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AMPA, not NMDA, activates RhoA GTPases and subsequetly phosphorylates moesin

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Abbreviations: AMPA, a-Amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; ERM, ezrin-radixin-moesin; NMDA, N-methyl D-Aspartate; ROK, Rho kinase

Abstract

Glutamate induced rapid phosphorylation of moesin, one of ERM family proteins involved in the ligation of membrane to actin cytoskeleton, in rat hippocampal cells (JBC, 277:16576-16584, 2002). However, the identity of glutamate receptor has not been explored. Here we show that a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is responsible for glutamate-induced RhoA activation and phosphorylation of moesin. Glutamate induced phosphorylation at Thr-558 of moesin was still detectible upon chelation of Ca^{2+}, suggesting involvement of AMPA receptor instead of N-methyl D-Aspartate (NMDA) receptor in this phosphorylation of moesin. AMPA but not NMDA-induced moesin phosphorylation was independent of Ca^{2+}. Both AMPA and NMDA but not Kainate induced moesin phosphorylation at similar levels. However, the kinetics of phosphorylation varied greatly between AMPA and NMDA where AMPA treatment rapidly increased phosphomoesin, which reached a maximum at 10 min after treatment and returned to a basal level at 30 min. In contrast, NMDA-induced phosphorylation of moesin reached a maximum at 30 min after treatment and was remained at higher levels at 60 min. A possible involvement of RhoA and its downstream effector, Rho kinase in the AMPA receptor-triggered phosphorylation of moesin was also explored. The kinetics for the glutamate- induced membrane translocation of RhoA was similar to that of moesin phosphorylation induced by AMPA. Moreover, Y-27632, a specific Rho kinase inhibitor, completely blocked AMPA-induced moesin phosphorylation but had no effect on NMDA-induced moesin phosphorylation. These results suggest that glutamate-induced phosphorylation of moesin may be mediated through the AMPA receptor/RhoA/Rho kinase pathway.

Keywords: AMPA; Moesin; NMDA; Rho GTPase; ROK; phsophorylation

Introduction

Moesin is one of ERM family proteins and is expressed ubiquitously. Major proposed function of moesin is ligation of membrane to actin cytoskeleton (Bretscher *et al*., 2002). When phosphorylated at Thr-558 of moesin, intramolecular association between N-terminus and C-terminus of moesin is disrupted. Subsequently N-terminal FERM domain of moesin is exposed, which in turn links C-terminal tail to membrane proteins such as I-CAM and actin cytoskeleton. ROK and PKC are reported to phosphorylate moesin. Previously, we reported phosphorylation of moesin by electroconvulsive shock and suggested glutamate-mediated RhoA-ROK activation might be an underlying mechanism (Jeon *et al*., 2002).

Involvement of Rho GTPase in glutamate receptor signaling has also been reported from other laboratories (Husi and Grant, 2001). Citron, a Rho GTPase target has been reproted to interact with PSD-95 in postssynaptic density underlying NMDA receptor in thalamus (Furuyashiki, *et al*., 1999; Zhang *et al*., 1999). Glutamate receptor and Rho GTPase have been proposed to be essential to dendrite growth in Xenopus (Sin *et al*., 2002). A Rho-GAP, RICS (Okabe *et al*., 2003) as well as several Rac/Cdc42-specific Rho-GEF, such as Kalirin-7 (Penzes *et al*., 2001), dPix (Parnas *et al*., 2002) and bPIX (Park *et al*., 2003), have been reported to play a role in dendrite remodeling. These reports are focusing on the involvement of Rho GTPases in dendritic morphogenesis through NMDA receptor and suggest that Rac and

Cdc42 activities enhance dendritic remodeling while RhoA activity inhibits (Wong *et al*., 2000; Li *et al*., 2002; Sin *et al*., 2002). Thus, RhoA would be inactive in NMDA receptor-mediated dendrite remodeling. However, our previous results indicated that glutamate stimulation led to activation of RhoA-ROK pathway, in hippocampal progenitor cells, H19-7/IGF-IR cells. One possible explanation about this discrepancy might be that RhoA is activated through glutamate receptor subfamily other than NMDA receptor. Thus so far no evidence on direct involvement of RhoA in AMPA or kinate signaling has been reported. In this study, we sought to determine which ionotropic glutamate receptor subfamily could activate RhoA-ROK pathway and subsequently phosphorylate moesin.

Materials and Methods

Materials

H19-7/IGF-IR cells were obtained from the American Type Culture Collection (Manassas, VA). Mouse monoclonal RhoA antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Y27632 was purchased from Tocris (Ellisville, MO).

Cell culture

H19-7/IGF-IR cells were maintained in DMEM containing 10% fetal bovine serum, 200 ㎍/ml G418, and 1 ㎍/ml puromycin at 34°C under 5% CO_2. H19-7/IGF-IR cells were seeded at 1×10^6 in a 60 mm dish. For AMPA or Kainate treatment, the cells were cultured in serum-free DMEM for 20 h and incubated with Kreb's buffer (126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 25 mM $NaHCO_3$, pH 7.4). And then the cells were stimulated with 100 ㎛M glutamate, AMPA (a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) or Kainate for various periods (2, 5, 10, 30, and 60 min). And the cells were incubated with Mg^{2+} free Kreb's buffer to treat the cells with NMDA or glutamate. Because NMDA receptors are blocked with Mg^{2+} ion. To chelate Ca^{2+} ion, the cells were incubated with Kreb's buffer containing 5 mM EGTA for 90 min before the glutamate, AMPA, or NMDA treatments. To examine the involvement of Rho kinase in glutamate-induced moesin phosphorylation, we used a Rho kinase inhibitor, Y-27632. Before the glutamate treatment, H19-7/IGF-IR cells were incubated with Kreb's buffer containing 30 ㎛M Y-27632 for 1 h. The cells were rinsed out with cold phosphate buffered saline and then lysed in 1×Laemmli buffer. The samples were boiled at 100°C for 10 min.

Subcellular fractionation

H19-7/IGF-IR cells were seeded at 1.25×10^6 in 100 mm dishes. For glutamate treatment, the cells were cultured in serum-free DMEM for 20 h and incubated with Kreb's buffer. And then the cells were stimulated with or without 100 ㎛M glutamate for various period (2, 5, 10, 30, 60 min). Then the cells were rinsed with cold phosphate-buffered saline and lysed in detergent-free buffer (50 mM TrisCl, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM $NaVO_3$, 10 ㎍/ml Leupeptin, 10 ㎍/ml aprotinin, 1 mM PMSF, pH 8.0) by passing 15 times through a 25 gauge needle. The lysed cells were centrifuged at 1,000 *g* for 10 min. The supernatants were centrifuged at 100,000 *g* for 1 h. The supernatants were used as cytosol, and the pellets as crude membrane. The supernatants were added with 5×Laemmli buffer and the pellets were melted in 1×Laemmli buffer. Then the samples were boiled at 100°C for 10 min.

Western blots

To analyze the amount of phosphorylated moesin, the samples were separated by SDS-polyacrylamide gel electrophoresis (10%) and then electrophoretically transferred from gels onto nitrocellulose membranes. To analyze the amount of translocated RhoA, we used 15% polyacrylamide gels and polyvinylidene fluoride membrane. Blots were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween-20 for 1 h, incubated with 1:3,000 rabbit antisera in 5% milk followed by 1:5,000 peroxidase-conjugated donkey anti-rabbit IgG in 5% milk or incubated with 1:5,000 RhoA antibody in 5% BSA followed by 1:5,000 peroxidase-conjugated goat anti-mouse IgG in 5% milk. Blots were developed using enhanced chemiluminescence (ECL).

Results and Discussion

Phsphorylation of moesin at Thr-558 in H19-7/IGF-IR cells after glutamate, AMPA, NMDA, and Kainate treatments

As shown in Figure 1A and B, glutamate stimulation induced rapid phosphorylation of moesin at Thr-558. Glutamate-induced moesin phosphorylation reached a peak at 10 min after stimulation and lasted up to 60 min. Glutamate is known to mediate its signal through 3 ionotropic subtypes and metabotropic subtype. In order to explore which glutamate receptor subtype mediates moesin phosphorylation, 3 ionotropic glutamate receptor agonists, NMDA, AMPA and Kinate were tested (Figure 1C, D, E). With treatment of AMPA, amount of phospho-moesin was rapidly increased, but returned to a pre-stimulated basal level after 30 min

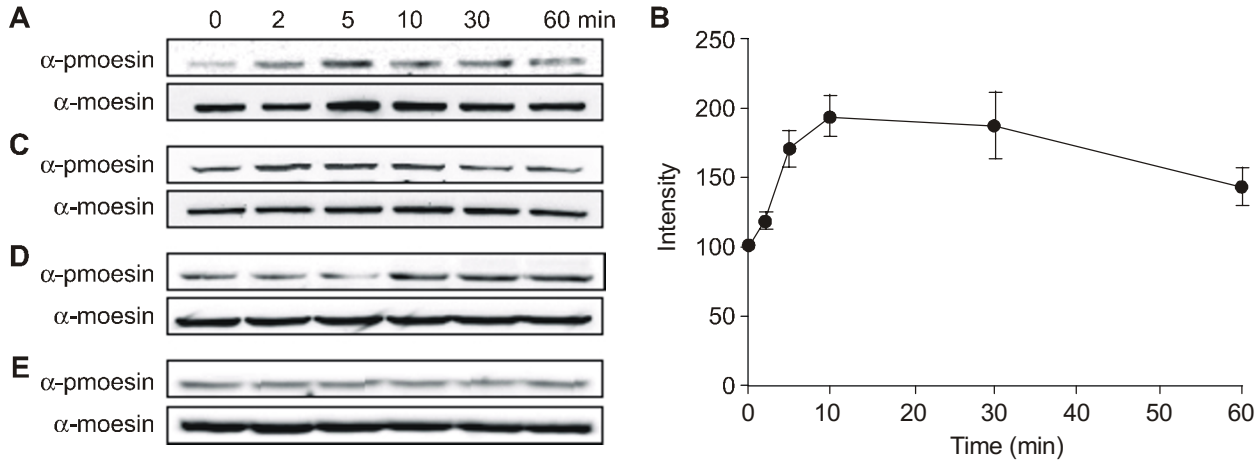

Figure 1. Phosphorylation of moesin upon stimulation with glutamate and glutamate receptor agonists in H19-7/IGF cells. (A) Phosphorylation of moesin was detected at 2 min after glutamate stimulation and lasted up to 60 min. (B) Intensities of phosphomoesin bands were plotted over time after normalized against the intensity of corresponding total moesin band. (C-E) Serum starved H19-7/IGF-IR cells were stimulated with AMPA(C), NMDA(D) or Kinate(E).

(Figure 1C). Since NMDA receptors are blocked in the presence of Mg^{2+} ion, H19-7/IGF-IR cells were incubated with Mg^{2+}-free Kreb's buffer and then treated with NMDA. Phosphorylation of moesin was also induced by NMDA but the time course of phosphorylation was totally different from that induced by AMPA. NMDA-induced phophorylation of moesin was observed at 10 min and last over 60 min much longer than AMPA-induced phosphorylation (Figure 1D). The amplitude of phosphorylation of moesin was quite comparable, about 50% over basal level on between AMPA and NMDA stimulation. Kinate stimulation had no effect on phosphorylation of moesin (Figure 1E) although kinate-induced seizures could activate intracellular signaling pathway in hippocampus (Lee *et al*., 2002). From the results of the moesin phosphorylation pattern on glutamate stimulation appears to be the summation of phosphorylation of moesin on both AMPA and NMDA stimuli. Taken together, both AMPA and NMDA are found capable of phosphorylating moesin in H19-7/IGFR cells but in a different time course.

Effect of Ca^{2+} on glutamate-induced phosphorylation of moesin

Among the three major subtypes of glutamate receptors, NMDA receptor would likely be the major participants because of its possible relation with short-term memory. Major ion required for NMDA receptor function is suggested to be Ca^{2+} ion where Ca^{2+}-mediated signaling cascade is reported to be crucial to NMDA-mediated long-term potentiation. Thus, the effect of Ca^{2+} ion on the phorphorylation of moesin upon glutamate stimulation was examined. As shown in Figure

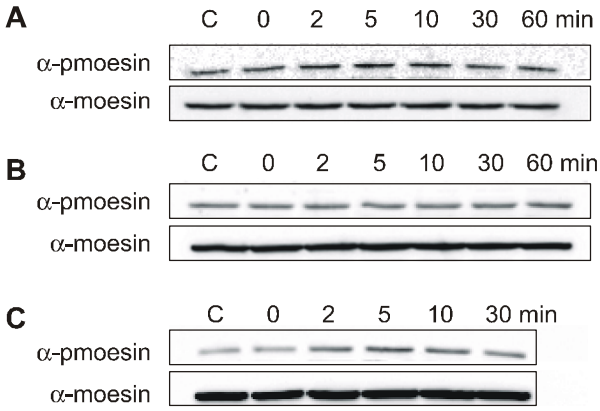

Figure 2. Effect of Ca^{2+} on phosphorylation of moesin by gluR agonists. Serum-starved H19-7/IGF-IR cells were pre-incubated with EGTA. EGTA-treated cells were stimulated with glutamate (A), NMDA (B) or AMPA (C).

2A, phosphorylation of moesin returned to basal levels at 30 min after glutamate stimulation in the absence of Ca^{2+} ion, much faster than in the presence of Ca^{2+} ion, which indicated that early phase phosphorylation of moesin was independent of Ca^{2+} ion while late phase phosphorylation of moesin was dependent of Ca^{2+} ion. Based on such observation, the glutamate stimulation of moesin phosphorylation may involve both AMPA receptor at early phase and NMDA receptor at late phase. To confirm this idea, the effect of calcium chelation on phosphorylation of moesin was tested on both AMPA and NMDA receptors. As shown in Figure 2C, pretreatment of cells with EGTA for 90 min completely blocked NMDA-induced phosphorylation of moesin, suggesting that

phosphorylation of moesin was totally dependent on Ca^{2+} influx through NMDA receptor. However, AMPA could still phosphorylate moesin in the absence of Ca^{2+} ion in medium even though AMPA receptor is known to allow passage of Na^{+}, K^{+}, and Ca^{2+} (Figure 2C). Metal ion requirement experiments support that moesin phosphorylation on glutamate stimulation was likely the summation effect of both AMPA and NMDA. NMDA phosphorylated moesin in a delayed, Ca^{2+}-dependent manner, suggesting that NMDA-induced moesin phosphorylation might occur in the subcellular location other than postsynaptic density and through a Ca^{2+}-dependent downstream pathway. However, rapid and transient nature of AMPA-induced moesin phosphorylation suggests that it might occur in postsynaptic density and through direct downstream pathway to AMPA receptor.

AMPA, not NMDA, phosphorylated moesin through RhoA-ROK pathway

Since glutamate-induced moesin phosphorylation was inhibited by a ROK inhibitor (Jeon *et al*., 2002), it would be a choice experiment to define major path for respective receptors, either AMPA or NMDA or both to be linked with RhoA small GTPase in the glutamate-induced moesin phosphorylation. Figure 3A shows the glutamate induced membrane translocation of RhoA. The amount of membrane-translocated RhoA was increased at 2 min after AMPA stimulation and returned to basal levels at 30 min. The kinetics of RhoA translocation exactly matched that of moesin phosphorylation by AMPA, suggesting that glutamate-induced membrane translocation of RhoA might be through AMPA receptor and AMPA-induced moesin phosphorylation might be conducted by ROK. When ROK was blocked with Y27632, a ROK inhibitor, AMPA-induced moesin phosphorylation was totally blocked. However, NMDA-induced mosesin phosphorylation was not effected at all (Figure 3B, C). Follow up experiment of Moesin phosphorylation for longer time period showed that NMDA-induced moesin phosphorylation was terminated at 90 min after stimulation.

These observations indicated that AMPA, not NMDA, induced activation of RhoA-ROK pathway and it was ROK that phosphorylated moesin. Involvement of RhoA in AMPA signaling was not totally unexpected since Rac and Cdc42 GTPase have already been proposed to be involved in NMDA signaling as described in introduction. Our result that NMDA didn't activate RhoA also matched to the previous reports in which Rac and Cdc42 GTPase, not RhoA, might be involved in NMDA-induced dendrite remodeling (Penz *et al*., 2001; Park *et al*., 2003). Possible involvement of RhoA GTPase signaling has been proposed in metabotropic glutamate receptor signaling

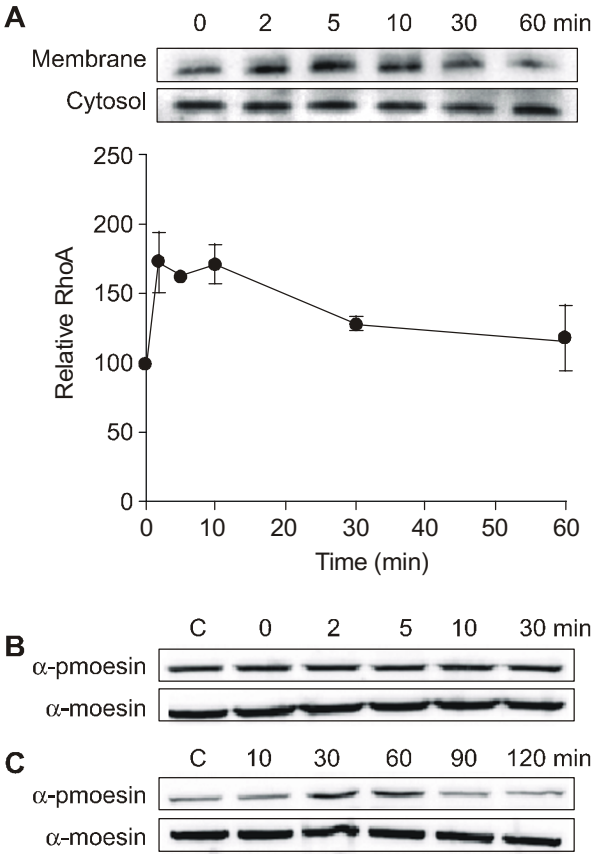

Figure 3. Membrane translocation of RhoA by glutamate and effect of Y27632 on the phosphorylation of moesin at Thr-558. A. Serum-starved H19-7/IGF-IR cells were stimulated with glutamate. Whole cell lysates were fractionated into membrane and cytosol fractions. The amount of RhoA in each fraction was determined by Western blot (top). The intensities of RhoA band in membrane fractions were plotted over time (bottom). B and C. Serum-starved H19-7/IGF-IR cells pre- incubated with Rho kinase inhibitor, Y-27632, were stimulated with AMPA (B) or NMDA (C). Lysates were then processed as described above.

(Kanumilli *et al*., 2001) and in glutamate signalling in hippocampal cell line, H19-7 cells (Jeon *et al*., 2002).

Since almost all the reports suggested involvement of Rac and Cdc42 GTPases in glutamate signaling instead of RhoA GTPase and reciprocal action between Rac and RhoA in dendrite remodeling, one can doubt what is the possible role of activation of RhoA and subsequent phosphorylation of moesin by AMPA. One possible hint might come from the report that C3 exotoxin, an inhibitor of RhoA, inhibited run-down of NMDA receptor channels (Nreberg *et al*., 1999), in which authors suggested that ligation of NMDA receptor and actin cytoskeleton by RhoA might be crucial for regulation of NMDA receptor activity but failed to show actual activation of RhoA. In this context, our result might be a missing puzzle fragment, that is, AMPA activated RhoA leading to polymeriz-

ation of actin cytoskeleton and also phosphorylate moesin by RhoA-ROK leading to ligation of AMPA or NMDA receptor to underlying actin cytoskeleton since phopshorylation of moesin has been reported to be crucial in memebrane-cytoskeleton ligation (Bretscher *et al*., 2002).

Recent study that a myosin/moesin phosphatase inhibitor, CPI-17 is crucial for cerebellar long-term depression through AMPA receptor (Eto *et al*., 2002) might be another clue for the role of moesin in AMPA receptor signaling. In this study, silencing CPI-17 by si-RNA blocked long-term depression (LTD) induction although significance of moesin phosphorylation in LTD is not directly proved. So it would be interesting to investigate what effect AMPA-activated RhoA and moesin might have on NMDA receptor activity since transient depolarization of membrane induced by AMPA is known to be crucial in NMDA receptor opening.

In conclusion, AMPA stimulation activated RhoA and in turn ROK followed by phosphorylation of moesin, while NMDA stimulation also phosphorylated moesin but in a different pathway other than RhoA-ROK pathway.

Acknowledgment

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