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Primary cultures of nervous system cells from the larva of the ascidian *Ciona intestinalis*

Laura Zanetti [a,*], Filomena Ristoratore [a], Maria Francone [b], Stefania Piscopo [b], Euan R. Brown [b]

[a] *Biochemistry and Molecular Biology Laboratory, Stazione Zoologica 'Anton Dohrn', Villa Comunale, I-80121 Naples, Italy*
[b] *Neurobiology Laboratory, Stazione Zoologica 'Anton Dohrn', Naples, Italy*

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Abstract

The ascidian *Ciona intestinalis* is a useful model for the study of nervous system development and function. The larva of this animal represents a 'primitive' vertebrate form that contains only about 100 neurons in the CNS. Although embryos can be easily subjected to genetic manipulation, the nervous system cells are not easily accessible for neurophysiological study at the larval stage. To remedy this problem, we have developed a method to obtain primary cell cultures from the larval stage of *Ciona*. Light microscopy and electrophysiology discriminate several types of cells including neurons and photoreceptors. The results show that in *Ciona* primary cultures different types of neurons as well as neurite sprouting and synapse formation can be visualised. *Ciona* primary cell cultures will be very useful to study the biochemical, molecular and biophysical properties of individual cells in the larval nervous system of *C. intestinalis*.

Keywords: Primary cell culture; Neurons; Chordate; Excitability

1. Introduction

Cell culture is an important tool in a variety of scientific disciplines including biological and medical sciences. *In vitro* applications may also be used as alternative tools for animal experimentation, for biotechnological applications and pathological investigations. Invertebrates may be regarded as a major source for such applications and, within them, marine invertebrates represent potentially rich sources of cell and tissue types that significantly differ from one group to another. Many cell types from a variety of invertebrates possess extensive morphogenetic potential (multipotency, totipotency, including neoplasia) (Rinkevich and Rabinowitz, 1994, 1997; Rosenfield et al., 1994). This leads *in vivo* to a high plasticity of shapes, structures, rate of cell replacement, proliferation and cell lineage in invertebrate taxa, sometimes differing significantly between systematically related groups of organisms.

Although attempts to maintain and grow proliferative invertebrate cells *in vitro* were made quite early in the history of tissue culture (there are more than 200 cell lines established from insects and ticks, Mitsuhashi, 1989), efforts to develop permanent and proliferative cell cultures from marine invertebrates have so far been unsuccessful. From the point of view of basic neuroscience however, primary culture of marine invertebrate cells, particularly neurons have been a useful tool to understand synaptic function (Kaczmarek et al., 1979).

Until now attempts to develop cell cultures from tunicates are far from optimal. A few studies have focused only on epithelial cell cultures (Kawamura and Fujiwara, 1995; Rinkevich and Rabinowitz, 1997) or on embryo-derived cell cultures (Rinkevich and Rabinowitz, 1994) from budding or colonial tunicates. Tunicates, the most primitive group of the phylum Chordata, have the potential to reveal the basic mechanisms underlying important neurobiological, immunological, and developmental processes. Tunicates are emerging as important model organisms because of their relative simplicity and their position as a potential sister group of the vertebrates (Delsuc et al., 2006). The basic features of the chordate body plan remain recognizable in the ascidian larvae, although composed of only ~2500 cells (Satoh, 1994). Of these cells only 300 constitute the nervous system which contains around 100 neurons (Meinertzhagen et al., 2004). Apart from basic developmental

* Corresponding author. Tel.: +39 081 5833251; fax: +39 081 7641355.
E-mail address: lzanetti@szn.it (L. Zanetti).

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similarities to vertebrates (Meinertzhagen and Okamura, 2001), nervous system functions such as motor-network activity (Brown et al., 2005) indicate strong similarities at the functional level. Recent detailed morphological studies (Imai and Meinertzhagen, 2007a,b) have shown that there are several readily distinguishable classes of neurons at the light microscope level. This suggests that a close study of neural mechanisms will yield important information to aid interpretation of the processes operating in tunicates and vertebrates.

However, as previously pointed out (Meinertzhagen and Okamura, 2001), the nervous system is almost inaccessible to neurophysiological study because of the tough outer tunic of the larva. To address this issue we have developed a system for the maintenance of primary cell cultures of the ascidian *Ciona intestinalis* by systematically establishing: mechanical plus enzymatic digestion techniques, the nature of media in term of essential nutrients, and the nature of the substrate to obtain cells that conserve their functionality. In addition we have evaluated, by morphology and electrophysiology, cell viability and form.

2. Materials and methods

For all experiments, conditions were aseptic, using sterile glass and plasticware. All media and reagents were purchased from Sigma–Aldrich unless otherwise indicated.

2.1. Biological material

Adult *C. intestinalis* were collected in the Bay of Naples by the Fishing Service of the Stazione Zoologica. Gametes were used for *in vitro* fertilization. Before fertilization, eggs were dechorionated chemically (Locascio et al., 1999). After fertilization, the resulting embryos were cultured until the larval stage in filtered (0.22 μm, GP Express PLUS Membrane Stericup, Millipore Corporation, Massachusetts) sea water at 16–18 °C containing penicillin–streptomycin (20,000 U/ml–50 mg/ml).

2.2. Primary cell cultures from Ciona embryos

Embryos at larval stage were collected by hand centrifugation (pellet ∼30 μl) and cell dissociation was carried out in 1 ml of sterile artificial sea water (ASW) (Hochner et al., 2003) containing 1 mg/ml collagenase P (Roche) and 1 mg/ml trypsin (Type II-S) with 0.1% DNaseI (Roche), for 30 min at room temperature (a technique adapted from Inoue et al., 1994). Embryos were carefully triturated with a sterile glass pipette, the solution was diluted ten times in order to block enzyme activity and filtered through a 40 μm nylon membrane. Cell suspension was centrifuged at 1000 rpm for 5 min and the pellet resuspended in 2 ml of cell culture medium (L15 with L-glutamine adjusted to sea water salt concentration) and antibiotics (penicillin, 100 U/ml and streptomycin, 0.1 mg/ml) (Hochner et al., 2003), with or without 10% FBS (fetal bovine serum). Cells (500 μl) were plated onto sterile *WillCo-dish* (22 mm) or onto glass coverslips, placed in plastic plates (35 mm) previously coated with 0.1 mg/ml poly-L-lysine in 0.1 M sodium borate (pH 8.2) (Ready and Nicholls, 1979). Culture medium was added to obtain a volume of 2 ml in both cases. Plates were then incubated at 15 °C. For cultures longer than 2 days, the medium was aspirated to remove additional debris, rinsed once with L15 serum free media and replaced with fresh complete medium to equal the original volume. The medium was changed every 2 days.

2.3. Haemalum and eosin staining of cell cultures

Cells plated on glass coverslips were fixed in buffered paraformaldehyde (4%, v/v, in 0.1 M MOPS, pH 7.4 containing 0.5 M NaCl), stained with haemalum and eosin (Prentø, 1985), passed through a series of graded ethanols and xylene, and embedded in mounting medium.

2.4. Electrophysiology

Glass coverslips with adherent cells were removed from the culture dishes and placed directly in a chamber on an inverted microscope. The chamber was continuously perfused at 2 ml/min with a standard filtered seawater solution. For voltage clamp, 3–5 MΩ borosilicate glass pipettes were brought into direct contact with the cell surface by micromanipulation. GΩ cell attached seals were obtained by gentle suction (1–10 cm H_2O) and whole-cell access by rupturing the GΩ seal patch with direct suction. The internal solution was:400 mM KCl, 20 mM NaCl, 10 mM Hepes-Na, 1 mM $MgCl_2$, 0.5 mM EGTA, in bidistilled water pH 7.2. Using an Axoclamp 2B amplifier and a 0.1 Ul headstage, capacity transients and feedback resistance were adjusted optimally and the cell clamped to −60 mV. Current and voltage outputs from the amplifier were digitized by a Digidata 1200 digital acquisition system and stored on an IBM PC. Voltage and current steps generated by Clampex software (Axon instruments, Inc., California, USA) were delivered through the Digidata board to the cell via the amplifier.

3. Results and discussion

3.1. Cell cultures

3.1.1. General observations

Dissociated cells from *C. intestinalis* settled and adhered to the culture dish or on treated glass coverslips placed in the Petri dish within 1 h. Some cells showed signs of neuronal morphology immediately after dissociation, others initially resembled small spheres and only adopted neuronal form later. Over the following 48 h sprouting neurities with growth cones were visualised in a large proportion of cells (Fig. 1A). Cells and projections were defined as 'neuronal' by appearance, when they showed prominent oval, triangular or polygonal cell bodies with single fine axons and dendritic arborizations (Fig. 1B and C). Axons often extended over several 100's of μm within a few days (Fig. 1D and E). Frequently, neurites appeared to have navigated to make contact with other neurons (Fig. 2A), or with presumptive muscle fibres (visualized as flat polynucleated spindle-shaped cells) (Fig. 2B). Where they occurred, neurites from *Ciona* cells were of narrow diameter (about 0.5 μm). Mean cell body diameter was about 10 μm.