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Localization of aromatase in the nervous system, Hatschek's pit and gonad of amphioxus by in situ hybridization and immunocytochemistry *

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Abstract Aromatase activity has been found in the brain and pituitary as well as gonads of vertebrates, but no information is available on the specific localization in the brain and Hatschek's pit (primitive pituitary) of amphioxus (*Branchiostoma belcheri*). Using immunocytochemistry and *in situ* hybridization, we studied definitive tissue specific localization of aromatase in the nervous system (brain and spinal cord), wheel organ, Hatschek's pit and gonads at young and adult female and male amphioxus at different stages of gonadal development. Aromatase protein and its mRNA were abundant in the forebrain, midbrain, spinal cord, wheel organ and Hatschek's pit, but not abundant in the hindbrain or early ovary and testis. No aromatase expression was detected in both irregular-shaped cells and ciliated mucous cells in the Hatschek's pit or in the mature ovary and testis. Aromatase immunoreactive material was distributed in the cytoplasm with negative nuclei. The aromatase distribution pattern in the nervous system, Hatschek's pit and gonad closely is similar to that observed in vertebrates. Especially, aromatase expression was found in the neuroendocrine center regulating the secretory activity of Hatschek's pit. Therefore, amphioxus has a primitive brain-aromatase regulatory system similar to that of vertebrates. These results strongly suggest that the high level of aromatase activity in the brain and Hatschek's pit of amphioxus may play a key role in mediating aromatization of local *in situ* synthetic testosterone, and influence the brain aromatase system, which participates in regulating the secretory activity of Hatschek's pit [*Acta Zoologica Sinica* **49** (6) : 800 - 806, 2003].

Key words Amphiosus (*Branchiostoma belcheri*), Aromatase, Nervous system, Hatschek's pit, Gonads

芳香化酶在文昌鱼神经系统、哈氏窝和性腺特异性定位：原位杂交和免疫细胞化学研究 *

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摘 要 芳香化酶活性发现在脊椎动物脑、脑垂体和性腺中，但在文昌鱼脑和哈氏窝的组织特异性定位尚无可利用资料。本文用免疫细胞化学和原位杂交技术，首次发现芳香化酶活性组织特异性定位在幼年和性腺发育不同时期雌、雄文昌鱼神经系统（脑和脊髓）、轮器、哈氏窝和性腺中。芳香化酶蛋白和转录物在前脑、中脑、脊髓、轮器和哈氏窝十分丰富，而后脑、早期卵巢和精巢不够丰富；没有芳香化酶表达的部位是哈氏窝另两种细胞（不规则形细胞和带纤毛粘液细胞）以及成熟卵巢和精巢；芳香化酶免疫活性物质分布在胞质，核为阴性。芳香化酶在文昌鱼神经系统、哈氏窝和性腺的分布模式与低等脊椎动物中的分布模式极为类似，尤其是芳香化酶在脑内调节哈氏窝分泌活动的神经内分泌中枢表达，并形成类似脊椎动物的文昌鱼原始的脑 - 芳香化酶调节系统。这些结果有力地证明，文昌鱼脑和哈氏窝高水平的芳香化酶活性像在其它脊椎动物中一样，对局部介导睾酮芳香化起着关键作用，同时还可能影响脑 - 芳香化酶系统参与调节哈氏窝的分泌活动 [动物学报 **49** (6) : 800～806, 2003]。

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Brief introduction to the first author Male. Research area: protochordata reproductive endocrinology.

关键词 文昌鱼 芳香化酶 神经系统 哈氏窝 性腺

Aromatase is the terminal enzyme in the steroidogenic pathway that converts androgens (e. g. , testosterone) into estrogen (e. g. , estradiol) . It plays a key role in the control of sex differentiation and gonadal development , as well as the feedback regulation to brain and pituitary in vertebrates (Trant *et al*. , 2001; Hong *et al*. , 2000; Callard *et al*. , 1981a , b; Callard , 1983) . The specific localization and distribution of aromatase is typically restricted to the tissues including the gonad , brain , spinal cord , neuroendocrine , retinal and pituitary (Callard *et al*. , 1981a , b; Callard , 1983; Young *et al*. , 1983; Olivereau *et al*. , 1985; Callard *et al*. , 1993; Trant *et al*. , 1997) . It has been shown that the cephalochordate amphioxus (*Branchiostoma belcheri*) , which is in an intermediate evolutionary position between vertebrate and invertebrates , displays aromatase activity in the gonad but not in the brain and other segments (Callard *et al*. , 1984) . Previous work (Fang , 1999) indicates that a primitive system of reproductive neuroendocrine regulation and brain-Hatschek 's pit-gonad axis already emerges in amphioxus. Radioimmunoassay demonstrated that sex steroid hormones (androgen , estrogen and progestin) exist in the gonad (ovary and testis) of amphioxus (Zhang *et al*. , 1985; Fang *et al*. , 1993) . Levels of estradiol-17β peaked during vitellogenesis and rapidly declined during postvitellogenesis and the mature stage of ovary (Fang *et al*. , 1993 , 1994) , while 17α-methyltestosterone can promote spermatogenesis in testis of amphioxus (Fang *et al*. , 1991) . Recently , it has been shown that estrogen and androgen immunoreactive cells and their receptors exist in the brain vesicle and Hatschek 's pit of amphioxus (Fang *et al*. , 2001; Weng *et al*. , 2001a) , suggesting that aromatase immunoreactive cells exist in the both regions.

Thus , it is necessary to re-examine the specific localization and distribution of aromatase protein and mRNA in the nervous system , Hatschek 's pit and gonad of amphioxus in order to understand the role of aromatase on neuroendocrine regulation of the brain and Hatschek 's pit and the feedback regulation of sex hormones. In addition , from an evolutionary viewpoint , the occurrence of aromatase activity in amphioxus would provide a new clue to the relationship between cephalochordates and vertebrates.

1 Material and methods

1.1 Animals

Amphioxus were collected from the Qiotou coast of Xiamen , Fujian Province in South China. The animals were reared for one week in the laboratory at 24 ℃ where they were fed on cultured microscopic algae once every day. A total of 32 amphioxus individuals , ranging from 18.0 - 51.8 mm in length of both sexes were used in the study , with male and female 14 respectively. According to the gonadal classification described by Fang *et al*. (1990) , the animals belonged to the young stage (no gonad) , small growth stage (previtellogenic stage) , large growth stage (vitellogenic stage) and mature stage.

1.2 Sample for immunocytochemistry and in situ hybridization

The whole body of each animal was immersed in freshly prepared Bouin 's fluid without acetic acid for 24 h. After fixation , specimens were cut and the head region (from the front part to the anterior of the first gonad) and the middle region (from the beginning of the first pair of gonads to the end) separated. The tissue was dehydrated through a graded ethanol series then embedded in paraffin. Blocks were serially sectioned (6 μm thick) and mounted on poly-L-lysine-coated slides. Sections with the structure of the Hatschek 's pit and gonadal sections at different developmental stages (female and male) were identified under a microscope and selected for immunostaining.

The excised head and middle region from amphioxus of different developmental stages for *in situ* hybridization were fixed in the fresh 4 % paraformaldehyde solution for 24 h at 4 ℃ overnight , then dehydrated at 4 ℃ in ethanol of serial concentrations prepared using 10 % diethylpyrocarbonate (DEPC)-treated water , finally , treated by xylene overnight at 4 ℃, embedded in paraffin , and serial cross sections of 6 μm were mounted on glass slides.

1.3 Immunocytochemical staining

The paraffin sections were de-waxed , hydrated and incubated in methanol-H_2O_2 for 10 min to remove endogenous peroxidase. They were then stained according to the immunocytochemical avidin-biotin-peroxidase complex (ABC) method. Tissue sections were incubated at 4 ℃ for 24 h in the primary antibodies , mouse anti-human placental aromatase antibodies (1 ∶ 400 dilution) , washed three times in PBS , incubated for 30 min at room temperature with goat anti-mouse IgG (diluted 1 ∶100 with PBS) , rinsed with PBS , incubated for 30 min at room temperature with an avidin-biotin-peroxidase complex (1 ∶100 dilution) . Final visualization of the antigen was achieved with diaminobenzidine (DAB) / H_2O_2 solution for 10 - 30 min.

1.4 In situ hybridization and detection

Tissue sections were deparaffined with xylene , dehydrated through graded ethanol to PBS with preparation of DEPC-treated water and incubated for

2 min at 37 ℃in a fresh preparation of 3 % citric acid containing diluted pepsin after which they were prehybridized at 37 ℃for 4 h. The sections were incubated in hybridization buffer solution containing synthetic fish aromatase oligonucleotide probe (1.5 μg/ml) (purchased from Wuhan Boster Biological Technology Company) in a sealed humid box at 42 ℃ overnight. After hybridization, the slides were washed at 37 ℃for 15 min by 2 ×ssc, 0.5 ×ssc and 0.2 ×ssc respectively. The biotin mouse anti-Dig antibody (1 ∶500 dilution) was then applied, followed by incubation at room temperature for 2 h in a humid box. The slides were washed four times in 0.5 mol/L PBS for 5 min, and incubated in the strept avidin-biotin complex (SABC) solution for 20 min at 37 ℃. Finally sections were incubated in biotin-peroxidase at 37 ℃for 20 min. The hybridization signal was visualized after 20 to 30 min incubation with diaminobenzidine(DAB) solution.

The negative controls were processed as described above but in the absence of labeled probes, and were incubated with normal goat serum instead of the biotin mouse anti-Dig labeled antibody.

2 Results

2.1 Immunocytochemical localization

Immunochemistry showed aromatase positive cells dark brown, on a light yellow or unstained background, the immunopositive cells were easily identified and two negative controls showed negative reactions. Expression of aromatase was highly tissue-specific in the nervous system, Hatschek's pit and gonad in young and different stages of gonadal development of both sexes.

2.2 Nervous system

The nervous system of amphioxus consists of brain and spinal cord (nerve tube). The brain of amphioxus is divided into three regions: forebrain, midbrain (corresponding to the diencephalon of vertebrates) and hindbrain. Immunoreactivity of aromatase showed in the forebrain, midbrain and infundibulum (the brain-Hatschek's pit connection), hindbrain and nerve tube. The cytoplasm of nerve cells and its nerve fiber in these regions showed a immunopositive reaction, but the nuclei of nerve cells showed negative staining. Immunoreactive cells of aromatase were distributed along both sides from dorsal to ventral. The reaction intensity showed no difference in these regions in young and adult amphioxus (Plate Ⅰ:1 - 5).

2.3 Hatschek's pit

The Hatschek's pit consists of three kinds of cells: (1) epithelial cells on the basal parts of Hatschek's pit; (2) multilateral or irregular shape cells located below the basal epithelial cells; (3) ciliated mucous cells located near the oral cavity. Immunoreactivity of aromatase is located in the basal part of the epithelial cell of Hatschek's pit and ciliated cell of the wheel organ. There was a strong immunopositive reaction in young and adult amphioxus (Plate Ⅰ:6,7). The other two kinds of Hatschek's pit cells showed immunonegative staining.

2.4 Gonad

Immuno-activity of aromatase was expressed in ovary follicle cells in amphioxus, the activity become stronger with the development from small growth phase (previtellogenic phase) to large growth phase (Plate Ⅰ:8; Plate Ⅱ:9). The follicle cells at the mature phase revealed a negative immuno-reaction. In the testis, aromatase activity was localized in the close germinal epithelium of early spermatogenic cells, such as, spermatogonia, primary and secondary spermatocyte and Sertoli cell (Plate Ⅰ:10), but spermatids and spermatozoa were immunonegative.

2.5 Localization of *in situ* hybridization

The pattern of aromatase expression observed in immunocytochemical studies was further confirmed by *in situ* hybridization with a specific fish aromatase Dig-labelled oligonucleotide probe. The strongest mRNA hybridization signal was observed in the nervous system, including forebrain, midbrain, infundibulum, nerve tube, Hatschek's pit and wheel organ. Hybridization signal in the hindbrain and oocytes of early ovaries was obviously weak, and no hybridization signal showed in the mature oocytes and spermatozoa. The pattern of tissue specific expression did not change in young and adult amphioxus (Plate Ⅰ: 11 - 16).

3 Discussion

Our previous studies have revealed that there are different systems regulating the secretory activity of Hatschek's pit epithelial cells, such as GnRH neuron and its receptor (Fang *et al*., 1999a, b), neurotransmitter and regulatory peptides (Xia *et al*., 1999; Weng *et al*., 2001b, 2002), 5-hydroxytriptamine, norepinephrine, neuropeptide Y and β-endorhine in the midbrain, as well as dopamine immunoreactive neural cells in the infundibulum. All these together constitute the neuroendocrine center to regulate secretory activity of Hatschek's pit.

Although Callard *et al*. (1984) indicated that aromatase activity was detected in the gonads, not in brain or other part of amphioxus, little is known about the feedback regulatory mechanism of androgen created from *in situ* synthesis or circulation in brain of amphioxus. Our data indicated that aromatase was expressed in the neuroendocrine center. It was strongly believed that probably two regulatoary modes exist for androgen regulation on brain and Hatschek's

pit. First, androgen is converted to estrogen by aromation in brain and then act directly on GnRH neuron system and influences the secretory activity of Hatschek's pit indirctly. Second, androgen converted to estrogen in Hatschek? s pit and regulates the secretory activity of Hatschek's pit directly. It is suggested that regulatory system of brain-aromatase may be established in amphioxus, that is similar to the vertebrates, e. g. teleost (Olivereau *et al*., 1985), frog (Callard *et al*., 1978) and rodent (Pasmanik *et al*., 1985). The aromatase activity is distributed on the growth hormone-secreting cells of sculpin pituitary and somatolactotropes of rodent pituitary (Callard *et al*., 1983). The aromatase immunoreactive cells is distributated on the basal part of Hatschek's pit in amphioxus. It would reveal the conservation of aromatase in the evolution to trace the relationship between amphioxus and vertebrate.

Large amounts of protein and transcript of aromatase were expressed in neural cells of various parts of brain and neural tubes during the development of amphioxus, as well as in oocytes of vitellogenesis and early spermatogenic cells. It is identical with the capacity of production of estrogen in brain and gonads (Zhang *et al*., 1985; Fang *et al*., 1993, 2001; Weng *et al*., 2001a). High activity of aromatase was found in the forebrain, midbrain, hindbrain of avians (Foidart *et al*., 1998; Silverine *et al*., 2000) and teleosts (Lee *et al*., 2000; Callard *et al*., 1981b). It may promote local production of estrogen and influence sexual differentiation in vertebrate (Lephart, 1996). We have seen a very strong signal from aromatase-immunocytochemistry staining and *in situ* hybridization in the younger forebrain and midbrain. It was suggested that aromatase of brain in amphioxus maybe has another function, such as influencing sex differentiation.

Aromatase activity was located on the granulosa cells in amago salmon (Young *et al*., 1983). Though Callard *et al*. (1984) indicated that the separated ovary of amphioxus was able to convert androgen into estrogen, it was unknown that what kind of cells performed the function. In this study, the immuno-activity of aromatase was found in ovary follicle cells of amphioxus. It is indicated that the conversion was completed in follicle cells. In previous study (Welsch *et al*., 1997), it was indicated that steroid hormones were synthesized in ovary follicle cells in amphioxus. These data would provide some new evidence that the function of follicle cells in amphioxus would be same with the granulose cells in fish.

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Explanation of Plates

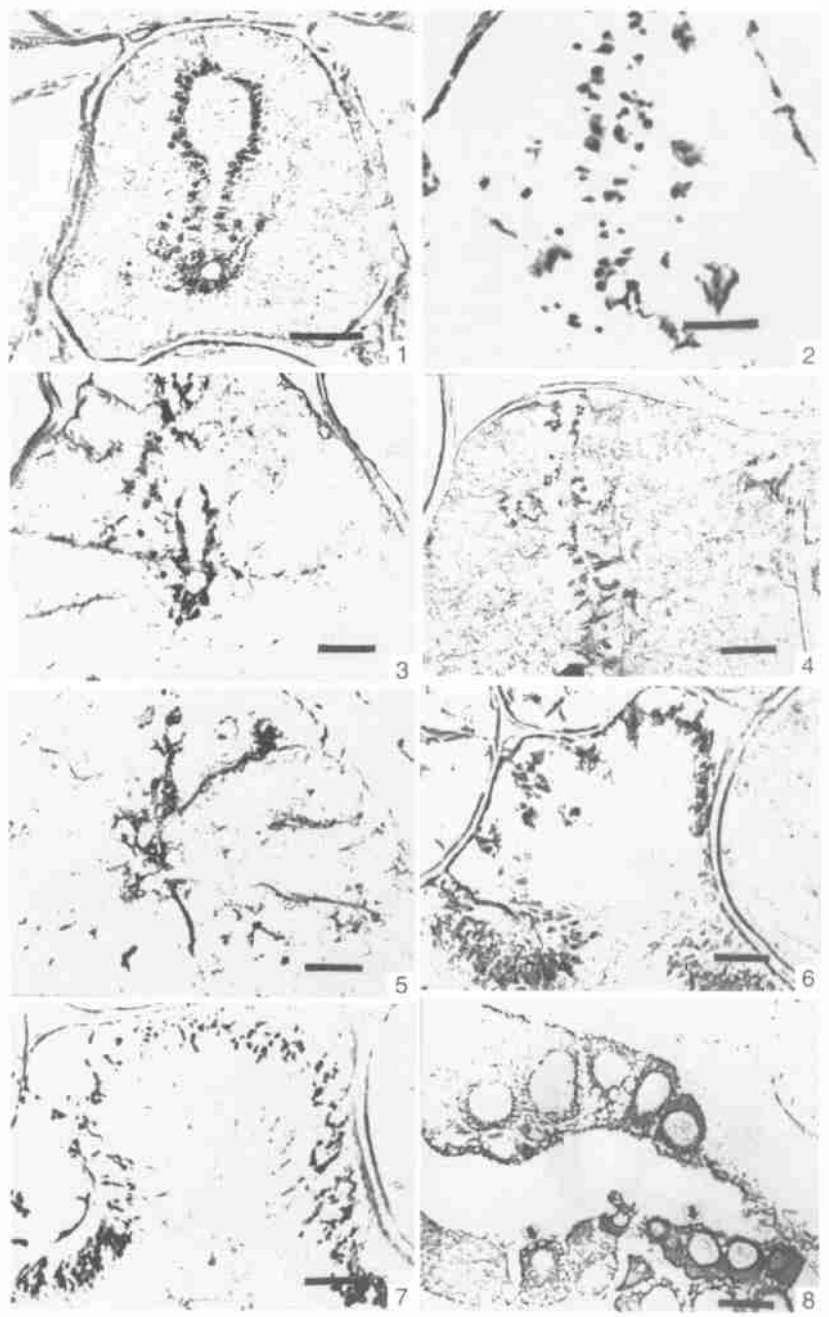
Plate Ⅰ

1. Aromatase-immunoreactive nerve cells in the forebrain of young amphioxus showed strong positive reaction
2. Aromatase-immunoreactive nerve cells in the midbrain of young amphioxus showed strong positive reaction
3. Aromatase-immunoreactive nerve cells and fibers in the midbrain of adult female amphioxus showed strong positive reaction
4. Aromatase-immunoreactive nerve cells in the hindbrain of adult male amphioxus showed weak positive reaction
5. Aromatase-immunoreactive nerve cells in the nerve tube of adult amphioxus showed strong positive reaction
6, 7. The epithelial cells of Hatschek's pit and wheel organ showed strong aromatase immunoreactivity
8. At the Ⅰ- Ⅱ phase of ovary, aromatase immunoreactivity was located in the follicle cells (arrow)

Plate Ⅱ

9. At the vitellogenic phase, aromatase immunoreactivity was located in the follicile cells (arrow) and showed strong immunoreaction
10. In early testis, aromatase immunoreactivity was located in the spermatogonia (thin arrow), spermatocyte (thick arrow) and Sertoli cell (long arrow)
11. Aromatase mRNA hybridization signal was detected in the brain vesicle of adult female amphioixus
12. Aromatase mRNA hybridization signal was detected in the infundibulum
13. Aromatase mRNA hybridization signal was detected in the nerve tube
14. Aromatase mRNA hybridization signal was detected in the Hatschek's pit and wheel organ
15. Aromatase *in situ* hybridization (ion signal was detected in the early ovary
16. Aromatase mRNA hybridization signal was detected in the early testis

Plate Ⅰ: 1 - 7 and 11 - 16, scale bar = 25 μm Plate Ⅰ: 8 and Plate Ⅱ: 9, scale bar = 50 μm



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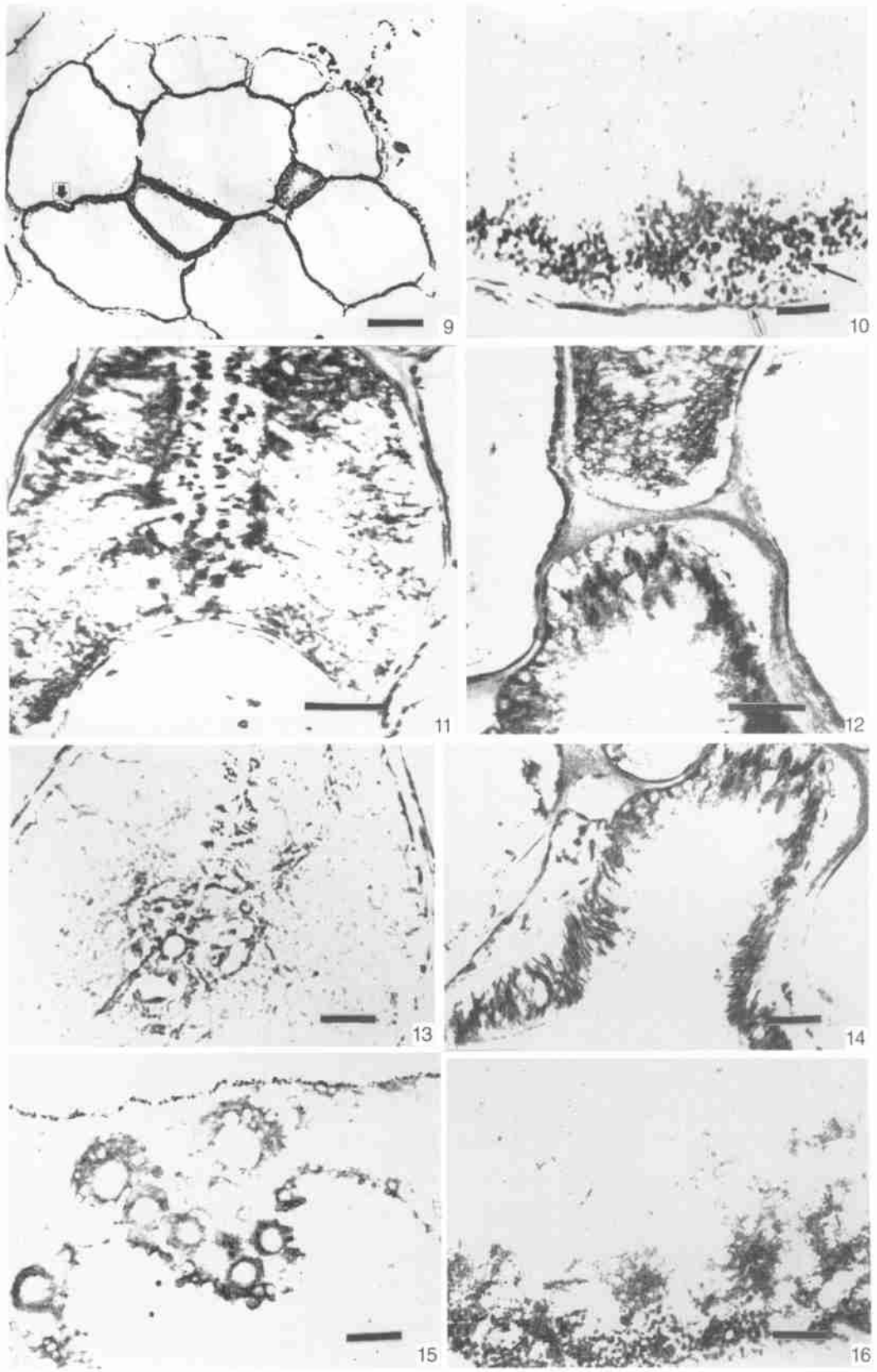

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