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Dephosphorylation of Cofilin in Parotid Acinar Cells[1]

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Cofilin is an actin-depolymerizing protein, whose depolymerizing activity is supposed to be regulated in part by phosphorylation and dephosphorylation. Thus, we studied the phosphorylation states of cofilin in rat parotid acinar cells during stimulation for amylase exocytosis. Isoproterenol and carbachol induced rapid and extensive dephosphorylation of cofilin; 60-70% dephosphorylation was clearly detectable within 1 min. Membrane-permeable cyclic AMP (CPS-cAMP), phorbol ester (PMA), and Ca ionophore A23187 mimicked the effect of isoproterenol and carbachol. Protein phosphatase inhibitors (calyculin A or FK506 plus cyclosporin A) did not block the dephosphorylation in response to isoproterenol or carbachol. Furthermore, calyculin A alone strongly dephosphorylated cofilin. Although no exogenous protein phosphatases tested dephosphorylated cofilin in the homogenate, the cofilin that was isolated by immunoprecipitation was clearly dephosphorylated by protein phosphatases 1, 2A, and 2C.

Key words: amylase exocytosis, cofilin dephosphorylation, parotid gland, protein phosphatase.

Amylase release from parotid acinar cells is a typical model of cyclic AMP-mediated exocytosis (*1*). We recently demonstrated that the catalytic subunit of cyclic AMP-dependent protein kinase induces amylase release from streptolysin O-permeabilized parotid acinar cells (*2*), although the phosphoprotein(s) responsible for amylase exocytosis has not identified. It has been repeatedly observed that isoproterenol or cAMP increases the phosphorylation of several proteins in parotid acini (*3-9*). Although some of those phosphoproteins (ribosomal S6 protein, and 21- and 26-kDa membrane proteins) were purified and characterized, their roles in amylase exocytosis remain uncertain (*10-14*). In contrast, a decrease in protein phosphorylation was also detected in parotid acini during stimulation with isoproterenol (*5, 8, 15*). In pancreatic acini, the dephosphorylation of some proteins correlated well with the onset of amylase release stimulated by carbachol and cholecystokinin (*16, 17*). Furthermore, cyclosporin A, an inhibitor of Ca and calmodulin-dependent protein phosphatase, was found to inhibit amylase release stimulated by those Ca-mobilizing agonists (*18*). Thus, we have recently focussed our attention on proteins dephosphorylated by secretory stimuli in parotid acini.

Actin microfilaments between the lumenal plasma membrane and secretory granules have long been considered to play an important role in exocytosis (*19*). According to the prevailing hypothesis (*20*), the machinery for membrane fusion in regulated exocytosis is mostly common to that in constitutive exocytosis or other intracellular vesicle traffic, but there are specific components that clamp membrane fusion until the arrival of secretory stimuli in regulated exocytosis. The actin microfilament is supposed to act as one such fusion clamp (*21*), since disassembly of actin filaments by exogenously added β-thymosin caused amylase release from streptolysin O-permeabilized pancreatic acini.

The assembly and disassembly of actin filaments is controlled by a range of actin binding proteins. Cofilin is a low molecular mass actin-depolymerizing protein originally isolated from porcine brain by DNase I affinity chromatography (*22*). Its actin-depolymerizing activity is pH-dependent (*23*), but Ca-independent, and is supposed to be regulated in part by phosphorylation and dephosphorylation as mentioned first for actin-depolymerizing factor (ADF), a protein closely related to cofilin (*24*). Recently, Davidson and Haslam (*25*) reported that cofilin was dephosphorylated in human platelets stimulated by thrombin. Thus, we examined the phosphorylation state of cofilin in parotid acini during amylase exocytosis evoked by isoproterenol and carbachol.

EXPERIMENTAL PROCEDURES

Materials—FK506 and cyclosporin A were gifts from Fujisawa (Osaka) and Sandoz (Basel, Switzerland), respectively. Collagenase (CLS II) was purchased from Worthington (Freehold, NJ, USA). Isoproterenol, carbachol, hyal-

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Abbreviations: cps-cAMP, 8-chlorophenylthio-cAMP; PMA, phorbol 12-myristate 13-acetate; PP, protein phosphatase; MEM; minimum essential medium; ISO, isoproterenol; CCH, carbachol.

uronidase (type I-S), trypsin (type III), trypsin inhibitor (type II-S), leupeptin, orthovanadate, Nonidet P-40, and phosphate-free minimum essential medium (MEM) were obtained from Sigma (St. Louis, MO). Normal MEM, and Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution were from GIBCO BRL (Gaithersburg, MD). [^{32}P]Orthophosphoric acid was from Dupont-Daiichi (Tokyo). Protein phosphatase 1 (rabbit skeletal muscle) and recombinant PP1γ were from Upstate Biotechnology (Lake Placid, NY), and Life Technologies (Gaithersburg, MD), respectively. 8-Chlorophenylthio-cAMP was from Boehringer Mannheim (Tokyo). Pefabloc SC was from Merck (Darmstadt, Germany). Polyvinylidene difluoride (PVDF) membranes (Immobilon) were from Millipore (Bedford, MA). The enhanced chemiluminescence (ECL) kit was from Amersham (Little Chalfont, England). Protein A-Sepharose CL-4B was from Pharmacia (Uppsala, Sweden). Calyculin A was from Wako (Osaka). All other chemicals utilized were of the highest grade commercially available.

Preparation of Parotid Acini—Rat parotid acini (small acini) were prepared as described previously (*26*). Parotid glands were minced finely and then incubated with trypsin (0.5 mg/ml) for 10 min at 37°C in minimum essential medium buffered with 20 mM Na-Hepes (pH 7.4) containing 0.1% BSA (MEM-H) under 100% O_2 in a metabolic shaker. The tissue was then washed once with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 2 mM EGTA, and subsequently incubated for 5 min with a trypsin inhibitor (0.5 mg/ml) in the same medium. After the tissue had been washed once with MEM-H, it was further incubated for 20 min in MEM-H containing collagenase (130 units/ml) and hyaluronidase (0.25 mg/ml). After digestion, the dispersed acini were filtered through two layers of medical gauze, washed 4 times with MEM-H, and then suspended in the same medium.

Protein Phosphorylation—Parotid acini were washed twice with phosphate-free MEM buffered with 20 mM Na-Hepes (pH 7.4) containing 0.1% BSA, and then incubated for 60 min at 37°C in the same medium containing [^{32}P]orthophosphoric acid (0.2-0.3 mCi/ml). The acini were washed once with the same medium without [^{32}P]-orthophosphoric acid, preincubated for 5-20 min with various phosphatase inhibitors, and further incubated for up to 10 min after the addition of isoproterenol, carbachol or another agonist.

Immunoprecipitation and Immunoblotting—After incubation as above, acini were pelleted and boiled for 5 min in 100 μl of lysis buffer comprising 140 mM NaCl, 20 mM Na-Hepes (pH 7.4), 10 mM EDTA, 1% SDS, 1% NP-40, 0.2 mM Pefabloc SC, and 10 μg/ml leupeptin. The lysate was diluted to 0.1% SDS with the lysis buffer without SDS, and then centrifuged at 15,000 rpm for 5 min at 4°C. An aliquot of the supernatant was removed and mixed with Laemmli buffer (*27*) to examine the total protein phosphorylation. The remaining supernatant was mixed with anti-cofilin antibody MAB-22 (*28*) and then rotated overnight at 4°C. The cofilin-MAB-22 complex was recovered with protein A-Sepharose beads and washed 5 times with the above buffer without SDS. The complex was dissolved in Laemmli buffer and boiled for 5 min.

The immunoprecipitate obtained with MAB-22 was resolved by SDS-PAGE on a 5-20% gradient gel (Atto, Tokyo). Proteins in the gel were transferred to a PVDF membrane at 100 mA per mini-gel (90×73×1 mm) for 60 min in a semi-dry blotter using 0.1 M Tris-0.192 M glycine buffer containing 5% methanol. The membrane was washed with water, and then the radioactivity of the membrane was determined with a radioactive image analyzer, BAS2000 (Fuji Film). Cofilin was visualized by immunoblotting with MAB-22 and the ECL system.

In Vitro Dephosphorylation of Cofilin in Homogenates—Acini prelabeled with [^{32}P]orthophosphoric acid as described above were homogenized with 100 mM Na-Hepes (pH 7.4), 1 mM EDTA, 0.2 mM Pefabloc SC, and 10 μg/ml leupeptin in a Teflon-glass homogenizer at 0°C. The homogenate was centrifuged at 123,000×g for 30 min. An aliquot (100 μl) of the supernatant was incubated at 37°C for up to 30 min with either 1 unit of protein phosphatase 1 (PP1), 8.3 μg of rabbit liver PP2A purified as described previously (*29*), 13 μg of bovine brain PP2B (*30*) with 10 μg of calmodulin and 2 mM $CaCl_2$, or 1.35 μg of recombinant PP2C (*31, 32*) with 10 mM $MgSO_4$. Calyculin A (1 μM) was included in the reaction mixture containing PP2B or PP2C. After incubation, an aliquot of each reaction mixture was removed and boiled with Laemmli buffer to examine the effects of protein phosphatases on total cytosolic proteins. Cofilin in the remaining reaction mixture was immunoprecipitated and immunoblotted as described above.

In Vitro Dephosphorylation Using Immunoprecipitated Cofilin—Acini prelabeled with [^{32}P]orthophosphoric acid for 60 min were lysed with 1% NP-40, 140 mM NaCl, 20 mM Na-Hepes (pH 7.4), 0.2 mM Pefabloc SC, and 10 μg/ml leupeptin. The lysate was centrifuged at 15,000 rpm for 5 min. Cofilin in the supernatant was immunoprecipitated with MAB-22 and protein A-Sepharose beads. The beads were washed 5 times with the lysis buffer and twice with the buffer without NP-40, and then suspended in the same buffer. The immunoprecipitated cofilin (30 μl) was incubated at 37°C for 30 min with the same concentrations of protein phosphatases as described above. After incubation the reaction mixture was boiled with a Laemmli cocktail for 5 min and then the radioactivity of cofilin was analyzed as above.

Assaying of PP2B Activity—Ca and calmodulin-dependent protein phosphatase (PP2B) activity in the supernatant fraction of the parotid gland was measured with [^{32}P]-RII peptide (type II regulatory subunit of cAMP-dependent protein kinase) as a substrate. The reaction mixture (50 μl) comprised 50 mM Mops (pH 7.4), 15 mM 2-mercaptoethanol, 0.5 μM okadaic acid, and 5 mM EGTA or 1 mM $MnCl_2$ plus 4 μg of calmodulin. After incubation at 30°C for 25 min, the reaction was terminated by the addition of 40 μl of 40% trichloroacetic acid. BSA (20 μl of 50 mg/ml) was immediately added as a carrier. The phosphate released was converted to its phosphomolybdic complex, and then extracted with isobutylalcohol/toluene (1 : 1, v/v) (*33*) and quantified with a liquid scintillation counter. For examination of the inhibitory effect of FK506 or cyclosporin A, the PP2B activity was measured with various concentrations of these compounds.

Amylase Release—Suspensions of parotid acini (1 ml) prepared as above were pipetted into 1.5-ml microcentrifuge tubes, each containing 10 μl of one of various secretagogues, and then incubated at 37°C for 20 min. After incubation, each tube was mixed and centrifuged at 10,000

rpm for 1 min, and then the supernatant was used for the amylase assay. For measurement of total amylase activity, acini were incubated with 0.2% Triton X-100 as above. Amylase activity was measured by the method of Bernfeld (*34*), and the released amylase activity is given as a percentage of the total activity.

RESULTS

Dephosphorylation of Cofilin by Secretory Agonists— When ^{32}P-prelabeled slices of parotid gland are incubated with isoproterenol (ISO), the dephosphorylation of certain proteins occurs, as was previously reported by several laboratories, although the molecular masses of the proteins seem to differ slightly from one laboratory to another (*5, 8, 15*). The parotid slices employed were composed of many different types of cells, including myoepithelial cells and cells of the connective tissue. Thus, we first confirmed the existence of dephosphorylated proteins in parotid acinar cells prepared by trypsin/EGTA/collagenase digestion (*26*). When the 123,000 × *g* supernatant fraction of the cell homogenate was resolved by SDS-PAGE, we detected a 20-kDa protein weakly dephosphorylated in response to ISO or carbachol (CCH) (data not shown).

To examine whether or not the protein dephosphorylated by ISO and CCH in parotid acini is cofilin, we immunoprecipitated cofilin with MAB-22 and analyzed its radioactivity with a BAS2000. As shown in Fig. 1, the radioactivity of the 20-kDa protein on 5-20% gradient gels was clearly decreased by 1 μM ISO and 10 μM CCH, and the radioactive bands were at exactly the same position as cofilin visualized by immunoblotting. Although a minor radioactive band was sometimes seen below the cofilin band, MAB-22 did not recognize this minor band. Thus we did not analyze this band further. The cofilin protein did not decrease in amount on treatment with ISO or CCH, indicating that the decrease in the radioactivity is not due to a decrease in the protein. This observation also implies that MAB-22 recognizes both the phosphorylated and dephosphorylated forms of cofilin.

The dephosphorylation of cofilin elicited by ISO and CCH was very rapid and clearly detected within 1 min after the addition of either agonist (Fig. 2). The dephosphorylation was more extensive on CCH (70-80%) than on ISO (60-70%) treatment. To clarify the intracellular second messengers of these agonists, we incubated parotid acini with 0.5 mM 8-chlorophenylthio-cAMP (cps-cAMP), 1 μM

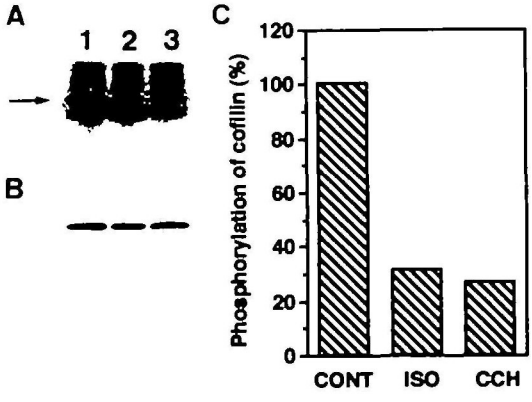

Fig. 1. **Effects of isoproterenol (ISO) and carbachol (CCH) on cofilin dephosphorylation.** Parotid acini were prelabeled with [^{32}P]orthophosphoric acid for 1 h, and then incubated with 1 μM ISO or 10 μM CCH for 10 min. Cofilin was immunoprecipitated with MAB-22, resolved by SDS-PAGE on 5-20% gradient gels, and then transferred to a PVDF membrane. The radioactivity of the membrane was analyzed with a BAS2000, and cofilin was visualized by immunoblotting with MAB-22 and the use of an ECL system. A: Autoradiograms. The arrow indicates the position of cofilin. Lanes: 1, control; 2, ISO; and 3, CCH. B: Immunoblot of cofilin on the same membrane as that used for autoradiography. C: Radioactivity of cofilin, as measured with the BAS2000.

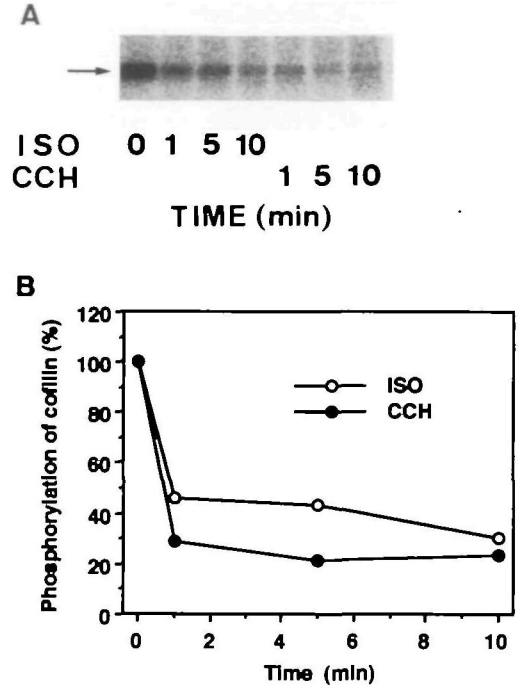

Fig. 2. **Time course of cofilin dephosphorylation by isoproterenol and carbachol.** ^{32}P-prelabeled parotid acini were incubated for 1-10 min with 1 μM ISO or 10 μM CCH. Cofilin in the acini was immunoprecipitated with MAB-22 and then analyzed with a BAS2000 as described in the legend to Fig. 1. A: Autoradiograms. The arrow indicates the position of cofilin. B: Radioactivity of cofilin.

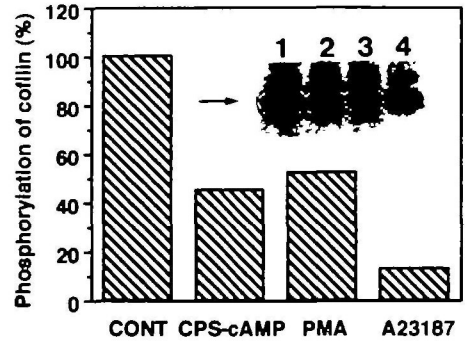

Fig. 3. **Effects of cAMP, phorbol ester and A23187 on cofilin dephosphorylation.** ^{32}P-prelabeled parotid acini were incubated for 10 min with 0.5 mM cps-cAMP, 1 μM PMA, or 1 μM A23187. Cofilin in the acini was immunoprecipitated with MAB-22 and then analyzed with a BAS2000. The insets are autoradiograms. The arrow indicates the position of cofilin. Lanes: 1, control; 2, cps-cAMP; 3, PMA; and 4, A23187.

phorbol 12-myristate 13-acetate (PMA), or 1 μM A23187 for 10 min. As can be seen in Fig. 3, all these agents induced cofilin dephosphorylation, A23187 showing the most prominent effect among them.

Effects of Protein Phosphatase Inhibitors—To identify the protein phosphatase engaged in the dephosphorylation of cofilin, we pretreated parotid acini with some protein phosphatase inhibitors and then incubated the acini with secretory agonists. As shown in Fig. 4, calyculin A, a specific inhibitor of PP1 and PP2A, by itself strongly stimulated cofilin dephosphorylation, although the phosphorylation states of many other proteins were markedly elevated, as reported previously (*35*).

To inhibit Ca and calmodulin-dependent protein phosphatase (PP2B) we preincubated acini with 1 μM FK506+1 μM cyclosporin A for 20 min (*18*), and then incubated them for an additional 10 min after the addition of 10 μM CCH or 1 μM ISO. One micromolar FK506 and 1 μM cyclosporin A separately inhibited PP2B activity by 50 and 72%, respectively, in the cytosolic fraction of rat parotid gland. Although these inhibitors appear to stimulate cofilin phosphorylation slightly (~30%), they did not block the dephosphorylation of cofilin by CCH (Fig. 4) or ISO (Fig. 5).

Effects of Agonists and Phosphatase Inhibitors on Amylase Release—As can be seen in Fig. 6, amylase exocytosis from parotid acini was stimulated by many agonists to various extents. The release caused by ISO or cps-cAMP was more than two times greater than that by CCH or PMA. The effect of A23187 was the weakest: less than one-half of that of CCH (Fig. 6A). On the other hand, calyculin A markedly inhibited amylase release stimulated by ISO or CCH (Fig. 6B), as observed previously (*35*). Preincubation with cyclosporin A plus FK506 for 20 min did not inhibit the amylase release stimulated by ISO, suggesting that PP2B is unlikely to be involved in cAMP-mediated amylase release, although the same treatment slightly attenuated the response to CCH (Fig. 6C). Thus, the magnitude of cofilin dephosphorylation was not necessarily correlated with that of amylase release.

In Vitro Dephosphorylation of Cofilin—To obtain direct evidence concerning the protein phosphatase responsible for the dephosphorylation of cofilin, we incubated a ^{32}P-labeled cytosolic fraction with various exogenous protein phosphatases. Although we used fairly high concentrations of enzymes and incubated them for up to 30 min, neither PP1, PP2A, PP2B, nor PP2C dephosphorylated cofilin in the presence of cytosolic proteins (Fig. 7). When total cytosolic proteins were analyzed, PP2B and PP2C clearly dephosphorylated some proteins other than cofilin (data not shown), indicating that the concentrations of protein phosphatases used were sufficient for dephosphorylation of these proteins. In the cell homogenate, calyculin A did not induce cofilin dephosphorylation. In addition, cofilin was not

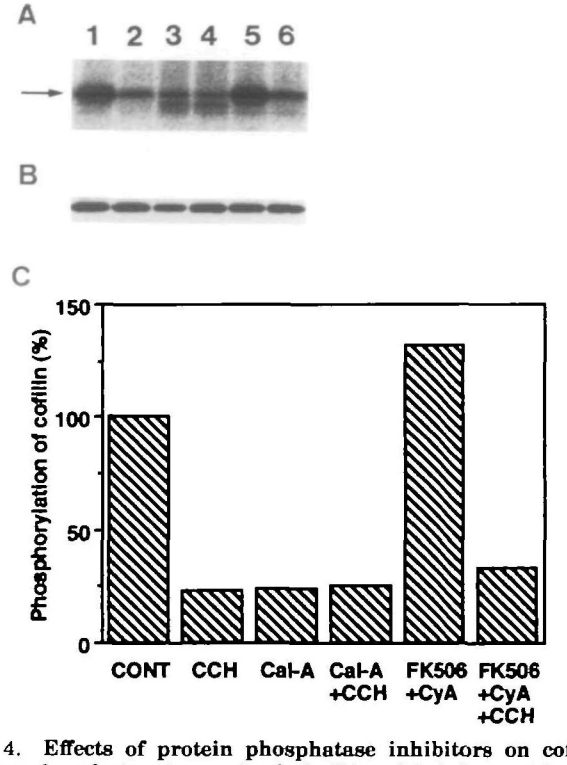

Fig. 4. **Effects of protein phosphatase inhibitors on cofilin dephosphorylation by carbachol.** ^{32}P-prelabeled parotid acini were preincubated for 5 min with 100 nM calyculin A or for 20 min with 1 μM FK506 plus 1 μM cyclosporin A, and then further incubated for 10 min after the addition of 10 μM CCH. A: Autoradiograms. The arrow indicates the position of cofilin. Lanes: 1, control; 2, CCH; 3, calyculin A (Cal-A) alone; 4, calyculin A and CCH; 5, FK506+cyclosporin A (CyA); and 6, FK506+cyclosporin A and CCH. B: Immunoblot of cofilin on the same membrane as that used for autoradiography. C: Radioactivity of cofilin.

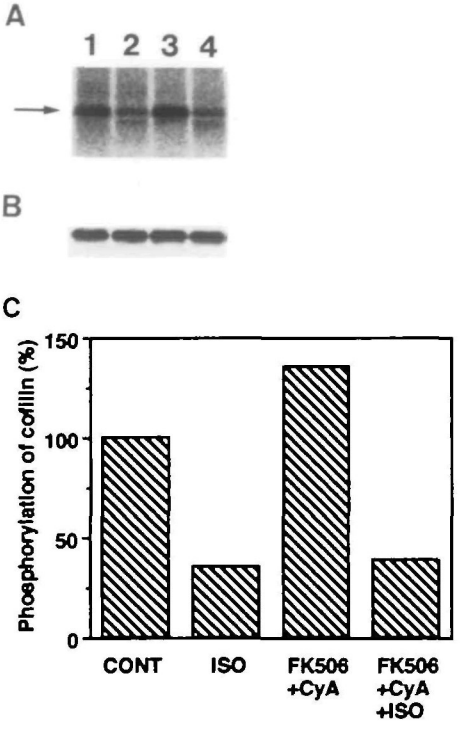

Fig. 5. **Effects of FK506 and cyclosporin A on cofilin dephosphorylation by isoproterenol.** ^{32}P-prelabeled parotid acini were preincubated for 20 min with 1 μM FK506 plus 1 μM cyclosporin A, and then further incubated for 10 min after the addition of 1 μM ISO. A: Autoradiograms. The arrow indicates the position of cofilin. Lanes: 1, control; 2, ISO; 3, FK506+cyclosporin A (CyA); and 4, FK506+cyclosporin A and ISO. B: Immunoblot of cofilin on the same membrane as that used for autoradiography. C: Radioactivity of cofilin.

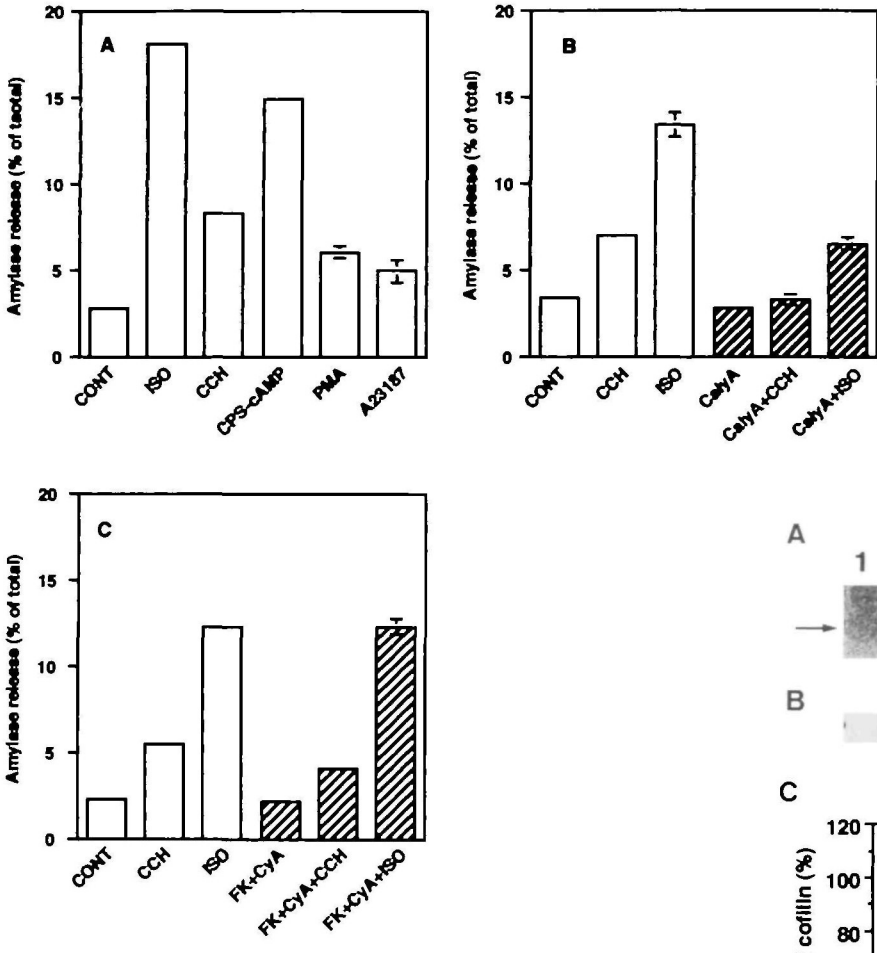

Fig. 6. **Effects of various agonists and protein phosphatase inhibitors on amylase release from parotid acini.** (A) Acini were incubated at 37°C for 20 min with 1 μM ISO, 10 μM CCH, 1 mM cps-cAMP, 1 μM PMA, or 1 μM A23187. (B) Acini preincubated for 5 min in the presence or absence of 100 nM calyculin A (CalyA) were further incubated for 20 min after the addition of 10 μM CCH or 1 μM ISO. (C) Acini were preincubated with 1 μM FK506 (FK) plus 1 μM cyclosporin A (CyA) for 20 min, and then incubated with CCH or ISO for another 20 min. The data shown are means ± SD (n = 3).

dephosphorylated on the addition of protein kinase A or Ca to the homogenate containing cold 1 mM Mg-ATP (data not shown).

To eliminate unknown factors influencing protein phosphorylation and dephosphorylation in the cytosolic fraction, we isolated cofilin by immunoprecipitation and then incubated it with protein phosphatases as above. As can be seen in Fig. 8, PP1, PP2A, and PP2C clearly dephosphorylated cofilin in the absence of cytosolic proteins. The effect of PP2B was fairly weak, if any, compared with the other phosphatases.

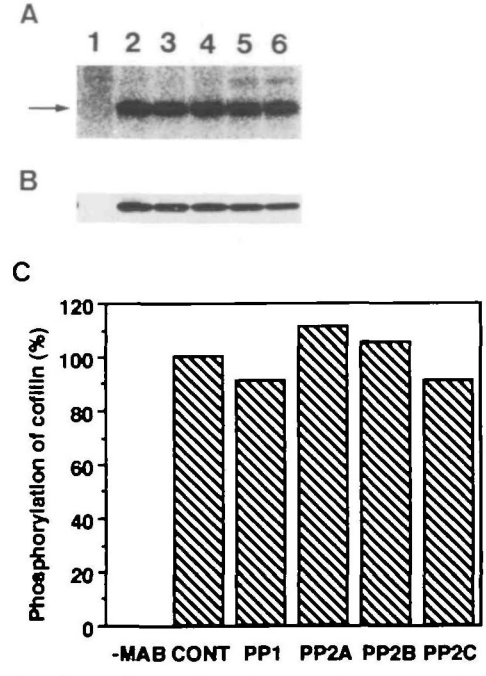

Fig. 7. ***In vitro* effects of various protein phosphatases on cofilin dephosphorylation in the soluble fraction of parotid acinar cells.** ^{32}P-prelabeled parotid acini were homogenized and then centrifuged at 123,000 × g for 30 min. The supernatant was incubated for 30 min at 37°C with either PP1, PP2A, PP2B or PP2C. After incubation, cofilin was immunoprecipitated with MAB-22 and then analyzed with a BAS2000. A: Autoradiograms of immunoprecipitates. The arrow indicates the position of cofilin. Lanes: 1, control sample without addition of MAB-22; 2, control immunoprecipitated with MAB-22; 3, PP1; 4, PP2A; 5, PP2B; and 6, PP2C. B: Immunoblot of cofilin on the same membrane as that used for autoradiography. C: Radioactivity of cofilin.

DISCUSSION

Dephosphorylation of Cofilin—In the present study, we have clearly shown that cofilin was extensively dephosphorylated by various secretory agonists, including ISO, CCH, cAMP, PMA, and Ca, in parotid acinar cells *in vivo*. Cofilin dephosphorylation was first observed when fibroblasts were exposed to heat shock at 43°C or to 10% dimethyl sulfoxide (DMSO) (*36*). Thereafter, cofilin dephosphorylation was detected in various cells in response to a wide variety of stimuli (*25, 37-40*). The significance of cofilin dephosphorylation was proposed from the results of a study on actindepolymerizing factor (ADF), a protein closely related to cofilin (*24*). Namely, phosphorylated ADF could not bind to actin, and hence was unable to depolymerize actin filaments or to prevent actin polymerization through the sequestration of actin monomers. Therefore, the phosphorylated ADF is regarded as an inactive form, and ADF is thus activated by dephosphorylation. Although direct evidence has not been reported yet, a similar interpretation of cofilin dephosphorylation is well compatible with several lines of circumstantial evidence, as described below.

Although cofilin was markedly dephosphorylated through various secretory stimuli, the magnitude of the dephosphorylation was not necessarily correlated with that of amylase

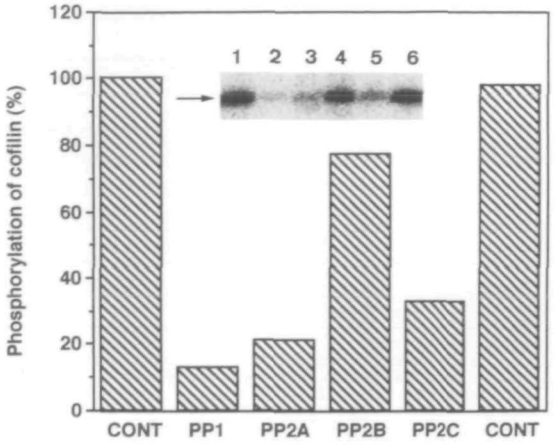

Fig. 8. **Effects of various protein phosphatases on cofilin dephosphorylation in the absence of cytosolic proteins.** ^{32}P-prelabeled cofilin was immunoprecipitated with MAB-22 and protein A-Sepharose beads. The cofilin immunoprecipitated was incubated for 30 min at 37°C with either PP1, PP2A, PP2B, or PP2C. After incubation, the radioactivity of cofilin was analyzed with a BAS2000. The insets are autoradiograms. The arrow indicates the position of cofilin. Lanes: 1 and 6, control; 2, PP1; 3, PP2A; 4, PP2B; and 5, PP2C.

exocytosis, as can be seen in Fig. 6, but rather well correlated with the extent of the morphological change induced by these stimuli. CCH is a moderate stimulus for amylase release, but strongly induces morphological changes, which would be related to electrolytes and fluid secretion (*19, 41*). As reported previously (*35*), calyculin A produces cytoplasmic vacuoles and large blebs on the basolateral plasma membrane in addition to hyperphosphorylation of cytokeratins. In contrast, ISO is the most potent agonist for amylase release, but morphological changes during exocytosis are relatively small. In human platelets, cofilin dephosphorylation caused by thrombin corresponded better to platelet aggregation than to 5-hydroxytryptamine release (*25*). In addition, in thyroid cells, cofilin dephosphorylation and rearrangement of cytoskeletal proteins were concomitantly induced by thyrotropin, cAMP and phorbol ester (*38*). These results suggest that cofilin dephosphorylation is deeply involved in the morphological changes accompanying the rearrangement of actin filaments.

Actin microfilaments are supposed to serve as fusion-clamps for regulated exocytosis, since partial disruption of actin filaments by β-thymosin evoked amylase release from streptolysin O-permeabilized pancreatic acini (*21*). In those experiments, however, excessive actin depolymerization showed a rather inhibitory effect, suggesting that actin filaments have an additional function (a positive role) in exocytosis. Thus, the magnitude of exocytosis and that of actin depolymerization are not simply correlated. In the present study, treatment of parotid acini with CCH, A23187, or calyculin A might have induced hyper-depolymerization of actin filaments for amylase exocytosis, since ISO-induced amylase release was greatly reduced by calyculin A (*35*) or carbachol (*42*).

Protein Phosphatase Responsible for Cofilin Dephosphorylation—To identify the protein phosphatase responsible for cofilin dephosphorylation, we utilized two separate approaches, *i.e.* an *in vivo* study involving protein phosphatase inhibitors and an *in vitro* study involving various exogenous protein phosphatases. Calyculin A, a specific inhibitor for PP1 and PP2A, did not prevent the dephosphorylation of cofilin, but rather stimulated the dephosphorylation by itself. PP1 and PP2A were really inhibited by calyculin A, since the phosphorylation state of many other proteins was markedly elevated. This suggests two possibilities: (1) the inhibition of PP1 and PP2A is insufficient to block cofilin dephosphorylation, and (2) calyculin A increases the phosphorylation of some protein(s) directly or indirectly involved in the dephosphorylation of cofilin. Similarly, cyclosporin A and FK506, specific inhibitors of PP2B, did not block the cofilin dephosphorylation. In addition, these inhibitors did not prevent cAMP-mediated amylase exocytosis, although cyclosporin A inhibited Ca-induced amylase release from pancreatic acini (*18*). Thus, PP2B is unlikely to be involved in the regulation of cAMP-mediated amylase release. It is presently unknown why these compounds slightly increased the phosphorylation of cofilin. In contrast to the effect of calyculin A, among the total proteins resolved by SDS-PAGE we could detect no protein whose phosphorylation was increased by the PP2B inhibitors. This suggests that the phosphoproteins dephosphorylated by PP2B in the parotid acini are rather limited.

Although cofilin in intact cells was rapidly dephosphorylated through various stimuli, cofilin in the homogenate was very stable and hardly dephosphorylated by the exogenous protein phosphatases tested. Furthermore, cofilin was scarcely dephosphorylated on the addition of calyculin A, protein kinase A or Ca to the homogenate containing cold 1 mM Mg-ATP. However, the cofilin that was isolated by immunoprecipitation was clearly dephosphorylated by PP1, PP2A, and PP2C (Fig. 8). The dephosphorylation by PP2B was rather weak, if any. These results suggest that cofilin and its phosphatases are closely associated in intact cells but not in the homogenate. It is also suggested that the phosphorylation state of cofilin in the homogenate is stabilized by some cytosolic components. Although PP1, PP2A, and PP2C dephosphorylated cofilin *in vitro*, it is presently unknown which protein phosphatase(s) are really engaged in the dephosphorylation in intact cells. Since cofilin is dephosphorylated even in the presence of calyculin A, PP2C or closely related phosphatases are very likely to be involved in this process.

In Conclusion—We here clearly demonstrated that various secretory stimuli cause the dephosphorylation of cofilin in parotid acini, although the magnitude of amylase exocytosis and that of cofilin dephosphorylation are not necessarily correlated. Cofilin was rapidly dephosphorylated through various stimuli *in vivo*, but hardly dephosphorylated by any of the protein phosphatases tested in the homogenate. In the absence of cytosolic proteins, however, PP1, PP2A, and PP2C clearly dephosphorylated cofilin. Further study is necessary to identify the protein phosphatase(s) responsible for cofilin dephosphorylation in response to secretory stimuli.

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