Brief Communications

Rho-Kinase Accelerates Synaptic Vesicle Endocytosis by Linking Cyclic GMP-Dependent Protein Kinase Activity to Phosphatidylinositol-4,5-Bisphosphate Synthesis

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Rho-kinase plays diverse roles in cell motility. During neuronal development, Rho-kinase is involved in neuronal migration, and in neurite outgrowth and retraction. Rho-kinase remains highly expressed in mature neurons, but its physiological roles are poorly understood. Here we report that Rho-kinase plays a key role in the synaptic vesicle recycling system in presynaptic terminals. Vesicles consumed by excessive exocytosis are replenished by accelerating vesicle endocytosis via a retrograde feedback mechanism involving nitric oxide released from postsynaptic cells. This homeostatic control system involves presynaptic cyclic GMP-dependent protein kinase (PKG) and a plasma membrane phospholipid, phosphatidylinositol-4,5-bisphophate (PIP_2). We found that application of a Rho-kinase inhibitor, a PKG inhibitor or both, reduced the PIP_2 content in Wistar rat brainstem synaptosomes to a similar extent. Likewise, application of the Rho-kinase inhibitor into the calyx of Held presynaptic terminal slowed vesicle endocytosis to the same degree as did application of the PKG inhibitor. This endocytic slowing effect of the Rho-kinase inhibitor was canceled by coapplication of PIP_2 into the terminal. By contrast, a RhoA activator increased the PIP_2 content and reversed the effect of the PKG inhibitor in brainstem synaptosomes. The RhoA activator, when loaded into calyceal terminals, also rescued the endocytic slowing effect of the PKG inhibitor. Furthermore, intraterminal loading of anti-PIP_2 antibody slowed vesicle endocytosis and blocked the rescuing effect of the RhoA activator. We conclude that Rho-kinase links presynaptic PKG activity to PIP_2 synthesis, thereby controlling the homeostatic balance of vesicle exocytosis and endocytosis in nerve terminals.

Introduction

At chemical synapses, an action potential arriving at the nerve terminal induces Ca^{2+} influx into the cell, thereby triggering exocytic release of neurotransmitter by fusion of synaptic vesicle membranes into the plasma membrane of the terminal. After releasing neurotransmitter, vesicle membrane is retrieved from the plasma membrane by endocytosis, and new vesicles are reformed and refilled with neurotransmitter to be reused in another round of synaptic transmission (Schweizer and Ryan, 2006; Jung and Haucke, 2007; Royle and Lagnado, 2010).

Clathrin-mediated endocytosis is a major vesicle-recycling pathway (Granseth et al., 2006; Jung and Haucke, 2007; Dittman and Ryan, 2009; Haucke et al., 2011). In clathrin-mediated endocytosis, the adaptor protein complex 2 binds to clathrin, synaptotagmin, and stonin 2, together with phosphatidylinositol-4,5-bisphophate (PIP_2) in the plasma membrane, to promote clathrin coat formation (McPherson et al., 1996; Jost et al., 1998; Itoh et al., 2001; Dittman and Ryan, 2009; Royle and Lagnado, 2010; Haucke et al., 2011). PIP_2 is also thought to be involved in clathrin uncoating (Cremona et al., 1999). Furthermore, PIP_2 binds to the GTPase dynamin, possibly assisting tubular invagination and vesicle formation (Zheng et al., 1996). At hippocampal synapses in culture (Micheva et al., 2003) and at calyx of Held synapses in rodent brainstem slices (Eguchi et al., 2012), retrograde activation of cyclic GMP-dependent protein kinase (PKG) by nitric oxide (NO) released from postsynaptic cells accelerates vesicle endocytosis by elevating the level of PIP_2 in the presynaptic terminal. However, it is not known how PKG is linked to PIP_2.

Rho-kinase is a coiled-coil-forming *serine/threonine* protein kinase and a major effector of the small GTPase RhoA. Rho-kinase is involved in cell motility, such as migration and proliferation, as well as in cell survival (Riento and Ridley, 2003; Mueller et al., 2005). In neurons, Rho-kinase contributes to neurite outgrowth (Bito et al., 2000) and neurite retraction (Nakayama et al., 2000; Riento and Ridley, 2003; Mueller et al., 2005; Sunico et al., 2010). At synapses, Rho-kinase activity reportedly participates in transmitter release (González-Forero et al., 2012) and in presynaptic plasticity (Ota et al., 2010). The NO/PKG signaling cascade upregulates RhoA expression in vascular tissue

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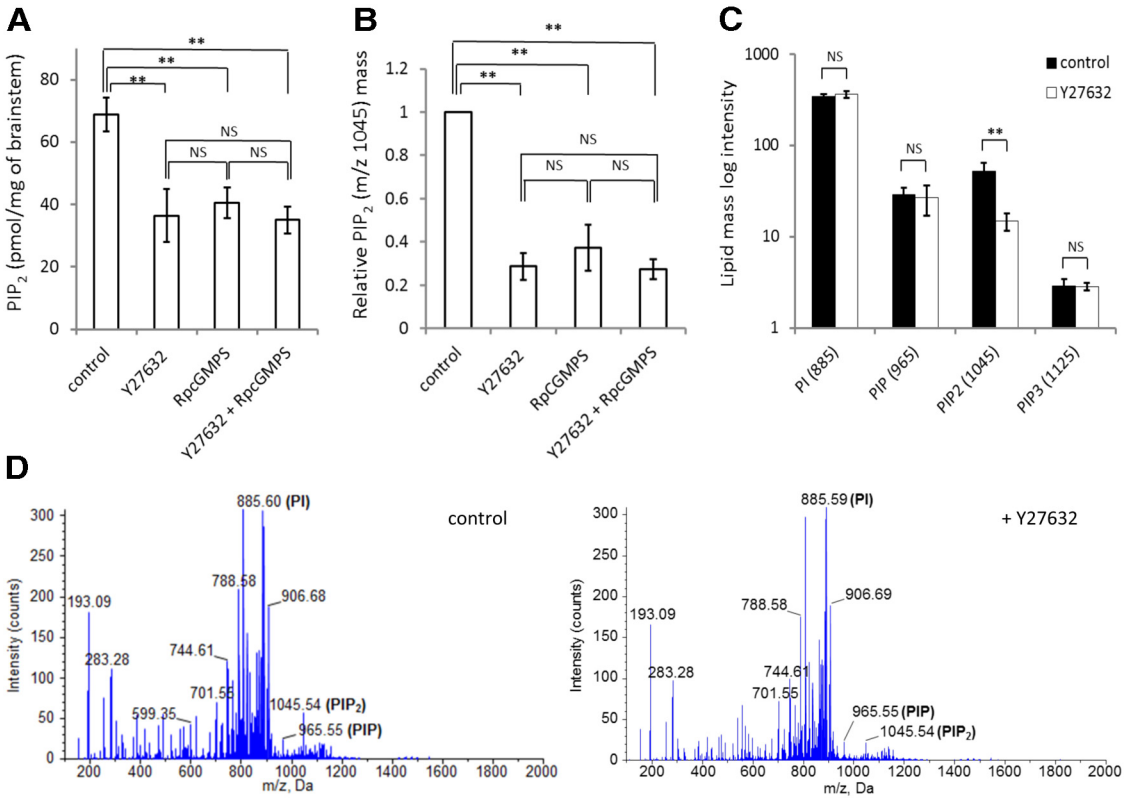

Figure 1. Rho-kinase inhibitor decreased PIP_2 level in brainstem synaptosomes. $PI(4,5)P_2$ levels in brainstem synaptosomes assayed using ELISA (***A***) and tandem mass spectrometry (***B***). Brainstem tissue was incubated with Y27632 (4 μM) or/and with Rp-cGMPS (3 μM) for 15 min at 37°C. ***C***, The inhibitory effect of Y27632 (4 μM) on the levels of PI, PIP, PIP_2, and PIP_3 (m/z 885, 965, 1045, and 1125 Da, respectively) assayed using tandem mass spectrometry. ***D***, Representative mass spectrometry profiles of acidic lipids from rat brainstem synaptosomes control and Y27632-treated tissues ($n = 6$). NS, not significant. $^{**}p < 0.01$.

(Sauzeau et al., 2003). In cell lines, RhoA reportedly enhances production of PIP_2 from PIP, by activating phosphatidylinositol-5-phosphate kinases (PIP5K) (Shibasaki et al., 1997; Weernink et al., 2000, 2004).

Does Rho-kinase link PKG activity to PIP_2 synthesis in the presynaptic terminal? We addressed this question using membrane capacitance measurements at calyx of Held presynaptic terminals in rat brainstem slices, together with quantitative mass spectrometry and ELISA assays of PIP_2 content in brainstem synaptosomes. Our results have revealed a physiological role of Rho-kinase in the homeostatic control of synaptic vesicle recycling, by linking PKG activity to presynaptic PIP_2 levels.

Materials and Methods

All experiments were performed in accordance with guidelines of the Physiological Society of Japan and animal experiment regulations at the Okinawa Institute of Science and Technology Graduate University.

Materials. The Rho-kinase inhibitor, trans-4-[(1*R*)-1-aminoethyl]-*N*-4-pyridinylcyclohexanecar boxamide dihydrochloride (Y27632) and l-α-phosphatidylinositol 4,5-diphosphate sodium salt from bovine brain (PIP_2) were obtained from Sigma-Aldrich. L-α-Phosphatidylinositol-4-phosphate ammonium salt from porcine brain (PIP) was purchased from Avanti Polar Lipids. The PKG inhibitor guanosine 3′,5′-cyclic monophosphorothioate, β-phenyl-1,N^2-etheno-8-bromo-, Rp-Isomer, sodium salt (Rp-cGMPS) was from Calbiochem. Cytotoxic necrotizing factor (CNF) catalytic domain-derived protein Rho activator II was from Cytoskeleton. A mouse monoclonal anti-PIP_2 antibody was from Abcam.

Biochemistry. For synaptosome isolation and phospholipid extraction, brainstems were isolated from postnatal day (P) 13–15 Wistar rats of either sex killed by decapitation under halothane anesthesia. After isolation, brainstem tissues were incubated with or without drugs for 5, 15, or 60 min at 37°C in artificial CSF (aCSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, 3 myo-inositol, 2 sodium pyruvate, 0.5 ascorbic acid, 1.25 NaH_2PO_4, 26 $NaHCO_3$ (310–315 mOsm, pH 7.3, when saturated with 95% O_2/5% CO_2). Synaptosomes were isolated as described previously (Villasana et al., 2006). Briefly, tissues were homogenized in 0.32 M sucrose, 4 mM HEPES-NaOH buffer (pH 7.3 kept at < 4°C), supplemented with EDTA-free Protease Inhibitor Cocktail (Roche), Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich), and kinase inhibitor sodium orthovanadate (Sigma-Aldrich). Filtered homogenates were then clarified by 15 min centrifugations at 10,200 × g. To extract acidic phosphatidyl lipids, an ice-cold methanol/chloroform/HCl 12N (80/40/1) mixture was added to the remaining tissue pellets, vortexed thoroughly, and centrifuged at 1,500 rpm for 5 min. A chloroform/0.1N-HCl (1/2) nonpolar/polar solvent mixture was added to create a phase split to separate lipids from water-soluble constituents. Phospholipids were collected and evaporated using a vacuum centrifuge (Concentrator 5301, Eppendorf). For quantification of PIP_2 by ELISA, lipid samples were dissolved in PBS containing the PIP_2 sensor provided in the $PI(4,5)P_2$ Mass ELISA kit (K-4500, Echelon). PIP_2 content in each sample was then quantified following the manufacturer's instructions. Luminometric analyses were performed by measuring the final signal absorbance at 450 nm using a microplate reader (Benchmark Plus 170–6930J1, Bio-Rad). PIP_2 levels were normalized to the wet weight of brainstem tissue. For quantification of inositol phospholipids by mass spectrometry, lipids samples were diluted with isopropanol/acetonitrile (60/40, v/v). After mixing 10 μl of diluted sample with 10 μl of 9-aminoacridine hemihydrate matrix (Acros Organics), prepared at 10 mg/ml dissolved in isopropanol/acetonitrile, 0.5 μl of the mixture was spotted on a MALDI Opti-TOF stainless steel plate (Applied Biosystems). MALDI-ToF MS and MS/MS analyses were performed using a QSTAR Elite QqTOF mass spectrometer, equipped with a 1 kHz Nd/YAG pulsed laser (355 nm) and an electrostatic mirror (Applied Biosys-

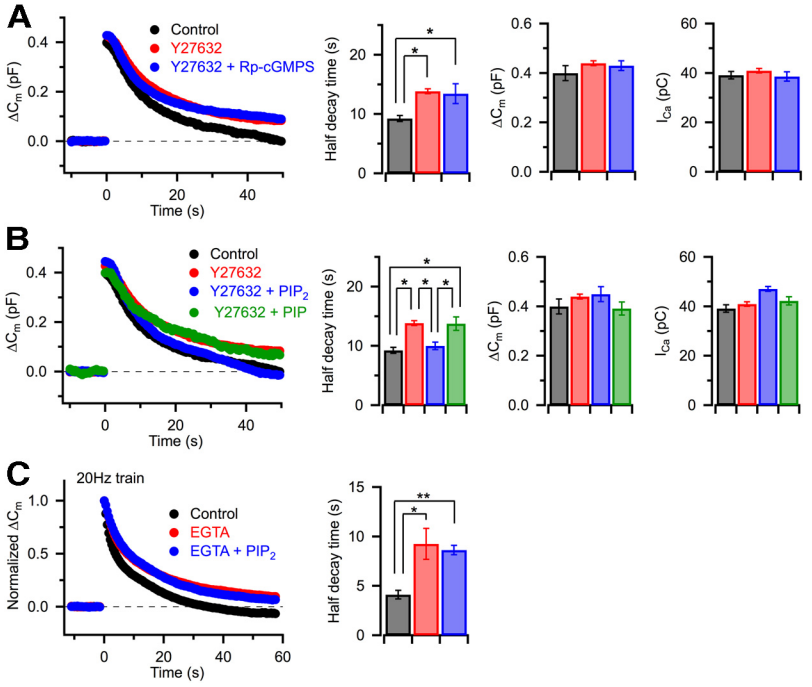

Figure 2. Rho-kinase inhibitor slowed vesicle endocytosis via the PKG/PIP_2 pathway in calyx of Held synapses. Membrane capacitance (C_m) change evoked by a 20 ms depolarizing pulse (from −80 to +10 mV). ***A***, Direct injection of Y27632 (10 μM) into calyceal terminals (red trace and bars) significantly slowed endocytosis. The effect of coloading Y27632 and Rp-cGMPS (3 μM) (blue) into calyces was indistinguishable from the effect of Y27632 alone (red). The averaged traces of C_m records are derived from 6–10 terminals. Summary bar graphs of Y27632 effect on endocytic half-decay time, magnitudes of exocytic C_m change (ΔC_m), and Ca^{2+} current amplitude (I_{Ca}). ***B***, PIP_2 (5 μM), when coloaded with Y27632, rescued the slowing effect of the latter (blue trace, superimposed). Bar graphs are the same as in ***A***. In contrast, PIP (5 μM) had no such effect (green). ***C***, C_m changes induced by a train of 20 ms depolarizing pulses (20 times) at 20 Hz in the presence of 0.5 mM EGTA (control, black), 10 mM EGTA (red), or 10 mM EGTA and 5 μM PIP_2 (blue) coloaded into P13–P14 calyceal terminals. Traces are averaged C_m records derived from 6–10 terminals, normalized at the peak. Bar graphs represent endocytic half-decay times. *$p < 0.05$. **$p < 0.01$.

tems). Data were acquired in negative ion reflectron mode at an accelerating potential of −15 kV. Phospholipids spectra and signal-to-noise ratios were determined using Analyst QS 2.0 software (Applied Biosystems). Integrated areas of lipid peaks were corrected against peak areas of internal standards (giving a response ratio for each lipid) and normalized to an external standard, phosphatidylglycerol, PG (18:0/18:0) added during the lipid extraction procedure (Avanti Polar Lipids). For purification of anti-PIP_2 antibody, sodium azide and newborn calf serum were removed using the Antibody Purification Kit Protein A (Abcam) according to the manufacturer's instructions.

Electrophysiology. Methods for preparing auditory brainstem slices, containing the medial nuclei of trapezoid bodies from Wistar rats (P13-P15), have been described previously (Yamashita et al., 2010; Eguchi et al., 2012). Briefly, rats were killed by decapitation under halothane anesthesia. Before recording, brainstem slices were incubated at 37°C for 1 h in aCSF. Membrane capacitance measurements were made from calyx of Held presynaptic terminals in whole-cell configuration at 26–27°C (Yamashita et al., 2010; Eguchi et al., 2012). For recordings, aCSF contained 10 mM tetraethylammonium chloride, 0.5 mM 4-aminopyridine, 1 μM tetrodotoxin, 10 μM bicuculline methiodide, and 0.5 μM strychnine hydrochloride. The pipette solution contained 118 mM Cs gluconate, 30 mM CsCl, 10 mM HEPES, 0.5 mM EGTA, 1 mM $MgCl_2$, 12 mM disodium phosphocreatine, 3 mM Mg-ATP, and 0.3 mM Na-GTP (pH 7.3–7.4 adjusted with CsOH, 315–320 mOsm). Data were acquired at a sampling rate of 100 kHz, using an EPC-10 patch-clamp amplifier controlled by PatchMaster software (HEKA Elektronik) after on-line filtering at 5 kHz. Calyceal terminals were voltage-clamped at a holding potential of −80 mV, and single-step depolarization (to +10 mV, 20 ms) was used for inducing presynaptic Ca^{2+} currents (I_{Ca}). Drugs were infused from whole-cell pipettes into calyceal terminals by diffusion. Care was taken to maintain access resistance <14 MΩ to allow diffusion of drugs into the terminal within 4 min after membrane rupture. Data were analyzed using IGOR Pro 6 (WaveMetrics). All values are given as mean ± SEM. Differences were considered statistically significant at $p < 0.05$ in Student's unpaired *t* test or ANOVA with Tukey *ad hoc* test.

Results

Downregulatory effect of a Rho-kinase inhibitor on PIP_2 content in brainstem synaptosomes

The PIP_2 level in brainstem tissue and at calyces of Held of rats after hearing onset can be reduced by application of an NO scavenger or a PKG inhibitor (Eguchi et al., 2012). To test whether Rho-kinase is involved in this signaling pathway, we examined the PIP_2 level in brainstem synaptosomes after application of the Rho-kinase-specific inhibitor Y27632 (Uehata et al., 1997) using ELISA and mass spectrometry. Incubation of brainstem synaptosomes with Y27632 (4 μM) for 15 min at 37°C reduced the PIP_2 content by twofold to threefold in ELISA (Fig. 1*A*) and mass spectrometry (Fig. 1*B*) assays. Likewise, the PKG inhibitor Rp-cGMPS (3 μM) incubated with brainstem synaptosomes for 15 min at 37°C, reduced the PIP_2 content in ELISA (Fig. 1*A*) and mass spectrometric assays (Fig. 1*B*), as previously reported for ELISA experiments of brainstem tissue extracts (Eguchi et al., 2012). Coapplication of Y27632 (4 μM) and Rp-cGMPS (3 μM) did not further reduce the PIP_2 content (Fig. 1*A*,*B*), suggesting that PKG and Rho-kinase operate in series in a signaling pathway upregulating PIP_2. The inhibitory effect of Y27632 was surprisingly selective for PIP_2 (1045 Da), with little effect on the levels of the precursors phosphatidylinositol (PI) (885 Da), phosphatidylinositol-mono-phosphate (PIP) (965 Da), or the downstream product phosphatidylinositol-tri-phosphate (PIP_3) (1125 Da) in tandem mass spectrometry analysis (Fig. 1*C*,*D*; see below for discussions).

Inhibitory effect of Rho-kinase inhibitor on vesicle endocytosis at the calyx of Held presynaptic terminal

At calyces of Held of developing rats, the expression of PKG increases, and PKG accelerates vesicle endocytosis through a PIP_2-dependent mechanism after hearing onset (Eguchi et al., 2012). To test whether Rho-kinase might be involved in this mechanism, we loaded Y27632 (10 μM) directly into calyceal terminals from whole-cell patch pipettes (Fig. 2*A*,*B*). Depolarizing pulses (from −80 to +10 mV) of 20 ms duration induced an exocytic ΔC_m jump of ~0.4 pF, followed by a slower endocytic decay, with a half-time ($\tau_{0.5}$) of 9.25 ± 0.51 s ($n = 6$). Y27632 loading significantly slowed the endocytic $\tau_{0.5}$ to 13.9 ± 0.42 s ($n = 10$), with no effect on the exocytic ΔC_m amplitude or I_{Ca} (Fig. 2*A*,*B*).

As in biochemical assays (Fig. 1), coloading of Rp-cGMPS (3 μM) and Y27632 (10 μM) into calyces had no greater effect than their individual loadings on the endocytic $\tau_{0.5}$ (13.5 ± 1.67 s, $n = 10$) (Fig. 2*A*), suggesting that Rho-kinase operates downstream from PKG in the same pathway, rather than in a parallel pathway.

Unlike bath-applied Rho-kinase inhibitor at hippoglossal synapses (González-Forero et al., 2012), Y27632 directly loaded into the calyx presynaptic terminal had no effect on ΔC_m or Ca^{2+} currents (I_{Ca}) associated with transmitter release (Fig. 2*A*).

The slowing effect of the Rho-kinase inhibitor on vesicle endocytosis was reversed by coapplication of PIP_2 (5 μM) into calyceal terminals (Fig. 2*B*), suggesting that a reduction in endogenous PIP_2 by the Rho-kinase inhibitor could be rescued by exogenous PIP_2 loading into presynaptic terminals. By contrast, intraterminal loading of the PIP_2 precursor PIP together with Y27632 had no effect, suggesting that the latter inhibited PIP5K for the production of PIP_2 from PIP. Furthermore, PIP_2 loading had no effect on vesicle endocytosis slowed by the Ca^{2+} chelator EGTA (10 mM) (Yamashita et al., 2010), suggesting that it has no general rescuing effect on the rate of vesicle endocytosis (Fig. 2*C*). These results, together with no effect of loading PIP_2 alone on endocytic rate (Eguchi et al., 2012), suggest that exogenous PIP_2 can be incorporated into an endocytic accelerating mechanism downstream of Rho-kinase cascade, only when endogenous PIP_2 level declines, thereby rescuing the endocytic rate of synaptic vesicles (see below).

Rho-kinase activator counteracted the negative regulatory effects of PKG inhibitor on the PIP_2 level in brainstem synaptosomes and on the rate of vesicle endocytosis

We next examined the effect of activating Rho-kinase using the cytotoxic necrotizing factor (CNF)-derived peptide Rho activator II (Flatau et al., 1997). Incubation of brainstem synaptosomes with Rho activator II (5 μg/ml, 15 min at 37°C) significantly increased the synaptosomal PIP_2 content and counteracted the inhibitory effect of the PKG inhibitor, Rp-cGMPS (3 μM), in ELISA (Fig. 3*A*) and tandem mass spectrometric assays (Fig. 3*B*). These results, together with those of the Rho-kinase inhibitor (Fig. 1), strongly suggest that Rho-kinase mediates the upregulatory effect of PKG on the PIP_2 level in the brainstem.

Rho activator II (5 μg/ml), when coloaded with Rp-cGMPS into the calyx of Held presynaptic terminal, rescued the slowing effect of the PKG inhibitor on vesicle endocytosis (8.0 ± 0.5 s, $n = 4$) (Fig. 4*A*). Rho activator II, when loaded alone, had no significant effect on endocytic rate (8.2 ± 0.4 s, $n = 7$), like single PIP_2 loading (Eguchi et al., 2012), suggesting that endogenous PIP_2 is close to a saturating level in normal presynaptic terminal membrane. Neither the exocytic ΔC_m nor I_{Ca} was affected by the Rho activator (Fig. 4*A*). These results suggest that presynaptic Rho-kinase accelerates vesicle endocytosis, operating downstream from the NO/PKG signal cascade.

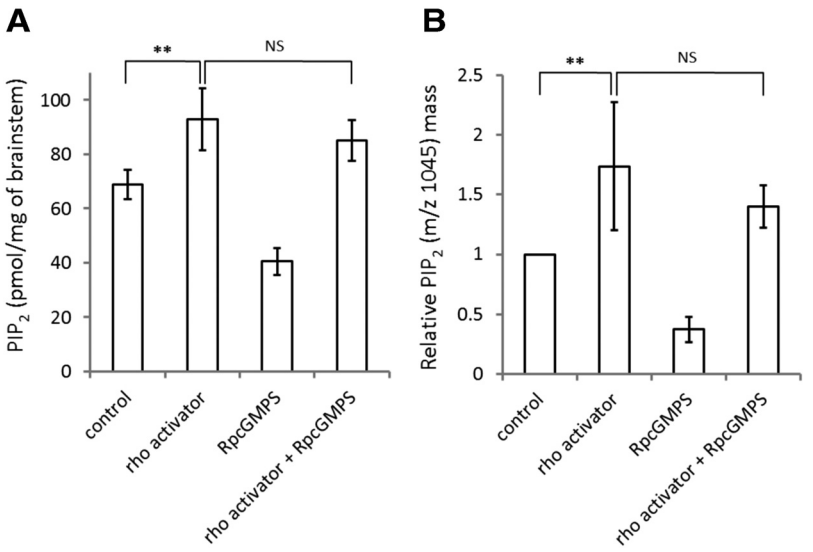

Figure 3. Rho activator increased PIP_2 level and rescued the effect of the PKG inhibitor. Assessment of PIP_2 level in brainstem synaptosomes by ELISA (***A***) and tandem mass spectrometry (***B***). Treatment of brainstem tissue with Rho activator II (CNF derived) (5 μg/ml) for 15 min at 37°C, significantly increased the PIP_2 content of synaptosomes. Bathing the tissue with Rho activator rescued the inhibitory effect of Rp-cGMPS (3 μM) on PIP_2 level ($n = 6$).

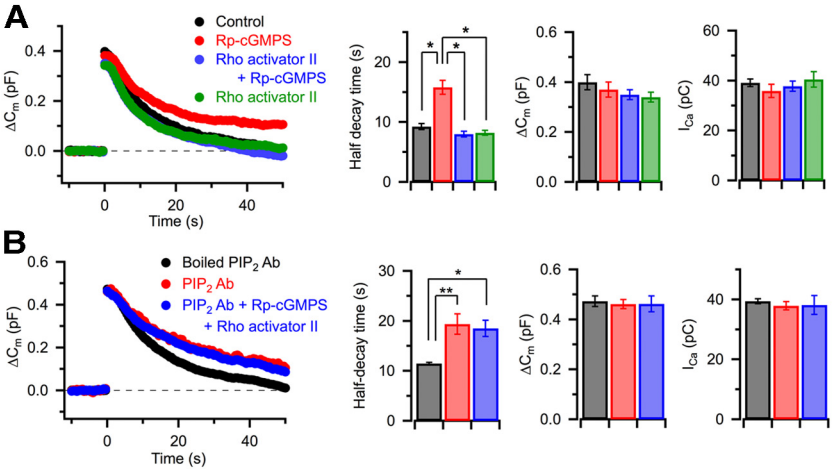

Figure 4. Rho activator II reversed the slowing effect of PKG inhibitor on vesicle endocytosis via increasing PIP_2 level. ***A***, Intraterminal loading of Rho activator II (CNF derived) (5 μg/ml) into calyceal terminals had no effect on endocytosis (green trace and bars). Coloading of Rho activator II with Rp-cGMPS (3 μM) reversed (blue) the inhibitory effect of Rp-cGMPS (red) on endocytosis. Data are from 6–10 calyces. ***B***, Anti-PIP_2 antibody (20 μg/ml, red trace and bars) loaded into the presynaptic terminals significantly slowed endocytic C_m changes, whereas boiled anti-PIP_2 antibody (20 μg/ml, black) had no effect. In the presence of anti-PIP_2 antibody, Rho activator II no longer rescued the inhibitory effect of Rp-cGMPS (blue). Data are from 4–6 calyces.

To examine whether the rescuing effect of the Rho activator on endocytic rate is indeed mediated by endogenous PIP_2 production, we loaded anti-PIP_2 antibody into calyceal terminals. This antibody, when loaded alone, slowed vesicle endocytosis (19.4 ± 2.1 s, $n = 5$), whereas boiled antibody had no effect, suggesting that the antibody reduced available PIP_2 (Fig. 4*B*). In the presence of anti-PIP_2 antibody in presynaptic terminals, the Rho activator no longer rescued the endocytic rate slowed by Rp-cGMPS (18.5 ± 1.6 s, $n = 4$) (Fig. 4*B*).

Discussion

In the present study, inhibition of Rho-kinase activity with Y27632 specifically decreased PIP_2 content in the synaptosomal fraction of brainstem tissue and slowed the time course of vesicle endocytosis recorded using capacitance measurements at calyces

of Held presynaptic terminals. In contrast, enhancement of Rho-kinase activity with Rho activator II increased synaptosomal PIP_2 levels and counteracted the slowing effect of the PKG inhibitor on vesicle endocytosis. When vesicle endocytosis is slowed, replenishment of releasable vesicles through recycling is retarded. This distorts the fidelity of synaptic transmission, particularly in response to prolonged high-frequency inputs (Eguchi et al., 2012). In this respect, Rho-kinase plays a key role in the molecular cascade providing homeostatic control of high-frequency information transfer through neuronal circuits. This cascade operates when postsynaptic NMDA receptors are activated by glutamate released from presynaptic terminals and accelerates endocytosis via PIP_2 production, in an activity-dependent manner, according to the magnitude of exocytosis, thereby coupling vesicle recycling with neuronal activity (Micheva et al., 2003; Eguchi et al., 2012). The retrograde linkage from postsynaptic NMDA receptors to presynaptic PKG is mediated by NO, but the linkage from PKG to PIP_2 in presynaptic terminals remained elusive (Micheva et al., 2003; Eguchi et al., 2012). In this study, we have shown that Rho-kinase mediates the molecular cascade from PKG to PIP_2. Although the present study was conducted at room temperature (26–27°C), endocytic rate is faster at physiological temperature by a factor of >1.4 (Fernandez-Alfonso and Ryan, 2004; Micheva and Smith, 2005; Renden and von Gersdorff, 2007). Likewise, at physiological temperature, activities of various kinases and NMDA receptors (Steinert et al., 2010) involved in this retrograde cascade are higher. Thus, this exoendocytic coupling mechanism would operate more efficiently for the maintenance of high-fidelity, high-frequency synaptic transmission *in vivo*.

PKG directly binds to the GTPase RhoA (Kato et al., 2012) and negatively controls Rho-kinase activity for vascular smooth muscle contraction (Sauzeau et al., 2000; Sandu et al., 2001; Kato et al., 2012) and stress fiber formation (Sawada et al., 2001). In contrast, PKG can positively control RhoA expression (Sauzeau et al., 2003; Rolli-Derkinderen et al., 2005) and Rho-kinase activity possibly via phosphorylation of RhoGEF17 (Lutz et al., 2013). The downstream effector, Rho-kinase, also binds to and activates PIP5K (Weernink et al., 2000, 2004; Yang et al., 2004), which produces PIP_2 through phosphorylation of PIP. Thus, the upregulatory effect of the RhoA activator on the PIP_2 content of brainstem synaptosomes (Fig. 3) is likely mediated by activation of PIP5K. Although Rho-kinase might also increase the availability of PIP to the PIP5 kinase (Yamamoto et al., 2001), intraterminal loading of PIP did not rescue the slowing effect of Rho-kinase inhibitor on vesicle endocytosis unlike PIP_2 loading (Fig. 2*B*), suggesting that PIP5K is likely the main target of Rho-kinase in the nerve terminal. Our phospholipids mass spectrometry analyses have shown that the Rho-kinase inhibitor Y27632 specifically lowers the PIP_2 content in brainstem synaptosomes with little effect on the PIP content (Fig. 1*C*,*D*). These results are consistent with reports that the main locus of cellular PIP is in the Golgi membrane of cells (Wang et al., 2003) and that the PIP pool in the plasma membrane contributes little to *de novo* PIP_2 synthesis (Hammond et al., 2012).

In conclusion, we have elucidated a novel physiological function of Rho-kinase, which positively controls synaptic vesicle endocytosis by upregulating plasma membrane PIP_2. This function is linked via the retrograde signal cascade of NO and PKG that is essential for maintenance of high-fidelity synaptic transmission in the CNS.

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