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Activation of Transforming G Protein-Coupled Receptors Induces Rapid Tyrosine Phosphorylation of Cellular Proteins, Including p125^{FAK} and the p130 v-*src* Substrate

J. Silvio Gutkind* and Keith C. Robbins

Laboratory of Cellular Development and Oncology, National Institute of Dental Research, NIH, Bethesda, Maryland 20892

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We have used the family of human muscarinic receptors (mAChRs) as a model for receptors coupled to G proteins and have shown that genes for certain mAChR subtypes can behave as potent agonist-dependent oncogenes. Futhermore, transforming mAChRs can transduce mitogenic signals in transfected NIH 3T3 cells. In this study, we show that in cells expressing m1 mAChRs, the cholinergic agonist carbachol, induces a rapid and dose-dependent increase in tyrosine phosphorylation of cellular proteins which are different from those induced by PDGF. Interestingly, carbachol, but not PDGF, induces an increase in tyrosine phosphorylation of the p125^{FAK} and p130 v-*src* substrates. Thus, growth promoting pathways activated by receptors coupled to G proteins might involve tyrosine phosphorylation of a small set of cellular proteins previously identified as substrates for oncogene-encoded tyrosine kinases.

A number of cell surface receptors transduce signals through the activation of heterotrimeric GTP binding proteins (G proteins). The increasing list of such receptors includes those that are activated by polypeptides or neurotransmitters such as thrombin, bombesin, vasopresin, endothelin, angiotensin, bradykinin, serotonin, adrenaline or acetylcholine (see 1 for review). Although G protein-coupled receptors are generally associated with differentiated function, they have also been implicated in embryogenesis, tissue regeneration, growth stimulation, and neoplasia (1). Receptors of this class lack an intrinsic protein tyrosine kinase activity which is commonly found in most receptors for polypeptide growth factors such as platelet-derived growth factor (PDGF) (2), and in several oncogenes (3-5). Thus, tyrosine

*To whom correspondence should be addressed.

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phosphorylation is commonly associated with growth stimulation and cellular transformation (2-5).

We have used the family of human muscarinic receptors (mAChRs) for the neurotransmitter acetylcholine as a model to study receptors coupled to G proteins (6), and we have recently shown that genes for certain mAChR subtypes can induce agonist-dependent neoplastic transformation when transfected into mouse fibroblasts. Those mAChRs stimulating PI hydrolysis (m1, m3, m5) are agonist-dependent transforming proteins, whereas those that inhibit adenylyl cyclase (m2, m4) are not (6). In addition, transforming mAChRs can also transduce potent mitogenic signals (7). In the present study, we investigated whether growth stimulation by the mAChR class of G protein-coupled receptors might involve tyrosine phosphorylation of cellular substrates.

MATERIALS AND METHODS

Cells: NIH 3T3 cells as well as NIH 3T3 cells transfected with an expression plasmid carrying human mACHRs genes (6) or the v-*src* oncogene and a dominant selectable marker, *neo*, (8) have been previously described. Cultures were maintained in Dulbecco's modified Eagle medium (DMEM)(GIBCO), supplemented with 10% calf serum (Advanced Biotechnologies Inc.). Platelet derived growth factor (PDGF) was from Upstate Biotechnology. All other chemicals were purchased from Sigma.

Immunoblotting: Confluent cells, cultured for 24 hs in DMEM without serum were lysed at 4° as described. Protein extracts were fractionated either directly or after immunoprecipitation with the aid of Rabbit anti-mouse (Cappel) coated protein A-sepharose (Pharmacia), by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting (9). Antiphosphotyrosine monoclonal antibody PY-20 was from ICN, monoclonal antibodies against phospholipase C-γ were a generous gift from Dr. S.G. Rhee (NIH, Bethesda), and monoclonal antibodies specific for v-*src* substrates p85 (4F11), p120 (2B12), p125 (2A7) and p130 (AF4) were generously provided by T. Parsons (University of Virginia) (10). Rabbit anti-mouse IgG was utilized to enhance binding of ^{125}I-protein A (Amersham).

Phosphatidylinositol kinase assays: Phosphatidylinositol (PI) kinase activity was assayed on anti-PY immunoprecipitates essentially as described (8), using PI (Sigma) as a substrate at a final concentration of 0.2 mg/ml, and [γ-^{32}P]ATP and $MgCl_2$ to final concentrations of 1 μM and 5 mM, respectively.

RESULTS AND DISCUSSION

We have used clones of NIH 3T3 cells transfected with an m1 human mAChR expression plasmid. These clones express more then 600 fmols of [^{3}H]N-methylscopolamine binding sites/mg protein (around 20,000 receptors per cell), and in these cells carbachol potently induces reinitiation of DNA synthesis (7). Upon prolonged exposure,

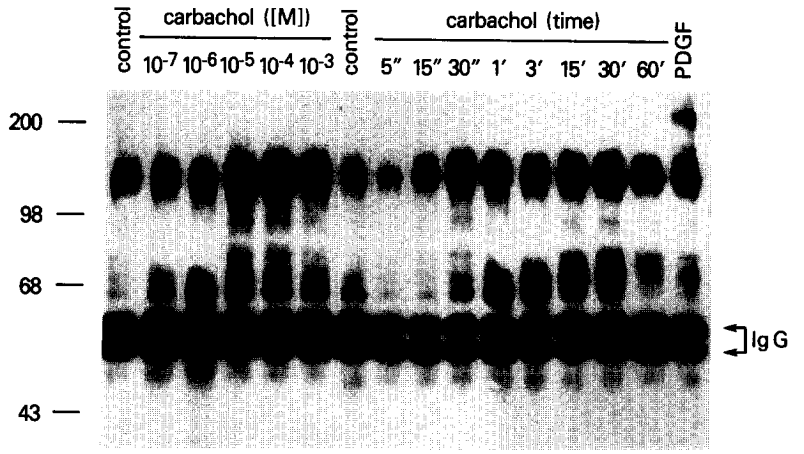

Fig. 1. **Protein-tyrosine phosphorylation in response to carbachol treatment of cells expressing m1 mAChRs.** Quiescent clonal NIH 3T3 cells expressing approximately 20,000 m1 mAChR per cell were treated with the indicated concentrations of carbachol for 30 sec, or for the indicated time with 10 μM carbachol. Cell extracts were prepared, and aliquots containing 500 μg of total protein were immunoprecipitated with 1 μg of anti-PY antibody (PY20) as described under "Materials and Methods". Immunocomplexes were recovered with the aid of protein A-sepharose coated with rabbit-antimouse IgG antiserum, fractionated by SDS-PAGE and analyzed by immunoblotting using anti-PY as a probe. Cells treated with PDGF (10 ng/ml) served as controls. Autoradiograph represents three independent experiments using one clonal NIH 3T3 transfectant. Similar results were obtained when using each of 5 different clonal NIH 3T3 cells expressing 20,000 to 200,000 m1 mAChRs per cell (data not shown). Arrows indicate bands corresponding to the immunoglobulin used for immunoprecipitation.

the agonist efficiently induces focus-formation (6). As shown in Fig. 1, carbachol induced rapid tyrosine phosphorylation of cellular proteins as detected by immunoblotting with anti-PY antibodies. Tyrosine phosphorylation was observed as soon as 15 seconds after agonist addition and increased with time, reaching a maximum between 1-30 min. The signal decreased in intensity by 60 minutes after stimulation. The tyrosine phosphorylation response to carbachol was dose dependent (Fig. 1), and concentrations effective in inducing tyrosine phosphorylation were similar to those required to induce DNA synthesis or cellular transformation (6,7). No tyrosine phosphorylation was observed in cells expressing mAChRs linked to inhibition of adenylyl cyclase (data not shown), a fact consistent with their inability to induce DNA synthesis or cellular transformation.

We observed that tyrosine phosphorylated species appearing as a result of mAChR activation were different from those induced by PDGF, a natural growth factor for these cells (Fig. 1, see below).

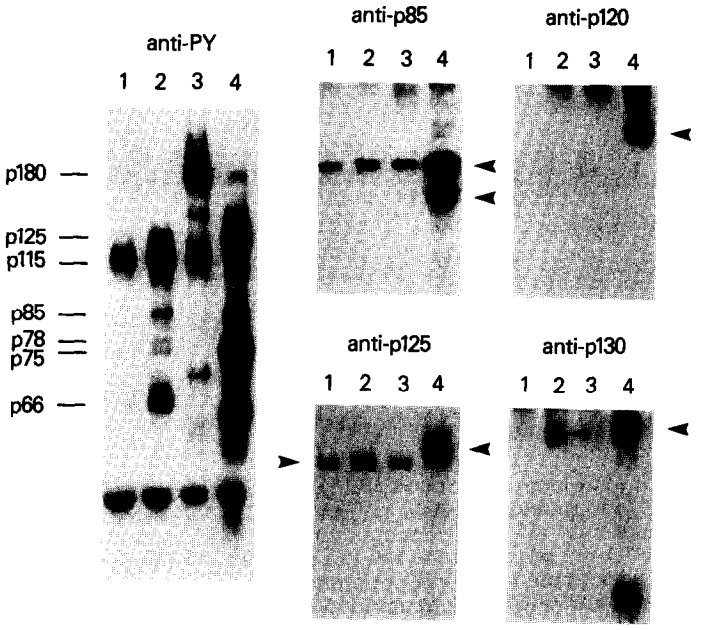

Fig.2 **Effect of carbachol on tyrosine phosphorylation of known substrates for v-*src* or PDGF tyrosine kinases.** Lysates containing 2 mg of protein extracted from quiescent NIH 3T3 expressing m1 mAChRs (lane 1), that were treated with carbachol (10 μM for 30 sec) (lane 2), PDGF (10 ng/ml for 5 min) (lane 3), or from v-*src*-transformed NIH 3T3 cells (lane 4) were immunoprecipitated with the indicated antibodies, fractionated by SDS-PAGE and analyzed by immunoblotting using the anti-PY monoclonal PY 20. In this particular set of experiments, 20 μl of agarose-conjugated PY-20 beads (ICN) was used for each antiphosphotyrosine immunoprecipitation. Prominent tyrosine phosphorylated species are indicated. The position of the corresponding v-*src* substrates are marked with arrowheads.

Such differences were more prominent when larger amounts of cellular lysate were precipitated using agarose conjugated anti-PY (Fig. 2). Using these conditions, bands corresponding to proteins of 66 kDa or a 115-125 kDa, as well as less prominent species of 75, 78, and 85 kDA were immunodetected from lysates of carbachol treated cells. In contrast, tyrosine phosphorylation of the PDGF receptor itself (180 kDa), as well as proteins of 70, 110 and 145 kDa were observed in response to PDGF. Lysates of NIH 3T3 cells transformed by v-*src* exhibited a high level of phosphotyrosine-containing cellular proteins some of which were similar in size to those observed in carbachol treated cells (Fig. 2).

To determine whether proteins tyrosine phosphorylated in response to carbachol might also be phosphorylated by the PDGF-receptor or v-*src*, we examined the effect of carbachol on the state of phosphorylation of known v-*src* or PDGF-receptor substrates.

Mouse monoclonal antibodies that recognize proteins tyrosine phosphorylated in v-*src* transformed chicken fibroblasts have been described (10). As expected, such antibodies against p85 or p120 immunoprecipitated heavily tyrosine phosphorylated proteins from lysates of v-*src* transfected cells (Fig. 2). No tyrosine phosphorylated p120 was detected in m1 transfected cells treated with PDGF or carbachol (Fig. 2). A tyrosine phosphorylated band of 85 kDa was detected in anti-p85 immunoprecipitates from control as well treated cells, although, its state of phosphorylation was not affected by the agonists. In contrast, anti-p125 and anti-p130 antibodies immunoprecipitated corresponding tyrosine phosphorylated proteins from v-*src* transformed cells, and their level of tyrosine phosphorylation increased in response to the cholinergic agonist but not to PDGF (Fig. 2). Known PDGF substrates were not tyrosine phosphorylated in response to carbachol. As an example, anti-phospholipase C-γ antibodies recognized an 145 kDa protein in lysates from NIH 3T3 cells, and in anti-PY immunoprecipitates from cells treated with PDGF but not with carbachol (Fig. 3). Furthermore, high anti-PY recovable PI3K activity was immunoprecipitated from PDGF treated or v-*src* transformed NIH 3T3 cells, but was nearly undetectable in untreated cells. Carbachol addition slightly increased PI3K activity, an effect that was prevented by pretreating cells with atropine.

Carbachol is a potent mitogen for NIH 3T3 cells transfected with transforming mAChR subtypes (7), and we view this system as a useful model for the elucidation of signal transduction pathways leading to normal cell growth and oncogenic transformation. The induction of tyrosine phosphorylation by activation of G protein coupled receptors has been previously documented (11-13). However, this study represents the first demonstration that fully transforming receptors of this class can mediate tyrosine phosphorylation of a set of cellular proteins different from those phosphorylated by growth factor tyrosine kinase receptors. Furthermore, this study has identified two proteins, p125 and p130, whose tyrosine phosphorylated state increases in response to activation of transforming G protein-coupled receptors. Since both proteins were initially identified by virtue of their tyrosine phosphorylation in v-*src* transformed cells (10), this observation links G protein coupled receptors with oncogene-encoded tyrosine kinases. Interestingly, the p125 v-*src* substrate has been recently cloned and found to be itself a tyrosine-kinase, $p125^{FAK}$ (14). We

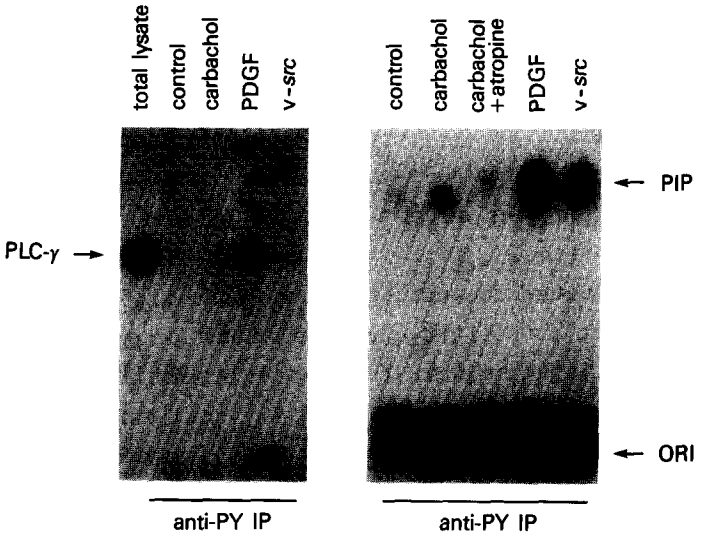

Fig.3 **Effect of carbachol on tyrosine phosphorylation of PLC-γ and antiphosphotyrosine recoverable PI3K activity.** Protein extracts were prepared from untreated, carbachol (10 μM for 30 sec) or PDGF (10 ng/ml for 5 min) treated quiescent m1 transfectants, or from v-*src* transformed NIH 3T3 cells. Aliquots containing 1 mg of protein were immunoprecipitated with PY-20 (anti-PY IP), fractionated by SDS-PAGE and immunoblotted using anti-PLC-γ antibodies (left panel), or assayed for PIK activity (right panel) as described in the text (right panel). A sample containing 40 μg of total cellular protein (total lysate) was used as control for anti-PLC-γ immunodetection. In one case (carbachol + atropine) the effect of carbachol was prevented by pretreating cells with the muscarinic antagonist atropine (10 μM, 10 min before carbachol addition). ORI, chromatographic starting point.

are currently investigating the molecular basis for $p125^{FAK}$ phosphorylation, as well as whether its kinase activity is affected by carbachol treatment.

Although the role of m1-mediated tyrosine phosphorylation is not known, it correlates well with agonist-induced DNA synthesis and transformation (6,7) and is likely to be an integral part of mitogenic signalling through G protein-coupled receptors. Complete elucidation of the role of agonist-induced tyrosine phosphorylation warrants further investigation.

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