Animal Cells and Systems
Vol. 14, No. 3, September 2010, 155–160

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ERK1/2 activation by the *C. elegans* muscarinic acetylcholine receptor GAR-3 in cultured mammalian cells involves multiple signaling pathways

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(*Received 31 March 2010; received in revised form 9 June 2010; accepted 10 June 2010*)

Extracellular signal-regulated kinases 1/2 (ERK1/2) play important roles in a variety of biological processes including cell growth and differentiation. We have previously reported that GAR-3 activates ERK1/2 via phospholipase C and protein kinase C, presumably through pertussis toxin (PTX)-insensitive Gq proteins, in Chinese hamster ovary (CHO) cells. Here we provide evidence that GAR-3 also activates ERK1/2 through PTX-sensitive G proteins, phosphatidylinositol 3-kinase (PI 3-kinase), and Src family kinases in CHO cells. We further show that in human embryonic kidney (HEK293) cells, epidermal growth factor receptor and Ras are required for efficient ERK1/2 activation by GAR-3. Taken together, our data indicate that GAR-3 evokes ERK1/2 activation through multiple signaling pathways in cultured mammalian cells.

Keywords: *C. elegans*; ERK1/2; GAR-3; muscarinic acetylcholine receptor

Abbreviations: *C. elegans*, *Caenorhabditis elegans*; CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; mAChR, muscarinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; RTK, receptor tyrosine kinase

Introduction

Extracellular signal-regulated kinases (ERKs) are members of the mitogen-activated protein kinase (MAPK) family that are highly conserved in eukaryotes (Widmann et al. 1999). ERK1/2 are known to play crucial roles in diverse biological functions such as cell proliferation and cell death (Mebratu and Tesfaigzi 2009). In addition, ERK1/2 have been reported to regulate memory formation (Kelleher et al. 2004) and microRNA biogenesis (Paroo et al. 2009).

ERK1/2 activation is typically initiated by receptor tyrosine kinases (RTKs) as follows (Egan et al. 1993; Burgering and Bos 1995). Ligand-stimulated RTKs interact with GRB2, which is associated with Sos. The guanine nucleotide exchange factor Sos then catalyzes the exchange of GTP for GDP on Ras, a member of the small G protein superfamily. GTP-bound Ras binds to Raf and triggers sequential phosphorylation events: the serine/threonine kinase Raf phosphorylates MAPK/ERK kinases (MEK1/2), which subsequently phosphorylate and activate ERK1/2. Although the ERK1/2 pathway is mainly induced by RTKs, later studies established that G protein-coupled receptors (GPCRs) are also important regulators of ERK1/2 activity (Marinissen and Gutkind 2001; Rozengurt 2007).

Muscarinic acetylcholine receptors (mAChRs) are GPCRs implicated in various nervous functions such as perception, emotion, learning and memory (Nathanson 1987). Five subtypes of mAChRs (M1-M5) exist in mammals (Bonner et al. 1987, 1988) and these subtypes have been shown to activate ERK1/2 through distinct pathways. In COS-7 cells, M1- and M2-mediated ERK2 activation was protein kinase C (PKC)-dependent and -independent, respectively (Crespo et al. 1994). On the other hand, M3-mediated ERK1/2 activation was PKC-independent in rat thyroid epithelial cells (Jiménez et al. 2002), but PKC-dependent in CHO and HEK293 cells (Wotta et al. 1998; Wylie et al. 1999; Slack 2000). Presumably, biochemical mechanisms by which mAChRs activate ERK1/2 vary depending on receptor subtypes and cell types.

From the nematode *Caenorhabditis elegans* (*C. elegans*), we identified three G protein-coupled acetylcholine receptors (GAR-1, GAR-2, and GAR-3) and found that GAR-3 is structurally and pharmacologically most similar to mammalian mAChRs (Hwang et al. 1999; Lee et al. 1999,

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ISSN 1976-8354 print/ISSN 2151-2485 online

DOI: 10.1080/19768354.2010.504341
http://www.informaworld.com

2000). Recent studies from other research groups showed that GAR-3 controls *C. elegans* behaviors such as feeding, mating, and locomotion (Steger and Avery 2004; You et al. 2006; Liu et al. 2007; Dittman and Kaplan 2008). To help understand the biochemical mechanisms underlying these behaviors, we have been investigating the signaling pathways modulated by GAR-3 using cultured mammalian cells. Previously we have shown that GAR-3 activates ERK1/2 via phospholipase C (PLC) and PKC (Kim et al. 2008). In the current work we found that GAR-3-mediated ERK1/2 activation occurs through PTX-sensitive G proteins, PI 3-kinase, Src family kinases, epidermal growth factor receptor (EGFR) and Ras.

Materials and methods

Materials

LY294002, pertussis toxin (PTX), carbamoylcholine chloride (carbachol), and atropine were purchased from Sigma. PP1 analog and PD168393 were from Calbiochem.

Cell culture

The CHO cell line stably expressing GAR-3 (GAR-3/CHO) (Hwang et al. 1999) was used in this study. GAR-3/CHO cells or HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) at 37°C with 5% CO_2. Culture media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 75 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Media for the culture of stably transfected cells (GAR-3/CHO cells) were supplemented with 250 μg/ml G418. Culture media, fetal bovine serum, G-418, and antibiotics were purchased from Gibco.

Transient expression in cultured cells

For transient expression of a dominant negative Ras (H-RasS17N), GAR-3/CHO cells were seeded into six-well plates at a density of 4×10^5 cells and transfected with 3 μg of cDNA encoding H-RasS17N by using the PolyFect transfection reagent (QIAGEN). HEK293 cells were transfected with 3 μg of cDNA encoding GAR-3 and 3 μg of cDNA encoding H-RasS17N. The transfected cells were grown for 36 h in DMEM containing 10% fetal bovine serum, and serum-starved for 12–18 h prior to ERK1/2 assay. The cDNA encoding H-RasS17N was obtained from Guthrie-cDNA Resource Center.

ERK1/2 assay

ERK1/2 activation was determined as described previously (Kim et al. 2008). Briefly, after drug treatment, cell lysates were prepared and analyzed by SDS-PAGE. The fractionated protein bands were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore), and the membrane was treated with anti-phospho-ERK1/2 monoclonal antibody. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody, and immunoreactive bands were visualized using the WEST-ZOL plus detection system (Intron, Korea). To detect total ERK1/2, the same membranes were stripped and reprobed with anti-ERK1/2 polyclonal antibody and HRP-conjugated anti-rabbit IgG antibody. The densities of the bands (44- and 42-kDa for ERK1 and ERK2, respectively) were measured by densitometry. ERK1/2 phosphorylation was normalized by total ERK1/2. All antibodies were from Cell Signaling Technology, Inc.

Results and discussion

GAR-3 activates ERK1/2 in a PTX-sensitive manner

Recently we have shown that in GAR-3/CHO cells, carbachol treatment induces ERK1/2 activation, which appears to be PLC-dependent (Kim et al. 2008). Since GAR-3-mediated PLC stimulation is not affected by PTX treatment (Park et al. 2003), it is likely that the PLC-dependent ERK1/2 activation occurs through PTX-insensitive Gq proteins. It has been reported, however, that mammalian mAChRs can couple to both PTX-insensitive and -sensitive G proteins for ERK activation (Crespo et al. 1994; van Biesen et al. 1996; Wotta et al. 1998; Wylie et al. 1999). We thus tested whether GAR-3-mediated ERK1/2 activation also occurs via PTX-sensitive G proteins. When GAR-3/CHO cells were treated with PTX, which ADP-ribosylates and inactivates Gi/o proteins, the effect of carbachol was reduced by more than 50% (Figure 1). These results strongly suggest that GAR-3 activates ERK1/2 by coupling to Gi/o proteins. Since no or little endogenous Go proteins are expressed in CHO cells (Dell'Acqua et al. 1993; Cowen et al. 1996), albeit controversial (van Biesen et al. 1996), Gi proteins seem to play a major role in the pathway of carbachol-stimulated ERK1/2 activation.

GAR-3-mediated ERK1/2 activation involves PI 3-kinase and Src family kinases

Gi-coupled receptors, including M2 mAChR subtype, have been shown to require PI 3-kinase for efficient ERK activation (Hawes et al. 1996; Lopez-Ilasaca et al. 1997). So we next determined whether PI 3-kinase is involved in GAR-3-mediated ERK1/2 activation by treating GAR-3/CHO cells with the PI 3-kinase inhibitor LY294002.

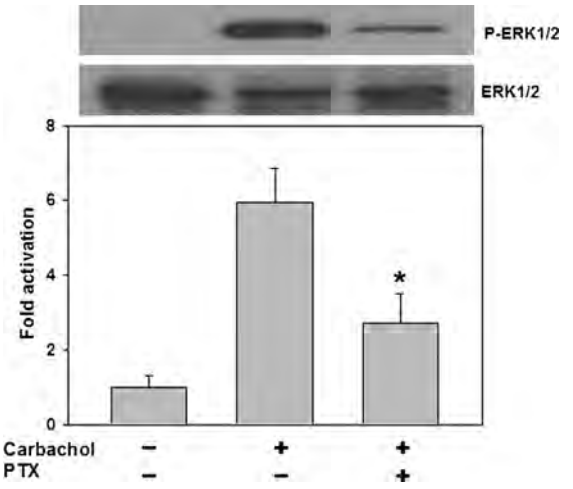

Figure 1. GAR-3 activates ERK1/2 via PTX-sensitive G proteins. GAR-3/CHO cells were serum-starved for 12 h in the presence of 100 ng/ml PTX, and stimulated with 1 mM carbachol for 5 min. Samples were immunoblotted with anti-phospho-ERK1/2 antibody (P-ERK1/2) and anti-ERK1/2 antibody (ERK1/2). A representative Western blot is shown in the upper panel, and the data for ERK1/2 activation (fold activation compared with untreated control GAR-3/CHO cells, mean ± SEM) are shown in the lower panel. $P < 0.05$ (*) compared with carbachol-treated control using Student's *t*-test.

This treatment markedly inhibited the carbachol-stimulated ERK1/2 activation (Figure 2A), indicating a critical role for PI 3-kinase in the process.

Previous studies showed that Src family kinases are important regulators of ERK1/2 activation upon stimulation of Gi-coupled receptors (Luttrell et al. 1997; Igishi and Gutkind 1998; Singer et al. 2002). Moreover, physical association of Src family kinases with PI 3-kinase has been reported (Gutkind et al. 1990; Yamanashi et al. 1992; Pleiman et al. 1994). To assess a role of Src family kinases in GAR-3-mediated ERK1/2 activation, we treated GAR-3/CHO cells with PP1 analog, a Src family kinase-specific inhibitor. As shown in Figure 2B, PP1 analog treatment diminished carbachol-stimulated ERK1/2 activation, supporting the idea that Src family kinases participate in the activation of ERK1/2.

GAR-3-mediated ERK1/2 activation occurs via EGFR and Ras in HEK293 cells

Receptor tyrosine kinases (RTKs), such as EGFR, often serve as effector molecules in the ERK1/2 activation pathway initiated by GPCRs (Daub et al. 1996; Marinissen and Gutkind 2001; Rozengurt 2007). In fact, mammalian mAChRs were shown to activate EGFR in the process of ERK1/2 activation (Daub et al. 1997; Slack 2000). We therefore examined whether EGFR is involved in the pathway of GAR-3-mediated ERK1/2 activation. For this purpose, we used HEK293 cells instead of CHO cells because CHO cells do not express endogenous EGFR (Heimbrook et al. 1990; Tzahar et al. 1996). When HEK293 cells were transiently transfected with *gar-3* cDNA, carbachol evoked robust ERK1/2 activation (Figure 3A). Untransfected control HEK293 cells showed little, if any, ERK1/2 activation upon carbachol treatment (data not shown). We observed that PD168393, an EGFR inhibitor, substantially blocked carbachol-stimulated ERK1/2 activation. These results suggest that EGFR is transactivated in the ERK1/2 activation pathway. On the other hand, when GAR-3/CHO cells were treated with the

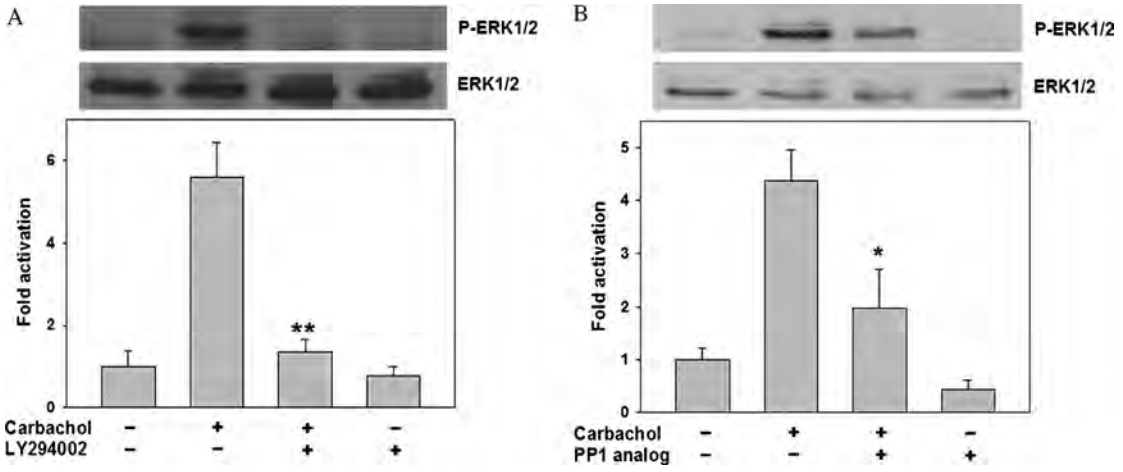

Figure 2. GAR-3-mediated ERK1/2 activation involves PI 3-kinase and Src family kinases. GAR-3/CHO cells were grown in a serum-free medium for 12 h, and treated with 100 μM LY294002 (inhibitor of PI 3-kinase) (A) or 10 μM PP1 analog (inhibitor of Src family kinases) (B) for 30 min before addition of 1 mM carbachol for 5 min. Samples were analyzed as described in Figure 1. $P < 0.01$ (**) and $P < 0.05$ (*) compared with carbachol-treated control using Student's *t*-test.

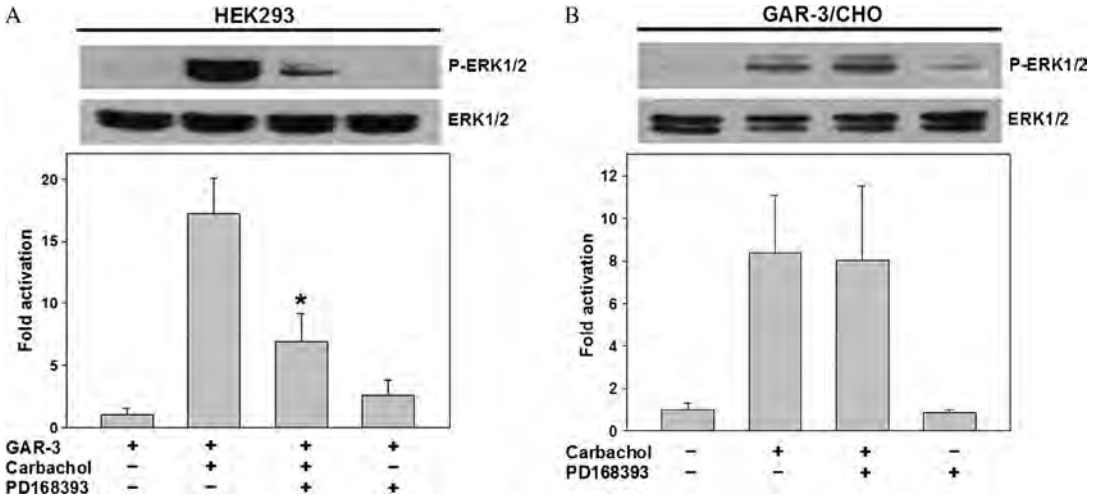

Figure 3. GAR-3-mediated ERK1/2 activation is EGFR-dependent in HEK293 cells. (A) HEK293 cells were transiently transfected with cDNA encoding GAR-3 and allowed to grow for 36 h. The cells were serum-starved for 12–18 h, pretreated with 1 μM PD168393 (EGFR inhibitor) for 30 min, and stimulated with 1 mM carbachol for 5 min. Samples were analyzed as described in Figure 1. $P < 0.05$ (*) compared with carbachol-treated control using Student's *t*-test. (B) GAR-3/CHO cells were grown in a serum-free medium for 12–18 h, pretreated with 1 μM PD168393 for 30 min, and stimulated with 1 mM carbachol for 5 min. Samples were analyzed as described in Figure 1.

same inhibitor, no significant effect on the ERK1/2 activation was observed (Figure 3B). This observation is consistent with the lack of EGFR in CHO cells.

Following GPCR-induced transactivation of RTK, ERK1/2 activation usually occurs via Ras. Ras is a GTP binding protein, and mutations of this protein have been implicated in diverse types of cancers (Bos 1989; Downward 2003). To ask whether Ras is involved in GAR-3-mediated ERK1/2 activation, we expressed a dominant negative mutant of Ras (RasS17N) in HEK293 cells. Since this mutant protein cannot bind GTP effectively, overexpression of this protein results in the inhibition of the endogenous wild-type Ras activity (Feig and Cooper 1988). As shown in Figure 4A, RasS17N significantly reduced carbachol-induced ERK1/2 activation in HEK293 cells. By contrast,

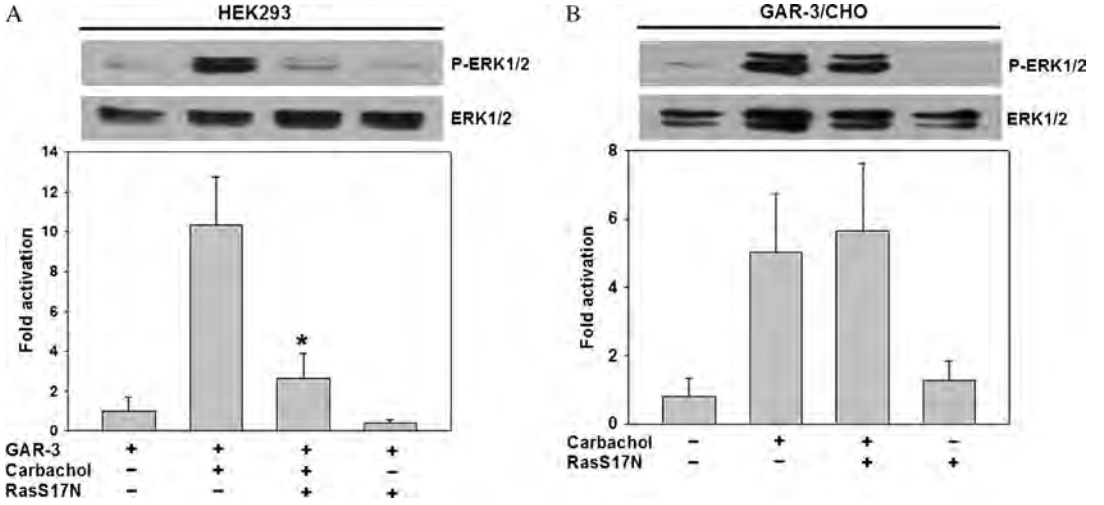

Figure 4. GAR-3-mediated ERK1/2 activation involves Ras in HEK293 cells. (A) HEK293 cells were transiently transfected with cDNA encoding GAR-3 and allowed to grow for 36 h (the two right samples were also transfected with cDNA encoding RasS17N while the two left samples were transfected with the empty vector pcDNA3.1). The cells were serum-starved for 12–18 h and stimulated with 1 mM carbachol for 5 min. Samples were analyzed as described in Figure 1. $P < 0.05$ (*) compared with carbachol-treated control using Student's *t*-test. (B) GAR-3/CHO cells were transiently transfected with cDNA encoding RasS17N (two right samples) or with the empty vector pcDNA3.1 (two left samples) and allowed to grow for 36 h. The cells were serum-starved for 12–18 h and stimulated with 1 mM carbachol for 5 min. Samples were analyzed as described in Figure 1.

RasS17N did not decrease the ERK1/2 activity in GAR-3/CHO cells (Figure 4B). Collectively, these results indicate that GAR-3 activates ERK1/2 through EGFR and Ras in HEK293 cells, but not in CHO cells.

This study, combined with our previous report (Kim et al. 2008), presents evidence that multiple signaling pathways exist linking GAR-3 stimulation to ERK1/2 activation in cultured mammalian cells. First, GAR-3 activates ERK1/2 via PLC and PKC, probably by coupling to PTX-insensitive Gq proteins. Second, GAR-3 interacts with PTX-sensitive G proteins (presumably Gi proteins) and activates ERK1/2 via PI 3-kinase and Src family kinases. Third, Ras-dependent EGFR transactivation is likely to be involved in the process of GAR-3-mediated ERK1/2 activation. Fourth, cAMP production elicited by GAR-3 leads to ERK1/2 inhibition via protein kinase A (PKA). This last pathway may serve as a feedback circuit to maintain proper levels of ERK1/2 activity. It will be interesting to see how these diverse signaling pathways contribute to the worm's behaviors controlled by GAR-3.

Acknowledgements

This work was supported by the Korea Research Foundation grant (KRF-2005-070-C00118) funded by the Korean Government and by the research grant of the Chungbuk National University in 2008.

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