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Protein phosphatase inhibitor calyculin A induces hyperphosphorylation of cytokeratins and inhibits amylase exocytosis in the rat parotid acini

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Calyculin A, a protein phosphatase inhibitor with a chemical structure completely different from that of okadaic acid, reproduced the inhibitory effect of okadaic acid on cyclic AMP-mediated amylase release from rat parotid acinar cells. Calyculin A markedly enhanced phosphorylation of cytokeratins in the cytoskeletal fraction of the cells, whereas cAMP had apparently no effect on the phosphorylation. Microscopic observations showed that parotid acini incubated with 100 nM calyculin A for 15 min had large vacuoles in the cytoplasm and conspicuous blebs on the basal plasma membrane. K252a, a nonselective protein kinase inhibitor, clearly reduced calyclin A-induced phosphorylation of cytokeratins, and it markedly blocked the inhibition of amylase release and morphological changes evoked by calyculin A. These results suggest that hyperphosphorylation of cytokeratins profoundly affects the morphology and secretory activity of parotid acinar cells.

Protein phosphatase inhibitor; Amylase secretion; Calyculin A; cAMP-mediated exocytosis; Cytokeratin phosphorylation

1. INTRODUCTION

Protein phosphorylation has been generally thought to play a central role in the regulatory mechanisms of diverse cellular functions [1,2]. In the regulation of exocytosis, however, the role of protein phosphorylation has yet to be established, although almost all secretory stimuli affect protein phosphorylation [3,4]. Amylase exocytosis from parotid acinar cells has been extensively studied as a useful model of cAMP-mediated exocytosis [3,5]. Several lines of evidence support the involvement of cAMP-dependent protein kinase in amylase exocytosis [6–8]. Nevertheless, previous studies using protein kinase inhibitors (H-8 and peptide fragments of heat-stable protein kinase inhibitor) suggested that cAMP-dependent protein phosphorylation was not directly involved in the exocytosis, since the inhibitors markedly inhibited protein phosphorylation without decreasing amylase release [6,9]. Furthermore, okadaic acid, a potent selective inhibitor of protein phosphatase types 1 and 2A, increased protein phosphorylation but inhibited, rather than enhanced, cAMP-mediated amylase release [10].

Recently okadaic acid and its related protein phosphatase inhibitors have been found to provoke hyperphosphorylation of vimentin, a cytoskeletal protein of intermediate filaments, in human and mouse fibroblasts [11,12]. Although it has long been postulated that cytoskeletal proteins play important roles in the process of exocytosis [13–16], the relationship between phosphorylation states of cytoskeletal proteins and amylase exocytosis has not been examined so far. Thus, for the first time, we have studied the effects of cAMP and protein phosphatase inhibitors on the phosphorylation of cytoskeletal proteins in rat parotid acini.

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2. EXPERIMENTAL

2.1. *Materials*

Okadaic acid was generous gift from Dr. Y. Tsukitani (Fujisawa Pharmaceutical, Tokyo, Japan). Calyculin A and K252a were purchased from Wako Pure Chemicals (Osaka, Japan) and Kyowa Medex (Tokyo), respectively. [γ-^{32}P]ATP was from Dupont-Daiichi (Tokyo). Monoclonal antibodies to cytokeratins and actin were from Labsystems Japan (Tokyo) and Amersham Japan (Tokyo), respectively. All other reagents were of the highest grade commercially available.

2.2. *Amylase release*

Parotid acini were prepared by enzyme digestion, and amylase release from intact and saponin-permeabilized acini was measured as described [6,8].

2.3. *Phosphorylation of cytoskeletal proteins*

Parotid acini were preincubated for 5 min at 37°C with 20 μg/ml saponin, ~ 0.2 mCi/ml [γ-^{32}P]ATP in Ca-free medium composed of 120 mM KCl, 40 mM K-HEPES (pH 7.2), 1 mM EGTA, 2 mM $MgCl_2$, 1 mg/ml bovine serum albumin, and 10 μg/ml Phenol red and further incubated for 15 min after addition of calyculin A and/or cAMP. After incubation, the medium was removed and the cells were homogenized in 0.3 M sucrose, 10 mM K-phosphate (pH 6.8), 10 mM EDTA, and 5 mM EGTA in a Teflon–glass homogenizer. For preparation of the cytoskeletal fraction, the homogenates were treated at 4°C for 10 min with 1% Triton X-100, 0.6 M KCl, 10 mM EDTA, and 10 mM K-phosphate (pH 6.8), washed twice with the same medium

and twice with the homogenizing medium as above, and the resulting pellets were boiled for 10 min in 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 70 mM Tris-HCl (pH 6.8).

2.4. *Immunoblotting and immunoprecipitation*

Proteins in the cytoskeletal fractions were separated by SDS-PAGE using 10% running gel and 4% stacking gel, and were transferred to a polyvinylidene difluoride (PVDF) membrane. Cytokeratins and actin on the membrane were immunologically identified by the avidin–biotin–peroxidase complex method using mouse monoclonal antibodies to cytokeratins and actin as the first antibodies.

The cytoskeletal proteins were solubilized by boiling for 10 min with 3% SDS, 10 mM HEPES–Tris (pH 7.2), 0.14 M NaCl, 1% Triton X-100, and 1 mM EDTA, and were diluted to 0.1% SDS with the same solution without SDS. Aliquots of the solubilized proteins were incubated with anti-cytokeratin antibody and protein A sepharose. The immunoprecipitate was separated by SDS-PAGE, and cytokeratins were detected by autoradiography.

2.5 *Light microscopy*

Parotid acini were fixed with 2% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) containing 0.2% tannic acid for 1 h at 4°C, and post-fixed with 1% OsO_4 in distilled water for 30 min at room temperature. The preparations were dehydrated with acetone and embedded in Spurr resin. One-micrometer sections were cut and stained with 1% Toluidine blue.

3. RESULTS

Fig. 1 shows the effect of calyculin A, a potent inhibitor of protein phosphatase types 1 and 2A [17], on amylase release from intact parotid acini. Calyculin A strongly inhibited amylase release stimulated by 10^{-6} and 10^{-8} M isoproterenol, the maximum and half-maximum doses, respectively. In contrast, calyculin A alone slightly increased amylase release. The inhibitory and stimulatory effects were clearly observed at 0.1 μM calyculin A, indicating this drug to be 10 times more potent than okadaic acid [10]. The inhibitory effect of calyculin A was similarly observed in saponin-permeabilized parotid acini incubated with exogenous cAMP (Fig. 2), suggesting that the inhibition is not due to damage to the plasma membrane or β-receptor–adenylate cyclase system. In this experiment, the 10-fold difference in the potency of the two inhibitors was clearly confirmed. Since these protein phosphatase inhibitors apparently enhance basal activities of all protein kinases in the cell, we examined the effect of K252a, a nonselective protein kinase inhibitor. Fig. 3 shows that 0.3–3 μM K252a dose-dependently blocked the inhibitory effect of 100 nM calyculin A on cAMP-mediated amylase release.

Next we examined the effects of cAMP and calyculin A on the phosphorylation states of cytoskeletal proteins in the 1% Triton X-100 insoluble fraction of parotid acini. The fraction contained three major protein bands between 40 and 60 kDa, and the upper two bands were heavily phosphorylated by 100 nM calyculin A (Fig. 4). Immunoblotting studies using monoclonal antibodies against cytokeratins and actin revealed that the upper two phosphorylated proteins were very likely to be cytokeratins and the third band was actin (Fig. 5A). Since the parotid acini appeared to contain some minor cytokeratin subunits besides the two major cytokeratin bands (Fig. 5A, lane 2), cytokeratins were further characterized by immunoprecipitation, Fig. 5B shows that the anti-cytokeratin antibody clearly precipitated the two phosphoproteins. As shown in Fig. 6, 3 μM K252a markedly inhibited the phosphorylation of cytokeratins induced by 100 nM calyculin A. K252a also reduced basal phosphorylation of cytokeratins.

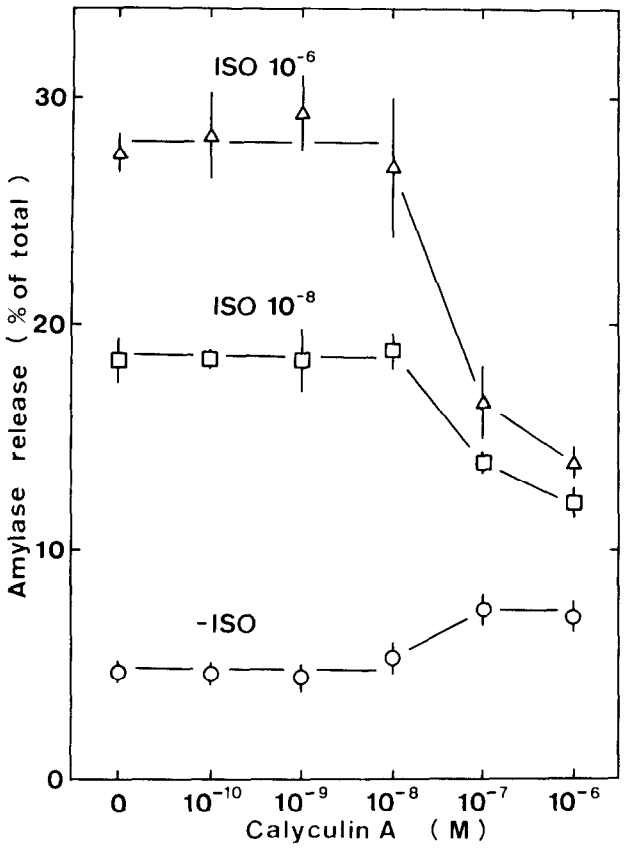

Fig. 1. Effect of calyculin A on amylase release from intact parotid acini. Rat parotid acini were incubated at 37°C for 15 min in regular Hanks' balanced salt solution containing various concentrations of calyculin A and 0, 0.01, or 1 μM isoproterenol (ISO). Data shown are means ± S.D ($n = 4$).

Finally we examined the effect of calyculin A on the morphological structure of parotid acini. For that purpose, we incubated intact acini with 100 nM calyculin A and/or 1 μM isoproterenol for 15 min. As seen in Fig. 7, calyculin A induced drastic changes in the cell structure; large vacuoles were observed in the cytoplasm and conspicuous blebs appeared on the surface of the basal plasma membrane (photos 3 and 4 in Fig. 7). Although small blebs were occasionally seen in control acini, isoproterenol treatment did not increase their size (photos 1 and 2). Okadaic acid (1 μM) also induced similar morphological changes in the acini (data not shown). K252a (3 μM) had apparently no effect on the morphol-

ogy of parotid acini, but clearly prevented the morphological changes induced by 100 nM calyculin A (photos 5 and 6). During these experiments, we monitored the leakage of lactic dehydrogenase (LDH) as an index of damage to the plasma membrane by calyculin A; calyculin A slightly increased LDH leakage from the control value of 2.5 ± 0.2 (% of the total activity in homogenates) to 4.3 ± 0.3% at 100 nM calyculin A during a 15-min incubation.

4. DISCUSSION

The present study revealed that calyculin A clearly reproduced the effect of okadaic acid on amylase exocytosis from parotid acini: strong inhibition of cAMP-mediated amylase release and weak stimulation by itself. The dual effects were detectable above 10^{-8} M calyculin A, indicating that calyculin A is 10 times as potent as okadaic acid [10]. Since the two compounds share only biological activity but not the same chemical structure, it is highly possible that the effects of calyculin A and okadaic acid on amylase release are provoked by the inhibition of protein phosphatases, which in turn increases protein phosphorylation. This hypothesis was strongly supported by the fact that K252a blocked the inhibitory effect of calyculin A. The small increase in amylase release by the protein phosphatase inhibitors suggests that at least a limited portion of the exocytosis depends upon increase in protein phosphorylation.

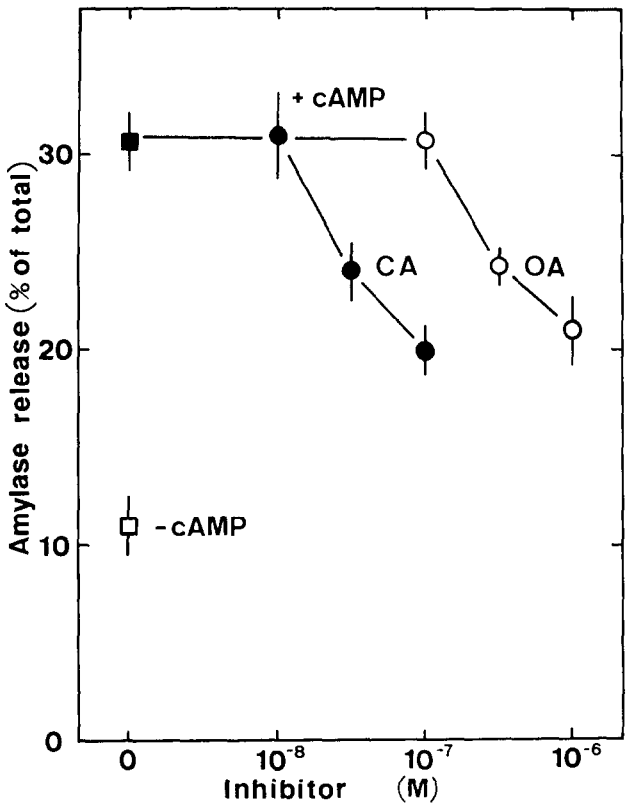

Fig. 2. Effects of calyculin A and okadaic acid on amylase release from saponin-permeabilized parotid acini. The acini were incubated for 15 min with 1 mM cAMP and various concentrations of calyculin A (CA) or okadaic acid (OA) in Ca-free KCl medium containing 20 μg/ml saponin. Data shown are means ± S.D. (n = 6).

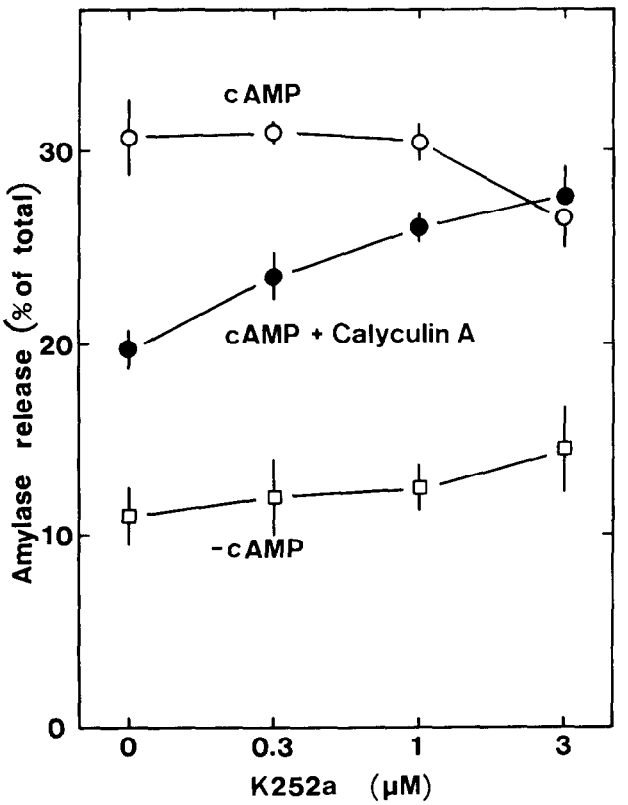

Fig. 3. Effects of calyculin A and K252a on amylase release from saponin-permeabilized parotid acini. The acini were incubated for 15 min with 1 mM cAMP, 100 nM calyculin A, and various concentrations of K252a in Ca-free medium containing 20 μg/ml saponin. Data shown are means ± S.D. (n = 6).

The 10-fold difference between the effects of calyculin A and okadaic acid indicates that the inhibition of protein phosphatase type 1 is a rate-limiting step for the present phenomena, since the inhibitory effects of the two compounds on protein phosphatase type 2A are almost identical (IC_{50} = 0.5–1 nM), while the IC_{50} of calyculin A for type 1 phosphatase is approximately 100 times lower than that of okadaic acid [17]. In this study, however, we could not detect any effect of calyculin A up to 10 nM, although its IC_{50} for type 1 phosphatase is 2 nM. This discrepancy may be due to the fact that the IC_{50} values were determined in a cell-free system using purified enzymes and substrates; whereas in intact and even in permeabilized acini, nonspecific adsorption and diffusion barriers decrease accessibility of these compounds to protein phosphatases, and such hindrances are especially critical for lower concentrations of the reagents. In addition, it has been reported that the cellular concentration of protein phosphatases is much

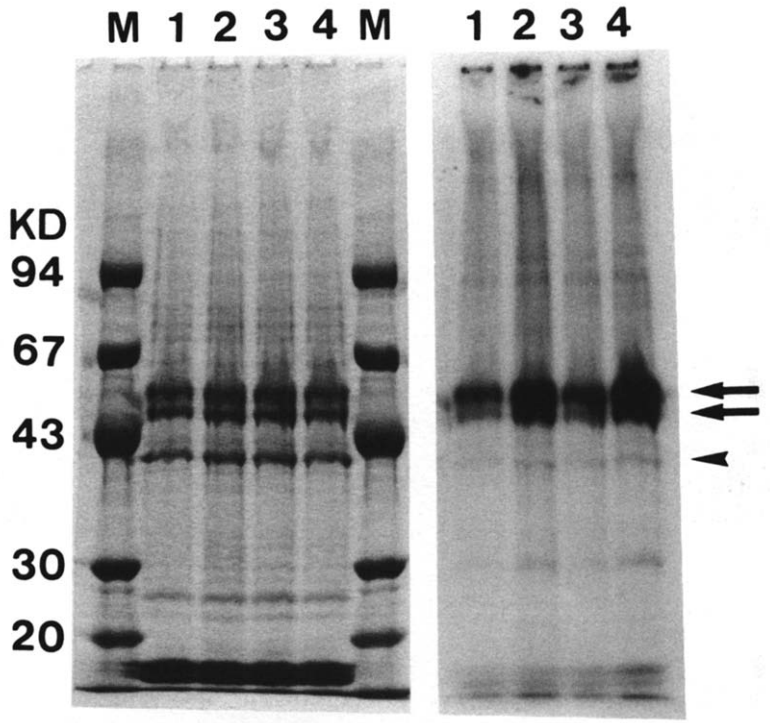

Fig. 4. Effects of calyculin A and cAMP on protein phosphorylation in the cytoskeletal fraction of parotid acini. Proteins were separated by SDS-PAGE (4–20% gradient gel), and phosphorylated proteins were detected by autoradiography. (Left) The gel stained with Coomassie blue. (Right) The autoradiogram. Lane 1, control; lane 2, 100 nM calyculin A; lane 3, 1 mM cAMP, lane 4, calyculin A plus cAMP. M, molecular markers. Arrows and the arrowheads indicate the major protein bands.

higher than the IC_{50} values of calyculin A for those phosphatases [18].

In the present study calyculin A strongly enhanced

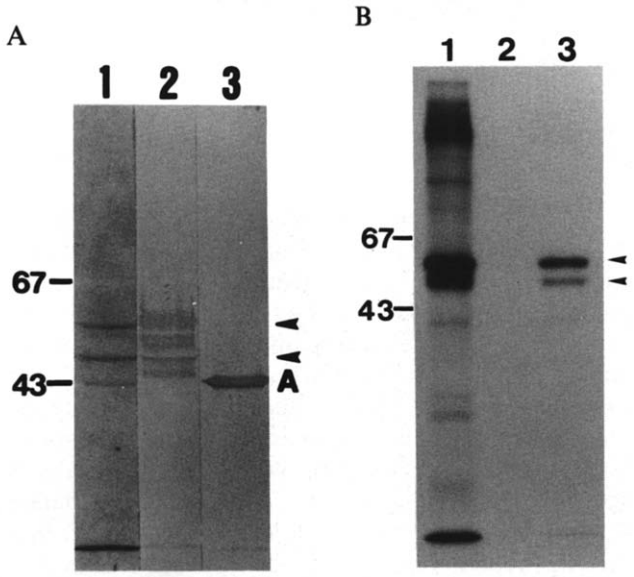

Fig. 5. (A) Western blotting analysis of the cytoskeletal proteins. Lane 1, the gel stained with Coomassie blue; lanes 2 and 3, the membranes incubated with monoclonal anti-cytokeratin and anti-actin antibodies, respectively. Arrowheads indicate the phosphorylation bands induced by calyculin A. (B) Immunoprecipitation of cytokeratins. Autoradiograms were prepared from SDS-PAGE (10%). Lane 1, proteins in the cytoskeletal fraction; lanes 2 and 3, immunoprecipitates with control and anti-cytokeratin antibodies, respectively.

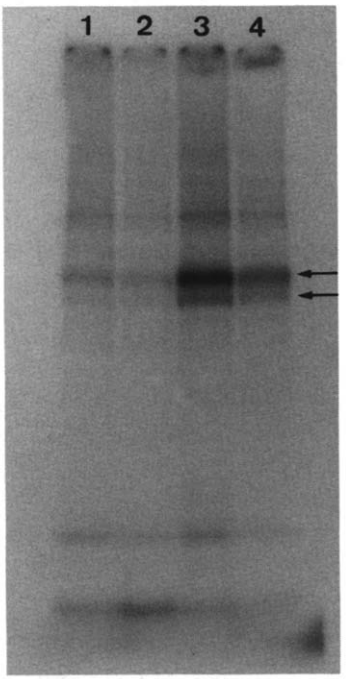

Fig. 6. Effects of K252a and calyculin A on protein phosphorylation in the cytoskeletal fraction of parotid acini. Proteins were separated by SDS-PAGE (5–20% gradient gel), and phosphorylated proteins were detected by autoradiography. Lane 1, control; lane 2, 3 μM K252a; lane 3, 100 nM calyculin A; lane 4, 3 μM K252a plus 100 nM calyculin A. Arrows indicate positions of the major cytokeratin bands.

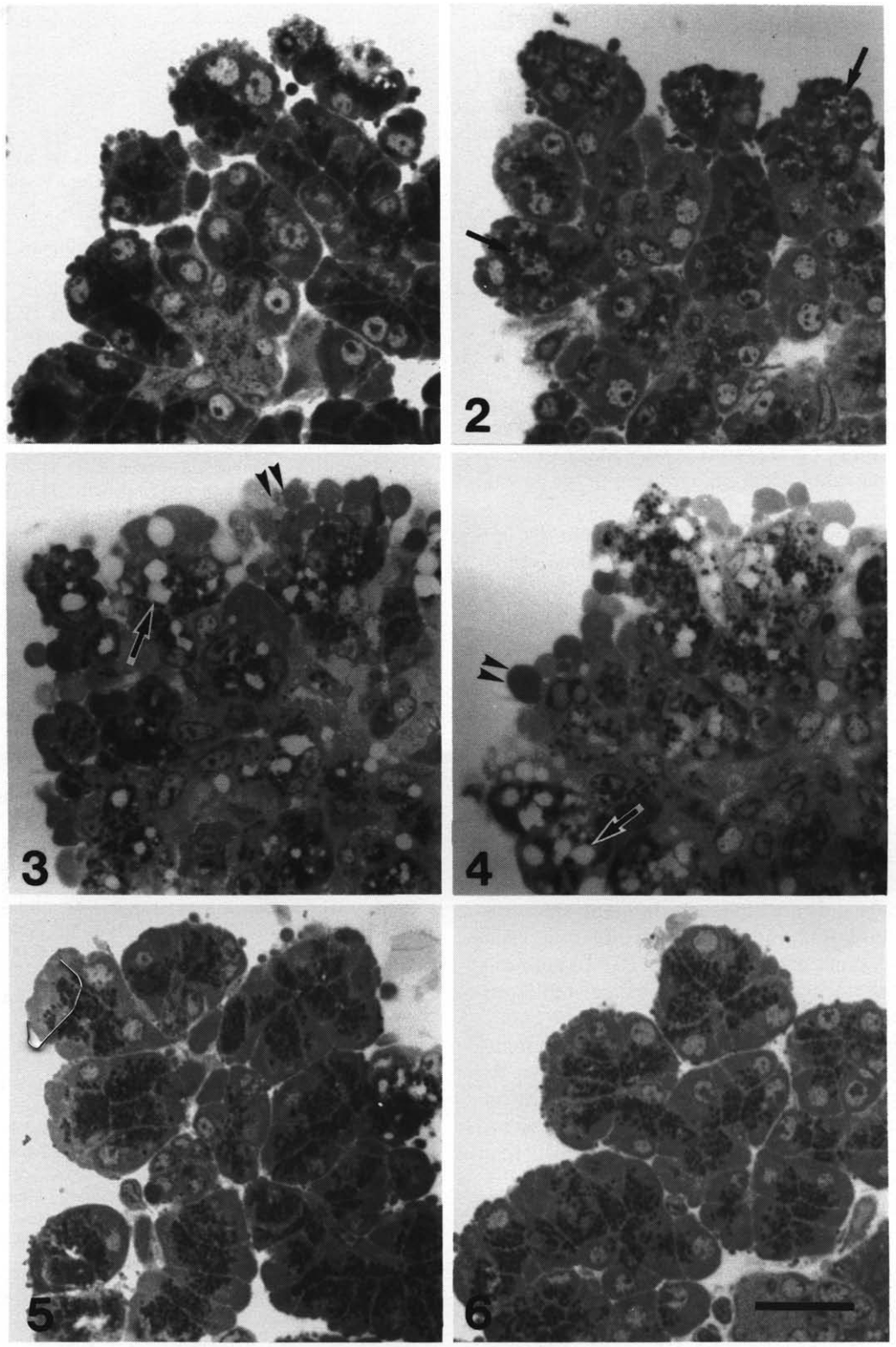

Fig. 7. Effect of calyculin A on the morphology of parotid acini. (1) Control; (2) 1 μM isoproterenol; (3) 100 nM calyculin A; (4) 1 μM isoproterenol plus 100 nM calyculin A; (5) 3 μM K252a; (6) 3 μM K252a plus 100 nM calyculin A. Simple arrows (in photo 2) show acinar lumina as they appear during exocytosis. Bars = 20 μm.

phosphorylation of cytokeratins in the 1% Triton X-insoluble fraction of parotid acinar cells. In contrast, cAMP had apparently no effect on protein phosphorylation in the cytoskeletal fraction, suggesting that the phosphorylation of cytokeratins is not involved in the normal process of cAMP-mediated amylase exocytosis. It has been amply demonstrated that the phosphorylation states of nonhelical terminal domains of intermediate filament proteins, including those of vimentin, desmin, and lamins, regulate the assembly and disassembly of these filaments [19–21]. Furthermore, hyperphosphorylation of cytokeratins has also been observed concurrently with reorganization of cytokeratin filaments in various cells [22–25] and in a cell-free system [26]. In this study K252a clearly inhibited both hyperphosphorylation of cytokeratins and morphological changes induced by calyculin A. Thus, the hyperphosphorylation of cytokeratins is very likely to be involved in the morphological changes seen in the parotid acini.

Previously, cytochalasin D, a microfilament-disrupting agent, inhibited amylase release and induced similar vacuoles in the rat parotid acini as seen in this study [14,15]. When acini were incubated with cytochalasin D and isoproterenol, however, the number of secretory granules decreased and the vacuoles were filled with secretory materials. Thus, it is recognized that the vacuoles are formed by enlargement of acinar lumina or intercellular canaliculi, and that the disruption of microfilaments itself does not inhibit exocytosis [14,15]. In contrast, even when acini were incubated with isoproterenol and calyculin A for 30 min, the vacuoles were not filled with the secretory materials. Furthermore, the origins of the vacuoles produced by calyculin A and cytochalasin D are obviously different, since protein phosphatase inhibitors induced cytoplasmic vacuoles in various types of single cells [12,27]. In addition, it has been well documented that colchicine, a microtubule- disrupting agent, does not inhibit amylase exocytosis from parotid glands stimulated by isoproterenol [13,28]. Taken together, these findings suggest that the reorganization of intermediate filaments induced by hyperphosphorylation of cytokeratins causes more harmful effects on cAMP-mediated amylase exocytosis than the disruption of microfilaments and microtubules. Further study is necessary to elucidate the precise role of intermediate filaments in exocytosis.

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