Muscarinic receptor-stimulated cytosol-membrane translocation of RhoA

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Abstract Receptor-mediated phospholipase D activation in human embryonic kidney (HEK) cells stably expressing the m3 muscarinic acetylcholine receptor (mAChR) apparently involves the small G protein RhoA. Here, activation of RhoA was examined by measuring cytosol-membrane translocation, which is a sign of RhoA activation. RhoA translocation was induced by guanosine 5′-*O*-(3-thio)triphosphate in digitonin-permeabilized HEK cells, and in intact cells by the agonist-activated mAChR and by direct activation of heterotrimeric G proteins. RhoA translocation was also induced by the phosphotyrosine phosphatase inhibitor pervanadate, while the tyrosine kinase inhibitors tyrphostin 23 and genistein inhibited the mAChR-induced RhoA translocation. These data suggest that translocation and thus activation of RhoA by the G protein-coupled m3 mAChR in HEK cells apparently involves a tyrosine kinase-dependent reaction.

Key words: RhoA; Muscarinic receptor; Tyrosine phosphorylation; Phospholipase D

1. Introduction

Rho family GTPases, a subgroup of the Ras superfamily of low molecular weight guanine nucleotide-binding proteins (G proteins), play essential roles in various cellular functions, predominantly in the organization of the actin cytoskeleton [1–4] and cell growth and transformation [5–9]. These GTPases serve as molecular switches which are inactive in the GDP-bound state and active in the GTP-bound state. Cycling between these states is controlled by several regulatory proteins, such as guanine nucleotide dissociation inhibitors (GDIs), guanine nucleotide exchange factors and GTPase-activating proteins [1].

RhoA is one of the most abundant members of the Rho family and present in all tissues so far examined [1,10]. In unstimulated cells, RhoA is localized predominantly in the cytosol with usually only a small fraction associated with the plasma membrane [11–14]. The cytosolic location is apparently due to binding of RhoA to RhoGDI, a protein which inhibits GDP dissociation as well as GTP hydrolysis [15–17]. The process of RhoA activation by GDP/GTP exchange is closely associated with the translocation of RhoA from the cytosol to the plasma membrane [1,14]. It is not clear, however, whether translocation is a consequence of activation or whether translocation of RhoA to the plasma membrane is a prerequisite for interaction with its guanine nucleotide exchange factor(s) [18]. When Rho returns to the inactive, GDP-bound state, it is supposed to form a new complex with RhoGDI and regains its cytosolic localization. Thus, the activation-inactivation cycle of RhoA by GTP binding and hydrolysis is closely related to a cytosol-membrane translocation cycle.

Previous studies with Rho-inactivating toxins demonstrated that RhoA is involved in receptor activation of phospholipase D (PLD) [19,20] and phospholipase C [21,22] in various cell types. The aim of the present study was to study receptor-induced RhoA activation, by measuring translocation of RhoA from the cytosolic to the membrane fraction, in human embryonic kidney (HEK) cells, stably expressing the human m3 muscarinic acetylcholine receptor (mAChR). The data presented indicate that agonist activation of the m3 mAChR induces RhoA translocation and that this G protein-coupled receptor action apparently involves a tyrosine kinase-dependent process.

2. Materials and methods

2.1. Materials

RhoA antibody was purchased from Santa Cruz (Heidelberg, Germany). Genistein and tyrphostin 23 were from Biomol (Hamburg, Germany) and nitrocellulose membranes from Microdevices (Alabama, India). All other materials were from previously described sources [19,23].

2.2. Cell culture

Culture conditions of HEK cells stably expressing the human m3 mAChR were as reported in detail before [23]. For experiments, cells subcultured in Dulbecco's modified Eagle's medium/F-12 medium were grown to near confluence (175 cm^2 culture flasks).

2.3. Translocation assays

For measurement of RhoA translocation, culture medium was discharged, and cells were carefully rinsed with phosphate-buffered saline (PBS), containing 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 6.5 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2, before detachment from the culture flasks. Cells were washed once in PBS and then resuspended in PBS at a density of about 5×10^7 cells/ml. In some experiments, cells were washed and resuspended in Mg^{2+}- and Ca^{2+}-free PBS (composition see above, except that Mg^{2-} and Ca^{2-} were not included). 50 μl of the cell suspension (2×10^6 cells) was incubated at 37°C for varying periods of time with or without stimuli as indicated in the figure legends. Thereafter, 950 μl of permeabilization buffer, containing 20 mM HEPES, pH 7.2, 135 mM KCl, 5 mM $NaCO_3$, 5.6 mM D-glucose, 2 mM ATP, 4 mM $MgCl_2$, 5 mM EGTA, 1.5 mM $CaCl_2$ (corresponding to 40 nM free Ca^{2+}) and 8 μM digitonin, was added, followed by a further incubation period of 10–60 min. Routinely and if not indicated otherwise, permeabilization was performed for 10 min. Afterwards, cells were pelleted by centrifugation at $15000\times g$ for 1 min, and RhoA contents of supernatant and pellet fractions were analyzed as follows: a 300 μl aliquot of the supernatant (corresponding to about 60 μg protein) was precipitated with 6% (mass/vol., final concentration) trichloroacetic acid and

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Abbreviations: G protein, guanine nucleotide-binding protein; GDI, guanine nucleotide dissociation inhibitor; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HEK, human embryonic kidney; mAChR, muscarinic acetylcholine receptor; PBS, phosphate-buffered saline; PLD, phospholipase D

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0.0125% (mass/vol., final concentration) deoxycholate, and precipitated proteins were subjected to SDS/PAGE on gels containing 15% (mass/vol.) acrylamide. The cell pellet, representing either a crude membrane fraction or intact cells, was resuspended in 200 μl Laemmli buffer, incubated at 95°C for 10 min, and an aliquot (20 μl, corresponding to about 60 μg protein) was subjected to SDS/PAGE.

2.4. Immunoblot analysis

Following SDS/PAGE, proteins were transferred from polyacrylamide gels onto nitrocellulose filters and analyzed by standard procedures. The blots were blocked for 2 h with 5% (mass/vol.) bovine serum albumin and incubated with the polyclonal rabbit anti-RhoA antibody (dilution 1:1000). Afterwards, the blots were incubated with a goat anti-rabbit IgG-peroxidase conjugate (1:4000 dilution, Sigma), before bound antibodies were detected by enhanced chemiluminescence (Amersham). The staining of immunoblots was quantified by densitometry (QuantiScan program, Biosoft).

2.5. Data presentation

The results shown are characteristic of at least three similar experiments.

3. Results

Treatment of m3 mAChR-expressing HEK cells during digitonin permeabilization with the stable GTP analog guanosine 5′-*O*-(3-thio)triphosphate (GTPγS), induced translocation of RhoA from the cytosolic to the membrane fraction (Fig. 1). Permeabilization of HEK cells with digitonin markedly reduced RhoA content of the crude membrane fraction compared to non-permeabilized cells, and this effect was almost complete when permeabilization was for 60 min (Fig. 1A). However, when GTPγS (100 μM) was present during permeabilization, loss of RhoA during permeabilization was fully

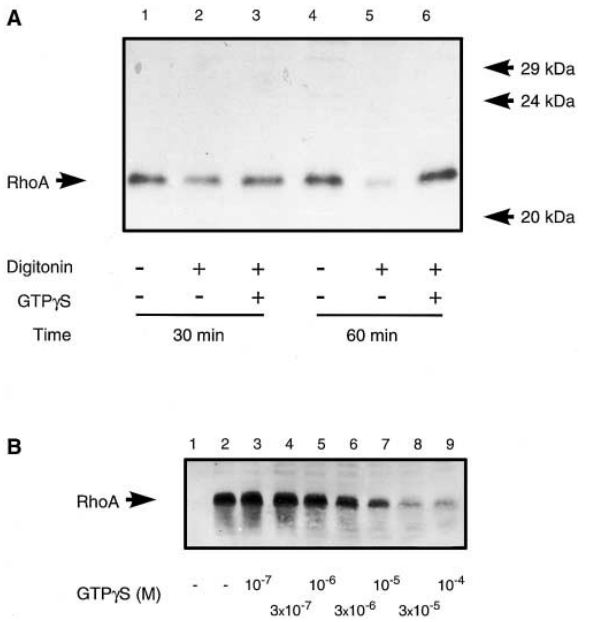

Fig. 1. GTPγS-stimulated translocation of RhoA from the cytosol to the membrane fraction. A: HEK cells were permeabilized with digitonin in the absence and presence of 100 μM GTPγS for 30 min (lanes 1–3) or 60 min (lanes 4–6), and after centrifugation the pellet fraction was analyzed for RhoA content as described in Section 2. Lanes 1 and 4: non-permeabilized cells. Molecular weight markers are indicated on the right. B: Digitonin permeabilization of HEK cells was performed for 30 min at the indicated concentrations of GTPγS. Subsequently, the supernatant was analyzed for RhoA content. Lane 1: non-permeabilized cells.

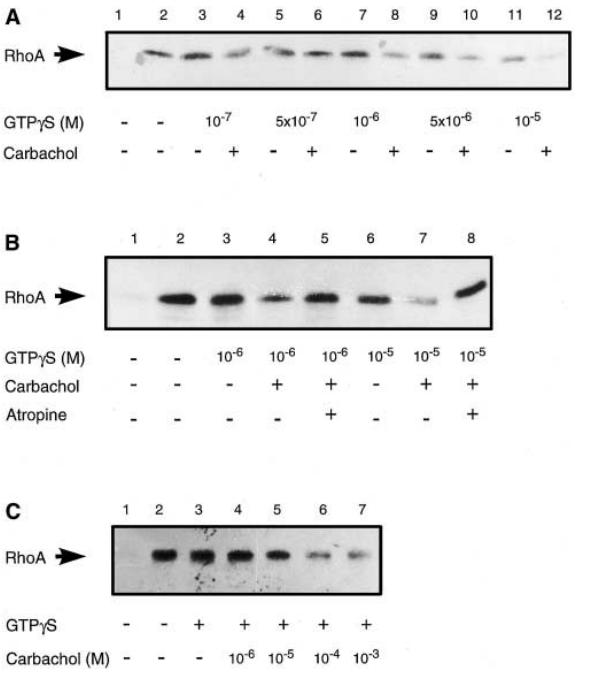

Fig. 2. mAChR-stimulated RhoA translocation. A: m3 mAChR-expressing HEK cells were treated with or without 100 μM carbachol for 3 min prior to permeabilization with digitonin (10 min) in the presence of the indicated concentrations of GTPγS. B: HEK cells were treated with or without 100 μM carbachol in the presence or absence of 100 μM atropine for 3 min prior to permeabilization by digitonin in the presence of 1 μM or 10 μM GTPγS. C: HEK cells were treated with the indicated concentrations of carbachol for 3 min prior to digitonin permeabilization in the presence of 1 μM GTPγS. The RhoA content of supernatant fractions was determined. Lanes 1 in A–C: non-permeabilized cells.

prevented. RhoA contents of non-permeabilized cells and of the crude membrane fraction of HEK cells permeabilized in the presence of GTPγS were virtually identical, strongly suggesting that the stable GTP analog induced translocation of cytosolic RhoA to the membrane fraction. This translocation of RhoA was reflected in a GTPγS-induced decrease in cytosolic RhoA, released during permeabilization of HEK cells with digitonin (Fig. 1B). Under the experimental conditions used, half-maximal and maximal effects on RhoA release were achieved with about 3 μM and 100 μM GTPγS, respectively, as determined by densitometric quantification. Thus, RhoA translocation could be detected both as increase in membrane-bound RhoA and decrease in cytosolic RhoA. In order to minimize RhoA degradation possibly occurring during prolonged digitonin permeabilization required for full release of RhoA, cytosolic and crude membrane fractions were routinely prepared after 10 min permeabilization of HEK cells with digitonin. Under this condition, about 60% of cellular RhoA in unstimulated HEK cells was pelleted with the crude membrane fraction. Therefore, in the following translocation of RhoA is illustrated as decrease in release of cytosolic RhoA.

Next, we studied whether agonist activation of the stably expressed m3 mAChR can induce RhoA translocation. For this, intact HEK cells were first stimulated for 3 min with and without carbachol, followed by digitonin permeabilization in the presence of GTPγS. When GTPγS was not added to the permeabilization buffer, a receptor-stimulated RhoA translocation was not observed under standard assay conditions (but

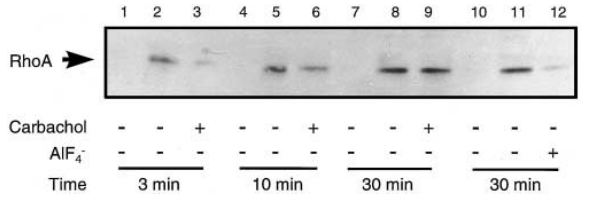

Fig. 3. Time course of carbachol-induced RhoA translocation; influence of AlF_4^-. m3 mAChR-expressing HEK cells washed and resuspended in Mg^{2+}- and Ca^{2+}-free PBS were treated with or without 100 μM carbachol or AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$) for 3 min (lanes 1–3), 10 min (lanes 4–6) or 30 min (lanes 7–12) as indicated prior to permeabilization by digitonin (10 min). The supernatant fractions were analyzed for RhoA content. Lanes 1, 4, 7 and 10: non-permeabilized cells.

see below). However, pre-stimulation of HEK cells with carbachol markedly potentiated GTPγS-induced RhoA translocation. As shown in Fig. 2A, stimulation of HEK cells with 100 μM carbachol, followed by digitonin permeabilization in the presence of 0.1–10 μM GTPγS, strongly increased the inhibitory effect of GTPγS on RhoA release compared to cells treated with GTPγS alone. This potentiating effect of carbachol on GTPγS-induced inhibition of RhoA release was fully prevented when carbachol (100 μM) pretreatment of HEK cells was performed in the presence of the muscarinic receptor antagonist atropine (100 μM) (Fig. 2B). The potentiating effect of carbachol on inhibition of RhoA release was concentration-dependent. In the presence of 1 μM GTPγS, which by itself had only a small effect on RhoA release, half-maximal potentiation of inhibition of RhoA release was observed at about 20 μM carbachol (Fig. 2C).

mAChR-stimulated RhoA translocation in HEK cells could also be demonstrated, even without addition of GTPγS to the permeabilization buffer, when the cells were incubated in Mg^{2+}- and Ca^{2+}-free PBS. As illustrated in Fig. 3, under this condition, treatment of HEK cells with carbachol induced a biphasic RhoA translocation. In cells pretreated for 3 min with carbachol (100 μM), release of RhoA into the supernatant was almost fully prevented. Upon prolonged treatment of HEK cells, the inhibitory effect of carbachol was reduced and fully lost in cells treated for 30 min with carbachol. These data, additionally, demonstrate that receptor-induced inhibition of RhoA release is not due to RhoA degradation. As signal transduction of the mAChR is mediated by heterotrimeric G proteins, we examined whether direct activation of these G proteins can also induce RhoA translocation. Preincubation of HEK cells with AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$) prior to permeabilization strongly inhibited RhoA release (Fig. 3, lanes 10–12). Similar to the mAChR agonist action, AlF_4^--regulated RhoA translocation was only observed when cells were incubated in Mg^{2+}- and Ca^{2+}-free PBS (data not shown). In contrast to carbachol, however, the inhibitory effect of AlF_4^- on RhoA release was retained even after 30 min stimulation of HEK cells with this direct activator of heterotrimeric G proteins.

Since evidence has been provided that a tyrosine kinase is involved in receptor signaling to RhoA [24], we studied the effects of tyrosine kinase and phosphotyrosine phosphatase inhibitors on RhoA translocation in HEK cells. As shown in Fig. 4A, treatment of HEK cells for 30 min with the phosphotyrosine phosphatase inhibitor pervanadate [25,26] caused a concentration-dependent decrease in RhoA release. Inhibition of RhoA release was almost complete in cells pretreated with 4 mM pervanadate. On the other hand, the tyrosine kinase inhibitors, tyrphostin 23 and genistein, effectively inhibited carbachol-induced RhoA translocation. Preincubation of HEK cells with 100 μM tyrphostin 23 for 30 min before stimulation with carbachol (100 μM, 3 min) nearly completely prevented the agonist-induced potentiation of inhibition of RhoA release by GTPγS (1 μM) (Fig. 4B, compare lanes 3 and 4 with lanes 7 and 8). Similarly, preincubation of HEK cells with genistein (100 μM, 30 min) also markedly reduced the inhibitory agonist effect on RhoA release (Fig. 4B, compare lanes 3 and 4 with lanes 11 and 12).

4. Discussion

Although there is ample evidence that Rho proteins are crucial for the regulation of the actin cytoskeleton organization and also have critical functions in the control of cell proliferation, until now, most information on the role of Rho in receptor signaling pathways has been obtained by rather indirect methods, using either constitutively active and dominant-negative Rho protein mutants or Rho-inactivating toxins. Since GDP/GTP exchange-dependent activation of RhoA is coupled with a translocation of this G protein from the cytosol to the plasma membrane, we examined in the present study the subcellular redistribution of RhoA in response to various stimuli to measure RhoA activation. Translocation of RhoA was quantified in HEK cells stably expressing the human m3 mAChR, in which previous studies, using the Rho-inactivating toxins, toxin B and C3 exoenzyme, demonstrated that Rho proteins, most likely RhoA, are involved in receptor signaling to PLD and phospholipase C [19,21].

Similar as reported in other cell types [18], incubation of permeabilized HEK cells with the stable GTP analog GTPγS

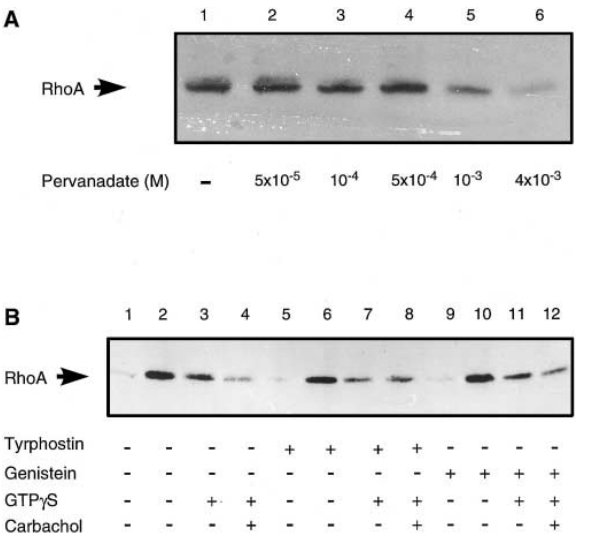

Fig. 4. Regulation of RhoA translocation by tyrosine phosphorylation. A: HEK cells were preincubated with the indicated concentrations of pervanadate for 30 min prior to permeabilization by digitonin (10 min). B: HEK cells preincubated for 30 min with or without 100 μM tyrphostin 23 or genistein were treated with or without 100 μM carbachol for 3 min, followed by digitonin permeabilization (10 min) in the presence or absence of 1 μM GTPγS as indicated. Lanes 1, 5 and 9: non-permeabilized cells. RhoA content of supernatant fractions is demonstrated.

led to a concentration-dependent translocation of cytosolic RhoA to the particulate fraction. The translocation could be demonstrated both as a GTPγS-induced decrease in release of cytosolic RhoA and as an increase in RhoA content of the particulate fraction. As studies in other cell types demonstrated that RhoA is predominantly localized in the cytosol and/or at the plasma membrane [11–14], the translocation of RhoA to the particulate fraction of HEK cells probably reflects association of RhoA with the plasma membrane.

Translocation of RhoA was not only induced by the stable GTP analog, most likely by direct binding to RhoA. Incubation of intact HEK cells with the mAChR agonist, carbachol, and AlF_4^-, a direct activator of heterotrimeric G proteins, also induced RhoA translocation. Complete inhibition of carbachol-stimulated translocation by concomitant incubation with atropine proves receptor specificity. Interestingly, reproducible effects of both the receptor agonist alone and nonselective activation of heterotrimeric G proteins by AlF_4^- on RhoA translocation could only be demonstrated when the cells were incubated under Mg^{2+}- and Ca^{2+}-free conditions. This may be explained by moderate lowering of cytosolic Mg^{2+} concentrations leading to facilitated GDP/GTP exchange, as reported for Rab5 proteins [27].

When cells prestimulated with carbachol were permeabilized in the presence of submaximal concentrations of GTPγS, there was a concentration-dependent, receptor agonist-induced potentiation of RhoA translocation, suggesting that mAChR activation facilitates GTPγS binding to RhoA. This finding contrasts with the lack of agonist effect on PLD activity measured in permeabilized HEK cells in the presence of GTPγS [23,28]. One possible explanation might be that for receptor-mediated and RhoA-dependent PLD stimulation additional components are required which cannot be assembled in permeabilized cells. mAChR-induced translocation of RhoA was transient, with maximal effects being achieved within 3 min, and completely abolished upon prolonged agonist activation. A similar transient agonist (lysophosphatidic acid)-induced RhoA translocation has been reported in rat1 fibroblasts [20]. Interestingly, mAChR-induced PLD stimulation in HEK cells exhibits similar kinetics. Maximal PLD stimulation is rapidly reached and is then fully desensitized upon prolonged agonist treatment [28].

Tyrosine kinase inhibitors have been reported to inhibit mAChR-induced PLD stimulation in HEK cells [23] as well as lysophosphatidic acid-induced and RhoA-mediated formation of focal adhesions and actin stress fibers in Swiss 3T3 fibroblasts [24]. These findings prompted us to investigate the role of tyrosine phosphorylation in translocation of RhoA in HEK cells. The phosphotyrosine phosphatase inhibitor pervanadate [25,26], which induced strong tyrosine phosphorylation of various proteins in HEK cells [23] (data not shown), effectively induced RhoA translocation, suggesting that a tyrosine kinase/phosphotyrosine phosphatase cycle is maintained in a basal activity state in HEK cells similar as reported for other cells [3,25,26]. In support of the data obtained with pervanadate, suggesting that a tyrosine phosphorylation-dependent event is involved in RhoA activation, we observed that the tyrosine kinase inhibitors, tyrphostin 23 and genistein, effectively inhibited mAChR-stimulated RhoA translocation. These data strongly suggest that an as yet unidentified tyrosine kinase(s) participates in translocation and thus activation of RhoA by the G protein-coupled m3 mAChR in HEK cells.

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