Evidence for Opioid Receptor-Mediated Activation of the G-proteins, Gα and Gβγ, in Membranes of Neuroblastoma x Glioma (NG108–15) Hybrid Cells*

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In membranes of neuroblastoma x glioma (NG108–15) hybrid cells, the photoreactive GTP analog, [α-32P]GTP azidoanilide, was incorporated into 39–41-kDa proteins comigrating in urea-containing sodium dodecyl sulfate-polyacrylamide gels with immunologically identified G-protein α-subunits, i.e. a 39-kDa Gα, an α-subunit, a 40-kDa Gβγ α-subunit, and a 41-kDa Gα subunit of an unknown subtype. The synthetic opioid, D-Ala²,D-Leu⁵-enkephalin (DADLE), stimulated photolabeling of the 39–41-kDa proteins. In the presence of GDP, which increased the ratio of agonist-stimulated to basal photolabeling, DADLE at a maximally effective concentration stimulated photolabeling of the 39- and the 40-kDa protein 2–3-fold. Somatostatin, adrenaline, and bradykinin were less potent than DADLE and, to varying degrees, stimulated photolabeling of the 40-kDa protein more than that of the 39-kDa protein. Prostaglandin E₁ was inactive. The present data represent direct evidence for an activation of endogenous Gα and Gβγ via opioid receptors and other receptors in the native membrane milieu.

In neuronal cells, activated opioid receptors couple via PTX³-sensitive G-proteins (1) to various effectors including adenylylcyclase and ion channels for K⁺ and Ca²⁺ (2–4). Recent data suggest that opioids inhibit adenylylcyclase via Gα₁, a member of the Gi family (6, 7). The inhibitory modulation of voltage-dependent Ca²⁺ channels and the stimulatory modulation of K⁺ channels via opioid receptors and other inhibitory receptors may be mediated by the G-protein, Gα₁ (6, 7).

Similar to neurons, neuroendocrine, and pituitary cells (8), NxG cells possess subtypes of Gα and Gβγ (5, 9). Activation of opioid receptors in NxG cells results in the inhibition of adenylylcyclase (10) and the inhibition of voltage-dependent Ca²⁺ channels (4, 11). In PTX-treated NxG cells, the opioid-induced inhibition of Ca²⁺ currents is efficiently restored by intracellular application of purified Gα and Gβγ (4). Evidence for coupling of opioid receptors to Gα and Gβγ comes from reconstitution experiments with the µ-opioid receptor purified from rat brain and G-proteins purified from porcine and rat brain (12). While the data obtained in reconstitution experiments demonstrate the ability of activated opioid receptors to couple to exogenous Gα and Gβγ, they do not necessarily imply that coupling to these G-proteins occurs in the native plasma membrane.

In order to identify endogenous G-proteins activated via opioid receptors, we studied in membranes of NxG cells the effects of the synthetic δ-opioid receptor agonist, DADLE, and, for comparison, of other receptor agonists on photolabeling of G-proteins with the photoreactive GTP analog, [α-32P]GTP azidoanilide.

EXPERIMENTAL PROCEDURES

Photolabeling of Membrane Proteins—Synthesis and purification of [α-32P]GTP azidoanilide were performed as described (13, 14). For photolabeling of G-protein α-subunits with [α-32P]GTP azidoanilide, membranes from NxG cells (50 µg of protein/assay tube) were incubated at 30 °C in a buffer consisting of 0.1 mM EDTA, 5 mM MgCl₂, 1 mM benzamidine, 100 mM NaCl, 30 µM GDP, 0.1 adenosine 5′-[γ-32P]triphosphate, and 30 mM Hepes (pH 7.4) in the absence or presence of receptor agonists. After 3 min of preincubation, samples were incubated for another 3 min with 5–30 nM [c-32P]GTP azidoanilide (15 kBq/tube). Variations from this protocol are indicated in the figure legends. The final assay volume was 60 µl. The reaction was stopped by cooling the sample on ice. After centrifugation at 12,000 × g for 5 min at 4 °C (for removal of unbound [α-32P]GTP azidoanilide), the obtained membrane pellets were resuspended in 60 µl of a modified GDP-free incubation buffer supplemented with 2 mM dithiothreitol. Membrane suspensions were then transferred onto Parafilm, which they formed a pellet, and were irradiated at 300 nm for 10 min at 4 °C with a 254 nm/150 watts/7 × 30-cm UV light source (Vilber Lourmat, Torcy, France). The distance of the light source from the samples was 5 cm. Thereafter, membrane suspensions were retransferred into the assay tubes, again centrifuged, and prepared for SDS-PAGE by adding sample buffer (15).

SDS-PAGE, Immunoblotting, and Antisera—SDS-PAGE (18), blotting of proteins onto nitrocellulose filters, and autoradiography of gels were performed as described (16) with the following modifications. The separating gels contained 8% (w/v) acrylamide and 4.3 mM urea (17). In some instances, the 39–41-kDa regions of the dried gels were cut out and shaken in 1 ml of 30% (v/v) H₂O₂ for at least 1 h, and the incorporated radioactivity was counted after the addition of 5 ml of scintillant. Nitrocellulose filters were incubated with antisera generated against synthetic peptides corresponding to confined regions of G-protein α-subunits. Properties of the employed antisera and immunostaining of filters have been described elsewhere (9, 13).

Miscellaneous, Reproducibility of Data—Protein was determined according to Lowry et al. (18). Cell culture, differentiation of NxG cells with dibutyryl cAMP, and preparation of membranes were performed as described (4, 9). Autoradiograms were scanned with a laser densitometer (LKB 2202 Ultrascan). The experiments shown are representative for three or more independently performed exper-
Photolabeling of 39-, 40-, and 41-kDa proteins was observed in membranes of NxG cells. Membranes were photolabeled with [α-32P]GTP-azidoanilide in the presence of 1 µM bradykinin as described under "Experimental Procedures" (bradykinin was employed in order to increase incorporation of [α-32P]GTP-azidoanilide into the 41-kDa protein; see "Results"). Thereafter, proteins were separated by SDS-PAGE in the presence of urea and transferred onto a nitrocellulose filter. The filter was cut into stripes which were autoradiographed and then incubated with different antisera; filter-bound antibodies were visualized by an antibody-phosphatase color reaction (4 central lanes). Figures on the left panel margin indicate molecular masses of marker proteins (kDa). AA-GTP, [α-32P]GTP-azidoanilide; αc, αcommon peptide antiserum; αo, α peptide antiserum; α1, α2, α3 peptide antiserum; DF, dye front.

RESULTS

When membranes of NxG cells were incubated with [α-32P]GTP-azidoanilide and subsequently exposed to UV light, photolabeling of 39-, 40-, and 41-kDa proteins was observed (Figs. 2–5). For identification of these proteins, membranes were photolabeled with [α-32P]GTP-azidoanilide; subsequently, proteins were separated by SDS-PAGE and blotted onto nitrocellulose filters. The filters were first autoradiographed (outer lanes) or incubated with various antisera; bound antibodies were visualized by an antibody-phosphatase color reaction (4 central lanes). Figures on the left panel margin indicate molecular masses of marker proteins (kDa). AA-GTP, [α-32P]GTP-azidoanilide; αc, αcommon peptide antiserum; αo, α peptide antiserum; α1, α2, α3 peptide antiserum; DF, dye front.

Further data support the assumption that the photolabeled proteins in the 40-kDa region represent a-subunits of PTX-dependent G-proteins. Modification of G-protein α-subunits by PTX leads to a decreased mobility of G-protein α-subunits in membranes. Different membrane batches and different batches of [α-32P]GTP-azidoanilide were employed in the photolabeling studies.

Photolabeling of 39–41-kDa proteins increased with the time elapsed after addition of [α-32P]GTP-azidoanilide to the reaction mixture (Fig. 3). DADLE stimulated photolabeling at all incubation times (ranging from 0.5 to 12 min). At incubation times of 0.5–1.5 min, the 39- and 40-kDa proteins incorporated approximately equal amounts of radioactivity in the absence of DADLE; in the presence of DADLE, the 39-kDa protein was the preferentially photolabeled protein. At incubation times of 3–6 min, the 39- or 40-kDa protein was preferentially photolabeled, depending on whether or not DADLE was present in the reaction mixture. Only at long incubation times (12 min) was the 40-kDa protein the preferentially photolabeled protein, irrespective of the absence and presence of DADLE. The findings indicate that at incubation times of 0.5–6 min, DADLE promotes nucleotide exchange on the 39-kDa protein more than that on the 40-kDa protein. Since [α-32P]GTP-azidoanilide is a poorly hydrolyzable GTP analog (20), [α-32P]GTP-azidoanilide-ligated G-proteins (i.e. activated G-proteins) will accumulate, in contrast to the intact cell. Thus the photolabeling pattern ob-

2 F.-J. Klinz, personal communication; S. Offermanns, A. Schmidt, K. Spicher, K.-D. Hinsch, unpublished experiments.
DLE was half-maximally and maximally active at concentrations of 10-100 nM and 1 pM, respectively (not shown). DADLE dose dependently labeled G-protein interaction. Yatani and Brown (21) provided values for G-protein interaction a second. Therefore, photolabeling patterns observed after short incubation intervals are more likely to reflect the receptor/G-protein interaction in vivo. In further experiments, an incubation time of 3 min was chosen since it (i) allowed sufficient incorporation of radioactivity and (ii) the ratio of agonist-stimulated to basal photolabeling was satisfactory. Under the conditions described under "Experimental Procedures," DADLE dose dependently stimulated photolabeling of 39-41-kDa proteins (Fig. 4). Densitometric evaluation of autoradiograms revealed that DADLE was half-maximally and maximally active at concentrations of 10-100 nM and 1 μM, respectively (not shown). Similar concentrations were reported for maximal and half-maximal effects of DADLE on GTPase and adenylcyclase activity in membranes of NxB cells (22). Employed at a maximally effective concentration, DADLE increased the incorporation of radioactivity into the 39-41-kDa proteins about 2-3-fold (see Fig. 2-3). Photolabeling of the 39-kDa protein was slightly more stimulated than that of the 40-kDa protein. A quantitative evaluation of the stimulatory effect of DADLE on photolabeling of the 41-kDa protein was not possible. DADLE did not stimulate photolabeling of proteins modified by PTX (not shown). The stimulatory effect of DADLE was abolished by the opioid receptor antagonist, naloxone (see Fig. 4), indicating that the effect of DADLE was due to activation of opioid receptors.

Other receptor agonists were employed to compare their effects on photolabeling of membrane proteins with that of DADLE. Prostaglandin E1, a potent stimulator of adenyllycyclase in membranes of NxB cells (23), had no effect on photolabeling of 39-41-kDa proteins (see Fig. 4). In contrast, somatostatin, adrenaline, and bradykinin, which activate pertussis toxin-sensitive G-proteins in membranes of NxB cells (10, 24-26), stimulated photolabeling of 39-41-kDa proteins (Fig. 5). None of these receptor agonists was as potent as DADLE. Somatostatin, adrenaline, and particularly bradykinin showed a preference for the 40-kDa protein at all concentrations tested. Maximal stimulations of these agonists on photolabeling of the 40-kDa protein were comparable (about 1.5-fold). The differences among the employed receptor agonist were highly reproducible with different batches of membranes or [α-32P]GTP azidoanilide. All receptor agonists stimulated photolabeling of the 41-kDa protein (see Figs. 3 and 4). However, photolabeling of the 41-kDa protein was too small to be well documented by densitometric scanning of autoradiograms (see Fig. 5).

Small amounts of [α-32P]GTP azidoanilide were also incorporated into a 32-kDa protein (see Fig. 4). Photolabeling of this protein was not affected by DADLE or GDP and occurred in the absence of Mg++. Its mobility in urea-containing SDS-polyacrylamide gels was not affected by PTX (not shown). In addition, photolabeled 32-kDa proteins in NxB and RINm5F

**Fig. 3.** Time course of photolabeling of 39-41-kDa proteins in membranes of NxB cells. Membranes were incubated with [α-32P]GTP azidoanilide for the times indicated on the abscissa in the absence (open circles) and presence (closed circles) of 1 μM DADLE. After SDS-PAGE and autoradiography, the 39-41-kDa regions of lanes were counted for radioactivity (ordinate). Values are mean values ± S.D. (n = 3). The inset shows the 39-41-kDa region of a representative autoradiogram obtained from an SDS gel.

**Fig. 4.** Influence of DADLE, naloxone, and prostaglandin E1 on photolabeling of 39-41-kDa proteins in membranes of NxB cells. Membranes were photolabeled as described under "Experimental Procedures." Shown are autoradiograms of SDS gels. Panel A shows the dose-response relation for DADLE, panel B effects of 100 μM naloxone (N), 1 μM DADLE (D), and of naloxone plus DADLE (D/N), and panel C effects of prostaglandin E1 (PGEl) employed at two concentrations. Figures on the left indicate molecular masses of marker proteins (kDa). c, controls (no receptor agonist or antagonist); DF, dye front. The experiment shown in panel B was performed independently of the one shown in panels A and C.

**Fig. 5.** Influence of various concentrations of DADLE, adrenaline, bradykinin, and somatostatin on photolabeling of 39-40-kDa proteins in membranes of NxB cells. Membranes were photolabeled with [α-32P]GTP azidoanilide as described under "Experimental Procedures." Shown are densitometric scans of autoradiograms obtained from SDS gels. c, controls (no receptor agonist). Numbers with arrowheads indicate molecular masses of photolabeled proteins (kDa).
The present data indicate that the 3-opioid receptor couples to G-proteins of the G1 and G2 families. Evidence for the activation of different types of G-proteins by a single receptor has been provided in previous reports. Studies with membranes on adenylcyclase regulation and on binding of receptor agonists suggest that β-adrenoceptors can couple not only to G2, but also to G1 (27, 28). In membranes of HL-60 cells, formyl peptides stimulate the choleratoxin-catalyzed ADP-ribosylation of two G2 subtypes (22). In addition, data obtained in reconstituted systems suggest that β-adrenoceptors, μ-opioid receptor, and muscarinic receptors interact with multiple G-proteins (12, 30, 31).

The method described here may be advantageous if compared with other experimental approaches designed to study receptor/G-protein interactions. (i) In contrast to the antagonist-sensitive ADP-ribosylation catalyzed by cholera toxin, it does not require the absence of guanine nucleotides, the natural ligands of G-proteins. Instead, it is based on a physiological response of G-proteins to receptor-induced activation, i.e., the increase in the exchange of guanine nucleotides on the α-subunits. (ii) It appears to be more generally applicable than agonist-sensitive ADP-ribosylation. In membranes of HL-60 cells, leukotriene B4 stimulates photolabeling of G-protein α-subunits (13) but does not affect cholera toxin-catalyzed ADP-ribosylation (32). Since the affinities of [α-32P]GTP azidoanilide to G1, G2, and G4 are very similar (33), the method should be suitable for examining the interaction of receptors with at least these families of G-proteins. (iii) It does not depend on a reconstituted system in which the interaction of signal transduction components may lack specificity (see the Introduction). (iv) In contrast to methods based on the determination of enzyme activities, it allows identification of receptor-activated G-proteins.

The observed differences among receptor agonists on photolabeling of G-protein α-subunits may reflect differences in their ability to regulate certain effectors. DADLE, somatostatin, and adrenaline (in the order of decreasing maximal activity) inhibit voltage-dependent Ca2+ currents in XNG cells (4, 11, 34) and stimulate photolabeling of the 39-kDa protein comigrating with a G2 α-subunit. Bradykinin does not inhibit Ca2+ currents in XNG cells in a pertussis toxin-sensitive manner. Compared with its ability to stimulate photolabeling of the 40-kDa protein, bradykinin has little effect on photolabeling of the 39-kDa protein. Considering the ability of G2 to reconstitute the inhibitory modulation of Ca2+ currents in various PTX-treated neuronal cells (4, 35–37) and the finding that antibodies against the G2 α-subunit attenuate the Ca2+ current inhibition by noradrenaline in XNG cells (34) and by dopamine in snail neurons (36), the present data are consistent with the hypothesis that G2 couples activated inhibitory receptors (e.g., the 3-opioid receptor) to neuronal voltage-dependent Ca2+ channels.

All the employed receptor agonists stimulate effectively photolabeling of the 40-kDa protein, apparently representing the G2 α-subunit. Previously published data indicate that all these agonists inhibit adenylcyclase in membranes of XNG cells (10, 22, 24–26). Thus the present data support the notion that a G-protein of the G2 family, possibly G2 (5), is involved in the receptor-mediated inhibition of adenylcyclase.

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