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Adenylyl cyclase inhibition and altered G protein subunit expression and ADP-ribosylation patterns in tissues and cells from $G_{i2}\alpha-/-$ mice

(signal transduction/gene inactivation/penetrance/pertussis toxin)

UWE RUDOLPH*‡, KARSTEN SPICHER†, AND LUTZ BIRNBAUMER*†

*Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030 and †Departments of Anesthesiology and Biological Chemistry, School of Medicine, and Molecular Biology Institute, University of California, Los Angeles, CA 90095-1778

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ABSTRACT **The inhibition of $\alpha_{i2}-/-$ mouse cardiac isoproterenol-stimulated adenylyl cyclase (AC; EC 4.6.1.1) activity by carbachol and that of $\alpha_{i2}-/-$ adipocyte AC by phenylisopropyladenosine (PIA), prostaglandin E_2, and nicotinic acid were partially, but not completely, inhibited. While the inhibition of cardiac AC was affected in all $\alpha_{i2}-/-$ animals tested, only 50% of the $\alpha_{i2}-/-$ animals showed an impaired inhibition of adipocyte AC, indicative of a partial penetrance of this phenotype. In agreement with previous results, the data show that G_{i2} mediates hormonal inhibition of AC and that G_{i3} and/or G_{i1} is capable of doing the same but with a lower efficacy. Disruption of the α_{i2} gene affected about equally the actions of all the receptors studied, indicating that none of them exhibits a striking specificity for one type of G_i over another and that receptors are likely to be selective rather than specific in their interaction with functionally homologous G proteins (e.g., G_{i1}, G_{i2}, G_{i3}). Western analysis of G protein subunit levels in simian virus 40-transformed primary embryonic fibroblasts from $\alpha_{i2}+/+$ and $\alpha_{i2}-/-$ animals showed that α_{i2} accounts for about 50% of the immunopositive G protein α subunits and that loss of the α_{i2} is accompanied by a parallel reduction in $G\beta_{35}$ and $G\beta_{36}$ subunits and by a 30–50% increase in α_{i3}. This suggests that $G\beta\gamma$ levels may be regulated passively through differential rates of turnover in their free vs. trimeric states. The existence of compensatory increase(s) in α_i subunit expression raises the possibility that the lack of effect of a missing α_{i2} on AC inhibition in adipocytes of some $\alpha_{i2}-/-$ animals may be the reflection of a more pronounced compensatory expression of α_{i3} and/or α_{i1}.**

Disruption of the murine gene encoding the α subunit of G_{i2} protein (α_{i2}) causes mice to develop an inflammatory bowel disease and adenocarcinomas of the colon resembling what in humans is referred to as ulcerative colitis (1). Analysis of T cells in $\alpha_{i2}-/-$ mice showed them to be hyper-responsive to T-cell receptor stimulation triggered by anti-CD3, exhibiting both a hyperproliferative response and excessive production of interleukin 2, tumor necrosis factor, and interferon γ. However, it is not known whether these changes are responsible for the development of the observed phenotype.

To gain further insight into changes that occur in mice deficient in α_{i2} we have now studied receptor-mediated inhibition of adenylyl cyclase (AC; EC 4.6.1.1) in homogenates of heart and free fat cells and explored by Western blotting compensatory changes that occur in transformed embryonic fibroblasts in the relative abundance of other G protein subunits. Previous studies from Malbon and collaborators (2) had shown that suppression of α_{i2} can lead to apparently unrelated changes such as enhancement of phospholipase C signalling. Using pertussis toxin (PTX)-catalyzed [^{32}P]ADP-ribosylation, we demonstrate the absence of the α_{i2} subunit from several tissues from $\alpha_{i2}-/-$ mice and also the absence of a detectable amount of the α_o subunit from adipocyte membranes, a controversial topic (3, 4).

MATERIALS AND METHODS

$\alpha_{i2}-/-$ Mice. Age-matched crossbred mice $\alpha_{i2}+/+$ or $\alpha_{i2}-/-$ (5) between 5 and 13 weeks of age were used.

Primary Embryonic Fibroblasts. 129Sv inbred $\alpha_{i2}-/-$ embryos were obtained at 14 days of embryonic development and disaggregated mechanically by repeated passage (three to five times) through a 20 gauge needle in 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (HyClone), at glucose 4.5 mg/ml, penicillin at 100 units/ml, and streptomycin at 100 μg/ml. The mixture was placed into a 10-cm Petri dish with 10–15 ml of medium. Culture was at 37°C in an atmosphere of 5% CO_2/95% air. Nonattached cells and debris were aspirated the following day, and cultures were continued with fresh medium. Cells were subcultured every 3–4 days.

Embryonic 129Sv Fibroblasts Immortalized by Simian Virus 40 (SV40) T Antigen. Primary embryonic fibroblasts at passage 5 were transfected with plasmid pWTSV40 (a gift from John A. Lednicky), which contains the complete genome of wild-type SV40 by the calcium phosphate/glycerol shock method (6). Colonies were picked into 24-well plates and expanded by growing in DMEM containing a high concentration of glucose (4.5 mg/ml) and supplemented with 10% (vol/vol) fetal calf serum, 100 μM 2-mercaptoethanol, penicillin at 100 units/ml, streptomycin at 100 μg/ml, and 2 mM L-glutamine. Cultures were at 37°C in an atmosphere of 5% CO_2.

Homogenate and Membrane Preparations. *Embryonic fibroblasts.* T-antigen-transformed embryonic 129Sv fibroblasts were grown to confluence in 10-mm dishes, washed once with balanced salt solution containing 5 μM $CaCl_2$, 98 μM $MgCl_2$, 540 μM KCl, 120 mM NaCl, 14.5 mM Tris·HCl at pH 7.5, and 0.01% glucose and were removed from dishes with a rubber policeman in the presence of Dulbecco's phosphate-buffered saline (PBS) containing 1 mM EDTA and collected by centrifugation. Cells collected from one 10-cm plate were homogenized in 1.0 ml of homogenization medium [25% (wt/vol) sucrose/1 mM EDTA/10 mM Tris·HCl, pH 7.4) either by using a Dounce homogenizer with a tight pestle (10 strokes) or by passing the suspension 25 times through a 26 gauge needle to ensure thorough shearing of DNA.

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Abbreviations: AC, adenylyl cyclase; SV40, simian virus 40; PTX, pertussis toxin; PIA, phenylisopropyladenosine; PGE_2, prostaglandin E_2.

‡Present address: Institute of Pharmacology, University of Zürich, Winterthurstrasse 190, CH-8057 Zürich, Switzerland.

Thymus. Thymi were excised, placed in 1 ml of homogenization buffer (see above), and homogenized with 10 strokes in a Dounce homogenizer before [^{32}P]ADP-ribosylation.

Heart. Hearts were excised, placed for up to 20 min on ice, and then homogenized with the aid of a Polytron (Brinkmann) with a PT-10ST generator probe at a setting of 5 for 10 sec in 1–2 ml of homogenization buffer (see above). The resulting homogenate was subjected to 10 strokes with a Dounce homogenizer and subsequently diluted 1:3 with homogenization buffer and tested for AC activity without further processing. For [^{32}P]ADP-ribosylation experiments a 1:10 homogenate was used.

Adipose cells. Adipocytes from abdominal, perirenal, and peri-ovaric or epididymal fat from individual animals were prepared by the collagenase method of Rodbell (7). Cells were then suspended in 1 ml of ice-cold adipocyte homogenization medium [10 mM potassium phosphate, pH 8.0/1% defatted bovine serum albumin (BSA; Sigma A 6003)/1 mM NaEDTA/1 mM dithiothreitol/40 μM leupeptin/1 μg of soybean trypsin inhibitor per ml] and homogenized with 20 strokes in a Dounce homogenizer. The homogenate was transferred to a 5-ml polypropylene tube and filled with adipocyte homogenization medium and centrifuged in a Sorvall HB4 rotor at 13,000 rpm (27,000 $\times$ *g*) for 15 min at 4°C. The pellet was resuspended in a 10:1 mixture of adipocyte suspension buffer (25 mM Tris·HCl, pH 7.5/1 mM NaEDTA/1% BSA/40 μM leupeptin/1 μg of soybean trypsin inhibitor per ml) and adipocyte homogenization medium, with the aid of a 1-ml disposable polypropylene syringe fitted with a 22 gauge needle and assayed for AC activity or subjected to ADP-ribosylation.

AC Assays. AC activities were assayed as described by Levy *et al.* (8) in a final volume of 50 μl in the presence of 0.1 mM ATP, 1 mM EDTA, and 2 mM $MgCl_2$. Activity in fibroblast and cardiac homogenates was assayed at 10 and 50 μM GTP, respectively. For adipocyte membranes the assay mixtures contained 100 μM GTP, 100 mM NaCl, and adenosine deaminase (Boehringer Mannheim) at 0.5 unit/ml. Other additives are indicated in the legends to the figures. Incubations were at 32°C for 20 min for adipocyte and fibroblast assays and 30 min for heart assays.

[^{32}P]ADP-Ribosylation Reactions. Homogenates or membranes in 10 μl were [^{32}P]ADP-ribosylated for 30 min at 32°C with preactivated PTX at 8–10 μg/ml and 1 μM [^{32}P]NAD^+ (2–4 $\times$ 10^6 cpm) in a final volume of 30 μl as described (9). The reactions were stopped by the addition of 2× Laemmli's sample buffer (10) containing 10 mM NAD^+. Aliquots of these mixtures were placed directly into the sample wells of polyacrylamide gels and subjected to 4–8 M urea gradient/9% polyacrylamide gel electrophoresis as described previously (11). After electrophoresis the slabs were stained with Coomassie blue, destained, dried, and autoradiographed. Photographs of these autoradiograms are shown in the figures.

Western Analysis of G Protein Subunits. Immunoblotting was performed as described (12), using sample amounts that give signals proportional to the amounts of protein subjected to electrophoretic separation. Antibodies bound to the filters were visualized with iodinated staphylococcal protein A (100,000 cpm/ml final concentration). Specificities of antibodies have been described (13–15).

RESULTS

ADP-Ribosylation Experiments. Labeling of G_i/G_o proteins by ADP-ribosylation followed by electrophoretic separation by SDS/PAGE is probably the most common test used to obtain an initial insight as to the type of PTX-sensitive G proteins that are expressed in a cell. When the ADP-ribosylation is carried out in the presence of [^{32}P]NAD, the PTX substrates become radioactively labeled and are detected by autoradiography. The assay is very sensitive, fast, and easy to perform. The use of a high concentration of urea at constant polyacrylamide or of a double urea and polyacrylamide gradient during the electrophoresis allows for a partial separation of the various ADP-ribosylated PTX substrates. In the case of cells expressing the three G_i α subunits, autoradiograms typically show two bands representing α_{i2} (lower) and the combination of α_{i1} and α_{i3}. In cells expressing in addition α_{o1} and α_{o2}, α_{o1} either comigrates with α_{i2} or migrates between the upper two G_i α subunits and the (lower) α_{i2}, overlapping with the latter, and α_{o2} migrates ahead of α_{i2} (for examples see ref. 11). For the present studies, rather than preparing purified membranes from the tissues we wished to analyze for PTX substrates, we ADP-ribosylated crude homogenates and obtained a semiquantitative measure of relative amounts of the various PTX substrates.

Total cardiac ventricle homogenates displayed a single ADP-ribosylated band migrating between brain α_{i1} and brain α_{o2}, used as standards (Fig. 1). The identity of the cardiac band as α_{i2} was established by it's being absent from homogenates from $\alpha_{i2}-/-$ animals. As seen in whole homogenates, G_{i2} is the predominant PTX substrate in heart. Studies on isolated and washed membranes have shown, however, that cardiac membranes contain, in addition to G_{i2}, G_{i1}/G_{i3} and both G_{o1} and G_{o2} (11, 16). In purified dog ventricle membranes α_{o2} exceeds α_{o1} by a factor of 2 when probed by Western blotting.

Crude adipose cell membranes (of the same type as used in AC assays presented below) showed two ADP-ribosylated bands corresponding to α_{i2} plus the combination of α_{i3} plus α_{i1} (Fig. 2). A similar result was obtained with thymus homogenates (Fig. 3). Adipocytes as well as blood-borne and epithelial cells are thought not to express α_{i1}, so that it is likely that the upper band detected in these ADP-ribosylation experiments represents α_{i3}. Although the ADP-ribosylations were not carried out under conditions where 100% of the substrates were labeled, the conditions were such that the intensity of labeling was proportional to the abundance of the substrate, allowing for some quantitative inferences as to relative abundance of the different substrates in the samples. We found that in adipocytes the labeling intensities of the type 2 and 3 G_i α subunits were approximately equal. On the other hand, in thymus α_{i2} exceeded α_{i3} by a factor of approximately 3 (Figs. 2 and 3). More interesting, however, was the comparison of

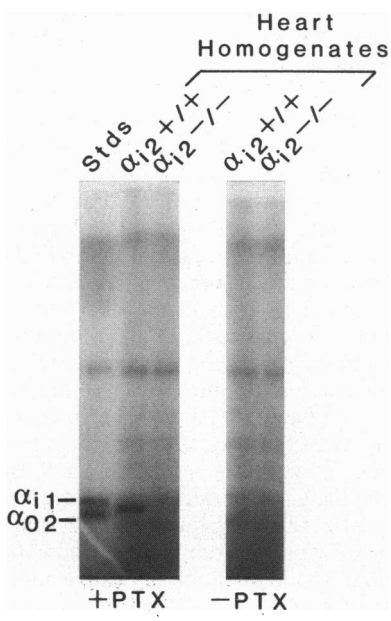

FIG. 1. [^{32}P]ADP-ribosylation of heart homogenates. A mixture of G proteins partially purified from bovine brain which contains α_{i1}, α_{o1}, and α_{o2} was included as a standard. Reactions without PTX served as controls to identify nonspecific labeling.

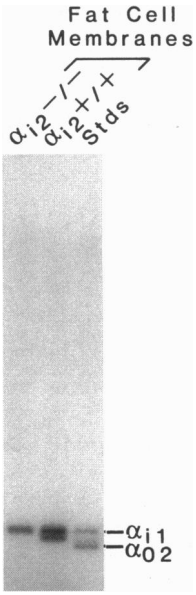

FIG. 2. PTX substrates in adipocyte membranes. G proteins partially purified from bovine brain (see legend to Fig. 1) were used as standards.

labeling intensities obtained in $\alpha_{i2}+/+$ and $\alpha_{i2}+/-$ samples, for it became clear that expression of α_{i2} is sensitive to gene dosage. Thus, both in thymus homogenates and in homogenates from primary embryonic fibroblasts, the labeling intensity of the α_{i2} band appeared to be reduced with respect to that of the α_{i3} band in $\alpha_{i2}+/-$ as compared with $\alpha_{i2}-/-$ samples (Fig. 3). Labeling of skeletal muscle membranes showed two bands in $\alpha_{i2}+/+$ membranes and only one (the upper) in $\alpha_{i2}-/-$ membranes, ruling out the presence of an α_o species (not shown).

Changes in G Protein Subunit Levels Secondary to α_{i2} Gene Disruption in Transformed Fibroblasts. Transformed embryonic fibroblast lines were obtained from both $\alpha_{i2}+/+$ and $\alpha_{i2}-/-$ mice by transfecting the complete SV40 genome into primary embryonic fibroblasts. Individual cell clones were isolated and expanded. For the experiments reported here cells were grown to subconfluence, harvested, and homogenized. Proteins from total homogenates were then separated by SDS/PAGE, transferred to nitrocellulose filters, and probed with subunit-specific antibodies (Fig. 4). A parallel reduction in the abundance of G α (probed with an anti-α_{common} antibody) and G β subunits (probed with a β_{common} antibody that recognizes $\beta 1$ and $\beta 2$) to about 50% of $\alpha_{i2}+/+$ control was seen (Fig. 4*A*) as well as a 30–50% increase in α_{i3} in $\alpha_{i2}-/-$ cell homogenates as compared with $\alpha_{i2}+/+$ cell homogenates (Fig. 4*B*). This indicates that the disruption of the α_{i2} gene can be expected to cause pleotypic changes in cell homeostasis. The findings were identical in two pairs of SV40-transformed fibroblast clones.

AC Regulation Through G_i-Coupled Receptors in G_{i2}-Deficient Mice. Agonist-stimulated inhibition of AC was measured in total homogenates of hearts, isolated adipocytes, and SV40-transformed embryonic fibroblasts. In cardiac homogenates from control $\alpha_{i2}+/+$ animals, carbachol inhibited isoproterenol-stimulated AC by 31% ($n = 12$). This was blunted by 60–70% in homogenates from $\alpha_{i2}-/-$ animals (inhibition by carbachol = 11%; $n = 12$). As shown in Fig. 5, the blunting of the response in $\alpha_{i2}-/-$ homogenates was the result of a loss of maximal effect and not due to a shift in sensitivity to carbachol. A very similar result was obtained when inhibition of fat cell AC was measured in crude membranes prepared from fat cells obtained by collagenase treatment from mesenteric, perirenal, and epididymal or peri-ovaric fat, only in these membranes the responses to three agonists [phenylisopropyladenosine (PIA), acting through A1 adenosine receptors; prostaglandin E_2 (PGE_2), acting through EP3 prostaglandin receptors; and nicotinic acid, acting through nicotinic acid receptors] were measured. As shown in Fig. 6, maximal inhibitions through each of these receptors differed. Inhibition was maximal at 10 μM PIA and PGE_2. In contrast, even at 1

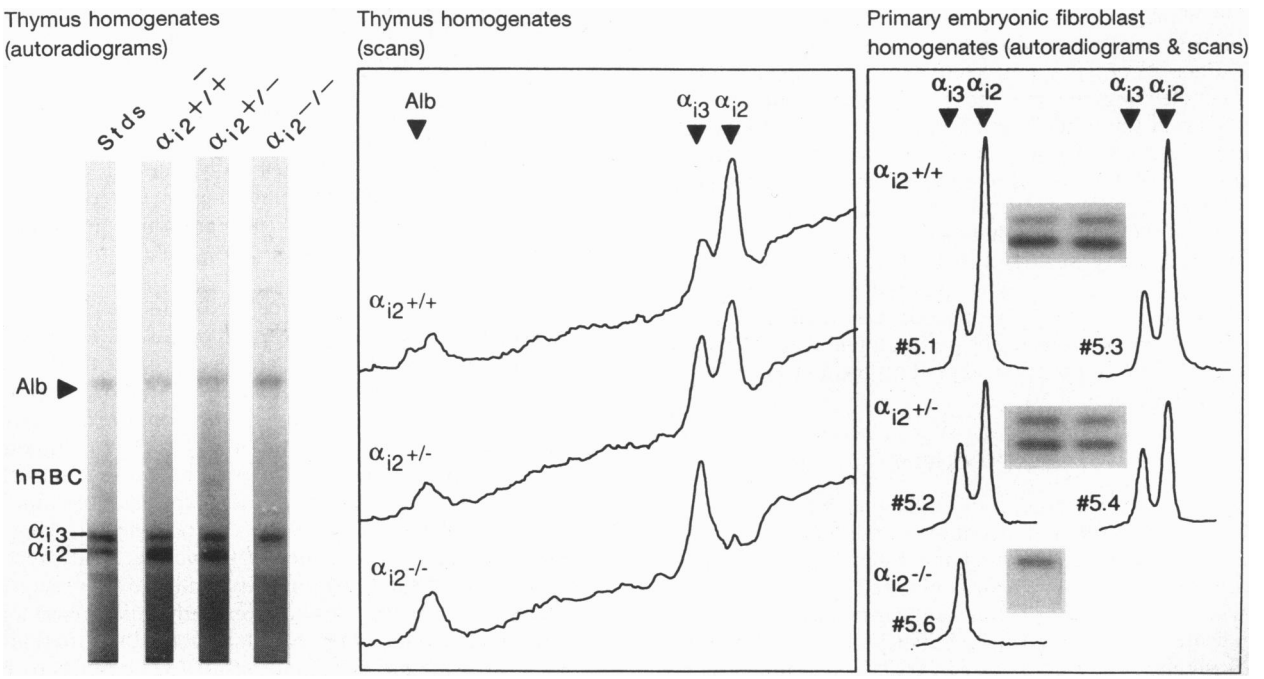

FIG. 3. [^{32}P]ADP-ribosylation of thymus homogenates and primary embryonic fibroblast homogenates. (*Left*) Thymi from genotyped $\alpha_{i2}+/+$, $\alpha_{i2}+/-$, and $\alpha_{i2}-/-$ mice were homogenized and analyzed for PTX substrates. Human red blood cell membranes containing α_{i2} and α_{i3} were used as standards. (*Center*) The autoradiogram shown in *Left* was scanned. The positions of the marker albumin (Alb), α_{i1}, and α_{i3} are indicated. (*Right*) Primary embryonic fibroblasts were obtained from 14-day embryos and subjected to ADP-ribosylation. The regions of interest of an autoradiogram and corresponding scans are shown.

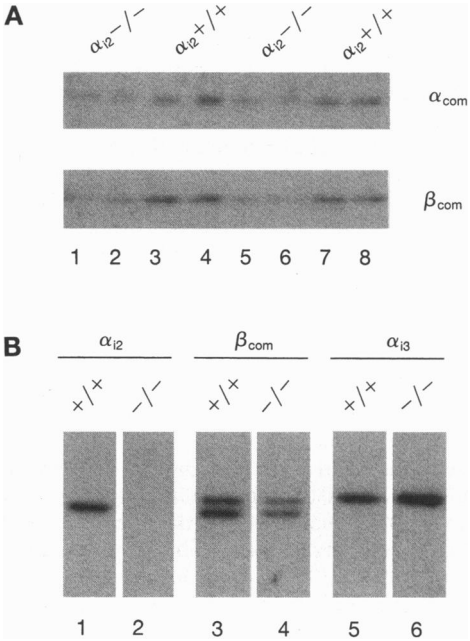

FIG. 4. Comparison of the amount of G protein α and β subunits in homogenates of $\alpha_{i2}-/-$ and $\alpha_{i2}+/+$ embryonic fibroblasts. (*A*) Duplicate samples of either 40 μg (upper gel) or 100 μg (lower gel) homogenate protein from $\alpha_{i2}-/-$ cells (lanes 1 and 2, clone 5; lanes 5 and 6, clone 11) and $\alpha_{i2}+/+$ cells (lanes 3 and 4, clone 7; lanes 7 and 8, clone 10) were subjected to SDS/9% PAGE, transferred to nitrocellulose membranes, and probed with anti-α_{common} antibody AS 8 (upper) or anti-β_{common} antibody AS 11 (lower). Exposure times were 4 days (upper) and 6 days (lower). (*B*) Immunoblotting was performed on 50 μg of protein of $\alpha_{i2}-/-$ and $\alpha_{i2}+/+$ embryonic fibroblast membranes per lane after separation by urea gradient SDS/PAGE (11). After transfer of proteins, nitrocellulose filters were probed with anti-α_{i2} antibody AS 64 (lanes 1 and 2), anti-β_{common} antibody AS 11 (lanes 3 and 4), and anti-α_{i3} antibody AS 86 (lanes 5 and 6). Exposure time to x-ray films was 1 day.

mM, the highest concentration used, the response to nicotinic acid was still increasing. Disruption of the α_{i2} gene resulted in two distinct phenotypes: one, seen in 5 of 10 animals, showed no effect, leaving the inhibitory responses unaltered. The other, seen in the other 5 animals, showed a blunting of the inhibitory response that was about equal for all three receptor systems. All of the $\alpha_{i2}-/-$ animals showing no effect on inhibition of AC in fat cells had a blunted carbachol-induced inhibition of AC in cardiac homogenates.

In parallel assays we found that AC of fibroblasts derived from $\alpha_{i2}+/+$ and $\alpha_{i2}-/-$ are inhibited up to 40–50% in a dose-dependent manner by lysophosphatidic acid and that this inhibition is almost abolished by pretreatment with PTX (data not shown). This supports further that G_{i3} and G_{i1} mediate hormonal inhibition of AC.

DISCUSSION

Three PTX-sensitive families of G proteins have been implicated in receptor-initiated mediation of inhibition of AC: G_{i1}, G_{i2}, and G_{i3}. This is based on the following: (*i*) receptor-mediated inhibition of AC is 90% inhibited by pretreatment of cells with PTX (17–20); (*ii*) transient expression of mutationally activated α_i subunits ($\alpha_{i1/i3}$[Q204L] and α_{i2}[Q205L], but not of similarly activated α_o (α_o[Q205L]), inhibits cAMP accumulation (21); (*iii*) a highly specific antibody against the C terminus of α_{i2} interferes with inhibition of platelet membrane AC by α_2-adrenergic receptors (22); and (*iv*) recombinant guanosine 5′-[γ-thio]triphosphate-activated α_{i1}, α_{i2}, and α_{i2} inhibit AC activity (23). Taken together, these data identify

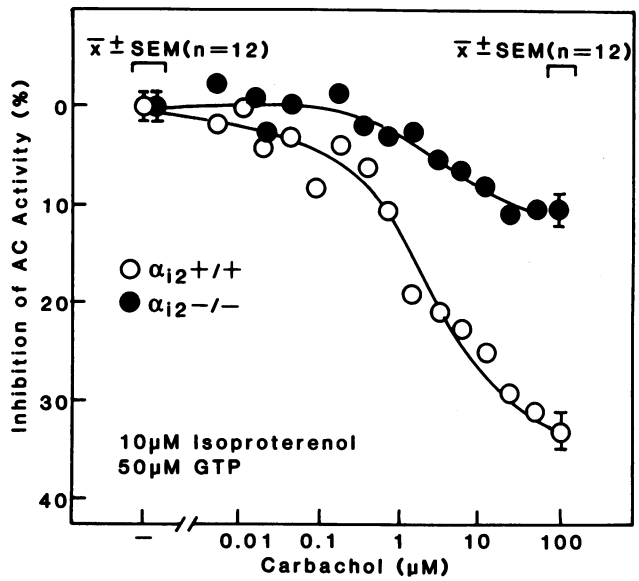

FIG. 5. Inhibition of AC in heart homogenates of $\alpha_{i2}+/+$ and $\alpha_{i2}-/-$ mice. Dose–response curves for inhibition of AC by carbachol were determined in homogenates from four individual mice of each genotype. In addition, isoproterenol-stimulated activity was determined in the absence and presence of saturating concentrations of carbachol in homogenates of eight additional animals of each genotype. The relative inhibition of AC activity is plotted against the concentration of carbachol. ○, $\alpha_{i2}+/+$ mice; ●, $\alpha_{i2}-/-$ mice. The endpoints of each curve were determined in triplicate.

G_{i2} as mediator of AC inhibition and suggest similar roles for G_{i1} and G_{i3} proteins. However, in contrast to G_{i2}, the direct involvement of either G_{i3} or G_{i1} in receptor-mediated inhibition of AC has not been shown for the native proteins. Immunolocalization experiments showed α_{i3} to localize to intracellular membranes, and α_{i3} was proposed to appear on plasma membranes only after overexpression, while α_{i2} was located primarily on what appeared to be cell surface membranes, raising the possibility that α_{i3} may not be physiologically involved in signal transduction at the plasma membrane (24). The present studies demonstrate that α_{i2} couples receptors to the inhibition of AC and that other G proteins do not fully substitute for it *in vivo*. Since the lack of α_{i2} leads to an only partial loss of inhibition of AC, other PTX-sensitive G_i proteins must mediate AC inhibition in genetically altered "native" tissues. On the basis of migration of the remaining PTX substrate in fat cell membranes, this G protein is either G_{i1} or G_{i3} or both.

The high degree of specificity exhibited by M4 and SST receptors for interaction with isoforms of the G_o protein in rat pituitary cells (25–27) stands in contrast to the rather remarkable lack of such specificity exhibited by a series of G protein-coupled receptors that, when expressed in *Xenopus* oocytes, are all able to activate through a G protein-coupled process the oocyte's phospholipase C, a feature that has been used by numerous investigators for expression cloning purposes. Examples of this are the thyrotropin-releasing hormone receptor (28), the metabotropic glutamate receptor (29), the thrombin receptor (30), and the V1-vasopressin receptor (31). Functional studies in mammalian cells indicate further that receptors may even interact with more than one class of G proteins. Suprenant *et al.* (32) described that a point mutation of the α_2 adrenergic receptor uncouples its effect to activate G_o and inhibit Ca^{2+} currents but not to activate G_i and hence K^+ channel stimulation. Likewise, Ashkenazi *et al.* (33) distinguished effects of transfected receptors through PTX-sensitive and nonsensitive G proteins.

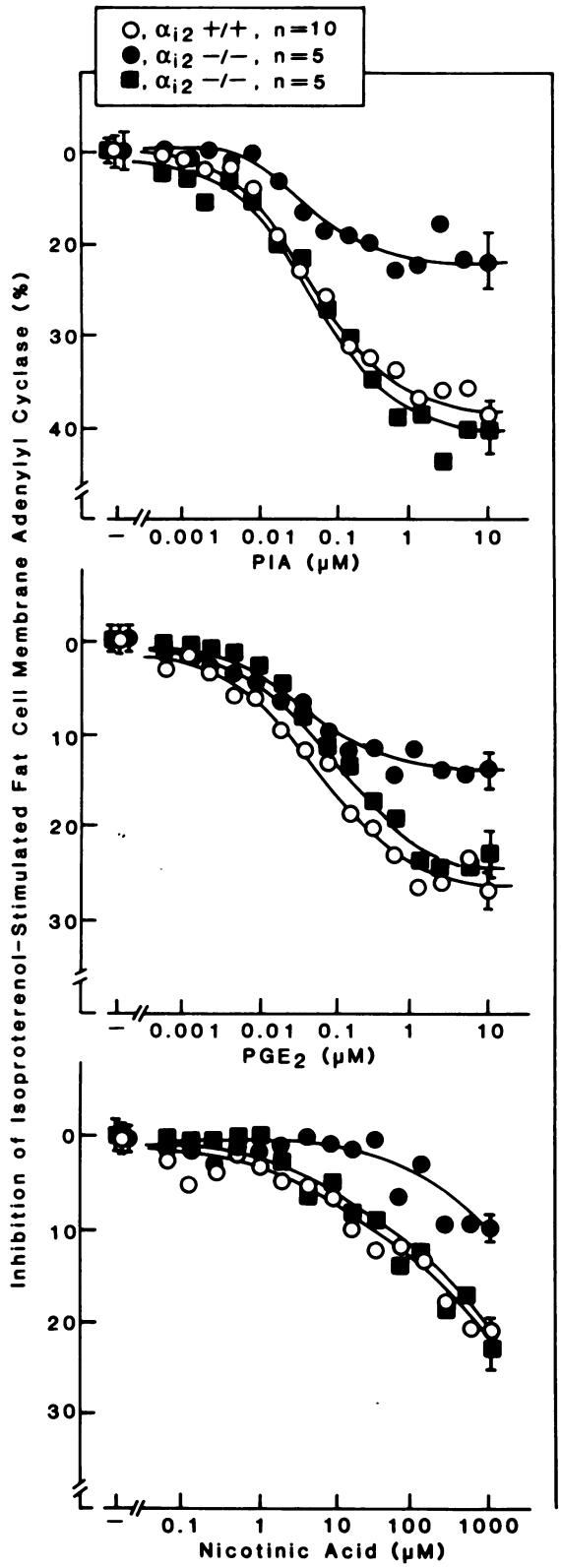

FIG. 6. Inhibition of AC in adipocyte membranes of α_{i2}+/+ and α_{i2}−/− mice. AC activity was determined in 10 mice of each genotype. The stimulated enzyme (10 μM isoproterenol, 100 μM GTP) was inhibited with the indicated hormones. Inhibition of AC activity is plotted against the concentration of the inhibitory hormone. ○, α_{i2}+/+ mice; ■, α_{i2}−/− mice ("nonresponders"); ●, α_{i2}−/− mice ("responders," since they respond to the lack of α_{i2}). The endpoints of each curve were determined in triplicate; bars indicate SEM.

For four receptors we functionally demonstrated their coupling to α_{i2} as well as to other G_i proteins. These findings may indicate either that different subunits may share common receptor recognition determinants or that receptors exhibit subunit selectivity rather than specificity. One possibility is that receptor–G protein interactions are selective, rather than specific, and that selectivity was overcome in previous studies by overexpression; the other is that, within a given subfamily of G proteins, receptors, like effectors, are not very selective and that the selectivity found by Kleuss and collaborators (25–27) is exceptional. It is also possible that the subcellular distribution of the G proteins that a receptor interacts with may help to program specific G protein–effector interactions so that specificity may be achieved at the subcellular level.

We noted that in fibroblasts with reduced total $G\alpha$ there was a parallel reduction in $G\beta\gamma$ (Fig. 4). A similar phenomenon, but in the reverse direction, was described by Spiegel and collaborators (34), who found a coordinated increase in expression of β35 and β36 upon overexpression of G_i and G_q α subunits in NIH 3T3 cells. One explanation for this phenomenon is that $\beta\gamma$ levels are regulated passively by a decrease in degradation rate when combined with α subunits. This mechanism would have the advantage of precluding accumulation of free $\beta\gamma$ dimers and undesired activation of the type 2 and 3 phospholipase C enzymes or over stimulation of the type 2 and 4 ACs every time they are activated by $G_s\alpha$.

An unforseen but perhaps not surprising finding was that lack of a functional G_{i2} prompts compensatory reactions, in our case increased synthesis of α_{i3}, and that phenotypes that result as a consequence of gene inactivation need to be interpreted with caution. The variable penetrance we observed in the effect that inactivation of the α_{i2} gene has on inhibitory regulation of fat cell AC may be due to variable (compensatory) synthesis of α_{i3} (and or α_{i1}). Animals showing a blunting of hormonal inhibition may have compensated less than those in which hormonal inhibition was unaltered, so that in effect the animals with no apparent phenotype at the level of AC inhibition were more affected than those that showed the altered phenotype. The influence of the crossbred genetic background (129SvEv × C57BL/6J) on this phenomenon needs to be determined.

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