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ULTRACYTOCHEMICAL LOCALIZATION OF CALCIUM IONS IN THE PINEAL ORGAN OF THE DOMESTIC TURKEY

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Abstract

Calcium ions are involved in several processes occurring in the avian pineal, including the regulation of melatonin secretion. The aim of study was to investigate distribution of calcium ions in the pineal organ of domestic turkey at the level of electron microscopy. The study was performed on 12 female turkeys housed in a cycle of 12L : 12 D (light intensity 300 lux; 07:00 – 19:00), starting from the third week of life. At the age of 12 weeks, the birds were anesthetized with halothane and sacrificed by decapitation at 14:00 and at 02:00 (in darkness). The pineals were fixed using the potassium pyroantimonate method, which enables visualization of calcium ions in the form of electron dense precipitates.

Extremely numerous, large precipitates of calcium pyroantimonate were observed between collagen fibres of the connective tissue surrounding the pineal follicles. The number of precipitates in the follicles was much lower than in the neighbouring stroma. Precipitates were numerous in intercellular spaces between cells forming the follicular wall. In contrast, they were infrequently observed in the follicular lumen. In pinealocytes, large amounts of precipitates were present in the nucleus, mitochondria and short, wide cisterns of the smooth endoplasmic reticulum. Precipitates were sparse in the apical prolongations of rudimentary-receptor pinealocytes. The content of precipitates did not differ prominently between individual pinealocytes. Precipitates in the cytosol of both rudimentary-receptor pinealocytes and secretory pinealocytes were much more numerous in the organs taken *ex vivo* during nighttime than during daytime. Supporting cells contained much fewer precipitates than pinealocytes.

Key words: calcium, pineal organ, cytochemistry, pyroantimonate, turkey.

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ULTRACYTOCHEMICZNA LOKALIZACJA JONÓW WAPNIOWYCH W SZYSZYNCE INDYKA

Abstrakt

Jony wapniowe uczestniczą w wielu procesach zachodzących w szyszynce ptaków, w tym m.in. w regulacji wydzielania melatoniny. Celem badań było określenie rozmieszczenia jonów wapniowych w szyszynce indyka domowego na poziomie mikroskopu elektronowego. Badania przeprowadzono na 12 indyczkach utrzymywanych od 3. tygodnia życia w warunkach cyklu świetlnego 12 h światła : 12 h ciemności (światło o natężeniu 300 lx na poziomie podłogi; od 07:00 do 19:00). W wieku 12 tygodni ptaki poddano eutanazji o godzinie 14:00 oraz 02:00. Szyszynki utrwalono w mieszaninie zawierającej piroantymonian potasu, co umożliwiło wizualizację jonów wapniowych w postaci elektronowo gęstych precypitatów.

Szczególnie liczne duże precypitaty piroantymonianu wapnia występowały między włóknami kolagenowymi tkanki łącznej otaczającej pęcherzyki szyszynki, natomiast ich liczba w pęcherzykach była znacznie mniejsza. Liczne precypitaty występowały w przestrzeniach międzykomórkowych między komórkami budującymi ścianę pęcherzyków, zaś tylko pojedyncze obserwowano w świetle pęcherzyków. W pinealocytach bardzo liczne precypitaty były obecne w jądrze komórkowym, mitochondriach oraz krótkich, szerokich cysternach siateczki śródplazmatycznej gładkiej. W wypustkach wierzchołkowych pinealocytów szczątkowo-receptorowych występowała niewielka liczba precypitatów. Zawartość precypitatów nie różniła się wyraźnie w poszczególnych pinealocytach. Precypitaty występujące w cytozolu pinealocytów szczątkowo-receptorowych i pinealocytów wydzielniczych były znacznie liczniejsze w szyszynkach pobranych podczas nocy niż w trakcie dnia. Komórki podporowe zawierały znacznie mniej precypitatów niż pinealocyty.

Słowa kluczowe: wapń, szyszynka, cytochemia, piroantymonian, indyk.

INTRODUCTION

The avian pineal organ, probably due to its intermediate evolutionary position between the pineals of lower vertebrates and mammals, is characterized by a very intricate internal structure and complex functional organization (Vollrath 1981, Prusik, Lewczuk 2008a, b). The pineal parenchyma in birds is composed of three cell populations: pinealocytes, supporting cells and neurons (Vollrath 1981, Prusik et al. 2006, Prusik, Lewczuk 2008a). Fundamental differences in the ultrastructure of avian pinealocytes lead to their classification into receptor pinealocytes, rudimentary-receptor pinealocytes and secretory pinealocytes. Supporting cells are represented by ependymal-like cells and astrocyte-like cells. The histological structure of the avian pineal organ shows prominent interspecies differences, therefore three main forms of the organ are distinguished: saccular, tubulofollicular and solid (Vollrath 1981, Prusik, Lewczuk 2008a). Recently, an intermediate solid-follicular type has been acknowledged (Ohshima, Hiramatsu 1993, Haldar, Bishnupuri 2001).

Avian pinealocytes are directly photoreceptive due to the presence of two photopigments: pinopsin and melanopsin (Okano et al. 1994, 1997, Chaurasia et al. 2004, Holthues et al. 2005). Moreover, they contain a circadian oscillator, composed by a set of clock genes, that is the genes responsible for generation of an endogenous circadian rhythm of pinealocyte activity (Murakami et al. 1994, Okano, Fukada 2003, Csernus et al. 2005). Thus, pinealocytes in birds, unlike in mammals, are more or less autonomous (depending on the species and the stage of postembryonic development) in the creation and entrainment of the diurnal rhythm of melatonin secretion (Barrett, Underwood 1992, Prusik 2005, Prusik, Lewczuk 2008b). In the vast majority of species, this autonomy is partially limited by the sympathetic innervation, which belongs to the multisynaptic pathway connecting the pineal organ with the hypothalamus and the retina (Cassone et al. 1990, Prusik, Lewczuk 2008b). Norepinephrine released from the sympathetic nerve fibers, acting via α_2-adrenoceptors, inhibits melatonin secretion during the photophase (Prusik 2005, Prusik, Lewczuk 2008b).

It is generally considered that calcium ions are involved in several processes occurring in avian pinealocytes, including photoreception, regulation of melatonin secretion and exocytosis of microvesicles; nevertheless, many aspects of the mechanisms of Ca^{2+} action in these cells are still poorly recognized (Zatz 1989, 1992, Zatz, Mullen 1988b, D'-Souza, Dryer 1994, 1996, Zatz, Heath 1995, Alonso-Gomez, Iuvone 1995, Pablos et al. 1996, Nikaido, Takahashi 1998, Agapito et al. 1998), for instance, subcelullar localization of Ca^{2+} in parenchymal cells has been not described in birds yet.

The present study was undertaken to investigate the distribution of calcium ions in the pineal organ of domestic turkey at the level of electron microscopy. The turkey pineal organ was chosen as the subject of our investigations because 1) it represents the tubulofollicular type of the pineal parenchyma organization, which is the most common in birds (Przybylska-Gornowicz et al. 2005); 2) it contains well-developed rudimentary-receptor pinealocytes (with the prominent apical prolongations and regular, stratified distribution of organelles) and simultaneously numerous secretory pinealocytes (Lewczuk 2000, Prusik, Lewczuk 2008a); 3) turkey pinealocytes are photosensitive, possess an effective endogenous oscillator and are influenced by the sympathetic innervation (Prusik 2005, Prusik, Lewczuk 2008b); 4) the diurnal rhythm of melatonin secretion by turkey pinealocytes is characterized by a high amplitude (Prusik 2005, Zawilska et al. 2006, Prusik, Lewczuk 2008b), which seems to be important in studies on day-night differences in Ca^{2+} distribution.

The study was performed using the potassium pyroantimonate ultracytochemical method, which enables visualization of calcium ions in the form of electron dense precipitates and is commonly used for ultracytochemical localization of calcium ions in biological samples (Klein et al. 1972, Appleton, Morris 1979, Krstič 1985, Lewczuk et al. 1994, 2007, Mentre, Escaig 1988,

PIZARRO et al. 1989a,b, THERON et al. 1989, TUTTER et al. 1991, WICK, HEPLER 1982). Studies on chemical composition of pyroantimonate deposits have demonstrated that Ca is the predominant (beside small amounts of K, Na, Mg) or the only element associated with antimony (SUZUKI, SUGI 1989, MENTRE, ESCAIG 1988). The potassium pyroantimonate technique is the only one sensitive enough to demonstrate intracellular calcium ions at the level of electron microscopy in mammalian pinealocytes (TUTTER et al. 1991).

MATERIAL AND METHODS

The study was performed on 12 females of the domestic turkey (*Meleagris gallopavo*). The birds were housed in a cycle of 12 hours light : 12 hours dark, starting from the third week of life. During the photophase (between 07:00 and 19:00), fluorescent lamps provided light of the intensity of 300 lx at the floor level. The birds had free access to standard food and water. At the age of 12 weeks, turkeys were anesthetized with halothane and sacrificed by decapitation at 14:00 and at 02:00. All procedures (including euthanasia, tissue preparation and fixation) during the scotophase were made in darkness with the help of personal noctovisors. The experiment was performed in compliance with the Polish law.

The pineal organs were removed immediately after death, divided into small pieces and fixed in a solution containing 2% glutaraldehyde, 2% potassium pyroantimonate and 0.735% potassium acetate for 2 hours at 4ºC (pH 7.5). After the first step of fixation, the pieces were rinsed three times (10 min each) with 0.735% potassium acetate in distilled water, incubated for 2 hours in 1% aqueous solution of osmium tetroxide containing 2% potassium pyroantimonate and 0.735% potassium acetate. Next, the pieces were washed in potassium acetate solution to remove any unreacted potassium pyroantimonate and to prevent nonspecific precipitation, dehydrated and embedded in Epon 812. Semithin sections were cut from each block of the tissue, stained with toluidine blue and examined under a light microscope in order to choose the sites for preparation of ultrathin sections. Both contrasted (with lead citrate and uranyl acetate) and uncontrasted ultrathin sections were examined in the Tecnai G2 Spirit BioTwin transmission electron microscope (FEI, USA) equipped with two digital cameras: Veleta (Olympus, Japan) and Eage 4k (FEI, USA).

For the control purposes, pieces of the pineal organs were prefixed for 30 minutes in a mixture of 2% glutaraldehyde and 0.735% potassium acetate, and then incubated (at 40ºC) for 60 minutes in 0.735% potassium acetate solution with 2.5% EGTA or without EGTA, prior to the above described pyroantimonate procedure.

RESULTS

Ultrastructure of the turkey pineal organ

Parenchyma of the turkey pineal consisted of follicles containing a round or oval lumen, variable in size (Figure 1*a*). The follicles were surrounded by the thick basement membrane and separated from one another by the connective tissue septa, penetrating inside the organ from the capsule. The follicular wall was composed by rudimentary-receptor pinealocytes and ependymal-like supporting cells, which together created a single layer of elongated cells bordering the lumen, and by secretory pinealocytes and astrocyte-like supporting cells forming one or more layers of oval cells located at the periphery of the follicle (Figure 1*b*). Rudimentary-receptor pinealocytes possessed highly regular organization, with stratified distributions of organelles in the form of four zones: 1) perinuclear zone, 2) zone with the rough endoplasmic reticulum, 3) zone with the Golgi apparatus and 4) zone with mitochondria (Figure 1*b*). These cells created the apical prolongation extending into the follicular lumen and the basal process ending close to the basement membrane. Secretory pinealocytes were characterized by an irregular distri-

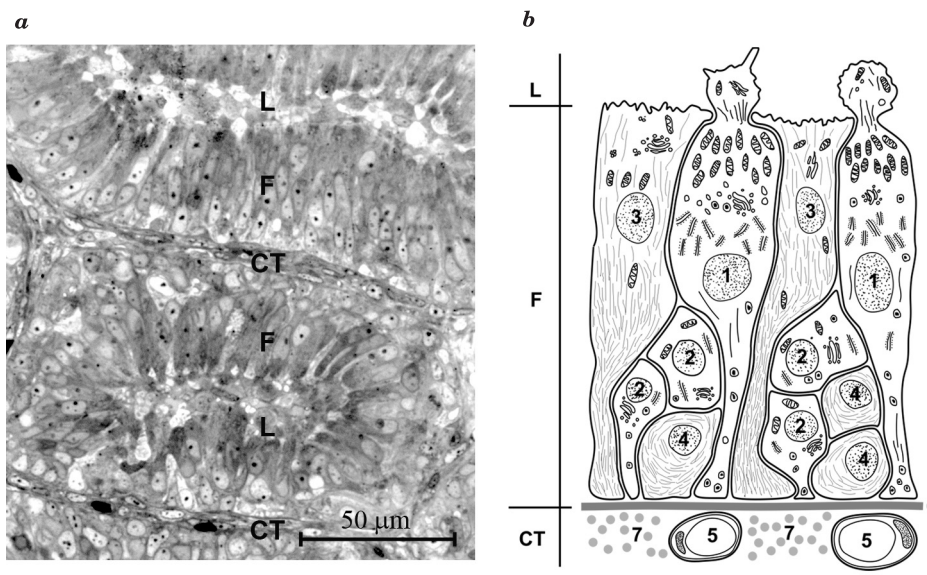


Fig. 1*a*. Fragment of the semithin section through the turkey pineal organ stained with toluidine blue showing the follicles (F) and the connective tissue stroma (CT). Note pseudo-stratified distribution of cells forming the follicular wall. L – follicular lumen; Fig. *b* – diagram illustrating the ultrastructure of the turkey pineal organ prepared on the basis of electron microscopic studies: L – follicular lumen, F – follicular wall, CT – connective tissue stroma, 1 – rudimentary-receptor pinealocytes, 2 – secretory pinealocytes, 3 – ependymal-like supporting cells, 4 – astrocyte-like supporting cell, 5 – capillary vessels, 6 – the basement membrane, 7 – collagen fibres

bution of organelles and possessed different numbers of cell processes (Figure 1*b*). Supporting cells contained numerous intermediate filament filling the basal part of ependymal-like cells and almost whole cytoplasm of astrocytes-like cells (Figure 1*b*).

Distribution of pyroantimonate precipitates in the pineal organs fixed during day-time

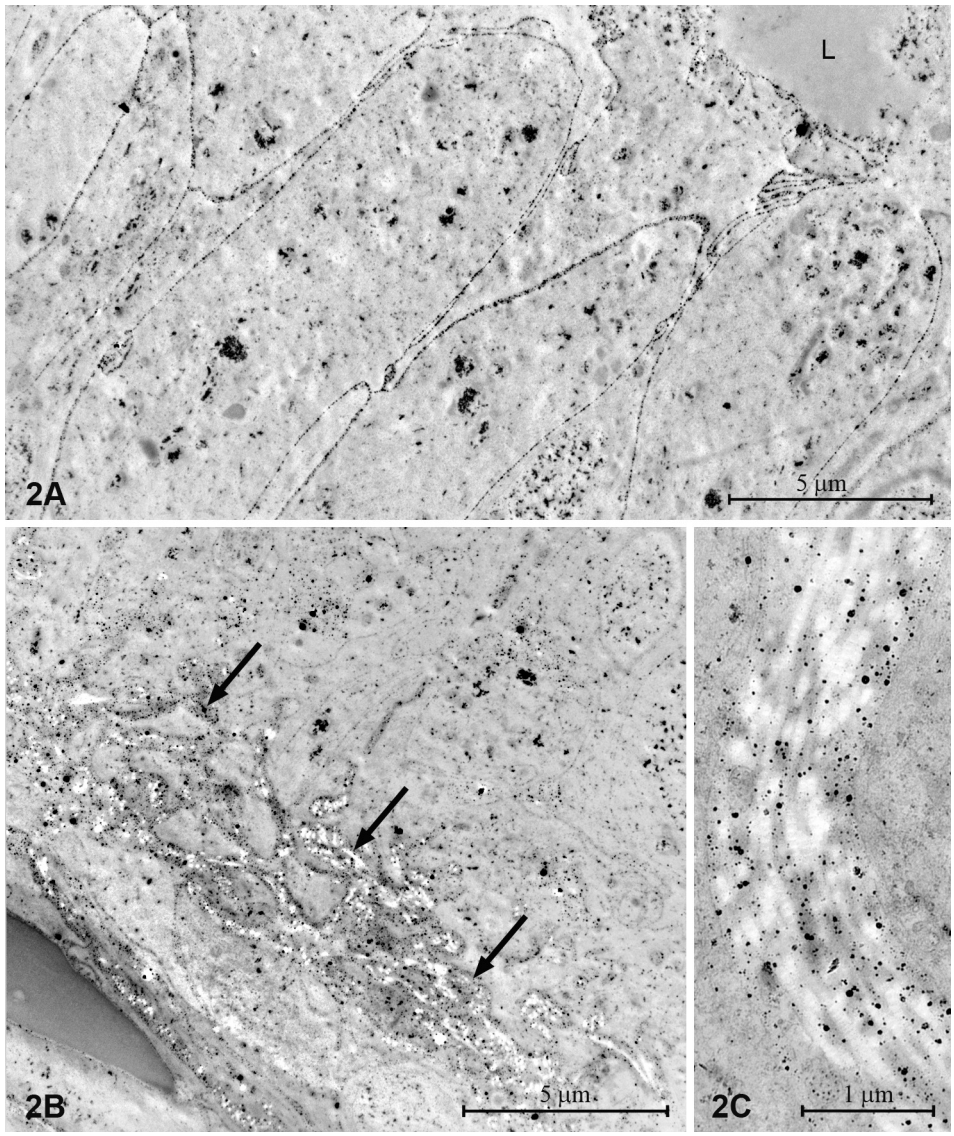
Extremely numerous, usually large, precipitates of calcium pyroantimonate were observed between collagen fibres of the connective tissue surrounding the pineal follicles (Figure 2*b, c*). The number of precipitates in the follicles was much lower than in the neighbouring stroma (Figure 2*a*). Precipitates were numerous in intercellular spaces between the cells forming the follicular wall (Figure 2*a*). In contrast, they were infrequently observed in the follicular lumen (Figure 2*a*, 3*b*). A clear difference in the amount of precipitates was observed between cells forming the wall of the follicle. Rudimentary-receptor pinealocytes (Figure 3*a–e*, 4*a–d*) and secretory pinealocytes (Figure 5) contained much more calcium precipitates than the supporting cells (Figure 6*a–e*).

In rudimentary-receptor pinealocytes, precipitates were present mainly in the nucleus (Figure 3*c*) and mitochondria (Figure 3*a,b,d*, 4*a*). Numerous precipitates were also found in short, wide cisterns located in the upper part of the cells (Figure 4*b*), whereas other components of the reticulum were sparse in precipitates (Figure 3*a*,*e*). Abundant precipitates were also observed in myelin-like structures surrounding the lipid droplets (Figure 4*c*). A few precipitates occurred in the endings of basal processes, where they were located close to a clear and granular vesicle as well as in the cytosol (Figure 4*d*). Precipitates were also sparse in apical prolongations extending into the follicular lumen, except for mitochondria, which – as usual – contained numerous precipitates (Figure 3*a*,*b*). There were no prominent differences in the amount of precipitates between individual pinealocytes of the rudimentary-receptor type.

In secretory pinealocytes, calcium pyroantimonate precipitates were accumulated mainly in the nucleus, mitochondria and some cisterns of smooth endoplasmic reticulum (Figure 5). Less numerous precipitates was found in cytosol. Amounts of precipitates did not differ markedly between individual secretory pinealocytes.

Calcium precipitates in ependymal-like cells were present mainly in the nucleus and in the upper part of cytoplasm, where they were localized mainly in mitochondria, cisterns of smooth endoplasmic reticulum and in the cytosol (Figure 6*a*). Precipitates were infrequently observed in the areas of cytoplasm filled with intermediate filaments (Figure 6*b*,*c*). Astrocyte-like supporting cells contained a small number of precipitates located mainly in the nucleus and mitochondria (Figure 6*d*,*e*).

a



b *c*

Fig. 2. Distribution of calcium pyroantimone precipitates in intercellular spaces between cells forming the follicular wall (Fig. *a*) and in the connective tissue surrounding the pineal follicle (Fig. *b, c*) in organs taken *ex vivo* during daytime. Intercellular spaces between the adjacent cells in the follicular wall are filled by precipitates (see Fig. *a*). Note the presence of numerous precipitates between collagen fibres (in Fig. *b* – arrows and Fig. *c*) and a few precipitates inside the follicular lumen (L in Fig. *a*).

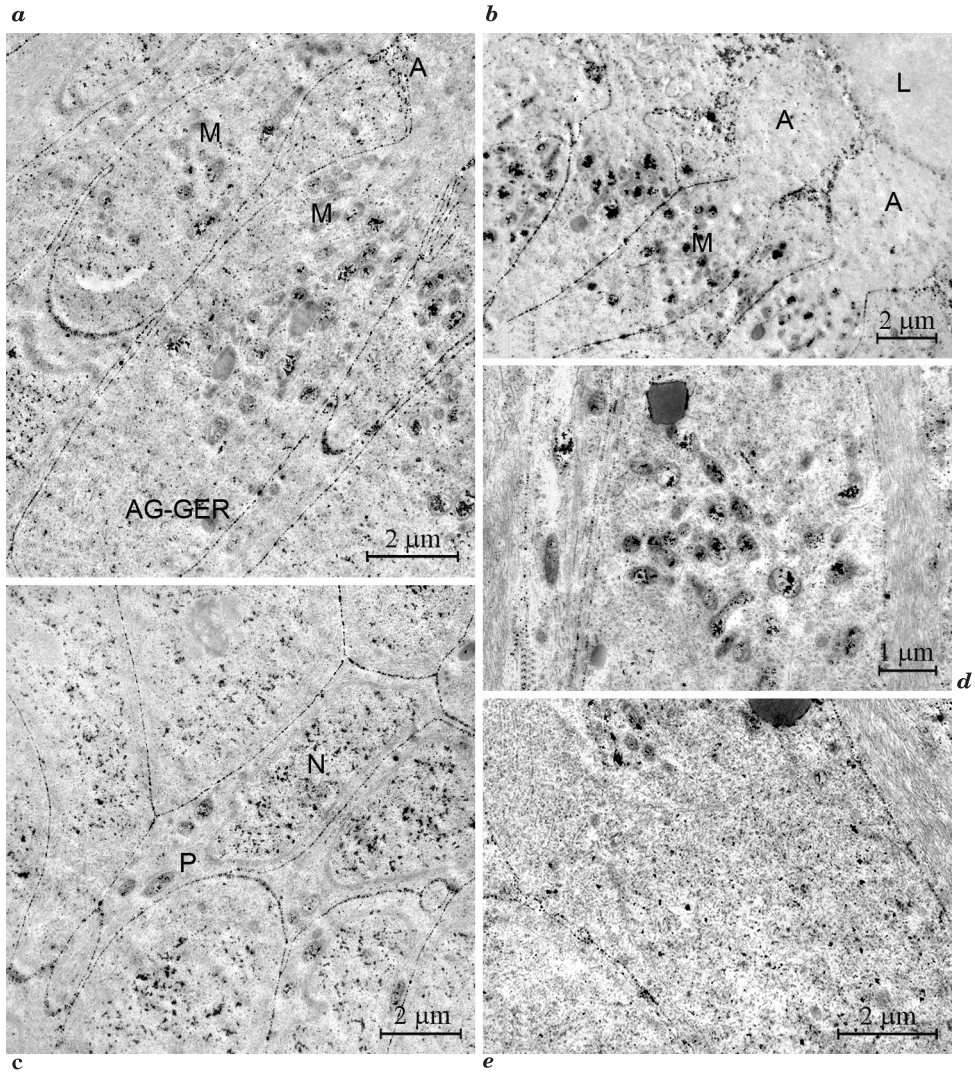


Fig. 3. Distribution of calcium pyroantimone precipitates in various parts of rudimentary-receptor pinealocytes in the pineal organs taken *ex vivo* during daytime: *a, b* – precipitates in the apical prolongations (A), the zones with mitochondria (M) and the zones containing the Golgi apparatus and granular endoplasmic reticulum (AG-GER), L – follicular lumen, *c* – precipitates in the cell nucleus (N) and the basal process (P), *d* – precipitates in the zone containing mitochondria, *e* – precipitates in the zones containing the Golgi apparatus and granular endoplasmic reticulum. Note the presence of numerous precipitates in mitochondria and cell nuclei as well as a low content of precipitates in the apical prolongations and the areas containing the granular endoplasmic reticulum

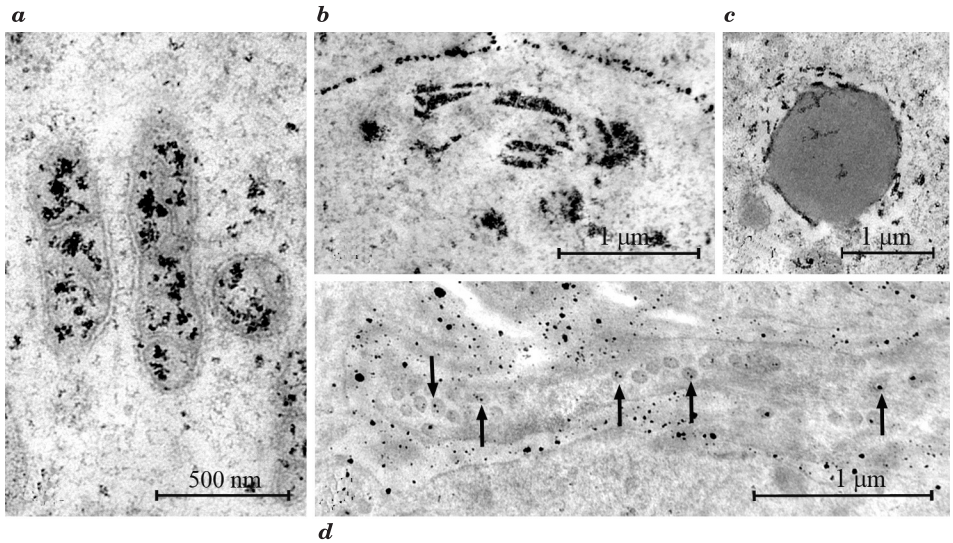


Fig. 4. Subcellular distribution of calcium pyroantimone precipitates in rudimentary-receptor pinealocytes in organs taken *ex vivo* during daytime: *a* – precipitates in the mitochondrial matrix, *b* – precipitates in wide cisterns of smooth endoplasmic reticulum, *c* – precipitates in myeloid-like structures surrounding lipid droplets, *d* – precipitates inside or close to clear vesicles (arrows)

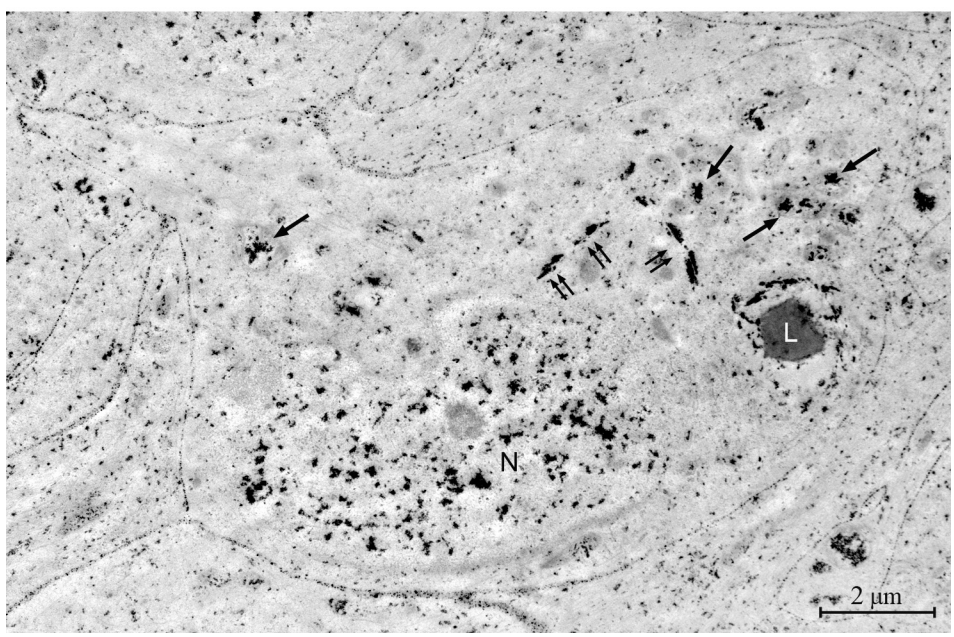


Fig. 5. Localization of calcium pyroantimone precipitates in secretory pinealocytes in organs taken *ex vivo* during daytime. Note the presence of numerous precipitates in the nucleus (N), mitochondria (arrows), wide cisterns of the smooth endoplasmic reticulum (double arrows) and around the lipid droplet (L)

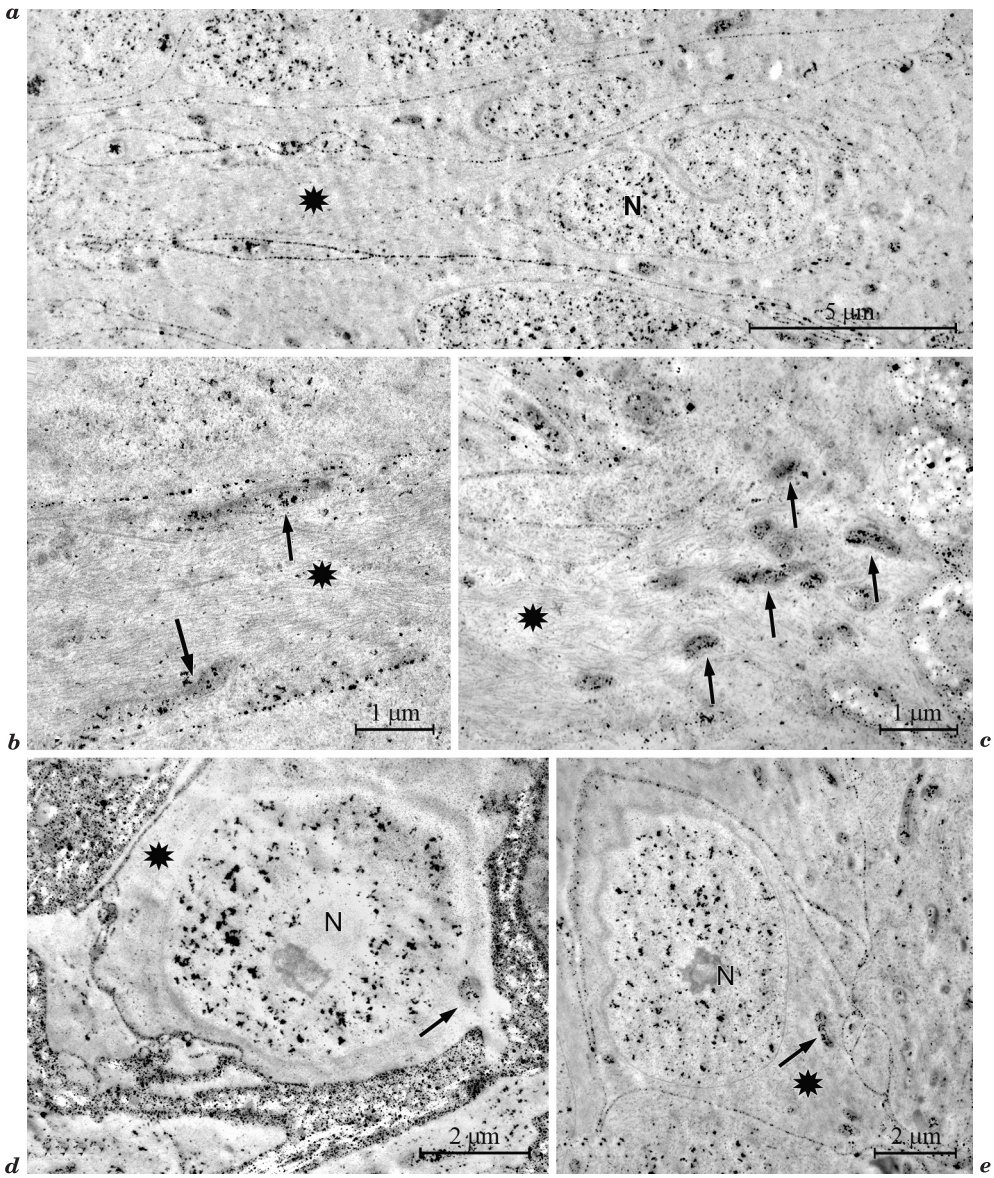


Fig. 6. Distribution of calcium pyroantimone precipitates in ependymal-like supporting cells (*a, b, c*) and astrocyte-like supporting cells (*d, e*) in organs taken *ex vivo* during daytime. Precipitates are present mainly in the nuclei (N) and in mitochondria (arrows). They are sparse in areas filled with filaments (asterisks)

Distribution of pyroantimonate precipitates in the pineal organs fixed during night-time

General patterns of calcium precipitate distribution in the pineals fixed during the scotophase were similar to those in the organs fixed during the photophase. The most important difference concerned the presence of much more numerous precipitates in the cytosol of rudimentary-receptor pinealocytes (Figure 7*a*) and secretory pinealocytes (Figure 7*b*) taken *ex vivo* during nighttime than during daytime.

Control

Pretreatment of tissues in a solution containing EGTA – divalent cations chelator – resulted in a complete lack of precipitates. Analogous incubation without the use of EGTA did not affect the presence of precipitates.

DISCUSSION

Calcium ions together with cAMP molecules play a crucial role in the regulation of melatonin secretion in avian pinealocytes, but the precise mechanisms of Ca^{2+} influence on the pineal hormone synthesis and the interactions between these two second messengers are poorly recognized (Zatz, Mullen 1988a, Zatz 1989, 1992, Alonso-Gomez, Iuvone 1995, Pablos et al. 1996, Agapito et al. 1998). Two types of calcium channels have been described in the plasma membrane of chicken pinealocytes: voltage-dependent calcium channels of L-type (Harrison, Zatz 1989) and long-open-time cationic channels I_{LOT} (D'-Souza, Dryer 1996). Activator of the L-type channels (Bay K 8644) increases the nocturnal peak of melatonin secretion from chicken pinealocytes, whereas antagonists (nitrendipine and nifedipine) decrease the nocturnal rise in the pineal hormone level (Zatz 1989, Harrison, Zatz 1989). None of these drugs influences the synchronization of the circadian rhythm of the pineal secretory activity with the light-dark cycle (Zatz, Mullen 1988b). Bay K 8644 and nitrendipine alter the cAMP level in avian pinealocytes (Zatz 1992a). D'-Souza and Dryer (1996) investigating chick pinealocytes with the patch-clump technique demonstrated the presence of cationic channels with an unusually long open time and for that reason they were termed as I_{LOT}. These channels are not gated by voltage or soluble second messengers. They are cyclically synthesized and open for calcium ions at night and degraded during the day (D'-Souza, Dryer 1996). The rhythm of I_{LOT} channels activity persists in chicken pinealocytes kept in continuous darkness, therefore these channels are considered as being dependent on the endogenous oscillator function. Inhibitors of protein synthesis abolish the circadian rhythm in I_{LOT} channels activity. Summing up, the onset of nocturnal darkness in chick pinealocytes is associated with two phenomena: 1) depo-

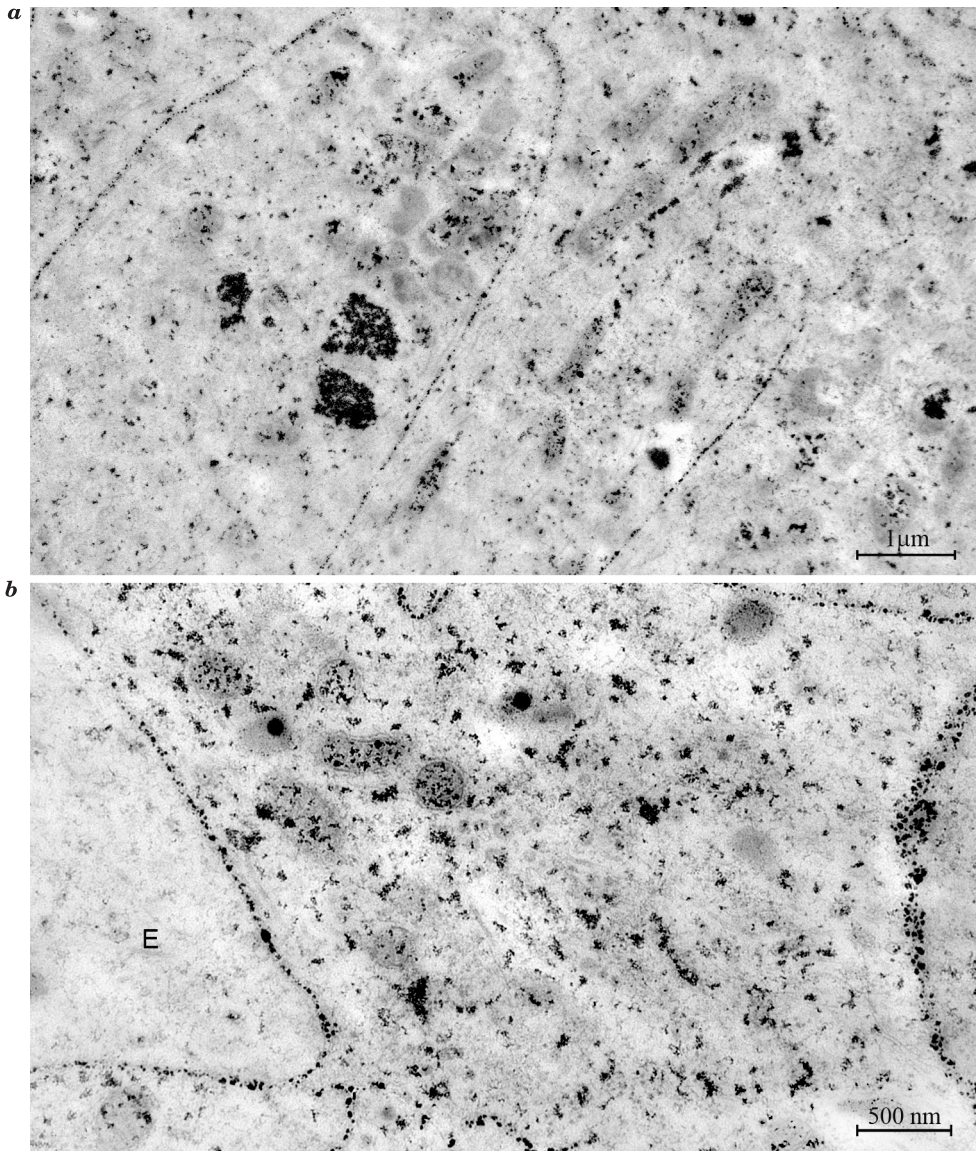


Fig. 7. Localization of calcium pyroantimone precipitates in rudimentary-receptor pinealocytes (*a*) and secretory pinealocytes (*b*) in the pineal organ taken *ex vivo* during night-time. Note the presence of numerous precipitates in cytosol of both types of pinealocytes. Only single precipitates are present in the cytoplasm of the ependymal-like supporting cell (E)

larization of plasma membrane and opening of voltage-dependent calcium channels, and 2) a dramatic increase in the number of I_{LOT} channels. These events are probably responsible for a large rise in intracellular free calcium in chicken pinealocytes at night. Elevation of Ca^{2+} influences the activity of calcium/calmodulin-stimulated adenylate cyclase and results in an increase (additional to the one caused by the biosynthesis of adenylate cyclase controlled by clock genes) in cAMP production in pinealocytes. Cyclic-AMP is the main factor controlling the level and activity of serotonin N-acetyltransferase (an enzyme limiting the melatonin secretion) in pinealocytes (NATESAN et al. 2002).

Substances affecting the calcium output from the intracellular stores, in contrast to membrane calcium-channels activators and antagonists, influenced the activity of the endogenous oscillator controlling the melatonin secretion in chick pinealocytes. Caffeine at high concentrations, increasing the calcium output from intracellular reserves, induced the phase shift in the rhythm of melatonin secretion similar to the effects evoked by light impulses (ZATZ, HEATH 1995). Cyclopiazonic acid, an inhibitor of calcium release from intracellular stores, synchronized the melatonin rhythm in the way resembling the effect of darkness (NATESAN et al. 2002). The cited data suggest that calcium ions released from the intracellular reserves participate in regulation of the chick pineal clock. It is worth noticing that spontaneous fluctuations of free calcium ions concentration were observed in about 10% of a population of chicken pinealocytes kept in monolayer culture (D'-SOUZA, DRYER 1994).

Until now, ultracytochemical localization of calcium ions has been investigated in the pineals of poikilothermic vertebrates (VIGH, VIGH-TEICHMANN 1989, VÍGH et al. 1998) and mammals (KRSTIČ 1985, THERON et al. 1989, PIZARRO et al. 1989a,b, TUTTER et al. 1991, LEWCZUK et al. 1994, 2007). As concerning the pineal organ in birds, the distribution of Ca^{2+} at the level of electron microscopy was described only in the areas of the formation of calcified concrements (VÍGH et al. 1998, PRZYBYLSKA-GORNOWICZ et al. 2009). Localization of calcium ions in pinealocytes and supporting cells of the avian pineal is reported for the first time in the present study.

Electron dense precipitates of calcium pyroantimonate were found in two components of the turkey pineal organ, separated from each other by the continuous basal membranes: the connective tissue stroma and the parenchyma formed by the pineal follicles. The amount of calcium ions differed markedly between these compartments. The connective tissue contained very numerous, frequently large calcium pyroantimonate precipitates and this observation well agrees with our previous studies concerning the formation of calcified concretions in the turkey pineal (PRZYBYLSKA-GORNOWICZ et al. 2009). In the follicles, precipitates were much less abundant comparing to the stroma. They were relatively numerous in the intercellular spaces separating the lateral domains of the adjoining cells, moderate in number

in pinealocytes and supporting cells, and sparse inside the follicular lumen. These observations implicate that diffusion of calcium ions from the stroma to the extracellular spaces of the follicular wall and later to the follicular lumen is partially limited or undergoes some control mechanisms. This suggestion seems to be very interesting, although it could not be excluded that the low number of precipitates inside the follicular lumen may have its source in restricted penetration of pyroantimonate ions through the follicular wall. Explanation of this problem requires subsequent studies.

Both rudimentary-receptor and secretory pinealocytes contained much more precipitates than supporting cells. According to the results, the highest concentrations of calcium ions in pinealocytes occur in the nucleus, mitochondria and some cisterns of the smooth endoplasmic reticulum. High levels of Ca^{2+} in nuclei and mitochondria were reported in numerous cell types including pinealocytes (KRSTIČ 1985, PIZARRO et al. 1989a,b, WELSH 1984, LEWCZUK et al. 1994, 2007). It is reasonable to suspect that short cisterns of endoplasmic reticulum, present in both types of pinealocytes and distinguished in our study by the occurrence of numerous precipitates, are the main intracellular calcium storage sites related to the regulatory functions of this second messenger. Generally, distribution of calcium ions in turkey pinealocytes is similar to the one described in mammalian pinealocytes, where calcium pyroantimonate precipitates were found in nuclei, mitochondria, vesicles and cisterns of the smooth endoplasmic reticulum (including subsurface cisterns), the Golgi apparatus and cytosol (KRSTIČ 1985, PIZARRO et al. 1989a,b, WELSH 1984, LEWCZUK et al. 1994, 2007).

The results show that both rudimentary-receptor and secretory pinealocytes form homogenous subpopulations concerning the amount and distribution of calcium ions. Prominent differences in the presence and localization of Ca^{2+} between pinealocytes were reported in mammals, based on the investigations performed with the use of potassium pyroantimonate method, e.g. PIZARRO et al. (1989a,b) distinguished two kinds of rat pinealocytes: type I corresponding to the classic light pinealocytes with almost complete absence of calcium pyroantimonate precipitates and type II with the dense cyplasmic matrix and very abundant coarse precipitates inside the cytoplasm and the nucleus. Two types of pinealocytes, differing by the amount of calcium ions, were distinguished also in the domestic pig (LEWCZUK et al. 2007). The first type included cells containing a small or moderate amount of precipitates. These cells were usually characterized by low electron density of their cytoplasm. Pinealocytes classified as the second type were characterized by a large or very large content of precipitates both in the cell nucleus and the cytoplasm. They were characterized by the electron dense cytoplasm and the presence of numerous dense bodies – the structures specific for swine pinealocytes. Differences in the amount of precipitates were also observed between distinct forms of gerbil pinealocytes (KRSTIČ 1985, WELSH 1984).

Our study demonstrated a low concentration of calcium ions inside and around the apical prolongations of rudimentary-receptor pinealocytes. These structures contain a photopigment, pinopsin, thus they are considered as being involved in the light reception and analogous to the outer segments of classical photoreceptor cells (PRUSIK, LEWCZUK 2008a). It should be noted that numerous precipitates of calcium pyroantimonate were found in the outer segments of photoreceptor cells in the frog pineal gland (VIGH, VIGH-TEICHMANN 1989). Similarly to the pineal, precipitates of calcium pyroantimonate were numerous in the rod outer segments in the frog retina. In the cone outer segments, they were much less abundant (VIGH, VIGH-TEICHMANN 1989). Calcium pyroantimonate precipitates in frogs adopted to darkness were present on both cytoplasmic and intradiscal sides of the rod photoreceptor membranes, whereas in light adopted animals precipitates were concentrated inside the discs (VIGH et al. 1998). Numerous precipitates of calcium pyroantimonate were also reported in the outer segments of retinal rod photoreceptor cells in the rat (VAN REEMPTS et al. 1984) and the rabbit (VIGH et al. 1998). The above data show that there are important differences in amounts of calcium ions between the apical prolongations of avian pinealocytes and the outer segments of photoreceptor cells occurring in the pineals of poikilothermic vertebrates and in the retinas. Our results suggest that despite many similarities in the photoreception mechanisms between avian pinealocytes and classical photoreceptor cells, these are also important distinctions. It is worth remembering that the apical prolongations of rudimentary-receptor pinealocytes and the outer segments of photoreceptor cells show completely different structural organization. In secretory pinealocytes, which are also photosensitive, pinopsin is present in the processes penetrating between neighbouring cells (PRUSIK, LEWCZUK 2008a). In the present study, we did not find any calcium-rich processes outgrowing from or located in the vicinity of secretory pinealocytes. Summing up, the light-sensitive structures of turkey pinealocytes are characterized by a low content of calcium ions.

The study on day-night differences in the distribution of Ca^{2+} in the turkey pineal demonstrated that precipitates of calcium pyroantimonate in the cytosol of both rudimentary-receptor and secretory pinealocytes were more numerous in the organs taken *ex vivo* at 2.00 than at 14.00. Although in our opinion the potassium pyroantimonate fixation is not a valid method for quantitative comparisons, the differences in the occurrence of precipitates in the cytosol could be considered as reflecting the diurnal rhythm in the level of free calcium ions in cytosol of pinealocytes. The present data are in agreement with the results of physiological studies on chicken pinealocytes suggesting that the influx of calcium ions via two types of calcium channels occurs during scotophase (see the first part of discussion). We were not able to observe any day-night variations in the amount of calcium precipitates in short, wide cisterns of smooth endoplasmic reticulum. Two explanations of the lack of these differences should be taken into consideration. One is that visualization of changes was impossible because the level

of Ca^{2+} in cisterns of the smooth endoplasmic reticulum was too high to notice the changes in the amount of precipitates related to the release of ions into the cytosol and their re-uptake. The second explanation is related to the discrepancy between the time when animals were sacrificed and occurrence of the nadir and peak in a putative diurnal rhythm of Ca^{2+} release from these stores. It should be underlined that in contrast to pinealocytes, no variations in the amount and distribution of precipitates were observed in ependymal-like and astrocyte-like supporting cells.

Until now, day-night differences in the localization of calcium ions have been reported in the pineal glands of mammals. Using the potassium pyroantimonate technique, THERON et al. (1989) described a prominent shift in Ca^{2+} distribution in baboon pinealocytes. During the dark phase, the location of calcium ions was predominantly intravesicular and intravacuolar, whereas during the photophase calcium ions were present mainly in cytosol. Contrary data were obtained in the gerbil pineal gland (TUTTER et al. 1991). The accumulation of precipitates occurred in all compartments of the endoplasmic reticulum including subsurface cisterns in pinealocytes of gerbils sacrificed during daytime but they were not present anywhere in the endoplasmic reticulum when the animals were euthanized at night. Analyzing the data obtained in mammals and birds, it is necessary to remember that the mechanisms regulating the level of calcium ions in pinealocytes of both groups of vertebrates are completely different.

CONCLUSIONS

1. The potassium pyroantimonate method enables visualization of the Ca^{2+} distribution at the level of electron microscopy in two compartments of the turkey pineal organ: the parenchyma formed by the follicles and the connective tissue stroma.

2. Concentration of calcium ions in the pineal follicles is markedly lower than in the surrounding extracellular matrix of the connective tissue. The follicular lumen is characterized by a low level of Ca^{2+}.

3. Rudimentary-receptor and secretory pinealocytes contain more calcium ions than the ependymal-like and astrocyte-like supporting cells.

4. The highest concentrations of free Ca^{2+} in both rudimentary-receptor and secretory pinealocytes are present in the nuclei, mitochondria and some cisterns of the smooth endoplasmic reticulum. Calcium ions are not accumulated in the apical prolongations of rudimentary-like pinealocytes and in the processes of secretory pinealocytes.

5. There are no differences in the amounts and distribution of Ca^{2+} between individual cells belonging to subpopulations of rudimentary-like pinealocytes and secretory pinealocytes.

6. Concentration of calcium ions in the cytosol of rudimentary-like pinealocytes and secretory pinealocytes is higher during the scotophase than during the photophase.

7. No day-night differences in the concentration of calcium ions in the cytosol occur in astrocyte-like and ependymal-like supporting cells.

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