The Cross Talk Between Protein Kinase A– and RhoA-Mediated Signaling in Cancer Cells

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The cross talk between cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and RhoA-mediated signal transductions and the effect of this cross talk on biologic features of human prostate and gastric cancer cells were investigated. In the human gastric cancer cell line, SGC-7901, lysophosphatidic acid (LPA) increased RhoA activity in a dose-dependent manner. The cellular permeable cAMP analog, 8-chlorophenylthio-cAMP (CPT-cAMP), inhibited the LPA-induced RhoA activation and caused phosphorylation of RhoA at serine^{188}. Immunofluorescence microscopy, Western blotting, and green fluorescent protein (GFP)-tagged RhoA location assay in live cells revealed that RhoA was distributed in both the cytoplasm and nucleus of SGC-7901 cells. Treatment with LPA and/or CPT-cAMP did not induce obvious translocation of RhoA in the cells. The LPA treatment caused formation of F-actin in SGC-7901 cells, and CPT-cAMP inhibited the formation. In a modified Boyden chamber assay, LPA stimulated the migration of SGC-7901 cells, and CPT-cAMP dose-dependently inhibited the stimulating effect of LPA. In soft agar assay, LPA stimulated early proliferation of SGC-7901 cells, and CPT-cAMP significantly inhibited the growth of LPA-stimulated cells. In the prostate cancer cell line, PC-3, LPA caused morphologic changes from polygonal to round, and transfection with plasmid DNA encoding constitutively active RhoA(63L) caused a similar change. Treatment with CPT-cAMP inhibited the changes in both cases. However, in PC-3 cells transfected with a plasmid encoding mutant RhoA188A, LPA induced rounding, but CPT-cAMP could not prevent the change. Results of this experiment indicated that cAMP/PKA inhibited RhoA activation, and serine^{188} phosphorylation on RhoA was necessary for PKA to exert its inhibitory effect on RhoA activation. The cross talk between cAMP/PKA and RhoA-mediated signal transductions had significant affect on biologic features of gastric and prostate cancer cells, such as morphologic and cytoskeletal change, migration, and anchorage-independent growth. The results may be helpful in implementing novel therapeutic strategies for invasive and metastatic prostate and gastric cancers. Exp Biol Med 230:731–741, 2005

Key words: PKA; RhoA; signal transduction; cross talk; cancer cell

Introduction

RhoA is the most extensively studied member of Rho protein family and is involved in biologic processes requiring coordinated rearrangement of the actin cytoskeleton, including cell adhesion and motility (1). RhoA activation is generally associated with invasive growth and metastasis. Although no activating RhoA mutations have been found in tumors, RhoA overexpression is found in many human cancers, with RhoA likely contributing to the loss of growth control and the invasive phenotype of cancer cells (2, 3). Whereas RhoA inhibition decreases tumor cell invasion and metastasis, both effects may be mediated by the RhoA target protein, Rho-kinase (ROCK; Refs. 1, 3–7).

The cyclic adenosine monophosphate (cAMP)-dependent, protein kinase A (PKA or A-kinase) is a tetramer of two regulatory (R; RI and RII) and two catalytic (C) subunits. It dissociates and releases active C subunits after cAMP binding to the R subunits (8). Two types of A-kinases differ in subcellular localization: type I A-kinase containing RI is predominantly cytoplasmic, whereas type II A-kinase containing RII is targeted to intracellular sites through association with A-kinase–anchoring proteins (8–11). Protein kinase A seems to inhibit RhoA functions in many different cell types. For example, cAMP inhibits RhoA-induced cytoskeletal changes, smooth muscle contraction, and endothelial and tumor cell migration (12, 13–18). Activation of PKA inhibited RhoA-related stress fiber and focal adhesion complexes (FACs) formation (19–22). We propose this may occur partially through PKA inhibition of RhoA.

To our knowledge, no previous studies address PKA regulation of RhoA activity and function in prostate and gastric cancers despite the importance of both signaling pathways for anchorage-independent growth and metastasis.

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However, because pharmacological agents designed to modulate Rho- and PKA-mediated signaling are becoming available and entering clinical trials (23–25), a better understanding of the regulation of RhoA by PKA may lead to novel therapeutic strategies for invasive and metastatic human prostate and gastric cancers.

Materials and Methods

Cell Lines. The human gastric epithelial cell line, SGC-7901, was provided by the Institute of Cell Biology (Shanghai, China). The human prostate cancer cell line, PC-3, was a kind gift from Dr. Renate Pilz, University of California at San Diego, San Diego, CA.

Reagents. Dulbecco's Modified Eagle Media (DMEM) and DMEM/F-12 culture media was from GIBCO (Grand Island, NY). Fetal calf serum (FCS) and new-born calf serum (NBCS) were from Minhai Bio-engineering C. (Lanzhou, China). Antibodies against RhoA, Rho-GDI, Rock2, and p-53 were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against RhoA phosphorylated at serine[188] was from Calbiochem (San Diego, CA). The antibody against α-tubulin was from Boster Biological Technology (Wuhan, China). The horseradish peroxidase (HRP)-conjugated secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). The cell transfection reagent, Lipofectamin 2000, was from Invitrogen (Carlsbad, CA). The cellular permeable cAMP analog, 8-chlorophenylthio-cAMP (CPT-cAMP), was from Calbiochem. Lysophosphatidic acid (LPA) was from Sigma (St. Louis, MO). Electrochemiluminescence (ECL) reagents were from Amersham Biosciences (Buckinghamshire, England). The Nuclear/Cytosol Fractionation Kit was from BioVision (Mountain View, CA). All plasmid DNA constructs used in this experiment were kind gifts from Dr. Renate Pilz, University of California, San Diego.

Construction of the Expression Vectors of Green Fluorescent Protein (GFP)–Tagged RhoA and its Application in Observing RhoA Location in Live Cells. The 550 base pair (bp) RhoA cDNA was cut from expression vector pcDNA3/EE-tagged RhoA with *Bam*HI and *Xba*I and inserted into the *Bam*HI/*Xba*I site downstream to GFP in a modified pcDNA3.1(+)-containing GFP sequence. The insertion of RhoA cDNA was identified by restriction enzyme analysis and DNA sequencing. Both the pcDNA3(+)/GFP and pcDNA3(+)/GFP-RhoA expression vectors were used to transfect SGC-7901 cells, and the locations of GFP and GFP-tagged RhoA in live cells were observed with a confocal laser scanning microscope (Radiance 2100TM; Bio-Rad, Hercules, CA).

Cell Culture and Transfection. The SGC-7901 cells and PC-3 cells were cultured in culture medium supplied with 10% serum. The medium was changed every second day and the cells were subcultured at confluence. For transfection, the cells were subcultured the day before the process. The seeding amount of cells was adjusted to attain a density of 80–90% confluence on the day of transfection. Transfection was performed according to the instruction of the manufacturer.

Preparation of Rhotekin-GST. The plasmid DNA encoding the RhoA-binding domain (RBD) of Rhotekin fused to glutathione-S-transferase (GST) was transfected into *Escherichia coli*. The bacteria were cultured at 37°C overnight and induced with isopropyl thiogalactoside (IPTG) at 30°C for 2 hrs to express protein. The bacteria cells were lysed with lysis buffer containing 50 m*M* Tris-HCl, pH 7.4; 1% Nonidet P-40 (NP-40); 150 m*M* NaCl; 5 m*M* $MgCl_2$; 1 m*M* dithiothreitol (DTT); 10 μg/ml of aprotinin; 10 μg/ml of leupeptin; and 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The lysate was centrifuged and the supernatant was incubated with glutathione beads at 4°C for 2 hrs. The beads were washed several times with washing buffer containing 50 m*M* Tris-HCl, pH 7.4; 0.5% Triton X-100; 150 m*M* NaCl; 5 m*M* $MgCl_2$; and 1 m*M* DTT. After the final washing, the beads were suspended in washing buffer containing 10% glycerol and kept at –70°C until use.

RhoA-GTP Pull-Down Assay. Rho activity was measured according to the method from Ren *et al.* (26). Briefly, 3×10^6 cells were seeded on a 10-cm dish. After different treatment, the cells were washed with Tris-buffered saline (TBS) and lysed with 400 μl of lysis buffer containing 50 m*M* Tris-HCl, pH 7.4; 1% NP-40; 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS); 200 m*M* NaCl; 1 m*M* $MgCl_2$; 10 μg/ml leupeptin; 10 μg/ml aprotinin; and 1 m*M* PMSF. The cell lysate was centrifuged to get rid of the cell debris. Ten microliters of the supernatant was kept for loading control and the rest of the supernatant was incubated with GST-Rhotekin-glutathione beads at 4°C for 45 mins, with continuous shaking. The beads were washed three times with a buffer containing 50 m*M* Tris-HCl, pH 7.4; 2% NP-40; 200 m*M* NaCl; and 10 m*M* $MgSO_4$. After the final washing, 20 μl of 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added to the beads, and the beads were boiled for 5 mins to release proteins.

Preparation of Nuclear and Cytosolic Proteins. Cells on plates were washed with phosphate-buffered saline (PBS) and treated with trypsin. The nuclear and cytosolic proteins were prepared with a Nuclear/Cytosol Fractionation Kit from BioVision, following the protocol provided by the manufacturer. The nuclear and cytosolic fractions were identified by blots against p53 and α-tubulin, respectively.

Western Blotting. Gels for SDS-PAGE of different concentrations were cast according to the molecular size of target proteins. Sample proteins were accumulated with a voltage of 8 V/cm and separated with a voltage of 15 V/cm on the gel. After electrophoresis, the proteins on the gel were transferred to polyvinyl difluoride (PVDF) membrane, and the membrane was blocked with 3% (w/v) of bovine serum albumin (BSA) in TBS-Tween-20 for 1 hr at room temperature (RT). The incubation with the first antibody

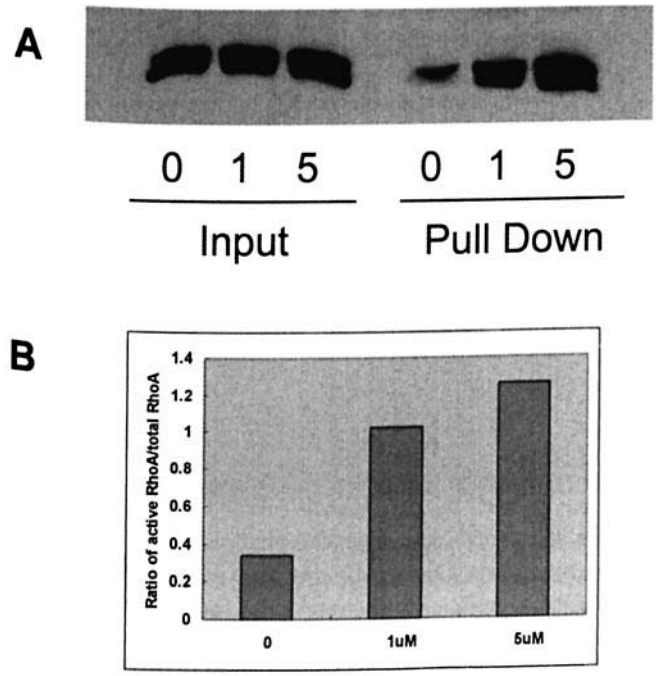

Figure 1. Rho activity in SGC-7901 cells treated with LPA. Cell lysate (2.5% input) and pull-down assay product containing active RhoA (pull-down) were analyzed by Western blotting with an anti-RhoA antibody. (A) Western blotting: SGC-7901 cells were stimulated with LPA of different concentrations for 10 mins. Lane 0, cells without stimulation; Lane 1, cells stimulated with 1 μM LPA; Lane 5, cells stimulated with 5 μM LPA. (B) Western blotting results were analyzed by GeneSnap/Gene Tool software and the raw volume ratio of active RhoA to RhoA input (pull-down/input) was calculated and presented. The result shown is representative of three experiments.

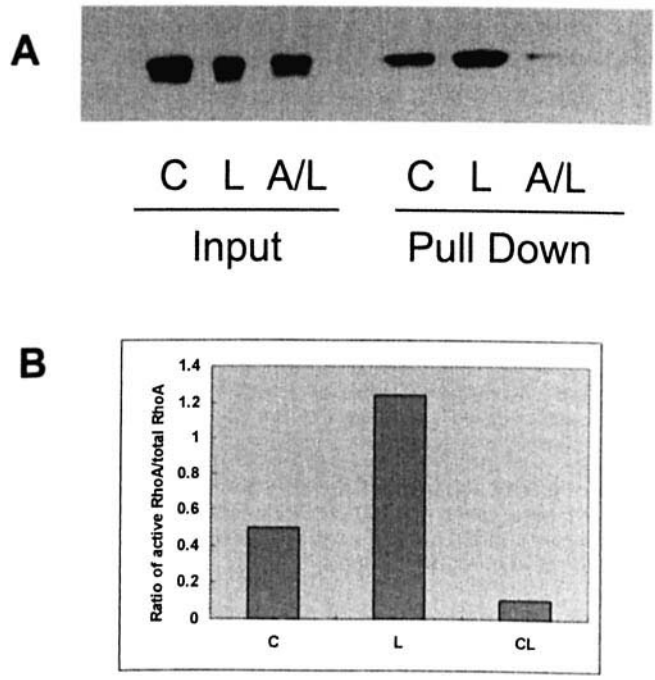

Figure 2. Rho Activity in SGC-7901 cells treated with CPT-cAMP followed by LPA. (A) Western blotting, SGC-7901 cells were treated with 100 μM CPT-cAMP and/or 1 μM LPA. Lane C, control; Lane L, cells stimulated with LPA alone for 10 mins; Lane A/l, cells stimulated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 10 mins. (B) Western blotting results were analyzed by GeneSnap/Gene Tool software and the raw volume ratio of active RhoA to RhoA input (pull-down/input) was calculated and presented. The result shown is representatives of three experiments.

was overnight at 4°C, and the incubation with the secondary antibody was 50 mins at RT, with three washes after each incubation. Electrochemiluminescence reagents were used to show the positive bands on the membrane. Briefly, same volumes of solution A and solution B were mixed and added to the protein side of the membrane. The incubation was 1 min at RT. The exposure time of the first film was 15 secs. The exposure time of the second film was adjusted according to the extensity of the signal on the first film. The bands on film were analyzed with GeneSnap/Gene Tool software from Syngene (Cambridge, UK).

Immunofluorescence Microscopy. The cells grown on coverslips were fixed with freshly prepared 2% paraformaldehyde in PBS. After penetratration with 0.3% Triton X-100 and washing with PBS, the cells were incubated with the first antibody for 2 hrs at RT and then with a fluorescein isothiocyanate (FITC)- or tetrarhodamine isothiocyanate (TRITC)-conjugated second antibody for 1 hr at RT, with three washes after each incubation. The morphologic changes of the cells were observed with a fluorescence microscope.

Cell Migration Assay. A modified Boyden Chamber Assay was applied to assess cell migration (27). Briefly, cells were trypsinized and counted, and 5×10^4 cells were placed in the upper compartment and allowed to migrate through the pores of the membrane into the lower compartment. After 8 hrs of incubation, the membrane between the two compartments was fixed and stained, and the number of cells that had migrated to the lower side of the membrane was determined by microscopy.

Anchorage-Independent Growth Assay. (28) The base agar was prepared by mixing equal volumes of 1% agar and 2× DMEM + 20% NBCS. The top agar was prepared by mixing equal volumes of 0.7% agar (DNA-grade agarose) and 2× DMEM + 20% NBCS, with 5000 cells/30-mm plate suspended in the agar. The plate was incubated at 37°C, 5% CO_2 in a humidified incubator. The cell proliferation was observed and recorded under a reverse microscope.

Statistics. All data were expressed as mean ± standard deviation (SD). Statistical significant difference was tested with Student's t test, and $P < 0.05$ was assumed a significant difference.

Results

Effect of LPA and CPT-cAMP on RhoA Activity in SGC-7901 Cells. Treating SGC-7901 cells with LPA increased RhoA activity in a dosage-dependent manner (Fig. 1). Pretreating cells with the membrane-permeable cAMP analog, CPT-cAMP, inhibited RhoA activation induced by LPA (Fig. 2). Similar results were found when SGC-7901

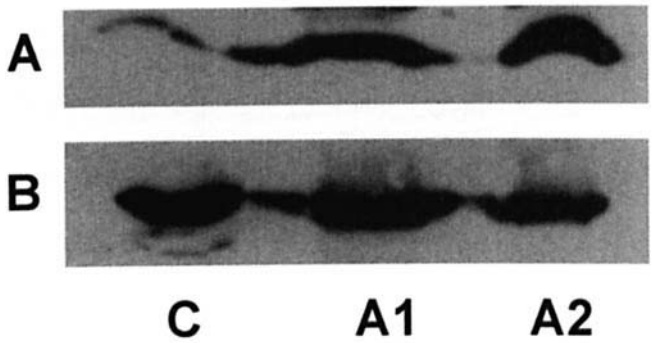

Figure 3. RhoA phosphorylation at serine[188] induced by CPT-cAMP/PKA. The SGC-7901 cells were treated with CPT-cAMP, and the cell lysate was detected by Western blotting with an antibody against RhoA phosphorylated at serine[188]. Panel A, Western blot with an antibody against RhoA phosphorylated at serine[188]. Panel B, Western blot with an antibody against RhoA. Lane C, control cells; Lane A1, cells treated with 100 μM CPT-cAMP for 30 mins; Lane A2, cells treated with 250 μM CPT-cAMP for 30 mins. The result shown is representative of two experiments.

cells were transfected with epitope-tagged RhoA (data not shown).

RhoA Serine[188] Phosphorylation Caused by CPT-cAMP. Research data showed that PKA exerts an inhibitory effect on RhoA through phosphorylating serine[188] of RhoA. To detect the serine[188] phosphorylation of RhoA induced by CPT-cAMP/PKA in SGC-7901 cells, an antibody against RhoA phosphorylated at serine[188] was used in this experiment. Western blot results showed that, in control cells, nearly no phosphorylation of RhoA was detected. However, in cells treated with CPT-cAMP, the phosphorylation increased dramatically (Fig. 3).

RhoA Distribution in SGC-7901 Cells Treated With LPA and CPT-cAMP. Confocal laser scanning microscopy was used to observe the location of GFP-tagged RhoA in live SGC-7901 cells. Results showed that GFP-tagged RhoA was located in both the cytoplasm and nucleus, whereas GFP alone was located in the cytoplasm (Fig. 4A–D). Western blotting detected RhoA in both cytosolic and nuclear proteins from the cells (Fig. 4E). Immunofluorescence microscopy also revealed that RhoA protein was interspersed in the cytoplasm, whereas it was accumulated in several areas within the nucleus (Fig. 5A). Treatment of cells with LPA or CPT-cAMP, or CPT-cAMP followed by LPA, did not cause obvious translocation of RhoA (Fig. 5B–D), suggesting that, in SGC-7901 cells, the locational change was not related to the RhoA activation induced by LPA, or to the inhibition of RhoA activation induced by CPT-cAMP.

Effect of CPT-cAMP and LPA on Morphology of PC-3 Cells. Because our preliminary result showed that PC-3 cells changed their configuration much more obviously than SGC-7901 cells when they were treated with LPA, the morphologic experiment was carried out with PC-3 cells. Treating PC-3 cells with LPA changed the cell configuration from polygonal to round (Fig. 6A and B). In contrast, treating the cells with CPT-cAMP followed by LPA did not change the configuration of the cells (Fig. 6C). Cells co-transfected with plasmid DNA encoding GFP and wild-type RhoA had a polygonal morphology under fluorescence microscopy (Fig. 6D). Transfecting cells with plasmid DNA encoding constitutively active RhoA(63L) induced cell rounding and contraction (Fig. 6E). Treatment with CPT-cAMP induced an intermediate phenotype in RhoA(63L)-expressing cells (Fig. 6F). When the cells were transfected with plasmid DNA encoding RhoA188A, LPA induced the cells to change from polygonal to round, but CPT-cAMP could not further antagonize the effect of LPA (Fig. 6G and H).

Effect of CPT-cAMP and LPA on Cytoskeletal Changes of SGC-7901 Cells. To examine cytoskeletal changes induced by CPT-cAMP and/or LPA, we stained F-actin with rhodamine-conjugated phalloidin. The SGC-7901 cells growing on glass coverslips showed little F-actin (Fig. 7A); LPA significantly increased the formation of F-actin. (Fig. 7B). Pretreating cells with CPT-cAMP not only changed cell conformation, but also prevented LPA-induced F-actin formation (Fig. 7C and D). To show the structure in more detail, the cells were scanned with a confocal laser scanning microscope. In cells treated with LPA, fiber-like structures could be seen clearly; in cells treated with CPT-cAMP followed by LPA, the fiber-like structures did not appear (Fig. 7E and F).

Effect of CPT-cAMP on SGC-7901 Cell Motility Induced by LPA. In a modified Boyden chamber assay, a few untreated SGC-7901 cells migrated from the top side of the membrane to the bottom side of the membrane within 8 hrs. Treating the cells with 1 μM and 10 μM LPA promoted the migration of the cells, with 1 μM LPA exerting a more obvious stimulating effect (Fig. 8A). Treatment with CPT-cAMP inhibited the LPA-induced cell migration in a dosage-dependent manner (Fig. 8B).

Effect of CPT-cAMP and LPA on Anchorage-Independent Growth of SGC-7901 Cells. Because the effect of LPA on colony formation of SGC-7901 cells in soft agar was not obvious (preliminary data, not shown), the short time effect of LPA on cell proliferation in soft agar was investigated. Twenty-four hours after seeding, cell clusters containing several cells were seen in soft agar. The clusters became bigger as the incubation continued. In cells treated with LPA, there were more cell clusters than in untreated cells. Treatment with CPT-cAMP prevented the stimulating effect of LPA on the formation of cell clusters. To quantify the proliferation, 10 microscopic fields were randomly selected and the total cell numbers (representing the seeded cells) and the number of cell clusters (representing the proliferation of the cells) in the fields were counted. The ratio of cell clusters (number of cell clusters divided by number of seeded cells) was calculated and used to represent the anchorage-independent growth of the cells (Fig. 9).

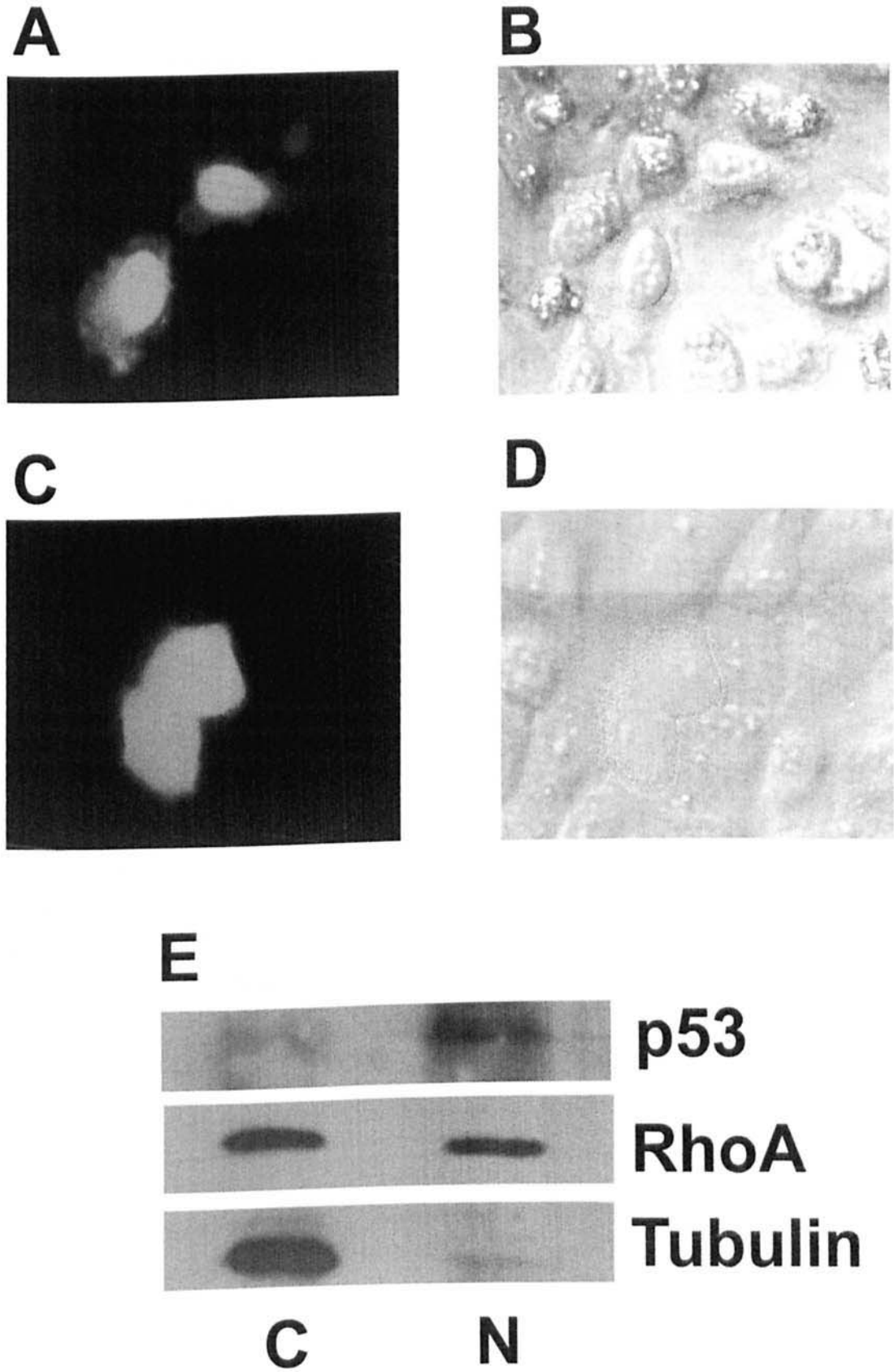

Figure 4. Distribution of RhoA in SGC-7901 cells. (A–D) Images from confocal laser scanning microscopy. (A) Laser scan of the cells transfected with plasmid DNA encoding GFP-tagged RhoA. (B) Overlay of the scanning image showing GFP-tagged RhoA and the scanning image showing the cytoplasm and the nucleus of the cells. (C) Laser scan of the cells transfected with plasmid DNA encoding GFP. (D) Overlay of the scanning image showing GFP and the scanning image showing the cytoplasm and the nucleus of the cells. (E) Result of Western blotting. Cytosolic and nuclear fractions were probed with antibodies against p-53 and α-tubulin to show the efficiency of the fractionation. The fractions were also probed with an antibody against RhoA to show the distribution of the protein. Lane C, cytosolic protein of SGC-7901 cells; Lane N, nuclear protein of SGC-7901 cells.

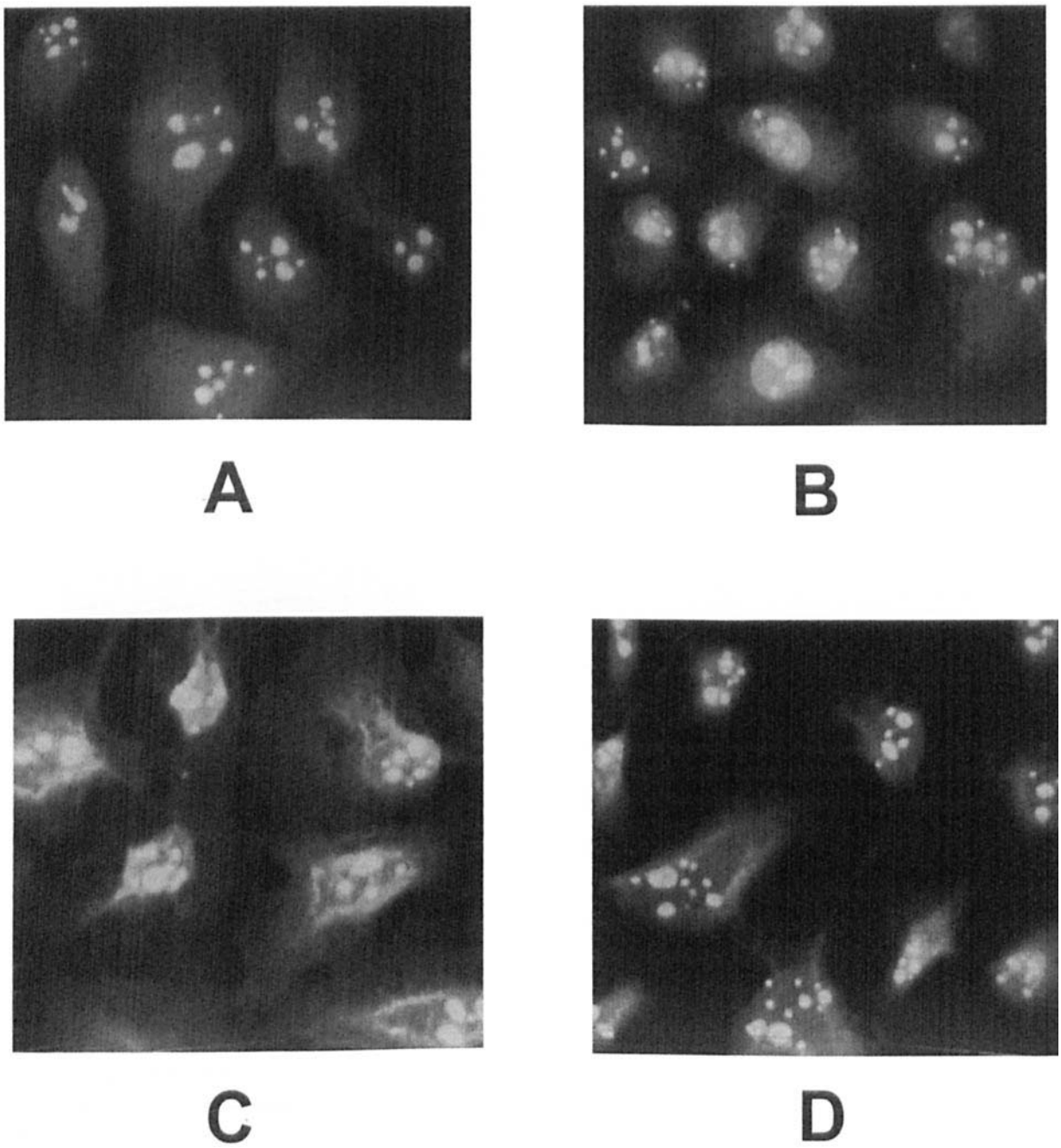

Figure 5. Effect of LPA and CPT-cAMP on RhoA distribution in SGC-7901 cells. Pictures from immunofluorescence microscopy. The cells were fixed with freshly prepared 2% paraformaldehyde and penetrated with 0.3% Triton X-100. The staining process included incubation with an anti-RhoA antibody for 2 hrs at RT and then with TRITC-conjugated secondary antibody for 1 hr at RT. (A) SGC-7901 cells without stimulation. (B) SGC-7901 cells stimulated with 1 μM LPA for 5 mins. (C) SGC-7901 cells treated with 100 μM CPT-cAMP for 30 mins. (D) SGC-7901 cells treated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 5 mins. Original magnification: ×1000 for all four photos.

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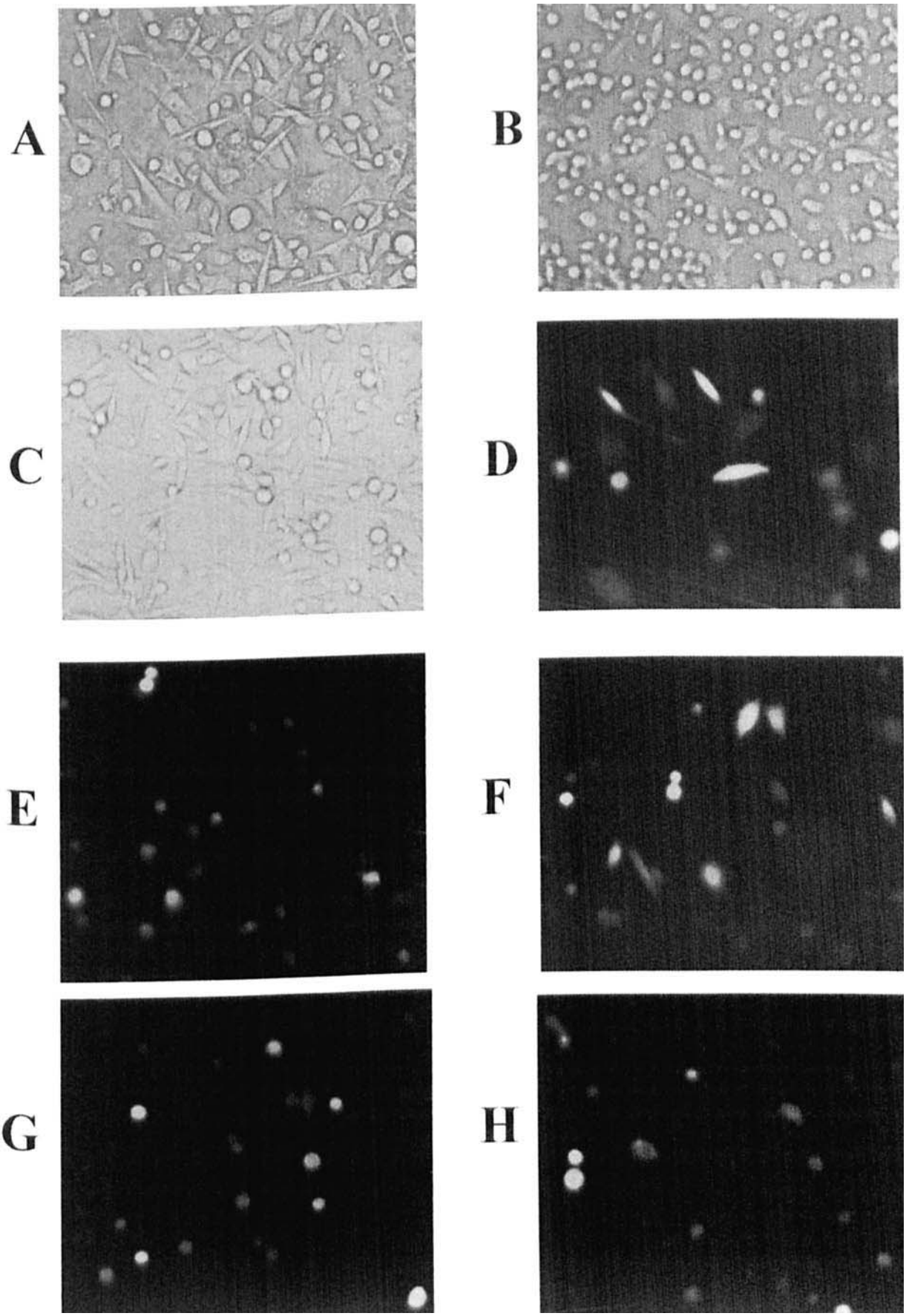
Figure 6. Morphologic changes of PC-3 cells induced by LPA and CPT-cAMP. (A–C) Phase-contrast images. (A) Untransfected PC-3 cells without stimulation. (B) Untransfected PC-3 cells stimulated with 1 μM LPA for 5 mins. (C) Untransfected PC-3 cells treated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 5 mins. (D–H) Reverse-fluorescence microscopy images. (D) PC-3 cells were transfected with plasmid DNA encoding wild-type RhoA, without stimulation. (E) PC-3 cells were transfected with plasmid DNA encoding RhoA(63L), without stimulation. (F) PC-3 cells transfected with plasmid DNA encoding RhoA(63L) and treated with 100 μM cAMP for 30 mins. (G) PC-3 cells transfected with plasmid DNA encoding RhoA188A and treated with 1 μM LPA for 5 mins. (H) PC-3 cells transfected with plasmid DNA encoding RhoA188A and treated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 5 mins. Original magnification: ×200 for all photos.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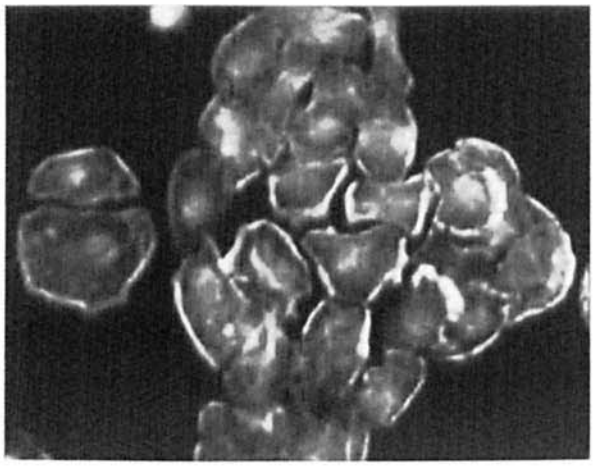

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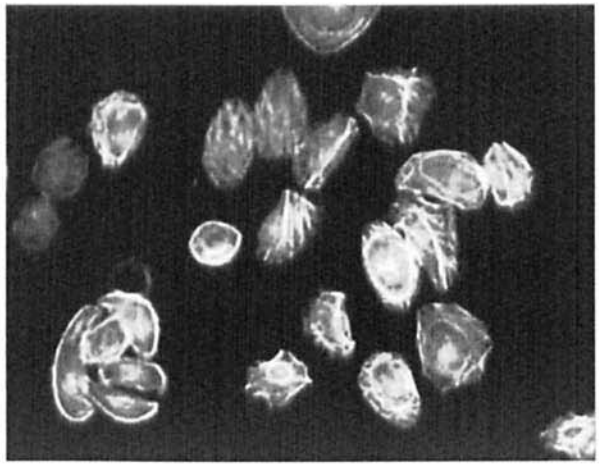

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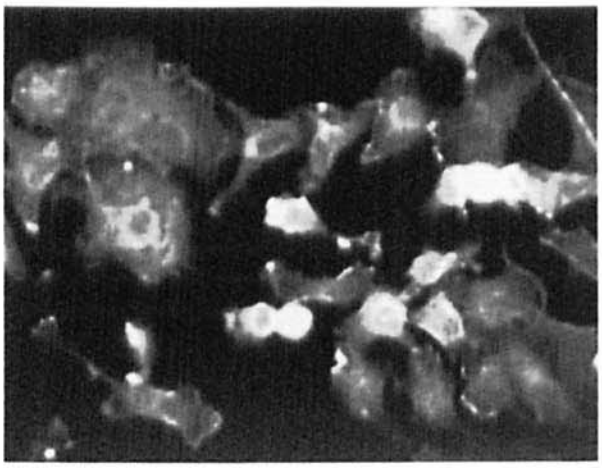

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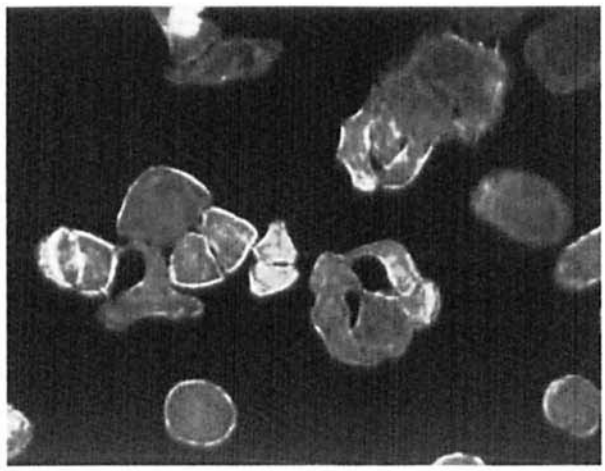

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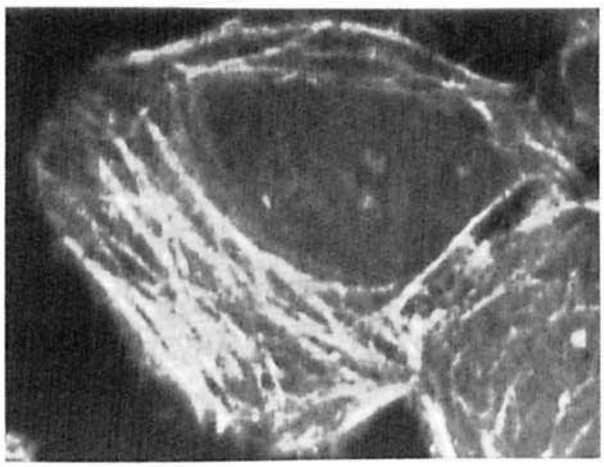

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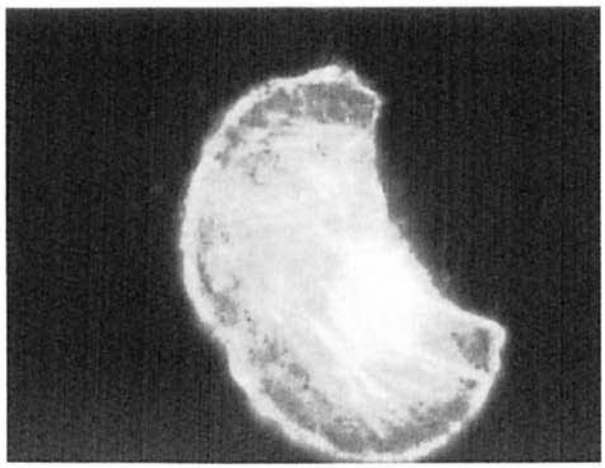

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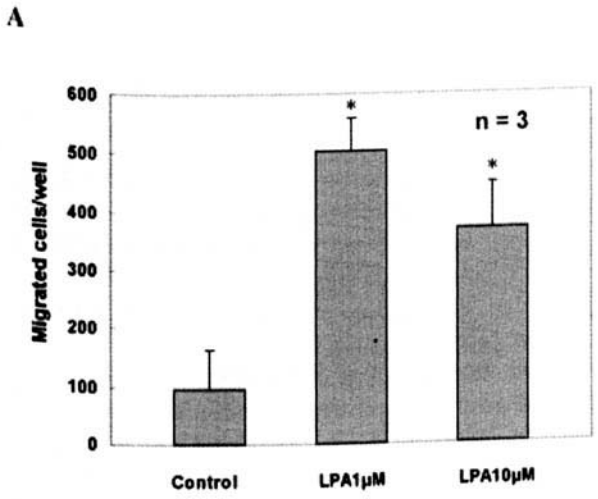

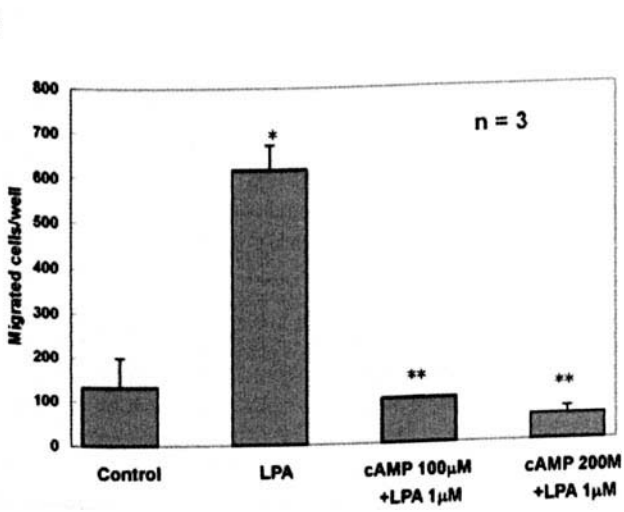

Figure 8. Migration of SGC-7901 cells treated with LPA and CPT-cAMP. In modified Boyden chamber assay, the migrated cells on the lower side of the membrane of the chamber were counted under microscope, presented as migrated cells per well. (A) The migration of SGC-7901 cells treated with LPA (1 μM, 5 μM). (B) The inhibition of CPT-cAMP (100 μM, 250 μM) on the LPA (1 μM) induced migration of SGC-7901 cells. The data shown were means ± SD from three independent experiments, each performed in duplicate. $^*P < 0.05$ compared with control. $^{**}P < 0.05$ compared with treatment with LPA alone.

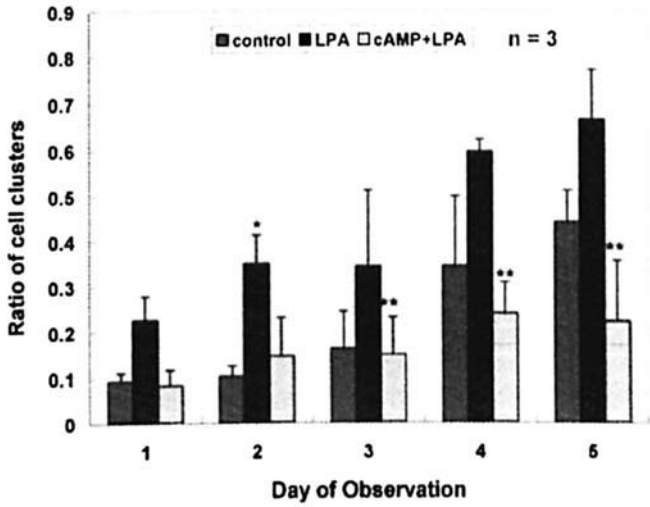

Figure 9. Change of cell cluster formation in SGC-7901 cells treated with LPA and CPT-cAMP. Cells were cultured in soft agar. Ratio of cell clusters (number of clusters divided by number of total seeded cells in the randomly selected field) was calculated to represent the proliferation of the cells. The data were means ± SD from three independent experiments. $^*P < 0.05$ compared with control. $^{**}P < 0.05$ compared with LPA treatment.

Discussion

Inhibition of RhoA by PKA occurs in many cell types, and involves inhibition of stress fiber and FAC formation with tyrosine dephosphorylation of FAC proteins (19–22). Treating tumor cells with cAMP-elevating agents can reverse the malignant phenotype, including a return to a more normal morphology and an inhibition of anchorage-independent growth and invasion (29). It has been proposed this may occur partially through PKA inhibition of RhoA. Although negative regulation of RhoA by PKA could provide novel pharmacologic approaches to inhibit tumor cell growth, invasion, and metastasis, relatively little is known about how PKA inhibits RhoA. Research data revealed that PKA phosphorylated RhoA on Ser^{188} *in vitro* and probably also *in vivo*. Although results are conflicting, it is likely that the *in vivo* phosphorylation enhances the RhoA association with RhoGDI, prevents RhoA activation, uncouples RhoA from interactions with downstream effectors, and dissociates RhoA from the plasma membrane (13, 14, 19, 22, 30, 31). Our results show that pretreating SGC-7901 cells with CPT-cAMP prevented the activation of RhoA induced by LPA, and that CPT-cAMP treatment caused obvious $serine^{188}$ phosphorylation of RhoA. This confirms that phosphorylation of $serine^{188}$ on RhoA is essential for PKA to exert its inhibitory effect in gastric cancer cells. In a separate experiment, we investigated the effect of LPA and CPT-cAMP on the association between RhoA and RhoGDI. Co-immunoprecipitation results showed that there was no obvious change of interaction between the two proteins when SGC-7901 cells were treated with LPA for different times (data not shown), indicating

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Figure 7. F-actin staining in SGC-7901 cells treated with LPA and/or CPT-cAMP. The cells were fixed with freshly prepared 2% paraformaldehyde, penetrated with 0.3% Triton X-100, incubated with TRITC-conjugated phalloidin for 1 hr at RT, and observed with a fluorescence microscope. (A) In SGC-7901 cells without stimulation, nearly no F-actin could be seen. (B) In SGC-7901 cells stimulated with 1 μM LPA for 5 mins, obvious formation of F-actin is seen. (C) In SGC-7901 cells stimulated with 100 μM cAMP for 30 mins, no F-actin staining is seen. (D) In SGC-7901 cells stimulated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 5 mins, no obvious F-actin staining is seen. The cells were also scanned with a confocal laser scanning microscope: (E) cells treated with 1 μM LPA for 5 mins; (F) cells treated with 100 μM CPT-cAMP for 30 mins followed by 1 μM LPA for 5 mins. Original magnification: ×400 for fluorescence microscopy photos.

that LPA did not increase the RhoA association with RhoGDI when it activated RhoA. On the other hand, treatment with CPT-cAMP did not change the interaction between RhoA and RhoGDI either (data not shown). The interaction between RhoA and its downstream kinase, ROCK, was also intensively investigated in our laboratory. Owing to several reasons, the interaction between RhoA and ROCK was not satisfactorily detected in this experiment. However, our preliminary immunofluorescence microscopy result showed that ROCK had a similar location to RhoA in SGC-7901 cells (data not shown). Therefore, in these cells, it was likely that a change in RhoA association with ROCK might be a result of RhoA phosphorylation induced by CPT-cAMP/PKA. Our results also showed that RhoA was located within both the cytoplasm and nucleus in SGC-7901 cells, and that LPA and/or CPT-cAMP did not cause detectable translocation of the protein. The unique location of RhoA in these cells was confirmed by immunofluorescence microscopy, Western blotting, and assay of expression of GFP-tagged RhoA in live cells. Because our results are inconsistent with most present data concerning RhoA location, and few of the previous data were from gastric and prostate cancer cells, our data might provide new knowledge about the location and function of this protein in cancer cells. Extensive investigation is being carried out in our laboratory to elucidate this matter.

The effect of RhoA activation on biological features of cancer cells has attracted much research attention. In a murine prostate cancer model, RhoA inhibition led to slowed tumor growth, reduced cellular motility, and decreased metastatic potential, whereas transfection of activated RhoA increased cellular proliferation (32). In the highly invasive human prostate cancer cell line, PC-3, inhibition of ROCK decreased cell motility *in vitro* and metastatic growth in immunocompromised mice *in vivo* (33). A similar function of RhoA was also observed in gastric cancers. For example, in gastric cancer tissue specimens, the mRNA expression levels of *RhoA* were significantly higher than those in the adjacent nontumorous tissue specimens (34). RhoA and its effector, Rhotekin, mediated nuclear factor-κB (NF-κB) activation, leading to cell survival, which may play a key role in gastric tumorigenesis (35). Both *RhoA*-specific siRNA and dominant-negative *RhoA* expressions could significantly inhibit the proliferation and tumorigenicity of the gastric adenocarcinoma cell line, AGS (36). On the other hand, agents that increase intracellular cAMP in prostate cancer cells generally inhibit proliferation and reduce invasive potential (37–40). The growth of PC-3 cells *in vitro* and in nude mice is severely inhibited by antisense inhibition of the RI-subunit of PKA. This leads to increased free C subunit activity of PKA, supporting a role for PKA in prostate cancer growth control (41). In several prostate cancer cell lines, cAMP-elevating agents or transfection of the free C subunit of PKA induce a loss of mitotic activity and some degree of neuroendocrine differentiation, including extension of neurite-like processes and expression of neurotensin and chromogranin A (36, 37, 42, 43). In the human gastric adenocarcinoma cell line, AGS, a direct increase of the intracellular cAMP level resulted in a dose-dependent growth inhibition (44).

Although it is clear that RhoA- and cAMP/PKA-mediated signal transductions play an important role in cancer growth, invasion, and metastasis, and that there is cross talk between the two signal transduction pathways, to our knowledge, there are no studies addressing the role of cross talk between RhoA- and cAMP/PKA-mediated signal transductions in regulating the biological activities of prostate and gastric cancer. In this experiment, the effect of cross talk between RhoA- and cAMP/PKA-mediated signal transduction on cancer cell biologic activities, such as morphologic change, migration, and anchorage-independent growth, was investigated. The results revealed that PKA activation inhibits RhoA function in both prostate cancer cells and gastric cancer cells. In PC-3 cells, LPA induced cell rounding and contraction, and transfecting cells with constitutively active RhoA(63L) induced similar morphologic changes. Treatment with CPT-cAMP inhibited the morphologic changes induced by activation of both endogenous and exogenous RhoA. In the SGC-7901 gastric cancer cells, CPT-cAMP inhibited both the migration and anchorage-independent growth induced by LPA. These results revealed that cross talk between the cAMP/PKA- and RhoA-mediated signal transductions had an important effect on biologic features of cancer cells. This will be helpful to further our knowledge about the mechanisms of the migration and the invasive growth of cancer cells, and may potentially lead to novel therapeutic approaches.

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