if they could modify haemocyte activities. Haemocyte parameters including granulocyte percentage, aminopeptidase activity and phagocytosis were significantly reduced in ASW. However, esterase activity and cell viability were enhanced in this artificial medium. Serum-starvation has been shown to induce a stress-resistant state in cells leading to over-expression of proteins [28; 29] and apoptosis [30]. Changes in nutrient supply influences metabolic rates and cell level activities [31, 32]. It is of major concern to define cell status when calcium homeostasis is studied with fluorescent calcium probes in different incubation media. Cells incubated in H exhibited more efficient phagocytic activity compare to cells incubating in ASW. Therefore, if the aim of the study is to investigate a potential link between cytoplasmic calcium and phagocytosis, H seems to be the best extracellular environment. However, the objective of the study should be considered before selecting a particular medium.

In the present study, fluo-3/AM was used for the first time to detect intracellular free calcium in oyster haemocytes using flow cytometry. This probe allowed the identification of positive cells interpreted as cells containing detectable free calcium in both haemolymph and physiological saline solution (ASW). Only, a small percentage of haemocytes (approximately 18 to 20%) were found to be fluorescent after incubation with Fluo-3/AM. Intracellular

calcium level may be low in mollusc cells. It has been found in *Mytilus edulis* haemocytes [33] with spectrofluorimetry and Fluo-3/AM an intracellular calcium concentration around 71 nM. This is lower than cytosolic calcium found in mammalian cells that is typically around 100 nM [34]. Fluo-3/AM is usually used in vertebrate cells in well-defined experimental conditions. Such conditions may not be encountered in oyster haemocytes. In this study, in both media, haemocytes mortality is low and haemocyte activities depend on incubation medium. However, for both incubation media, the high osmolarity and salinity of intracellular environment may induce modifications of biochemical and spectroscopic properties of the probe. It has been demonstrated that Kd (association/dissociation coefficient) is modulated by a number of factors including temperature, pH, and buffer composition [35, 36]. In order to explain the small percentage of fluorescent cells in our study, fluo-3/AM Kd determination appears also necessary. Further calcium fluorescent dyes with higher fluorescence emission intensity could be tested to optimise the assay.

In spite of the small percentage of fluorescent haemocytes, free intracellular calcium can be detected using flow cytometry and Fluo-3/AM. In order to confirm these results, two calcium modulators, Bapta/AM and ryanodine were tested on oyster haemocytes. The chelator Bapta/AM induced a significant decrease of the percentage of Fluo-3/AM positive cells when incubated in ASW but not in H whereas ryanodine induced a significant increase in both media. Fluorescence in cells incubated with the chelator showed a significant difference between both media H and ASW. Haemolymph components may interfere with this chelator and also affect the affinity of ryanodine for calcium. Results may also indicate the presence of a ryanodine receptor: this calcium channel has already been reported in mammalian inflammatory cells and is supposed to mediate calcium release from intracellular stores [37]**.**

Existing specific antibodies against this calcium channel may be used to confirm these preliminary results.

This preliminary study has demonstrated that flow cytometry may be useful in studying intracellular calcium in *C. gigas* haemocytes using Fluo-3/AM. Artificial media may modify oyster haemocyte activities. However, these media contain no organic component but have a defined ionic composition. In these conditions, they may facilitate the use of calcium modulators and signal detection for studying different aspects of cellular calcium. Futher research have to be conducted to investigate calcium homeostasis. However, defining the impact of the incubation medium on *C. gigas* haemocytes also appears to be a necessary step when investigating calcium flux.

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Figure 7[18]