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Kakigi, Akinobu
Okada, Teruhiko
Takeda, Taizo
Uehara, Natsumi
Nibu, Ken-ichi

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Endocytosis of CF in marginal cells of stria vascularis regulated by ROCK and MLCK signaling cascade, but not G-proteins

Running title: Regulation of CF endocytosis in marginal cells by ROCK and MLCK signaling cascade

Akinobu Kakigi, MD, PhD[1)], Teruhiko Okada, PhD[2)], Taizo Takeda, MD, PhD[3)], Natsumi UeharaMD, PhD[1)], Ken-ichi Nibu, MD, PhD[1)]

1) Department of Otolaryngology-Head & Neck Surgery, Kobe University, Graduate School of Medicine, Hyogo, Japan
2) Department of Anatomy, Kochi Medical School, Nankoku, Kochi, Japan
3) Department of Otolaryngology, Kochi Medical School, Nankoku, Kochi, Japan

Address correspondence to: Akinobu Kakigi, M.D., PhD.
Department of Otolaryngology-Head & Neck Surgery, Kobe University, Graduate School of Medicine
7-5-1 Kusunokicho Chuo-ku, Kobe, Hyogo 650-0017 Japan

Tel; +81-78-382-6024

e-mail: kakigia@gmail.com

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Abstract

Objective: The endocytosis of cationized feritin (CF) via a clathrin-mediated pathway is regulated by a signaling network. Marginal cells showed the active endocytosis of CF via a clathrin-mediated pathway. The internalization of receptors through this clathrin-mediated pathway is an important regulatory event in signal transduction. Numerous kinases are involved in endocytosis, and each endocytic route is subjected to high-order regulation by cellular signaling mechanisms. In this study, we investigated whether ROCK and MLCK signaling cascades and G-proteins regulate the endocytosis of CF in marginal cells of the stria vascularis.

Methods: CF was infused into the cochlear duct with pertussis toxin (PTX), *Clostridium botulinum* C3 toxin (BTX), guanosine(g-thio)-triphosphate (GTP-γS), ML-7, Y-27632. Endocytic activity was measured at 30 minutes after the start of infusion under an electron microscope.

Results: In marginal cells, CF was internalized via a clathrin-mediated pathway that depends on F-actin and microtubules (MT). Its processes were controlled by myosin light chain kinase (MLCK) and Rho-associated kinase (ROCK), but not affected by G-protein-coupled receptor (GPCR) or the RhoA signaling cascade.

Conclusion: Our previous study showed that the main endocytotic pathway of microperoxidase (MPO) did not depend on the Rho/ROCK molecular switch or actin/myosin motor system, but was mainly regulated by the RhoA signaling cascade. The present study results indicate that these signaling cascades regulating CF internalization completely differ from the cascades for MPO internalization.

Key words: endocytosis, stria vascularis, marginal cell, CF, signaling network, MLCK, ROCK

Introduction

The stria vascularis (SV) plays a pivotal role in cochlear fluid homeostasis, ion transport, the production of endocochlear potential (EP), and maintaining the function of the organ of Corti [1, 2]. Marginal cells of SV take up Na+ from endolymph and secrete K+ [3-5]. It was observed in electron microscopic studies that marginal cells have many apical vesicles (100-200 nm) in the apical cytoplasm. In 1968, Christoph von Ilberg was the first to demonstrate the ultrastructural pinocytotic and endocytotic uptake of thorotrast into and through cells of the cochlear walls of the perilymphatic and endolymphatic fluid spaces following intracochlear thorotrast perfusion and/or injection [6]. Marginal cells showed active clathrin-mediated endocytosis and clathrin-independent endocytosis at the apical plasma membrane to internalize endolymph from the cochlear duct [4, 7, 8]. Mammalian cells internalize the plasma membrane, receptors, ion channels, ion pumps, ligand receptors, and solutes via several endocytic pathways [9].

Endocytosis plays an important role in the activation and propagation of signaling. The classical view of the role of endocytosis in cell signaling is that it down regulates signal responses by internalizing receptors. However, in recent views on the role of

endocytosis, ligands bind to their receptors on the plasma membrane, initiate signal transduction events, and induce receptor internalization [10].

In our previous report, marginal cells in the stria vascularis showed very active CF endocytosis via a clathrin-mediated pathway. In a CF endocytic pathway, the transport of the endocytic carrier vesicles (ECVs) loaded with CF was dependent on F-actin and MT [4]. In the present report, we investigated the roles of G-protein, ROCK, MLCK, and myosin phosphatase in the CF endocytosis of marginal cells using specific inhibitors in order to understand how to regulate the endocytosis in stria vascularis.

Materials and Methods

Animals and chemicals:

Thirty Hartley white guinea pigs (250-350 g) were obtained from Japan SLC Inc. (Hamamatsu, Japan) and divided into six groups each consisting of five animals (Table 1). Cationized ferritin (CF), pertussis toxin (PTX), *Clostridium botulinum* C3 toxin (BTX), and guanosine(γ-thio)-triphosphate (GTP-γS) were obtained from Sigma-Aldrich. ML-7 and Y-27632 were obtained from Calbiochem-Novabiochem.

The care and use of the animals in this study were approved by the Kochi Medical School Animal Care and use Committee.

Assay of endocytic activity:

Endocytic activity was examined using methods described previously [4]. Briefly, the solution containing CF and an inhibitor dissolved in artificial endolymph were infused into the cochlear duct of guinea pigs for 30 minutes. We set the infusion period as 30 minutes because the largest number of ECVs loaded with CF was observed at 30 minutes of infusion of the CF solution [7]. After infusion, the stria vascularis fixed in glutaraldehyde was harvested, postfixed in buffered osmium tetraoxide for one hour,

and embedded in Spurr's resin. The thin sections were examined by electron microscopy, and ECVs labeled with CF were counted.

The concentrations of all the agents are based on published data: PTX at 0.25 μg/mL [13], BTX at 2 μg/mL [14], GTP-γS at 40 μM [15, 16], Y-27632 at 30 μM [17], and ML-7 at 20 μM [18].

Electron microscopy and quantitation of endocytic activity:

Ultrathin sections were examined with a Hitachi H-7000H Electron Microscope. The endocytic activity was measured by counting the number of ECVs labeled with CF in marginal cells. Marginal cells at the center of cells were shown at 9,000 times magnification. The number of ECVs loaded with CF was counted in images magnified 20,000 times and expressed as the number of ECVs/cell. In Table 1, values in brackets represent the number of cells analyzed and not the number of images. Statistical analysis was performed using Dunnett's test to evaluate the differences in means compared with the control, and P-values <0.05 were considered significant.

Results

1) Endocytosis of CF in marginal cells of control animals

CF (1 mg/mL) in artificial endolymph was infused into the cochlear duct for 30 minutes. CF was internalized via clathrin-coated pits and vesicles, and transported to the early sorting endosomes. Invagination of the plasma membrane is the first step of endocytosis, creating a concave area with clathrin under the plasma membrane. Many ECVs labeled with CF were observed in the apical cytoplasm of marginal cells and the early sorting endosomes were labeled with CF (Figure 1, Table 1).

2) Effects of PTX on endocytosis of CF

PTX is a G-protein ADP-ribosylation inducer. CF (1 mg/mL) and PTX (0.25 μg/mL) in artificial endolymph were infused into the cochlear duct for 30 minutes. The internalization of CF was not inhibited. The number of ECVs labeled with CF was not different from that in the control. The number of invaginations of the plasma membrane was similar to that in the control. (Figure 2, Table 1).

3) Effects of BTX on endocytosis of CF

BTX is a low-molecular-weight GTP-binding protein that inactivates RhoA by the ADP-ribosylation, and results in inactivation of ERK signaling cascade. CF (1 mg/mL) and BTX (2 μg/mL) were infused into the cochlear duct for 30 minutes. The endocytosis of CF was not affected by BTX. The number of invaginations of the plasma membrane was similar to that in the control. (Figure 3, Table 1).

4) Effects of GTP-γS on the CF endocytosis

GTP-γS is a nonhydrolyzable analog of GTP and an activator of G-proteins. GTP-γS (40 μM) in artificial endolymph was infused into the cochlear duct for 30 minutes. CF endocytosis was moderately inhibited. Many invaginations of the plasma membrane were observed (Figure 4, Table 1).

5) Effects of Y27632 on endocytosis of CF

Rho-associated kinase (ROCK) phosphorylates a number of substrates, such as LIM kinase1/LIM kinase2, regulator myosin light chain (MLC), and MLC phosphatase. Through phosphorylation events, ROCK promotes the stabilization of F-actin and increases myosin ATPase activity, leading to the formation of contractile actin-myosin bundles (stress fibers), and integrin-containing focal adhesions. A specific inhibitor of

ROCK, Y27632 (30 μM), and CF (1 mg/mL) in artificial endolymph were infused into the cochlear duct for 30 minutes. The endocytosis of CF was markedly inhibited by Y27632. The number of ECVs loaded with CF was 25% of the control. The number of invaginations of the plasma membrane was similar to that in the control. (Figure 5, Table 1).

6）Effects of ML-7 on endocytosis of CF

Phosphorylation of MLC plays a critical role in controlling actomyosin contractivity. MLC phosphorylation is regulated by the balance of MLC kinases (MLCK) and MLC phosphatase (MLCP). A specific inhibitor of MLCK, ML-7 (20 μM), and CF (1 mg/mL) in artificial endolymph were infused into the cochlear duct for 30 minutes. The endocytosis of CF was markedly inhibited. The number of ECVs loaded with CF was decreased to 50% of the control. This means that the increased MLC phosphorylation inhibits vesicular transport along F-actin. Many invaginations of the plasma membrane were observed (Figure 6, Table 1).

Discussion

Marginal cells of the stria vascularis internalize CF that is infused into the cochlear duct via the clathrin-mediated endocytic pathway. Internalized CF is transported from the plasma membrane to the early sorting endosomes by smooth endocytic carrier vesicles (ECVs) (70 nm in diameter). These processes are dependent on MT and the F-actin cytoskeleton [4]. The most widely used mechanism for intracellular transport within vesicles involves motor proteins, myosins along F-actin, and kinesins and dyneins along MT. The activity of motor proteins is controlled by G-protein signaling pathways [19]. The largest number of ECVs loaded with CF was observed at 30 minutes after starting endocytic infusion of the CF solution [7].

In this report, CF endocytosis in marginal cells was not inhibited by PTX. PTX induces ADP-ribosylation of the Gα subunit of GPCR, inhibits GPCR signaling transduction, and inactivates ERK and JNK activities [20]. GPCRs are the main receptors in the plasma membrane. There are over 900 members known as GPCRs [11]. GPCRs are mainly internalized via a clathrin-, dynamin-dependent pathway. GPCR signalings are regulated by receptor internalization, rapid desensitization, and receptor trafficking [11].

CF endocytosis in marginal cells may not involve internalizing GPCRs or ERK/JNK activity.

In the present report, BTX did not inhibit CF endocytosis in marginal cells of the stria vascularis. BTX recognizes a cell-surface glycoprotein, translocates an ADP-ribosyltransferase to the cytoplasm, and inactivates RhoA and ERK signaling [21]. CF endocytosis in marginal cells may not involve the glycoprotein or RhoA/ERK signaling. Our previous study showed that the main endocytotic pathway of microperoxdase (MPO) is mainly regulated by the RhoA signaling cascade [22]. The signaling cascades regulating CF internalization are different from the cascades for MPO internalization.

In the present report, GTP-γS interference in CF endocytosis was moderate, but coated invaginations of the plasma membrane were observed frequently. GTP-γS inhibits rapid and slow endocytosis in clomaffin cells and arrests the fission of coated vesicles, but does not inhibit coated pit invagination [12]. Marginal cells of the stria vascularis may have similar mechanisms of endocytosis in clomaffin cells. The increase in the number of coated invaginations of the plasma membrane may be due to a decrease in the number of ECVs. The process from coated pits to ECV may be inhibited by GTP-γS and then the coated pits may accumulate.

In the present study, Y-27632 markedly inhibited CF endocytosis without the accumulation of coated pit invaginations. A selective ROCK inhibitor, Y27632, disrupts the F-actin network, and reduces the F-actin cytoskeleton, especially at the cell center. Y-27632 also inhibits the internalization of an intercellular adhesion molecule (ICAM-1) [23]. Inhibition of the internalization of ICAM-1 may explain why the number of coated pit invaginations did not increase. CF endocytosis in marginal cells may be regulated by ROCK. Our previous study showed that the main endocytotic pathway of MPO was not dependent on the Rho/ROCK molecular switch [22]. The signaling cascades regulating CF internalization are different from the cascades for MPO internalization.

In the present report, ML-7 moderately inhibited CF endocytosis, but coated invaginations of the plasma membrane were frequently observed. The manner of inhibition of ML-7 is similar to that of Y-27632. An MLCK-specific inhibitor, ML-7, induces the disassembly of F-actin, and inhibits lamellipodia formation. However, Y27632 causes lamellipodia formation. The reported results and our own indicate that Y-27632 and ML-7 inhibit MLCK via distinct signaling cascades. We are clearly on the verge of understanding the role of endocytosis in marginal cells, and so further investigations are required.

[Disclosure Statement]

The authors are not aware of any conflict of interest or similar issue that may be relevant to the present work.

[Acknowledgement]

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Legends

Figure 1. Endocytosis of CF in marginal cells of control animals.

CF (1 mg/mL) in artificial endolymph was infused into the cochlear duct for 30 minutes. ECVs were labeled with CF (arrowhead) in the apical part of the cell. Early sorting endosomes (star) were labeled with CF. Invaginations of the plasma membrane (arrow) were observed. Magnified image of the rectangular area in this figure indicates an invagination of the plasma membrane. (Bar: 0.5 μm)

Figure 2. Endocytosis of CF in marginal cells of PTX-treated animals.

CF (1 mg/mL) and PTX (0.25 μg/mL) in artificial endolymph were infused into the cochlear duct for 30 minutes. Many ECVs were labeled with CF (arrowhead), with little CF present in the early sorting endosomes (star). The number of invaginations (arrow) of the plasma membrane was similar to the control. (Bar: 0.5 μm)

Figure 3. Endocytosis of CF in marginal cells of BTX-treated animals.

CF (1 mg/mL) and BTX (2 μg/mL) in artificial endolymph were infused into the cochlear duct for 30 minutes. Many ECVs labeled with CF were observed. Early sorting

endosomes (star) were labeled with CF. The number of invaginations (arrow) of the plasma membrane was similar to the control. (Bar: 0.5 μm)

Figure 4. Endocytosis of CF in marginal cells of GTP-γS-treated animals. CF (1 mg/mL) and GTP-γS (40 μM) in artificial endolymph were infused into the cochlear duct for 30 minutes. A few ECVs were labeled with CF (arrowhead), and little CF is present in the early sorting endosomes (star). Many invaginations of the plasma membrane (arrow) were observed. (Bar: 0.5 μm)

Figure 5. Endocytosis of CF in marginal cells of Y-27632-treated animals.
CF (1 mg/mL) and Y-27632 (30 μM) in artificial endolymph were infused into the cochlear duct for 30 minutes. Few ECVs were labeled with CF (arrowhead), and little CF was present in the early sorting endosomes (star). The number of invaginations (arrow) of the plasma membrane was similar to that in the control. (Bar: 0.5 μm)

Figure 6. Endocytosis of CF in marginal cells of ML-7-treated animals.
CF (1 mg/mL) and ML-7 (20 μM) were infused into the cochlear duct for 30 minutes. Few ECVs were labeled with CF (arrowhead), and little CF was present in the early

sorting endosomes (star). Many invaginations of the plasma membrane (arrow) were observed. (Bar: 0.5 μm)

Table 1. Quantitation of ECV labeled with CF

ECVs labeled with CF were counted as described in Materials and Methods. The average number/cell and standard deviation were calculated. Values in brackets show the number of cells counted. Statistical analysis was performed with Dunnett's test to evaluate differences in means compared with the control, and P-values <0.05 were considered significant. *: P<0.05, **: P<0.01

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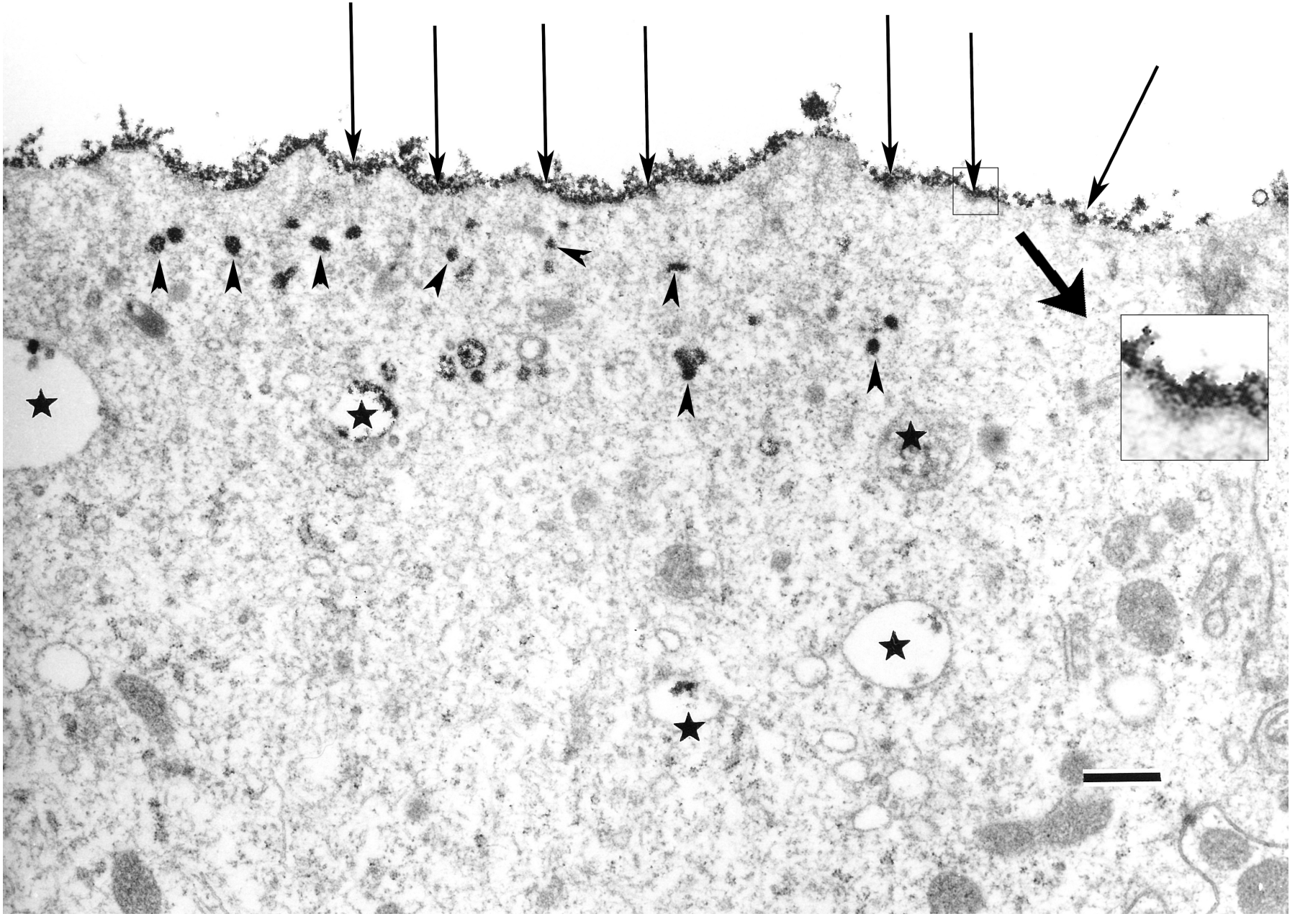

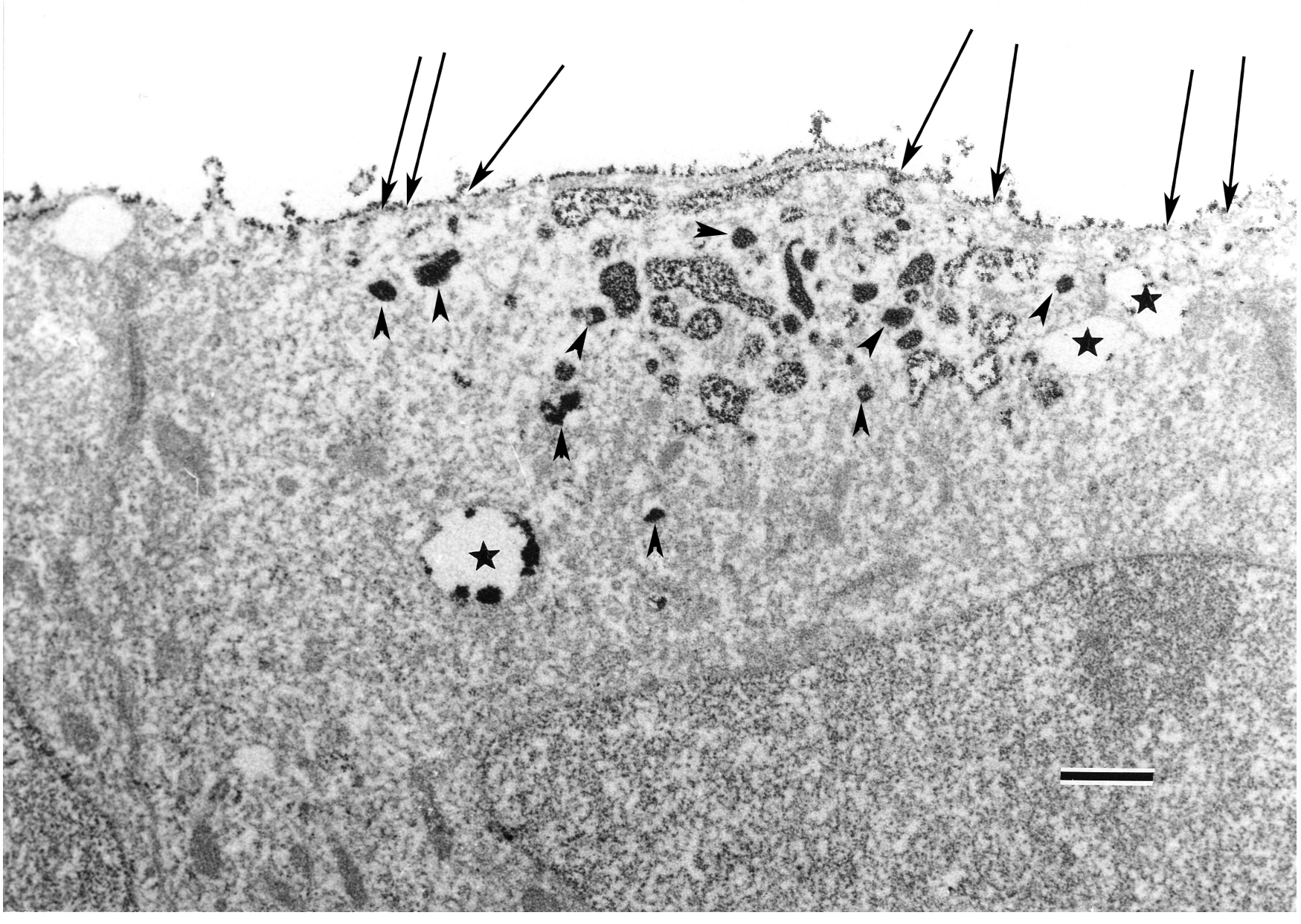

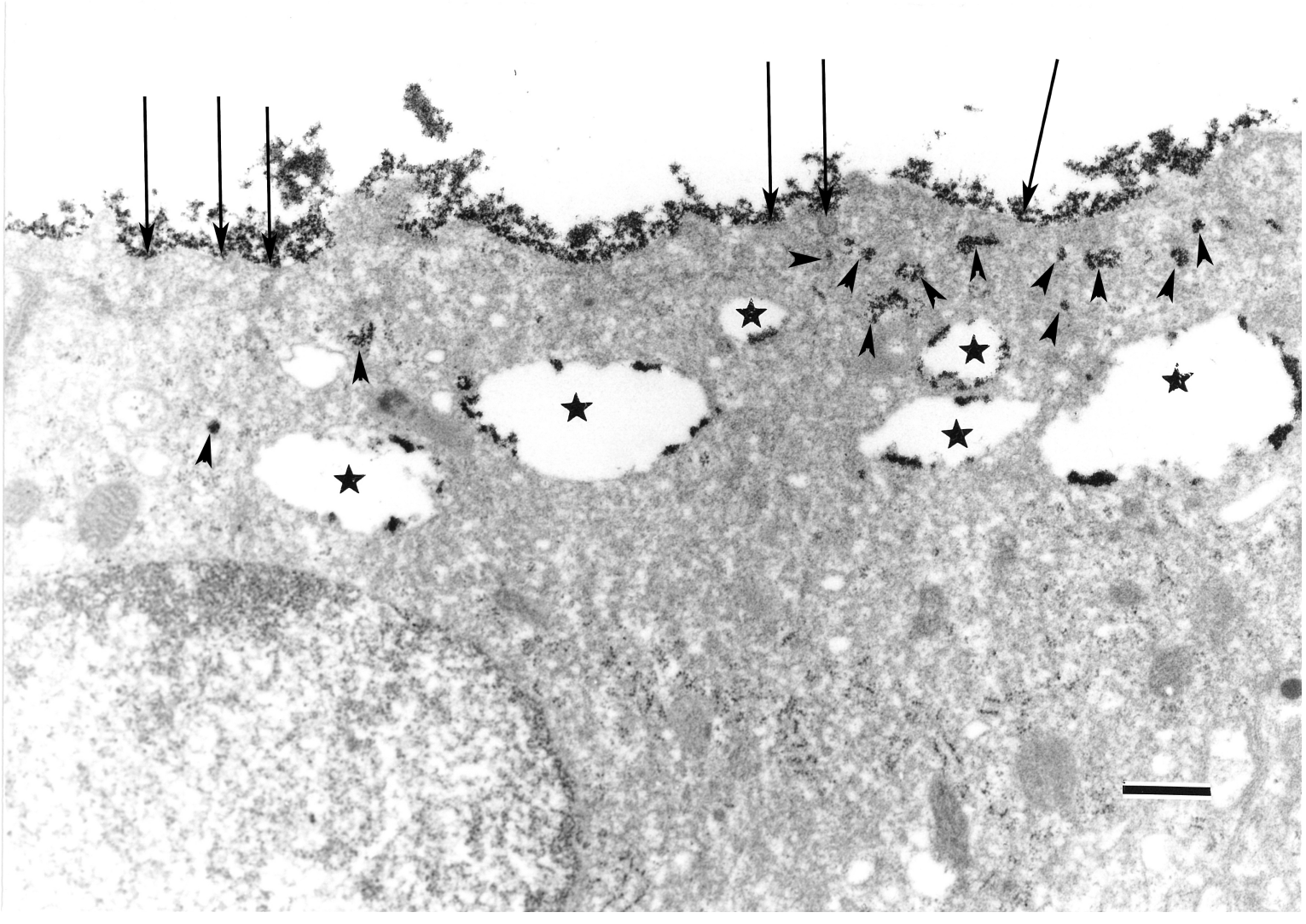

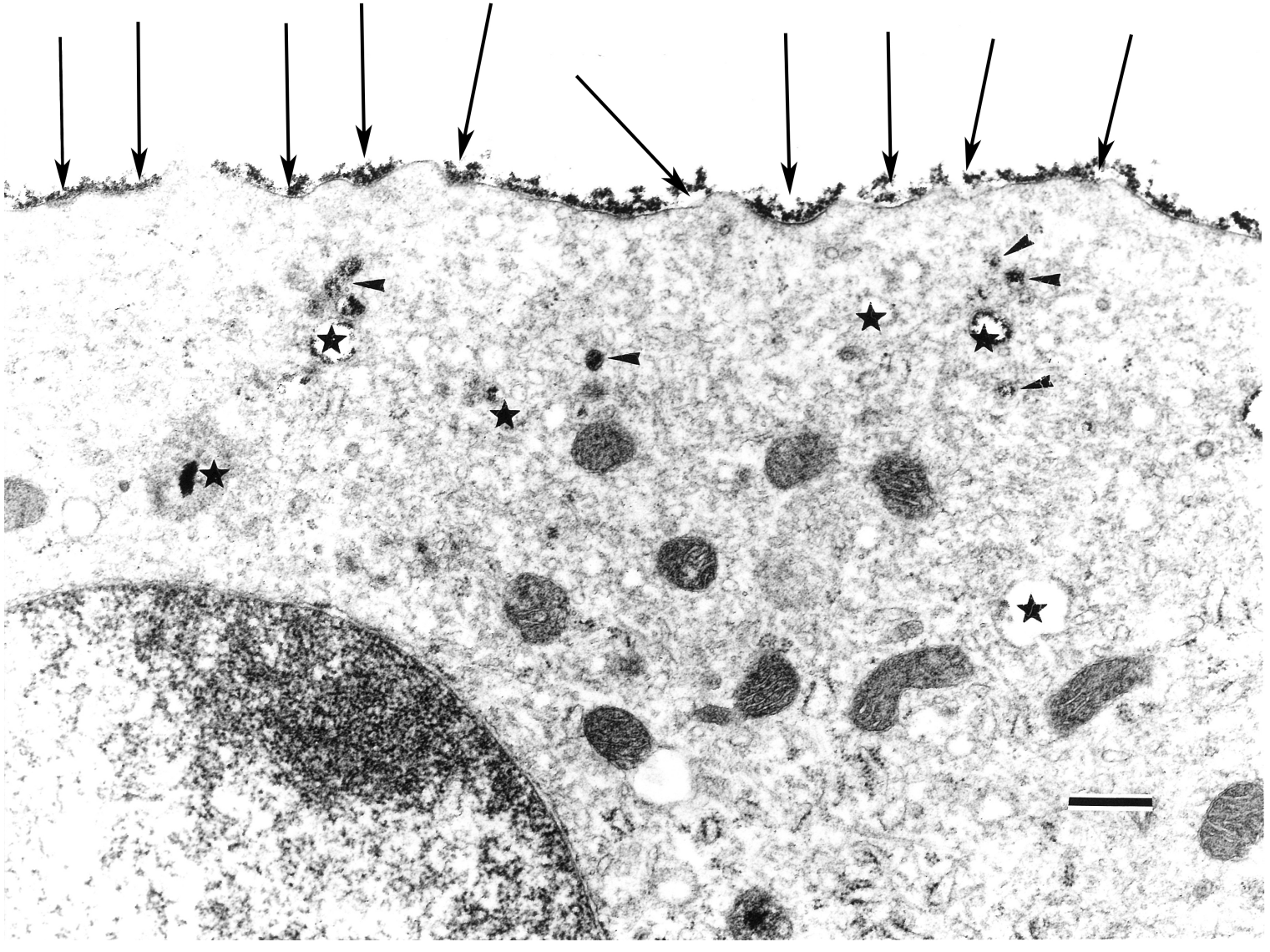

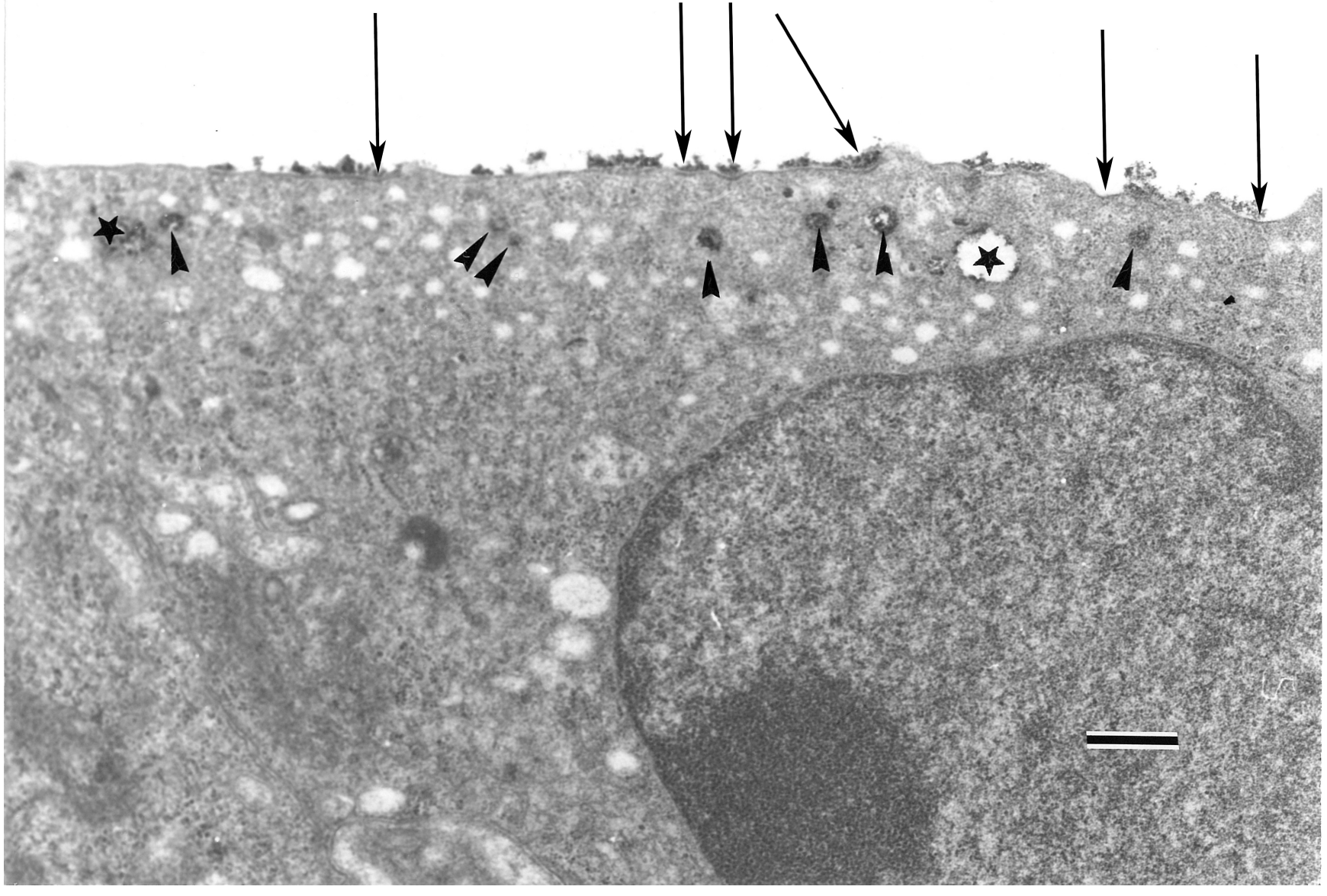

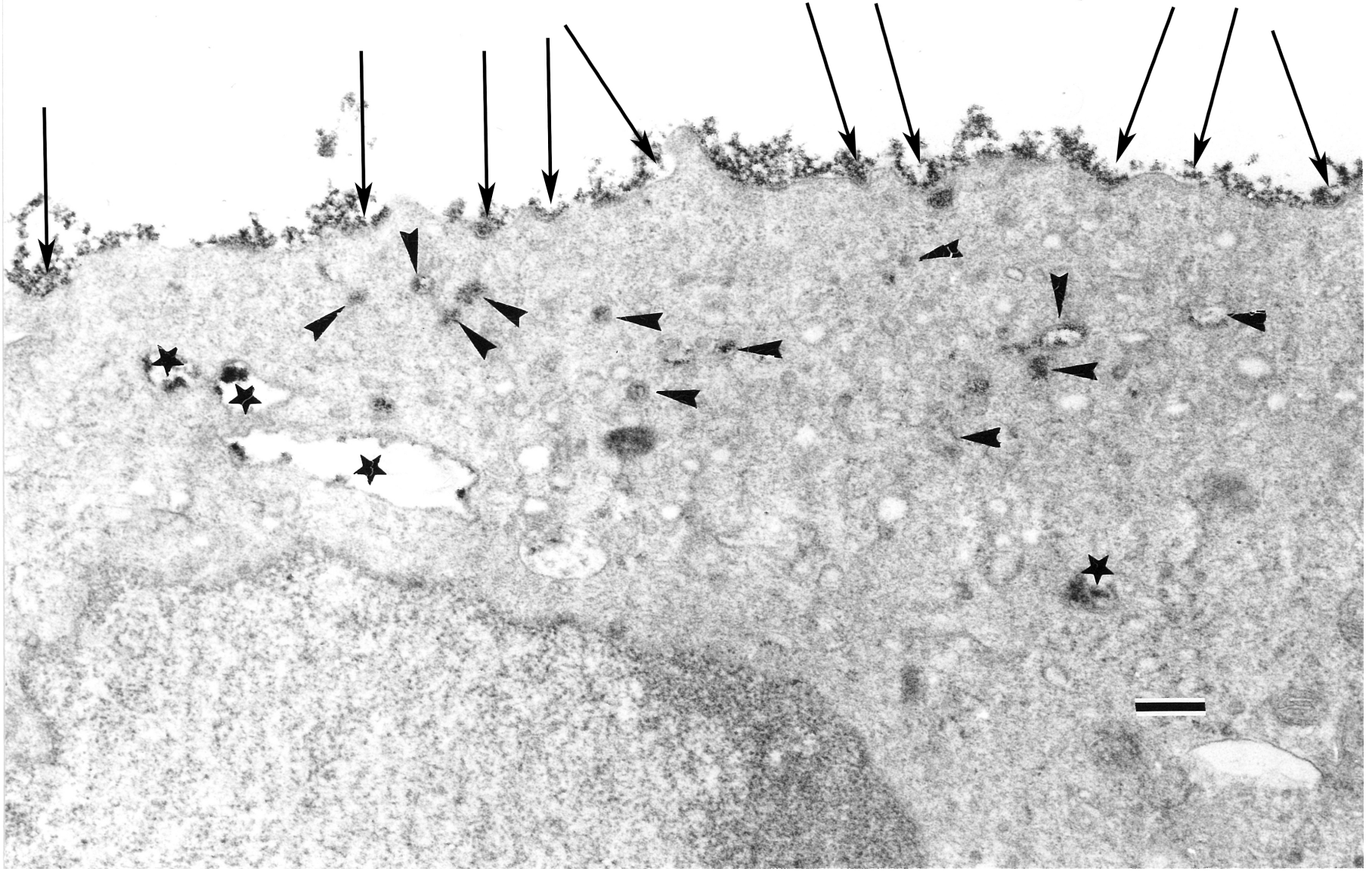

Table 1

	control	inhibitor				
		PTX	BTX	GTP-γS	Y-27632	ML-7
Number of ECVs labeled with CF (cells)	21.3 ± 8.48 (158)	18.8 ± 9.34 (141)	19.9 ± 8.43 (74)	14.1 ± 4.62* (62)	5.5 ± 3.52** (100)	11.6 ± 5.44* (114)
n	5	5	5	5	5	5