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Partial Knockdown of $G\alpha_{i2}$ Protein Is Sufficient to Abolish the Coupling of PYY Receptors to Biological Response in Renal Proximal Tubule Cells[1]

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Peptide YY (PYY)-preferring receptors are expressed in the renal proximal tubule cell clone Cl.10 isolated from the PKSV-PCT cell line. They mediate PYY-inhibited cAMP production through coupling with pertussis-sensitive Gi proteins. Previous $G\alpha_i$ RNA antisense experiments demonstrated the exclusive coupling of the PYY receptor to the Gi2 protein. Here we characterized a clone stably expressing $G\alpha_{i2}$ antisense RNA which exhibited only a partial decrease in $G\alpha_{i2}$ content (#60 %) as estimated by Western blot. When compared to control Cl.10 cells, this clone, referred to as Cl.10(t), exhibited : (i) an increase in the dissociation constant of PYY receptors (6.42 vs 0.63 nM) ; (ii) a complete absence of inhibition of [^{125}I]PYY binding by GTPγS and GTP ; (iii) the failure of PYY to inhibit basal and forskolin-stimulated cAMP levels ; (iv) the failure of PYY to stimulate [^{35}S]GTPγS binding to membranes. These findings show that partial knockdown of $G\alpha_{i2}$ expression in Cl.10 cells completely abolish the coupling of PYY receptors to biological response.

Peptide YY (PYY)-preferring receptors are expressed in PKSV-PCT cells (1), derived from microdissected proximal convoluted tubules of kidneys from transgenic mice harboring the simian virus 40 (SV40) large T antigen placed under the control of the rat L-type pyruvate kinase 5′-regulatory sequence (2). In PKSV-PCT cells PYY receptors mediate inhibition of cAMP production and stimulation of cell growth by coupling to a pertussis toxin-sensitive G protein.

$G\alpha_i$ antisense RNA expression recently demonstrated the exclusive coupling of PYY receptors to the Gi2 protein in a clone, Cl.10, isolated from PKSV-PCT cells (3). Indeed, after transfection of Cl.10 cells with antisense $G\alpha_{i2}$ expression vectors, a clone Cl.10/$G\alpha_{i2}$- was isolated. It showed a 90% decrease of $G\alpha_{i2}$ associated with an increase of the dissociation constant of PYY receptors, an absence of inhibition of PYY binding by GTPγS and of inhibitory effect of PYY on basal and forskolin-stimulated cAMP levels (3). A similar study was conducted with $G\alpha_{i3}$ protein (3) and a Cl.10/$G\alpha_{i3}$- clone in which the synthesis of the $G\alpha_{i3}$ protein was specifically down-regulated by expression of antisense $G\alpha_{i3}$ RNA was selected. This clone did not exhibit any modification in the dissociation constant of PYY receptor or in the sensitivity to GTPγS further indicating that $G\alpha_{i3}$ is not coupled to PYY receptors in Cl.10 cells.

In the present work, we have investigated a new clone, referred to as Cl.10(t) clone, which exhibited only a partial decrease (#60%) in $G\alpha_{i2}$ content after stable transfection of antisense $G\alpha_{i2}$ DNA in Cl.10 cells. By studying receptor affinity, regulation of ligand binding by GTPγS

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and GTP, GTPγS binding and inhibition of cAMP production, we show that partial knockdown of Gα_{i2} protein is sufficient to uncouple PYY receptors to biological response in Cl.10 cells.

MATERIALS AND METHODS

Materials. Synthetic porcine PYY was purchased from Neosystem (Strasbourg, France). [^{125}I]Na and [^{3}S]GTPγS were from Amersham (Les Ulis, France). Geneticin (G418) and culture media DMEM and HAM's F12 were purchased from Gibco BRL (Cergy Pontoise, France). All highly purified chemicals used were purchased from Sigma Chemical Co. (St Louis, MO). BSA (Pentex, Fraction V) was obtained from Miles Laboratories (Elkart, NJ). Anti-α_{i2}/α_{i1} (AS7), anti-α_{i3}/α_{o} (EC2) antibodies were from N.E.N. (Boston, MA). [^{125}I]Tyr^{36}-monoiodo PYY (referred to as [^{125}I]PYY below) was prepared and purified as described (1).

Cultured cells. Cl.10 cells were cultured in a standard culture medium (DMEM : HAM's F12, 1 : 1 (v/v)) supplemented with various compounds as described (1, 3). All studies were performed between the 4th and 12th passages on sets of cells seeded on plastic culture flasks (25 cm^2 or 75 cm^2 surface). The cells were routinely passaged every 7 days.

Transfection with antisense Gα_{i2} subunit expression vector. The 39 bases of the 5′-noncoding region immediately upstream of end including the ATG translation initiation codon of Gα_{i2} (5′-GCGTGTGGG-GGCCAG-GCCGG-GCCGGCGGACGGCAGGATG-3′) was inserted into the EcoRV cloning site of the polylinker of pcDNA3 vector as described (3). Cl.10 cells were transfected by electroporation using a gene pulser as described (3). 48 hours after electroporation, transfected cells were selected by addition of geneticin to a final concentration of 400 μg/ml for 3 weeks and cells resistant to geneticin were subsequently cloned by limiting dilution allowing to isolate the Cl.10(t) clone.

Preparation of particulate fraction of cultured cells. Control and Cl.10(t) cells grown in 75 cm^2 plastic culture were harvested and a particulate fraction from cell homogenates, referred to as membrane preparation, was prepared as described (1).

Immunoblotting of Gα_{i2}- and Gα_{i3}- subunits of Gi proteins. Membranes (50 μg protein) were solubilized and proteins were separated on a 10 % polyacrylamide gel. Proteins were transferred to nitrocellulose and the nitrocellulose sheets were incubated with anti-α_{i2}/α_{i1} (AS7, dilution 1/1,000) or anti-α_{i3}/α_{o} (EC2, dilution 1/1,000) followed by washing and incubation with [^{125}I]labelled goat antibodies to rabbit IgG as described (4). Autoradiograms of the dried immunoblots were scanned with a Macintosh Onescanner densitometer in order to estimate the relative amount of G protein subunits (1).

Binding of [^{125}I]PYY to membrane-bound receptors. Binding of [^{125}I]PYY to membrane preparations was conducted as previously described (1, 3, 5). All binding data were analyzed using the LIGAND computer program developed by Munson and Rodbard (6).

Cyclic AMP measurement. Cellular cAMP content was assayed as described (1). The cAMP was then measured by radioimmunoassay (5). Data are reported as pmoles of cAMP per mg protein. Cell protein determinations were made in parallel wells by the method of Bradford using BSA as a standard (7).

Binding of GTPγS to membranes. Binding of [^{35}S]GTPγS to membrane preparations was conducted as described (8). Briefly, membranes (200 μg protein / ml) were incubated for 120 min at 30°C in 250 μl of incubation buffer (20 mM Tris-HCl (pH 8), 0.1 % lubrol, 1 mM EDTA, 1mM dithiotreitol, 100 mM NaCl, 30 mM $MgCl_2$, 2% BSA and 0.1% bacitracin) containing 0.4 nM [^{35}S]GTPγS (1,013 Ci/mmol) with or without 100 μM unlabelled GTPγS in the presence or the absence of 0.1 μM PYY. At the end of the incubation, 500 μl ice-cold incubation buffer were added. Bound and free [^{35}S]GTPγS were separated by centrifugation at 20,000 $\times$ g for 10min. The radioactivity in the membrane pellets was measured by scintillation counting with a β counter. The nonspecific binding represented about 5 % of total binding. The results were expressed as counts / mg protein.

RESULTS AND DISCUSSION

After transfection of Cl.10 cells with the pcDNA3/Gα_{i2}- expression vector, a clone, referred to as Cl.10(t) clone, was isolated exhibiting a partial decrease in Gα_{i2} content as compared to parent Cl.10 cells. Figure 1 shows the G protein subunit profile in control Cl.10 cells and Cl.10(t) cells as determined by Western blot. A partial decrease in the expression of Gα_{i2} was observed in Cl.10(t) cells whereas Gα_{i3} (Figure 1), Gα_{s} and Gβ (not shown) were expressed at the same level as in Cl.10 cells transfected with vector alone. Densitometric scanning of the blot indicated that Cl.10(t) cells displayed a 60% down-regulation in the expression of Gα_{i2} protein. As previously reported (3), Cl.10 cells do not express Gα_{i1}.

PYY receptors in Cl.10 and Cl.10(t) cells were investigated by studying the competitive

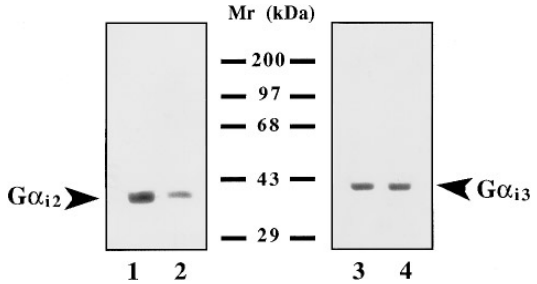

FIG. 1. Western blot analysis of $G\alpha_{i2}$- and $G\alpha_{i3}$- subunits of G proteins in control Cl.10 and Cl.10(t) cells. Membrane proteins (50 μg per lane) of Cl.10 cells transfected with vector alone (lanes 1 and 3) and of Cl.10(t) (lanes 2 and 4) were subjected to 10% acrylamide slab gel electrophoresis. After transfer onto nitrocellulose sheets, bands were revealed as described in Materials and Methods. Gels were calibrated with myosin (200 kDa), phosphorylase b (97 kDa), BSA (68 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa).

inhibition of [^{125}I]PYY binding by unlabelled PYY. These experiments revealed a marked decrease in [^{125}I]PYY binding in Cl.10(t) cells. Scatchard analysis gave a straight line in both cell clones (Figure 2) with a marked increase of the dissociation constant in Cl.10(t) cells (6.42 $\pm$ 1.48 nM) as compared to Cl.10 cells (0.63 $\pm$ 0.20 nM) without change in the binding capacity i.e. 128 $\pm$ 18 and 145 $\pm$ 22 fmol/mg protein in Cl.10(t) and Cl.10 cells, respectively. These data demonstrated that a partial loss of $G\alpha_{i2}$ (60%) resulted in the conversion of all PYY receptors to a low affinity state. The binding parameters of PYY in Cl.10(t) cells were identical to those observed in Cl.10 cells which had been preincubated overnight with pertussis toxin (data not shown) and in Cl.10/$G\alpha_{i2}$- with >90% decrease in $G\alpha_{i2}$ content as previously described (3).

We investigated the effect of GTP and GTPγS in inhibiting [^{125}I]PYY binding to Cl.10(t)

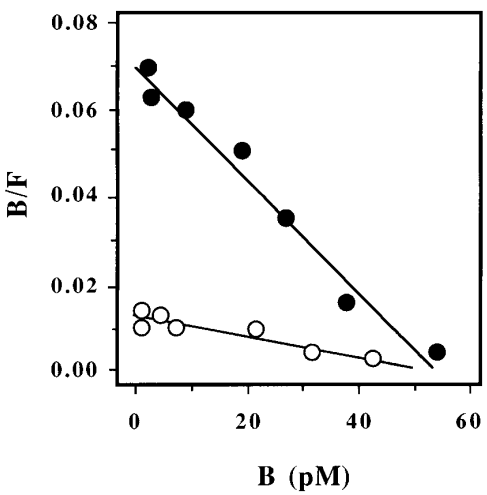

FIG. 2. Scatchard analysis of PYY binding to control Cl.10 cells and Cl.10(t) cells. Saturation analysis was conducted as described in Materials and Methods in the presence of a fixed concentration of [^{125}I]PYY (0.05 nM) and increasing concentrations of unlabelled PYY. Nonspecific binding was determined in the presence of 1 μM unlabelled PYY. Binding experiments were performed on membranes prepared from control Cl.10 cells (●) or Cl.10(t) cells (○). Results shown are from a typical experiment. Two other experiments gave similar results.

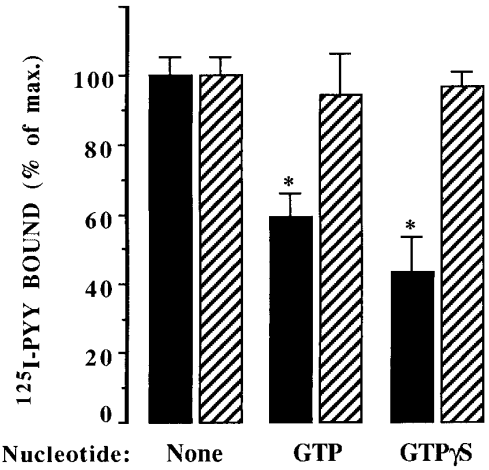

FIG. 3. Effect of guanine nucleotides on PYY binding to membranes from control Cl.10 cells and Cl.10(t) cells. Effects of GTP or GTPγS on PYY binding were measured on Cl.10 cells (solid bars) and Cl.10(t) cells (hatched bars). Experiments were carried out in the presence of a fixed concentration of [^{125}I]PYY (0.05 nM) and a fixed concentration of nucleotide (10 μM). Nonspecific binding was determined in the presence of 1 μM unlabelled PYY and substracted from total binding. *$p < 0.001$ versus control without nucleotide.

and Cl.10 cell membranes (Figure 3). GTP (10 μM) and GTPγS (10 μM) inhibited 42% and 57% of [^{125}I]PYY binding in Cl.10 cells, respectively. In contrast, GTP and GTPγS had no effect in Cl.10(t) cells. Conversely the effect of PYY in stimulating nonhydrolysable GTPγS analog binding was investigated in Cl.10(t) and Cl.10 cell membranes. Experiments were carried out in the presence of 0.4 nM [^{35}S]GTPγS in the absence or the presence of 0.1 μM PYY and data expressed as specific GTPγS binding in the presence of PYY minus specific GTPγS binding in the absence of PYY. Since we previously demonstrated that PYY receptors in Cl.10 cells were exclusively coupled to Gα_{i2} (3), such measurement estimates PYY effect on GTPγS binding to Gα_{i2} and not to other Gα proteins. As shown in figure 4, PYY stimulated

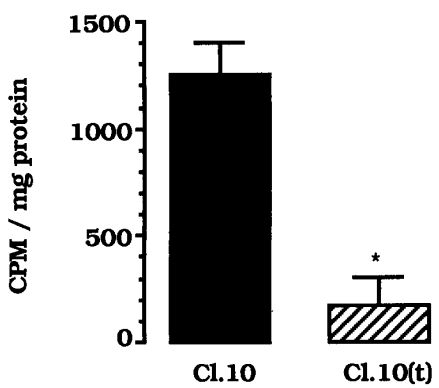

FIG. 4. Effect of PYY on [^{35}S]GTPγS binding to membranes from control Cl.10 cells and Cl. 10(t) cells. PYY effect on specific GTPγS binding was measured on Cl.10 cells (solid bar) and Cl.10(t) cells (hatched bar). Experiments were carried out as described in Materials and Methods. Results are expressed as specific GTPγS binding in the presence of PYY minus specific GTPγS binding in the absence of PYY. Each value is the mean ± SE of four determinations. *$p < 0.001$ versus control Cl.10 cells.

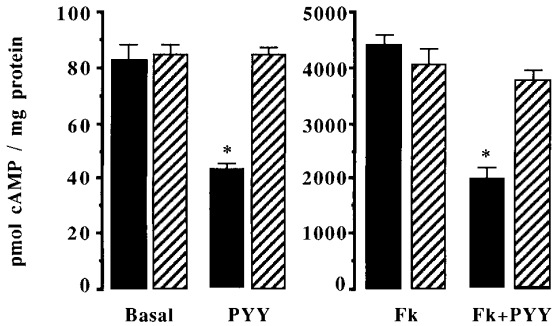

FIG. 5. Effect of PYY on basal and forskolin-stimulated cAMP levels in control Cl.10 cells or Cl.10(t) cells. PYY effect on cAMP production was investigated in control Cl.10 cells (solid bars) and Cl.10(t) cells (hatched bars). Cells were incubated with or without 10 μM forskolin in the absence or in the presence of 1 μM PYY for 30 min at 37°C. The cellular cAMP content was then determined as described in Materials and Methods. Each value is the mean ± S.E. of three determinations. *$p < 0.001$ versus control without PYY

GTPγS binding to Cl.10 cell membranes whereas there was no significant specific binding stimulated by PYY in Cl.10(t) cell membranes.

Finally, we investigated the effect of PYY on cAMP production in Cl.10 and Cl.10(t) cells. As shown in Figure 5, PYY inhibited both basal and forskolin-stimulated cAMP production in control Cl.10 cells. In contrast, PYY failed to alter basal and forskolin-stimulated cAMP levels in Cl.10(t) cells.

The present investigation shows that Cl.10(t) cells in which the synthesis of the $G\alpha_{i2}$ protein was 60% down-regulated by expression of antisense $G\alpha_{i2}$ RNA exhibit a total uncoupling of PYY receptors to Gi proteins. This was demonstrated in experiments exploring a direct interaction between PYY receptors and Gi2 proteins (Figures 3 and 4) and also more distally at the cAMP level (Figure 5). It is worth pointing out that knockdown of almost all $G\alpha_{i2}$ proteins (3) or only 60% (this paper) results in the same functional uncoupling of PYY receptors.

In view of the fact that G protein subunits are generally considered to be expressed in large excess over individual G protein-coupled receptors (9), it was intriguing to observe that a partial decrease of the expression of $G\alpha_{i2}$ in Cl.10 cells totally abolished the regulation of PYY binding by GTPγS as well as PYY receptor-mediated inhibition of cAMP production. It can be hypothesized that a threshold amount of $G\alpha_{i2}$ protein is necessary to interact significantly with PYY receptors and for signal propagation. This has been previously suggested for G protein coupling to other receptors (10). This would be consistent with a clinical observation indicating that a 50% loss of $G\alpha_s$ subunits results in a lack of parathormone action in the kidneys in pseudohypoparathyroidism (11). The situation is more complex in ob/ob mouse where adipocyte $G\alpha_{i1}$ and $G\alpha_{i3}$ are reduced twofold without a corresponding change in $G\alpha_{i2}$ (12, 13). This results in no significant effect on the ability of adenosine to inhibit adenylylcyclase and lipolysis (12, 13). However, since the A1-adenosine receptor can couple to $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$ (14), it can be hypothesized that the different $G\alpha_i$ subunits are interchangeable giving an axplanation for the absence of alteration of adenosine effect in adipocytes of ob/ob mice. The situation is quite different for PYY receptors in Cl.10 cells which couple exclusively to the Gi2 protein.

In conclusion, our antisense RNA technology studies suggest that a threshold amount of $G\alpha_{i2}$ is necessary for PYY receptor mediated- signal propagation in the renal proximal tubule cell clone Cl.10. They further demonstrate that Gi proteins are not interchangeable in this model. More generally, our data pintpoint the fact that when the coupling of a given receptor

to a specific G protein is exclusive (3), the amount of a subunit of this G protein can be crucial for signal propagation.

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