Wortmannin, a specific inhibitor of phosphatidylinositol-3 kinase, blocks osteoclastic bone resorption

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Abstract The biological role of phosphatidylinositol (PI)-3 kinase was examined in osteoclast-like multinucleated cells (OCLs) formed in co-cultures of mouse osteoblastic cells and bone marrow cells. The expression of PI-3 kinase in OCLs was confirmed by Western blot analysis. Wortmannin (WT), a specific inhibitor of PI-3 kinase, inhibited PI-3 kinase activity in OCLs both in vitro and in vivo. WT also inhibited pit-forming activity on dentine slices and disrupted a ringed structure of F-actin-containing dots (an actin ring) in OCLs in a dose-dependent manner. The inhibitory profiles of WT for pit and actin ring formation were similar to that for PI-3 kinase activity in OCLs. Electron microscopic analysis revealed that OCLs treated with WT did not form ruffled borders. Instead, numerous electron lucent vacuoles of differing sizes were found throughout the cytoplasm. These results suggest that PI-3 kinase is important in osteoclastic bone resorption.

Key words: Osteoclast; Phosphatidylinositol-3 kinase; Wortmannin; Ruffled border

1. Introduction

Phosphatidylinositol (PI)-3 kinase is one of the key enzymes activated in signaling pathways of growth factors [1,2]. It catalyzes phosphorylation of PI to PI-3-P, PI-4-P to PI-3,4-P_2, and PI-4,5-P_2 to PI-3,4,5-P_3, respectively [3–5]. The roles of PI-3 kinase have been studied extensively over the past few years. Involvement of PI-3 kinase in the action of S6 kinase [6], membrane ruffling [7,8], neurite formation [9], activation of protein kinase C (PKC) [10], actin polymerization [11,12], and transportation of proteins [13,14] has been suggested. However, it is still not clear how phosphatidylinositides produced by PI-3 kinase work in these systems.

Osteoclasts are primary bone-resorbing cells that play a critical role in bone remodeling [15]. After adhesion to the bone surface, osteoclasts exhibit highly polarized cytoplasmic organizations such as ruffled borders and clear zones. The clear zone serves as the attachment site of osteoclasts to the bone surface through the specific adhesion machinery called podosomes which are composed of F-actin and other adhesion molecules.

Recently, it has been shown that the signaling mediated by protein tyrosine kinases may play an important role in osteoclastic bone resorption. Targeted disruption of the *c-src* gene in mice induced osteopetrosis, a disease characterized by reduced osteoclastic bone resorption [16]. Indeed, osteoclasts have been reported to express a high level of $pp60^{c\text{-}src}$ [17,18]. Careful examination of osteoclasts in *c-src*-deficient mice revealed that they lacked ruffled borders [19]. PI-3 kinase is one of the enzymes that may be activated by the *c-src* tyrosine kinase [20,21]. In the present study, we examined whether PI-3 kinase is involved in osteoclastic bone resorption, using wortmannin (WT), a specific inhibitor of PI-3 kinase [22,23]. We report here that PI-3 kinase is involved in ruffled border formation and actin rearrangement in osteoclasts.

2. Materials and methods

2.1. Materials

Polyclonal antibody against the 85 kDa subunit of PI-3 kinase (p85) was prepared as described previously [24]. Wortmannin, a gift from Dr. Y. Matsuda (Kyowa Hakko Kogyo Co., Japan), was dissolved in dimethyl sulfoxide at 1 mM, stored at −20° in the dark, and diluted with distilled water just before use. 1α,25-Dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] was purchased from Wako Pure Chemical Co. (Osaka, Japan). [γ-^{32}P]ATP (111 TBq/mmol) was obtained from New England Nuclear Products (Boston, MA).

2.2. Cells and cell cultures

Mouse osteoclast-like multinucleated cells (OCLs) were prepared using ddY mice, as reported previously [25]. Primary osteoblastic cells obtained from newborn mouse calvaria and bone marrow cells obtained from tibiae of 7- to 9-week-old male mice were co-cultured in α-MEM containing 10% fetal bovine serum and 10 nM 1α,25$(OH)_2D_3$ on culture dishes precoated with 4 ml of 0.2% collagen gel matrix (Nitta Gelatin Co., Osaka, Japan). OCLs were formed within 6 days of culture, and released from dishes by treating with 4 ml of 0.2% collagenase (Wako), and collected by centrifugation at 250 × *g* for 5 min. The purity of OCLs in this fraction was about 5% (crude OCL preparation). For the PI-3 kinase assay, they were further purified with pronase according to the method described previously [26]. In the final preparation (purified OCL preparation), more than 80% of the cells remaining on the dishes were TRAP-positive multinucleated and mononuclear cells, and more than 95% of the total protein was estimated to be derived from OCLs.

2.3. Western blot analysis

Osteoblastic cells, crude OCL preparations, and purified OCL preparations were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.8, 1% (v/v) Nonidet P-40, 20 mM EDTA, 0.1% sodium dodecyl sulfate, 0.1% sodium deoxycholate, 2 mM sodium orthovanadate, 10 mM sodium fluoride and aprotinin at 10 μg/ml). RIPA extracts were prepared by centrifugation at 12,000 × *g* for 15 min. Samples containing equal amounts of protein were suspended in Laemmli sample buffer, and electrophoresed on SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to Immobilon-P (Millipore Co., Bedford,

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MA). Immunostaining with anti-p85 antibody was performed using ECL Western blotting detection reagents (Amersham International plc., Amersham Place, UK).

2.4. Assay for PI-3 kinase

PI-3 kinase was assayed as described before [21]. Briefly, PI-3 kinase was immunoprecipitated from purified OCL preparations with polyclonal anti-p85 antibody and washed thoroughly. PI-3 kinase activity was measured by incubating immunoprecipitates with PI and [γ-^{32}P]ATP. Phosphorylated products of PI were separated by thin layer chromatography.

2.5. Assay for pit-forming activity of OCLs on dentine slices

To assess bone-resorbing activity of OCLs, pit-forming activity of OCLs on dentine slices was determined by the method previously reported [27]. Crude OCL preparations were placed on dentine slices and incubated for 48 h with or without WT. Since the activity of WT declined within 5 h [9], fresh WT was supplied every 5 h. At the end of the culture period, the dentine slices were cleaned by ultrasonication to remove adherent cells. The slices were then stained with Mayer's hematoxylin solution. To quantitate pit-forming activity of OCLs, the areas of resorption lacunae were measured with an image analysis system (LA-525; PIAS Co., Tokyo). The results are expressed as the percentage of resorbed area with respect to the whole surface area of a dentine slice.

2.6. Podosome formation in OCLs

Podosomes formed in OCLs were visualized by F-actin staining with rhodamine-conjugated phalloidin as described before [26]. Crude OCL preparations placed on culture dishes for 3 h were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min, and permeated by 0.1% Triton X-100 in PBS for 5 min. To identify OCLs, cells were stained for TRAP. After washing with PBS, F-actin was stained with 0.3 μM rhodamine-conjugated phalloidin. Distribution of F-actin was detected under a fluorescent microscope (Olympus BX-FLA, Osaka).

2.7. Preparation of ultrathin sections

Crude OCL preparations were cultured on dentine slices for 3 h and then treated with or without 100 nM WT for 60 min, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). They were postfixed with 1.5% potassium ferrocyanide-reduced 1% osmium tetroxide, block-stained with 1% uranyl acetate, dehydrated with a graded ethanol series, and embedded in Quetol 812 (Nisshin EM, Tokyo). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-12A electron microscope at 75 kV.

3. Results and discussion

Fig. 1a shows a Western blot analysis of PI-3 kinase in lysates from mouse osteoblastic cells (lane 1), crude OCL preparations (lane 2), and purified OCL preparations (lane 3). PI-3 kinase was detected in the three preparations. These results demon-

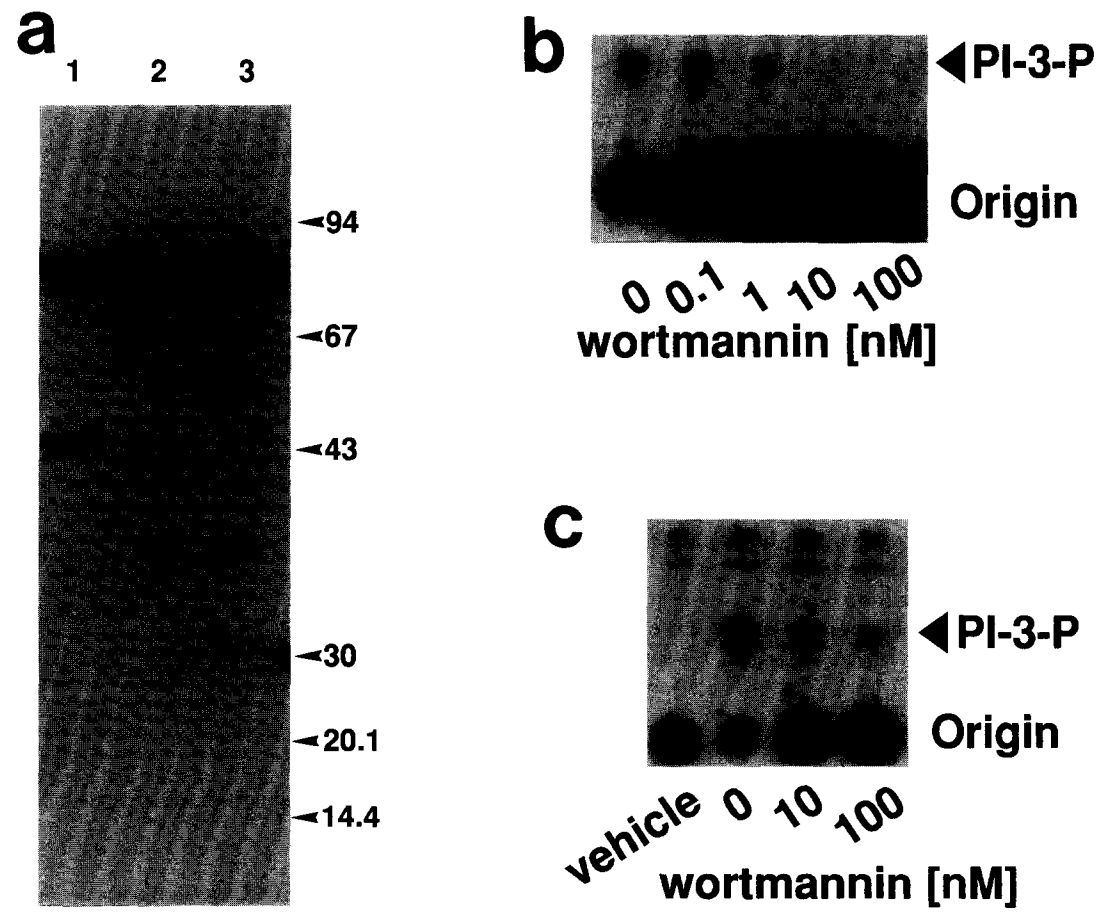

Fig. 1. Effects of WT on PI-3 kinase activity in OCLs in vitro and in vivo. (a) Western blotting of PI-3 kinase. Expression of PI-3 kinase in lysates from mouse osteoblastic cells (lane 1), crude OCL preparations (lane 2), and purified OCL preparations (lane 3) was analyzed by Western blotting. The numbers on the right indicate the positions of molecular weight markers. (b) Examination of PI-3 kinase activity in anti-p85 immunoprecipitates prepared from purified OCL preparations. After treatment of the immunoprecipitates with WT for 10 min, PI-3 kinase activity was analyzed. (c) Effect of WT on PI-3 kinase activity in vivo. After purified OCL preparations were pretreated with WT for 60 min, PI-3 kinase was immunoprecipitated with anti-p85 antibody and the activity in the immunoprecipitates was analyzed. Positions of the origin and PI-3-P spots are shown on the right in panels b and c.

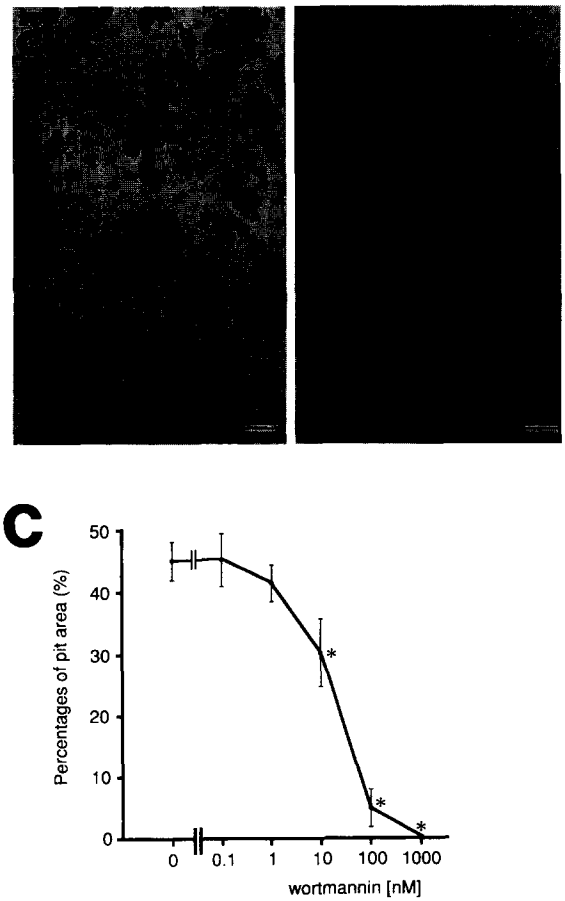

Fig. 2. Effect of WT on pit-forming activity of OCLs on dentine slices. Crude OCL preparations were cultured on dentine slices with graded concentrations of WT. After culture for 48 h, resorption pits formed on dentine slices were stained with Mayer's hematoxylin and quantitated using an image analysis system. (a) Pits on dentine slices in the control culture. (b) Pits on dentine slices in cultures treated with 100 nM WT. (c) Effect of WT on pit-forming activity of OCLs (means ± S.D.; $n = 5$). *Significantly different from the group treated without WT, $P < 0.01$. Bars = 200 μm.

strate that not only osteoblastic cells but also OCLs express PI-3 kinase. To determine the effect of WT on PI-3 kinase activity in OCLs, PI-3 kinase was immunoprecipitated from purified OCL preparations with anti-p85 antibody and the activity was determined in the presence or absence of WT. The immunoprecipitates exhibited PI-3 kinase activity, which was inhibited dose-dependently by WT (Fig. 1b). The activity of PI-3 kinase was completely inhibited by WT at 100 nM. The effect of WT on PI-3 kinase in intact OCLs was examined as well. Pretreatment of OCLs with WT for 60 min inhibited PI-3 kinase activity in a dose-dependent manner (Fig. 1c). PI-3 kinase activity was almost completely inhibited by 100 nM WT.

OCLs placed on dentine slices formed resorption pits within 48 h (Fig. 2a). When crude OCL preparations were treated with WT, pit-forming activity of OCLs was inhibited dose-dependently (Fig. 2c). The concentration of WT for the complete inhibition of pit-forming activity was 100 nM.

When OCLs were placed on culture dishes, more than 80% of the OCLs spread out and formed ringed structures of F-actin (actin rings) within 3 h (Fig. 3c and e). When WT was added at 100 nM, actin rings in OCLs disappeared almost completely within 60 min, resulting in the distribution of the actin-containing dots throughout the cytoplasm (Fig. 3d). WT, however, did not appear to cause distinct structural changes in the stress fibers of F-actin in osteoblastic cells contaminating the crude OCL preparation (Fig. 3d, arrows). The inhibitory profiles of WT for the pit-forming activity by OCLs and for the actin ring formation in OCLs were similar to that for PI-3 kinase activity in vivo (Figs. 1c, 2c and 3e). It may be that WT might be inhibiting other enzymes such as the myosin light chain kinase. However, it has been well established that a much higher concentration of WT is required to inhibit these enzymes [22,28]. Moreover, the effect of WT on PI-3 kinase activity in OCLs exactly corresponded with that observed in other types of cells reported in previous studies [22,23]. Although there is no direct evidence that myosin light chain kinase is not inhibited by 100 nM WT, it is likely that PI-3 kinase is involved in both osteoclastic bone resorption and actin rearrangement in podosome formation.

At an ultrastructural level, OCLs treated with 100 nM WT did not form ruffled borders (Fig. 4). Instead, many pale vacuoles of differing sizes were distributed throughout the cytoplasm (Fig. 4a). Although fusion of adjacent vacuoles frequently occurred (Fig. 4 arrow heads), these vacuoles never fused with apical membranes facing the dentine surface (Fig. 4b). Control OCLs formed ruffled borders on dentine slices in the absence of WT (data not shown). These results suggest that PI-3 kinase is involved in ruffled border formation in osteoclasts as well.

It has been shown that $pp60^{c\text{-}src}$ is required for the ruffled border formation of osteoclasts [16–19]. Since it is suggested that PI-3 kinase is activated by $pp60^{c\text{-}src}$ [20,21], it is natural to speculate that PI-3 kinase is involved in osteoclastic bone resorption. The present study suggests that PI-3 kinase is involved in osteoclastic bone resorption through the ruffled border formation. PI-3 kinase may be involved in actin rearrangement and cytoskeleton formation [8,11,12]. In our experiments, the inhibitory profiles of WT for actin ring formation and for PI-3 kinase activity in vivo were very similar. This suggests that PI-3 kinase plays a regulatory role in the rearrangement of actin cytoskeletons in osteoclasts as well. In contrast, stress fibers in osteoblastic cells were not affected by WT (Fig. 3d, arrows). The reason why the actin rearrangement was abolished by WT in one cell type but not in the other is not clear at present. Different cytoskeletal structures may be present in OCLs and osteoblastic cells. The actin ring formation appears to be a prerequisite for the ruffled border formation in osteoclasts, because the disruption of actin rings by calcitonin, herbimycin A and bisphosphonates prevented ruffled border formation and inhibited osteoclastic bone resorption (unpublished results). There could be a direct or indirect link between the actin ring formation and the ruffled border formation. Further studies are required to substantiate this hypothesis.

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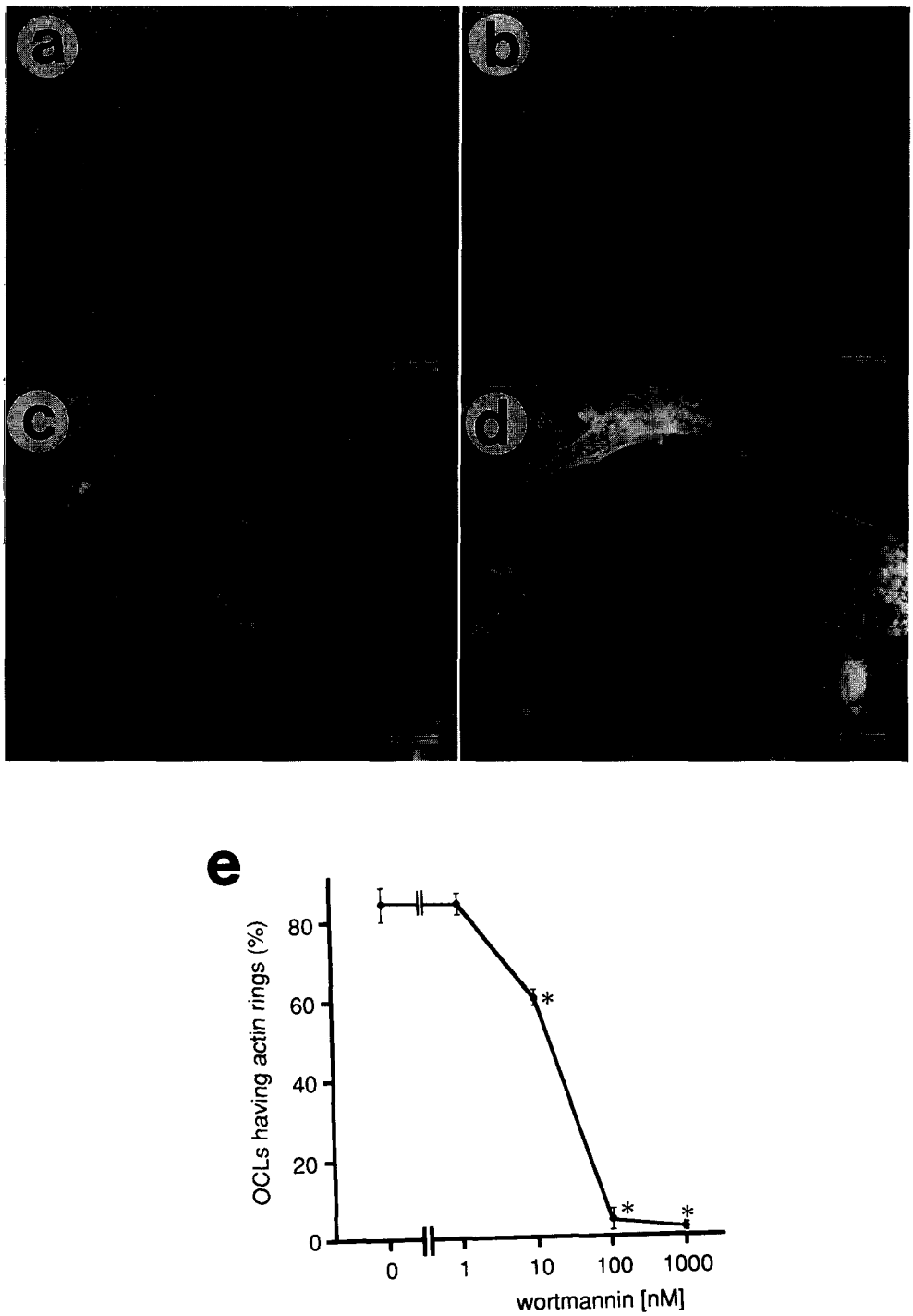

Fig. 3. Disruption of F-actin rings in OCLs by WT. Crude OCL preparations were placed on culture dishes. After culture for 3 h, they were treated with (b and d) or without (a and c) 100 nM WT for 60 min. Light micrographs (a and b) and fluorescent micrographs (c and d) were prepared of OCLs stained for TRAP followed by rhodamine-conjugated phalloidin staining. Arrows in panel d indicate stress fibers in osteoblastic cells. (e) Effect of WT on F-actin rings in OCLs (means ± S.D.; $n = 4$). More than 150 OCLs were evaluated in each group. *Significantly different from the group treated without WT, $P < 0.01$. Bars = 40 μm.

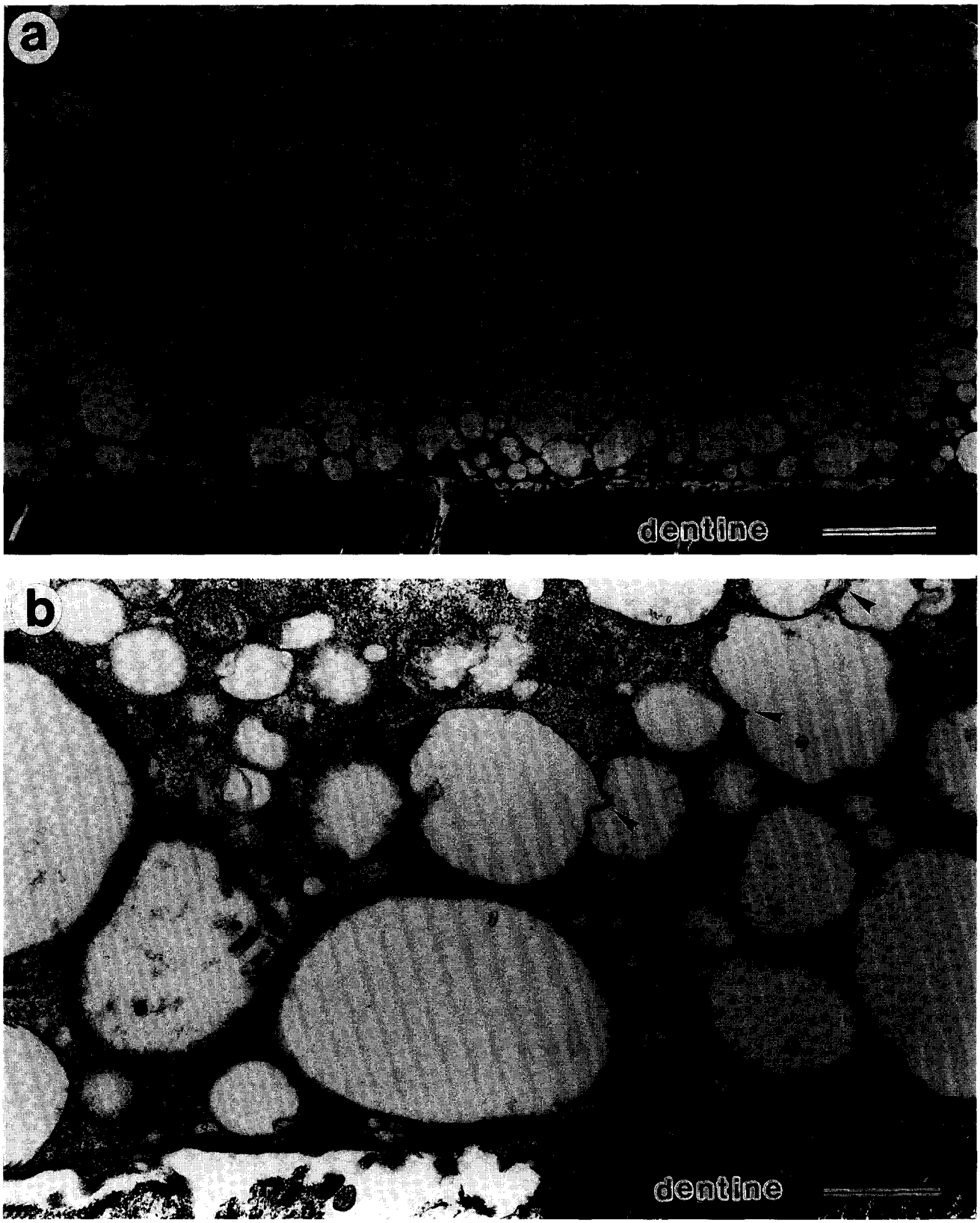

Fig. 4. Morphological changes in OCLs treated with WT. (a) Electron micrograph of an OCL placed on a dentine slice in the presence of 100 nM WT. (b) A high-power view of a portion of the apical membrane in another OCL on a dentine slice. Arrow heads indicate fusion of adjacent vacuoles. Bars in a = 5 μm; in b = 1 μm.

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