Signalling pathway leading to an activation of mitogen-activated protein kinase by stimulating M_3 muscarinic receptor

Jee-Young KIM*, Myung-Soon YANG*, Chun-Do OH*, Kyong-Tai KIM†, Mahn Joon HA‡, Shin-Sung KANG* and Jang-Soo CHUN*[1]

*Department of Biology, Kyungpook National University, Taegu 702-701, Korea, †Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea, and ‡Laboratory of Medical Genetics, Institute for Medical Sciences, Ajou University, Suwon 441-749, Korea

The signalling pathway leading to an activation of mitogen-activated protein (MAP) kinase subtypes Erk-1 and -2 upon stimulation of muscarinic receptor with carbachol in human neuroblastoma SK-N-BE2(C) cells was investigated. Carbachol activated Erk-1/-2 by stimulating M_3 muscarinic receptor, as determined by specific antagonists for individual muscarinic receptors. The activation of Erk-1/-2 by carbachol was blocked by the inhibition or down-regulation of protein kinase C (PKC). Among the multiple PKC isoforms expressed in SK-N-BE2(C) cells, only PKCϵ was activated by the treatment of carbachol, and selective down-regulation of PKCϵ was sufficient to block Erk-1/-2 activation. Carbachol treatment induced activation of the serine/threonine protein kinase Raf, and an inhibition of Raf blocked Erk-1/-2 activation. Ectopic expression of inhibitory small GTPase Ras, RasN17, blocked the carbachol-induced Raf activation without affecting the activation of PKCϵ, while the inhibition of PKC blocked the Raf activation. Thus, these results suggest that carbachol-induced activation of PKCϵ mediates Erk-1/-2 activation by a sequential activation of Ras, Raf and MAP kinase kinase.

Key words: carbachol, mitogen-activated protein (MAP) kinase, protein kinase C, Raf, Ras.

INTRODUCTION

Muscarinic receptors, which are linked to a variety of intracellular second messenger systems through G-proteins, can be divided into five distinct subtypes (i.e., M_1, M_2, M_3, M_4, and M_5), depending on their binding affinities for specific agonists and antagonists [1,2]. Of the five muscarinic receptor subtypes, M_1, M_3 and M_5 are known to couple preferentially with phospholipase C (PLC), which induces phosphoinositide turnover [3–6]. Hydrolysis of PtdIns leads to the formation of diacylglycerol and InsP_3, which may result in the activation of protein kinase C (PKC) and mobilization of Ca^{2+} respectively [7]. These three receptors can also activate adenylate cyclase and ion channels [5,6,8]. On the other hand, activation of the other two subtypes, M_2 and M_4, inhibits adenylate cyclase via G_i- or G_o-proteins and does not stimulate PLC [5].

Stimulation of muscarinic receptors is also known to induce activation of mitogen-activated protein (MAP) kinase subtypes Erk-1 and -2 [9–11]. Activation of Erk by the stimulation of M_2 muscarinic receptor with carbachol is mediated by $G_{\beta\gamma}$-dependent activation of phosphoinositide 3-kinase (PI 3-kinase) [9]. Signalling from PI 3-kinase to Erk requires a tyrosine kinase, adapter proteins Shc and Grb2, guanine-nucleotide exchange factor Sos, small GTPase Ras and serine/threonine protein kinase Raf. Similarly to M_2 muscarinic receptor, a stimulation of M_3 muscarinic receptor with carbachol induces Erk activation [11,12]. However, apart from the fact that PKC is required for Erk activation [11,12], the signalling pathway from M_3 muscarinic receptor to Erk is not very well understood. The present study was undertaken to elucidate a signalling pathway leading to the activation of Erk-1 and -2 in human neuroblastoma SK-N-BE2(C) cells upon a stimulation of muscarinic receptor with carbachol. We report herein that PKCϵ mediates Ras, Raf, and MAP kinase kinase (MEK)-dependent activation of Erk-1 and -2 upon stimulation of M_3 muscarinic receptor through a signalling pathway independent of the activity of PI 3-kinase.

EXPERIMENTAL

Cell culture

Human neuroblastoma SK-N-BE2(C) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated bovine-calf serum, 50 μg/ml of streptomycin, and 50 units/ml of penicillin (Gibco–BRL, Gaithersburg, MD, U.S.A.) in a humidified atmosphere of air/CO_2 (19:1) at 37 °C as described previously [6].

Mobility-shift assay of Erk

Activation of Erk-1 and -2 upon stimulation of cells with carbachol was examined by mobility-shift assay as described previously [13]. Briefly, total cell lysates were prepared by extracting proteins with 50 mM Tris, pH 7.4, containing 150 mM NaCl, 1% Nonidet P40, 0.2% SDS, 0.1% deoxycholate, 10 μg/ml of leupeptin, 10 μg/ml of pepstatin A, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 5 mM NaF, 1 mM sodium orthovanadate and 1 mM 4-nitrophenyl phosphate. The cell lysates were separated by 0.1%-SDS/10%-PAGE. Erk-1 and -2 were detected with antibodies purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Alternatively, activation of Erk-1 and -2 was examined by determining their phosphorylation using antibody specific to phosphorylated Erk-1 and -2 (New England Biolabs. Inc., Beverly, MA, U.S.A.). The

Abbreviations used: MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; MEKK, MEK kinase; PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; ECL® enhanced chemiluminescence (Amersham).

[1] To whom correspondence should be addressed (e-mail jschun@kyungpook.ac.kr)

blots were incubated with a peroxidase-conjugated secondary antibody, and proteins were revealed by the enhanced chemiluminescence (ECL®; Amersham) system. Relative amounts of phosphorylated Erk-1 or -2 were quantified by densitometry using X-ray film.

Raf kinase assay

SK-N-BE2(C) cells, untreated or treated with carbachol, were extracted with a buffer [50 mM Tris (pH 7.5)/150 mM NaCl/1 % Triton X-100/2 mM EDTA/1 mM EGTA/10 μg/ml of leupeptin/10 μg/ml pepstatin A/10 μg/ml aprotinin]. A 500 μg portion of protein was incubated with 1 μg of anti-Raf polyclonal antibody (Santa Cruz Biotechnology) for 2 h at 4 °C. The immune complex was incubated with Protein A beads (Pierce, Rockford, IL, U.S.A.) for 1 h at 4 °C and washed five times with a washing buffer [20 mM Tris (pH 7.5)/0.5 mM EDTA/0.5 mM EGTA] and protease inhibitors. It was then incubated for 30 min at 30 °C with 0.5 μg of unphosphorylated MEK-1 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), and 1 μCi of [γ-^{32}P]ATP in a 30 μl of reaction buffer [25 mM Tris (pH 7.4)/10 mM $MgCl_2$/0.5 mM EGTA/10 μg/ml leupeptin/10 μg/ml pepstatin A/10 μg/ml aprotinin/5 mM NaF/1 mM sodium orthovanadate/1 mM 4-nitrophenyl phosphate. The reaction was stopped by adding SDS sample buffer, and proteins were separated by SDS/8 %-PAGE. Phosphorylation of MEK-1 with [γ-^{32}P]ATP was detected by autoradiography.

Cell fractionation

Separation of cytosolic and particulate membrane fractions was performed as described previously [13,14]. SK-N-BE2(C) cells were scraped into buffer A (20 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA and protease inhibitors as described above). The cells were sonicated twice for 5 s using microtips at 30 % of maximum power of the sonifier (Sonics and Materials Inc, Danbury, CT, U.S.A.) and centrifuged for 1 h at 100000 ***g***. The supernatant was designated 'cytosolic fraction'. The pellet was incubated with buffer B (20 mM Tris/HCl, pH 7.5, containing 1 % SDS, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA and protease inhibitors as described above). The incubation mixture was centrifuged for 15 min at 15000 ***g***, and the supernatant was saved as 'particulate membrane fraction'.

Western-blot analysis

Proteins (30 μg) separated by SDS/PAGE were transferred to nitrocellulose membrane, and the nitrocelluose sheet was incubated with 3 % (w/v) non-fat dry milk in Tris-buffered saline. PKC isoforms were detected with anti-PKC monoclonal antibodies specific for the β, δ, ϵ, θ, λ/ι, and μ isoforms (Transduction Laboratories, Lexington, KY, U.S.A.) or with polyclonal antibodies for α, γ, and ζ (Santa Cruz Biotechnology) and η (Biomol, Plymouth Meeting, PA, U.S.A.). panRas and c-Raf-1 were detected with antibodies purchased from Santa Cruz Biotechnology. The blots were incubated with a peroxidase-conjugated secondary antibody, and proteins were visualized by the ECL® system [13,15].

Expression of RasN17

The expression vectors containing cDNA for the dominant-negative mutant of Ras, RasN17, have been described previously [16]. The expression vector was introduced into SK-N-BE2(C) cells using LipofectaminePLUS (Gibco–BRL) by the procedure recommended by the manufacturer. The transfected cells were cultured in complete medium for 24 h and were used for the assay of Erk-1/-2, Raf and PKC.

RESULTS

Carbachol activates Erk-1/-2 by the stimulation of M_3 muscarinic receptor in SK-N-BE2(C) cells

Activation of Erk-1/-2 by stimulating SK-N-BE2(C) cells with carbachol was examined either by mobility-shift assay or by determining phosphorylation using antibody specific to phosphorylated Erk-1 and -2. Both assay methods suggested an activation of Erk-1 and -2, as shown in Figures 1(A) and 1(B). Therefore the mobility-shift assay was routinely employed in the following experiments. Carbachol-induced Erk activation was blocked when the cells were pretreated with non-specific antagonist of muscarinic receptors, such as 10 μM atropine (Figure 2A). Among the antagonists sensitive to individual muscarinic receptors [17–19], *p*-fluorohexahydrosiladifenidol, an inhibitor sensitive to M_3 muscarinic receptor, was sufficient to block the carbachol-induced activation of Erk-1 and -2 (Figure 2A), suggesting that M_3 muscarinic receptor mediated carbachol-induced Erk-1 and -2 activation in SK-N-BE2(C) cells. This was further confirmed by treating cells with various concentrations of antagonists, with carbachol and by investigating the order of potency of the inhibitory effects on carbachol-induced Erk-1/-2 activation. As shown in Figure 2(B), the inhibition of Erk-2 activation was concentration-dependent with each antagonist. The order of potency of the inhibitory effects was: atropine = *p*-fluorohexahydrosiladifenidol > pirenzepine = methoctramine. Similar effects of antagonists on the activation of Erk-1 were observed, and the antagonists themselves had no effect on Erk-1/-2 activation (results not shown). We therefore concluded that carbachol-induced Erk activation was primarily mediated by M_3 muscarinic receptor in SK-N-BE2(C) cells.

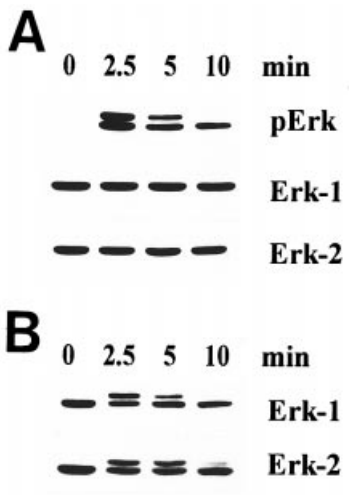


Figure 1 Carbachol induces activation of Erk-1 and -2 in SK-N-BE2(C) cells

(**A**) The cells were treated with 1 mM carbachol for the indicated time periods. Activation of Erk-1 and -2 was examined by determining its phosphorylation using antibody specific to phosphorylated Erk-1 and -2 (pErk). Upper and lower bands represent phosphorylated Erk-1 and -2 respectively. Protein levels of total Erk-1 and -2 were determined by Western blotting using the same lysate separated by 7.5 %-polyacrylamide gel. Note that both Erk-1 and -2 appear as a single band under these conditions. (**B**) Activation of Erk-1 and -2 was determined by mobility-shift assay in 10 %-polyacrylamide gel as described in the Experimental section.

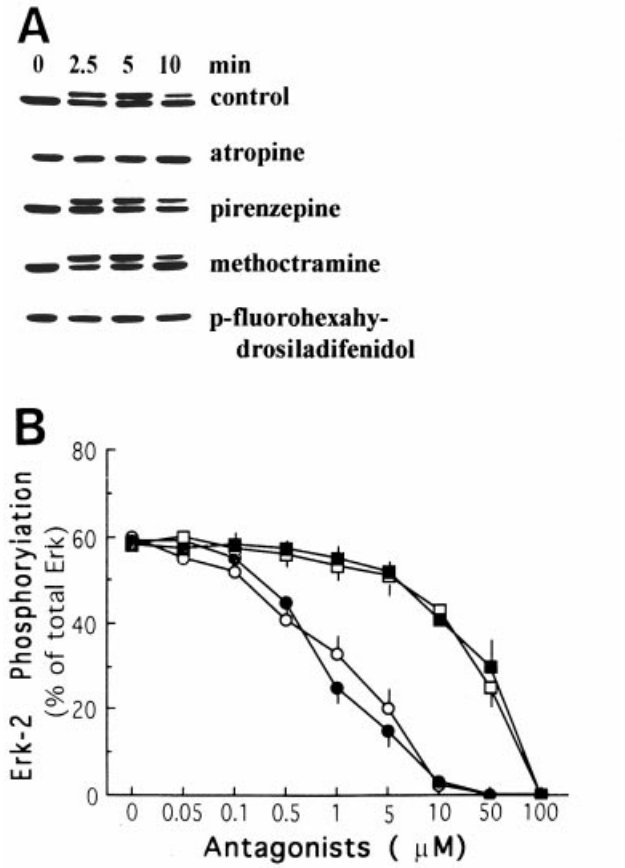


Figure 2 M_3 muscarinic receptor mediates carbachol-induced Erk-1/-2 activation

(**A**) SK-N-BE2(C) cells were treated with vehicle alone as a control, 10 μM atropine (non-specific muscarinic receptor antagonist), pirenzepine (M_1 antagonist), methoctramine (M_2 antagonist) or *p*-fluorohexahydrosiladifenidol (M_3 antagonist) for 2 min. The treated cells were stimulated with 1 mM carbachol for the indicated time periods. Activation of Erk-2 was determined by mobility-shift assay. The data represent a typical experiment conducted more than five times with comparable results. (**B**) The cells were treated with indicated concentration of antagonist for 2 min, followed by stimulation with 1 mM carbachol for 5 min. Relative amounts of phosphorylated Erk-2 were quantified by densitometry from three independent experiments. ○, Atropine; ●, *p*-fluorohexahydrosiladifenidol; □, pirenzepine; ■, methoctramine.

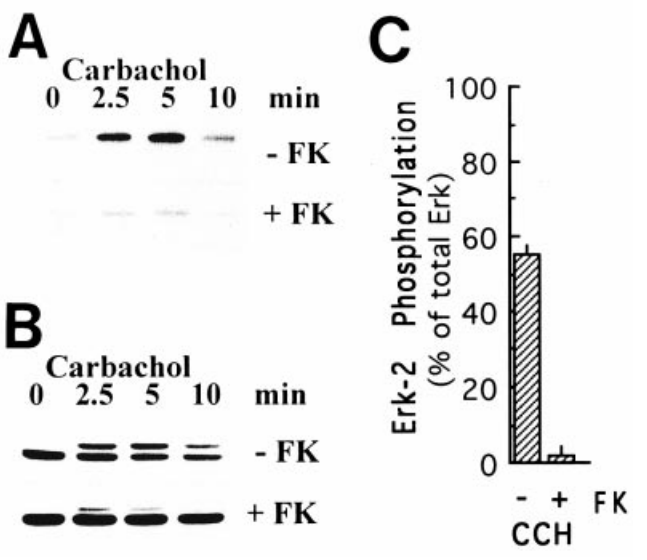


Figure 3 Raf is required for carbachol-induced Erk-2 activation

(**A**) SK-N-BE2(C) cells were treated with vehicle alone (−) or 50 μM forskolin (FK) (+) for 5 min prior to the stimulation of cells with 1 mM carbachol (CCH). Following incubation for the indicated periods, Raf kinase activity was determined by immune complex kinase assay using MEK-1 as a substrate as described in the Experimental section. (**B**) The same cell lysates (50 μg) described in (**A**) were used to determine Erk-2 activation by mobility-shift assay. The data in (**A**) and (**B**) are highly reproducible results of a typical experiment conducted four times. (**C**) The cells were treated with vehicle alone or forskolin (− and + FK) for 5 min and relative amounts of phosphorylated Erk-2 in the cells treated with carbachol for 5 min were quantified by densitometry ($n = 6$).

Carbachol-induced Erk-1/-2 activation depends on Ras, Raf and MEK

In an attempt to elucidate signalling pathways leading to the activation of Erk-1/-2, we first examined the role of MEK, which is known to directly activate Erk by dual phosphorylation on threonine and tyrosine residues [20]. Carbachol-induced activation of Erk-1/-2 was inhibited in a dose-dependent manner by pretreatment of the cells with a specific inhibitor of MEK, PD98059 ([21]; results not shown). Since MEK is known to be activated by phosphorylation via MEK kinase (MEKK) [20,22], we next examined the role of c-Raf-1, the best characterized MEKK [23]. Raf kinase activity in the cells treated with carbachol was directly measured using immunoprecipitated Raf and MEK-1 as a substrate for Raf. As shown in Figure 3(A), stimulation of the cells with carbachol induced MEK-1 phosphorylation, indicating that Raf was activated by carbachol. We further examined the role of Raf in MAP kinase signalling by treating the cells with forskolin prior to the stimulation of the cells with carbachol, since forskolin is known to induce inhibitory phosphorylation of Raf by activating protein kinase A [24,25]. Under the present conditions, carbachol-induced activation of Raf (Figure 3A) and Erk-2 (Figure 3B) was significantly inhibited. The amounts of immunoprecipitaed or expressed c-Raf-1 in the absence or presence of forskolin were practically the same (results not shown). Taken together, the results indicated that Raf activity was required for MEK-dependent Erk-2 activation.

It has been known for some time that stimulation of M_2 muscarinic receptor by carbachol leads to the activation of Erk via PI 3-kinase and Ras [9,10]. We therefore posed the question of whether M_3 muscarinic receptor used the same signalling molecules for the activation of Erk-1 and -2 by an ectopic expression of inhibitory Ras, RasN17, since expression of RasN17 has been demonstrated to block Ras-mediated growth-factor signalling [16]. As Figures 4(A) and 4(B) show, carbachol-induced phosphorylated Erk-2 in RasN17-expressing cells was approx. 30% of that in the control, suggesting that Ras was required for Erk activation. The partial inhibition of Erk-2 activation by RasN17 might be due to incomplete transfection of cDNA, although the expression of Ras increased more than 10-fold (Figure 4C). Ras activity also appeared to be required for the activation of Raf, because the expression of RasN17 blocked carbachol-induced Raf activity (Figure 4D). The expression of RasN17 did not affect the expression level of Erk-2 nor Raf (results not shown). In contrast with the role of Ras, carbachol-induced Erk signalling was not significantly affected by the treatment of the cells with specific inhibitors of PI 3-kinase {400 nM wortmannin [26] and 50 μM LY294002 ([27]; data not

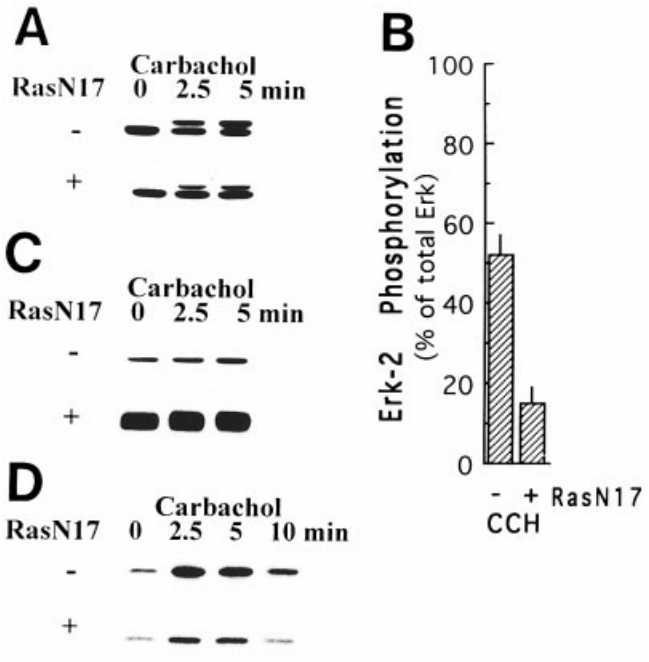


Figure 4 Ras is required for the Raf-dependent Erk activation

(**A**) Overexpression of RasN17 inhibits carbachol-induced Erk-2 activation. SK-N-BE2(C) cells were transiently transfected with empty vector (−) or vector containing cDNA for RasN17 (+). Following incubation for 24 h, the cells were stimulated with 1 mM carbachol (CCH) for the indicated time periods, and activation of Erk-2 was determined by mobility-shift assay. (**B**) Relative amounts of phosphorylated Erk-2 in cells treated with carbachol for 5 min was quantified from four independent experiments. (**C**) Overexpression of RasN17. Expression of RasN17 was determined by Western blotting from the same cell lysate described in (**A**). (**D**) Overexpression of RasN17 inhibits carbachol-induced Raf activation. The cells were transfected with control vector (−) or cDNA for RasN17 (+). Following incubation for 24 h the cells were stimulated with 1 mM carbachol, and Raf kinase activity was determined by immune complex kinase assay. The data in (**A**), (**C**) and (**D**) represent results of a typical experiment conducted at least four times.

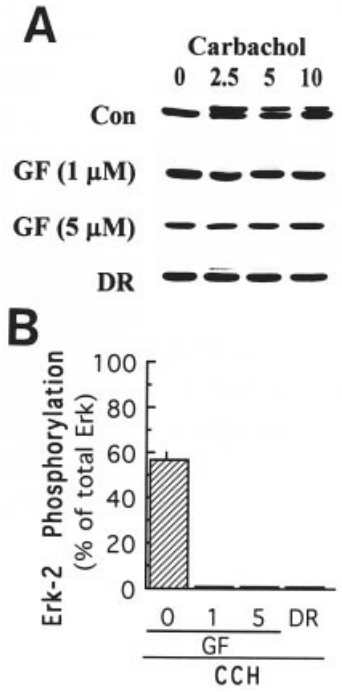


Figure 5 PKC is required for carbachol-induced Erk activation

(**A**) SK-N-BE2(C) cells were treated with vehicle alone (Con), indicated concentrations of GF109203X for 5 min to inhibit PKC (GF), or 1 μM of PMA for 16 h to down-regulate PKC (DR). Following treatment with carbachol (1 mM) for the indicated time periods, activation of Erk-2 was determined by mobility-shift assay. The data represent results of a typical experiment conducted four times with high reproducibility. (**B**) Relative amounts of phosphorylated Erk-2 in cells treated with carbachol (CCH) for 5 min was quantified by densitometry ($n = 4$).

shown)}, suggesting that M_3 muscarinic receptor activated Erk in a PI 3-kinase-independent pathway.

PKCε mediates Ras, Raf and MEK-dependent Erk activation

In human SH-SY5Y neuroblastoma cells [11] and oligodendrocytes [12] it has been suggested that PKC is required for Erk activation upon the stimulation of M_3 muscarinic receptor. We, therefore, examined whether carbachol activated Erk in SK-N-BE2(C) cells by modulating PKC activity. Carbachol-induced activation of Erk-2 was completely inhibited by preincubation of the cells with GF109203X, an inhibitor of PKC [28], or down-regulation of PKC with prolonged treatment of PMA (Figure 5).

PKC is a multigene protein kinase family composed of 11 known isoforms [7]. SK-N-BE2(C) cells expressed α, ϵ, ζ, λ/ι, and μ isoforms of PKC (Figure 6A), while other PKC isoforms (i.e., β, γ, δ, θ, and η) were hardly detectable by Western blotting (results not shown). We therefore examined which PKC isoforms were activated in Erk signalling in carbachol-treated cells. Activation of PKC was determined by examining translocation of cytosolic PKC to a particulate membrane fraction, because PKC activation involves a stable association of PKC with membranes [29,30]. As shown in Figure 6(A), PKCα and PKCζ were detected predominantly in the cytosolic fraction of the untreated cells, whereas the ϵ, λ/ι, and μ isoforms were mainly in the cytosolic fraction with a significant amount in the particulate membrane fraction. As expected, treatment of the cells with PMA induced translocation of cytosolic α and ϵ isoforms to the particulate membrane fraction with a concomitant decrease from the cytosolic fraction. The distributions of PKCζ, PKCλ/ι and PKCμ were not altered by PMA treatment. When the cells were treated with carbachol, relative distributions of PKCα, PKCζ, PKCλ/ι and PKCμ were not changed. However, the level of PKCϵ in the cytosolic fraction was significantly decreased, with a concomitant increase in the membrane fraction (Figure 6A). Redistribution of PKCϵ was detectable as early as 2.5 min after the carbachol treatment (Figure 6B), as determined by measuring time-dependent translocation of cytosolic PKC isoforms to the particulate membrane. Distribution of PKCα (Figure 6B) and other isoforms (results not shown) was not affected throughout the time periods examined. The results indicated that only PKCϵ, among the expressed multiple PKC isoforms, was specifically activated by carbachol treatment and might be involved in Erk-1/-2 activation.

The role of PKCϵ in carbachol-induced Erk activation was further examined by selective down-regulation of PKC isoforms. As shown in Figure 7(D), chronic exposure of SK-N-BE2(C) cells to PMA or thymeleatoxin induced down-regulation of PKCα and PKCϵ, and the activation of Erk was significantly blocked under the conditions used (Figure 7C). Prolonged treatment of the cells with ingenol 3,20-dibenzoate induced selective down-regulation of PKCϵ without affecting other isoforms (Figure 7D), and down-regulation of PKCϵ alone was sufficient to block Erk-2 activation (Figure 7C). In addition,

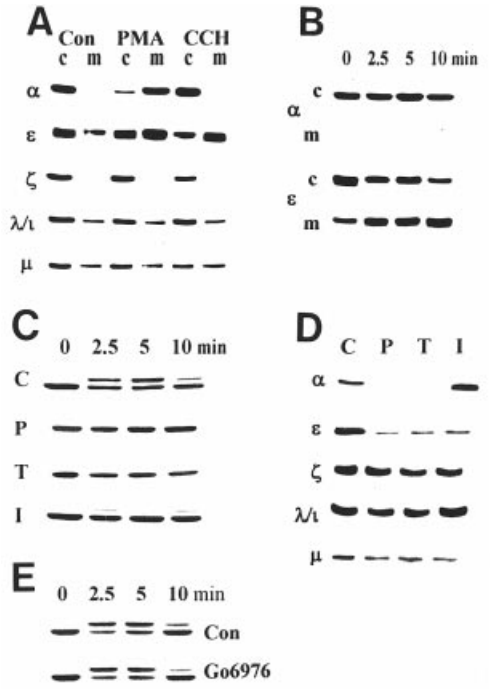


Figure 6 PKCε mediates carbachol-induced MAP kinase activation

(**A**) Activation of PKCε by carbachol or PMA. SK-N-BE2(C) cells treated with vehicle alone (Con), 200 nM PMA (PMA), or 1 mM carbachol (CCH) for 10 min were fractionated to cytosolic (c) and particulate membrane (m) fractions. Distribution of PKC isoforms was detected by Western blotting. (**B**) Time-dependent translocation of PKCε. The cells were treated with carbachol for the indicated time periods, and translocation of PKC isoforms were detected by Western blotting. (**C**) Down-regulation of PKCε was sufficient to block Erk-2 activation. SK-N-BE2(C) cells were treated for 16 h with vehicle alone (C), 1 μM of PMA (P), thymeleatoxin (T) or ingenol 3,20-dibenzoate (I) to down-regulate PKC, and the cells were then treated with 1 mM carbachol for the indicated periods. Activation of Erk-2 was determined by mobility-shift assay. (**D**) Down-regulation of PKC isoforms. The cells were treated for 16 h with vehicle alone (C), 1 μM of PMA (P), thymeleatoxin (T), or ingenol 3,20-dibenzoate (I). The levels of PKC isoforms were examined by Western blotting. (**E**) The cells were treated with 1 μM Go6976 for 5 min to inhibit conventional PKCα prior to the stimulation of the cells with carbachol (1 mM). Activation of Erk-2 was determined by mobility-shift assay. The data (**A**–**E**) represent a typical experiment performed at least four times.

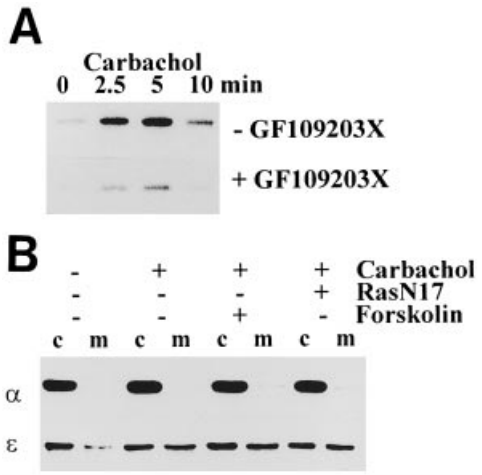


Figure 7 PKCε acts upstream of Ras, Raf and MEK

(**A**) SK-N-BE2(C) cells were treated with vehicle alone (−) or with 1 μM GF109203X (+) for 5 min, and then stimulated with 1 mM carbachol. Raf activity was determined as described in the Experimental section. (**B**) The cells were pretreated with vehicle alone (−) or 50 μM forskolin (+) for 5 min. Alternatively, the cells were transfected with vector containing cDNA for RasN17 and cultured for 24. The treated and transfected cells were stimulated with vehicle alone (−) or 1 mM carbachol (+), and were fractionated to cytosolic (c) and particulate membrane (m) fractions. Distribution of PKCα and ε isoforms was determined by Western blotting. The data represent a typical experiment conducted four times.

treatment of the cells with Go6976, which inhibits conventional PKC isoforms such as PKCα and PKCβ [31], did not affect Erk-2 activation (Figure 7E). Taken together, our results strongly suggest that PKCε is the responsible isoform for carbachol-induced Erk activation in SK-N-BE2(C) cells.

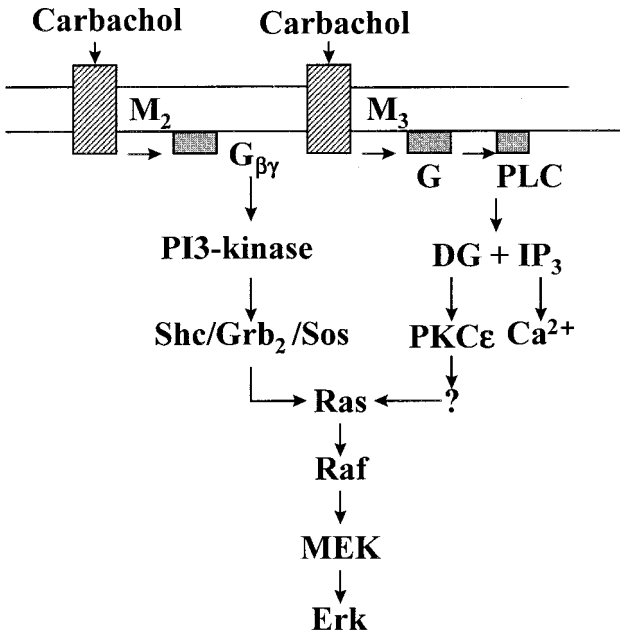
Finally, we examined at which step in the carbachol-treated cells PKCε activated Erk-1 and -2. As Figure 7(A) shows, carbachol-induced activation of Raf was blocked by the inhibition of PKC with GF109203X, but the inhibition of Raf with forskolin did not affect translocation of PKCε (Figure 7B), although Erk-2 activation was blocked (Figure 3). This indicated that PKCε acted upstream of Raf in the MAP kinase signalling cascade. In addition, carbachol-induced translocation of PKCε was not affected by the expression of RasN17 (Figure 7B), and Ras was required for the activation of Raf (Figure 4D). These results suggested that PKCε was activated before the sequential activation of Ras, Raf and MEK.

DISCUSSION

Erk-1 and -2, members of the MAP kinase family, are common intermediates in intracellular signalling cascades involved in diverse cellular functions [20,22]. MAP kinase is activated by phosphorylation on both threonine and tyrosine by MEK upon stimulation of various cell-surface receptors such as receptor tyrosine kinase, G-protein-linked receptors and cytokine receptors [20,22]. The receptor tyrosine kinase is relatively well characterized and requires sequential action of Grb2/Sos, Ras, Raf and MEK for the activation of MAP kinase [20,22]. Additionally, PKC appears to regulate MAP kinase signalling, because a direct stimulation of PKC with phorbol ester leads to the activation of MAP kinase [32,33].

In the present study we elucidated a signalling pathway leading to the activation of Erk-1 and -2 upon stimulation of G-protein-linked M_3 muscarinic receptor. As summarized in Scheme 1, our data indicate that PKCε mediates Ras-, Raf- and MEK-dependent activation of MAP kinase. The signalling pathway from the activation of PKCε to Ras activation has not yet been clarified. However, PI 3-kinase does not seem to be involved in MAP kinase signalling in SK-N-BE2(C) cells, because inhibition of PI 3-kinase with wortmannin and LY294002 does not affect carbachol-induced Erk-2 activation (results not shown). Furthermore, we cannot detect immunochemically any association between Shc, Grb2, and Sos during carbachol stimulation of SK-N-BE2(C) cells (results not shown): These signalling molecules (i.e., PI 3-kinase, Shc, Grb2 and Sos) are required for the $G_{\beta\gamma}$-dependent activation of MAP kinase by the stimulation of M_2 muscarinic receptor ([9,10]; Scheme 1).

Our results, summarized in Scheme 1, indicate that PKCε was activated before the activation of Ras. The evidence included the observations that: (1) carbachol-induced translocation of PKCε was not affected by the expression of RasN17 (Figure 7B); (2) overexpression of RasN17 significantly decreased carbachol-induced Raf activation (Figure 4D); and (3) carbachol-induced activation of Raf was blocked by the inhibition of PKC with GF109203X, but the inhibition of Raf with forskolin did not affect translocation of PKCε (Figure 7B). However, activation of



Scheme 1 Comparison of signalling pathway leading to Erk activation initiated by M_2 and M_3 muscarinic receptors

Erk activation by a stimulation of M_2 muscarinic receptor with carbachol is mediated by $G_{\beta\gamma}$-dependent activation of PI 3-kinase that sequentially activates Shc, Grb2, Sos, Ras, Raf, and MEK [9]. In contrast, stimulation of M_3 muscarinic receptor with carbachol induces activation of G-protein-linked PLC that produces diacylglycerol and $Ins P_3$ [6]. Diacylglycerol in turn selectively activates PKCϵ among the expressed PKC isoforms. The activated PKCϵ stimulates Ras via an as-yet-unknown signalling pathway to sequentially activate Raf, MEK and Erk-1/2.

Raf and Erk-2 was not completely inhibited by the overexpression of RasN17. Although this raises the possibility that PKCϵ may directly activate Raf, we are inclined to believe that this is due to incomplete transfection of cDNA. Indeed, transient transfection resulted in an overexpression of RasN17 in about 70% of cells as determined by immunofluorescence microscopy (results not shown), although the total level of Ras increased more than 10-fold as determined by Western blotting (Figure 4C).

PKC is a large family composed of 11 known isoforms, each of which exhibits unique characteristics [7,29,30]. Individual PKC isoforms are activated in different ways, which variably depend on Ca^{2+} and various membrane lipid mediators, and they play distinct roles in the control of physiological functions. The multiplicity of PKC isoforms suggests that they are responsible for a wide variety of specialized physiological processes; however, direct evidence assigning any specific function to any specific isoform is scarce. The difficulty lies in the fact that most cells express multiple isoforms which may be regulated individually and which may interact or 'cross-talk'. SK-N-BE2(C) cells expressed multiple PKC isoforms such as α, ϵ, ζ, λ/ι, and μ. Among the expressed isoforms, only PKCϵ was found to be activated by the treatment of carbachol (Figure 6). Activation of PKC in carbachol-treated SK-N-BE2(C) cells was expected, because carbachol stimulation induced activation of PLC to generate diacylglycerol and to mobilize Ca^{2+} [6,7], and only Ca^{2+}-independent PKCϵ was activated by carbachol treatment (Figure 6). Regulation of PKC activation is a complex process that requires generation of second messengers, including diacylglycerol and Ca^{2+}, phosphorylation of PKC by a putative kinase, and targeting proteins [7,29,30]. This might explain why Ca^{2+}-dependent PKCα was not activated in carbachol-treated cells, although carbachol increased the cytosolic free Ca^{2+} level [6]. Alternatively, it is possible that carbachol affected the activity of other PKC isoforms; however, this is not detected by the classical translocation assay. The observation that selective down regulation of PKCϵ is sufficient to block carbachol-induced MAP kinase activation (Figure 6) strongly indicates that PKCϵ is the only responsible isoform in carbachol-induced MAP kinase activation in SK-N-BE2(C) cells.

We are grateful to Dr. Woon Ki Paik of one of our institutions (Ajou University) for a critical reading of the manuscript before its submission. This work was supported by grants from Korea Ministry of Education (BSRI-97-4402 and '97 Genetic Engineering Foundation).

REFERENCES

1 Bonner, T. I. (1989) Trends Neurosci. **12**, 148–151
2 Wess, J. (1993) Trends Pharmacol. Sci. **14**, 783–789
3 Liao, C. F., Schilling, W. P., Birnbaumer, M. and Birnbaumer, L. (1990) J. Biol. Chem. **265**, 11273–11284
4 Matthews, G. (1991) Trends Pharmacol. Sci. **121**, 245–247
5 Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandra, J. and Capon, D. J. (1988) Nature (London) **334**, 434–437
6 Suh, B.-C. and Kim, K.-T. (1995) J. Neurochem. **64**, 2500–2508
7 Nishizuka, Y. (1995) FASEB J. **9**, 484–496
8 Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N. and Jan, L. Y. (1993) Nature (London) **364**, 802–806
9 Lopez-Ilasaca, M., Crepso, P., Pellici, P. G., Gutkind, J. S. and Wetzker, R. (1997) Science **275**, 394–397
10 Gutkind, J. S., Crepso, P., Xu, N., Teramoto, H. and Cosco, O. A. (1997) Life Sci. **60**, 999–1006
11 Offermanns, S., Bombien, E. and Schultz, G. (1993) Biochem. J. **294**, 545–550
12 Larocca, J. N. and Almazan, G. (1997) J. Neurosci. Res. **50**, 743–754
13 Kim, S.-J., Chang, Y.-Y., Kang, S.-S. and Chun, J.-S. (1997) Biochem. Biophys. Res. Commun. **237**, 336–339
14 Chun, J.-S., Ha, M.-J. and Jacobson, B. S. (1996) J. Biol. Chem. **271**, 13008–13012
15 Chang, S.-H., Oh, C.-D., Yang, M.-S., Kang, S.-S., Lee, Y.-S., Sonn, J.-K. and Chun, J.-S. (1998) J. Biol. Chem. **273**, 19213–19219
16 deVries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J. and Bos, J. L. (1992) Nature (London) **357**, 602–604
17 Akiba, I., Kubo, T., Maeda, A., Bujo, H., Nakai, J., Mishina, M. and Numa, S. (1988) FEBS Lett. **235**, 257–261
18 Buckley, N. J., Bonner, T. I. and Brann, M. R. (1988) J. Neurosci. **8**, 4646–4652
19 Doods, H. N., Mathy, M. J., Davidesko, D., van Charlorp, K. J., de Jonge, A. and van Zwieth, P. A. (1987) J. Pharmacol. Exp. Ther. **242**, 257–262
20 Cohen, P. (1997) Trends Cell Biol. **7**, 353–361
21 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) J. Biol. Chem. **270**, 27489–27494
22 Robinson, M. J. and Cobb, M. H. (1997) Curr. Opin. Cell Biol. **9**, 180–186
23 Morrison, D. K. and Culter, R. E. J. (1997) Curr. Opin. Cell Biol. **7**, 174–179
24 Cook, S. J. and McCormick, F. (1993) Science **262**, 1069–1072
25 Wu, J., Dent, P., Jelineck, T., Wofman, A., Weber, M. J. and Sturgill, T. W. (1993) Science **262**, 1065–1069
26 Arcaro, A. and Wymann, M. P. (1993) Biochem. J. **296**, 297–301
27 Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) J. Biol. Chem. **269**, 5241–5248
28 Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F. et al. (1991) J. Biol. Chem. **266**, 15771–15871
29 Newton, A. C. (1997) Curr. Opin. Cell Biol. **9**, 161–167
30 Goodnight, J., Mischak, H. and Mushinski, J. F. (1994) Adv. Cancer Res. **64**, 159–209
31 Martiny-Baron, G., Kazanietz, M. G., Mischack, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D. and Schachtele, C. (1993) J. Biol. Chem. **268**, 9194–9197
32 Chao, T.-S. O., Foster, D. A., Rapp, U. R. and Rosner, M. R. (1994) J. Biol. Chem. **269**, 7337–7341
33 Ueda, Y., Hirai, S.-I., Osda, S.-I., Suzuki, A., Mizuno, K. and Ohno, S. (1996) J. Biol. Chem. **271**, 23512–23519

Received 22 June 1998/6 October 1998; accepted 30 October 1998