Ga11 Induces Caspase-mediated Proteolytic Activation of Rho-associated Kinase, ROCK-I, in HeLa Cells*

Hiroshi Ueda‡, Rika Morishita‡, Hiroshi Itoh§, Shuh Narumiya¶, Katsuhiko Mikoshiba‡, Kanefusa Katot, and Tomiko Asanot

Expression of the constitutively active mutant of Ga11 (Ga11QL) induces the formation of vinculin-containing focal adhesion-like structures in HeLa cells. This was found to be inhibited by Y-27632, a specific inhibitor of Rho-associated kinases (ROCK), but not by co-expression with a dominant negative mutant of RhoA, suggesting Rho-independent activation of ROCK by Ga11QL. Investigation of trypan blue exclusion and immunocytochemistry with an antibody against cleaved caspase revealed the cellular phenotype of Ga11QL-expressing cells to be identical to that displayed by cells undergoing apoptosis, and the caspase inhibitor zVAD-fmk blocked all morphological changes induced by Ga11QL. Transfection of Ga11QL induced cleavage of ROCK-I, and this proteolysis was also prevented by zVAD-fmk. ROCK-I C-terminally truncated at its authentic caspase sites also induced the formation of vinculin-containing focal adhesion-like structures. In addition, cleavage of ROCK-I was observed when cells overexpressing m1 muscarinic acetylcholine receptors were stimulated with carbachol. These results suggest that Ga11 induces proteolytic activation of ROCK-I by caspase and thereby regulates the actin cytoskeleton during apoptosis.

Heterotrimeric GTP-binding proteins (G proteins) play key roles as modulators in intracellular signal transduction from cell surface G protein-coupled receptors (1, 2). The α subunits of G proteins comprising four major families, Ga, Gb, Gc, and Gd, have a guanine nucleotide-binding site and intrinsic GTPase activity. When the associated receptors are activated by stimuli, exchange of a guanine nucleotide from GDP to GTP occurs which then interact with and regulate effectors, such as adenyl cyclase and phospholipase Cβ as well as K+- and Ca2+-channels (1, 2).

Recent studies have shown that G proteins regulate cell shape and cell motility through reorganization of the actin cytoskeleton by activating Rho family GTP-binding proteins (3–7). In cultured fibroblasts (3, 4) and epithelial cells (6), Ga12 and Ga13 subunits of the Gaq family G proteins and Gb2 subunits induce the formation of focal adhesions and stress fibers through activation of Rho to facilitate cell adhesion to the extracellular matrix via integrins. Gaq, as well as Ga12 and Ga13, induce Rho-dependent neurite retraction in differentiated PC12 cells (8).

Apoptosis is characterized by morphological changes including cell shrinkage, dynamic membrane blebbing, chromatin condensation, activation of specific proteases and endonucleases, and DNA fragmentation (9). In blebbing cells, myosin light chain phosphorylation is increased, and myosin light chain kinase inhibition decreases membrane blebbing (10). It has been established that myosin light chain phosphorylation is regulated by a small GTP-binding protein, Rho, and its effectors, Rho-associated coiled-coil forming protein serine/threonine kinases (ROCKs) such as ROCK-I (p160ROCK) and ROCK-II (11–16). The botulinum C3 exoenzyme, which inactivates Rho, inhibits apoptotic membrane blebbing, supporting a role for the Rho pathway in this process (10). Recent reports have indicated that enhancement of Gaq activity induces apoptosis of cultured cells and cardiac myocytes in transgenic mice (17–19). Transfection of a constitutively active mutant of Gaq into COS-7 and CHO cells was further found to cause apoptosis through a protein kinase C-dependent mechanism (17). In cardiac myocytes, expression of a constitutively active mutant of Gaq produces initial hypertrophy, which rapidly progresses to apoptotic cell death (18). However, the underlying mechanisms are not entirely clear.

We have previously demonstrated that Gb2 and a constitutively active mutant of Ga12 caused the formation of actin stress fibers and focal adhesions in a Rho-dependent manner in HeLa cells (6). In the same report, we demonstrated that expression of constitutively active mutants of Gaα5, Gaα92, and Ga11 (Ga11QL), a member of the Gaq family, does not induce stress fibers, though Ga11QL appeared to be associated with formation of thin actin fibers judging by the bright staining of cells with rhodamine phalloidin (6). In the present study, we found Ga11QL to strongly induce vinculin containing focal adhesion-like structures, which was mediated by a ROCK-dependent but Rho-independent pathway. We here present evidence that Ga11 induces proteolytic activation of ROCK-I by caspase, and this results in regulation of the actin cytoskeleton.
were co-transfected with Gα11,QL induced formation of vinculin-enriched plaques or Gβγ-induced focal adhesion assembly. HeLa cells were transfected with Gα11,QL (A, B, C) or co-transfected with Gβ6 and Gγ11, (F, I, D) and treated with 10 μM Y-27632 (E, F) or 100 μM zVAD-fmk (zVAD) (G, H) for 48 h. In C and D, cells were co-transfected with Gα11,QL and a dominant negative mutant of Rho (RhoDN) (C) or with Gβ6, Gγ11, and RhoDN (D). The cells were stained with the antibody against vinculin. Those expressing Gα11,QL and Gβ6 were identified by staining with the respective antibodies as indicated by arrowheads. The results shown are representative of three independent experiments. Scale bar, 50 μm.

**EXPERIMENTAL PROCEDURES**

**Materials**—pCMV5-Gα11,QL, pCMV5-Gγ11, pCMV5-Gβ6, pCMV5-FLAG-RhoAT19N, the pCMV5-m1 muscarinic acetylcholine receptor, and pCAG-Myc-ROCK-I were constructed as detailed earlier (7, 20–22). To construct mutants with stop codons substituted for Ser-1100, Gly-1114, and Lys-1159 of ROCK-I, polymerase chain reaction was performed with pCAG-Myc-ROCK-I as a template using primers (forward primer: 5'-CAGGCTCTTTAAACATGTTGGACAA-3', reverse primers: 5'-CGGGATATCGTGCAGTTAATCCGAGAGGTCCAAAAGTT-3', 5'-CGGGATATCGTGCAGTTAATCCGAGAGGTCCAAAAGTT-3', 5'-CGGGATATCGTGCAGTTAATCCGAGAGGTCCAAAAGTT-3', 5'-CGGGATATCGTGCAGTTAATCCGAGAGGTCCAAAAGTT-3', 5'-CGGGATATCGTGCAGTTAATCCGAGAGGTCCAAAAGTT-3'). XhoI fragment of pCMV5-Myc-ROCK-I was ligated to a plasmid pBluescript SK-I to produce pBluescript-ROCK-I-XhoI. Polymerase chain reaction products digested with HindIII and EcoRV were inserted into XhoI-EcoRV fragment of pBluescript-ROCK-I-XhoI. These products were digested with XhoI and inserted into a XhoI fragment of pCMV5-Myc-ROCK-I, respectively. The antibody against the Gβ subunit generated by us has been described previously (23). A rabbit polyclonal antibody against ROCK-I and ROCK-II (ROKAB5) was purchased from Transduction Laboratories, mouse monoclonal antibodies against ROCK-I and ROCK-II (ROK) from Sigma, a mouse monoclonal antibody against human vinculin and FLAG from Transduction Laboratories, mouse monoclonal antibodies against human Fas (CH-11) from MBL. Antibodies against phosphospecific p38 MAP kinase and cleaved caspase-3 were obtained from Cell Signaling Technology. A mouse monoclonal antibody against Myc epitope (9E10) was purchased from Roche Molecular Biochemicals and Alexa Fluor 488 anti-rabbit IgG, Alexa Fluor 568 anti-mouse IgG and Alexa Fluor 568 phalloidin were obtained from Molecular Probes. Ro-31-8220, Gö 6983, BAPTA/AM, or 2-aminoethoxydiphenyl borate were stimulated with 100 μM carbachol. Cells were then fixed and immunostained with antibodies against vinculin or subjected to immunoblotting. In experiments with other apoptotic stimuli, cells were transfected with ROCK-I, cultured for 16 h, and stimulated with staurosporine, an antibody against Fas plus cyclohexamide, phorbol 12-myristate 13-acetate, or A23187 for 4 h. For immunoblotting, they were lysed with 1% SDS in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and lysates were subjected to SDS-polyacrylamide gel electrophoresis using 7.5% and 10% polyacrylamide gels for detection of ROCK and other proteins, respectively. Immunoblotting was performed employing a chemiluminescence reagent (PerkinElmer Life Sciences), and densitometry was achieved using the LAS-1000 system (Fujifilm).

**Transfection and Immunofluorescence Staining**—HeLa cells were grown in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum at 37°C. Transient transfection with LipofectAMINE plus reagent was accomplished according to the manufacturer’s instructions (Life Technologies, Inc.). Immediately after transfection, zVAD-fmk and Y-27632 were added to final concentrations of 100 and 10 μM, respectively. Cells were cultured for 16 or 48 h, washed with phosphate-buffered saline, and fixed in 4% paraformaldehyde in phosphate-buffered saline. Fixed cells were then immunostained using antibodies against Gα11, Gβ6, vinculin, and cleaved caspase-3 followed by secondary antibodies, Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 568 anti-mouse IgG. The cells were also stained for F-actin with Alexa Fluor 568 phalloidin. After coverslipping, slides were examined under a laser-scanning microscope (FLUOVIEW Olympus) equipped for fluorescence.

Cell Stimulation and Immunoblotting—HeLa cells were co-transfected with m1 muscarinic acetylcholine receptor and ROCK-I plasmids (plus pEGFP-C3 for immunocytochemistry), cultured for 16 h, and 30 min after addition of atropine, Y-27632, zVAD-fmk, Ro-31-8220, Gö 6983, BAPTA/AM, or 2-aminoethoxydiphenyl borate were stimulated with 100 μM carbachol. Cells were then fixed and immunostained with antibodies against vinculin or subjected to immunoblotting. In experiments with other apoptotic stimuli, cells were transfected with ROCK-I, cultured for 16 h, and stimulated with staurosporine, an antibody against Fas plus cyclohexamide, phorbol 12-myristate 13-acetate, or A23187 for 4 h. For immunoblotting, they were lysed with 1% SDS in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and lysates were subjected to SDS-polyacrylamide gel electrophoresis using 7.5% and 10% polyacrylamide gels for detection of ROCK and other proteins, respectively. Immunoblotting was performed employing a chemiluminescence reagent (PerkinElmer Life Sciences), and densitometry was achieved using the LAS-1000 system (Fujifilm).
**Results and Discussion**

We have previously reported that expression of Gβγ induces the formation of thick actin stress fibers and focal adhesions in HeLa cells (6). Co-transfection of a dominant negative mutant of RhoA and a specific inhibitor of ROCK, Y-27632 have inhibited Gβγ-induced focal adhesion assembly (Fig. 1, B, D, and F) and stress fiber formation (data not shown), indicating that these morphological changes are Rho- and ROCK-dependent (6). Because cells were cultured in medium containing 10% fetal calf serum in the present study, focal adhesions were also observed in surrounding untransfected cells, but to a much lesser extent than in Gβγ-expressing cells (Fig. 1B). Expression of Gα11QL induced the formation of vinculin-enriched plaques and somewhat contractile shapes of cells (Fig. 1A). Punctate vinculin staining was observed within as well as at the edges of
Fig. 4. Truncated form ROCK-I, with high intrinsic kinase activity, induces the formation of vinculin-enriched plaques. A, HeLa cells were transfected with Mock, wild-type Myc-ROCK-I (WT), truncated forms of Myc-ROCK-I, D1, D2, and D3, or wild-type Myc-ROCK-I plus G11QL (WT + G11QL), and incubated for 16 h. Lysates were immunoprecipitated with the antibody against ROCK-I. B, cells transfected with Mock, wild-type Myc-ROCK-I, or D2 in the presence of 100 μM zVAD-fmk were immunoprecipitated with the antibody against Myc. Immunoprecipitates were subjected to immunoblotting with the antibody against ROCK-I, showing similar amounts of ROCK-I to be precipitated (left panel). The same immunoprecipitates were assayed for MBP kinase activity in vitro (right panel). The intensities of phosphorylated MBP were quantified, and the relative densities (means ± S.D. of data from three experiments) are shown below each panel.

Fig. 5. Cleavage of ROCK-I by caspase on stimulation of the m1 muscarinic acetylcholine receptor. A, HeLa cells were co-transfected with the m1 muscarinic acetylcholine receptor and Myc-ROCK-I, and incubated for 16 h. Then cells were stimulated with 100 μM carbachol for the indicated times, and lysates were immunoblotted with the antibody against ROCK-I. B, cells were co-transfected with the m1 muscarinic acetylcholine receptor and Myc-ROCK-I, incubated for 16 h, and then stimulated with 100 μM carbachol in the presence or absence of 10 μM atropine (atro) or 100 μM zVAD-fmk (zVAD) for 4 h. Lysates were immunoblotted with the antibody against ROCK-I. The intensity of the 130-kDa band was quantified by densitometry and relative intensities are shown as means ± S.D. of data from four experiments.

cells and was more dense and greater in area than that in Gβ7γ2-expressing cells (Fig. 1, A and B). Gα11QL-induced formation of vinculin-enriched plaques, however, was not inhibited by co-transfection of the dominant negative mutant of Rho (Fig. 1C), whereas it was blocked by Y-27632 (Fig. 1E), suggesting Gα11QL-induced formation of vinculin-enriched plaques to be Rho-independent but ROCK-dependent.

Because previous reports showed that a constitutively active mutant of Gαq induced apoptosis in several cultured cells (17-19), we examined whether this was also the case for Gα11QL in HeLa cells. First, the trypan blue exclusion method revealed that expression of Gα11QL increased the population of trypan blue-positive cells (Fig. 2A). Treatment with zVAD-fmk, a specific inhibitor of caspases, prevented this increase. Next, determination of protein content as a parameter for detached cells indicated that Gα11QL promoted detachment, but not in the presence of zVAD-fmk (Fig. 2B). In a third experiment, immunocytochemical staining with an antibody against cleaved caspase-3 showed many positive Gα11QL-expressing cells, and treatment with zVAD-fmk prevented cleavage of these (Fig. 2C). In addition, zVAD-fmk inhibited Gα11QL-induced vinculin-enriched plaque formation but not Gβ7γ2-induced focal adhesion formation (Fig. 1, G and H). These results strongly indicate that Gα11QL induces apoptosis in HeLa cells, and this involves ROCK activation.

It has been shown that one Rho effector, a fatty acid- and Rho-activated serine/threonine kinase, is cleaved and activated by caspase-3 in Jurkat and U973 cells during apoptosis (26). In addition, evidence that deletion of the C-terminal region of ROCK results in kinase activation (22, 27, 28) and transfection with C-terminally truncated mutants induces the formation of vinculin-enriched plaques in HeLa cells (22) suggests the possibility that ROCK is cleaved and activated by caspase. Cell extracts were therefore immunoblotted with the antibody against ROCK-I, and indeed a fragment with a molecular mass of ~130 kDa was observed, as well as the native protein with a molecular mass of ~160 kDa, in Gα11QL-expressing cells (Fig. 3A). This fragment was not detected in the presence of zVAD-fmk, indicating the cleavage of ROCK-I to be caspase-dependent (Fig. 3A). In contrast, immunoblotting with the antibody against ROCK-II revealed that it was not a substrate of caspase (Fig. 3A). To confirm these observations obtained with endogenous ROCK-I, we co-transfected HeLa cells with Gα11QL and Myc-tagged ROCK-I. Gα11QL stimulated cleavage of overexpressed ROCK-I, and this was inhibited by zVAD-fmk (Fig. 3B). The expression of the dominant negative mutant of Rho did not affect Gα11QL-induced cleavage of ROCK-I, suggesting the cleavage to be Rho-independent (Fig. 3C).

In the present study, we used two separate antibodies to detect ROCK-I; one against a peptide (amino acid residues 906–1012) and the other against Myc for detection of ROCK-I tagged with Myc epitope at the N terminus (Fig. 3D). Because both antibodies reacted with the fragment with a molecular mass of ~130 kDa as well as native protein (Fig. 3B), it was concluded to have an intact N terminus. Previous studies have established that caspase cleaves carboxyl-terminally at sequences conforming to the consensus sequence DXDX (26, 29). Notably, around 130 kDa from the N terminus of ROCK-I protein are three potential caspase cleavage sites: the first comprising amino acid residues 1096–1099 and the second and third corresponding to amino acid residues 1110–1113 and 1155–1158, respectively (Fig. 3D). ROCK-I is probably cleaved at Asp-1099, Asp-1113, or Asp-1158, to constitutively active forms. To examine this hypothesis, we transfected HeLa cells with mutants of ROCK-I in which amino acid residues at 1100 (D1), 1114 (D2), and 1159 (D3) were substituted with stop codons. Immunoblot analyses of cell lysates revealed D2 to have the same apparent molecular mass as the ROCK-I fragment cleaved by the caspase, suggesting the cleavage site to be Asp-1113 (Fig. 4A). The D2 mutant was then subjected to an in vitro immunocomplex kinase assay to determine whether it is an active form, showing higher phosphorylation of MBP than full-length wild-type ROCK-I (Fig. 4B). Transfection with D2 induced contractile shapes of cells and the formation of vinculin-enriched plaques, which were more dense and greater in area than those in wild-type ROCK-I-expressing cells (Fig. 4, D and E). The caspase inhibitor did not affect the plaque formation induced by D2 (Fig. 4F). These results indicate that caspase-cleaved ROCK-I is the constitutively active form.

Because transfection of a constitutively active mutant induced sustained activation of Ga, the apoptosis and cleavage of ROCK-I in Gαi1-QL-expressing cells could have been due to secondary effects. To assess this possibility, we transfected cells with the m1 muscarinic acetylcholine receptor, which couples with Gq and G11, and examined whether cleavage of ROCK-I occurs upon stimulation of the receptor. A time course study showed carbachol-induced cleavage of ROCK-I, which was observed around 2 h after stimulation (Fig. 5A). Carbachol also increased the level of phosphorylation of p38 MAP kinase around 20 min after stimulation, indicating functioning of the expressed m1 muscarinic receptors (data not shown). Carbachol-stimulated cleavage of ROCK-I was prevented by the antagonist, atropine, showing the cleavage to be agonist-dependent (Fig. 5B). Small amounts of the fragment were also observed in unstimulated cells. Atropine diminished carbachol-enhanced cleavage to this level of untreated cells, whereas zVAD-fmk exerted almost complete blocking action (Fig. 5B). These results suggest that some apoptosis does occur in untreated HeLa cells, probably due to the 10% fetal calf serum, which contains various agonists capable of activating Gq/11, such as lysophosphatidic acid (30).

We next examined whether carbachol stimulation induces the formation of vinculin-enriched plaques in a ROCK-dependent but Rho-independent manner, as observed in Fig. 1. Upon stimulation of cells expressing the m1 muscarinic acetylcholine receptor by carbachol, vinculin staining increased in to cover greater areas and became more dense (Fig. 6, A and B). Carbachol-induced vinculin-enriched plaque formation was inhibited by atropine and Y-27632 but not by a dominant negative mutant of Rho (Fig. 6, C–E). In the presence of the caspase
these conditions (data not shown). These results suggest that nase C (33), indicating these inhibitors to be effective under protein kinase C inhibitors, Ro-31-8220 and Go
10
in the presence or absence of 0.5 μM carbachol in

1,4,5-trisphosphate-induced Ca2

sorine and anti-Fas plus cycloheximide effectively induced ROCK-I cleavage, HeLa cells were incubated with staurosporine, an antibody against Fas plus cycloheximide, phorbol 12-myristate 13-acetate, or the Ca2

Effects of inhibitors for protein kinase C and inositol 1,4,5-trisphosphate-induced Ca2

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inhibitor, vinculin staining was only partially decreased, though zVAD-fmk is expected to completely prevent carbachol-stimulated vinculin-enriched plaque formation (Fig. 6F). Incomplete inhibition could be explained as follows; m1 muscarinic acetylcholine receptors may couple with G12/13 as well as Gq/11 (31), and activated Go and Gβγ are released from these G proteins on stimulation of receptors. Both Gβγ and G12/13 induce focal adhesion assembly in a Rho- and ROCK-dependent manner, and this signal pathway may occur together with activation of ROCK-I by caspase-mediated cleavage on stimulation of m1 muscarinic acetylcholine receptor-expressing cells. This explanation is supported by the fact that the combination of the dominant negative mutant of Rho and zVAD-fmk completely inhibited carbachol-stimulated formation of vinculin-enriched plaques (Fig. 6G). These results thus indicate that activation of Gq11 by receptor stimulation induces caspase-mediated activation of ROCK-I.

It is well known that Gq stimulates phospholipase Cβ, resulting in an increase in protein kinase C activity and elevation of initial Ca2

sorine, an antibody against Fas plus cycloheximide, phorbol 12-myristate 13-acetate, or the Ca2

are released from these G proteins on stimulation of receptors. Both Gβγ and G12/13 induce focal adhesion assembly in a Rho- and ROCK-dependent manner, and this signal pathway may occur together with activation of ROCK-I by caspase-mediated cleavage on stimulation of m1 muscarinic acetylcholine receptor-expressing cells. This explanation is supported by the fact that the combination of the dominant negative mutant of Rho and zVAD-fmk completely inhibited carbachol-stimulated formation of vinculin-enriched plaques (Fig. 6G). These results thus indicate that activation of Gq11 by receptor stimulation induces caspase-mediated activation of ROCK-I.

In conclusion, Gq11 stimulates phospholipase Cβ, resulting in an increase in protein kinase C activity and elevation of initial Ca2

antibody against Fas plus 40 μg/ml cycloheximide (aFas), 50 nm phorbol 12-myristate 13-acetate (PMA) or 5 μM A23187 (A23). Lysates were immunoblotted with the antibody against ROCK-I. The intensity of the 130-kDa band was quantified by densitometry and relative intensities are shown as mean ± S.D. of data from three experiments.

This evidence suggests that the intracellular concentration of phosphatidylinositol 4,5-bisphosphate directly regulates the progression of apoptosis, and therefore, the decrease of phosphatidylinositol 4,5-bisphosphate by Gq11-stimulated phospholipase C may activate caspase pathways. Thus, Gq11 regulates concentrations of phosphatidylinositol 4,5-bisphosphate and Ca2

11-mediated caspase activation in HeLa cells. Cells were transfected with ROCK-I, cultured for 16 h, and incubated in the presence or absence (cont) of 1 μM staurosporine (ST), 0.5 μg/ml antibody against Fas plus 40 μg/ml cycloheximide (aFas), 50 nm phorbol 12-myristate 13-acetate (PMA) or 5 μM A23187 (A23). Lysates were immunoblotted with the antibody against ROCK-I. The intensity of the 130-kDa band was quantified by densitometry and relative intensities are shown as mean ± S.D. of data from three experiments.

To examine whether other common apoptotic stimuli induce ROCK-I cleavage, HeLa cells were incubated with staurosporine, an antibody against Fas plus cycloheximide, phorbol 12-myristate 13-acetate, or the Ca2

The present study as well as previous findings (17–19) suggests that enhanced signals to Gq11 may trigger apoptosis.

ROCK-I is known to regulate myosin light chain phosphorylation (16), and a previous study showed that this is critical for apoptotic membrane blebbing, also implicating Rho signaling in such active morphological changes (10). Although the physiological role of the vinculin-containing plaques observed in the present study is unclear, caspase-mediated ROCK-I activation seems to contribute to morphological changes such as cell contraction and membrane blebbing during apoptosis.

ROCK-I activation has also been found to contribute to stimulation of transcription by a serum response factor (36). Thus the constitutively active mutant of ROCK-I activates serum response factor-dependent gene expression. However, this activation is inhibited by the C3 exoenzyme, indicating that ROCK-I activation and an unidentified Rho-dependent pathway are necessary for stimulation of serum response factor-dependent gene expression (36). Sagi et al. (37) also demonstrated that a constitutively active mutant of Goq stimulated serum response factor-dependent transcription without activating Rho in COS-7 cells. Because the constitutively active mutant of Goq induces apoptosis of COS-7 cells (17), caspase-induced activation of ROCK-I may be involved in Goq-induced activation of serum response factor-dependent transcription.

In conclusion, Gαq induces proteolytic activation of ROCK-I by caspase and thereby regulates the actin cytoskeleton. The
results point to the existence of a novel pathway activating ROCK-I in a Rho-independent manner and may offer insights on some previously inexplicable observations related to Rho and ROCK-I.

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**REFERENCES**

Additions and Corrections


$\text{Ga}_{11}$ induces caspase-mediated proteolytic activation of Rho-associated kinase, ROCK-I, in HeLa cells.

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Page 42531: Fig. 6 is wrong. The correct figure and its legend are shown following.

**FIG. 6. Effects of atropine, the dominant negative mutant of Rho, Y-27632, and zVAD-fmk on carbachol-induced formation of vinculin-enriched plaques.** HeLa cells were co-transfected with pEGFP-C3 and the m1 muscarinic acetylcholine receptor (A–C, E, and F) plus the dominant negative mutant of Rho (RhoDN) (D and G) and incubated for 16 h. They were treated with 10 $\mu$M atropine (C), 10 $\mu$M Y-27632 (E), or 100 $\mu$M zVAD-fmk (zVAD) (F and G) for 30 min before addition of 100 $\mu$M carbachol. At 4 h after carbachol stimulation, cells were stained with the antibody against vinculin, and GFP-positive cells are indicated by arrowheads. The results shown are representative of three independent experiments. Scale bar, 50 $\mu$m.

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