Stimulation of tyrosine phosphorylation and mitogen-activated-protein (MAP) kinase activity in human SH-SY5Y neuroblastoma cells by carbachol

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Activation of the G-protein-coupled muscarinic (M_3) receptor in human neuroblastoma SH-SY5Y cells is known to lead to phosphoinositol hydrolysis and noradrenaline release. In this study, the effect of carbachol on tyrosine phosphorylation and mitogen-activated protein (MAP) kinase activity in SH-SY5Y cells was examined. Carbachol concentration-dependently induced tyrosine phosphorylation of several proteins, including one of 42 kDa. This tyrosine-phosphorylated 42 kDa protein co-eluted from a Mono Q anion-exchange column with MAP kinase activity and with immunologically detected MAP kinase. Stimulation of tyrosine phosphorylation and activation of MAP kinase were also observed after incubation of cells with phorbol 12-myristate 13-acetate (PMA) and epidermal growth factor (EGF). Down-regulation or inhibition of protein kinase C (PKC) abolished the stimulatory effects of both carbachol and PMA on MAP kinase activity, whereas EGF-stimulated MAP kinase activity remained unaffected. Thus carbachol acting through the muscarinic (M_3) receptor PKC-dependently induced tyrosine phosphorylation and activation of a 42 kDa MAP kinase in SH-SY5Y cells, whereas EGF-induced MAP kinase activation occurred independently of PKC.

INTRODUCTION

Tyrosine phosphorylation is regarded to be a regulatory mechanism associated with cell growth and mitosis. Receptors for many polypeptide growth factors [e.g. epidermal growth factor (EGF), platelet-derived growth factor and insulin-like growth factor I] possess protein tyrosine kinase activity which is indispensable for their growth-promoting activity (Ullrich and Schlessinger, 1990), and several peptide growth factors which act through G-protein-coupled receptors have recently been shown to increase the activity of non-receptor tyrosine kinases (Zachary et al., 1991; Huckle et al., 1992). However, G-protein-coupled receptors which do not mediate mitogenic effects can also induce activation of tyrosine kinases (Taniguchi et al., 1993), and tyrosine kinase activity can be found in non-proliferating cells such as platelets (Golden et al., 1986) and neurons (Sorge et al., 1984), indicating that the role of tyrosine phosphorylation may functionally not be restricted to growth-related processes.

Mitogen-activated protein (MAP) kinases, also described as extracellular signal-regulated kinases (ERK), are serine/threonine kinases activated by diverse stimuli, including growth factors acting through receptor tyrosine kinases and several agonists which bind to G-protein-coupled receptors (Cobb et al., 1991; Sturgill and Wu, 1991). MAP kinases require both threonine and tyrosine phosphorylation for full activation (Anderson et al., 1990; Boulton et al., 1991), and are particularly regarded to be involved in cell cycle regulation (Pelech and Sanghera, 1992; Thomas, 1992). Recently a MAP kinase kinase, which functions as an upstream regulator of MAP kinase leading to threonine/tyrosine phosphorylation and activation of MAP kinase, has been cloned (Crews et al., 1992; Wu et al., 1993).

The human neuroblastoma cell line SH-SY5Y is a well-established system in which to study the molecular events following activation of the muscarinic M_3 receptor, which mediates carbachol-induced phosphatidylinositol hydrolysis and noradrenaline secretion in this cell line (Lambert et al., 1991; Murphy et al., 1991). Since muscarinic M_3 receptors have the potency to lead to cell growth and transformation (Gutkind et al., 1991), we examined the effect of carbachol on tyrosine phosphorylation and MAP kinase activity in SH-SY5Y cells. We show here that in SH-SY5Y cells MAP kinase is activated via the heptahelical G-protein-coupled muscarinic receptor, as well as by EGF acting through its receptor tyrosine kinase. In addition, we provide evidence that protein kinase C (PKC) is involved in the carbachol-induced MAP kinase activation, but not in the EGF-dependent activation of MAP kinase.

EXPERIMENTAL

Materials

Carbachol, phorbol 12-myristate 13-acetate (PMA), EGF, pirenzepine and myelin basic protein (MBP) were from Sigma (Deisenhofen, Germany). Polyclonal anti-phosphotyrosine antisera were generated and affinity-purified as described by Kamps and Sefton (1988). Mouse anti-(MAP kinase) antibodies reacting with $p42^{mapk}$ and $p44^{mapk}$ were from Zymed Laboratories (San Francisco, CA, U.S.A.). SH-SY5Y cells originating from Dr. June L. Biedler (Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.) were given by Dr. F.-J. Klinz (Institut für Molekulare Neurochemie, Ruhr-Universität Bochum, Germany). 4-Diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and 2,2′,2″-[benzene-1,2,3-triyltris(oxy)]tris(*NNN*-triethylethanaminium) tri-iodide (gallamine) were from Research Biochemicals Inc. (Natick, MA, U.S.A.).

Cell culture

SH-SY5Y cells were grown as described by Laugwitz et al. (1993). For tyrosine-phosphorylation experiments, cells were seeded in 22 mm wells on Costar 12-well plates, and were used

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; MBP, myelin basic protein; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; MAP, mitogen-activated protein; PKC, protein kinase C.

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for experiments after reaching confluency. Before addition of agents, cells were washed once with a buffer containing 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose and 20 mM Hepes/NaOH (pH 7.4) and were allowed to equilibrate in this medium for about 30 min at 37 °C.

Immunoblotting of tyrosine-phosphorylated proteins

After exposure of cells to ligands at the concentrations and for the durations noted in the Figure legends, cells were lysed by addition of 100 μl of modified ice-cold RIPA buffer and prepared for SDS/PAGE as described by Offermanns et al. (1993). SDS/PAGE was performed on gels containing 9% (w/v) acrylamide. Transfer of proteins on nitrocellulose filters and detection of phosphotyrosine-containing proteins by the use of anti-phosphotyrosine antibodies and the chemiluminescence (ECL) Western-blotting detection system (Amersham, Braunschweig, Germany) have been described (Offermanns et al., 1992).

Partial purification and assay of MAP kinase

Cells were seeded in 60 mm dishes. After exposure of cells to ligands as described in the Figure legends, cells were scraped into 1 ml of homogenization buffer (25 mM NaCl, 0.1 mM Na_3VO_4, 2 mM EGTA, 1 mM dithiothreitol, 1 mM *p*-nitrophenyl phosphate, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 25 mM Tris/HCl, pH 7.5). Thereafter, cells were frozen and thawed three times in liquid nitrogen in order to disrupt the cells, and MAP kinase was partially purified as described by Anderson et al. (1991). Briefly, homogenates were centrifuged at 12000 *g* for 10 min, 900 μl of the supernatant was mixed with 100 μl of ethylene glycol to give a final concentration of 10% (v/v), and 75 μl of washed phenyl-Sepharose was added. After 5 min at 4 °C, phenyl-Sepharose was pelleted and subsequently washed with 1 ml of homogenization buffer containing 10% (v/v) ethylene glycol and 1 ml of homogenization buffer containing 30% ethylene glycol. To elute MAP kinase, 75 μl of homogenization buffer containing 60% ethylene glycol was added, and samples were kept for 5 min at 4 °C. After centrifugation, 50 μl of the supernatant was assayed for MAP kinase activity in a buffer containing 10 mM $MgCl_2$, 40 μM [γ-^{32}P]ATP (75 kBq/tube), 0.5 mg/ml MBP and 50 mM Tris/HCl (pH 7.5) for 15 min at 30 °C (100 μl final volume).

Column chromatography

Chromatography was performed as described by Gupta et al. (1992). Cells grown in 150 cm^2 culture flasks were incubated with different substances as described in the Figure legends and lysed in 1 ml of 0.5% (v/v) Triton X-100, containing 0.1 mM Na_3VO_4, 1 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 50 mM β-glycerophosphate (pH 7.5). Lysates were kept on ice for 5 min and centrifuged, and supernatants were normalized for protein and loaded on to a Mono Q HR 5/5 f.p.l.c. column equilibrated in 50 mM β-glycerophosphate (pH 7.5)/0.1 mM Na_3VO_4/1 mM EGTA/1 mM dithiothreitol. The column was developed with a 30 ml linear gradient of 0–0.5 M NaCl in the buffer described above at a flow rate of 1 ml/min; 1 ml fractions were collected. Samples (50 μl) of the fractions were assayed for MAP kinase activity as described above. The rest of the fractions was precipitated with 7.2% (v/v) trichloroacetic acid and 0.15% (w/v) sodium deoxycholate, washed twice with acetone, resuspended in electrophoresis sample buffer and subjected to SDS/PAGE and subsequent immunoblotting with anti-phosphotyrosine and anti-(MAP kinase) antibodies as described above.

Miscellaneous

[γ-^{32}P]ATP was synthesized as described by Johnson and Walseth (1979). Protein was determined using the BCA Protein Assay System (Pierce, Rockford, IL, U.S.A.).

RESULTS

Treatment of human neuroblastoma SH-SY5Y cells with carbachol resulted in an increased tyrosine phosphorylation of several proteins with relative molecular masses between 165 and 68 kDa as well as of a 42 kDa protein (Figure 1). Stimulation of tyrosine phosphorylation of all proteins was transient, reaching a maximum at about 2 min, except for the 42 kDa protein, which showed maximal stimulation of tyrosine phosphorylation after about 10 min (see Figure 1a). The effect of carbachol was concentration-dependent, being half-maximal at about 0.3 μM and maximal at 30 μM (see Figure 1b).

SH-SY5Y cells predominantly express the muscarinic M_3 receptor (Lambert et al., 1989), which has been shown to mediate carbachol-induced phosphoinositol hydrolysis in these cells (Lambert et al., 1991). Since phosphoinositol hydrolysis results in the release of inositol 1,4,5-trisphosphate, with consecutive increase in cytosolic Ca^{2+} and activation of PKC (Meldrum et al., 1991), we tested whether elevation of the intracellular Ca^{2+} concentration or activation of PKC led to tyrosine phosphorylation. Whereas ionophore-induced elevation of the cytosolic Ca^{2+} concentration had no influence on the level of tyrosine phosphorylation (result not shown), incubation of cells with the PKC activator PMA induced tyrosine phosphorylation of several proteins with identical molecular masses, as seen in response to carbachol (Figure 2). In addition, the polypeptide growth factor EGF effectively induced tyrosine phosphorylation of different proteins, including the 42 kDa protein which was also phosphorylated in response to carbachol and PMA (see Figure 2). Down-regulation of PKC by treatment of cells for 18 h with 1.5 μM PMA (Parrow et al., 1992) abolished the stimulatory effect on tyrosine phosphorylation of both PMA and carbachol, whereas tyrosine phosphorylation induced by EGF was not influenced by PMA treatment of cells.

Since various agents are known to stimulate tyrosine phosphorylation of a 42 kDa protein shown to be a MAP kinase, we measured the effect of carbachol, PMA and EGF on MAP kinase activity in SH-SY5Y cells. Extract supernatants from cells treated with carbachol, PMA or EGF were subjected to Mono Q ion-exchange chromatography. The results of assays of column fractions for MAP kinase activity, which was measured as MBP kinase activity (Erickson et al., 1990), are shown in Figure 3. All three agonists induced a several-fold increase in MAP kinase activity, which was almost undetectable in unstimulated cells. Samples of the fractions were subjected to immunoblotting with anti-(MAP kinase) and anti-phosphotyrosine antibodies. Immunologically detected MAP kinase of 42 kDa co-eluted with the peak of MBP kinase activity and with a 42 kDa protein reactive with the phosphotyrosine-specific antibody. A considerable level of phosphotyrosine in the 42 kDa protein was found only in fractions which exhibited an increased MBP kinase activity in response to the agonists (see Figure 3). The intensity of the 42 kDa band recognized by the anti-phosphotyrosine antibodies in fractions from activated cells did not exactly match

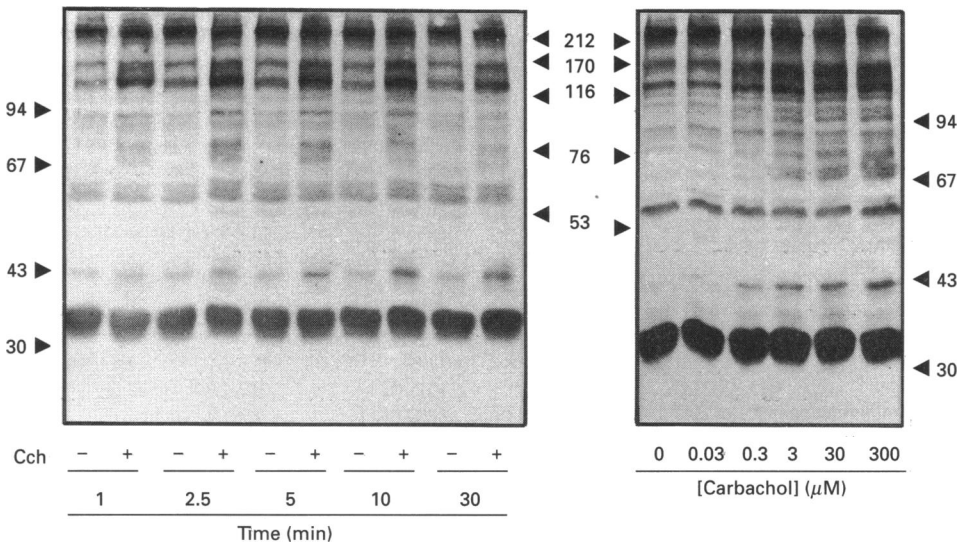

Figure 1 Time course and concentration-dependence of carbachol-induced tyrosine phosphorylation in SH-SY5Y cells

SH-SY5Y cells were incubated in the absence or presence of 100 μM carbachol (Cch) for the indicated time periods (**a**) or were incubated with carbachol at the indicated concentrations for 10 min (**b**). For detection of tyrosine-phosphorylated proteins, samples were subjected to immunoblotting with anti-phosphotyrosine antibodies as described in the Experimental section. Shown are autoluminograms of blots with the molecular masses (kDa) of marker proteins on the left and right.

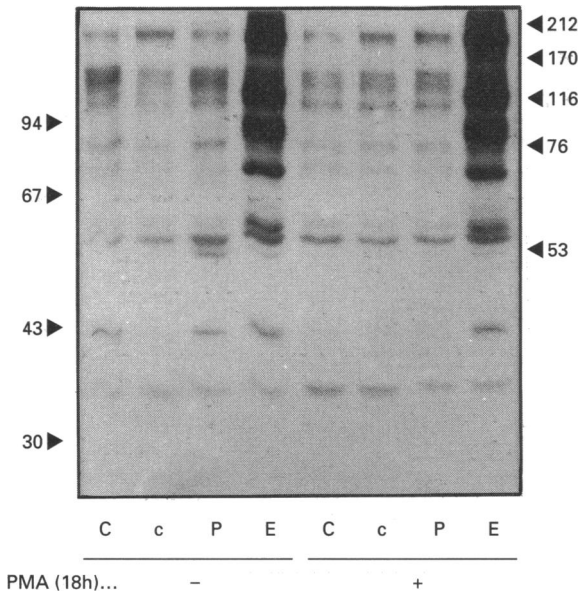

Figure 2 Influence of PKC depletion on stimulation of tyrosine phosphorylation by carbachol, PMA and EGF in SH-SY5Y cells

SH-SY5Y cells were pretreated for 18 h with 1.5 μM PMA (+) or vehicle (−) as indicated. Pretreated cells were incubated with 100 μM carbachol (C), 100 nM PMA (P) or 20 nM EGF (E) for 10 min, and tyrosine phosphorylation was monitored as described. Shown is an autoluminogram of a blot with the molecular masses (kDa) of marker proteins on the left and right; c, control (without agonist).

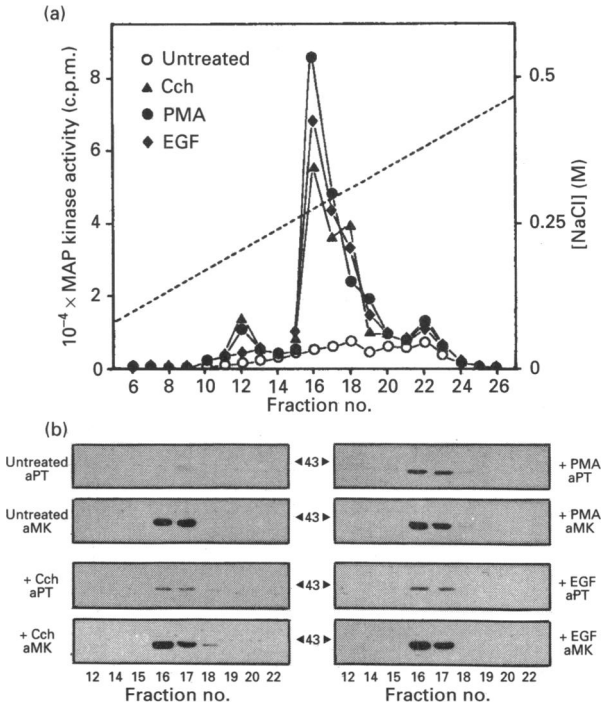

Figure 3 MAP kinase activity in cell lysates of stimulated SH-SY5Y cells

Lysates from untreated cells and from cells exposed for 10 min to 100 μM carbachol (Cch), 100 nM PMA or 20 nM EGF were separated by Mono Q chromatography as described in the Experimental section. (**a**) A portion of each fraction was assayed for MAP kinase activity with MBP as substrate. (**b**) Samples of fractions were analysed for phosphotyrosine and MAP kinase content by using anti-phosphotyrosine (aPT) and anti-(MAP kinase) (aMK) antibodies as described in the text. Shown are the 39–48 kDa regions of autoluminograms. Arrowheads point to the position of the 43 kDa marker protein. The broken line in (**a**) shows the predicted NaCl gradient (right ordinate). The experiment shown is representative of three independently performed experiments.

the level of MAP kinase activity measured in the same fractions. This is likely to be due to the chemiluminescence procedure used to detect bound antibodies, which does not allow absolute quantification as does a protein kinase assay based on counting

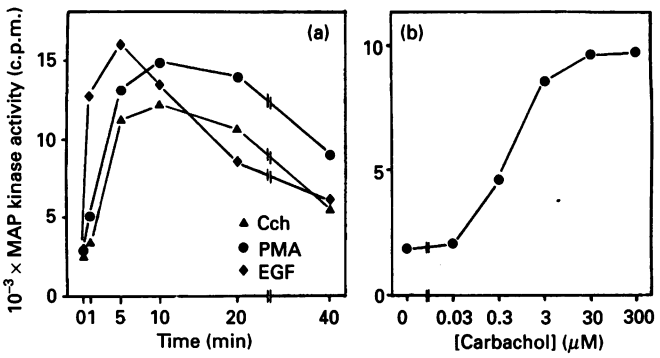

Figure 4 Time- and concentration-dependence of MAP kinase activation in SH-SY5Y cells

(**a**) 60 mm plates of cells were treated with 100 μM carbachol (Cch), 100 nM PMA or 20 nM EGF for the indicated time periods. (**b**) Plates were incubated for 10 min with the indicated concentrations of carbachol. After disruption of cells, MAP kinase was partially purified, and MAP kinase activity was determined as described in the Experimental section. Results are shown of single experiments which were repeated three times with similar results.

Table 1 Influence of PKC depletion and PKC inhibition on stimulation of MAP kinase activity by carbachol, PMA and EGF in SH-SY5Y cells

SH-SY5Y cells were pretreated 18 h with 1.5 μM PMA or for 30 min with 3 μM calphostin C. Pretreated cells were incubated with 100 μM carbachol, 100 nM PMA or 20 nM EGF for 10 min. Thereafter, cells were disrupted and MAP kinase activity was determined as described in the text. Results shown are the averages (±S.E.M.) of triplicate assays of MAP kinase activity. Results were repeated in three separate experiments.

	10^{-3} × MAP kinase activity (c.p.m.) from cells treated with:			
Preincubation	Carbachol (100 μM)	Untreated	PMA (100 nM)	EGF (20 nM)
None	13.6±1.1	2.8±0.6	15.2±1.3	14.8±1.7
PMA (18 h)	3.2±0.3	2.6±0.4	2.4±0.1	16.2±1.4
Calphostin C (30 min)	2.9±0.4	3.1±0.2	3.4±0.5	14.4±1.2

of radioactivity of labelled phosphate groups transferred to defined substrate molecules.

In further experiments, MAP kinase activity was measured after a mini purification procedure described by Anderson et al. (1991) (see the Experimental section). Stimulation of MAP kinase in response to carbachol and PMA occurred with similar time courses (Figure 4). Stimulation was clearly detectable after 5 min, reached a maximum after about 10 min and declined thereafter. Stimulation of MAP kinase activity by EGF was also transient, but occurred more rapidly after addition of the stimulus, reaching a maximum at about 5 min. Stimulation of MAP kinase activity by carbachol exhibited the same concentration-dependency as carbachol-stimulated tyrosine phosphorylation (see Figure 4) and was mediated by the muscarinic M_3 receptor, as shown by the effects of selective muscarinic-receptor antagonists (Figure 5). Whereas preincubation of cells with 4-DAMP (M_3-receptor antagonist) abolished carbachol-induced stimulation of MAP kinase as well as tyrosine phosphorylation of the co-eluting 42 kDa protein, preincubation with pirenzepine (M_1-receptor antagonist) or gallamine (M_2-receptor antagonist) had little effect.

Since PMA and carbachol appeared to have the same effects on MAP kinase activity, we tested whether the carbachol-induced stimulation of MAP kinase activity was solely due to activation of PKC. This was suggested by the finding that carbachol-induced tyrosine phosphorylation of p42 was mediated by PKC (see Figure 2). To test this, PKC was either down-regulated by 18 h treatment of cells with PMA or inhibited by preincubation

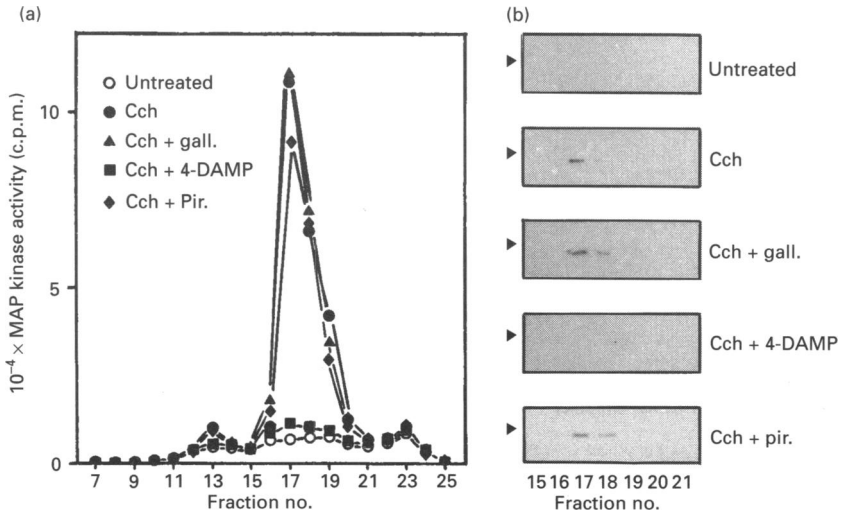

Figure 5 Effect of muscarinic-receptor antagonists on carbachol-induced MAP kinase activation in SH-SY5Y cells

SH-SY5Y cells were preincubated for 1 min without or with 100 μM of the muscarinic-receptor antagonists gallamine (gall.), 4-DAMP or pirenzepine (pir.) as indicated. Thereafter, 300 μM carbachol (Cch) was added and the reaction proceeded for 10 min. After exposure, cell lysates were prepared and subjected to Mono Q chromatography as described in the Experimental section. (**a**) A portion of each fraction was assayed for MAP kinase activity with MBP as substrate. The elution conditions were the same as in Figure 3(**a**). (**b**) Samples of fractions were analysed for phosphotyrosine content by using anti-phosphotyrosine antibodies as described in the text. Shown is the 37–48 kDa region of autoluminograms. Arrowheads point to the position of the 43 kDa marker protein. Similar results were obtained in two independent experiments.

with the specific PKC inhibitor calphostin C (Tamaoki, 1991). In both cases, stimulation of MAP kinase activity by carbachol or PMA was abolished, whereas activation of MAP kinase by EGF appeared to be unchanged (Table 1).

These data suggest that in SH-SY5Y cells carbachol activates MAP kinase in a PKC-dependent way, whereas activation of MAP kinase by EGF occurs independently of PKC.

DISCUSSION

In this study, we demonstrate that carbachol induces tyrosine phosphorylation of several proteins, including a 42 kDa species in SH-SY5Y cells (see Figures 1 and 2). A 42 kDa protein was also tyrosine-phosphorylated in response to the PKC activator PMA and EGF, which both have been shown to induce tyrosine phosphorylation and activation of 42/44 kDa MAP kinases in different cells (Rossomando et al., 1989; Gotoh et al., 1990; Anderson et al., 1991). Therefore we tested whether the 42 kDa protein tyrosine-phosphorylated in response to carbachol in SH-SY5Y cells is a MAP kinase (see Figure 3). Co-elution of increased MAP kinase activity, of enhanced tyrosine-phosphorylated 42 kDa protein and of immunologically detected 42 kDa MAP kinase strongly suggests that the 42 kDa protein tyrosine-phosphorylated in response to carbachol is a 42 kDa MAP kinase, likely to be $p42^{mapk}$. This is further supported by the finding that carbachol-induced tyrosine phosphorylation and MAP kinase activation occurred with very similar time- and concentration-dependencies (see Figures 1 and 4). This 42 kDa MAP kinase was the only protein recognized by the anti-(MAP kinase) antibody in human SH-SY5Y cells. The same antibody also recognized a single 42 kDa protein in human HL-60 cells (Ohta et al., 1992). In different rodent cell lines, the MAP kinase antibody recognized an additional 44 kDa protein likely to be $p44^{mapk}$ (results not shown). Whether the 42 kDa MAP kinase is the only MAP kinase activated in response to carbachol in SH-SY5Y cells is at present not known.

The role that MAP kinase may play in the physiological response to carbachol in SH-SY5Y cells is not clear. M_1 and M_3 muscarinic-receptor subtypes which stimulate phosphoinositol hydrolysis have the potency to mediate agonist-dependent transformation of cells when transfected into fibroblasts (Gutkind et al., 1991). However, muscarinic receptors expressed in neuronal cells are regarded to be involved in short-term neurotransmitter effects (Nathanson, 1987). Thus it remains to be elucidated whether activation of MAP kinase by carbachol mediates growth-promoting effects or whether it is involved in known cellular effects induced by carbachol in SH-SY5Y cells, e.g. carbachol-induced noradrenaline release (Murphy et al., 1991).

Activation of PKC in SH-SY5Y cells also led to tyrosine phosphorylation and activation of the 42 kDa MAP kinase, suggesting that PKC mediates the activation of MAP kinase in response to carbachol. In cells which were depleted of PKC or in which PKC was inhibited by calphostin C, carbachol as well as PMA had no influence on the phosphorylation and activation of MAP kinase (see Figure 2 and Table 1). Unspecific effects of down-regulation or inhibition of PKC leading to decreased responsiveness of cells towards carbachol or to a generally impaired activation of MAP kinase are unlikely, since carbachol was still able to mobilize Ca^{2+} (result not shown) and MAP kinase activation by EGF remained unchanged (see Table 1).

The different susceptibility of carbachol- and EGF-induced MAP kinase activation towards PKC depletion or inhibition points to different activation mechanisms. Whereas MAP kinase activation via the G-protein-coupled muscarinic receptor involved the stimulation of PKC, EGF working through a receptor tyrosine kinase apparently activated MAP kinase independently of PKC. The occurrence of PKC-dependent and -independent pathways leading to MAP kinase activation has been described in several systems (Gotoh et al., 1990; Anderson et al., 1991; Gallego et al., 1992). The entire activation mechanism of MAP kinases is at present not clear. It has been suggested that MAP kinase kinase, which can be activated by phorbol esters and different growth factors (Ahn et al., 1991; Rossomando et al., 1992), functions as an integration point for different pathways that lead to MAP kinase activation. Since MAP kinase kinase does not appear to be directly activated by PKC (Adams and Parker, 1992), carbachol- and EGF-induced MAP kinase activation pathways may converge upstream of MAP kinase kinase. One possible converging point of the two pathways could be the serine/threonine kinase $p74^{raf\text{-}1}$, which has been shown to be placed upstream of MAP kinase kinase in pathways leading to MAP kinase activation (Kyriakis et al., 1992; Dent et al., 1992; Howe et al., 1992) and which is activated by EGF and related growth factors in a predominantly PKC-independent way (Rapp, 1991) as well as directly by PKC (Sozeri et al., 1992). Involvement of $p74^{raf\text{-}1}$ in PKC- and EGF-induced MAP kinase activation is suggested by the recent finding that MAP kinase stimulation by PMA and EGF in COS-1 cells is potentiated by over-expression of $p74^{raf\text{-}1}$ (Howe et al., 1992).

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