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Regulation of CapZ, an Actin Capping Protein of Chicken Muscle, by Anionic Phospholipids†

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ABSTRACT: Chicken muscle CapZ, a member of the capping protein family of actin-binding proteins, binds to the barbed end of actin filaments and nucleates actin polymerization. No regulation of the capping protein family has been described. We report that micelles of phosphatidylinositol 4,5-bisphosphate (PIP_2) bind to CapZ and completely inhibit its ability to affect actin polymerization as measured by several independent assays. Higher concentrations of other anionic phospholipids also completely inhibit the activity of CapZ. Neutral phospholipids have no effect. Mixed vesicles of PIP_2 with phosphatidylcholine or phosphatidylethanolamine also inhibit CapZ, but addition of Triton X-100 both prevents and reverses PIP_2's inhibition of CapZ.

CapZ is an actin-binding protein that is located at the Z line in skeletal muscle and binds to the barbed ends of actin filaments in vitro (Casella et al., 1986). It is a member of the capping protein family which are α/β heterodimers that bind the barbed end and do not require Ca^{2+} for activity (Pollard & Cooper, 1986). Capping proteins have been found in all eukaryotic cells examined to date and have been purified from *Acanthamoeba* (Isenberg et al., 1980; Cooper et al., 1984), *Dictyostelium* (Schleicher et al., 1984), bovine brain (Kilimann & Isenberg, 1982), chicken skeletal muscle (Casella et al., 1986; Caldwell et al., 1989a), *Xenopus laevis* (Ankenbauer et al., 1989), and *Saccharomyces cerevisiae*.[1] Capping protein genes (two α and one β) are expressed in all of many chicken

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[1] J. F. Amatruda and J. A. Cooper, unpublished data (1991).

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muscle and nonmuscle tissues (Caldwell et al., 1989b; Cooper et al., 1991). Deletion of the gene for the β subunit of capping protein in yeast leads to an altered actin cytoskeleton (Amatruda et al., 1990).

CapZ accelerates the nucleation of actin polymerization from monomers and binds the barbed ends of actin filaments with high affinity, preventing actin monomer exchange at the end (Caldwell et al., 1989a). The actin filaments associated with CapZ in skeletal muscle cells are stable, but the actin filaments in nonmuscle cells are dynamic, expecially during locomotion or cell shape changes (Wang, 1985; Casella et al., 1981; Carson et al., 1986). Since capping protein is present in all nonmuscle cells, we hypothesize that regulation of capping protein may regulate actin polymerization. No method of regulation has been described for a member of the capping protein family. This study examines the regulation of CapZ activity by polyphosphoinositides and other phospholipids in vitro, including the effects of diluting phosphatidylinositol 4,5-bisphosphate (PIP_2)[2] in mixed vesicle and mixed micelle systems.

Materials and Methods

Except as stated otherwise, chemicals and chromatography resins were obtained from Sigma Chemical Co. (St. Louis, MO); solvents and supplies were from Fisher Scientific (St. Louis, MO).

Preparation of Actin. Actin was prepared from chicken skeletal muscle as described (Spudich & Watt, 1971), gel-filtered (MacLean-Fletcher & Pollard, 1980), lyophilized in Ca^{2+} buffer G (2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.1 mM DTT, and 1 mM NaN_3) with 2 mg of sucrose/mg of actin, and stored at −20 °C. Actin was dialyzed against Ca^{2+} buffer G overnight and centrifuged at 100000*g* for 30 min before use. Pyrene-labeled actin was prepared (Cooper et al., 1983) for use in fluorometer-based assays and stored lyophilized as described previously.

Preparation of CapZ. Chicken breasts were obtained from a local abattoir (Levin Poultry, St. Louis, MO), and CapZ was purified as previously described (Caldwell et al., 1989a). The CapZ was stored at −70 °C in equal volumes of buffer B (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1 mM 2-mercaptoethanol, 1 mM NaN_3, 0.5 mM EDTA, and 0.1 mM PMSF) and glycerol. Before use, the CapZ was dialyzed overnight versus buffer B. After dialysis, the CapZ retained about 80% of its activity, as determined by a depolymerization assay. CapZ was found to have significant adsorption to polypropylene when stored at low concentrations (≤600 nM) for several hours in buffer B, as measured by a depolymerization assay. The adsorption was eliminated by siliconizing the tubes or including 200 mM KCl in the buffer. Siliconized tubes adsorbed phospholipids, so experiments with phospholipids used short incubation times (≤5 min) in polypropylene tubes.

Preparation of Lipids. PIP_2, phosphatidylinositol 4-monophosphate (PIP), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA) were used without further purification. PC, PI, PE, PS, and PG, which were supplied as solutions in chloroform/methanol, were dried under a stream of nitrogen. Water was added to the samples, and they were sonicated for 15 min at 25 °C with a Virsonic 300 sonicator (Virtis Co., Gardiner, NY). PIP_2, PIP, and PA were dissolved in water and sonicated as above. The suspensions were frozen in aliquots in liquid nitrogen and stored at −70 °C. Before use, the aliquots were thawed and sonicated for 15 min at 25 °C in a water bath sonicator.

Gel Filtration Assay. One hundred eighty-five microliters of 2.75 μM CapZ, 861 μM PIP_2, or both in buffer B, was incubated for 15 min at room temperature. The mixtures were chromatographed on a 1 × 30 cm column of Superose 12 HR 10/30 on an FPLC system (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) at 0.5 mL/min at 4 °C; 0.5-mL fractions were collected and analyzed for the presence of CapZ by immunoblots (Towbin et al., 1979) from SDS–12.5% polyacrylamide gels (Laemmli, 1970). The nitrocellulose filter was blocked in 20 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.01% NaN_3, and 0.05% Tween 20 (TTBS) with 2% fish gelatin and 1% horse serum. The primary antibody was a goat polyclonal antiserum, prepared against purified CapZ α and β, diluted 1:5000 in blocking buffer.[3] Following incubation overnight at 4 °C, the filter was washed with TTBS and incubated with alkaline phosphatase conjugated, affinity-isolated swine anti-goat immunoglobulins (Tago Inc., Burlingame, CA) in TTBS and 1% swine serum for 1.5 h. The filter was washed in TTBS and developed as described (Ey & Ashman, 1986).

The fractions were also assayed for PIP_2 with a modification of a malachite green based inorganic phosphate assay (Selden & Pollard, 1983). Fifty microliters of each fraction was mixed with 25 μL of 10% $MgNO_3 \cdot H_2O$ in 95% ethyl alcohol in a borosilicate glass tube and dried down in a 110 °C oven overnight. The samples were ashed by holding over a Bunsen burner until the residue turned white. After being cooled, 300 μL of 1.2 N HCl was added to each tube, followed by 300 μL of AMMG reagent. The AMMG reagent was freshly made from 3 parts 0.045% malachite green in H_2O and 1 part 4.2% ammonium molybdate in 4 M HCl. After 5 min the absorbance at 660 nm was read.

Falling-Ball Viscometry Assay. The effect of PIP_2 on the interaction of CapZ with actin was measured by falling-ball viscometry (Pollard & Cooper, 1982). One hundred microliters of polymerized actin in buffer G with MKEI (0.1 M KCL, 2 mM $MgCl_2$, 1 mM EGTA, and 20 mM imidazole hydrochloride) was mixed with 80 μL of buffer B with or without CapZ and 10 μL of PIP_2 diluted in H_2O. The mixtures were incubated in glass capillary tubes for 40 min before the viscosity was determined. The final concentrations were 3.7 μM actin, 12 nM CapZ, and 0–48 μM PIP_2.

Nucleation Assay. The polymerization of actin from monomers was determined from the fluorescence of 5% pyrene-labeled actin as described (Cooper et al., 1983), using a FluoroMax spectrofluorometer (SPEX Industries, Edison, NJ). CapZ (61 nM) was incubated with the PIP_2 (0.18–18.0 μM) in buffer G in the fluorometer cuvette for 2 min before actin (5 μM) was added. After a further 5 min, MKEI was added from a 10× stock, and the pyrenyl actin fluorescence was measured over time.

Polymerization Assay. This assay is essentially like the nucleation assay except that the CapZ concentration was 20 nM and the PIP_2 (20 μM) was added to the cuvette either 100 or 300 s after the MKEI.

Depolymerization Assay. The assay was modified from prior published protocols (Bryan, 1986; Caldwell et al., 1989a)

[2] Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; PA, phosphatidic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[3] D. A. Schafer and J. A. Cooper, manuscript in preparation (1991).

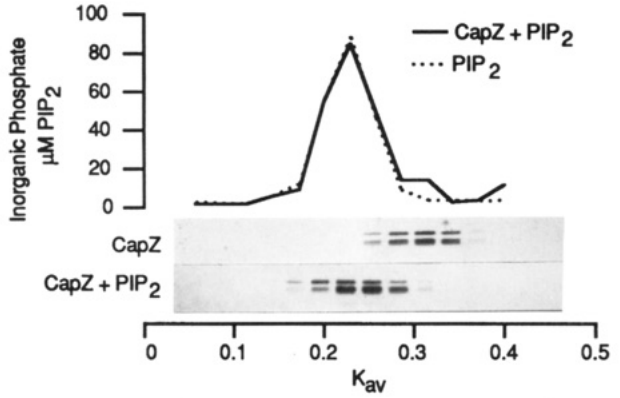

FIGURE 1: Binding of CapZ to PIP_2 micelles detected by gel filtration chromatography. The upper panel represents the elution profile for PIP_2, as measured by an inorganic phosphate assay, in the presence and absence of CapZ. The lower panel is an anti-CapZ immunoblot of the fractions for CapZ in the presence and absence of PIP_2. The upper band in each doublet represents the α subunit of CapZ, and the lower band represents the β subunit.

by diluting the actin into buffer G, which favors depolymerization. This allows the rapid assessment of capping activity by CapZ in the presence of various lipids. CapZ in buffer B was incubated with various lipids diluted in H_2O for 2–3 min before the mixture was added to an equal volume of polymerized actin in buffer G + MKEI. This mixture was incubated 5 min. During this incubation, the CapZ concentration was 60–62 nM, the actin concentration was 2.5 μM (25% pyrene-labeled), and the lipid concentrations were as shown for each experiment. At $t = 0$, the mixture was diluted 50-fold in buffer G (final actin concentration 0.05 μM), and the pyrene-labeled actin fluorescence was followed for at least 150 s.

The fraction of CapZ activity for the depolymerization assays was computed in the following manner. Within one experiment, all fluorescence curves were normalized to the same initial fluorescence to account for variability in pipeting the polymerized actin (typically within 15%). No capping activity was defined as the fluorescence signal at 150 s for depolymerization with no CapZ (F_0), because the actin was completely depolymerized within this time; 100% capping activity was defined as the fluorescence at 150 s with 60–66 nM CapZ and no lipids (F_{100}). "Fraction capped" was defined as (the fluorescence at 150 s $- F_0)/(F_{100} - F_0)$. A standard curve of "fraction capped" versus CapZ concentration was created to establish percent CapZ activity in the presence of phospholipids.

Results

Binding of CapZ to PIP_2. The binding of CapZ to PIP_2 was examined qualitatively by gel filtration chromatography (Figure 1) on Superose 12 in buffer B. In aqueous solutions, PIP_2 forms micelles with relative molecular mass 93 000 daltons, aggregation number 82 (Sugiura, 1981). PIP_2 eluted at K_{av} 0.23 as measured by an inorganic phosphate assay of the column fractions (upper panel). CapZ alone eluted with a peak at K_{av} 0.31 by immunoblot (lower panel) of column fractions. When CapZ was mixed with a 310-fold molar excess of PIP_2, the CapZ coeluted with the PIP_2 at K_{av} 0.24, as determined by immunoblot. The elution of the α subunit (M_r 36 000) of CapZ (upper band of each doublet) appeared to be shifted slightly further toward the void volume than the β subunit (M_r 32 000). This suggests that PIP_2 binds the α subunit and decreases the affinity of the subunits for each other.

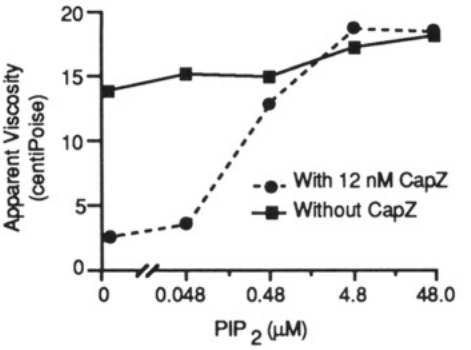

FIGURE 2: Effect of PIP_2 on the ability of CapZ to decrease the low-shear viscosity of filaments, measured by falling-ball viscometry. Apparent viscosity is plotted versus final concentration of PIP_2. The final concentrations were as follows: actin, 3.6 μM; CapZ, 12 nM.

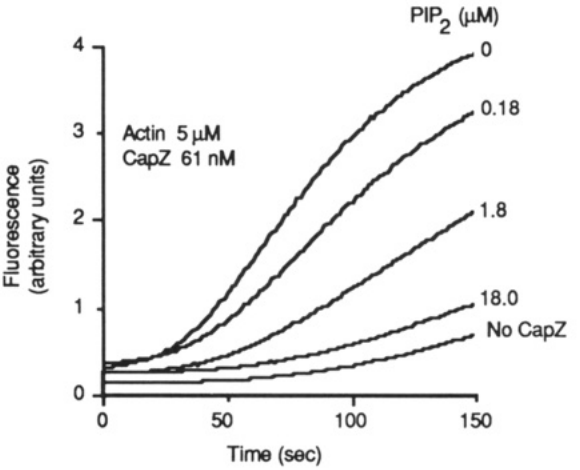

FIGURE 3: Effect of PIP_2 on the ability of CapZ to nucleate actin polymerization. 61 nM CapZ was incubated with PIP_2; then actin was added at time zero. The fluorescence of pyrene-labeled actin is plotted versus time. In a control experiment, PIP_2 had no effect on actin polymerization in the absence of CapZ (data not shown).

Effect of PIP_2 on the Ability of CapZ To Decrease the Viscosity of Actin Filaments. The ability of CapZ to decrease the average length of actin filaments in solution, thus reducing the apparent viscosity, was measured by falling-ball viscometry (Pollard & Cooper, 1982). A 40-fold molar excess of PIP_2 was found to substantially inhibit the viscosity-lowering property of 12 nM CapZ, and a 400-fold excess eliminated the effect of CapZ (Figure 2). The slight rise in viscosity with increased PIP_2, in the absence of CapZ, may have been due to trace contamination of the gel-filtered actin with CapZ (Casella & Maack, 1987).

Effect of PIP_2 on the Ability of CapZ To Nucleate Actin Polymerization. Previous work with CapZ (Caldwell et al., 1989a) and *Acanthamoeba* capping protein (Cooper & Pollard, 1985) showed an increased rate of polymerization from actin monomers that was consistent with an increased nucleation rate. When monomeric actin in buffer G was polymerized with MKEI, there was an initial lag period, which was decreased by CapZ (Caldwell et al., 1989a), but when PIP_2 was added to the CapZ before polymerization began, the ability of CapZ to accelerate nucleation was eliminated (Figure 3). This is consistent with the PIP_2 inhibiting the stabilization of actin nuclei by CapZ.

Effect of PIP_2 on the Ability of CapZ To Cap Actin Filaments. The capping of actin filaments was also measured with a depolymerization assay. In this assay, the actin filaments were capped with CapZ before being depolymerized in buffer G. Incubating the actin with 60 nM CapZ (Figure 4,

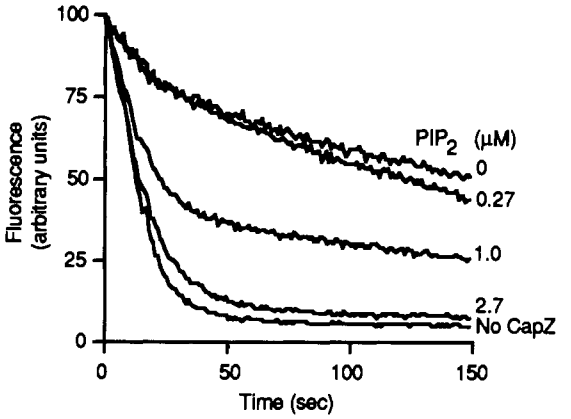

FIGURE 4: Inhibition of CapZ by PIP_2 in an actin depolymerization assay. The fluorescence of pyrene-labeled actin was plotted versus time, with each curve normalized to the same initial fluorescence. The PIP_2 concentrations shown represent the concentration in the final incubation before depolymerization. The CapZ concentration in this mixture was 62 nM, and the actin concentration was 2.5 μM.

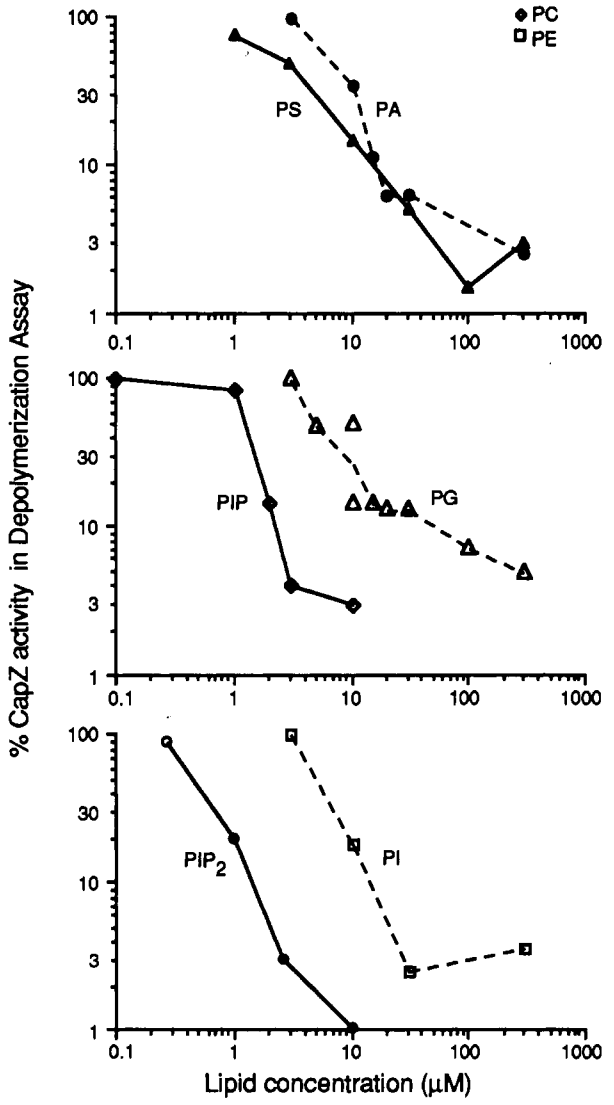

FIGURE 5: Comparison of the effect of different phospholipids on the activity of CapZ in the depolymerization assay. All concentrations shown are those in the final incubation before depolymerization. For each phospholipid, data like those in Figure 2 were used to calculate the fraction of CapZ activity as described under Materials and Methods.

upper curve) slowed depolymerization, and this effect was inhibited by first incubating the CapZ with PIP_2.

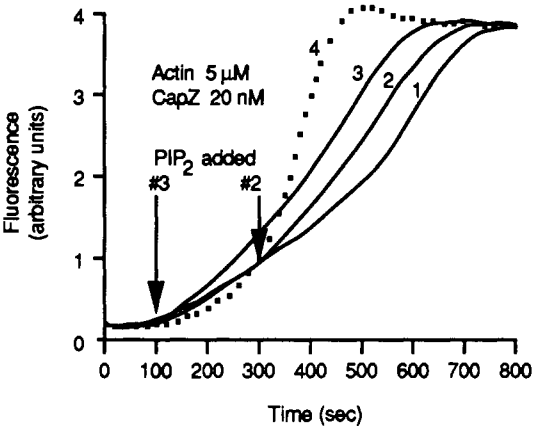

FIGURE 6: Ability of PIP_2 to remove CapZ bound to barbed ends. 20 nM CapZ and actin were in the cuvette when MKEI buffer was added at $t = 0$. Curve 1, no PIP_2 added; curve 2, 20 μM PIP_2 added at $t = 300$ s; curve 3, 20 μM PIP_2 added at $t = 100$ s; curve 4, actin alone. Curves were normalized to same final fluorescence. The solution was stirred continuously, which accounts for the rapid elongation phase of curve 4.

Comparison of Different Phospholipids. Other phospholipids were also tested with the depolymerization assay. Curves such as those in Figure 4 were compared to curves obtained with varying amounts of CapZ to calculate the percent CapZ activity in the presence of the different phospholipids (Figure 5). PIP is nearly as effective as PIP_2 at inhibiting CapZ, and other anionic phospholipids including PI, PS, PA, and PG can also inhibit the capping activity of CapZ, albeit at higher phospholipid concentrations. No activity was seen with the neutral phospholipids PC and PE.

Effect of PIP_2 on CapZ Bound to Actin Filaments. When actin is polymerizing onto free filaments, the majority of the growth occurs at the barbed end where the addition of subunits is highly favored (Pollard, 1986). CapZ should decrease the apparent polymerization rate by preventing subunit addition at the barbed end. In an experiment involving actin polymerization from monomers in the presence of CapZ, PIP_2 was added to the mixture after polymerization had begun. Though the CapZ decreased the lag period before polymerization began, the maximal rate of polymerization was decreased below that of actin alone (Figure 6, curve 1). When PIP_2 was added during polymerization, the rate of polymerization increased (Figure 6, curves 2 and 3). Also, in a depolymerization assay like that shown in Figure 4, the addition of PIP_2 to CapZ that had been incubated with the actin filaments resulted in a partial inhibition of CapZ activity (data not shown).

Reversibility of CapZ–PIP_2 Interaction. The inhibition of CapZ by PIP_2 can be reversed by Triton X-100. In a depolymerization experiment, 20 μM PIP_2 caused full inhibition of 66 nM CapZ activity (Figure 7). When 600 μM Triton X-100 was added after PIP_2 was allowed to bind CapZ, the CapZ regained the ability to cap actin filaments.

Dilution Effects of Other Phospholipids or Detergent on PIP_2 Inhibition of CapZ. PIP_2 inhibition of CapZ was also examined in the depolymerization assay with the PIP_2 present in mixed vesicles with PC, PE, or Triton X-100 (Figure 8). Dilution of PIP_2 with a 30-fold molar excess of PC or PE had a slight effect on its ability to inhibit CapZ. On the other hand, a 20-fold molar excess of Triton X-100 prevented inhibition of CapZ by PIP_2.

DISCUSSION

The results in this study provide the first description of regulation for capping protein, a ubiquitous actin-binding protein that caps barbed ends and nucleates filament forma-

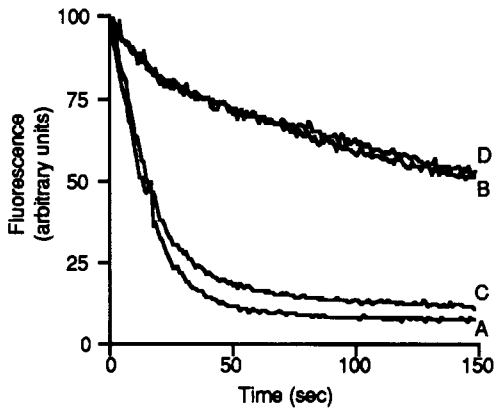

FIGURE 7: PIP_2 inhibition of CapZ is reversed by Triton X-100. In this depolymerization assay, 2.5 μM actin filaments (25% pyrene-labeled) were diluted to 0.05 μM in buffer G at $t = 0$. The pyrenylactin fluorescence is plotted versus time. The curves are (A) actin alone, (B) CapZ (66 nM) preincubated with actin filaments at $t = -5$ min, (C) PIP_2 (20 μM) mixed with CapZ at $t = -7$ min and then preincubated with actin filaments at $t = -5$ min, and (D) CapZ mixed with PIP_2 at $t = -12$ min [Triton X-100 (600 μM) added at $t = -7$ min] and then preincubated with actin filaments at $t = -5$ min. All concentrations are those in final incubation before depolymerization.

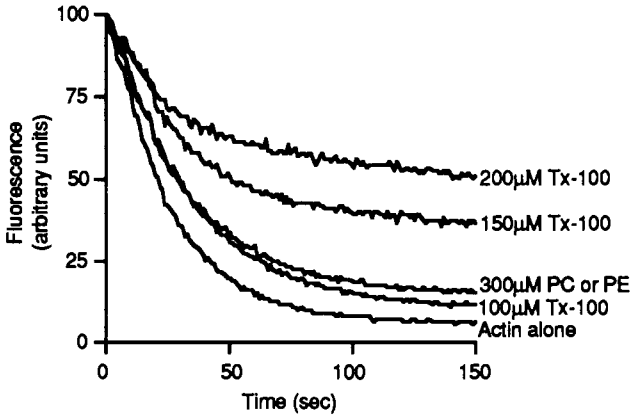

FIGURE 8: Dilution effects of other phospholipids or detergent on PIP_2 inhibition of CapZ. Triton X-100, PC, or PE were mixed individually with PIP_2 in water at molar ratios of 10:1 to 30:1 and sonicated. CapZ was added, yielding concentrations of 60 nM CapZ, 10 μM PIP_2, 0–300 μM Triton X-100, PC, or PE, and buffer B 20%. After 3 min, an equal volume of actin filaments (5 μM, 25% pyrene-labeled) in buffer G with MKEI was added. After an additional 5 min, the mixture was diluted 50-fold in buffer G, and the pyrenylactin fluorescence was measured over time. The curve for PIP_2 with 100 μM PC was the same as for actin alone, and in a control experiment, actin and CapZ without PIP_2 were the same as with PIP_2 and 200 μM Triton X-100 (data not shown).

tion. Anionic phospholipids completely inhibited the ability of CapZ (capping protein purified from chicken muscle) to affect actin polymerization. The nucleation and capping activities of CapZ were both inhibited, which suggests that PIP_2 inhibits the binding of actin to CapZ. Gel filtration chromatography showed that PIP_2 bound to CapZ. The effect of PIP_2 on CapZ was reversed by Triton X-100, which indicates that PIP_2 does not act by irreversibly denaturing CapZ. In most of the experiments, PIP_2 was incubated with CapZ before the addition of actin, indicating that PIP_2 can prevent the binding of CapZ to actin. In two types of experiments, PIP_2 was added after CapZ was allowed to bind actin filaments. A decrease in the amount of bound CapZ was observed. We do not know whether PIP_2 can bind to CapZ bound to actin (in a ternary complex) or whether PIP_2 simply binds free CapZ, which lowers the concentration of CapZ bound to actin by mass action.

All the anionic phospholipids tested, including PIP_2, PIP, PI, PS, PG, and PA, inhibited CapZ, but the neutral phospholipids PC and PE did not. PIP_2 was more effective than PIP, which was more effective than the other anionic phospholipids. These results suggest that the negative charge of the head group is important for the interaction with CapZ.

PIP_2 inhibition of CapZ may require binding of several PIP_2 molecules by each CapZ heterodimer. When PIP_2 was mixed and sonicated with PC or PE, which form large vesicles [200-nm diameter (Janmey & Stossel, 1989), containing approximately 100000 molecules (Vance & Vance, 1985)], the PIP_2 still inhibited CapZ at a PIP_2:PC or PIP_2:PE ratio of 1:30. However, when PIP_2 was mixed and sonicated with Triton X-100, which forms relatively small micelles, the PIP_2 had no inhibitory activity at PIP_2:Triton ratios less than 1:10 to 1:20. Perhaps a single mixed micelle of Triton with PIP_2 did not contain enough molecules of PIP_2 to inhibit CapZ, whereas a single vesicle of PC or PE with PIP_2 did.

The affinity of the binding between PIP_2 and CapZ is relatively high. In the depolymerization assay 2.7 μM PIP_2 inhibited 97% of the activity of 60 nM CapZ. PIP_2 was in the form of micelles, which contain approximately 80 molecules each; therefore, the concentration of PIP_2 micelles was 34 nM. Since nearly complete binding was observed between reactants in the nanomolar range, the K_d must also be in the nanomolar range. By comparison, the severing activity of 30 nM gelsolin is decreased 50% by 5 μM PIP_2 (Janmey & Stossel, 1989), and the nucleating activity of adseverin is decreased 50% by 10 μM PIP_2 (Maekawa & Sakai, 1990). Uncertainty about how many CapZ molecules can bind to one PIP_2 micelle precludes a more quantitative analysis.

Could this inhibition of CapZ occur in vivo with cellular membranes? Although the fraction of PIP_2 in cell membranes is relatively small, PIP_2 is active in a mixed vesicle. The other anionic phospholipids, which are not as effective as PIP_2, are present at relatively higher amounts in cell membranes. It has been reported that the phospholipid content of *Acanthamoeba castellanii* is 59 pg per cell (Korn & Bowers, 1969), and capping protein is 0.12% of the total cell protein (Cooper et al., 1984), or 0.59 pg per cell. If the phospholipids PI and PS, which inhibit CapZ in vitro, represent 10% of total cell phospholipid, they would be in 700-fold molar excess to capping protein. Thus, it is conceivable that these phospholipids could inhibit capping protein in vivo.

If CapZ is inhibited by membranes in vivo, then perhaps barbed ends of actin filaments are free in the vicinity of membranes. Some experiments with living cells indicate that polymerization of actin occurs at the periphery of the cytoplasm, where the barbed ends of actin filaments are adjacent to the plasma membrane (Forscher & Smith, 1988; Tilney, 1982; Wang, 1985). This polymerization is generally sensitive to cytochalasin (Forscher & Smith, 1988; Tilney, 1982), suggesting that it may occur at free barbed ends. In other cases, electron microscope images indicate that barbed ends of actin filaments contact the plasma membrane (Begg et al., 1979; Bennett & Condeelis, 1984; Mooseker, 1985). The nature of that interaction, in particular whether those barbed ends are free or capped, is not known. In one set of experiments, the barbed ends of microvilli of intestinal epithelial cells were capable of actin addition (Mooseker et al., 1982). One might speculate that an actin-binding protein, such as capping protein, binds to and caps the barbed ends of actin filaments at the membrane and that the capping protein is regulated by the phospholipid composition of the membrane, which varies spatially and temporally. In this manner, growth of actin

filaments might be regulated by signal transduction from the membrane.

A number of cell systems respond to extracellular signals by changes in phosphoinositide metabolism, actin polymerization, and cell motility [Fox et al., 1984; Rittenhouse, 1983; Schlessinger & Geiger, 1981; Dadabay et al., 1991; Pike & Eakes, 1987; reviews include Cooper (1991) and Stossel, (1989)]. This study shows that capping protein can be inhibited by phosphoinositides and other anionic phospholipids and polyphosphoinositides can regulate the activity of gelsolin (Janmey & Stossel, 1987, 1989; Janmey et al., 1987), villin (Janmey & Matsudaira, 1988), profilin (Lassing & Lindberg, 1985, 1988; Goldschmidt et al., 1990), adseverin (Maekawa & Sakai, 1990), cofilin, destrin, and deoxyribonuclease I (Yonezawa et al., 1990). The physiologic relevance of this regulation of actin-binding proteins is uncertain.

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