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Transfected $G_{o1}\alpha$ Inhibits the Calcium Dependence of β-Adrenergic Stimulated cAMP Accumulation in C6 Glioma Cells*

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Increasing evidence indicates that heterotrimeric G proteins, and in particular G_o, regulate ionic channel activities. In order to investigate the role of G_o proteins in the modulation of the Ca^{2+} influx, C6 glioma cells were stably transfected with α_{o1} cDNA. Expression of the $G_{o1}\alpha$ protein was checked by *Bordetella pertussis* toxin-catalyzed ADP-ribosylation and Western blots using one- and two-dimensional gel analyses. Three clones were selected based on their degree of $G_{o1}\alpha$ expression. In α_{o1}-transfected cells, cAMP accumulations, in response to isoproterenol or forskolin, were lower than in control cells. This inhibitory effect was a function of the amount of expressed $G_{o1}\alpha$. In contrast, $G_{o1}\alpha$ expression was not followed by a significant inhibition of isoproterenol- or forskolin-stimulated adenylyl cyclase activities in particulate fractions. In C6 parental cells, 50–60% of the isoproterenol-induced cAMP accumulation was dependent on external Ca^{2+} concentration. This Ca^{2+}-dependent cAMP accumulation was related to an induced transient Ca^{2+} influx. In transfected cells, expression of $G_{o1}\alpha$ inhibited the Ca^{2+} influx and the Ca^{2+}-dependent component of isoproterenol-induced cAMP accumulation. In conclusion, β-adrenergic agonists stimulate an entry of Ca^{2+} which exerts a positive feedback on cAMP production, and $G_{o1}\alpha$ blocks this positive feedback by inhibiting the Ca^{2+} influx.

GTP-binding proteins (G proteins) act as transducers by coupling membrane-bound receptors to intracellular effectors and ion channels (for reviews, see Gilman (1987), Neer and Clapham (1988), Birnbaumer *et al.* (1990), Bourne *et al.* (1991)).

G proteins are thought to be attached to the cytoplasmic face of membranes and consist of three subunits: α (39–52 kDa), β (35–36 kDa), and γ (8–10 kDa) (Simon *et al.*, 1991). It is believed that βγ subunits are interchangeable among different G proteins. In contrast, the distinctive α chains, which contain the guanine nucleotide-binding site and the GTPase activity, allow each G protein to interact with its own effectors and receptors (Bourne *et al.*, 1990).

One structurally very similar set of G protein α subunits can be covalently modified by *Bordetella pertussis* toxin. This set includes the two transducins that are localized in the retina, as well as two types of G proteins: G_i and G_o. The G_i proteins have been found in most of the cells studied, and one or several G_i proteins inhibit adenylyl cyclase (Birnbaumer *et al.*, 1990).

$G_o\alpha$ represents a 39-kDa species which is almost exclusively located in nervous tissue and excitable cells (Asano *et al.*, 1988; Bockaert *et al.*, 1990). Recently, $G_o\alpha$ has been shown to encode, by alternative splicing, two polypeptides $G_{o1}\alpha$ and $G_{o2}\alpha$ which only differ in the last hundred amino acids of their C-terminal region (Hsu *et al.*, 1990; Strathmann *et al.*, 1990; Tsukamoto *et al.*, 1991). We have previously identified these two isoforms of $G_o\alpha$ using two-dimensional gel analysis and immunoblotting with specific affinity-purified anti-peptide antibodies. Moreover, studies on the ontogenesis of both $G_o\alpha$ subunits in neuroblastoma cells and brains of mouse fetuses revealed that neuronal differentiation is responsible for an on-off switch of the expression of the two $G_o\alpha$ isoforms (Brabet *et al.*, 1990; Rouot *et al.*, 1991). However, the function of $G_o\alpha$ in signal transduction has not yet been fully clarified. Recent studies employing electrophysiological techniques indicate that it may regulate Ca^{2+} and K^+ channels and some types of phospholipases C (Harris-Warrick *et al.*, 1988; VanDongen *et al.*, 1988; Moriarty *et al.*, 1990; Kleuss *et al.*, 1991; Lledo *et al.*, 1992).

In order to determine the pathways and mechanisms of action of G_{o1} protein, we sought to express the $G_{o1}\alpha$ in a glial cell line (C6), where this protein is barely detectable (Brabet *et al.*, 1988a, 1988b) We therefore transfected these cells with a full-length cDNA for $G_{o1}\alpha$. We obtained several clones that transcribed the transfected cDNA from low to high levels. Here, we report that the expression of the $G_{o1}\alpha$ protein decreases the Ca^{2+} dependence of hormone-stimulated cAMP accumulation in intact transfected cells. Analysis of this phenomenon indicates that $G_{o1}\alpha$ appears to act by decreasing a cAMP-dependent Ca^{2+} influx, suggesting a possible role for $G_{o1}\alpha$ in the control of Ca^{2+} permeability in astrocytes.

MATERIALS AND METHODS

Cell Culture—C6 glioma cells were cultured as previously described (Lucas and Bockaert, 1977) with modifications. Briefly, C6 glioma cells were grown at 37 °C in OPTIMEM medium (Gibco Laboratories) containing 10% fetal calf serum supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin sulfate. Cells were maintained in the exponential growth phase by passage every 7 days. Cells used for experimentation were plated in multiwell culture dishes (Costar) or Falcon flasks at 10^4 cells/cm^2. They were used at confluency (10 days after seeding), and the medium was replenished every 2–3 days.

Construction of Expression Vector Containing Rat α_{o1} cDNA—The rat $G_{o1}\alpha$ cDNA (α_{o1}), isolated from a rat olfactory neuroepithelium cDNA library, was kindly provided by Dr. Reed (Jones and Reed, 1987). The *Eco*RI fragment of α_{o1} cDNA was inserted into the *Sma*I site in the polylinker region of the pMSG eukaryotic expression

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vector (Pharmacia LKB Biotechnology Inc.). The orientation of the insertion was determined by restriction enzyme mapping with *Bgl*II, *Nco*I, and *Xho*I. In this vector (pMSG-α_{o1}), the transcription of α_{o1} cDNA is directed by the promoter present in the mouse mammary tumor virus long terminal repeat. The vector also contains the *Escherichia coli* xanthine-guanine phosphoribosyltransferase (*gpt*) gene under the control of the SV40 early promoter and therefore provides a marker for selecting stable transformants in eukaryotic cells.

Transfection of C6 Cells—C6 glioma cells were transfected with pMSG-α_{o1} and pMSG control vector (mock-transfected) by the Ca^{2+} phosphate precipitation method (Wigler *et al.*, 1978). Cells that stably integrated pMSG-α_{o1} (and the *gpt* gene) were selected for their ability to grow in Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum, mycophenolic acid (78 μM), hypoxanthine (100 μM), xanthine (1.4 mM), thymidine (47 μM), aminopterine (4.5 μM), penicillin (50 IU/ml), streptomycin (50 μg/ml), and fungizone (2.5 μg/ml). After 3 weeks in the selective medium, resistant cells were subcloned by limited dilution in microwells. Microwells in which only one cell was observed were selected and clones were then amplified. C6 clones that contained the α_{o1} cDNA construct were termed C1, C2, etc.

Southern Blot Analysis—Total genomic DNA was isolated from parental C6 glioma cells (C6p) and stable pMSG or pMSG-α_{o1}-transfected clones. DNA samples (10 μg) were digested with *Eco*RI, electrophoresed in agarose gels, and subjected to Southern blot analysis (Southern, 1975). Filters were prehybridized in 0.05 M sodium phosphate, 0.75 M NaCl, 0.5% SDS, 2.5 mM EDTA, 10% Denhardt's solution, 50% deionized formamide, 5% dextran sulfate, and denatured salmon sperm DNA (50 μg/ml) for 2–4 h at 42 °C and then hybridized in the same buffer at 42 °C overnight with an α_{o1} cDNA probe radiolabeled by random primer extension. The blots were then washed for 5 min at room temperature with 2 × SSC, 0.5% SDS, for 15 min at room temperature with 2 × SSC, 0.1% SDS and then for two periods of 2 h and 1/2 h at 42 °C with 0.1 × SSC, 0.5% SDS before exposure to Kodak X-AR5 film with intensifying screens.

$G_{o1}\alpha$ Expression Analysis—Particulate fractions from control or transfected C6 glioma cells were prepared as previously described (Brabet *et al.*, 1990) and stored at −80 °C, at a final concentration of 0.5–1.0 mg/ml. Expression of $G_{o1}\alpha$ in transfected cells was checked by ADP-ribosylation with *B. pertussis* toxin (PTX),[1] and by immunoblotting proteins separated by one- and two-dimensional analyses. ADP-ribosylation with PTX toxin were conducted on aliquots of membranes containing 50 μg of protein and carried out as previously described (Brabet *et al.*, 1990). ADP-ribosylated proteins were separated by SDS-PAGE (Brabet *et al.*, 1990).

For immunoblotting, particulate fractions were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose sheet. After quenching and washing, nitrocellulose sheets were immunolabeled overnight at room temperature using 1/200 dilution of anti-G_o or anti-G_β antisera followed by ^{125}I-protein A incubation (Du Pont-New England Nuclear, 10^5 cpm/ml). Dried blots were exposed at −80 °C to Kodak X-AR5 film for 48 h with intensifying screens.

Two-dimensional analysis consisted in isoelectrofocusing and SDS-PAGE in first and second dimension, respectively. Isoelectrofocusing gels were performed according to the procedure of O'Farrell (1975) with a mixture of 2% ampholines at a ratio of 4:1 of pH 5–7 and pH 3.5–10. The established gradient in pH was linear between 5.0 and 6.4. Subsequent procedures were performed according to the method previously reported (Brabet *et al.*, 1990; Rouot *et al.*, 1992).

High Affinity GTPase Assay—GTPase activity in membranes from parental and α_{o1}-transfected C6 glioma cells (5–10 μg of protein/tube) was determined according to the method described by Cassel and Selinger (1976). Briefly, GTPase activity was measured in a reaction mixture (100 μl) containing 0.5 mM [γ-^{32}P]GTP (1.5–2 kBq/tube), 0.1 mM ATP, 0.5 mM App(NH)p, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol, 2 mM creatine phosphate, and creatine kinase (0.2 mg/ml), in 20 mM Tris-HCl, pH 7.5. Measurement of GTPase activity was initiated by the addition of radioactive GTP, and the reaction was carried out for 30 min at 30 °C. At the end of the incubation period, the reaction was stopped by adding 500 μl of ice-cold activated charcoal (5% w/v) in 20 mM sodium phosphate buffer, pH 2. The reaction tubes were then centrifuged for 10 min at 10,000 × *g* at 4 °C, and the radioactivity was determined in 100-μl aliquots of the supernatant. Nonspecific GTPase was assessed by parallel assays containing 100 μM GTP.

Whole Cell cAMP Assay—The cAMP content of cells was measured by the [^{3}H]adenine prelabeling technique as previously described (Shimizu *et al.*, 1969; Weiss *et al.*, 1985). After 2 h of incubation with 1 μCi/ml [^{3}H]adenine, the cultures were washed twice and incubated in 20 mM HEPES-buffered saline, pH 7.4, containing 150 mM NaCl, 4.2 mM KCl, 0.9 mM $CaCl_2$, 0.01% bovine serum albumin, 5 mM glucose, 0.5 mM $MgCl_2$, and 1 mM 3-isobutyl-1-methylxanthine (normal medium) or the same medium supplemented with 2.9 mM EGTA (Ca^{2+}-depleted medium). In Ca^{2+}-depleted medium, calculated Ca^{2+} free concentration was about 30 nM. Drugs were then added and incubation was carried out at 37 °C for the time indicated in the legends of the figures. The reaction was stopped by aspiration of the media and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were scraped and 100 μl of 5 mM ATP-cAMP was added to the mixture. Precipitated cellular proteins were centrifuged at 5000 × *g* for 15 min, and the supernatant was eluted through sequential chromatography on Dowex and alumina columns which permitted the [^{3}H]ATP and [^{3}H]cAMP separation (Salomon *et al.*, 1974). cAMP formation was expressed as:

$$\frac{[^3\text{H}]\text{cAMP}}{[^3\text{H}]\text{ATP} + [^3\text{H}]\text{ADP}} \times 1000 \qquad \text{(Eq. 1)}$$

Preparation of C6 Glioma Cell Membranes and Measurement of Adenylyl Cyclase Activity—Cells cultured in 150-cm^2 flasks were washed in 0.9% NaCl and scraped, and the cell suspension was centrifuged twice at 500 × *g*. The washed pellet was swollen for 10 min in cold lysing buffer (25 mM Tris-HCl, pH 8, 5 mM EDTA), homogenized in a Potter-Elvehjem, and centrifuged for 15 min at 40,000 × *g*. Each homogenate was resuspended in the same buffer and filtered over a silk screen (Nytrel TI, 150 μm) and stored at −80 °C at about 3–5 mg/ml protein until use. All these procedures were performed at 0–2 °C.

Adenylyl cyclase activity was determined as previously described (Homburger *et al.*, 1980). Briefly, particulate fractions (30 to 50 μg of protein) were incubated at 30 °C for 5 min in a total volume of 50 μl containing 100 mM Tris-HCl, pH 8, 5 mM $MgSO_4$, 1 mM cAMP, 0.2 mM ATP, 0.2 mg/ml creatine kinase, 20 mM phosphocreatine, 1 mM EDTA, and tracer amounts of [α-^{32}P]ATP (1–2 μCi), [^{3}H]cAMP (1–2 nCi), and effectors. The reaction was stopped by addition of 950 μl of 5.5 mM Tris-HCl, pH 7.6, 0.4 mM ATP, 0.6 mM cAMP, 10 mM $CaCl_2$, and 0.1 mM HCl, and the cAMP was isolated by sequential chromatography on Dowex and alumina columns.

Ca^{2+} Imaging Response in C6 Glioma Cells—Intracellular Ca^{2+} measurements were performed as previously described (Manzoni *et al.*, 1992). Briefly, C6 glioma cells were loaded with the fluorescent Ca^{2+} chelator fura-2AM, by incubation in OPTIMEM medium. After incubation, the cells were washed twice in HEPES-buffered saline containing NaCl (150 mM), KCl (4.2 mM), $CaCl_2$ (0.9 mM), glucose (5 mM), $MgCl_2$ (0.5 mM), and HEPES (20 mM, pH 7.4). The coverslips containing the loaded and washed cells were mounted for microscopic analysis. Cells were then incubated in the above medium or the same medium supplemented with 2.9 mM EGTA for 15 min at room temperature before adding indicated drugs. The cells were alternatively excited with 340 and 380 nm light, and the emission was measured at 500 nm (Grynkiewicz *et al.*, 1985), on a "Zeiss Axiovert 10" inverted microscope coupled to a MSP 21 microprocessor responsible for the filter changes. The images were taken by a low light level SIT LH 4036 camera from Lhésa (Paris) and digitized on line by a Quantel "Crystal" Imaging System. During the 2-s excitation period at each wavelength, we averaged 8 videoframes per digitalized image. Each image was coded on 512 × 576 × 8 bits. The camera dark noise was subtracted from the recorded crude images. $[Ca^{2+}]_i$ levels were estimated from the images using the procedure described by Grynkiewicz (Grynkiewicz *et al.*, 1985). The constants necessary for this procedure were obtained with fura-2 free acid standard solutions. The whole image treatments were carried out using homemade software.

RESULTS

Characterization of α_{o1}-transfected C6 Cells—After transfection with α_{o1} cDNA and selection, random cells were cloned and screened to determine the $G_{o1}\alpha$ protein concentration. 28 positive clones out of 30 were identified by PTX-catalyzed ADP-ribosylation and immunoblotting with anti-$G_o\alpha$ anti-

[1] The abbreviations used are: PTX, *Bordetella pertussis* toxin; PAGE, polyacrylamide gel electrophoresis; ISO, isoproterenol; FK, forskolin; App(NH)p, adenyl-5′-yl β,γ-imidodiphosphate; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

bodies (data not shown). Parental C6 cell line (C6p) or clones transfected with the vector alone were used as controls. At the sight of the α_{o1} expression, three clones were chosen (C3, C8, and C15) and characterized thereafter.

Fig. 1 shows the result of PTX ADP-ribosylation and immunoblotting. In C6p and α_{o1}-transfected cells, Western blots were probed with an antiserum that recognizes the $G_o\alpha$ protein. As previously described (Brabet *et al.*, 1990), in C6p cells, PTX ADP-ribosylated two proteins at apparent molecular masses of 40,000 and 41,000 corresponding to $G_{i2}\alpha$ and $G_{i3}\alpha$ (Fig. 1*A*, *lane 1*). In the same cells, immunoblot analysis did not reveal any immunoreactive band corresponding to $G_o\alpha$ protein (Fig. 1*B*, *lane 1*). In α_{o1}-transfected cells, ADP-ribosylation with PTX revealed three bands at 39,000, 40,000, and 41,000 corresponding, respectively, to the G_o, G_{i2}, and $G_{i3}\alpha$ proteins (Fig. 1*A*, *lanes 2* and *3* (clones C3 and C15, respectively)). Note that one α_{o1}-transfected clone (C8) did not express a detectable band at 39 kDa related to the $G_o\alpha$ protein (Fig. 1*A*, *lane 4*). Immunoblot with anti-$G_o\alpha$ antiserum confirms data from PTX-catalyzed ADP-ribosylation (Fig. 1*B*). In clones C3 and C15 (Fig. 1*B*, *lanes 2* and *3*, respectively), anti-G_o antiserum revealed one immunoreactive band corresponding to the purified $G_o\alpha$ protein (Fig. 1*B*, *lane 5*). No immunoreactive band related to the $G_o\alpha$ protein was detected in the C8 clone (Fig. 1*B*, *lane 4*).

Note that nearly all the immunoreactivity corresponding to $G_o\alpha$ is associated with membranes, since more than 90% of $G_o\alpha$ was found in a 100,000 × *g* pellet fraction (data not shown). Furthermore, the observations that the transfected $G_o\alpha$ subunit was present in membrane preparation and was a PTX substrate confirm that this subunit can interact with $\beta\gamma$ subunits to form a complex in cell membranes.

To assess the relative amounts of $G_o\alpha$ to $G\beta$ proteins in α_{o1}-transfected cells and parental cells, we performed quantitative immunoblotting with anti-$G_o\alpha$ and anti-$G\beta$ antisera as previously described (Brabet *et al.*, 1988a, 1988b). Fig. 2 shows a quantitative estimation of $G_o\alpha$ and $G\beta$ subunit content in C6 parental and three selected α_{o1}-transfected clones (C3, C8, C15). Results indicated that no detectable difference in the amount of β subunit in the transfected and parental cells was observed although they do express different amounts of $G_o\alpha$ protein. Furthermore, the amount of $G_o\alpha$ found in the highest expressing clone (C3) (Fig. 2*A*) is quite comparable to the amount of $G_o\alpha$ determined in primary cultures of neuronal or glial cells (about 50 pmol/mg of protein, see Brabet *et al.* (1988a, 1988b)).

To determine whether the $G_o\alpha$ protein expressed in the transfected cells was normally processed as the endogenous $G_o\alpha$, we performed two-dimensional gel electrophoresis and immunoblotting analysis with anti-$G_o\alpha$ antiserum on particulate fractions from the C3 transfected clone. We used particulate fractions of frontal cortex from 18-day-old fetuses as control, since this preparation contains $G_{o2}\alpha$ and $G_{o1}\alpha$ at about the same ratio (Rouot *et al.*, 1992). Fig. 3 shows the two spots corresponding to $G_{o2}\alpha$ and $G_{o1}\alpha$ in particulate fractions from frontal cortex focusing at a pH of 5.8 and 5.5, respectively (*upper panel*). In particulate fractions from the C3 clone, only one spot was revealed focusing at the same pI as the native $G_{o1}\alpha$ protein (Fig. 3, *lower panel*). Co-migration of particulate fractions derived from C6 cells and frontal cortex revealed no spot other than $G_{o1}\alpha$ and $G_{o2}\alpha$ (data not shown). Thus, α_{o1}-transfected clones expressed a $G_{o1}\alpha$ protein homologous to the endogenous $G_{o1}\alpha$.

Morphology of α_{o1}-transfected Cells—The phenotype of cells in lines containing detectable amounts of $G_{o1}\alpha$ (C3, C15) was altered compared to that of cells containing undetectable transfected proteins (C8, C6p). Many of these cells were spindle-shaped and not as flat as control cells (Fig. 4). The more transfected cells express $G_{o1}\alpha$, the less they adhere. Moreover, cells expressing $G_{o1}\alpha$ protein exhibited a decrease in their growth rates (data not shown).

Southern Blot Analysis of the Genomic Transfected Cell DNA—To examine the genomic integration of the transfected cDNA, Southern blot analysis was performed after *Eco*RI restriction digestion of DNA isolated from C6 glioma cells and several transfected clones. A common pattern of hybrid-

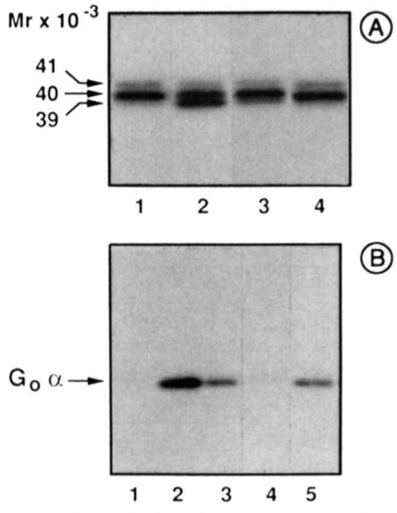

FIG. 1. **Expression of $G_{o1}\alpha$ in parental and α_{o1}-transfected C6 glioma cells.** *A*, PTX-catalyzed ADP-ribosylation. Membranes (50 μg of protein) from parental and α_{o1}-transfected (C3, C8, C15) glioma cells were subjected to ADP-ribosylation with PTX and [^{32}P]-NAD. Aliquots of ADP-ribosylated proteins were then separated on 10% SDS-polyacrylamide gels and autoradiographed as described under "Materials and Methods." A typical autoradiogram of the gel is shown: *lane 1*, C6p; *lane 2*, C3 clone; *lane 3*, C15 clone; *lane 4*, C8 clone. *B*, immunoblot assay of $G_o\alpha$. Membranes (50 μg of protein) and 50 ng of purified bovine brain $G_o\alpha$ were separated on 10% SDS-polyacrylamide gel electrophoresis. After transfer onto a nitrocellulose sheet, proteins were incubated with 1/200 dilution of anti-$G_o\alpha$ antiserum followed by ^{125}I-protein A. A typical autoradiogram of the gel is shown: *lane 1*, C6p; *lane 2*, C3 clone; *lane 3*, C15 clone; *lane 4*, C8 clone; *lane 5*, purified $G_o\alpha$ protein.

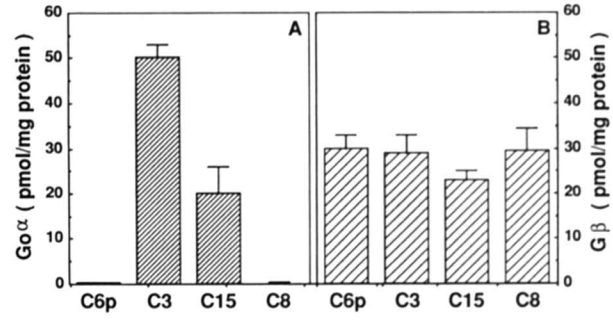

FIG. 2. **Quantitative estimation of $G_o\alpha$ and $G\beta$ subunit content in parental and α_{o1}-transfected cells.** Particulate fractions (50 μg of protein) were separated on 10% SDS-polyacrylamide gel electrophoresis. The presence of $G_o\alpha$ and $G\beta$ was assessed on autoradiograms after electrotransfer of the proteins from SDS-PAGE onto nitrocellulose sheets, successive incubations with anti-$G_o\alpha$ or anti-$G\beta$ sera, and then with radioiodinated protein A. Standard curves were made with purified $G_o\alpha$ and $G\beta$ subunits. The quantity of $G_o\alpha$ and $G\beta$ subunit content (*A* and *B*, respectively) in C6 parental and α_{o1}-transfected cells were determined from standard curves by cutting out the radioactive bands and counting by γ spectroscopy. The threshold of sensitivity in detecting $G_o\alpha$ and $G\beta$ subunits is equal to 10 ng. Results are expressed as the mean ± S.E. (n = 3) of three independent experiments.

ization in the α_{o1} cDNA is present in *Eco*RI restriction digests of nontransfected cells and transfected clones. In addition, transfected clones contain other bands besides the common pattern seen in control cells, indicating variations in the site of chromosomal insertion (data not shown).

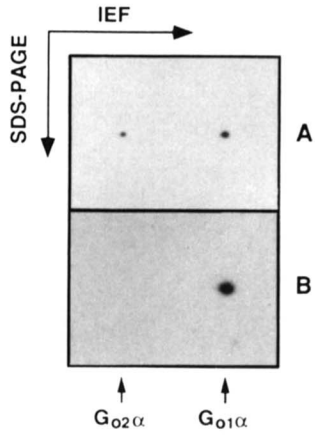

FIG. 3. **Two-dimensional immunoblotting analysis of the $G_o\alpha$ forms in α_{o1}-transfected cells.** Particulate fractions (100 μg of protein) from C3 line and frontal cortex of embryonic mice (18 days of gestation) were separated by two-dimensional electrophoresis followed by immunoblotting with anti-$G_o\alpha$ antibodies. *Arrows* indicate the positions of $G_{o1}\alpha$ and $G_{o2}\alpha$ identified by their respective pI, as previously described (Rouot *et al.*, 1992), in the particulate fraction from frontal cortex of 18-day-old fetuses (*panel A*). *Panel B* shows the only spot probed with anti-$G_o\alpha$ antibodies in particulate fractions of the C3 clone which exhibited the same pI as the endogenous $G_{o1}\alpha$ protein.

High Affinity GTPase Activity in Membranes from α_{o1}-transfected Cells—We measured high affinity GTPase activity in membranes from parental and α_{o1}-transfected cells to determine whether the $G_{o1}\alpha$ in transfected cells is able to bind and hydrolyze GTP. As shown in Table I, basal or mastoparan-stimulated GTPase activities were higher in $G_{o1}\alpha$-expressing cells (C15 and C3) than in cells containing undetectable transfected proteins (C6p and C8). This indicates that these higher values of GTPase activity in membranes of α_{o1}-transfected cells are related to $G_{o1}\alpha$ expression, suggesting that this protein is able to bind and hydrolyze GTP in intact cells.

Effect of $G_{o1}\alpha$ Expression on cAMP Production in Intact Cells—Basal cAMP levels were similar in wild type cells (C6p), mock-transfected (M1, M2, M3 clones) and α_{o1} cDNA-transfected clones (C3, C8, C15). In contrast, accumulation of cAMP stimulated by the β-adrenergic agonist, isoproterenol (ISO), was markedly depressed in the two $G_{o1}\alpha$-expressing clones (C15 and C3) compared to the control cells (wild type and mock-transfected clones). Note that the α_{o1}-transfected clone, C8, which did not express $G_{o1}\alpha$ protein at a detectable level, exhibits a similar β-adrenergic response compared to control cells (Fig. 5). In clone C15, the accumulation of cAMP following β-adrenergic stimulation was 51 $\pm$ 5% (n = 12) of the accumulation observed in parental cells. Thus, alteration in cAMP accumulation in $G_{o1}\alpha$-expressing clones is due to $G_{o1}\alpha$ expression and not to variability in cloning or transfection.

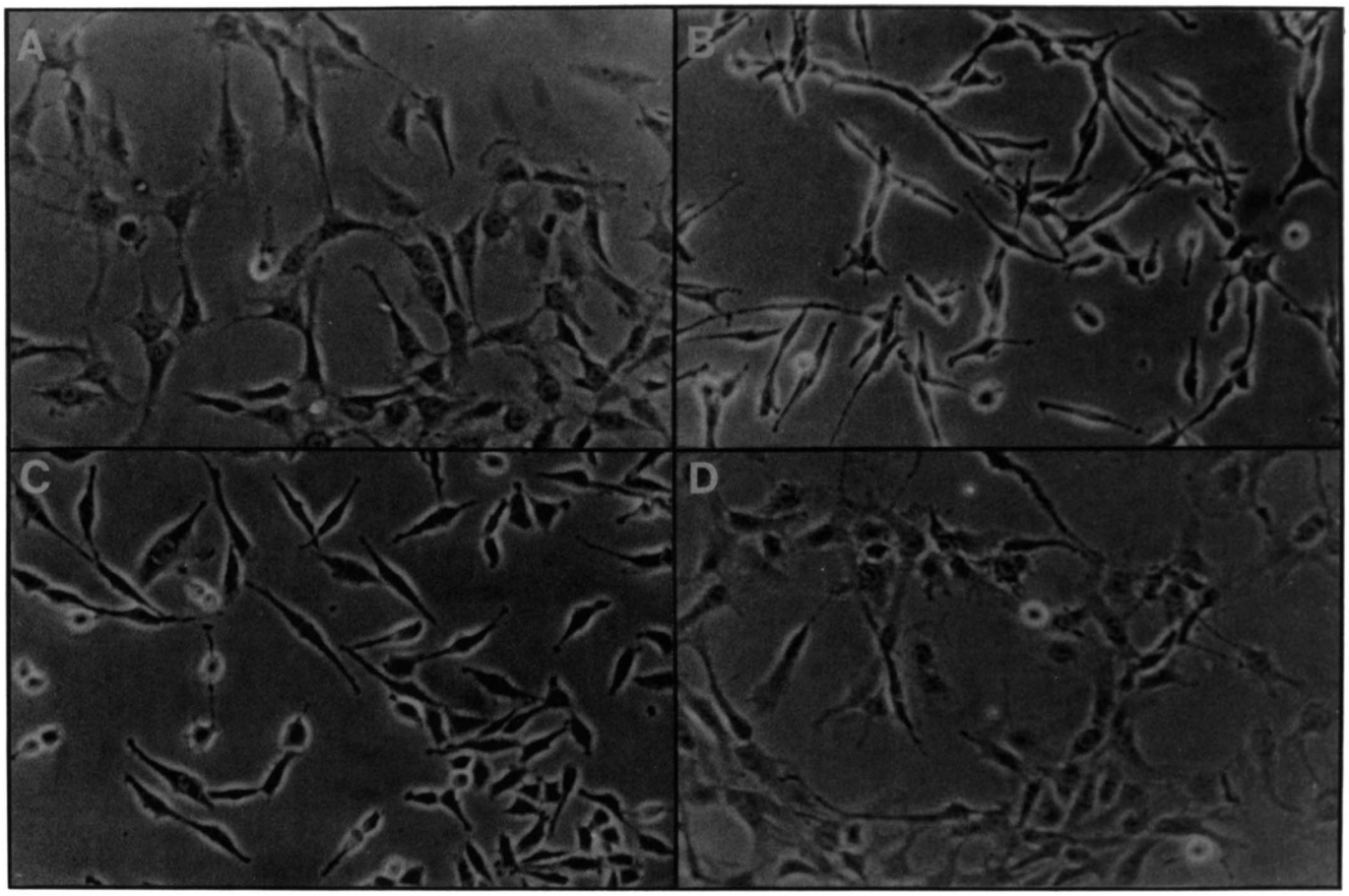

FIG. 4. **Morphological cellular changes associated with $G_{o1}\alpha$ expression in C6 glioma cells.** Parental cells and α_{o1}-transfected cells were grown in OPTIMEM medium and viewed by phase-contrast. Note that in $G_{o1}\alpha$-expressing clones, C3 and C15 (*B* and *C*, respectively) cells were exhibited as spindle-shape in contrast to C6 parental cells and C8 clone (*A* and *D*, respectively) which did not express $G_{o1}\alpha$ protein.

TABLE I

Effect of $G_{o1}\alpha$ expression on high affinity GTPase activity

Membranes from parental and α_{o1}-transfected C6 glioma cells were used to determine basal and mastoparan-stimulated high affinity GTPase activities as described under "Materials and Methods." Mastoparan is used at 25 μM. Results are means ± S.E. of (n) independent experiments performed in triplicate.

Clones	High affinity GTPase activity	
	Basal	Mastoparan
	pmol/min/mg protein	
C6p	40.9 ± 8.9 (4)[a]	51.3 ± 5.5 (4)
C8	52.3 ± 4.5 (3)	46.7 ± 5.5 (3)
C15	69.6 ± 6.5 (4)[a]	114.5 ± 5.1 (4)
C3	110.7 ± 15.7 (3)	187.8 ± 13.1 (3)

[a] A significant difference between two values ($p < 0.05$, t test).

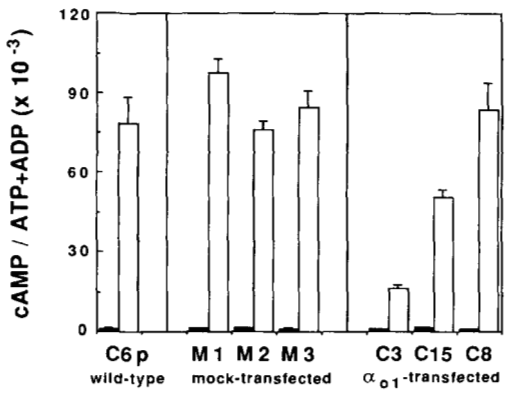

FIG. 5. **cAMP accumulation in C6 glioma cells and after transfection.** Intracellular cAMP accumulation was determined in a whole population of C6 parental cells (wild type), three clones transfected with the vector alone (mock-transfected cells) and three α_{o1}-transfected clones (C3, C8, C15). Cells prelabeled with [^{3}H]adenine were incubated without (■) or with 10 μM ISO (□) for 15 min. After addition of 5% trichloroacetic acid, determination of intracellular cAMP was carried out as described under "Materials and Methods." Values are the mean ± S.E. of three determinations. This experiment is representative of two others.

The time dependences of ISO-stimulated cAMP accumulation in intracellular and extracellular media from control parental cells and α_{o1}-transfected cells were examined (Fig. 6). A rapid increase in the cAMP content of C6p and the C8 clone was apparent during the first 20 min of incubation with ISO. This increase was followed by a decline in cAMP content which continued throughout the remaining 1-h incubation period, but was more pronounced in parental cells than in the C8 clone. In $G_{o1}\alpha$-expressing clones (C3 and C15), cells accumulated cAMP at different rates during the first 20 min of incubation, followed by a decline in cAMP content as in C6p and C8 cells. At 20 min, the cAMP contents of C3 and C15 cells were much lower than in C6p and C8 cells and in inverse correlation with the expression of the $G_{o1}\alpha$ protein, *i.e.* the higher the $G_{o1}\alpha$ expression, the lower the ISO-induced cAMP accumulation.

In contrast to the cAMP content in cells, the released cAMP into the extracellular medium increased slowly, but almost linearly with time for at least 1 h following ISO treatment in all the cells (Fig. 6). Thus, cAMP accumulation is due essentially to the cyclic nucleotide inside the cells at earlier time points, whereas at later time points it comes from the extracellular medium. As shown in Fig. 6, the decrease in cAMP content observed in C15 and C3 clones could not account for an increase in the cAMP released into the extracellular medium.

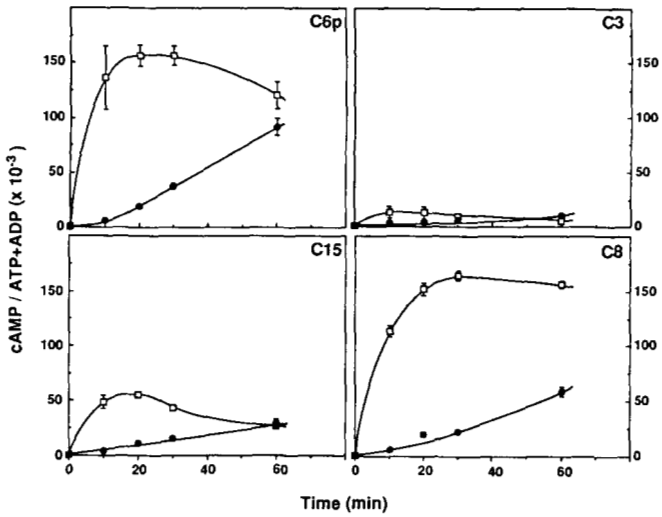

FIG. 6. **Time course of intra- and extracellular cAMP accumulation in parental and α_{o1}-transfected cells following addition of isoproterenol.** Confluent parental (C6p) and α_{o1}-transfected (C3, C8, C15) cells were prelabeled with [^{3}H]adenine and challenged with 10 μM ISO in OPTIMEM medium without serum. At the indicated times, 1-ml aliquots of medium were removed for determination of extracellular cAMP accumulation. Ice-cold trichloroacetic acid was added on dishes and extracellular medium (made 5% with respect to trichloroacetic acid). □, intracellular medium, ●, extracellular medium. Values are the mean ± S.E. of three determinations. This experiment is representative of two others.

The possibility that phosphodiesterase activity could be involved in the decrease in cAMP accumulation in cells expressing $G_{o1}\alpha$ was investigated. In addition to the phosphodiesterase inhibitor, isobutylmethylxanthine (1 mM), present in the incubation media, we added Ro20–1724 (100 μM). However, this treatment had no significant effect on cAMP accumulation in transfected cells (data not shown). Direct determination of the activity of phosphodiesterases (by measuring kinetics of cAMP degradation after stimulation with 1 μM ISO for 15 min followed by addition of 1 mM alprenolol to block further cAMP synthesis) indicated a cAMP disappearance rate which followed a first order process in control and transfected cells (not shown). Furthermore, although the degradation rate was slightly more rapid in $G_{o1}\alpha$-expressing clones (k_{deg} = 0.17 and 0.19 min^{-1} for C15 and C3 cells) than in control cells (k_{deg} = 0.14 and 0.16 min^{-1} for C6 and C8 cells), this does not explain the dramatic decrease in cAMP content after ISO stimulation. Thus, the results obtained strongly suggest an impairment in cAMP formation rather than enhancements in cAMP degradation and efflux.

Adenylyl Cyclase Activity in Particulate Fractions from α_{o1}-transfected Cells—To determine whether $G_{o1}\alpha$ expression alters ISO stimulation of adenylyl cyclase, we measured directly the activity of adenylyl cyclase on membranes. Fig. 7 compares ISO-stimulated adenylyl cyclase activity in particulate fractions from control (C6p and C8) and $G_{o1}\alpha$-expressing cells (C3 and C15). Basal and maximal ISO stimulation and EC_{50} for ISO were similar for all the clones and parental cells (Fig. 7). Three independent experiments gave values of 78 ± 6, 90 ± 4, 91 ± 6, and 108 ± 5 pmol/15 min/mg of protein for basal activities and 341 ± 7, 415 ± 38, 430 ± 21, and 336 ± 4 pmol/15 min/mg of protein for maximal activities (10 μM ISO) in C6p, C8, C15, and C3 clones, respectively. Adenylyl cyclase stimulated by 0.1 mM GTPγS, 10 mM sodium fluoride, or 10 μM forskolin was also similar among the different cells (not shown). Thus, it appears that transfection of α_{o1} does not modify the activity of adenylyl cyclase in membranes but

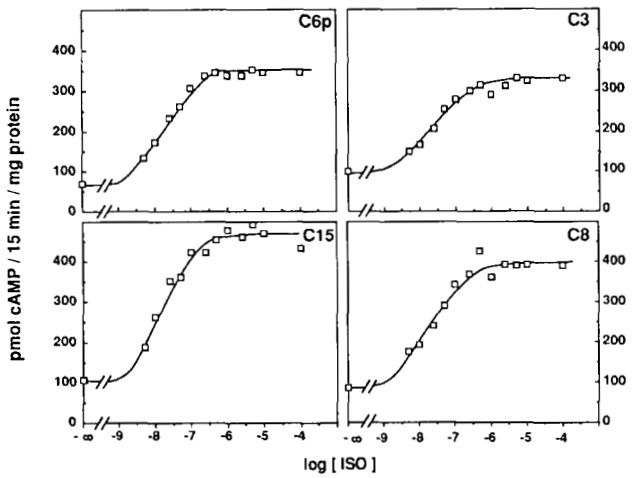

FIG. 7. **Isoproterenol concentration dependence of adenylyl cyclase activity in particulate fractions derived from parental and α_{o1}-transfected glioma cells.** Particulate fractions of confluent cells were prepared and determination of adenylyl cyclase activity stimulated by ISO was performed as described under "Materials and Methods." Values are the mean ± S.E. of three determinations. This experiment is representative of two others, each carried out in triplicate using different particulate fraction preparations.

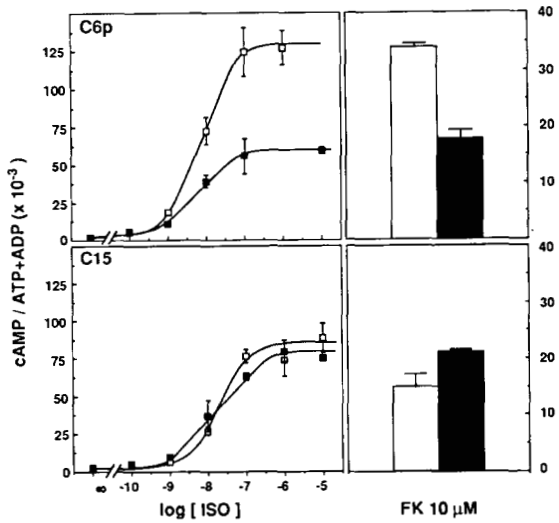

FIG. 8. **Ca^{2+} dependence of hormonal-stimulated cAMP accumulation in parental and $G_{o1}\alpha$-expressing cells.** Confluent parental (C6p) and α_{o1}-transfected cells (C15) were prelabeled with [^{3}H]adenine for 3 h. Cells were washed and incubated for 15 min at 37 °C in Ca^{2+}-depleted buffer as described under "Materials and Methods." After a 15-min incubation with increasing concentrations of ISO or 10 µM forskolin (FK) in normal (□) or Ca^{2+}-depleted media (■), reaction was stopped by removing extracellular medium and adding 5% ice-cold trichloroacetic acid. Intracellular cAMP content was determined. Values are the mean ± S.E. of three determinations.

only in intact cells suggesting that the integrity of the cells is necessary for the expression of the apparent inhibition of adenylyl cyclase activity observed in $G_{o1}\alpha$-expressing cells.

Effect of Ca^{2+} on Hormonal cAMP Accumulation in Intact and α_{o1}-transfected Cells—Since the G_o protein may control permeability of Ca^{2+} in mammalian cells (Kleuss *et al.*, 1991; Lledo *et al.*, 1992) and in *Xenopus* (Murphy and Mc Dermott, 1992), and because Ca^{2+} dependence of hormonal cAMP accumulation has been described in intact C6 glioma cells (Brostrom *et al.*, 1979), the effect of $G_{o1}\alpha$ on hormonal cAMP accumulation was investigated in normal (0.9 mM Ca^{2+}) or depleted media (30 nM Ca^{2+}) (see "Materials and Methods").

In parental C6 glioma cells, ISO induced a dose-response accumulation of cAMP with an EC_{50} at 10^{-8} M ISO (Fig. 8). In Ca^{2+}-depleted medium, the same cells exhibited a 50% decrease in the maximal response with no change in the EC_{50} for ISO (Table II and Fig. 8). If cells were first incubated in normal Ca^{2+} medium and then challenged in Ca^{2+}-depleted medium, comparable ISO dose-response curves to nonchallenged cells in Ca^{2+}-depleted medium were obtained (data not shown). Results were similar to those reported by Brostrom on Ca^{2+}-depleted or Ca^{2+}-restored cells, but we found that EGTA pretreatment of suspended cells, which depleted intracellular Ca^{2+} content, was not necessary. All our experiments were performed on confluent attached cells, and challenging the cells in Ca^{2+}-depleted medium was not followed by a change in the cytosolic free [Ca^{2+}] (see below).

In contrast, in transfected clones expressing $G_{o1}\alpha$, the Ca^{2+} dependence of cAMP accumulation is dramatically reduced or even absent. Fig. 8 shows the dose-response curve of cAMP accumulation in one of the transfected clones (C15) in normal or depleted Ca^{2+} media. ISO induced dose-response curves with EC_{50} of 10^{-8} M in the presence or absence of Ca^{2+}, values similar to those found in C6 parental cells. However, the Ca^{2+} dependency was considerably reduced and the maximal response was decreased by a factor of 2 compared to C6 parental cells (Fig. 8). Thus, $G_{o1}\alpha$ expression in C6 glioma cells led to a decrease in maximal ISO response and in the Ca^{2+} dependence of hormonal induced cAMP accumulation in intact cells. Similar results were obtained by direct stimulation of adenylyl cyclase with 10 µM forskolin, suggesting that the Ca^{2+} dependence on cAMP accumulation is not restricted to the activation of β-adrenergic receptors or G_s but is involved in the production of cAMP by adenylyl cyclase.

TABLE II

Effect of Ca^{2+} on isoproterenol-stimulated cAMP accumulation in parental (C6p) and α_{o1}-transfected (C8, C15, C3) C6 glioma cells

Cells were washed and incubated for 15 min in Ca^{2+}-depleted medium and then treated (with or without 10 µM ISO) for another 15-min period in normal or Ca^{2+}-depleted medium. Results represent the stimulated cAMP accumulation and are means ± S.E. of (n) independent experiments performed in triplicate.

Clones	cAMP content			
	Ca^{2+}-depleted medium		Normal Ca^{2+} medium	
	−ISO	+ISO	−ISO	+ISO
	% cAMP/cAMP + ATP (× 10^{-3})			
C6p	1.7 ± 0.3 (13)	61 ± 4 (21)[a]	1.8 ± 0.3 (13)	127 ± 9 (13)[a]
C8	1.0 ± 0.3 (3)	54 ± 5 (7)[b]	1.1 ± 0.1 (3)	118 ± 11 (7)[b]
C15	2.0 ± 0.5 (4)	76 ± 7 (12)	1.5 ± 0.6 (4)	76 ± 6 (12)
C3	1.1 ± 0.3 (3)	12 ± 0.9 (6)	1.5 ± 0.5 (3)	11 ± 1 (6)

[a, b] A significant difference between two values ($p < 0.01$, t-test).

Ca^{2+}-dependent cAMP Accumulation in Response to Hormones in Parental C6 Glial Cells—The time and concentration dependences of the Ca^{2+} component in the cAMP accumulation was then investigated. As shown in Fig. 9*A*, the Ca^{2+}-dependent component of ISO-stimulated cAMP accumulation increased with time to reach a maximal value at 15 min representing 68% of the total cAMP accumulation in the presence of 10 µM ISO.

The extracellular Ca^{2+} free concentration dependence of the cAMP content of the cells exposed to ISO for 15 min is shown in Fig. 9*B*. cAMP in unstimulated cells did not change with increasing $CaCl_2$ in the extracellular medium, whereas cAMP accumulation was dependent on external Ca^{2+} concentrations, with maximal rates being obtained at 1 mM free Ca^{2+}. As shown in Fig. 9*B*, about 70% of ISO-induced cAMP accumulation is dependent on external Ca^{2+}. In this latter phenomenon, two components were observed, one with an

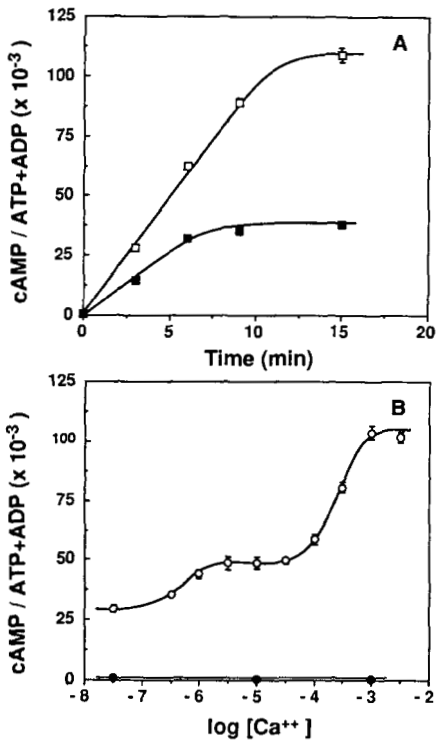

FIG. 9. **Time and concentration dependences of the Ca^{2+} effect on isoproterenol-induced cAMP accumulation in parental C6 glioma cells.** *A*, accumulation of cAMP with time in normal and Ca^{2+}-depleted media following addition of ISO. Confluent parental (C6p) cells were prelabeled with [^{3}H]adenine and then challenged for 15 min in normal (□) or Ca^{2+}-depleted media (■). Intracellular cAMP accumulation was followed with 10 μM ISO. At the indicated times, medium was removed, trichloroacetic acid was added, and intracellular cAMP accumulation was determined. This experiment is representative of two others, each performed in triplicate. *B*, extracellular Ca^{2+} concentration dependency of cAMP content in parental C6 glioma cells. Prelabeled cells were washed twice and incubated in a Ca^{2+}-depleted buffer for 15 min. Cells were then challenged with buffer containing the indicated free [Ca^{2+}] concentration with (○) or without (●) 10 μM ISO for 15 min. The reaction was then stopped and cAMP accumulation was determined. Under these conditions, basal cAMP accumulation (without ISO) did not change (0.9 ± 0.1 and 1.2 ± 0.1, $n = 3$ at 30 nM and 1.8 mM [Ca^{2+}]). This experiment is representative of two others, each performed in triplicate.

EC_{50} of 1 μM and the second of 3×10^{-4} M representing, respectively, 25% and 75% of the Ca^{2+}-dependent component of the ISO-induced cAMP accumulation (Fig. 9*B*).

Changes in $[Ca^{2+}]_i$ in Control and Transfected Cells after Isoproterenol Stimulation—Since cAMP accumulation is dependent on the external Ca^{2+} concentration, we investigated whether an influx of Ca^{2+} could be evoked by ISO stimulation using fura-2 Ca^{2+} imaging. To compare results between cAMP accumulation and videomicroscopy, we used the same conditions for both measurements (confluent cells, same media). As shown in Fig. 10, in confluent C6p cells, 10 μM ISO produced no change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the absence of external Ca^{2+}. In contrast, 10 μM ISO induced a rapid increase in $[Ca^{2+}]_i$ from 90 to 320 nM in the presence of 1.8 mM external Ca^{2+}. The response was transient, $[Ca^{2+}]_i$ peaked at 1 min and returned close to basal line by 4 min (Fig. 10). Direct stimulation of the catalytic subunit of adenylyl cyclase with the diterpene forskolin (FK) also resulted in an increase of $[Ca^{2+}]_i$ in parental C6p cells (Fig. 11, *left panel*). This suggests that cAMP is the relevant message for increased $[Ca^{2+}]_i$ by stimulation of a Ca^{2+} influx.

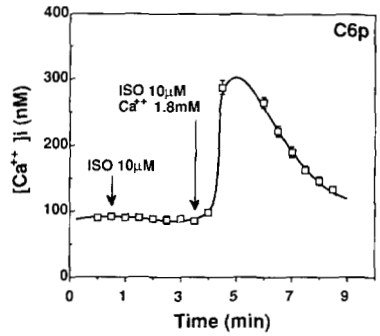

FIG. 10. **Isoproterenol-induced Ca^{2+} influx in C6 parental cells.** Fura-2 loading and $[Ca^{2+}]_i$ measurements were carried out as described under "Materials and Methods." Cells at confluency were washed twice and incubated in Ca^{2+}-depleted medium for 15 min. At the indicated time, 10 μM ISO was added, followed by addition of 10 μM ISO in a medium containing 1.8 mM $CaCl_2$. Traces represent typical responses of about 100 cells at 10 μM ISO.

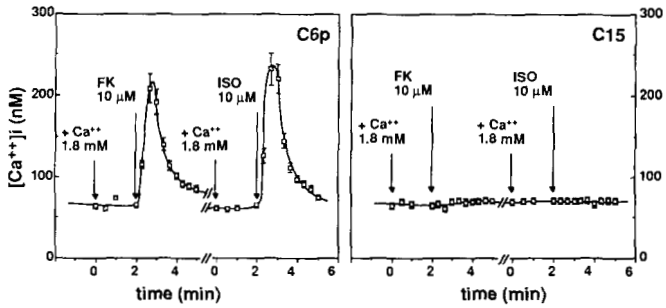

FIG. 11. **Changes in $[Ca^{2+}]_i$ following addition of isoproterenol or forskolin in parental and $G_{o1}\alpha$-expressing C6 glioma cells.** Fura-2 loading and $[Ca^{2+}]_i$ measurements were performed as in Fig. 10. Confluent cells (parental cells and C15 clone) were washed twice and incubated in a Ca^{2+}-depleted medium for 15 min. At the indicated time, they were challenged with buffer containing 1.8 mM Ca^{2+} followed by addition of 10 μM ISO or FK. *Traces* represent typical responses of about 80 cells. This experiment is representative of several others.

Futhermore, addition of K^+ up to 50 mM had no effect on production of a Ca^{2+} influx, suggesting that voltage-sensitive Ca^{2+} channels are not involved in the ISO-evoked Ca^{2+} influx.

The effect of ISO on Ca^{2+} influx was investigated in α_{o1}-transfected cells expressing $G_{o1}\alpha$, because there was a decrease in the Ca^{2+} dependence of cAMP accumulation. Parental C6 cells, preincubated in a Ca^{2+}-depleted medium exhibited no change in $[Ca^{2+}]_i$ when they were exposed to 1.8 mM $CaCl_2$ external medium (Fig. 11, *left panel*). In contrast, in this latter medium, addition of 10 μM FK or ISO induced a transient Ca^{2+} influx similar to that shown in Fig. 10. A similar result was obtained with an α_{o1}-transfected clone (C8) which did not express $G_{o1}\alpha$ (data not shown). In the C15 clone expressing $G_{o1}\alpha$, basal cytosolic $[Ca^{2+}]_i$ determined by fura-2 fluorescence was similar to C6 parental cells ($[Ca^{2+}]_i = 52 \pm 3$ nM and 55.5 ± 3 nM, $n = 78$ in C15 and C6 parental cells, respectively). Challenging the cells with 1.8 mM $CaCl_2$ evoked no change in $[Ca^{2+}]_i$ as in C6 parental cells. Furthermore, exposure to 10 μM FK or ISO was not followed by Ca^{2+} influx (Fig. 11, *right*). A similar result was obtained with the other α_{o1}-transfected clone expressing $G_{o1}\alpha$ (C3) (data not shown).

Thus, the absence of FK- or ISO-induced Ca^{2+} influx in α_{o1}-transfected clones expressing $G_{o1}\alpha$ strongly suggests that expression of $G_{o1}\alpha$ alters the entry of Ca^{2+} induced by cAMP.

DISCUSSION

Transfection of C6 glioma cells with pMSG-α_{o1} results in an expression of the $G_{o1}\alpha$ protein. In these cells, expression

of $G_{o1}\alpha$ alters cellular morphology, growth rate, hormonal formation of cAMP, and cAMP-induced Ca^{2+} influx.

Immunological analysis with anti-$G_o\alpha$ antibodies confirmed the presence of the $G_o\alpha$ protein. However, the level of expression of $G_{o1}\alpha$ was different from clone to clone, as indicated by Western blot analysis. We therefore selected three α_{o1}-transfected clones and determined the concentrations of $G_{o1}\alpha$ and β subunits and then compared them to the C6 parental cells and cells transfected with the plasmid alone. In all clones, the level of the β subunit, determined by quantitative blot using a standard curve with purified bovine $G\beta$ subunits, was between 25 and 30 pmol/mg.

Using the same method, we quantified the $G_{o1}\alpha$ content in α_{o1}-transfected cells. In these three clones, one of them did not express detectable $G_{o1}\alpha$ (C8), and the two others exhibited levels of 20 and 50 pmol/mg of protein in C15 and C3 clones, respectively. Note that these values are similar to those found in primary cultures of astrocytes and neurons (Brabet *et al.*, 1988a).

Thus, determination of $G\beta$ and $G_{o1}\alpha$ contents reveals that in C6 glial cells, expression of the $G_{o1}\alpha$ subunit is not accompanied by a concomitant expression of $G\beta$. Such a determination is important since $\beta\gamma$ participates in the equilibrium with α subunits, and that change in the β subunit or massive overproduction of $G_{o1}\alpha$ subunit could shift the equilibrium between α and β subunits.

Expression of the $G_{o1}\alpha$ protein in the α_{o1}-transfected cells was also shown by the appearance of a new radiolabeled band at 39 kDa on SDS-PAGE gel after PTX catalyzed [^{32}P]ADP-ribosylation of α subunits. A good correlation exists between immunoblot and ADP-ribosylation data, because in the C8 clone no $G_{o1}\alpha$ was detected and no new band was revealed by PTX-catalyzed ADP-ribosylation. Furthermore, in other clones, C15 and C3, the higher the level of $G_{o1}\alpha$, the stronger the band revealed on the autoradiogram after ADP-ribosylation. Since ADP-ribosylation requires the interaction of α with $\beta\gamma$ subunits (Neer *et al.*, 1984) and may reflect the affinity of a different α subunit for $\beta\gamma$ (Huff and Neer, 1985), this above result indicates that $G_{o1}\alpha$ can interact with $\beta\gamma$ on membranes.

Moreover, the expression of $G_{o1}\alpha$ does not modify the ADP-ribosylation of the other α subunits (α_{i3} and α_{i2}) by PTX although $G_{o1}\alpha$ was in excess to $\beta\gamma$. This suggests that either $G_{o1}\alpha$ ADP-ribosylation was not complete or that $\beta\gamma$ acted catalytically as described by Gilman and co-workers (Casey *et al.*, 1989).

In α_{o1}-transfected cells expressing $G_{o1}\alpha$, two-dimensional analysis followed by immunoblotting with anti-$G_{o1}\alpha$ antibodies revealed the apparition of one labeled spot co-migrating with the endogenous $G_{o1}\alpha$ protein of particulate fractions from embryonic mouse brain. This confirms that the $G_o\alpha$ form, focused at pH 5.55 and revealed by specific antibodies raised against a peptide derived from a unique sequence of $G_{o1}\alpha$, corresponds to $G_{o1}\alpha$ (Rouot *et al.*, 1992) and that the $G_{o1}\alpha$ subunit expressed in transfected cells was correctly processed as the endogenous $G_{o1}\alpha$.

Expression of $G_{o1}\alpha$ in C6 glioma cells was followed by an alteration in the morphology and a decrease in growth rate. Similar results were described by Bloch and co-workers (Bloch *et al.*, 1989). However, in C6 glioma cells, these alterations are in correlation with the level of expression of $G_{o1}\alpha$. Transfected cells which did not express the $G_{o1}\alpha$ protein exhibited characteristics similar to those of C6 parental cells. In contrast, the higher the expression of the $G_{o1}\alpha$ protein, the smaller the growth rate (not shown). Furthermore, C3 and C15 clones which expressed $G_{o1}\alpha$ were spindle-shaped and not as flat as parental cells.

In the C6 glioma cell line, cAMP has been described to be one of the effectors involved in the cell shape change (Oey, 1975) and in the growth rate (Mares *et al.*, 1981); this suggests that $G_{o1}\alpha$ may be able to alter production of this second messenger. However, if this alteration is due to $G_{o1}\alpha$, two mechanisms could be postulated. The first one involves the sequestering of $\beta\gamma$ subunits leading to a shift in the equilibrium between α and $\beta\gamma$ subunits (see below). The second one, directly or indirectly, implicates $G_{o1}\alpha$ in the production of cAMP in these cells. It is generally accepted that the GTP-bound α subunit allows G proteins to couple with effectors (Bourne *et al.*, 1991). In the case of transfected C6 glioma cells, the absence of known receptors permitting the exchange of GDP to GTP on $G_{o1}\alpha$, three mechanisms may be considered in the formation of the $G_{o1}\alpha$-GTP active species. Firstly, direct exchange of GDP by GTP, with a low efficacy but sufficient to generate some active $G_{o1}\alpha$. This hypothesis could be correct since a basal GDP-GTP replacement activity, with no activation of receptors, was observed in several systems where heterotrimeric G proteins are involved (Birnbaumer *et al.*, 1990). Secondly, GAP43, a neuronal protein associated to growth cone plasma membrane, has been shown to enhance guanyl nucleotide exchange on $G_o\alpha$ protein (Strittmatter *et al.*, 1991). Since this protein is detected in primary cultures of astrocytes (Vitkovic *et al.*, 1988) and in C6 glioma cells (not shown), such a protein could be used as a guanine nucleotide releasing protein in α_o-transfected cells permitting the binding of endogenous GTP. Thirdly, another guanine nucleotide releasing protein could be involved. In this context, it is interesting to note that particulate fractions derived from $G_{o1}\alpha$-expressing cells exhibited a greater basal and mastoparan-stimulated GTPase activity than particulate fractions derived from control cells.

Expression of $G_{o1}\alpha$ in C6 glioma cells had a marked effect on ISO and forskolin stimulation of adenylyl cyclase in intact cells. This effect was not due to the variability of responses from clone to clone, but was related to the expression of the $G_{o1}\alpha$ protein. This inhibition of cAMP accumulation could be the result of a decrease in adenylyl cyclase activity, an enhancement of cAMP phosphodiesterase activity, or an increase in the efflux of cAMP from the cells. This latter mechanism was described several years ago in C6 glioma cells and involves an energy-dependent mechanism (Doore *et al.*, 1975). However, direct measurement of extracellular cAMP indicated that $G_{o1}\alpha$-expressing cells did not exhibit an enhancement of the cAMP efflux. Activation of phosphodiesterases by cAMP has been described in C6 glioma cells. These phosphodiesterases are involved in desensitization of the β-adrenergic response (Browning *et al.*, 1974). Such an activation of phosphodiesterase by the $G_{o1}\alpha$ protein may be considered, but presence of phosphodiesterase inhibitors (isobutylmethylxanthine, Ro20–1724) did not restore the hormonal response in α_{o1}-transfected cells. Moreover, measurement of the phosphodiesterase activity in control and α_{o1}-transfected cells did not show a significant increase in cAMP degradation rates in $G_{o1}\alpha$-expressing cells. This might not explain the decrease in cAMP accumulation observed in these cells. Thus, the most reasonable explanation to account for the low accumulation induced by hormones and observed in $G_{o1}\alpha$-expressing cells is a decrease in the adenylyl cyclase activity. However, direct determination of stimulated adenylyl cyclase activity using ISO on particulate fractions derived from control and α_{o1}-transfected cells showed similar basal, maximal, and EC_{50} values.

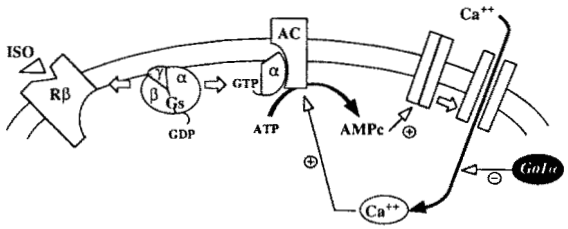

FIG. 12. **Schematic representation of the regulation of Ca^{2+} entry by cAMP and $G_{o1}\alpha$ in C6 glioma cells.** Occupancy of β-adrenergic receptors by ISO or direct activation of adenylyl cyclase by forskolin (FK) was followed by an increase in cAMP. In C6p cells, this increase in cAMP leads to a Ca^{2+} influx which is absent in α_{o1}-transfected cells expressing $G_{o1}\alpha$. This suggests that $G_{o1}\alpha$ may directly or indirectly regulate a Ca^{2+} influx in these cells.

Another possible mechanism, $\beta\gamma$ sequestration by the expression of $G_{o1}\alpha$, might be considered. Such a mechanism has been described recently by Gilman and co-workers (Tang and Gilman, 1991). They showed that $\beta\gamma$ subunits could stimulate type II adenylyl cyclase and inhibit type I adenylyl cyclase (a Ca^{2+}/calmodulin-dependent adenylyl cyclase) in the presence of activated recombinant $G_s\alpha$ proteins (Tang and Gilman, 1991). In this report, addition of $G_o\alpha$ loaded with GDP is able to reverse the effects of $\beta\gamma$. Under our conditions, results do not seem compatible with such a phenomenon, since no obvious difference exists between ISO dose-response curves in particulate fractions derived from control and transfected cells. Expressed $G_{o1}\alpha$ does not seem to modify the receptor-G_s coupling since the EC_{50} of ISO for adenylyl cyclase activation was not significantly modified between control and transfected cells. Furthermore, expression of $G_{o1}\alpha$ does not alter the ISO-induced stimulation of adenylyl cyclase on particulate fractions but inhibits this response on intact cells, suggesting that cell integrity is required in order to observe the inhibition of β-adrenergic induced cAMP accumulation.

Several years ago, the presence of a form of adenylyl cyclase sensitive to Ca^{2+} in C6 glioma cells was described by Brostrom and co-workers (Brostrom *et al.*, 1979). In this report, Ca^{2+} sensitivity was due to the formation of a Ca^{2+}/calmodulin complex which activates the enzyme, although the involvement of calmodulin in this response was the subject of a conflicting result (Katada *et al.*, 1982). Nevertheless, our results show that a Ca^{2+}-dependent adenylyl cyclase was present in our C6 parental glioma cells, confirming previous reports from Brostrom and Ui's groups using the same cell line (Brostrom *et al.*, 1979; Katada *et al.*, 1987). The Ca^{2+} dependence of adenylyl cyclase activity was observed when cells were stimulated by ISO or forskolin which elevated the levels of cAMP. This increase in cAMP was associated with a transient rise in $[Ca^{2+}]_i$ which occurs within 5 min. The rise in $[Ca^{2+}]_i$ is due mainly to the entry of external Ca^{2+} since it was absent in a Ca^{2+}-depleted medium. This was accompanied by an increase in cAMP accumulation, maximal after a 15-min incubation period in the presence of ISO (see Fig. 12). The difference in kinetics between $[Ca^{2+}]_i$ increase and activation of adenylyl cyclase activity suggests that the transient rise in Ca^{2+} induces a series of events which lead to a sustained activation of adenylyl cyclase activity. Thus, activation of an adenylyl cyclase is followed by a transient Ca^{2+} entry which leads to further activation of adenylyl cyclase. Under our experimental conditions, the contribution of the Ca^{2+}-sensitive adenylyl cyclase represents about 55% of total adenylyl cyclase activity. A similar result was reported by Brostrom's group several years ago (Brostrom *et al.*, 1979). We still do not know if it represents the same type of adenylyl cyclase or if it is the result of two types of adenylyl cyclases, one being insensitive to Ca^{2+} and the other sensitive. A Ca^{2+}/calmodulin-sensitive adenylyl cyclase could possibly be involved in this response, as proposed by Brostrom and colleagues (Brostrom *et al.*, 1979), but different calmodulin inhibitors, such as W7 or calmidazolium, had no effect at concentrations up to 10 μM.

Besides C6 glioma cells, a similar potentiating effect of Ca^{2+} on adenylyl cyclase has been reported in the nervous system (Cheung *et al.*, 1975), in some cell lines (Brostrom *et al.*, 1983), and in primary culture of astrocytes (Marin *et al.*, 1991). In C6 glioma cells, astrocyte-like cells, the maximal effect of Ca^{2+} on adenylyl cyclase required millimolar extracellular Ca^{2+} concentrations and did not appear to involve voltage-sensitive Ca^{2+} channels, because the Ca^{2+} entry was not blocked by dihydropyridines and because depolarization with a concentration of K^+ up to 50 mM did not produce any modification of $[Ca^{2+}]_i$ (not shown). Furthermore, using patch clamp techniques, we were unable to detect any Ca^{2+} currents after depolarization of our cells.

The major finding in this work is the fact that $G_{o1}\alpha$ expression blocks the Ca^{2+}-dependent component of hormonal induced cAMP accumulation in C6 glioma cells. Videomicroscopy analysis also indicated that cAMP evoked a Ca^{2+} influx which was absent in α_{o1}-transfected cells expressing $G_{o1}\alpha$. Thus, a possible mechanism involved in the blockade of the Ca^{2+}-sensitive adenylyl cyclase activity is the negative control of the Ca^{2+} influx by $G_{o1}\alpha$ (see Fig. 12).

This Ca^{2+} influx requires the initial production of cAMP, and, subsequently, the increase of $[Ca^{2+}]_i$ starts up a series of events which leads to a sustained activation of adenylyl cyclase activity. In our study, the possible contribution of $G_{o1}\alpha$ in blocking the initial production of cAMP (and therefore the transient Ca^{2+} entry) was ruled out. Firstly, because $G_{o1}\alpha$ did not interfere with the activation of adenylyl cyclase on membranes derived from $G_{o1}\alpha$-expressing cells, and, secondly, direct activation of adenylyl cyclase by forskolin did not evoke a transient Ca^{2+} rise in $G_{o1}\alpha$-expressing cells. Thus, the target of $G_{o1}\alpha$ seems to be the control of the influx of Ca^{2+}, similar to the control of voltage-sensitive Ca^{2+} channels by $G_o\alpha$, observed in cells after activation of some receptors (Harris-Warrick *et al.*, 1988; Kleuss *et al.*, 1991; Taussig *et al.*, 1992; Lledo *et al.*, 1992). However, several different features distinguish these two regulations. (i) In C6 glioma cells, the influx of Ca^{2+} does not seem to be mediated by voltage-sensitive Ca^{2+} channels. (ii) The transient $[Ca^{2+}]_i$ rise was induced by agents like ISO or forskolin which elevate cAMP. (iii) The blockade of the Ca^{2+} influx by the introduced $G_{o1}\alpha$ protein does not involve its activation by an identified receptor.

The nature and the exact mechanism, direct or indirect, by which $G_{o1}\alpha$ regulates the Ca^{2+} influx in C6 glioma cells, as well as the nature of the mechanism of Ca^{2+} entry which is under cAMP dependence, remain to be elucidated.

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