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Colocalization of pinopsin with two types of G-protein α-subunits in the chicken pineal gland

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Abstract Pinopsin is a photoreceptive molecule present in the outer segments of chicken pinealocytes. In this paper, the localization of α-subunits of G-proteins, rod transducin (Gt1) and Gq/11, was examined by immunoelectron microscopy to investigate whether these G-proteins colocalize with pinopsin in the outer segments. Ultrathin sections of the chicken pineal gland were double-immunolabeled with antibodies to pinopsin and either Gt1α or Gq/11α. As shown previously, the outer segments around the follicular lumen exhibited divergent morphology with ciliary, bulbous, or lamellate shapes, and most of them displayed pinopsin immunoreactivity. The majority (>90%) of pinopsin-immunopositive outer segments were labeled by anti-Gt1α and/or anti-Gq/11α antibodies. Application of double-immunolabeling to serial sections demonstrated that a large number of the pinopsin-immunopositive outer segments contained both Gt1α and Gq/11α immunoreactivities. These results suggest that Gt1α and Gq/11α are functionally coupled with light-activated pinopsin within a single outer segment.

Key words Rod transducin · Gq/11 · Phototransduction · Circadian clock · Pinealocyte · Immunoelectron microscopy · Chicken

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Introduction

The chicken pineal gland exhibits rhythmicity in melatonin production, which is controlled by the endogenous circadian clock. This circadian rhythmicity is maintained even when pineal cells are dissociated (Deguchi 1979a; Zatz and Mullen 1988; Takahashi et al. 1989) or when single cells are cultured individually (Nakahara et al. 1997), indicating the presence of both the oscillator and melatonin production (output) systems within an individual pinealocyte. An important aspect of the circadian clock system is the photoentrainment mechanism, by which the phase of melatonin production of the chicken pineal cells is shifted (Deguchi 1981). This light-input pathway also coexists with the oscillator and melatonin output systems in the pinealocyte (Nakahara et al. 1997). Light exhibits another effect on melatonin production, i.e., it acutely suppresses melatonin production by inhibiting the activity of *N*-acetyltransferase, which is the key enzyme in melatonin biosynthesis (Deguchi 1979b). These effects of light are triggered by the light absorption of endogenous photosensitive molecules such as pinopsin, a pineal-specific opsin in the chicken pineal gland (Okano et al. 1994).

Pinopsin is a member of the GTP-binding protein (G-protein)-coupled receptor family, and the light signal captured by pinopsin is most probably transduced by heterotrimeric G-protein. Recently, cDNAs for several G-protein α-subunits, viz., Gi2, Gi3, Go1, Go2, Gt1 (rod transducin), and G11, have been isolated from chicken pineal cDNA libraries (Okano et al. 1997b; Kasahara 1998). Among these, we have paid special attention to the α-subunits of Gt1 (Gt1α) and G11 (G11α), because both are localized in retinal rod outer segments (Peng et al. 1997) where the primary events of light-signal transduction take place. Regarding transducin, immunoreactivity of its α-subunit has been detected in the quail pineal gland at the level of the light microscope (van Veen et al. 1986; Foster et al. 1987). In the present study, ultrathin sections of the chicken pineal gland have been subjected to double-immunolabeling with antibodies against

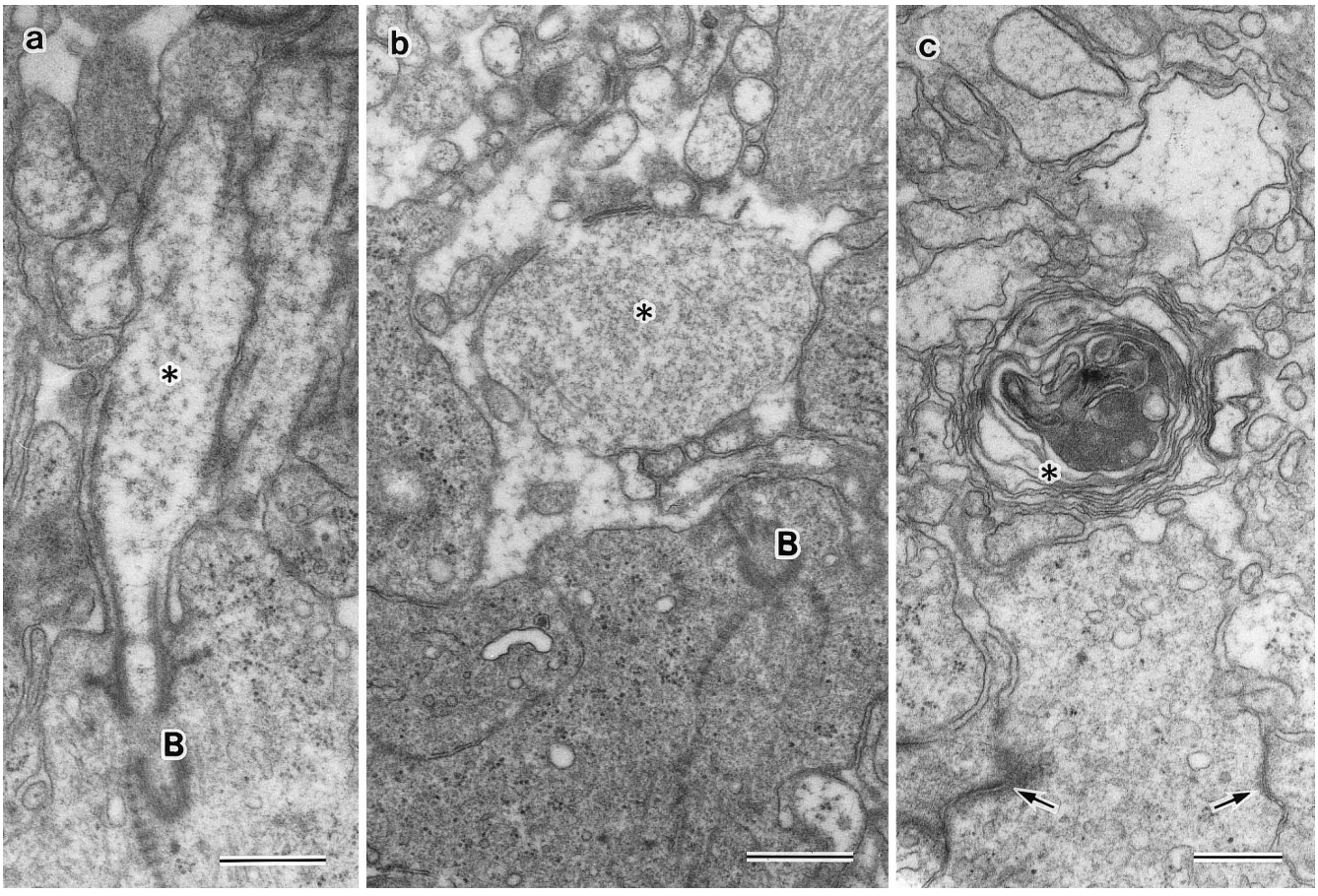

Fig. 1a–c Ultrastructure of the outer segments of follicular pinealocytes. **a** Ciliary-shaped outer segment. **b** Bulbous-shaped outer segment. **c** Concentric lamellate-shaped outer segment (*asterisks* outer segments, *arrows* cell junctions between pinealocytes, *B* basal body). *Bars* 0.5 µm

chicken pinopsin and either Gt1α or Gq/11α to determine whether these G-proteins are located in close proximity to pinopsin for functional coupling.

Materials and methods

Animals

Hatched chicks were kept under a light : dark (12 h:12 h) regime (light period: 5:00–17:00) prior to experiments. Light intensity was approximately 2000 lux at the level of the chicks' heads. The pineal glands were isolated from 7- to 14-day-old male chicks at 11:00 (light) and 23:00 (dark). Principles of laboratory animal care and specific national laws were followed.

Conventional electron microscopy

The pineal glands were cut into pieces and prefixed in 4% paraformaldehyde and 5% glutaraldehyde in 0.1 M sodium cacodylate buffer (CB) at pH 7.4 overnight at 4°C. The tissues were then postfixed with 2% OsO_4 in CB for 2 h at room temperature (25–27°C), dehydrated in a graded series of acetone (50%, 70%, 80%, 90%, 95%, and 100%), infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections were cut with a diamond knife and doubly stained with uranyl acetate and lead citrate for examination with a JEM 1200EX electron microscope.

Immunoelectron microscopy

The pineal glands were cut into pieces and fixed with 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB) at pH 7.4 for 2 h on ice and then with 4% paraformaldehyde and 0.2% picric acid in PB at 4°C overnight (Terakita et al. 1996). The tissues were rinsed in PB, dehydrated partially in a graded series of ethanol (50%, 70%, and 90%), and then embedded in LR White resin (London Resin, Berkshire, UK), which was polymerized at 4°C for 2 days in the presence of polymerization accelerator (Suzuki and Hirosawa 1994). Ultrathin sections (about 60 nm thick) were collected on nickel grids (T-300, Electron Microscopy Sciences, Fort Washington, Pa., USA).

The sections were first treated with aqueous saturated sodium metaperiodate at room temperature for 30 min. After being rinsed with distilled water, the sections were incubated with 5% normal goat serum (NGS) in PBS (10 mM Na-phosphate buffer, pH 7.4, 140 mM NaCl, 1 mM $MgCl_2$, and 0.9 mM $CaCl_2$) at room temperature for 90 min. The sections were then incubated with a mixture of two primary antibodies diluted with 2% NGS-containing PBS for 2 days at 4°C. The primary antibodies used were: mouse polyclonal anti-pinopsin (P9) specific to C-terminal tail region (Ile_{297}–Val_{351}) of the chicken pinopsin (1:2000 of the original antiserum; Okano et al. 1997a), rabbit polyclonal anti-bovine Gt1α (Ser_{153}–Tyr_{166}, anti-Gt1α, 1:1250; Kokame et al. 1993), and rabbit polyclonal anti-mouse Gq/11α (Phe_{341}–Val_{359}, 1:800, Santa Cruz, Calif., USA). The combinations of the primary antibodies were: (1) P9 plus anti-Gt1α and (2) P9 plus anti-Gq/11α. The binding of the primary antibody was detected by incubating the sections with a mixture of goat anti-mouse IgG conjugated to 5-nm colloidal gold (GAM IgG5 gold, 1:40, British Biocell International, Cardiff,

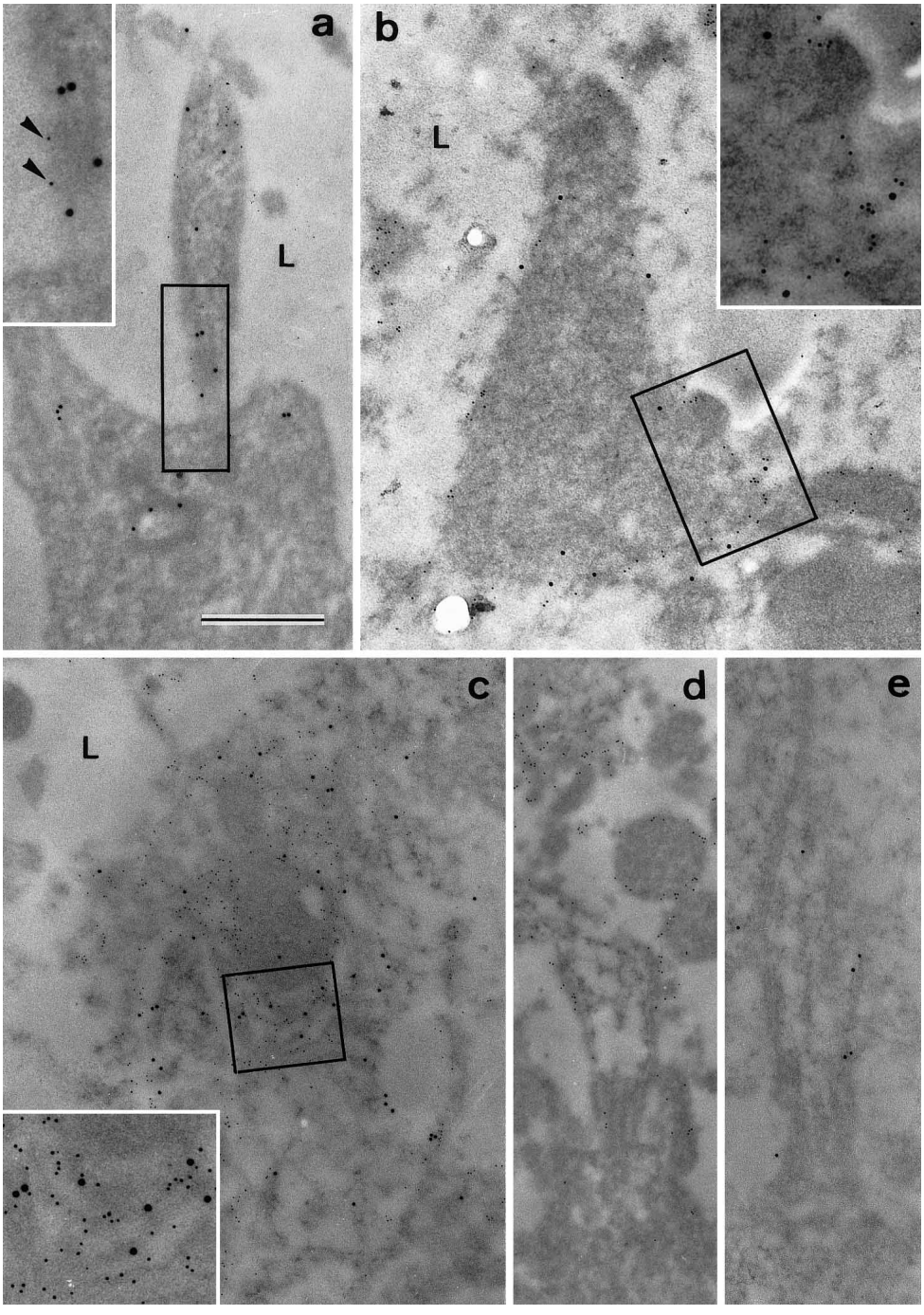

Fig. 2a–e Colocalization of immunoreactivities for pinopsin and Gt1α in the three types of the outer segments. **a–c** Three types of outer segments that are labeled by both anti-pinopsin (5-nm gold particles, *arrowheads*) and anti-Gt1α (15-nm gold particles) antibodies. *Boxed areas* are magnified in *insets*. **a** Ciliary-shaped outer segment at 11:00 (light). **b** Bulbous-shaped outer segment at 23:00 (dark). **c** Lamellate-shaped outer segment at 23:00 (dark).**d, e** Control labeling of ciliary-shaped outer segments. The sections were first incubated either with a mixture of anti-pinopsin plus normal rabbit serum (**d**) or anti-Gt1α plus normal mouse serum (**e**) and were subsequently incubated with a mixture of GAM IgG5 gold and GAR IgG15 gold (*L* follicular lumen). *Bar* 0.5 μm

UK) and goat anti-rabbit IgG conjugated to 15-nm colloidal gold (GAR IgG15 gold, 1:50, British Biocell) in PBS containing 0.02% fish gelatin (Sigma, St. Louis, Mo., USA) for 1 h at room temperature. The sections were rinsed several times with PBS containing 0.02% fish gelatin and subsequently with distilled water, stained with uranyl acetate, and examined with a JEM 1200EX.

In a control double-labeling experiment to confirm the specificity of each antibody, one of the primary antibodies was replaced by normal serum diluted in the same ratio as the primary antibody. The combinations were: (1) anti-pinopsin plus rabbit normal serum (1:1250 for anti-Gt1α and 1:800 for anti-Gq/11α), and (2) normal mouse serum (1: 2000) plus either anti-Gt1α or anti-Gq/11α. These control sections were similarly incubated with the mixture of GAM IgG5 gold and GAR IgG15 gold.

Results

In the chicken pineal gland, we identified three types of outer segments with ciliary, bulbous, and concentric lamellate structures around the follicular lumen (Fig. 1). Most of these outer segments showed anti-pinopsin immunoreactivity (Figs. 2, 3). The anti-pinopsin labeling was detected on the periphery of the ciliary- and bulbous-shaped structures (smaller gold particles in Figs. 2a, b, 3a, b) and on the lamellate membranes (Figs. 2c, 3c). These observations are consistent with our previous

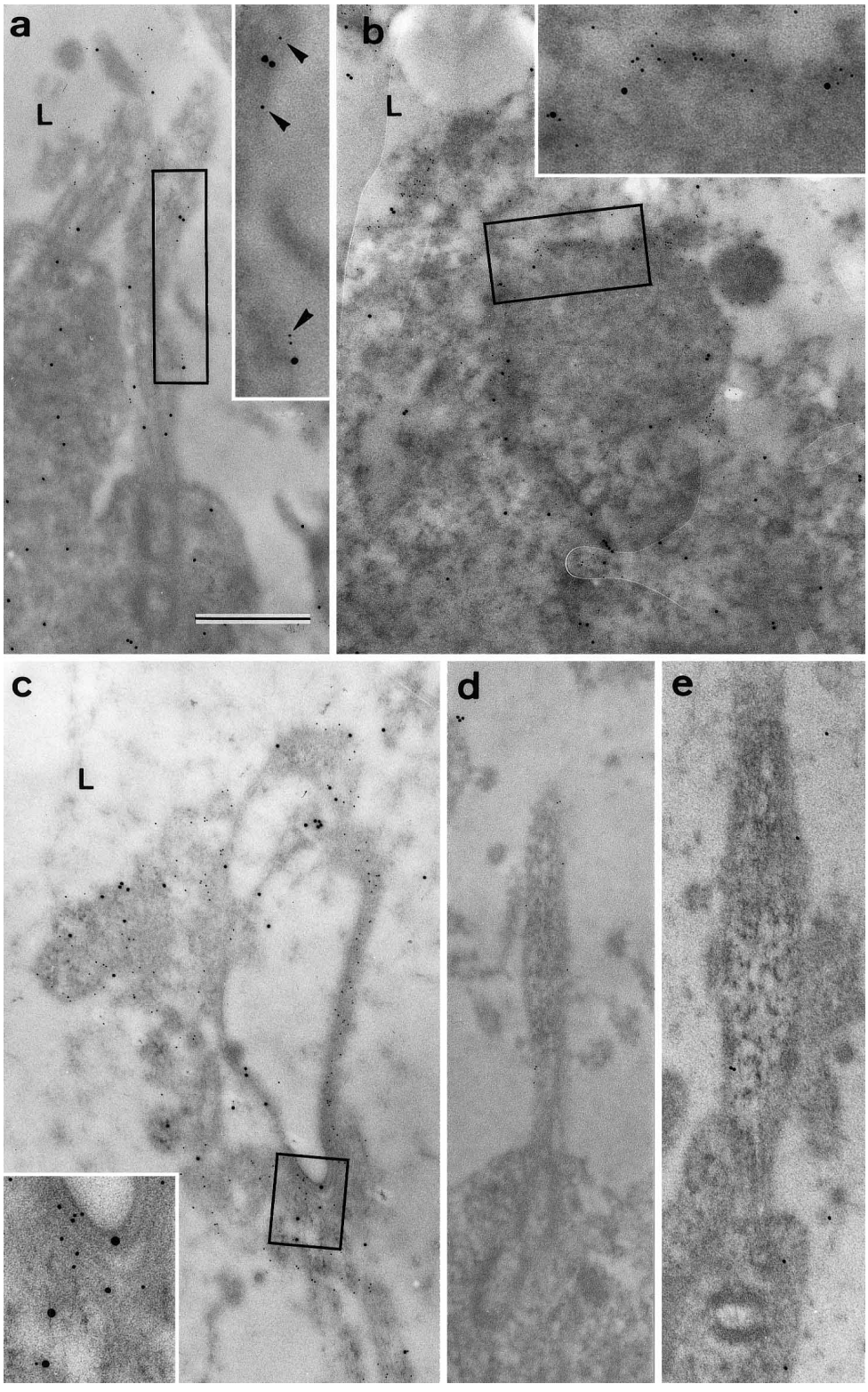

Fig. 3a–e Colocalization of immunoreactivities for pinopsin and Gq/11α in the three types of the outer segments. **a–c** Three types of outer segments that are labeled by anti-pinopsin (5-nm gold particles, *arrowheads*) and anti-Gq/11α (15-nm gold particles) antibodies. *Boxed areas* are magnified in *insets*. **a** Ciliary-shaped outer segment at 11:00 (light). **b** Bulbous-shaped outer segment at 23:00 (dark). **c** Lamellate-shaped outer segment at 11:00 (light). **d, e** Control labeling of ciliary-shaped outer segments. The sections were first incubated either with a mixture of anti-pinopsin plus normal rabbit serum (**d**) or anti-Gq/11α plus normal mouse serum (**e**) and were subsequently incubated with a mixture of GAM IgG5 gold and GAR IgG15 gold (*L* follicular lumen). *Bar* 0.5 μm

results obtained by pre-embedding immunocytochemical analyses (Hirunagi et al. 1997; Okano et al. 1997a).

Anti-Gt1α antibody labeled the majority of the pinopsin-immunopositive outer segments (larger particles in Fig. 2). Figure 2a–c shows typical examples of the outer segments with ciliary, bulbous, and lamellate structures that were immunolabeled simultaneously by anti-pinopsin and anti-Gt1α antibodies. Similarly, anti-Gq/11α antibody labeled a number of the pinopsin-immunopositive outer segments displaying the three different structural patterns (Fig. 3a–c). In addition to the outer segments, the inner segments of pinealocytes were labeled by anti-

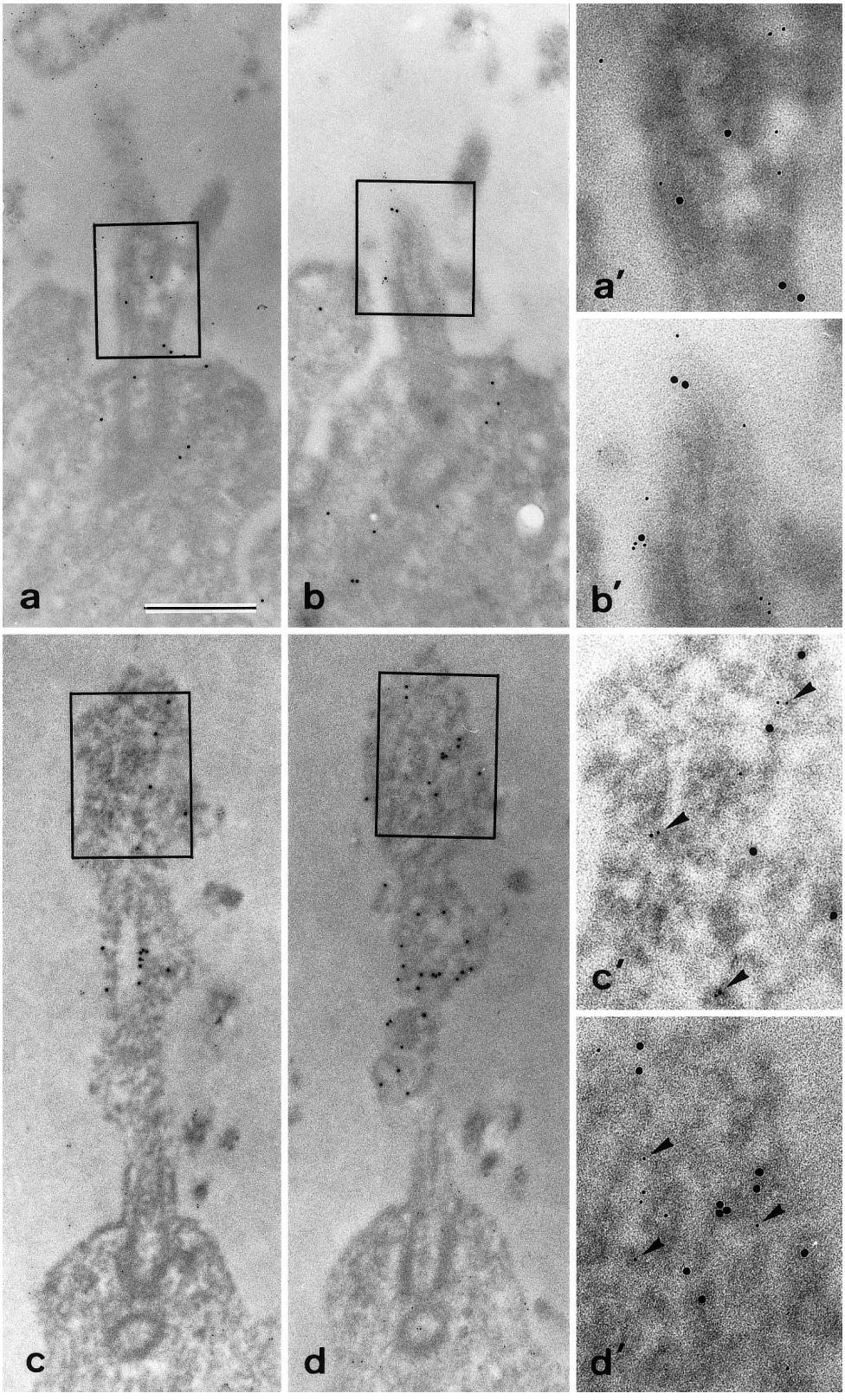

Fig. 4a–d Pinopsin-immunopositive outer segments labeled by both anti-Gt1α and anti-Gq/11α antibodies. **a, b** A single outer segment was serially sectioned and double-immunolabeled with anti-Gt1α (15-nm gold particles) plus anti-pinopsin (5-nm gold particles) in **a** or anti-Gq/11α (15-nm gold particles) plus anti-pinopsin (5-nm gold particles) in **b**. The sample was collected at 11:00 (light). *Boxed areas* in **a** and **b** are magnified in **a'** and **b'**, respectively. **c, d** A single outer segment was serially sectioned and double-immunolabeled with anti-Gq/11α (5-nm gold particles, *arrowheads*) plus anti-pinopsin (15-nm gold particles) in **c** or anti-Gq/11α (5-nm gold particles, *arrowheads*) plus anti-pinopsin (15-nm gold particles) in **d**. The sample was collected at 11:00 (light). *Boxed areas* in **c** and **d** are magnified in **c'** and **d'**, respectively. *Bar* 0.5 μm

Table 1 Characterization of immunoreactivities for Gt1α and Gq/11α in pinopsin-positive outer segments (– not labeled, + weakly labeled, ++ intensely labeled). Examples of 20 outer segments (*OS*) each from a chick pineal gland isolated at 11:00 (*Light*) or 23:00 (*Dark*)

Light			Dark		
#OS	Gt1α	G11α	#OS	Gt1α	G11α
1	++	++	21	++	++
2	++	++	22	++	++
3	++	–	23	+	++
4	+	+	24	–	++
5	++	–	25	++	++
6	++	++	26	++	++
7	–	++	27	–	++
8	+	++	28	++	+
9	++	++	29	++	+
10	–	–	30	++	+
11	++	++	31	++	–
12	–	++	32	++	++
13	+	–	33	–	++
14	–	+	34	++	++
15	+	+	35	++	+
16	–	+	36	++	+
17	++	++	37	++	–
18	–	++	38	++	++
19	++	–	39	–	++
20	–	–	40	–	++

Gt1α and Gq/11α antibodies (e.g., Figs. 2a, 3a); they may indicate transportation of newly synthesized proteins. Little labeling was detected in the proximal portion of the cell body in which the nucleus was located (data not shown). When one of the primary antibodies was replaced by normal serum in control experiments, gold particles of a particular size were detected, excluding a cross-reaction of the two secondary antibodies used in our immunocytochemical analyses (Figs. 2d, e, 3d, e).

Next, we localized immunoreactivities Gt1α and Gq/11α on serial sections to investigate whether the two α-subunits reside in a single pinopsin-positive outer segment or in different elements. We found that numerous ciliary-shaped pinopsin-positive outer segments were labeled by both Gt1α and Gq/11α antibodies (Fig. 4a, b). This was confirmed by exchanging particle sizes conjugated to the secondary antibodies (15-nm gold particles for detection of pinopsin, 5-nm particles for Gα; Fig. 4c, d). In spite of several trials, we were unable to detect serially sectioned bulbous or lamellate structures because of their low number.

To estimate the relative population of the pinopsin-positive outer segments with Gt1α and/or Gq/11α immunoreactivities, we evaluated labeling for both anti-Gt1α and anti-Gq/11α in serial sections as follows. We first searched for pinopsin-immunopositive outer segments (labeled by 5-nm gold particles) in every section. For each pinopsin-positive outer segment, we then determined whether it contained labeling for anti-Gt1α and/or anti-Gq/11α, based on the distribution pattern and the number of the 15-nm gold particles. When a significant number of the larger particles was detected in close proximity to the anti-pinopsin signal (smaller particles) within a single outer segment (e.g., Fig. 4), we termed the outer segment as being intensely labeled (++). When smaller numbers of particles were detected in the outer segment, this was termed as being weakly labeled (+). An outer segment with no particles was denoted as being not labeled (–). The percentage of the outer segments that were intensely labeled by both anti-Gt1α and anti-Gq/11α antibodies was 23.7±2.5% (mean ± SE) from four individuals (83 outer segments). Table 1 shows two examples of the evaluation results. There seemed to be no significant difference in the labeling pattern between the pineal sections prepared at 11:00 (in the light) and 23:00 (in the dark).

Discussion

In the present study, we have observed pinealocytes whose outer segments are immunoreactive to antibodies against pinopsin, Gt1α, and Gq/11α. About a quarter (23.7±2.5%) of the pinopsin-immunopositive outer segments are intensely labeled by both anti-Gt1α and anti-Gq/11α. In addition to this, 29.6±5.5% of the pinopsin-positive outer segments are labeled weakly by Gt1α/Gq/11α antibodies (combinations of ++/+, +/++, and +/+). Therefore, more than a half of the pinopsin-positive outer segments seem to contain both Gt1α and Gq/11α immunoreactivities. We consider this value as the lower limit of the population, because of the difficulties of preserving the antigenicities of Gt1α and Gq/11α in plastic-embedded sections. When we employed a milder fixative, we observed more intense labeling by Gα antibodies, but with more distinct degenerative changes in membrane morphology. Thus, it is reasonable to speculate that the majority of the pinopsin-positive pinealocytes contain both Gt1α and Gq/11α.

In chicken pinealocytes, the dual photic input pathways affecting melatonin production can be distinguished by their sensitivity to pertussis toxin (PTX). Thus, PTX inhibits the light-induced acute suppression of melatonin production but does not inhibit the light-dependent phase-shift of the melatonin rhythmicity (Zatz and Mullen 1988; Takahashi et al. 1989; Okano and Fukada 1997). Gt1α is probably PTX-sensitive and responsible for light signaling in the acute suppression pathway, because the present study suggests functional coupling of pinopsin and Gt1α, which are located in close proximity to each other in the outer segment of the pinealocytes. This is supported by in vitro experiments showing that recombinant pinopsin activates bovine retinal transducin in a light-dependent manner (Max et al. 1998; Nakamura et al. 1999).

On the other hand, the phase-shifting effect of light on rhythmic melatonin production is not blocked by PTX and suggests the contribution of a PTX-insensitive G-protein α-subunit (Zatz and Mullen 1988; Takahashi et al. 1989). The presence of G11α and phospholipase C-β4 immunoreactivities in bovine rod outer segments (Peng et al. 1997) has led us to consider the possible in-

volvement of a phosphoinositide pathway mediated by Gq family G-protein in the phase-shifting pathway. The present results showing colocalization of pinopsin and Gq/11α immunoreactivities are consistent with this hypothesis, although a functional coupling of pinopsin and Gq/11α remains to be elucidated. In Western blotting analyses, the anti-Gq/11α recognizes a single band of a 42-kDa protein in the chicken pineal homogenate, and the mobility of this protein is slightly higher than that of rat Gqα and is consistent with that of bovine G11α (Berstein et al. 1992). Together with the cDNA cloning of chicken pineal G11α (Kasahara 1998; unpublished), we expect that the Gq/11α immunoreactivity detected in this study reflects the presence of G11α. Another candidate for the G-protein mediating the phase-shifting pathway could be Gz (Max et al. 1998). Unfortunately, a commercially available anti-Gzα antibody (Santa Cruz) cross-reacted with several protein bands in Western blotting analysis of the chicken pineal homogenate, and therefore we were unable to evaluate Gzα localization by immunoelectron microscopy.

Iodopsin is another photoreceptive molecule identified in the chicken pineal gland (Okano et al. 1994). In our previous immunohistochemical investigations conducted at the level of the light microscope, iodopsin immunoreactivity was detected in membrane structures around the follicular lumen in fewer cases than pinopsin immunoreactivity (Okano et al. 1997a). In the present study, we have confirmed the existence of a minor population of iodopsin-positive ciliary-shaped outer segments of the pinealocytes (data not shown). Because of their low numbers, our effort was unsuccessful in identifying G-protein α-subunits that colocalize with iodopsin.

In conclusion, a significant number of pinopsin-positive outer segments of the chicken pinealocytes contain Gt1α and Gq/11α immunoreactivities, and it is suggested that light-activated pinopsin is coupled with Gt1 and Gq/11, which trigger dual (PTX-sensitive and insensitive) signal transduction pathways within a single cell. A detailed characterization of the G11-mediated signal transduction pathway may help us to understand the mechanism underlying the light-dependent phase-shift of the circadian oscillator in chicken pinealocytes.

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