**In vivo** Screening for Substrates of Protein Kinase A Using a Combination of Proteomic Approaches and Pharmacological Modulation of Kinase Activity

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**ABSTRACT.** Protein kinase A (PKA) is a serine/threonine kinase whose activity depends on the levels of cyclic AMP (cAMP). PKA plays essential roles in numerous cell types such as myocytes and neurons. Numerous substrate screens have been attempted to clarify the entire scope of the PKA signaling cascade, but it is still underway. Here, we performed a comprehensive screen that consisted of immunoprecipitation and mass spectrometry, with a focus on the identification of PKA substrates. The lysate of HeLa cells treated with Forskolin (FSK)/3-isobutyl methyl xanthine (IBMX) and/or H-89 was subjected to immunoprecipitation using anti-phospho-PKA substrate antibody. The identity of the phosphoproteins and phosphorylation sites in the precipitants was determined using liquid chromatography tandem mass spectrometry (LC/MS/MS). We obtained 112 proteins as candidate substrates and 65 candidate sites overall. Among the candidate substrates, Rho-kinase/ROCK2 was confirmed to be a novel substrate of PKA both in vitro and in vivo. In addition to Rho-kinase, we found more than a hundred of novel candidate substrates of PKA using this screen, and these discoveries provide us with new insights into PKA signaling.

**Key words:** Protein kinase A, Mass spectrometry, Phosphorylation, Rho-kinase

**Introduction**

Protein kinase A (PKA) is one of the first discovered kinases, and has been a central model for the study of signal transduction (Walsh *et al.*, 1968; Walsh and Van Patten, 1994). PKA is serine/threonine kinase activated in accordance with the cellular concentration of cAMP (Granot *et al.*, 1980). The PKA pathway begins extracellularly with an interaction between a ligand and its respective G protein coupled receptor (GPCR). When an external signaling molecule, such as adrenaline or dopamine, binds to a GPCR, the GPCR undergoes a conformational change (Ghanouni *et al.*, 2001). The Gs protein is activated by an active-state GPCR, which then activates adenylyl cyclase to increase the synthesis of cAMP (Pohl *et al.*, 1971; Tesmer *et al.*, 1997). This signaling cascade results in the activation of PKA, which in turns regulates a wide variety of physiological functions, such as contraction of the heart (Le Peuch *et al.*, 1979), during emotional behaviors (Self *et al.*, 1998) through the phosphorylation of various substrates including calcium channels and CREB (Holz *et al.*, 1986; Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989). Inactive PKA is a heterotetramer composed of a catalytic subunit dimer and a regulatory subunit dimer. The binding of cAMP to the regulatory subunit promotes a dissociation into a dimer of regulatory subunits and two active monomeric catalytic subunits (Granot *et al.*, 1980). The consensus phosphorylation motif of PKA is arginine-arginine-X-serine, where X is a hydrophobic amino acid (Kemp *et al.*, 1975, 1977). Although much is known regarding the downstream targets of PKA, there remains unidentified targets that could explain more of the physiological functions in which PKA participates. To expand on the knowledge surrounding PKA signaling, phosphoproteomics presents an ideal method for investigation.
Mass spectrometry has technically advanced to a point at which phosphorylation dynamics can be elucidated (Mann et al., 2002). Advances not only in instrumental development but also in the sample preparation technique prior to the ionization have contributed to the enhanced sensitivity and specificity. Among several approaches, the development of antibodies that can recognize and immunoprecipitate phosphorylated substrates specifically is a good example of improvements made in the investigation of downstream signaling (Moritz et al., 2010).

We report here a comprehensive phosphoproteomic approach for PKA substrate screening using FSK and IBMX, as well as the PKA inhibitor, H-89. The lysates of HeLa cells treated with FSK and IBMX and/or H-89 were subjected to immunoprecipitation using anti-phospho-PKA substrate Ab to enrich for phosphoproteins. The identity of the phosphoproteins and phosphorylation sites in the precipitants was determined by liquid chromatography tandem mass spectrometry (LC/MS/MS). We obtained 112 proteins as candidate substrates, including Rho-kinase, and 65 candidate sites overall.

**Materials and Methods**

**Reagents and chemicals**

The following antibodies and chemicals were purchased from commercial sources: GST-PKA-cat (Carna Biosciences, Kobe, Japan); anti-VASP antibody, anti-phospho-VASP (serine 157) antibody, rabbit monoclonal anti-phospho-PKA substrate (RRXS*/T*) (100G7E) antibody, rabbit monoclonal anti-phospho-Akt substrate (RRXS*/T*) (110B7E) antibody (Cell Signaling Technology, Danvers, MA, US); anti-GSK-3β antibody (BD Biosciences, San Jose, CA, US); anti-phospho-GSK-3β (serine 9) (R&D Systems, Minneapolis, MN, US); mouse monoclonal anti-Rho-kinase antibody (ab56661) (Abcam, Cambridge, MA, US); goat polyclonal anti-Rho-kinase antibody (C-20) (Santa Cruz Biotechnology, Dallas, TX, US); Alexa-555-conjugated secondary antibodies against rabbit IgG, Alexa-647-conjugated secondary antibodies against mouse IgG, CellTrackerTM Violet BMQC Dye and SYTO 16 green fluorescent nucleic acid stain (Life Technologies, Carlsbad, CA, US); [γ-32P]ATP (PerkinElmer, Waltham, MA, US); Forskolin (FSK), 3-Isobutyl-1-methylanxthine (IBMX) and H-89 (Tocris, St. Louis, MO, US) or pMal-C2 (New England Biolabs, Ipswich, MA). GST-fusion and MBP-fusion proteins were produced in *E. coli* and purified on Glutathione Sepharose 4B (GE Healthcare) and amylose resin (New England Biolabs, Ipswich, MA), respectively. The cDNAs of Rho-kinase-RB/PH-1342A, Rho-kinase-RB/PH-1361A, Rho-kinase-RB/PH-1362A, Rho-kinase-RB/PH-1365A, Rho-kinase-RB/PH-1366A, Rho-kinase-RB/PH-1374A, and Rho-kinase-RB/PH-1379A in which an alanine is substituted for the corresponding serine, were generated via site-directed mutagenesis and subcloned into the pEGFP-C1 plasmid.

**Cell culture and Immunoblot analysis**

COS-7 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO, US) with 10% FBS. All cell lines were grown in a humidified atmosphere of 5% CO2 at 37°C. Transfection was performed using the Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer’s instructions. Cells were grown over night. Cells were treated with dimethyl sulfoxide (DMSO) or H-89 for 30 min, and then treated with or without a Forskolin and IBMX mixture for 30 min without replacing the medium. The cells were lysed with SDS sample buffer and subjected to SDS-PAGE and immunoblot analysis with the indicated antibodies.

**Immunoprecipitation**

HeLa cells were seeded in a 100 mm dish at a density of 1.0×106 in DMEM with 10% FBS and cultured overnight at 37°C. Cells were treated with FSK, IBMX and/or H-89 as described above. The cells were gently washed with ice-cold PBS and scraped off the plate with ice-cold lysis buffer (20 mM Tris/HCl, pH 7.5, 1% NP-40, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl, 80 mM β-glycerolphosphate, 80 mM NaF, 4 μg/ml Leupeptin, 0.1 μM (p-Amidinophenyl) methanesulfonyl fluoride, 1 μg/ml aprotinin, 50 nM Calyculin A). The lysate was incubated for 30 min at 4°C with rotation and centrifuged at 17,000×g for 20 min at 4°C; the supernatant was used as the cell lysate. The indicated antibodies were incubated with the lysate for 1 hour at 4°C with rotation and then immobilized on Protein A Sepharose 4 Fast Flow beads (GE Healthcare) with rotation for 1 hour. The beads were then washed with lysis buffer twice and then with wash buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 150 mM NaCl) three times.

**LC/MS/MS**

The method of sample preparation for mass spectrometry was per-
formed as previously described (Nishioka et al., 2012). Briefly, after the immunoprecipitation with anti-phospho-PKA substrate Ab was performed, the bound proteins were extracted from the beads with a guanidine solution (50 mM Tris/HCl, 7 M guanidine), and the eluate was subjected to reduction, alkylation, demineralization, concentration and then digestion with trypsin. One fourth of the digest was passed through a SPE C-TIP (Nikkyo Technos, Tokyo, Japan) and introduced to LC/MS/MS for total peptide analysis, and the remaining sample was passed through a Titansphere® Phos-TiO Kit (GL Sciences, Tokyo, Japan) and SPE C-TIP for an enriched phosphopeptide analysis. LC/MS/MS was performed using the Q-Exact mass spectrometry (Thermo-Fisher Scientific Inc., Waltham, MA) system combined with an HTC-PAL autosampler and the Michrom nano-Advance UHPLC (Michrom BioResources Inc., CA) with a MonoCap C18 Nanoflow (0.1×150 mm) column (GL Science, Tokyo, Japan) and the Michrom’s ADCANCE CaptiveSpray Ionization Source. A peak list was generated and calibrated using MaxQuant software (version 1.2.2.5) (Cox and Mann, 2008). Database searches were performed against the complete proteome set of Homo sapiens in UniProtKB 2013_07 and concatenated with reversed copies of all the sequences (Peng et al., 2003). False discovery rates (FDRs) for the peptide, protein and site levels were set to 0.01. When the identified peptides satisfied the following criteria, they were regarded to be candidate substrates; the ion intensity of the identified peptides in the HeLa lysate treated with FSK/IBMX was more than twice as high as those in the lysate treated with other drug combinations; the ion intensity of the identified peptides decreased in accordance with the concentration of H-89. Phosphopeptide identifications with a localization probability of ≥0.75 were accepted.

Phosphorylation assay

The phosphorylation assay was performed as previously described (Amano et al., 1996b). The kinase reactions tested the activity of PKA on GST-fusion proteins (1 μM) and MBP-fusion proteins (1 μM) produced in E. coli and GFP-fusion proteins obtained from COS-7 cells. The reactions (50 μl total volume) consisted of a reaction mixture (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM MgCl₂, 100 μM [γ-32P] ATP [1 to 20 GBq/mmol]), 0.02 μM purified GST-PKA-cat and substrates and was carried out for 30 min at 30°C. The reaction mixtures were then boiled in SDS sample buffer and subjected to SDS-PAGE. The radiolabeled proteins were analyzed using an image analyzer, Typhoon FLA 9000 (GE healthcare).

Cortical neuron culture

Cortical neurons were prepared from E16 ICR mouse embryos using papain as previously described (Goslin and Banker, 1989; Inagaki et al., 2001). Briefly, neurons were seeded on dishes coated with poly-D-lysine and cultured for 72 h in neurobasal medium (Life Technologies) supplemented with B-27 (Life Technologies) and 1 mM GlutaMAX (Life Technologies). Neurons were treated with DMSO or 10 μM H89 for 30 min and then with or without 10 μM Forskolin and 50 μM IBMX for 30 min. The cell lysates were analyzed using immunoblot analysis with the indicated antibodies.

Preparation and incubation of striatal slices

Striatal slices were prepared from mice as described previously (Nishi et al., 1997). Male C57BL/6 mice at 6–8 weeks of age were decapitated. The brains were removed rapidly and placed in ice-cold, oxygenated Krebs-HCO₃ buffer (124 mM NaCl, 4 mM KCl, 26 mM NaHCO₃, 1.5 mM CaCl₂, 1.25 mM KH₂PO₄, 1.5 mM MgSO₄, and 10 mM D-glucose, pH 7.4). Coronal slices (350 μm) were prepared using a vibratome, VT1200S (Leica Microsystems, Nussloch, Germany). The striatum was dissected from the slices in ice-cold Krebs-HCO₃ buffer. Each slice was placed in a polypropylene incubation tube with 2 ml of fresh Krebs-HCO₃ buffer containing adenosine deaminase (10 μg/ml). The slices were pre-incubated at 30°C under constant oxygenation with 95% O₂/5% CO₂ for 60 min. The buffer was replaced with fresh Krebs-HCO₃ buffer after the 30 min of pre-incubation. Adenosine deaminase was included during the first 30 min of the pre-incubation. Slices were treated with or without 100 μM dopamine (Sigma-Aldrich) for 4 min. After the drug treatment, slices were transferred to Eppendorf tubes, frozen on dry ice, and stored at –80°C until further analysis. Prior to the immunoblot analysis, the slices were mixed with SDS sample buffer and boiled.

Cell culture and immunostaining

HeLa cells were seeded on 13-mm glass coverslips coated with poly-D-lysine in a 24-well plate at a density of 1.0×10⁴ cells in DMEM with 10% FBS and cultured overnight at 37°C. Cells were treated with FSK/IBMX and/or H-89 as described above. SYTO 16 was treated according to the manufacturer’s instructions. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed with PBS at room temperature. The cells were then incubated with the indicated antibodies overnight at 4°C. After washing, the samples were incubated with Alexa 555- and Alexa 647-conjugated secondary antibodies. Fluorescence was examined using a Zeiss LSM 780 laser scanning microscope (Carl Zeiss, Oberkochem, Germany) with a C-Apochromat 40× (NA 1.2) objective under the control of LSM software (Carl Zeiss).

Animals

All animal experiments were performed according to the guidelines of the Institute for Developmental Research. Male C57BL/6 mice and ICR mice were purchased from Japan SLC (Shizuoka, Japan).

Statistical analyses

Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, US). P<0.05 was considered statistically significant.
**Results**

**Isolation of PKA substrates in HeLa cells treated with FSK/IBMX and/or H-89**

A balance between cAMP levels and phosphodiesterase activity modulates the activity of PKA in cells. FSK is a known activator of adenylyl cyclase, and IBMX is a competitive non-selective phosphodiesterase inhibitor; both activate PKA. Conversely, H-89 competes with ATP for PKA binding, which results in an inhibition of PKA (Engh et al., 1996). To screen for potential substrates of PKA, we optimized the conditions of FSK, IBMX and H-89 in HeLa cells. Because VASP is known to be phosphorylated by PKA at serine 157 (Butt et al., 1994), we monitored its phosphorylation level. FSK alone was not enough to raise the phosphorylation level of VASP (data not shown), and so we treated HeLa cells with IBMX simultaneously. The phosphorylation of VASP was increased by this FSK and IBMX mixture, whereas this phosphorylation was prevented in an H-89 concentration-dependent manner (Fig. 1A). In contrast, the phosphorylation of GSK-3β at serine 9 was not affected under the same conditions.

Anti-phospho-PKA substrate Ab is directed against the motif RRXS*/T*, where phosphorylated serine (S*) or threonine (T*) is fixed at the 0 position and R is fixed at the −3 and −2 positions (Zhang et al., 2002). Anti-phospho-PKA substrate Ab detected the increase in phosphorylation levels of many proteins in the HeLa cell lysates treated with FSK and IBMX in addition to the decrease when treated with H-89 (Fig. 1B). Anti-phospho-Akt substrate Ab did not detect either the increase in phosphorylation levels by FSK/IBMX or the decrease by H-89 (Fig. 1B). Thus, anti-phospho-PKA substrate Ab could be employed to specifically detect both the activation and inactivation of PKA signaling under our conditions.

To enrich phosphorylated proteins, the HeLa cell lysates treated with H-89 and/or FSK and IBMX mixture were subjected to immunoprecipitation using anti-phospho-PKA substrate Ab. The proteins bound to anti-phospho-PKA substrate Ab were eluted with guanidine solution, followed by immunoblot analysis with anti-phospho-PKA substrate Ab. (Fig. 2A) The signal intensity of the precipitants changed in accordance with the level of PKA activation, suggesting that anti-phospho-PKA substrate Ab enriched phosphorylated PKA substrates under these drug conditions.

**LC/MS/MS analysis of phosphopeptides after immunoprecipitation**

To identify the proteins bound to anti-phospho-PKA substrate Ab, these proteins were extracted and digested by trypsin. For the enriched phosphopeptide analysis, three fourths of the digest was introduced to a TiO₂ column and were subsequently subjected to LC/MS/MS using a Q Exactive mass spectrometer (Fig. 2B). Using this enriched phosphopeptide analysis we identified 65 phosphorylation
sites derived from 46 proteins whose ion intensities in the sample treated with FSK/IBMX were more than twice as high as the others and decreased in accordance with the concentration of H-89 (Table I). Known phosphorylation sites, such as CAD (pS1859), CAMKK1 (pS458) and RAF (pS259) were detected using this analysis. As for the sub- 
strates of ARHGEF7 and EVL, the phosphorylation sites detected in this analysis were different from those that have been previously reported. The sequence alignment of the detected phosphopeptides was similar to the consensus motif of PKA (Fig. 2C).

Because the amount of proteins phosphorylated by PKA was increased by the stimulation of FSK/IBMX, the amount of precipitated protein using anti-phospho-PKA substrate Ab was also increased. Therefore, it was important to compare the intensity of total peptides without enrichment by the TiO$_2$ column. By total peptide analysis, we identified 81 proteins as phosphoproteins whose ion intensities in the sample treated with FSK/IBMX were more than twice as high as the others and decreased in accordance with the concentration of H-89. This study also identified several known substrates, such as ENAH, VASP and EVL (Table II). Of the identified proteins, 12 proteins were detected both by phosphopeptide analysis and total peptide analysis.

PKA phosphorylates Rho-kinase at Ser1379

After obtaining the mass spectrometry results, we focused on ROCK2/Rho-kinase (hereafter referred to as Rho-
kinase) because the relationship between PKA and Rho-kinase attracted our interest. Rho-kinase plays a key role in Rho signaling (Riento and Ridley, 2003; Amano et al., 2010). First, we examined whether PKA directly phosphorylates Rho-kinase in vitro. We performed an in vitro phosphorylation assay using three fragments of Rho-kinase; a kinase dead form of the Rho-kinase catalytic domain (GST-Rho-kinase-cat KD), a truncated region of the coiled-coil domain (GST-Rho-kinase-coil (421–701 aa)) and MBP-Rho-kinase-RB/PH(TT) (Fig. 3A).

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**Red words**: known phosphosites, **Underlined words**: those detected more than twice
the Rho-kinase RB/PH(TT) to a greater extent than the others, indicating that Rho-kinase is directly phosphorylated by PKA and that the RB/PH(TT) region contains the major site of phosphorylation (Fig. 3B).

We next made a long form of the Rho-kinase coiled-coil region containing the RB domain (GFP-Rho-kinase-RB/PH) and five fragments of the PH domain, each tagged with GFP, to narrow down the site of PKA phosphorylation. We found that Rho-kinase was phosphorylated within the 1261–1388 aa region (Fig. 3C). The GFP-Rho-kinase-coiled-coil region containing the RB domain was not phosphorylated by PKA. After scanning this region for the consensus sequence of PKA, we produced several mutant proteins including GST-Rho-kinase-RB/PH-1342A, -1361A, -1362A, -1365A, -1366A, -1375A, or -1379A, in which a serine or threonine was substituted with an alanine, and examined the phosphorylation efficiency (Fig. 3D). The phosphorylation level of GST-Rho-kinase-RB/PH-1379A was much lower than that of GST-Rho-kinase-RB/PH-WT (Fig. 3E). Serine 1366 of Rho-kinase has previously been reported as an autophosphorylation site (Chuang et al., 2012). Alanine substitution for serine 1366 (Fig. 3E) as well as for other serine residues besides serine 1379 (data not shown) did not affect the phosphorylation level, indicating that serine 1379 is the major site of phosphorylation by PKA.

We produced a polyclonal antibody that specifically recognizes Rho-kinase when it is phosphorylated at serine 1379 (anti-phospho-Rho-kinase (pS1379) Ab). An immunoblot analysis revealed that anti-phospho-Rho-kinase (pS1379) Ab recognized Rho-kinase phosphorylated by PKA in a dose-dependent manner (Fig. 4A). To examine whether PKA phosphorylates Rho-kinase at serine 1379 in vivo, an immunoblot analysis of HeLa cell lysates treated with a PKA activator/inhibitor was performed using anti-phospho-Rho-kinase (pS1379) Ab. It was difficult to detect a signal for this phosphorylation site under basal conditions. FSK and IBMX treatment enhanced the phosphorylation of Rho-kinase in HeLa cells, but H-89 inhibited the FSK- and IBMX-induced phosphorylation in a concentration-dependent manner (Fig. 4B). These results indicate that PKA phosphorylates Rho-kinase at serine 1379 in vivo.

ROCK1 is an isoform of Rho-kinase, but ROCK1 and Rho-kinase do not share sequence homology within the region surrounding serine 1379 of Rho-kinase (data not shown). Nevertheless, we examined whether PKA also phosphorylates ROCK1. HeLa cell lysates treated with or without FSK/IBMX and/or H-89 were subjected to immunoprecipitation using either anti-ROCK1 Ab or anti-Rho-kinase Ab. The bound proteins were eluted with SDS sample buffer and were then subjected to SDS-PAGE and an immunoblot analysis using anti-phospho-PKA substrate Ab. Although the phospho-signal for the proteins precipitated with anti-Rho-kinase Ab correlated with PKA activation/inactivation, there was no signal detected for the proteins precipitated by anti-ROCK1 Ab (Fig. 4C). Thus, it is possible that PKA specifically phosphorylates Rho-kinase.

It was reported that Rho-kinase is abundantly expressed in the brain, especially in neurons (Nakagawa et al., 1996; Iizuka et al., 2012). Therefore, we also examined whether this phosphorylation occurs in neurons. Dissociated cortical neuron cultures were treated with FSK/IBMX and/or H-89 and subsequently lysed with SDS sample buffer (Fig. 4D). The immunoblot analysis of these lysates using anti-phospho-Rho-kinase (pS1379) Ab suggested that Rho-kinase was also phosphorylated by PKA in cultured neurons. Next, we examined whether this phosphorylation was induced under more physiological conditions (Fig. 4E). To this end, we treated striatum slice cultures with dopamine, a natural agonist. Dopamine increased the phosphorylation of Rho-kinase at serine 1379 in the striatum. These results indicate that dopamine stimulates PKA signaling to phosphorylate Rho-kinase in neurons.

To visualize the localization of Rho-kinase when phosphorylated at serine 1379, we performed immunostaining of HeLa cells using anti-phospho-Rho-kinase (pS1379) Ab and mouse anti-Rho-kinase Ab (Fig. 5A). Treatment with FSK and IBMX increased the intensity of phosphorylated Rho-kinase in the cytosol partly as dot-like structures, suggesting that Rho-kinase was phosphorylated mainly in this cellular compartment. Adding H-89 together with FSK and IBMX decreased this phosphorylation. The signal intensity in the cytosol was almost identical in both the control and H-89 treated cells (Fig. 5B). The phospho-signal detected in the nucleus could be non-specific under our conditions because this nuclear signal was not affected by the knockdown of Rho-kinase (data not shown). Therefore, these data suggest that phosphorylated Rho-kinase is mainly localized in the cytosol.

**Discussion**

In this study, we identified a large number of known PKA substrates in addition to a number of novel candidate substrates. More than half of our identified candidate substrates and phosphosites have not been previously reported. We also found that PKA phosphorylates Rho-kinase at serine 1379 both in vitro and in vivo.

We employed two analyses depending on whether the immunoprecipitants were additionally passed through a TiO<sub>2</sub> column: enriched phosphopeptide analysis and total peptide analysis. For enriched phosphopeptide analysis, we could detect candidate substrates whose peptide sequences were identical to the consensus motif of PKA substrates. Because certain types of phosphopeptides are difficult to detect with mass spectrometry, we also used a total peptide analysis to screen for phosphoproteins that change in total
Fig. 3. Identification of a PKA phosphorylation site on Rho-kinase. (A) A schematic representation of Rho-kinase. The domain organization of Rho-kinase and its fragments are represented. “P” in an orange circle indicates the phosphorylation site. (B, C and E) Phosphorylation of Rho-kinase fragments by PKA. (B) GST-Rho-kinase-cat KD, GST-Rho-kinase-coil (421–701 aa) and MBP-Rho-kinase-RB/PH(TT) were produced and purified from E. coli. They were incubated with GST-PKA-cat and 100 μM [γ-32P] ATP for 30 min at 30°C. The reaction mixtures were subjected to SDS-PAGE and GST- and MBP-fused proteins were visualized by silver staining (lower panel). Phosphorylated proteins were imagmed using autoradiography (upper panel). Open arrowheads and arrows indicate the positions of the corresponding proteins and the auto phosphorylation of GST-PKA-cat, respectively. (C) Phosphorylation of immunoprecipitated Rho-kinase in COS-7 cells. GFP-Rho-kinase fragments were transiently expressed in COS-7 cells. They were subjected to immunoprecipitation with anti-GFP Ab. The precipitants were phosphorylated and subjected to SDS-PAGE. Silver staining is in the lower panel and autoradiography is in the upper panel. (D) Sequence of potential phosphorylation sites (blue) within the 1261–1388 aa region. The major phosphorylation site was identified as serine 1379 (red). (E) Phosphorylation of immunoprecipitated Rho-kinase mutants with amino acid substitutions. Transfection, immunoprecipitation and phosphorylation assays were described as above. Silver staining is in the lower panel and autoradiography is in the upper panel. Open arrowheads indicate the positions of the GFP-Rho-kinase-RB/PH and its mutants.
Fig. 4. Phosphorylation of Rho-kinase in vivo. (A) Specificity of the antibody against Rho-kinase phosphorylated at serine 1379 (anti-phospho-Rho-kinase (pS1379) Ab). MBP-Rho-kinase-RB/PH(TT) (500 fmol) containing the indicated amounts of phosphorylated MBP-Rho-kinase-RB/PH(TT) was subjected to SDS-PAGE, followed by immunoblot analysis with anti-phospho-Rho-kinase (pS1379) Ab. (B) Phosphorylation of Rho-kinase in HeLa cells. The HeLa cell lysates were analyzed using immunoblot analysis with anti-phospho-Rho-kinase (pS1379) Ab (upper panel) and mouse anti-Rho-kinase Ab (lower panel). Arrows indicate the positions of the corresponding proteins. (C) Immunoblot analysis of immunoprecipitated ROCK1 or Rho-kinase using anti-phospho-PKA substrate Ab. HeLa cells were treated with FSK, IBMX and/or H-89 as indicated. They were subjected to immunoprecipitation with anti-ROCK1 Ab or goat anti-Rho-kinase Ab. Immunoblot analysis was performed with these precipitants and detected with anti-phospho-PKA substrate Ab (upper panel), anti-ROCK1 Ab, or mouse anti-Rho-kinase Ab (lower panel). (D) Phosphorylation of Rho-kinase in dissociated cultured cortical neurons. Neurons were treated with DMSO or 10 μM H89 for 30 min and then with or without 10 μM Forskolin and 50 μM IBMX for 30 min. The cell lysates were analyzed using immunoblot analysis with anti-phospho-Rho-kinase (pS1379) Ab (upper panel) or mouse anti-Rho-kinase Ab (lower panel). (E) Phosphorylation of Rho-kinase in the murine striatum. Striatal slices were incised from male C57BL/6 mice at 6–8 weeks old. The slices were treated with or without 100 μM dopamine for 4 min. The slices were mixed with SDS sample buffer and subjected to immunoblot analysis with anti-phospho-Rho-kinase (pS1379) Ab (upper panel) or mouse anti-Rho-kinase Ab (lower panel).
amount according to PKA activation and inhibition. Indeed, we could not find the phosphopeptide containing serine 1379 of Rho-kinase in the enriched fraction by TiO2 column, even ion intensity in FSK/IBMX-treated sample was less than two times higher than control sample. We suppose that the predicted phosphopeptide containing serine 1379 of Rho-kinase (PpSR) would be hard to be detected under our experimental condition, due to the small m/z value. Total peptide analysis is expected to pick up such candidate substrates. We obtained many candidate substrates that were not detected in the phosphopeptide analysis. Thus, enriched phosphopeptide analysis and total peptide analysis can complement each other to improve the detection of phosphorylated proteins.

Among the candidate substrates, we found 10 ribosomal proteins in addition to several proteins involved in ribosomal RNA processing, such as BOP1 and Fibrillarin. It has been reported that ribosomal proteins are phosphorylated by PKA in vitro (Traugh and Porter, 1976; Wettenhall and Morgan, 1984). In particular, phosphorylation of the 40S ribosomal subunit by PKA inhibits the translation of poly(A,U,G) using a reconstituted protein synthesizing system (Burkhard and Traugh, 1983). PKA might also phosphorylate those ribosomal proteins detected in our assay to regulate protein synthesis.

Several proteins associated with the actin cytoskeleton were also detected. Activation of PKA has long been known to interfere with the RhoA/Rho-kinase signaling pathway. Upon the activation of RhoA by extracellular stimuli, Rho-kinase is activated by RhoA and regulates cellular contractility via the phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1) and myosin light chain (MLC) (Amano et al., 1996a). It was reported that PKA phosphorylates RhoA at its C-terminus and that treatment of cells with Bt2cAMP results in the translocation of membrane-associated RhoA toward the cytosol (Lang et al., 1996). Phosphorylation of RhoA by PKA in vitro also decreases the binding of RhoA to Rho-kinase (Dong et al., 1998). Phosphorylation of MYPT1 by PKA prevents its phosphorylation by Rho-kinase and therefore enhances the activity of myosin phosphatase (Wooldridge et al., 2004). Therefore, PKA signaling has an inhibitory effect on Rho.

Fig. 5. Localization of phosphorylated Rho-kinase in HeLa cells. (A) Immunostaining of HeLa cells treated with FSK/IBMX and/or H-89. HeLa cells were treated with/without 15 μM H-89 for 30 min and subsequently with/without 20 μM FSK/100 μM IBMX for 30 min. HeLa cells were fixed in 4% paraformaldehyde for 15 min at room temperature. After washing, the cells were immunostained with mouse anti-Rho-kinase Ab and anti-phospho-Rho-kinase (pS1379) Ab79 Ab. Colors indicate Rho-kinase (green), Rho-kinase pS1379 (red) and SYTO16 (blue). Scale bar, 20 μm. (B) The intensity ratios of Rho-kinase pS1379 to Rho-kinase in the cytosol of HeLa cells. Each intensity ratio represents the signal intensity of phosphorylated Rho-kinase at serine 1379 divided by the signal intensity of total Rho-kinase. The data represent the means±SD of more than three independent experiments. n=30 on each drug condition. *** p≤0.001 (Tukey’s multiple-comparison test). (C) Schematic illustration of PKA signaling associated with Rho signaling at multiple steps. PKA may inhibit Rho-kinase activity by phosphorylating at serine 1379 (red arrows).
signaling (Fig. 5C). Here, we found that PKA phosphorylates Rho-kinase in both HeLa cells and neurons, and we further tried several approaches to determine whether PKA signaling also has an inhibitory effect on Rho-kinase activity. First, we compared the activity of Rho-kinase immunoprecipitated from COS-7 cells treated by FSK/IBMX with that from control cells by an in vitro kinase assay but did not detect a significant difference (data not shown). Next, we examined the effect of phosphorylation on the intramolecular association between the catalytic and RB/PH regions of Rho-kinase; a closed configuration suppresses Rho-kinase function but active RhoA is supposed to disrupt this interaction (Amano et al., 1999). GST-Rho-kinase-cat-KD was co-expressed with GFP-Rho-kinase-RB/PH(TT) in COS-7 cells and pulled down with glutathione beads. The amount of co-precipitated GFP-Rho-kinase-RB/PH(TT) was not altered by the mutation of serine 1379 (data not shown). These results suggest that the phosphorylation of Rho-kinase by PKA does not change its basal kinase activity or intramolecular binding. From our immunostaining data, we found that PKA activation increased the intensity of phosphorylated Rho-kinase in the cytosol. The staining with phospho-Rho-kinase antibody displayed dot-like structures, and we could not clarify the structures. We previously examined the intracellular distribution of Rho-kinase (ROCK1 and ROCK2) in various mouse tissues both by light microscopy and electron microscopy (Iizuka et al., 2012). In this report, Rho-kinase (ROCK2) immunoreactivity was sometimes observed as clusters in the cytoplasm in addition to actin cytoskeleton and cell adhesion sites. We confirmed that these clusters were not associated with the cytoskeleton or any organelle, and their function remains unknown. Because serine 1379 resides at the C terminus of Rho-kinase close to the PH domain, phosphorylation by PKA might prevent Rho-kinase from binding to the membrane or actin cytoskeleton, thus translocating Rho-kinase to the cytosol. As a consequence, this phosphorylation might inhibit Rho-kinase activity in the cell (Fig. 5C). During the course of our study, Gerarduzzi et al., reported that PKA phosphorylates Rho-kinase at serine 1379 in synovial fibroblasts downstream of PGE2, and that expression of non-phosphorylated Rho-kinase mutant at serine 1379 prevents the PGE2-induced MLC dephosphorylation and cytoskeletal reorganization (Gerarduzzi et al., 2014), which is consistent with our hypothesis.

Although a comparison of the homology between Rho-kinase and ROCK1 shows that they have 92% identity in their kinase domains, the C terminal regions containing the PH domain of Rho-kinase and ROCK1 have only 66% sequence identity (Leung et al., 1996; Nakagawa et al., 1996). Furthermore, the sequence surrounding serine 1379 of Rho-kinase is different from that of ROCK1. It has been reported that the differences in activity between Rho-kinase and ROCK1 originate in, part, from the distinct lipid-binding preferences of their PH domains (Yoneda et al., 2005). Our data show that phosphorylation of Rho-kinase by PKA was detected with anti-phospho-PKA substrate Ab but that ROCK1 was not. Thus, the phosphorylation of Rho-kinase at serine 1379 by PKA could contribute to the differences observed in Rho-kinase and ROCK1 activity. The molecular mechanisms underlying Rho-kinase regulation and the differences between Rho-kinase and ROCK1 remain an issue for future investigation.

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