Gelsolin Modulates Phospholipase C Activity In Vivo through Phospholipid Binding

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Abstract. Gelsolin and CapG are actin regulatory proteins that remodel the cytoskeleton in response to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and Ca$^{2+}$ during agonist stimulation. A physiologically relevant rise in Ca$^{2+}$ increases their affinity for PIP$_2$ and can promote significant interactions with PIP$_2$ in activated cells. This may impact divergent PIP$_2$-dependent signaling processes at the level of substrate availability.

We found that CapG overexpression enhances PDGF-stimulated phospholipase C$_{\gamma}$ (PLC$_{\gamma}$) activity (Sun, H.-q., K. Kwiatkowska, D.C. Wooten, and H.L. Yin. 1995. J. Cell Biol. 129:147–156). In this paper, we examined the ability of gelsolin and CapG to compete with another PLC for PIP$_2$ in live cells, in semiintact cells, and in vitro. We found that CapG and gelsolin overexpression profoundly inhibited bradykinin-stimulated PLC$_{\gamma}$ inhibition occurred at or after the G protein activation step because overexpression also reduced the response to direct G protein activation with NaF. Bradykinin responsiveness was restored after cytosolic proteins, including gelsolin, leaked out of the overexpressing cells. Conversely, exogenous gelsolin added to permeabilized cells inhibited response in a dose-dependent manner.

The washout and addback experiments clearly establish that excess gelsolin is the primary cause of PLC inhibition in cells. In vitro experiments showed that gelsolin and CapG stimulated as well as inhibited PLC$_{\gamma}$ and only gelsolin domains containing PIP$_2$-binding sites were effective. Inhibition was mitigated by increasing PIP$_2$ concentration in a manner consistent with competition between gelsolin and PLC$_{\gamma}$ for PIP$_2$. Gelsolin and CapG also had biphasic effects on tyrosine kinase– phospholipase D (30), and phosphoinositide-specific phospholipase C (PLC) (1), phospholipase D (30), and phosphoinositide 3-OH kinase (30; Lu, P., A. Hsu, D. Wong, H. Yun, H.L. Yin, and C. Chen, manuscript submitted for publication). Therefore, gelsolin and CapG may be components of a signaling complex that transduces external stimuli to the cytoskeleton. The possibility of cross talk between PIP$_2$-regulated proteins provides a selective mechanism for positive as well as negative regulation of the signal transduction cascade.

Gelsolin (33) and CapG (36) are members of an actin filament severing and capping protein family. They are activated by Ca$^{2+}$ and inhibited by polyphosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (15, 33, 36). It is postulated that agonist-induced changes in Ca$^{2+}$ and plasma membrane PIP$_2$ levels alter the partitioning of gelsolin between the plasma membrane, cytosol, and actin filament ends (2, 3, 7, 8, 12, 19). These changes initiate actin filament severing and capping to remodel the cytoskeleton (14).

There is also emerging evidence to suggest that actin regulatory protein binding to PIP$_2$ may have implications beyond a direct effect on the cytoskeleton. In vitro, gelsolin modulates the activity of several important signaling enzymes through an effect on their interactions with PIP$_2$. It alters the activity of phosphoinositide-specific phospholipase C (PLC) (1), phospholipase D (30), and phosphoinositide 3-OH kinase (30; Lu, P., A. Hsu, D. Wong, H. Yun, H.L. Yin, and C. Chen, manuscript submitted for publication). Therefore, gelsolin and CapG may be components of a signaling complex that transduces external stimuli to the cytoskeleton. The possibility of cross talk between divergent PIP$_2$-binding proteins through regulation of substrate availability is particularly relevant as more PIP$_2$-binding proteins are identified. Many pleckstrin homology domain proteins with important signaling functions (27) bind PIP$_2$ with affinity comparable to that of the gelsolin family (18). We recently demonstrated that gelsolin and CapG affinity for PIP$_2$ is increased six- to eightfold by micromolar amounts of Ca$^{2+}$ (18). They can therefore compete effectively for PIP$_2$, particularly when cytosolic [Ca$^{2+}$] increases and PIP$_2$ level drops in agonist-stimulated cells. We reported that NIH3T3 clones expressing slightly more CapG than control clones have increased PDGF-
stirred PLC, activity (31), supporting this possibility. However, we did not anticipate a stimulatory effect since previous in vitro studies indicate that gelsolin and profilin, another PIP2-regulated actin-binding protein, inhibit PLCγ (1, 10).

In this paper, we use a variety of approaches to determine how overexpression affects another PLC, PLCγp, and the basis for interaction in vivo. We also compared the effects of CapG and gelsolin overexpression. Gelsolin and CapG are present in similar concentration (~0.7 μM) in NIH3T3 cells (9, 31). The gelsolin clones have a wider range of overexpression (9), and much more is known about gelsolin PIP2-binding sites (16, 34, 37). They have the same motility phenotype as CapG-overexpressing cells (9, 31), but their phosphoinositide metabolism has not been examined.

Materials and Methods

Cell Lines

NIH3T3 cell lines stably overexpressing human gelsolin were gifts of Drs. D.J. Kwiatkowski and C. Cunningham (Brigham and Women’s Hospital, Boston, MA). They were obtained by transfection of a human cytoplasmic gelsolin expression vector and elonantly selected with neomycin (9). Early passage cells that had been directly quantitated for gelsolin content were used, and their gelsolin expression was reconfirmed in our laboratory (see below). CapG overexpression cells were obtained as described in reference 31. Control (Ctrl) cells were transfected with the vector without a cDNA insert. Cells were maintained in DME with 10% FCS.

For inositol 1,4,5-triphosphate (IP3) and intracellular Ca2+ measurements, cell monolayers were starved in DME/0.2% serum for 24 h. They were switched to a completely defined 1:1 DME/F12 medium supplemented with 20 mM Hepes, pH 7.4, 0.5 mM MgCl2, 1 μg/ml insulin, and 1 μg/ml transferrin (Q-medium) for an additional 18 h before agonist addition. In some cases, cells were treated with 100 ng/ml pertussis toxin (List Biological Lab., Inc., Campbell, CA) overnight. Bradykinin and PDGF (AB chains) were from Sigma Chemical Co. (St. Louis, MO) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively.

Streptolysin O Permeabilization

Cells were trypsinized and resuspended in Chelex 100–treated buffer containing 100 mM KCl, 20 mM NaCl, 20 mM Hepes, pH 7.4. S. aureus, 5 × 106 cells were permeabilized in 0.4–0.6 U/ml streptolysin O (SLO) (Murex Diagnostics Limited, Dartford, England) with 1 mg/ml creatinine phosphate, containing 100 mM KCl, 20 mM NaCl, 20 mM Hepes, pH 7.4. 5 mM MgCl2, and 3 mM ATP (21). Cells were usually permeabilized for 4 min at 37°C before agonist application.

Gelsolin PIP2-binding Domains

Gelsolin NH2-terminal half domains containing zero to three PIP2-binding sites were expressed in Escherichia coli and isolated as described (35, 37). The final products were >99% pure, based on Coomassie blue staining of protein bands in sodium dodecyl sulfate polyacrylamide gels. Protein concentrations were determined by the method of Bradford (5). P2, a 22-mer synthetic peptide encompassing gelsolin residues 150–169 (CKHVP-NEVVQRLFQVKGR), amino-terminal cysteine added (16), was synthesized by solid phase methods. CapG and thymosin β4 were expressed in E. coli and purified as described previously (35, 38).

Ca2+ Measurements

Serum-deprived cells were washed and incubated with a buffer containing Fura 2 (2.5 μM), 1 mg/ml BSA, and 2 mg/ml glucose for 1 h at room temperature (31). Cells were trypsinized briefly and resuspended in a buffer containing BSA and glucose and warmed to 37°C. Fluorescence measurements were performed with 2 × 106 cells in 1.5 ml with slow stirring at 37°C, at excitation and emission wavelengths of 340 and 500 nm, respectively. [Ca2+]i was calibrated by adding 25 μg/ml digitonin and 0.25 mM MnCl2 to obtain Fmax and Fmin, respectively. A Kd,Ca2+ of 224 nM was used for calculation.

IP3 Content

Cell monolayers starved in Q-medium were stimulated with 5 μM bradykinin for 7 or 15 s, and the reaction was stopped by adding an equal volume of a 5% perchloric acid solution containing 10 mM EDTA and 1 μM ATP. The soluble extract was neutralized with 1:1 triton/soybean, and IP3 content was assayed by competition with exogenous [3H]IP3 to bind calf cerebellum microsomes (31). IP3 standards were extracted and assayed under identical conditions. The IP3 content of permeabilized cells was determined with cells in suspension under conditions identical to those used for Ca2+ measurements.

Immunoblotting

Cells were lysed in cold RIPA buffer containing a protease inhibitor cocktail as described previously (31). Lysate protein concentrations were determined by the micro-BCA method (Pierce Chemical Co., Rockford, IL). Proteins were electrophoresed on 5–10% polyacrylamide, discontinuous pH slab gels in the presence of SDS and transferred to nitrocellulose for Western blotting. Blots were probed with rabbit anti–mouse gelsolin, which recognizes the endogenous gelsolin (31), and/or a monoclonal anti–human gelsolin (clone 2C4), which recognizes human but not mouse gelsolin (6). Endogenous mouse and overexpressed human gelsolin were quantitated by comparing the intensity of gelsolin bands in cell lysates with that of purified mouse and human plasma gelsolins, respectively, as described in reference 9. In leak-out experiments, ctrl cells were permeabilized with streptolysin O at 37°C and centrifuged at 1,500 rpm in an Eppendorf microcentrifuge for 1 min at room temperature. Samples were blotted with rabbit anti–mouse gelsolin, rabbit anti-PLCγ3 (gift of P. Sternweis, University of Texas Southwestern Medical Center, Dallas, TX) (11), and monoclonal antiactin (clone C4; Boehringer Mannheim Corp., Indianapolis, IN).

PLC Assays

Recombinant PLCγ1 expressed in SF9 cells and purified to homogeneity was a gift of Dr. E. Ross (University of Texas Southwestern Medical Center) (4). Vesicles containing 100 μM phosphatidylethanolamine, 10 μM phosphatidyserine, 10 μM PIP2, and 0.028 μM [32P]ATP were made by sonicating in the PLC buffer (25 mM Hepes, 80 mM KCl, 3 mM EGTA, 0.5 mM DTT, pH 7.0). Vesicles were preincubated with gelsolin domains in the presence of 11 μM free [Ca2+]i for 4°C for 10 min, and enzyme reaction was initiated by adding 4 ng (per 60 μl) PLCγ3, and warming to 37°C. Reactions were terminated after 1–4 min by adding TCA and 3 mg/ml BSA. Precipitated proteins were removed by centrifugation, and radioactive IP3 in the TCA-soluble supernatant was determined by scintillation counting. Triplicate samples were assayed per condition. PIP2 hydrolysis was linear for 15 min under the conditions used. In some experiments, PIP2, ranging from 5–200 μM, was presented as micelles.

Phospholipase Cγ1, purified from bovine brain (26), was a gift of Dr. S.G. Rhee (National Institute of Health, Bethesda, MD). A preparation enriched in EGF receptor tyrosine kinase was prepared from A431 plasma membrane by Triton X-100 extraction and passage through a wheat germ agglutinin column with N-acetyl-glucosamine (32). The eluted receptor preparation was dialyzed and used at 3 μg/ml to phosphorylate 0.6 μg/ml PLCγ1, in the presence of 20 μM ATP and 3 μM EGF at 4°C for 1 h (22). Its activity was assessed by incubating PLCγ1 with [32P]ATP to detect EGF-dependent IP3 incorporation. PLCγ activity was assayed by using 6–12.5 ng PLCγ1/60 μl reaction mixture, and with vesicles containing 10 μM PIP2, 0.02 μM [32P]ATP/100 μM PE. The buffer conditions were as described above. The reaction was linear for at least 15 min at 37°C. Unphosphorylated PLCγ1 control was incubated with the kinase preparation in the absence of ATP and EGF.

Results

Ca2+ Signaling in CapG Overexpressing Cells

We showed previously that CapG overexpression increased Ca2+ and IP3 responses to PDGF (31). These results suggest that CapG enhanced PLCγ in vivo, although

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gelsolin (1) and CapG (see below) are predominantly inhibitory in vitro. We therefore examined the effect of overexpression on PLC\(_{\beta}\), which is activated through the bradykinin/heterotrimeric G protein pathway. 5 \(\mu\)M bradykinin stimulated ctrl cells to increase cytosolic Ca\(^{2+}\) in a sharp peak (Fig. 1 A). CapG clones had a reduced Ca\(^{2+}\) response (Fig. 1 A and Table I). Likewise, the IP\(_3\) response was also attenuated. Bradykinin-stimulated ctrl cells generated IP\(_3\) to a maximal level at 7 s (Fig. 1 B). The CapG clones generated less IP\(_3\) at 7 and 15 s. Ca\(^{2+}\) signal and IP\(_3\) response were inhibited to a similar extent, suggesting that CapG inhibited PLC\(_{\beta}\) and Ca\(^{2+}\) signaling by a similar mechanism (see Fig. 3 B).

To explore the basis for the opposite effects of CapG overexpression on PDGF- vs. bradykinin-activated PLC, we felt that studying cells with a wider range of overexpression and comparing cells overexpressing related proteins would be important. In spite of repeated attempts, we were not able to isolate stable CapG clones with higher overexpression. We therefore directed our effort to gelsolin-overexpressing clones, which have a wider range of overexpression (9).

**Effects of Gelsolin Overexpression on PIP\(_2\) Hydrolysis**

Western blotting with human gelsolin–specific antibody, which recognizes the transfected human gelsolin but not endogenous mouse gelsolin, confirmed that the clones had a range of gelsolin overexpression (Fig. 2, bottom), as described previously (9). C2 was not positive, indicating that it had lost the expression vector, and was not studied further. Blotting with anti–mouse gelsolin, which cross-reacts poorly with human gelsolin, showed that the endogenous gelsolin level in overexpressing cells was comparable to that of the ctrl cells (Fig. 2, top). Therefore, there was no compensatory change in endogenous gelsolin expression. Total gelsolin level, determined by quantitative Western blotting using purified human and mouse gelsolin standards, confirmed that the levels of expression were similar to that shown previously (9) (Table I).

Gelsolin overexpression modified the Ca\(^{2+}\) response to bradykinin. C3 that has 1.3 times as much gelsolin as ctrl was slightly more responsive to bradykinin and PDGF (Table I). In contrast, C6, C1, C4, and C5 that have higher overexpression had a striking decrease in bradykinin-induced Ca\(^{2+}\) signal (Fig. 3, Table I). The PDGF response was also reduced, but to a lesser extent (Table I). Since inhibition was observed with multiple independently isolated clones, the phenotype was attributed to gelsolin overexpression and was not an artifact of clonal selection. The stimulation we observed with the lowest overexpressing clone, C3, suggests that gelsolin may also increase signaling. However, because only one such clone was available, ruling out clonal variations unrelated to gelsolin was not possible.

**Table I. Effects of Overexpression on Ca\(^{2+}\) Signals**

| Clone | Fold overexpression* | Ca\(^{2+}\) (nM)
|-------|----------------------|-------------------|
|       |                      | PDGF       | Bradykinin |
| Cap G | ctrl                 | 232 ± 30 | 659 ± 95 |
| gK6   | 1.3                  | —         | 259 ± 58 |
| gK8   | 1.4                  | 323 ± 14 | 196 ± 21 |
| gK15  | 1.5                  | 339 ± 40 | 151 ± 57 |
| Gelsolin | Ctrl                | 289 ± 38 | 790 ± 146 |
| C3    | 1.3                  | 352 ± 20 | 1358 ± 251 |
| C6    | 1.4                  | 179 ± 25 | 270 ± 60 |
| C1    | 1.7                  | 125 ± 38 | 80 ± 18 |
| C4    | 2.1                  | 104 ± 10 | 30 ± 5 |
| C5    | 2.4                  | 125 ± 15 | 20 ± 2 |

*Gelsolin content was determined by scanning of Western blots, using mouse and human gelsolin as standards to quantitate overexpressed and endogenous gelsolins, respectively. Values were the means of three determinations. Cap G content was determined as described in reference 31.

†Peak Ca\(^{2+}\) concentration after stimulation with 50 ng/ml PDGF or 5 \(\mu\)M bradykinin. Values were the means ± SEM of three to five determinations.

**Figure 1.** Effect of CapG overexpression on response to bradykinin. Serum-deprived fibroblasts were stimulated with 5 \(\mu\)M bradykinin. (A) Ca\(^{2+}\) tracings from a representative experiment were shown. Fura 2 was used as an indicator. Top, ctrl; bottom, gk15. Arrows indicate time of bradykinin addition. (B) IP\(_3\) generation as a function of time after stimulation. Cells were extracted with PCA, and IP\(_3\) level was determined by a receptor binding assay. Results shown were from a single representative experiment performed in triplicate. Values were mean ± SEM.
The effect of gelsolin on PLC$_{\beta}$ activity per se was confirmed by quantitating IP$_3$. Ctrl and overexpressing clones had similar basal IP$_3$ levels (~10 pmol/mg cell protein). Gelsolin clones had reduced IP$_3$ response (Fig. 3B), and the extent of inhibition correlated with that of the Ca$^{2+}$ response. Overall, our results are consistent with the hypothesis that the gelsolin- and CapG-induced change in Ca$^{2+}$ signal was due to PLC$_{\beta}$ inhibition.

Characterization of the Gelsolin Effect on the Bradykinin Response

Since the bradykinin response was strikingly inhibited, we will focus on this aspect in the rest of the paper. Bradykinin stimulation of Ca$^{2+}$ release is a multistep process that involves bradykinin binding to receptors, activation of heterotrimeric G proteins, PLC$_{\beta}$ hydrolysis of PIP$_2$, and IP$_3$-induced release of Ca$^{2+}$ from intracellular stores. Ctrl cells responded to as low as 0.1 mM bradykinin, and Ca$^{2+}$ release was maximally stimulated at 1 mM (data not shown). C5 had a very small Ca$^{2+}$ peak, even at 50 mM bradykinin, suggesting that the problem was unlikely to be due to decreased receptor affinity. To identify the step compromised by gelsolin overexpression, NaF was used to activate G proteins directly, bypassing receptor involvement. NaF induced a slow Ca$^{2+}$ rise (Fig. 3A), which had a smaller amplitude than the bradykinin peak (320 ± 50 nM).

![Figure 2. Western blotting of gelsolin-overexpressing clones. (Top) Anti–mouse gelsolin (anti-mG), detecting endogenous gelsolin. (Bottom) Anti–human gelsolin (anti-hG), detecting the expression of human gelsolin in human gelsolin cDNA–transfected cells. ctrl, control-transfected NIH3T3 cells; C, gelsolin overexpressing clones.](image)

![Figure 3. Effect of gelsolin and CapG overexpression. (A) Ca$^{2+}$ tracings. Cells were stimulated with 5 mM bradykinin or 20 mM NaF. Tracings were from a representative experiment. Arrows indicate time of agonist addition. Ctrl, mock-transfected cells; C1, gelsolin-overexpressing cells. (B) Comparing the effects of gelsolin and CapG overexpression. Cells were stimulated with 5 mM bradykinin or 20 mM NaF. Ca$^{2+}$ spike and IP$_3$ generation were measured. The peak response of overexpressing cells was expressed as a percent of ctrl cells exposed to the same stimulus. Results shown were the average of two experiments. Filled circles, Ca$^{2+}$ signal after bradykinin stimulation; open circles, IP$_3$ generated in response to bradykinin; triangles, Ca$^{2+}$ signal after NaF treatment.](image)
Gelsolin overexpression reduced the NaF response, suggesting that it acted at or downstream of the heterotrimeric G protein activation step (Fig. 3 A). However, gelsolin did not inhibit the NaF response to as great an extent as the bradykinin response (Fig. 3 B). The Ca$^{2+}$ peak for C4 was reduced to 1.8 and 47.8% of ctrl after bradykinin and NaF stimulation, respectively. The difference may be due to activation of different PLCs. NaF activates all heterotrimeric G proteins, while bradykinin activates the alpha subunit (Go$_{q/11}$) (11, 29). This was confirmed with pertussis toxin that inhibits Gi but not Go$_{q/11}$. In ctrl cells, pertussis toxin reduced the NaF response by 45.7% but had minimal effect on the bradykinin response (data not shown). Our result suggested that Go$_{q/11}$-activated PLCs were more sensitive to gelsolin inhibition than Go$_{i}$-activated enzymes.

**Addition of Exogenous Gelsolin to Streptolysin O–permeabilized Ctrl Cells**

To determine if the change in PLC$_{b}$ activity is directly attributable to gelsolin, a gelsolin NH$_2$-terminal half polypeptide (45 kD, spanning gelsolin residues 1–406 [GS1-406]) that contains at least two PIP$_2$-binding sites (17, 35) was added to SLO-permeabilized ctrl cells. Under the permeabilizing conditions used, cells retained agonist responsiveness, as demonstrated previously for another type of cell (21). Fig. 4 showed the result of a representative experiment. The mean IP$_3$ response in the presence of 4 and 32 µM GS1-406 were 46.5 ± 4.0% (n = 8) and 6.4 ± 1.1% (n = 3) of control, respectively. Thus, exogenous gelsolin added to permeabilized cells mimicked the inhibitory effects of gelsolin overexpression.

Exogenous CapG also inhibited IP$_3$ rise, while thymosin $\beta_4$, an actin-binding protein that does not interact with PIP$_2$ (38), had no effect at 10 µM. (IP$_3$ response was 91.7 and 101.8% of control after bradykinin stimulation in two experiments.) These results suggested that inhibition of IP$_3$ generation in cells was linked to an increase in PIP$_2$-binding proteins.

**Regeneration of Bradykinin Response after Cytosol Leakage from Semiintact Cells**

Our data showed that gelsolin, either overexpressed in intact cells or added to semiintact cells, reduced bradykinin responsiveness. Removal of gelsolin should restore the bradykinin response. Cells were permeabilized for progressively longer intervals before bradykinin stimulation to allow gelsolin and other cytosolic proteins to leak out. After 3 min, gelsolin and actin were detected in the medium (Fig. 5 A). More proteins leaked out with longer treatment. In contrast, PLC$_{b3}$, the predominant PLC in NIH3T3 cells, did not leak out to a significant extent, as would be consistent with its stable plasma membrane association. There was no difference in the leakage of PLC$_{b3}$ between ctrl and C5.

The effect of cytosol leakage on IP$_3$ generation was determined by stimulating cells permeabilized for different intervals with bradykinin (Fig. 5 B). Both intact and permeabilized ctrl cells increased IP$_3$ after bradykinin stimulation. The semiintact cell’s response was more robust, suggesting that soluble inhibitors had leaked out or that other clamping mechanisms were disrupted. As shown above, intact C5 had a much lower IP$_3$ response than ctrl cells. At 2 min after SLO treatment, C5 was still inhibited relative to ctrl. Between 2 to 5 min, C5 response increased to a level comparable to that of ctrl. With longer permeabilization, both ctrl and C5 were less active, probably due to loss of membrane integrity and depletion of soluble cofactors such as GTP. The convergence of the IP$_3$ response between ctrl and C5 was not due to differential leakout of PLC$_{b3}$ (Fig. 5 A). Recovery of bradykinin-stimulated PLC activity indicates that reduced bradykinin responsiveness in intact overexpressing cells is unlikely to be due to bradykinin receptor defects. A similar conclusion was reached using NaF to stimulate G proteins directly (Fig. 3 A). Therefore, we conclude that gelsolin depresses PLC activity in intact C5, and reducing gelsolin concentration relieves the inhibition.

**Effects of Gelsolin on Phospholipase C In Vitro**

It has been reported that gelsolin inhibits several PLC isozymes in vitro (1). In light of our in vivo results, we re-examined this issue to determine if gelsolin and CapG inhibit PLC$_{b}$ and PLC$_{a}$ differentially, and whether inhibition is due to PIP$_2$ binding. Gelsolin GS1-406 that contains two identified PIP$_2$-binding sites increased Ca$^{2+}$-activated PLC$_{b}$ at 1 µM and inhibited at higher concentrations (Fig. 6 A). A biphasic effect has been reported previously (1), although the stimulatory phase was ignored. CapG stimulated and inhibited with a comparable dose response, consistent with its similar affinity as gelsolin for PIP$_2$ (18). Gelsolin also had a biphasic effect on PLC$_{a}$ phosphorylated with EGF receptor kinase. In contrast, gelsolin did...
not increase the activity of nonphosphorylated PLC\textsubscript{g} (Fig. 6\textit{B}) and inhibited it more strongly. The differential effects on phosphorylated and unphosphorylated PLC\textsubscript{g}, including activation of the phosphorylated enzyme at low concentration, were similar to that reported previously for profilin (10). A direct comparison between gelsolin and profilin showed that gelsolin was a stronger inhibitor (data not shown), consistent with their different affinity for PIP2 (18, 20). Although gelsolin was reported to bind PLC\textsubscript{g} (1), we were not able to detect such an interaction either with purified proteins or in cell lysates (data not shown). Therefore, gelsolin is not likely to act by binding PLC directly.

Gelsolin inhibited Ca\textsuperscript{2+}-activated PLC\textsubscript{b} more than phosphorylated PLC\textsubscript{g}. 2 \mu M gelsolin stimulated phosphorylated PLC\textsubscript{b}, but was located on the inhibitory arm of the PLC\textsubscript{b} dose-response curve (Fig. 6, \textit{A} and \textit{B}). Stimulation of phosphorylated PLC\textsubscript{b} may contribute to the increased PDGF response in clones overexpressing low levels of CapG and in the single low-level gelsolin-overexpressing clone (C3). The higher sensitivity of PLC\textsubscript{b} to inhibition may explain why PLC\textsubscript{b} and PLC\textsubscript{g} were affected in opposite directions in CapG clones and why the gelsolin clones showed more inhibition of PLC\textsubscript{b} than PLC\textsubscript{g}. Thus, the differential inhibitory effects of gelsolin on PLC\textsubscript{b} and PLC\textsubscript{g} in vitro recapitulated the in vivo effects of overexpression.

The inhibitory effect of GS1-406 was overcome by increasing PIP\textsubscript{2} concentration. As expected, PLC\textsubscript{b} generated more IP\textsubscript{3} as the substrate concentration increased. 5 \mu M GS1-406 completely inhibited PLC\textsubscript{b} activity at 5 and 10 \mu M PIP\textsubscript{2} but was progressively less effective at higher PIP\textsubscript{2} concentration (Fig. 6\textit{C}). No inhibition was observed at PIP\textsubscript{2} concentrations above 120 \mu M. The simplest interpretation was that gelsolin inhibited PLC\textsubscript{b} by substrate competition, as suggested for PLC\textsubscript{g} in an earlier study (1).

Experiments using gelsolin domains with and without PIP\textsubscript{2}-binding sites confirmed that gelsolin altered PLC activity through PIP\textsubscript{2} binding. GS1-406, encompassing gelsolin segments 1–3, contains at least two (and possibly three) PIP\textsubscript{2}-binding sites (Fig. 7 \textit{A}). The S1 and S2 PIP\textsubscript{2}-binding sites have been mapped precisely by deletion analyses (37) and peptide analog studies (16, 37). The existence of a third site is inferred from previous experiments after deleting the S2 site and is predicted to be in S3 (37). GS1-406, which binds PIP\textsubscript{2} with an estimated \( K_d \) of 2.9 \mu M and a stoichiometry of \( z = 3 \) (18), had the most activating and inhibitory effects (Fig. 7 \textit{B}). GS150-406 (S2-3), which binds with a similar \( K_d \) but with a stoichiometry of \( z = 2 \), was slightly less effective. GS150-267 that contains a single PIP\textsubscript{2}-binding site was markedly less effective, while GS171-267 with no PIP\textsubscript{2}-binding site had no effect. These results showed that gelsolin altered PLC activity through PIP\textsubscript{2} binding, and maximal effect was observed with the participation of two to three PIP\textsubscript{2}-binding sites. Binding to multiple PIP\textsubscript{2} may form a more stable complex that is less likely to be displaced by PLC, and the larger polypeptides are likely to be more effective in sterically hindering PLC access to its substrate.

**Figure 5.** Restoration of bradykinin response to gelsolin-overexpressing cells after SLO permeabilization. (\textit{A}) Time course of leakage of cytosolic proteins. Ctrl cells in suspension were permeabilized at 37°C with SLO and centrifuged for 1 min at room temperature. Pellets and supernatants were electrophoresed on SDS–polyacrylamide gels and blotted with anti–mouse gelsolin, antiaxin, or anti-PLC\textsubscript{b}. (\textit{B}). IP\textsubscript{3} generation after bradykinin stimulation. Ctrl cells and C5 were exposed to SLO for the amount of time indicated and then stimulated with bradykinin. The C5 Ca\textsuperscript{2+} peak was expressed as a percent of ctrl under identical conditions. Data shown were the means of two separate experiments.
P2, the PIP₂-binding peptide derived from gelsolin S2 (16), had no effect at low concentration and stimulated PLCβ by more than threefold at 53 μM (Fig. 7C). We were not able to use more P2 to determine if higher concentrations were inhibitory. P2 may stimulate by organizing PIP₂ in a more favorable conformation for PLC hydrolysis. It may be more stimulatory than the parent S2 domain because its positive effects are not neutralized by steric hindrance to block PLC access. However, in contrast to the marked stimulation in vitro, P2 had minimal effect on PLCβ activity in permeabilized cells. Semiintact cells treated with 100 μM P2 showed 72.5 ± 6.7% (n = 9) of the bradykinin-induced IP₃ response of untreated cells. No stimulation was observed between 5–100 μM (data not shown). We cannot explain why P2 had divergent effects in vitro and in vivo.

**Discussion**

In this paper, we examined the ability of gelsolin and CapG to compete with PLC for PIP₂ in live cells, in semiintact cells, and in vitro. The various approaches help to define the mechanism by which gelsolin and CapG modulate PLC activity in two independent signaling pathways. Our results suggest that these cytoskeletal proteins may participate in signaling by controlling the availability of PIP₂ substrates.

The CapG and gelsolin overexpressing cell lines were isolated independently by two different laboratories, using the same parent NIH3T3 cell line and expression vector. Their remarkably similar effects on PLCβ strongly support the conclusion that gelsolin and CapG bind PIP₂ in vivo to inhibit PLCβ. There are, however, differences with respect...
CapG clone gK8 and gelsolin clone C6 had increased and decreased response to PDGF, respectively. The divergent effects of gelsolin and CapG may be due to multiple factors. CapG has one PIP2-binding site, while gelsolin has two (37). CapG is half the size of gelsolin (36). Thus, CapG may be more readily displaced by activated PLCγ and may not sterically block PLCγ access to PIP2 as effectively. CapG is phosphorylated (23), and phospho-CapG may have a different affinity for PIP2 (Lu, P., A. Hsu, D. Wong, H. Yan, H.L. Yin, and C. Chen, manuscript submitted for publication). Another possibility may be different intracellular localization. CapG is a nuclear and cytosolic protein, while gelsolin is excluded from the nucleus (24). There is emerging evidence for phosphoinositide signaling pathways in nuclei (Lu, P., A. Hsu, D. Wong, H. Yan, H.L. Yin, and C. Chen, manuscript submitted for publication), and CapG may therefore act in the nucleus.

Another feature that emerges from these studies is that there is a difference in the sensitivity of PLCβ and PLCγ towards gelsolin and CapG. This may be explained in several ways. Phosphorylated PLCγ may have a higher affinity for PIP2 than PLCβ and is therefore better able to compete with gelsolin for PIP2. Differential sensitivity may account for the paradoxical finding that CapG-overexpressing cells have increased PLCγ (31) but reduced PLCβ activity. Another possibility is that PLC isozymes may use different pools of PIP2, and gelsolin/CapG have differential access to these pools. PIP2 pools with distinct turnover characteristics have been identified (25), although their re-
lation to the different PLC isozymes, gelsolin and CapG, has not been examined.

Inhibition of PLC is most likely due to competition of gelsolin and CapG with PLC for PIP2. Steric hindrance may also be a contributing factor. The mechanism by which PLC is enhanced by a small increase in CapG or gelsolin is not known. Since only gelsolin domains with PIP2-β-binding sites stimulate, this effect depends on PIP2 binding. The simplest hypothesis is that gelsolin and CapG improve the presentation of PIP2 to PLC by altering the phospholipid conformation. Among the gelsolin domains tested, P2, the small synthetic peptide containing a single PIP2-β-binding site, is most stimulatory in vitro. However, it does not have any effect on PLC in semintact cells. This peptide has been used by others to examine the relation between gelsolin and PIP2, in cells (8, 13). P2 added to semintact platelets blocks Rac1-induced uncapping of actin filament + ends, and this effect is overcome by adding PIP2 (13). Overexpression of P2 in a permanently transfected cell line reduces gelsolin association with the plasma membrane (8). Both experiments indicate that P2 competes with gelsolin for PIP2, although effects on other PIP2-β-requiring processes have not been ruled out. Our result suggests that PLC was not affected by increasing P2 levels in cells. Additional experiments will be required to determine if other parameters are altered by P2.

In conclusion, we show that gelsolin and CapG interact with PIP2 in vivo, and a modest increase in concentration has profound effects on two PIP2 signaling pathways. Overexpression is used as a tool to probe intracellular function. Our overexpression results have physiological relevance. While total gelsolin and CapG content do not change per se, cytosolic concentration and membrane binding are expected to fluctuate during signaling. Gelsolin and CapG bind to and are released from filament ends and the membrane PIP2 content changes due to hydrolysis and resynthesis. Ca2+ increases gelsolin and CapG affinity for PIP2 (18), enhancing their ability to compete with other PIP2-β-binding proteins at a time when membrane PIP2 content decreases. CapG and gelsolin can provide positive and negative inputs on PLC signaling pathways, and these pathways are modulated selectively. The data presented here indicate that there is significant cross talk between components of the transmembrane signal machinery and actin cytoskeleton at multiple levels, including that of the generation of important second messengers.

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