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PKC phosphorylates MARCKS Ser159 not only directly but also through RhoA/ROCK ☆

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Abstract

It is well recognized that phorbol 12,13-dibutyrate (PDBu)-activated PKC directly phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS), whose phosphorylation is used as a marker of PKC activation. However, in SH-SY5Y neuroblastoma cells, Western blotting analyses revealed that Rho-associated coiled-coil kinase (ROCK)-specific inhibitor H-1152 inhibited PDBu-induced phosphorylation, and that a small G-protein inhibitor, toxin B, also inhibited MARCKS phosphorylation. Furthermore, in GST pull-down assays, PDBu induced RhoA activation in SH-SY5Y cells, and this activation was inhibited by PKC inhibitor Ro-31-8220. Finally, we showed that the transfection of a dominant negative form of RhoA inhibited PDBu-induced MARCKS phosphorylation in immunocytochemistries. These findings suggest that some PDBu-induced MARCKS phosphorylation includes the RhoA/ROCK pathway in SH-SY5Y cells.

Keywords: MARCKS; PKC; RhoA; ROCK; Neuroblastoma; Phosphorylation

Myristoylated alanine-rich C kinase substrate (MARCKS) was cloned as a substrate of PKC, whose activity is regulated by PKC-dependent phosphorylation. MARCKS is used as a marker of PKC activation [1–3]. On the other hand, Nagumo et al. and we previously revealed that at least the Ser159 site of MARCKS was directly phosphorylated by Rho-associated coiled-coil kinase (ROCK) in vitro [4] or in lysophosphatidic acid (LPA)-stimulated neuroteratoma NT-2 cells [5].

LPA activates three types of LPA receptors (LPA1–3), which are coupled with distinct G-proteins, leading to a variety of cellular and biochemical responses. PKC and ROCK are activated through different cascades [6–8]. PKC is activated through PLCβ downstream of $G_{q/11}$, while ROCK is activated by GTP binding RhoA downstream from $G_{12/13}$. RhoA is regulated by three kinds of protein—guanine nucleotide exchange factor (GEF), GDP dissociation inhibitor (GDI), and GTPase-activating protein (GAP) [9]. Mehta et al. indicated that PKCα phosphorylates Rho-GDI and leads to RhoA activation in vascular endothelial cells [10]. Other groups also have reported that PKC activates RhoA [11,12]. Therefore, we investigated whether or not PKC induces MARCKS phosphorylation

☆ *Abbreviations:* MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C; ROCK, Rho-associated coiled-coil kinase; PDBu, phorbol 12,13-dibutyrate; LPA, lysophosphatidic acid; ECFP, enhanced cyan fluorescent protein; GST, glutathione *S*-transferase; MBS, myosin binding subunit.

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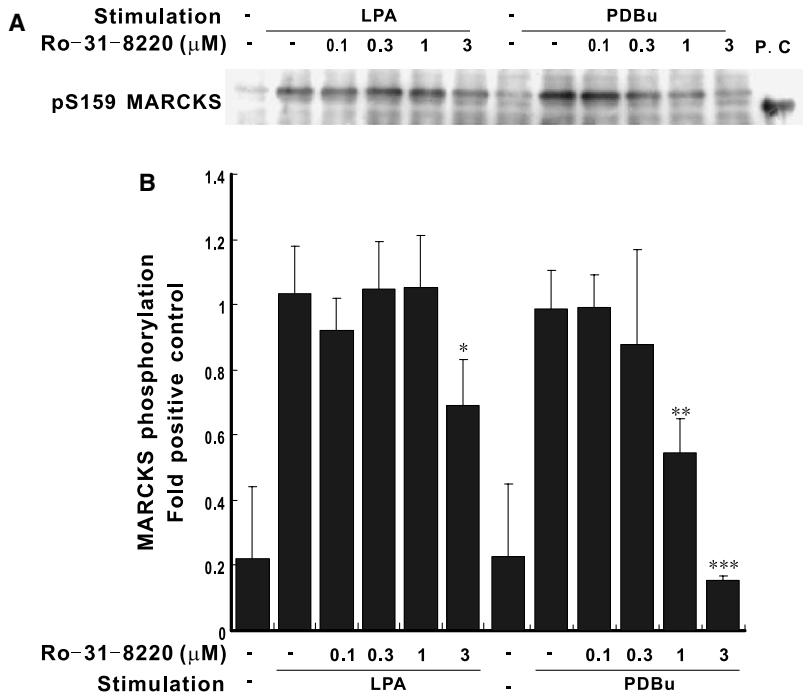

Fig. 1. PKC inhibitor Ro-31-8220 inhibited PDBu-induced but not LPA-induced MARCKS phosphorylation. Serum-starved SH-SY5Y cells were pretreated with or without Ro-31-8220 at indicated concentrations and then stimulated with 1 μM LPA for 2 min or 100 nM PDBu for 10 min. Phosphorylated MARCKS was determined by Western blotting analysis. (A) Representative immunoblotting. (B) Each point of LPA- or PDBu-induced MARCKS phosphorylation was normalized with a positive control (PKC-treated MARCKS). Data are means ± SE of four experiments. *$p < 0.05$ compared with LPA-stimulated cells, and **$p < 0.01$ and ***$p < 0.005$ compared with PDBu-stimulated cells.

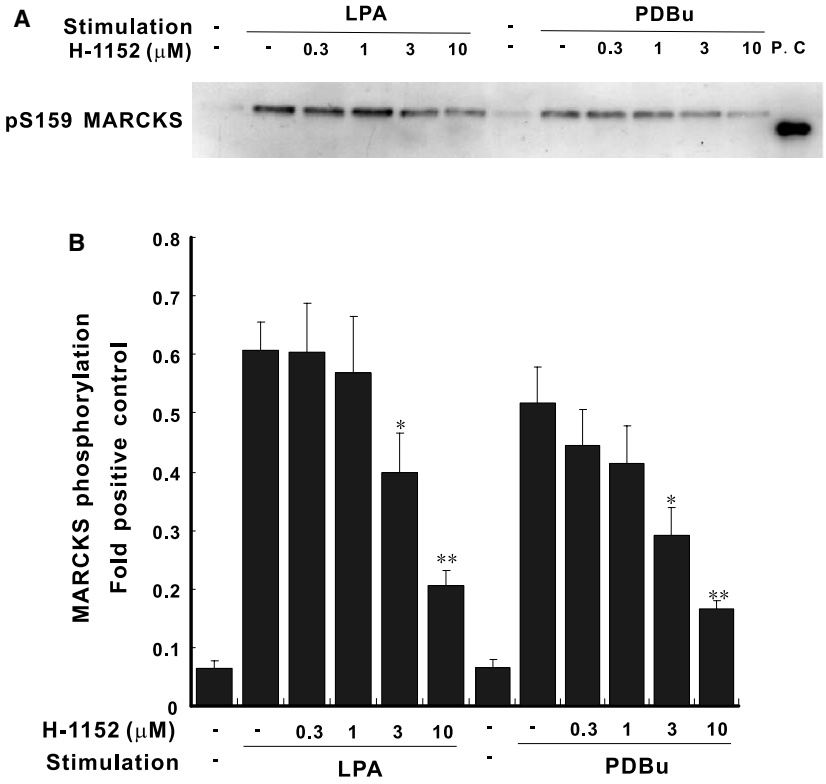

Fig. 2. ROCK inhibitor H-1152 inhibited both LPA- and PDBu-induced MARCKS phosphorylation. Serum-starved SH-SY5Y cells were pretreated with or without H-1152 at indicated concentrations and then stimulated with 1 μM LPA for 2 min or 100 nM PDBu for 10 min. Cell lysates were prepared, and phosphorylated MARCKS was determined by Western blotting analysis. (A) Representative immunoblotting. (B) Each point of LPA- or PDBu-induced MARCKS phosphorylation was normalized with a positive control (PKC-treated MARCKS). Data are means ± SE of four experiments. *$p < 0.05$ and **$p < 0.01$ compared with LPA- or PDBu-stimulated cells.

at least in part through the RhoA/ROCK pathway in neuronal cells.

In this paper, we showed that, besides direct phosphorylation, PKC phosphorylates MARCKS indirectly in neuroblastoma SH-SY5Y cells. MARCKS was alternatively phosphorylated by ROCK as the result of RhoA activation by PKC, although it is well recognized that PKC directly phosphorylates MARCKS.

Materials and methods

Plasmid construction. The cDNA of a dominant negative form of RhoA (T19N) with a *Bam*HI site at both ends was amplified by PCR as described previously [13] and subcloned into pECFP-N1 (BD Biosciences) at the site of *Bam*HI.

Toxin B treatment. Cells were incubated in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal bovine serum containing *Clostridium difficile* toxin B (Nacalai Tesque) for 7 h. The cells were then starved for 17 h in serum-free medium with toxin B.

Detection of phosphorylation using Western blotting. The serum-starved cells were pretreated with Ro-31-8220 (Calbiochem) or H-1152 [14] for 15 min and then simulated with 1 μM LPA (Sigma) for 2 min or 100 nM PDBu (Sigma) for 10 min. Trichloroacetic acid precipitants were subjected to Western blotting analyses as previously described [5]. The phosphorylation of MARCKS and myosin binding subunit (MBS) was detected with pS159-Mar-Ab [4] (1:5000 with Can Get Signal solution 1 (Toyobo)) and pT654-MBS-Ab [7] (1:50,000 with Can Get Signal solution 1), respectively. Signals were detected by Lumi-GLO (Cell Signaling Technology). Densitometric analyses were performed using the 1D Scientific Imaging System (Kodak) software.

GST pull-down assay. Activated RhoA was measured as described previously [13]. After 17 h of serum starvation, SH-SY5Y cells were stimulated and lysed for 30 min with cell lysis buffer (20 mM Tris (pH 7.5), 1% Triton X-100) containing 400 μg of GST-conjugated Rho binding domain of Rhotekin. The supernatants of cell lysates were incubated with glutathione–Sepharose beads for 1 h at 4 °C. After the beads were washed with cell lysis buffer, the bound proteins were subjected to 12.5% SDS–PAGE/Western blotting using a rabbit polyclonal anti-RhoA antibody (Santa Cruz Biotechnology, 1:200).

Transfection and immunocytochemistry. One microgram of plasmid was transfected into SH-SY5Y cells on cover glasses with 2 μg of Lipofectamine 2000 (Invitrogen). After serum starvation for 17 h, the cells were stimulated. Cell reactions were stopped by washing with ice-cold PBS(−), and the cells were fixed by 4% paraformaldehyde/4 mM EGTA/4% sucrose for 1 h at room temperature. The cells were permeabilized in 0.1% Triton X-100/PBS(−) for 10 min and incubated in 3% BSA/PBS(−) for 1 h to block nonspecific antibody binding. For immunocytochemical detection of phosphorylated MARCKS, cells were incubated with pS159-Mar-Ab (1:10) in 1% BSA/PBS(−) for 17 h at 4 °C followed by incubation with rhodamine-conjugated anti-mouse IgG Ab (Jackson Immuno Research, 1:100) for 1 h. The cells were observed with a Biozero fluorescence microscope (Keyence).

Results

PDBu-induced phosphorylation of MARCKS is inhibited by ROCK inhibitor H-1152 in SH-SY5Y cells

The phosphorylation of MARCKS peaks at 1 and 5 min after LPA- and PDBu-stimulation, respectively, and each peak is maintained for at least 20 min (data not shown). Accordingly, we determined the phosphorylation level of MARCKS at 2 and 10 min after LPA- and PDBu-stimulation, respectively. To investigate whether or not LPA and PDBu activate different kinase pathways in SH-SY5Y cells, first we performed Western blotting analyses of MARCKS phosphorylation. In neuroblastoma SH-SY5Y cells, PDBu-induced MARCKS phosphorylation was inhibited dose-dependently by Ro-31-8220, and LPA-induced MARCKS phosphorylation was scarcely inhibited even by 3 μM Ro-31-8220 (Fig. 1). On the other hand, ROCK-specific inhibitor H-1152 inhibited PDBu-induced MARCKS phosphorylation as well as LPA-induced phosphorylation (Fig. 2). Other ROCK inhibitors, Y-27632 and HA1077, showed similar results (data not shown). The results of the experiments using ROCK inhibitors showed the possibility that PDBu induces the phosphorylation of MARCKS through ROCK activation, although it is believed that MARCKS is directly phosphorylated by phorbol-ester-activated PKC.

To confirm this possibility, we tested whether or not PDBu stimulation activates ROCK in SH-SY5Y cells. In PDBu-stimulated SH-SY5Y cells, the myosin binding subunit (MBS) at Thr654, which is recognized as a ROCK substrate, was phosphorylated, and this phosphorylation was also inhibited by H-1152. MBS phosphorylation was also detected in LPA-stimulated cells, and H-1152 inhibited

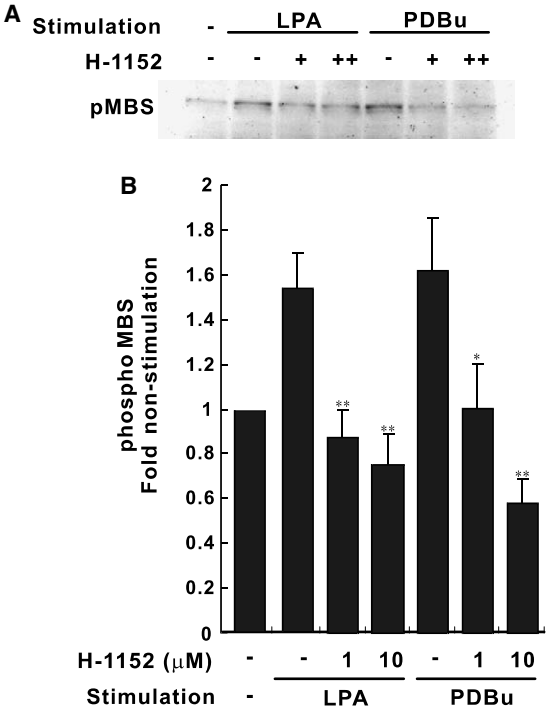

Fig. 3. ROCK inhibitor H-1152 inhibited both LPA- and PDBu-induced MBS phosphorylation. Serum-starved SH-SY5Y cells were pretreated with or without H-1152 at indicated concentrations and then stimulated with LPA or PDBu. Cell lysates were prepared, and phosphorylated MBS was determined by Western blotting analysis. (A) Representative immunoblotting. (B) Each point of LPA- or PDBu-induced MBS phosphorylation was normalized with phosphorylated MBS in non-stimulated cells. Data are means ± SE of three experiments. $*p < 0.05$ and $**p < 0.01$ compared with stimulated cells.

this phosphorylation (Fig. 3). These results suggest that ROCK works in PDBu-stimulated SH-SY5Y cells through a PKC activation pathway.

PDBu-induced phosphorylation of MARCKS is inhibited by small G-protein inhibitor Clostridium difficile toxin B

We used the small G-protein inhibitor *C. difficile* toxin B to test the involvement of the RhoA/ROCK pathway in PDBu-induced MARCKS phosphorylation. First, we checked the toxicity of toxin B on SH-SY5Y cells using the MTT assay. We found that toxin B at 24 h treatment did not affect the viability of SH-SY5Y cells at less than 200 pg/ml concentration (data not shown). Toxin B (200 pg/ml) inhibited both LPA- and PDBu-induced MARCKS phosphorylation (Fig. 4). These results mean that the small G-protein mediates PDBu-induced MARCKS phosphorylation in SH-SY5Y cells.

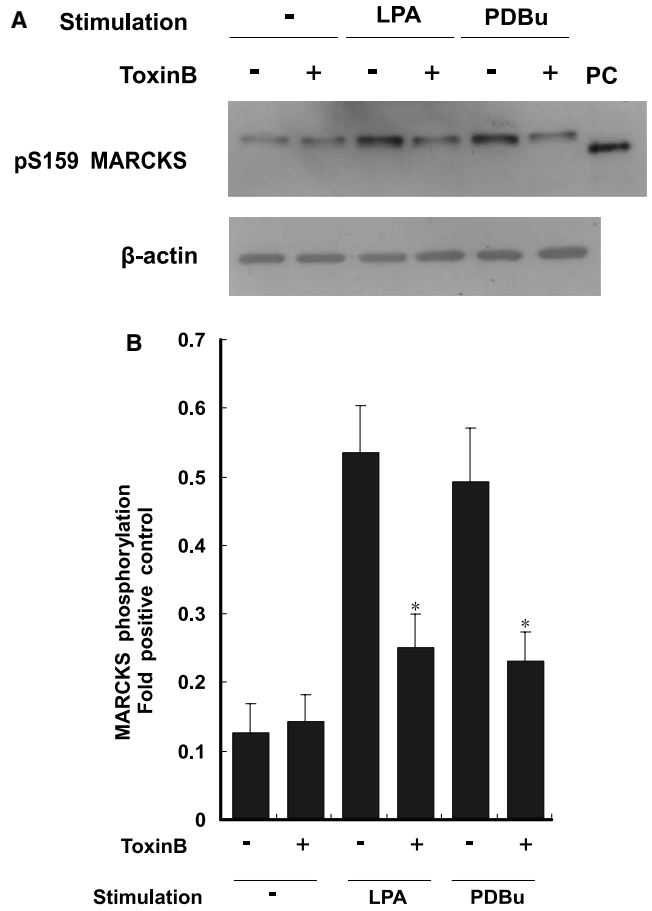

Fig. 4. Inhibition by toxin B of LPA- and PDBu-induced MARCKS phosphorylation in SH-SY5Y cells. SH-SY5Y cells were incubated with toxin B for 24 h and stimulated with LPA or PDBu. Cell lysates were prepared, and phosphorylated MARCKS was determined by Western blotting analysis. (A) Representative immunoblotting of phosphorylated MARCKS and β-actin. (B) Each point was normalized with positive control (PKC-treated MARCKS). Data are means ± SE of four experiments. *$p < 0.05$, compared with LPA- or PDBu-stimulated cells.

PKC inhibitor Ro-31-8220 inhibited the RhoA activation induced by PDBu

Next, we investigated whether PKC activates RhoA or not in SH-SY5Y cells (Fig. 5), because it was not obvious whether PKC activates the RhoA/ROCK pathway or RhoA/ROCK activates PKC in these situations. Activation of RhoA (GTP binding form) was detected in GST pull-down assays using GST-conjugated RhoA binding domain of Rhotekin. RhoA was activated in the PDBu-stimulated SH-SY5Y cells and Ro-31-8220 inhibited this activation. RhoA was activated in the LPA-stimulated SH-SY5Y cells as well as in the PDBu-stimulated cells, but Ro-31-8220 did not inhibit this LPA-induced activation of RhoA.

Dominant negative form of RhoA (RhoA-DN) inhibited PDBu-induced MARCKS phosphorylation

Finally, in order to observe whether or not RhoA mediates PDBu-induced MARCKS phosphorylation, we performed immunocytochemistries using the dominant negative form of RhoA (RhoA-DN). When SH-SY5Y cells

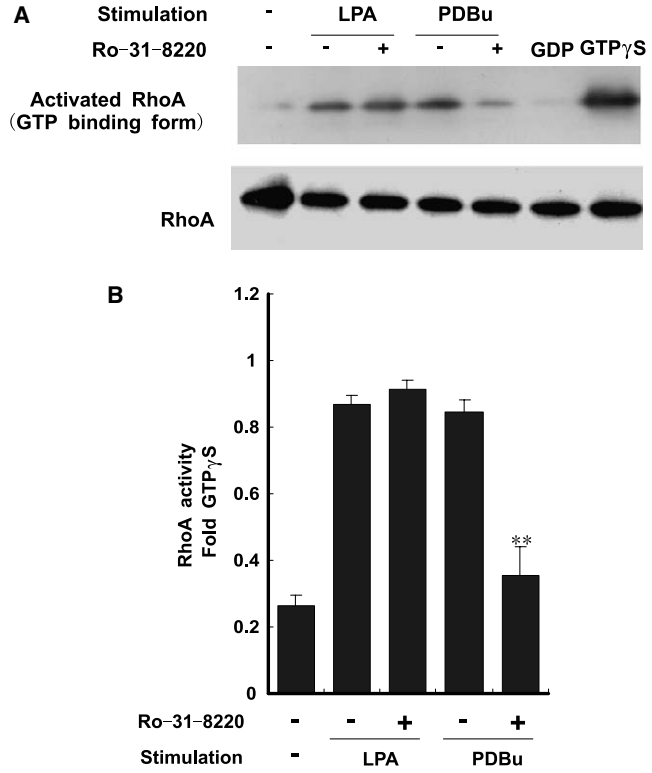

Fig. 5. Ro-31-8220 inhibited the RhoA activation induced by PDBu. Serum-starved cells were pretreated with or without 10 μM Ro-31-8220 for 15 min, and then the cells were simulated with LPA or PDBu. Cell lysate was incubated with GST-RBD and activated RhoA was pulled down. Western blotting with anti RhoA Ab was performed. (A) Representative immunoblotting. Upper panel, pulled down RhoA; lower panel, total RhoA. (B) Each point was normalized with no stimulation cells. Data are means ± SE of three or four experiments. **$p < 0.01$ compared with PDBu-stimulated cells.

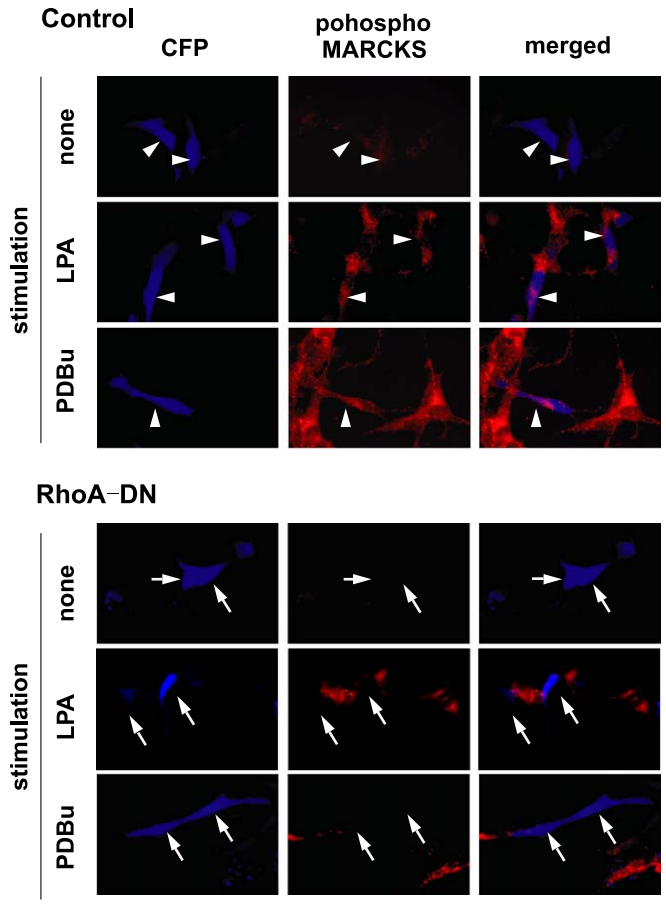

Fig. 6. Dominant negative form of RhoA (RhoA-DN) inhibited both LPA- and PDBu-induced MARCKS phosphorylation. ECFP-expressing plasmid (control) and ECFP-conjugated RhoA-DN expression plasmids (RhoA-DN) were transfected to SH-SY5Y. The cells were incubated for 24 h after transfection and serum-starved for 17 h. Then the cells were stimulated with LPA or PDBu. Phosphorylated MARCKS was visualized by pS159-Mar-Ab and Rhodamine-conjugated anti-mouse IgG Ab.

were stimulated with LPA or PDBu, MARCKS was phosphorylated even if the cells were transfected by the control vector (indicated by the arrow head). The transfection of ECFP-conjugated RhoA-DN (indicated by the arrow) inhibited the phosphorylation of MARCKS in both LPA- and PDBu-stimulated cells (Fig. 6). These results indicated that RhoA mediated PDBu-induced MARCKS phosphorylation at least in part.

Discussion

In this study, ROCK-specific inhibitor H-1152 unexpectedly inhibited not only LPA-induced phosphorylation of MARCKS Ser159 and MBS Thr654 but also PDBu-induced phosphorylation in SH-SY5Y cells (Figs. 2 and 3). Therefore, we assumed that ROCK is involved in PDBu-induced MARCKS phosphorylation in SH-SY5Y cells. In particular, it is interesting that PDBu induces MBS phosphorylation, suggesting the PDBu-induced activation of the ROCK pathway. However, low but distinct MARCKS phosphorylation, recognized as a basal phosphorylation, remained in the resting SH-SY5Y cells under some experimental conditions (Figs. 1–3), and ROCK inhibitor H-1152 did not completely inhibit the PDBu-induced phosphorylation of MARCKS and MBS (Figs. 2 and 3). Therefore, we could not exclude the possibility that PKC directly phosphorylates MARCKS in SH-SY5Y cells.

We considered that PDBu activates ROCK through PKC-activated RhoA, because several groups reported that PKC activates RhoA in non-neuronal cells [10–12]. According to the results obtained using several inhibitors (Figs. 4–6), we speculated that RhoA participates in PDBu-induced MARCKS phosphorylation downstream of PKC. However, the molecular mechanism of RhoA activation by PKC in neuronal cells remains unclear. Mehta et al. showed that PKC activated RhoA through Rho-GDI in human vascular endothelial cells [10]. We detected the expression of Rho-GDI mRNA in SH-SY5Y cells using RT-PCR (data not shown). Thus, it was possible that PKC phosphorylated Rho-GDI followed by RhoA activation in SH-SY5Y cells.

In this paper, we showed for the first time that, besides direct phosphorylation, PKC indirectly phosphorylates MARCKS in neuronal cells, whereas until now it was believed that PKC only directly phosphorylates MARCKS.

Acknowledgments

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