TISSUE AND CELL, 1994 **26** (3) 403–412

LUCIA MANNI, GIOVANNA ZANIOLO and PAOLO BURIGHEL

AN UNUSUAL MEMBRANE SYSTEM IN THE OOCYTE OF THE ASCIDIAN BOTRYLLUS SCHLOSSERI

Keywords: Porous membrane, annulate lamellae, ascidian oocytes, ultrastructure

ABSTRACT. During vitellogenesis, oocytes of *Botryllus schlosseri* always exhibit an unusual system scattered in the cytoplasm. It consists of an association between a single fenestrated endoplasmic reticulum cisterna and one or a few smooth vesicles (cisterna vesicle association: CVA) containing a dense core facing the cisterna itself. The latter is smooth and perforated by numerous small pores (about 25 nm in diameter) in the area of association; towards the periphery, it extends into several branches with ribosomes bound to their membranes. In the vesicles, fibrillar material radiates from the dense core and is sometimes organized into a long, dense lamina. The membranes of both cisterna and vesicles appear to be coupled, but are in fact separated by a constant narrow space occupied by short densities.

The presence in *B. schlosseri* of this unusual fenestrated membrane system contrasts with the absence of a typical porous cytoplasmic organelle, the annulate lamellae (ALs), which is widely distributed in female gametes. However, as in other animals, *B. schlosseri* oocytes possess intranuclear annulate lamellae (IALs) and vesicles. Comparative observations extended to the oocytes of the ascidian *Ciona intestinalis* have shown that the latter species exhibits typical ALs and IALs, but not the CVA.

The morphology of the CVA is analysed here in detail, and similarities and differences with ALs are pointed out. Hypotheses regarding CVA function are discussed in terms of possible relations with ALs.

Introduction

Porous membrane systems, such as the fenestrated cisternae of the sarcoplasmic reticulum, are quite widespread in tissues. Of these, the annulate lamellae (ALs) have recently been proposed as an actual cellular organelle (for review: Kessel, 1982, 1985, 1989, 1992).

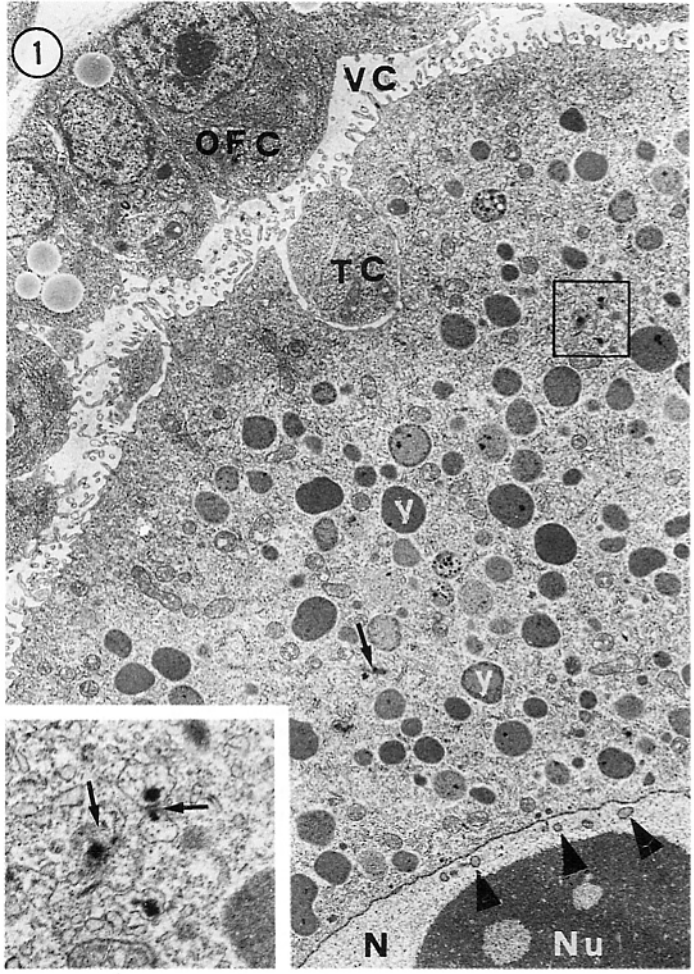
ALs consist of porous membranes, sometimes single but often arranged in parallel stacks, very abundant in the developing female gametes of a wide variety of organisms (Kessel, 1985). This membrane system is usually confined to the cytoplasm, but may also be located inside the nucleus in several cell types (intranuclear annulate lamellae—IALs) as single porous membranes, characteristically found in close proximity to the nuclear envelope.

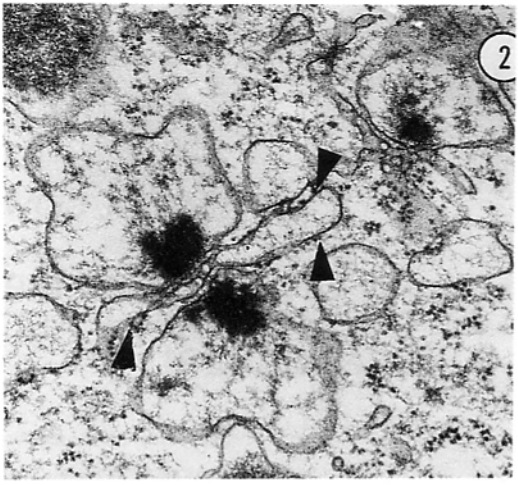
In ascidians, typical configurations of cytoplasmic ALs and IALs are very common in oocytes, as they have been reported in the solitary species so far examined such as *Ciona intestinalis*, *Styela plicata*, *Boltenia villosa* and others (Everingham, 1968a, b; Hsu, 1967; Kessel, 1964, 1965, 1968, 1983a; Mancuso, 1964).

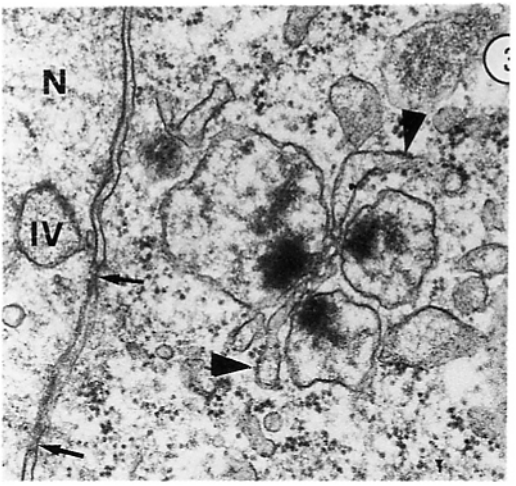
Nevertheless, during our studies on the oogenesis of the colonial ascidian *Botryllus schlosseri*, we never observed stacks or single cisternae of cytoplasmic ALs. Rather, in the cytoplasm of the oocyte in early vitellogenesis, we found particular associations between a fenestrated cisterna and one or a few vesicles with an electron-dense 'core' (cisterna-vesicles association: CVA) (Manni *et al.*, 1994). Because in this colonial ascidian

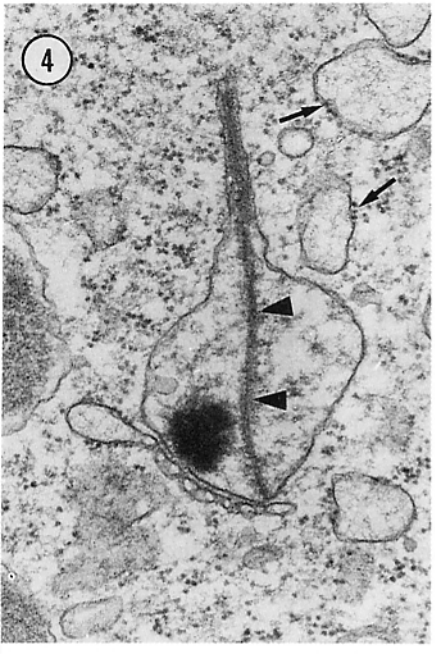
Dipartimento di Biologia, Università di Padova, via Trieste 75, 36121 Padova, Italy.

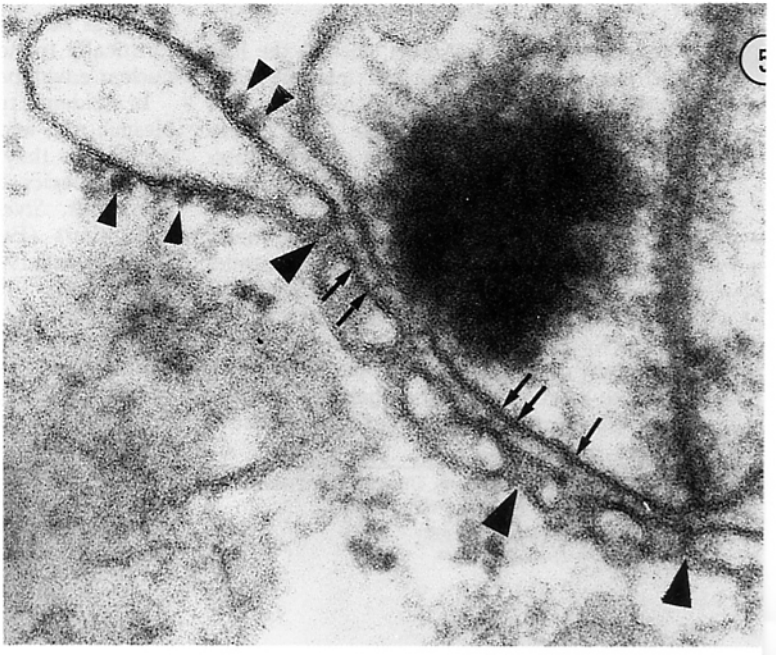
Received 29 November 1993
Accepted 21 January 1994


1
VC
OFC
TC
V
V
N
Nu


2


N
IV


4


5

cytoplasmic ALs are never found together with this porous structure, we analysed comparatively the oogenesis of the solitary species *Ciona intestinalis*, in which ALs have been found, to verify if the CVA were ever present concomitantly with ALs.

In the present paper we report detailed observations on the CVA in the oocytes of *B. schlosseri* and the results are discussed in terms of possible relations between the CVA and ALs.

Materials and Methods

Botryllus schlosseri (family Botryllidae, order Stolidobranchia) is a compound ascidian, colonies being made up of a great number of zooids. Colonies are routinely cultured in laboratory conditions (Sabbadin, 1960); their transparency makes it easy to follow the *in vivo* development of oocytes under the microscope, permitting the selection of zooids at appropriate stages.

The ripe specimens of *Ciona intestinalis* (family Cionidae, order Aplousobranchia) (Kott, 1990) used in this investigation were collected in spring from the Lagoon of Venice and dissected in the laboratory.

Sexually ripe zooids of *B. schlosseri*, cut with a small blade, and portions of the ovary of *C. intestinalis* containing oocytes at different stages of development, were fixed in 1·5% glutaraldehyde buffered with 0·2 M sodium cacodylate, pH 7·4, plus 1·6% NaCl. After washing in buffer and postfixation in 1% OsO_4 in 0·2 M cacodylate buffer, specimens were dehydrated and embedded in glycidether (Serva). Thick sections (1 μm) were stained with toluidine blue. Thin sections were contrasted with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope.

Observations

Porous membrane system in B. schlosseri

In the initial phases of vitellogenesis, the oocytes of *B. schlosseri* (about 120 μm in diameter) differentiate complex series of ovular envelopes (i.e. test cells encased in superficial depressions in the oocytes and covered by the fibrous acellular vitelline coat and, externally, inner and outer follicle cells) (Fig. 1). Small immature yolk granules, membrane-bound and varying in size and density, are scattered in the ooplasm. The rough endoplasmic reticulum (RER) vesicles are well developed and often contain fine homogeneous material, and the Golgi fields are numerous and active. The nucleus is large and possesses a nucleolus; dense granular material (nuage), not bound by membranes, is present in the perinuclear region.

A peculiar membrane system can be found formed of a porous cisterna among the yolk granules, and one or a few vesicles with dense cores associated with it (the CVA) (Figs 2–

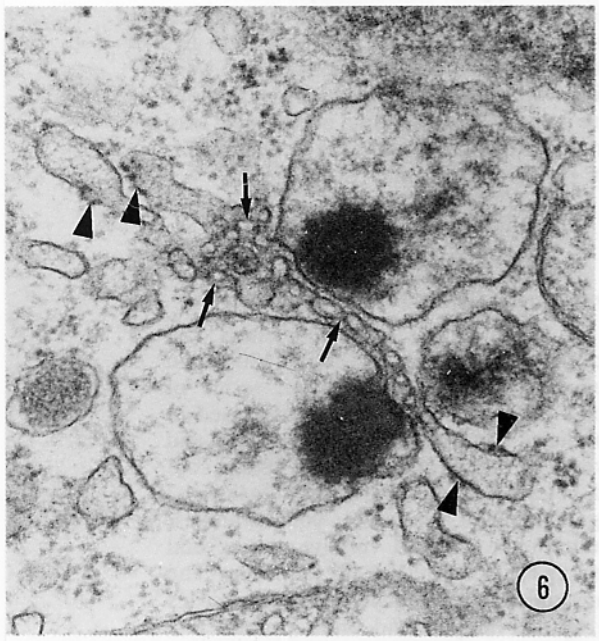
Figs 1–6. *Botryllus schlosseri* oocytes.

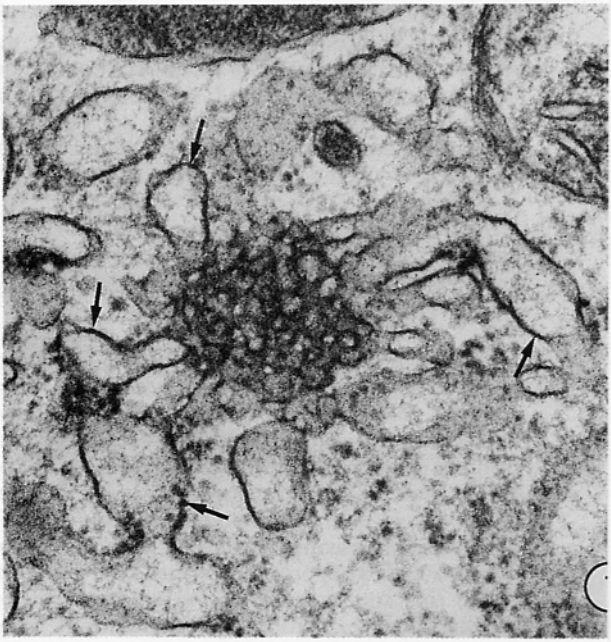
Fig. 1. Oocyte in early vitellogenesis, with maturing yolk granules (y) differing in size and density. Associations between a fenestrated cisterna and a few vesicles (arrows), shown in detail in inset, are scattered in ooplasm. Intranuclear vesicles (arrowheads) are also present. Outer follicle cells (OFC), test cells (TC) and area of vitelline coat (VC) are visible. N: nucleus; Nu: nucleolus. ×3,500; inset ×10,000.

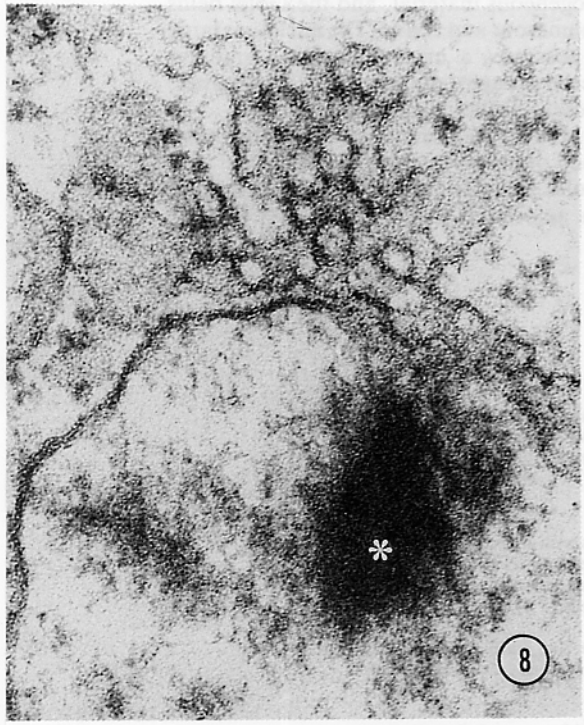
Figs 2–3. Transverse sections of CVA complexes. Note vesicles with strongly dense cores, lying above and beneath plane of cisterna and associated to its fenestrated area. Ribosomes (arrowheads) adhere at periphery of cisterna. CVA is sometimes found near nuclear envelope (Fig. 3). Arrows: nuclear pores. IV: intranuclear vesicle; N: nucleus. ×28,000

Fig. 4. Vesicles of CVA with dense cores, always facing cisterna, and fibrillar material, the latter occasionally organized in dense lamina (arrowheads) which lengthens deforming vesicles. Arrows: RER vesicles. ×32,500.

Fig. 5. A detail of Figure 4. Pores of cisterna (large arrowheads) are regularly arranged. Short feet between membranes of vesicle and cisterna are visible (arrows). Arrowheads: ribosomes adhering to cisterna. ×132,000.


6




8

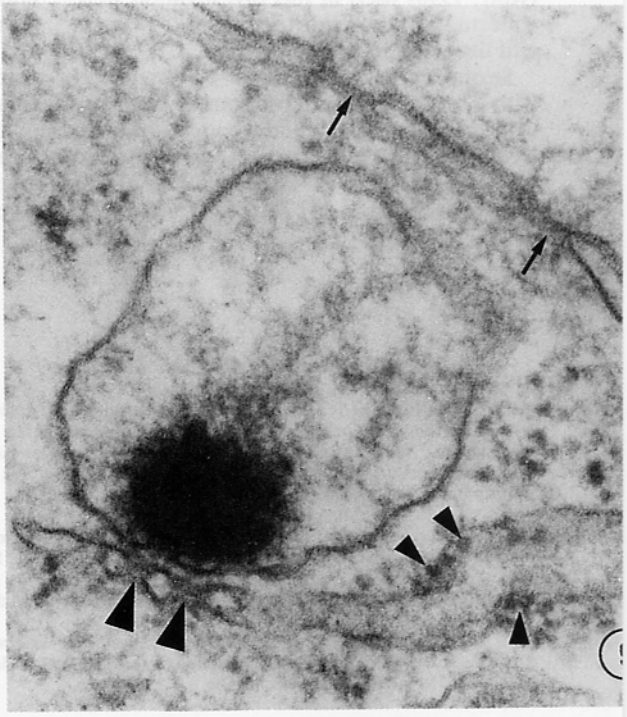


Table 1. *Main differences between CVA and nuclear pores*

	Nuclear pores	CVA pores
Diameter	60–80 nm	20–30 nm
Shape	Octagonal	Circular
Pore-associated material	Present	Absent
Association with nuage	Present	Absent
Association with nuclear lamina	Present	Absent
Association with vesicles	Absent	Present

4). This association is frequent during early vitellogenesis, but decreases in number and becomes occasional in the later stages of oogenesis. The CVA is widely scattered in the cytoplasm and often located in the perinuclear regions, although it does not show any precise orientation with respect to the nuclear envelope. No significant close spatial relationship of the CVA with other cytoplasmic components (mitochondria, Golgi complex, yolk granules, nuage) can be recognized.

Owing to the organization of the membrane system, the features of transverse sections and the '*en face*' views of the CVA are markedly different from each other. In transverse section, the central part of the cisterna is fenestrated and closely associated with large, smooth vesicles lying above and beneath the plane of the cisterna itself. At the periphery, the cisterna is prolonged into the surrounding cytosol by means of extensions having ribosomes bound to the membranes (Figs 2–5). In the '*en face*' view (Figs 6, 7) the cisterna appears to be laminar and its lateral extensions radiate all round in the form of sinuous 'arms'. The cisterna encloses a compartment containing loose fibrillar material, similar to the fine content of RER vesicles (Fig. 4). The pores of the central region of the cisterna are about 25 nm in diameter and are aligned in register (intervening distance 20–30 nm) (Figs 5, 8). They recall nuclear pores in distribution, but are smaller (Figs 3, 9) and also have other distinctive features (see Table 1). In tangential section, the CVA pores are circular in outline, apparently empty and not associated with material, nuage, free ribosomes or polyribosomes (Figs 6–8).

In transverse and oblique sections (Figs 6, 8) the fenestrated area of the cisterna is always associated with the vesicles but, when the section cuts the cisterna, giving an '*en face*' view, the vesicles are no longer visible because they lie outside the plane of the section (Fig. 7). The CVA vesicles contain fibrillar material which radiates from a dense, eccentric core facing the coupling membranes. This material is often organized into

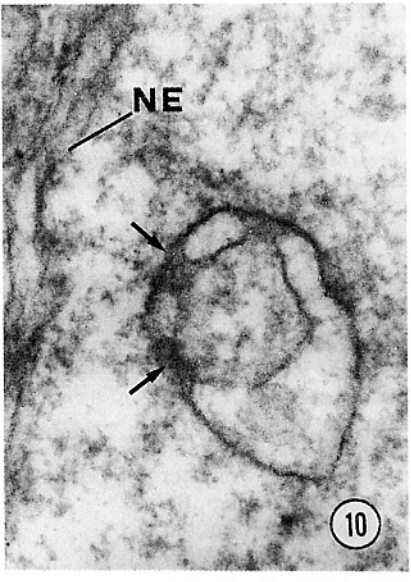
Figs 6–9. *B. schlosseri* oocytes.

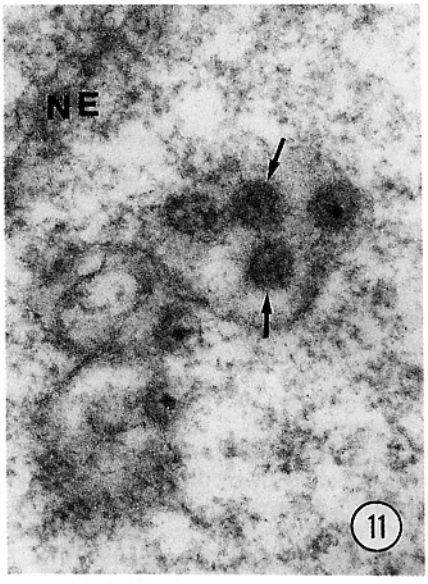
Fig. 6. Oblique section of a CVA complex. Part of cisterna is seen 'en face'. Arrows: pores of cisterna. Arrowheads: ribosomes. ×45,000.

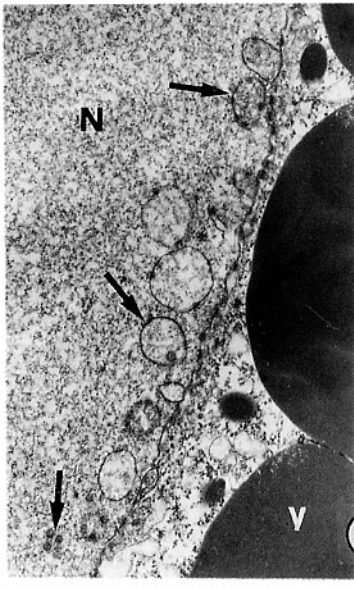
Fig. 7. Plane of section passes through cisterna, so that associated vesicles are not visible; note relation between fenestrated central area of cisterna and peripheral extensions of RER (arrows) which radiate from it. ×55,000.

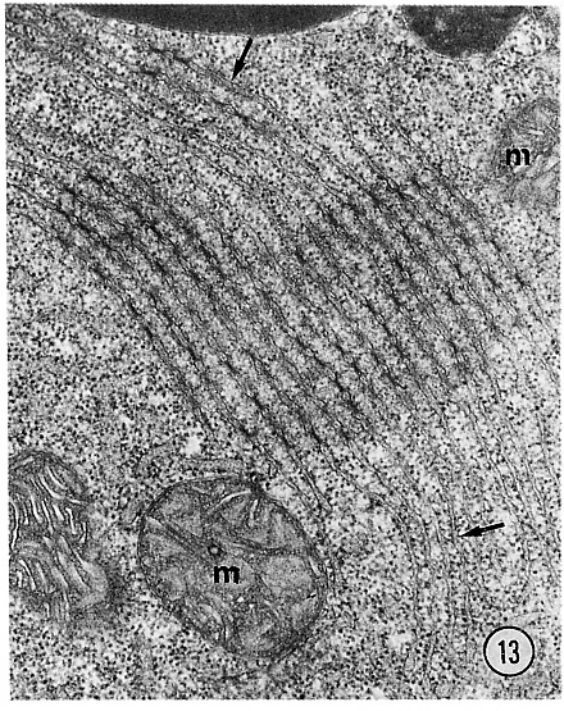
Fig. 8. Detail of a CVA in oblique section. Pores of cisterna appear circular in outline and are apparently empty. *Dense core of vesicles. ×125,000.

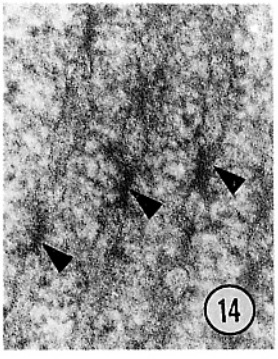
Fig. 9. Pores of nuclear envelopes (arrows) and CVA complex (large arrowheads) are shown in comparison. Arrowheads: ribosomes. ×88,000.

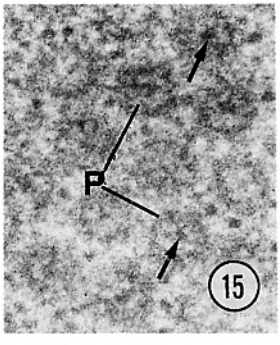

NE
10

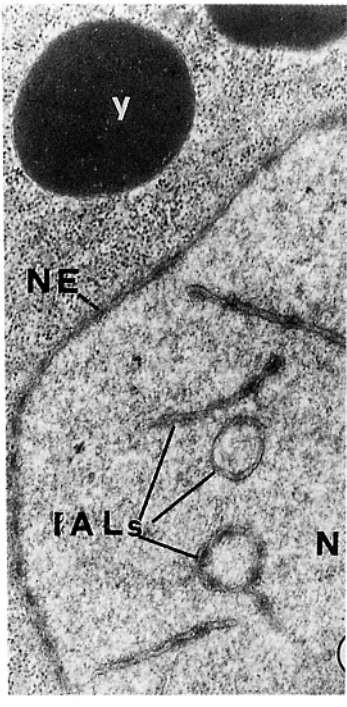

NE
11


N
V


m
m
13


14


P
15


V
NE
IALs
N

a fibrillar lamina which lengthens, deforming the vesicle (Figs 4, 5).

Frequently, the coupled membranes of the cisterna and adjoining vesicles run parallel to each other over a large area being separated by a mean distance of 11 nm. In several cases this narrow space is crossed by short, densities 14 nm wide, occurring at regular intervals of approximately 10 nm and separated by electron-lucent regions (Fig. 5).

Intranuclear porous membranes in B. schlosseri

During most stages of oogenesis a number of IALs are present (Figs 10–12), although they are lacking in very young oocytes and ripe eggs. They are commonly distributed in the peripheral nucleoplasm very close to the nuclear envelope, and are never observed in the central portion of the nucleus. The IALs are formed of a double membrane and have usually circular profiles. In tangential section their pores show central granules (Fig. 11) and are structurally similar to the nuclear pores.

Especially in early vitellogenesis, numerous intranuclear vesicles and blebs are also found close to the nuclear envelope (Figs 3, 10), formed of a single membrane, sometimes connected with its inner membrane. Densities occur along the membrane of these vesicles, recalling nuclear pores in diameter and distribution.

Porous membrane system in Ciona intestinalis

The vitellogenic oocytes of *C. intestinalis* often have conspicuous stacks of ALs (Fig. 13) in the cortical ooplasm, where yolk granules are occasional and mitochondria numerous. The ALs are formed of numerous (10–15) parallel cisternae extending toward the periphery with endoplasmic reticulum cisternae to which ribosomes adhere. Their pores (Figs 14, 15) are regularly arranged, octagonal in shape, about 70 nm in diameter, and usually associated with fibrillar material; in tangential section (Fig. 15) they often show dense central granules. ALs are never associated with vesicles with dense cores. Moreover, the oocytes never show associations between a fenestrated cisterna and vesicles with dense cores.

Porous membranes are also located inside the nucleus of the oocytes (Fig. 16). They represent solitary IALs with circular or curved configurations, in which the pores are apparently identical in structure to those of the nuclear envelope and cytoplasmic ALs.

Discussion

Our data show that *B. schlosseri* oocytes are provided with an unusual membrane system, composed of the association of a porous cisterna and some vesicles with dense cores (Fig. 17). This association does not represent an occasional finding, because it was constantly found in all examined oocytes. Since this membrane system was found only during a restricted phase of oocyte development, e.g. early vitellogenesis, when intense synthesis of yolk granules begins (Manni *et al.*,

Figs 10–12. *B. schlosseri* oocytes. Intranuclear annulate lamellae and vesicles (*large arrows*) in periphery of nucleoplasm (Fig. 12). IALs (Figs 10 and 11) are composed of double membranes and possess pores (*arrows*) structurally similar to nuclear pores. In Figure 11 pores of IALs (*arrows*) are shown in tangential section. Note central granule associated with some pores. N: nucleus; NE: nuclear envelope; y: yolk. Figure 10 ×27,000; Figure 11 ×54,000; Figure 12 ×9,000.

Figs 13–16. *Ciona intestinalis* oocytes.

Fig. 13. Stacks of annulate lamellae in vitellogenic oocyte. Note connection with RER (arrows) at periphery of porous cisternae. m: mitochondria. ×14,000.

Figs 14–15. Cross (Fig. 14) and tangential (Fig. 15) sections of pores (P) of ALs. Note fibrillar material (arrowheads) and central granules (arrows) associated with pores. ×75,000.

Fig. 16. Section of oocyte illustrating intranuclear annulate lamellae (IALs) at periphery of nucleoplasm. N: nucleus; NE: nuclear envelope; y: yolk. ×18,000.

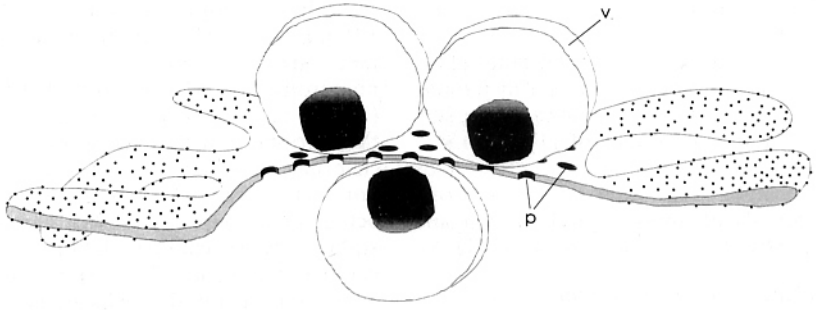


Fig. 17. Schematic drawing illustrating 3-dimensional structure of CVA. Note extensions of the cisterna in peripheral 'arms' with ribosomes bound to membrane. Smooth vesicles (v) with dense cores lie above and beneath fenestrated area of cisterna. Densities between vesicles and cisterna are omitted. p: pore.

1994), it may be assumed that the presence of the CVA is related to the particular processes occurring at this phase, but the precise significance of this structure is not immediately clear.

The CVA does undoubtedly look more like ALs than any other organelle or cytoplasmic membrane system. Despite the fact that ALs are also very common in the female gametes of solitary ascidians (Everingham, 1968a, b; Hsu, 1967; Kessel, 1964, 1965, 1968, 1983a; Mancuso, 1964), we never were able to identify them in *B. schlosseri*. Conversely, we never were able to identify the CVA in the oocytes of *Ciona intestinalis*, in which we confirmed the presence of ALs as the widespread cytoplasmic porous membrane system during vitellogenesis. Nevertheless, the nuclei of *B. schlosseri* oocytes possess vesicles and IALs, like other ascidian oocytes (Everingham, 1968b; Kessel, 1983a). IALs are considered to be the nuclear form of ALs and probably derive from blebbing of the nuclear envelope representing its amplification (Kessel 1992). So in *B. schlosseri* the contemporary presence of IALs and porous cytoplasmic membranes, the CVA, raise the question of whether the CVA is a particular form of ALs.

Although the CVA has characteristics in common with ALs (Table 2), such as fenestrations, peripheral continuity with RER,

Table 2. *Comparison between CVA and ALs, stressing main correspondences and differences*

	CVA	AL
Porous membrane system	+	+
Transitory presence	+	+
Cisternae organized in stacked array	−	+
Association with RER	+	+
Association with cytoplasmic organelles (except RER and dense-cored vesicles) or other components	−	+
Association with vesicles with dense cores	+	−
Similarity of pores with nuclear pores	−	+
Presence of pore associated material	−	+

+ presence; − absence.

and presence limited to definite periods of development, it also has several important differences (Table 2), especially regarding the size and structure of the pores. CVA pores (about 25 nm in diameter) are much smaller than those of the nucleus and ALs (60–80 nm) (Kessel, 1968, 1973, 1983b, 1985), and they do not show structural similarity with ALs pores because they lack the central granule and pore-associated material. Moreover, the CVA cisterna is always single, and the stacked, parallel array of ALs membranes is never encountered. It is always in relationship with the dense-cored vesicles and peripheral RER, but never shows significant relationships with other cytoplasmic components, as sometimes occurs for ALs. Also nuage, the dense material found in the germinal cells of many species (Eddy, 1975), is often found associated with ALs (Clèrot, 1979; Kessel and Beams, 1969), but we did not note any definite relationship between it and the CVA in *B. schlosseri*. Conversely, the ALs never exhibit a close association with vesicles with dense cores, as the CVA does.

In the light of the above, the CVA does not seem to correspond to ALs. However, considering the reciprocal presence/absence relationship in the two species studied, it is possible that the CVA takes over some of the functions of ALs. These functions are difficult to define, because the true significance of ALs is still enigmatic, despite a great number of studies and the variety of roles attributed to them (Kessel, 1989, 1992).

The presence of RER at the edges of the cisterna indicates that the CVA is involved in the production of proteins, which may be accumulated at the level of dense cores. In this phase of vitellogenesis, yolk granules are variable in texture and some of them possess dense areas, recalling the core of the CVA (Manni, *et al.* 1994). So some of the yolk granules may form with the contribution of vesicles from the CVA, although most granules form from previtellogenic bodies (Manni, *et al.* 1994).

As mentioned above, the CVA pores are very simple in structure, apparently empty, and unlike those of ALs. It is possible that, as in other cases, they represent a way of increasing the membrane surface for better exchange between the inner part of the cisterna and the cytosol in the area of association. A similar solution is known to be adopted by another peculiar form of endoplasmic reticulum, the fenestrated sarcoplasmic reticulum (Franzini-Armstrong, 1973). The CVA appears to be a highly polarized complex, with the cisterna at the periphery involved in protein synthesis, and the porous, smooth, central region involved in exchanges with the cytosol and/or contiguous vesicles. The densities in the area of membrane coupling may play an important role in anchoring the dense-cored vesicles to the central area of the cisterna, or they may be the way in which communication occurs among the components of this polarized complex. In this light, their resemblance to the 'feet' of the striated muscle involved in the electron-mechanical coupling between T-tubules and sarcoplasmic reticulum is noteworthy.

Lastly, our results demonstrate that the CVA is a specialization present only in *B. schlosseri*. So it is possible that this specialization, in addition to other characteristics regarding dimensions, amount of yolk and ovular envelopes development (see Sabbadin *et al.*, 1992; Manni *et al.*, 1993, 1994; Zaniolo *et al.*, 1987), differentiate the oocytes of this colonial ascidian from those of solitary species.

Acknowledgements

We thank Professor R. G. Kessel for useful discussion and Mr V. Miolo for technical assistance and Mr C. Friso for drawings. This investigation was supported by grants from MURST and CNR.

References

Clèrot, J. C. 1979. Les groupements mitochondriaux des cellules germinales des poissons Téléostéens cyprinidés. I. Etude ultrastructurale. *J. Ultrastruct. Res.*, **54,** 461–475.

Eddy, E. M. 1975. Germ plasm and the differentiation of the germ line. *Int. Rev. Cytol.*, **43,** 229–280.

Everingham, J. W. 1968a. Attachment of intranuclear annulate lamellae to the nuclear envelope. *J. Cell Biol.*, **37,** 540–550.

Everingham, J. W. 1968b. Intranuclear annulate lamellae in ascidian embryos. *J. Cell. Biol.*, **37,** 351–354.

Franzini-Armstrong, C. 1973. Membranous systems in muscle fibers. In *The Structure and Function of Muscle* (ed. C. H. Bourne) Vol. **II,** pp. 531–619. Academic Press, New York.

Hsu, W. S. 1967. The origin of annulate lamellae in the oocyte of the ascidian *Boltenia villosa* Stimpson. *Z. Zellforsch. Mikrosk. Anat.*, **82,** 376–390.

Kessel, R. G. 1964. Intranuclear annulate lamellae in oocytes of the tunicate *Styela partita*. *Z. Zellforsch. Mikrosk. Anat.*, **63,** 37–51.

Kessel, R. G. 1965. Intranuclear and cytoplasmic annulate lamellae in tunicate oocytes. *J. Cell Biol.*, **24,** 471–487.

Kessel, R. G. 1968. Annulate lamellae. *J. Ultrastruct. Res. Suppl.*, **10,** 1–82.

Kessel, R. G. 1973. Structure and function of the nuclear envelope and related cytomembranes. *Prog. Surf. Membr. Sci.*, **6,** 243–329.

Kessel, R. G. 1982. The structure and function of annulate lamellae: porous cytoplasmic and intranuclear membranes. *Int. Rev. Cytol.*, **82,** 181–303.

Kessel, R. G. 1983a. Intranuclear membranes (vesicles, lamellae, lamellae annulate) in oocytes of the ascidian *Styela partita*. *J. Submicrosc. Cytol.*, **15** (3), 773–785.

Kessel, R. G. 1983b. Structure and function of annulate lamellae. *Int. Rev. Cytol.*, **82,** 181–303.

Kessel, R. G. 1985. Annulate lamellae (Porous cytomembranes): with particular emphasis on their possible role in differentiation of female gamete. In *Developmental Biology* (ed. L. W. Browder). Vol. **1,** pp. 179–233. Plenum Publishing Corporation, New York.

Kessel, R. G. 1989. The annulate lamellae—from obscurity to spotlight. *Electron Microsc. Rev.*, **2,** 257–348.

Kessel, R. G. 1992. Annulate lamellae: a last frontier in cellular organelles. *Int. Rev. Cytol.*, **133,** 43–120.

Kessel, R. G. and Beams, H. W. 1969. Annulate lamellae and 'yolk nuclei' in oocytes of the dragonfly *Libellula pulchella*. *J. Cell Biol.*, **42,** 185–201.

Kott, P. 1990. The Australian Ascidiacea. Part 2, Aplousobranchia. *Mem. Queensland Mus.*, **29,** 1–298.

Mancuso, V. 1964. Ultrastructural changes in the cytoplasm of *Ciona intestinalis*. *Acta Embryol. Morphol. Exp.*, **177,** 129–146.

Manni, L., Zaniolo, G. and Burighel, P. 1993. Egg envelope cytodifferentiation in the colonial ascidian *Botryllus schlosseri*. *Acta Zool. (Stockholm)* **74,** 103–113.

Manni, L., Zaniolo, G. and Burighel, P. 1994. Ultrastructural study of oogenesis in the compound ascidian *Botryllus schlosseri* (Tunicata). *Acta Zool. (Stockholm)*, **75,** 101–113.

Sabbadin, A. 1960. Ulteriori notizie sull'allevamento e sulla biologia dei Botrilli in condizioni di laboratorio. *Arch. Ocean. Limn.*, **12,** 97–107.

Sabbadin, A., Burighel, P. and Zaniolo, G. 1992. Some aspects of reproduction in ascidians. In *Sex Origin and Evolution* (ed. R. Dallai). Selected Symposia and Monographs U.Z.I. Mucchi Modena, Vol. **6,** pp. 103–115.

Zaniolo, G., Burighel, P. and Martinucci, G. B. 1987. Ovulation and placentation in *Botryllus schlosseri* (Ascidiacea): an ultrastructural study. *Can. J. Zool.*, **65,** 1181–1190.