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The Neuropeptide Y/Peptide YY Y1 Receptor Is Coupled to MAP Kinase via PKC and Ras in CHO Cells

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The neuropeptide Y/peptide YY (PYY) Y1 receptor subtype mediates proliferative responses. This report identifies effector molecules which mediate mitogen-activated protein kinase (MAPK) phosphorylation by Y1 receptor activation in transfected CHO cells. Pertussis toxin pretreatment abolishes this effect, indicating involvement of G_i or G_o proteins. Inhibition of protein kinase C (PKC) also blocks PYY-induced MAPK phosphorylation. Additionally in this cell model PYY causes an increase in GTP binding to Ras protein, and cotransfection of dominant negative constructs for Ras and Raf blocks PYY effects on MAPK. These data suggest a novel mechanism for Y1 receptor coupling to MAPK, which is at once pertussis toxin-sensitive as well as PKC- and Ras-dependent.

The abundant brain peptide neuropeptide Y (NPY) and the closely related gut hormone peptide YY (PYY) have been shown to exert proliferative effects in vivo and in vitro (1 - 4). This mitogenic effect is seen in cells expressing the Y1 receptor subtype either constitutively or after transfection. The Y1 receptor is a member of the G protein-coupled superfamily and has been characterized by pertussis toxin-sensitive responses, supporting the assumption the Y1 subtype activates second messenger pathways via G_i and G_o proteins (5, 6). Growth-inducing ligands acting through G protein-coupled receptors (GPCRs) and receptor tyrosine kinases stimulate signalling cascades which converge on mitogen-activated protein kinase. Separate exclusive pathways involving either activation of Ras or activation of PKC and Raf have been identified as coupling GPCRs to MAPK. It is uncertain however how the Y1 receptor couples to MAPK and whether pertussis toxin-sensitive G proteins are involved. This report demonstrates that Y1 receptor activation rapidly stimulates MAPK phosphorylation in Y1 receptor-transfected CHO cells via a mechanism that is pertussis toxin-sensitive and which also uniquely depends on both protein kinase C and Ras.

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Abbreviations used: PYY, peptide YY; NPY, neuropeptide Y; MAPK, mitogen-activated protein kinase; Y1R, Y1 receptor; PKC, protein kinase C; GPCR, G protein-coupled receptor; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; PI3, phosphatidylinositol 3; MEK, MAPK kinase.

MATERIALS AND METHODS

Materials. Geneticin, D-*erythro*-sphingosine, tyrphostins, Ro-31-8220, pertussis toxin and wortmannin were from Calbiochem (San Diego, CA). Leupeptin, phenylmethylsulfonyl fluoride (PMSF), phorbol 12-myristate 13-acetate, and protein G-agarose were from Sigma (St. Louis, MO). PD98059 was from New England Biolabs (Beverly, MA). $^{32}P_i$ was from Dupont/NEN (Boston, MA). All other chemicals were of reagent grade.

Cell culture. CHO-K1 cells were transfected (LipofectAMINE, Gibco/BRL, Rockville) with cDNA encoding the human Y1 receptor (Y1R) (gift of Herbert Herzog, Garvan Medical Research Institute, Sydney) cloned into the pBK-CMV vector (Stratagene, LaJolla, CA). Individual clones from cells surviving 500 μg/ml geneticin were isolated by limited dilution. The Y1R-CHO-A1 line used in these studies was grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% streptomycin and penicillin G, and 500 μg/ml geneticin.

Cell lysis. Y1R-CHO-A1 cells are seeded at 2×10^5 cells/well in 6 well plates, grown for two days, and medium exchanged for serum-free medium 24 hours prior to use. After treatment the plates are placed on ice, washed twice with 2 ml ice-cold PBS, and harvested with 60 μl ice-cold lysis buffer (0.3 M NaCl, 25 mM Hepes, pH 7.7, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/ml leupeptin, and 100 μg/ml PMSF). The cells are scraped into chilled 1.5 ml tubes, incubated on a rotary shaker for 30 min at 4°C, and the supernatants recovered after centrifuging at 14,000 × g for 10 min at 4°C. Protein concentration of the lysates are determined by the method of Bradford (7), and the lysates are stored at −80°C.

Western blot analysis for total and phosphorylated MAPK. Samples (10-20 μg/lane) are separated by SDS-PAGE using 4-20% continuous gradient gels, transferred to PVDF paper, washed with 1X PBS for 5 min and then for 1 hr with blocking buffer (1× PBS with 0.1% Tween-

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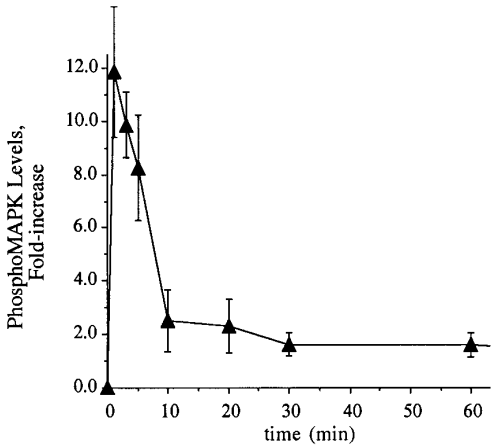

FIG. 1. PYY effects on MAPK phosphorylation in Y1R-CHO-A1 cells. Cells were incubated with 0.1 μM PYY for various times prior to measuring phosphoMAPK levels by Western blot. (mean ± SD, n = 2 - 10).

20 and 5% nonfat dry milk). Primary antibody (rabbit p44/p42 MAPK and phospho-specific p44/p42 MAPK antibodies, New England Biolabs, Beverly, MA) is diluted 1:1000 in 1× PBS with 0.05% Tween-20 and 5% bovine serum albumin and incubated with the blot for 18 hr at 4°C. The blot is washed three times for 5 min each with blocking buffer before incubation with secondary antibody (anti-rabbit IgG, alkaline phosphatase-linked, 1:1000 in blocking buffer) for 1 hr at 25°C. The blot is washed three times for 5 min with blocking buffer and twice with wash buffer (10 mM Tris HCl, pH 9.5, 10 mM NaCl, and 1 mM $MgCl_2$) before developing with a chemiluminescent detection method (Phototope-Star, New England Biolabs) and exposure to x-ray film. Films are quantitated using laser densitometry. Blots are reprobed after stripping (30 min at 70°C in 100 mM β-mercaptoethanol, 2% SDS, 67.5 mM Tris HCl, pH 6.7) so that signals obtained with the phospho-specific p44/p42 MAPK antibody could be normalized to the total p44/p42 MAPK signal per lane.

Determination of GTP binding to Ras protein. Cells made quiescent by incubation in serum-free medium (24 hr) are incubated for 2 hr with 100 μCi $^{32}P_i$/well in phosphate-free medium, 1 ml/well in 6-well plate. Cells are exposed to PYY or medium (control) for 3 min then placed on ice, washed twice with 2 ml ice-cold PBS and scraped into 100 μl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 1% NP-40, 10 mM NaF, 1 mM dithiothreitol, 10 μg/ml leupeptin, 0.1 mM PMSF) containing 2 μg anti-Ras antibody (R02120, Transduction Labs, Lexington , KY). After 30 min of solubilization at 4°C, the supernatants are collected by centrifugation and incubated for an additional 30 min at 4°C. Immunocomplexes are precipitated with 30 μl protein G-agarose (30 min incubation) and the beads washed four times with lysis buffer. The beads are washed with PBS, carefully aspirated dry, then resuspended in 20 μl 2 mM EDTA, 0.2% SDS, 2 mM DTT, heated to 95°C for 5 min, and cooled on ice. Phosphorylated Ras-GTP is separated from Ras-GDP by thin layer chromatography using PEI cellulose plates (J.T. Baker, Phillipsburg, N.J.) and KH_2PO_4 (pH 3.4) as the mobile phase. Autoradiography of the plates allow densitometric quantitation of radiolabeled Ras-GTP which is expressed as percent of total Ras activity (Ras-GTP + Ras-GDP).

RESULTS

The clonal cell line CHO-Y1R-A1 was used in the MAPK phosphorylation studies (75,000 receptors/cell, K_D = 0.1 nM). Figure 1 shows the time-dependent changes in levels of phosphorylated MAPK after exposure to PYY (0.1 μM). The response is biphasic with an initial rapid peak (1-3 min) and a prolonged elevation at 1.5 - 2 times basal levels for up to one hour, returning to baseline by 2 hours. This response is pertussis toxin-sensitive. Western blot shows that PYY-induced increases in phosphoMAPK levels are abolished by preincubating the cells with pertussis toxin (200 ng/ml) for 18 hours prior to PYY stimulation (0.1 μM for 3 min at 37°C) (Figure 2A). Measures to inhibit PKC activity also block PYY effects on MAPK phosphorylation. Prolonged incubation with phorbol ester (100 nM phorbol 12-myristate 13-acetate in DMSO for 24hr) to downregulate protein kinase C (PKC) results in significant attenuation of PYY-induced increases in phosphoMAPK levels (Figure 2B). No significant inhibitory effects are seen with prolonged exposure to vehicle (0.1 % DMSO) alone. Furthermore pretreatment of cells with PKC inhibitors sphingosine (5 μM for 60 min) and Ro-31-8220 (2 μM for 60 min) also significantly block PYY-stimulated elevations in phosphoMAPK (Figure 2B).

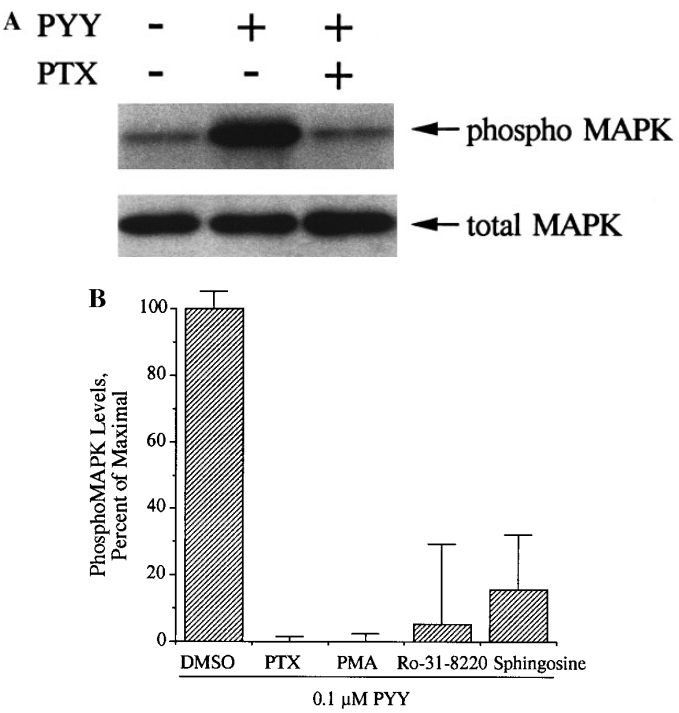

FIG. 2. Pertussis toxin and PKC inhibition block PYY-induced MAPK phosphorylation in Y1R-CHO-A1 cells. (A) Western blot showing pretreatment with pertussis toxin (PTX, 200 ng/ml for 24 hr) blocks PYY-induced (0.1 μM for 3 min) increases in phosphoMAPK levels. Reprobing blot for total MAPK shows equivalent amounts of MAPK loaded per lane. (B) Results of densitometric analysis of Western blots showing PYY-induced (0.1 μM for 3 min) increases in levels of phosphoMAPK blocked by pertussis toxin (PTX), prolonged incubation with phorbol ester (PMA), and PKC inhibitors Ro-31-8220 (2 μM for 60 min) and sphingosine (5 μM for 60 min) (mean ± SD, n = 2 - 4).

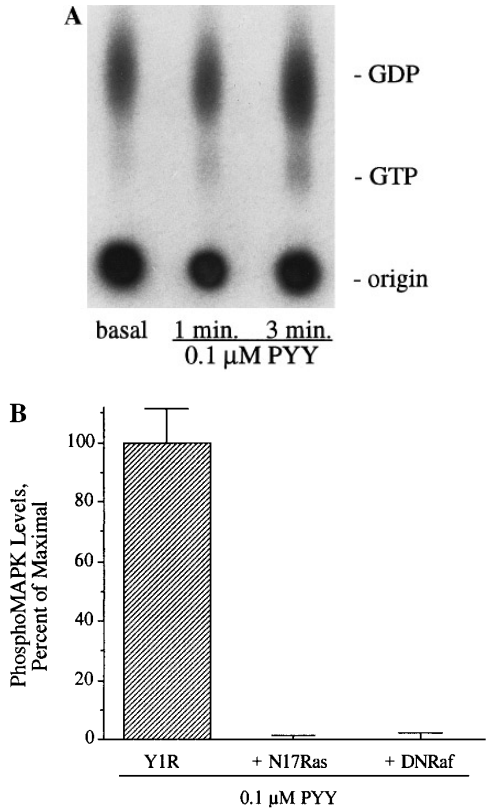

FIG. 3. PYY activates Ras protein, an important effector of MAPK phosphorylation in Y1R-CHO-A1 cells. (A) Autoradiogram of thin layer chromatography plate shows increase in GTP bound to Ras during exposure to 0.1 μM PYY. (B) Results of densitometric analysis of Western blots showing inhibition of PYY-induced (0.1 μM for 3 min) increases in levels of phosphoMAPK after transiently cotransfecting Y1R cDNA in the absence and presence of dominant negative constructs of Ras (N17Ras) and Raf (DNRaf) in CHO-K1 cells (mean ± SD, n = 2).

To determine if Y1R activation could also stimulate MAPK phosphorylation via alternative pathways involving Ras, Ras protein activation was studied by measuring PYY-induced incorporation of radiolabeled GTP. Figure 3A shows PYY causing a time-dependent increase in the amount of GTP bound to Ras, from 8% in unstimulated cells to 12% and 15% at one and three minutes respectively. Conversely, transiently cotransfecting CHO cells with the Y1 receptor in the presence and absence of dominant negative constructs of the Ras (N17Ras) and Raf (DNRaf) proteins (8) significantly inhibit Y1R-mediated MAPK phosphorylation (Figure 3B). To determine if putative upstream effectors of Ras activation are involved in coupling the Y1 receptor to MAPK phosphorylation, inhibitors of tyrosine kinase activity and phosphatidylinositol 3 (PI3)-kinase were assessed for their ability to block PYY-induced MAPK phosphorylation. Pretreatment (18 hr) of CHO-Y1R-A1 cells with the tyrosine kinase inhibitors tyrphostin A25 and B42 (20 μM) blocks the PYY-induced increase in phosphoMAPK (Figure 4). There is no similar inhibitory effect of vehicle alone (0.1% DMSO) or following pretreatment with the inactive isomer tyrphostin A1. Pretreatment with the phosphatidylinositol 3-kinase inhibitor wortmannin (50 nM for 30 min) partially blocks (40-50%) PYY effects on phosphoMAPK levels. Lastly the specific MAPK kinase (MEK) inhibitor PD98059 (100 μM for 30 min) is also able to significantly block PYY-induced phosphoMAPK accumulation.

DISCUSSION

These data show that in CHO cells the Y1 receptor is coupled to MAPK phosphorylation, an important convergence point for classical and putative growth factors. The inhibition of this this effect by pertussis toxin indicates that the Y1R interacts with a G_i or G_o protein. Furthermore the data suggest that the full stimulation of MAPK phosphorylation by Y1R activation is dependent on both protein kinase C and Ras as upstream effectors.

The receptors for peptide YY and neuropeptide Y belong to the G protein-coupled superfamily and are predominantly linked through pertussis toxin-sensitive mechanisms to second messengers and a variety of biologic responses. The Y1 and Y2 receptor subtypes have transduced proliferative responses to PYY and NPY, and the mouse Y1 receptor can activate MAPK (9). Many examples exist of GPCR coupling to mitogenic responses as well as stimulation of MAPK. The specific effector molecules involved in these pathways vary according to the cell- and receptor-type being studied. For instance, G protein α subunits alone can regulate cell

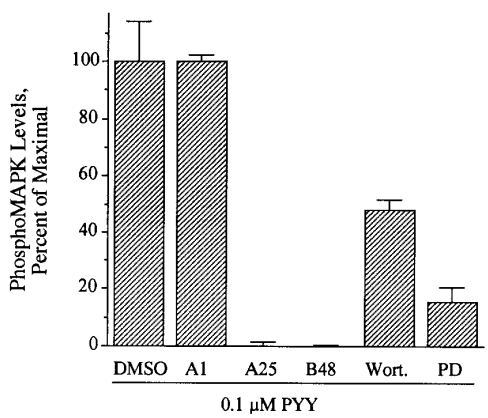

FIG. 4. Inhibition of tyrosine kinase activity, PI3-kinase, and MEK block PYY-induced MAPK phosphorylation in Y1R-CHO-A1 cells. Results of densitometric analysis of Western blots showing effects on PYY-induced (0.1 μM for 3 min) increases in levels of phosphoMAPK after pretreatment with tyrphostins A25, B48, and the inactive isomer A1 (20 μM for 18 hr), as well as the phosphatidylinositol 3-kinase inhibitor wortmannin (50 nM for 30 min) and the MAPK kinase (MEK) inhibitor PD98059 (100 μM for 30 min) (mean ± SD, n = 3).

proliferation because GTPase-deficient Gα mutants induce transformation in fibroblast cell lines (10), and inactivation of the $\beta\gamma$ subunit does not necessarily inhibit MAP kinase activation by GPCR ligands. However with other GPCRs, $\beta\gamma$ subunits play an important role in MAPK stimulation as seen with Shc-mediated activation of Ras by the α_{2A}-adrenergic receptor (11). Futher downstream, MAPK activation can involve Ras activation by pertussis toxin-sensitive G_i (M2 muscarinic and α_2-adrenergic receptors) or the pertussis toxin-insensitive G_q (thrombin and lysophosphatidic acid receptors) or even a Ras-independent but PKC-dependent pathway involving the α subunit of G_o (12). Another signalling molecule with a role in GPCR coupling to MAPK is phosphatidylinositol 3-kinase which can act along the receptor tyrosine kinase-Ras-MAPK pathway in various spots. GPCRs can activate PI3-K as well, and $\beta\gamma$ subunits appear to mediate this interaction (13, 14).

GPCR coupling to protein kinase C has usually involved the pertussis toxin-insensitive G_q protein, but at least one report demonstrates PKC activation via G_o protein (12) albeit in a Ras-independent manner. In our CHO cell model the Y1 receptor could couple through the pertussis toxin-sensitive G_o protein to activate PKC, consistent with van Biesen et al. (12), but since PYY stimulates GTP binding to Ras in Y1R-transfected CHO cells and the MAPK effects are blocked by Ras dominant negative constructs, the mechanism of PYY-induced MAPK phosphorylation cannot be said to be Ras-independent. Because Ras activation may involve interactions of phosphotyrosine consensus sites between PI3-K or so-called adaptor (Grb2, Sos) and docking (Shc, IRS-1) proteins, inhibitors of tyrosine kinase activity were used to disrupt formation of phosphotyrosines. Inhibition of PYY-induced phospho-MAPK accumulation by specific tyrphostins show that tyrosine kinase activity is necessary for the Y1R-MAPK cascade. Phosphatidylinositol 3-kinase may also be involved in the Y1R-MAPK pathway since pharmacologic inhibition of this kinase attenuates PYY effects on phosphoMAPK levels. Inhibitory effects of tyrphostins on MEK phosphorylation of MAPK tyrosine residues cannot be ruled out.

These data suggest that Y1R couples to MAPK in CHO cells by a novel mechanism which is simultaneously PKC- and Ras-dependent. It is possible that Y1R couples to both G_i and G_o proteins in CHO cells, activating parallel signalling cascades involving PKC and Ras. Perhaps the pathway diverges at the level of a single G protein, with differential effects exerted by α and $\beta\gamma$ subunits on downstream effectors. Perhaps PKC is an effector of Ras activation, stimulating tyrosine kinase activity as an intermediate and integrating PKC and Ras regulation of Raf activity. These results show that Y1R-transfected CHO cells are a useful and malleable model for defining the hierarchy of effector molecules in the Y1R-MAPK cascade and investigating the basis for receptor- and cell-specific mechanisms regulating MAPK activation and mitogenic responses.

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