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Gαolf Identification by RT-PCR in Purified Normal Pancreatic B Cells and in Islets from Rat Models of Non-insulin-Dependent Diabetes

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Recent reports using immunohistochemistry have shown that Gαolf which shares 88% homology with Gαs was expressed in pancreatic islets. To test the specificity of the expression of this G protein isotype in rat islet cells, B and non-B cells were separated by flow cytometry. The expression of Gαolf and adenylyl cyclases (AC) of types II, III, V, and VI was evaluated by reverse-transcriptase polymerase chain reaction (RT-PCR). Since alterations in the expression of AC III were recently reported in the GK rat (a model of non-insulin-dependent diabetes mellitus, NIDDM), we also have analyzed the mRNA expression of Gαolf and AC isoforms in pancreatic islets from GK rats and from adult rats neonatally treated by streptozotocin (nSTZ rats), another model of NIDDM. Southern blots of amplicons generated with specific primers of Gαolf revealed the presence of a 540-bp band only in B cells. AC of types II, III, V, and VI were expressed both in B and non-B cells. However, AC III mRNA was clearly more abundant in non-B than in B cells. Moreover, in B cells the expression of AC VI was higher than that of AC V, whereas equal expressions of AC V and AC VI were found in non-B cells. In GK rat islets, the mRNA expressions of Gαolf, AC II, and AC III were clearly increased and no change in AC V and AC VI was found. In nSTZ rat islets, Gαolf expression was barely detectable, but AC II and AC III mRNA levels were higher than those observed in controls. In conclusion, Gαolf mRNA appeared specifically expressed in islet B cells and was increased in GK islets. The steady-state mRNA levels of AC II and AC III were clearly increased in the islets of the two rat models of NIDDM. Thus, alterations in the expression of G protein isotypes and AC isoforms could contribute to the diabetic phenotype. © 1999 Academic Press

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Originally described in the neuro-olfactory epithelium coupled to the adenylyl cyclase (AC) of type III (1), Gαolf which shares 88% homology with Gαs was found subsequently in other nervous tissues (2, 3), and non-nervous tissues (4, 5). In our laboratory, using antibodies, the protein was localized by electron microscopy to insulin secretory granules (6, 7) and was already found in the developing human fetal pancreas (8). The recent invalidation of the Gαolf gene in mice suggest that Gαolf is required for olfactory signal transduction and may also function as an essential signaling molecule more centrally in the brain (9). These data did not exclude essential functions in other tissues and/or cells. As yet, identification of mRNA coding for Gαolf in isolated pancreatic islet cells is lacking. Nine mammalian adenylyl cyclase isoforms have been cloned (10), but their expression in islet B cells is also unknown. The mRNA for Type III AC was recently identified by *in situ* hybridization in the rat islet B cell (11). Furthermore, very recently, a new member (OL2) of the olfactory receptor subfamily was cloned (12) showing a high level of expression in the insulin-secreting cell line MIN-6. To ascertain a role of Gαolf in the secretion of insulin by the B cell, it can be hypothesize that some unknown ligand interacting with the serpentine receptor OL2 activates Gαolf, and then AC III to stimulate the insulin release by the B cell.

A potential role of Gαolf in the secretion of insulin may be inferred from alterations in the expression of this G protein isotype in animal models of diabetes. In the present study, (i) purified B and non-B cells were separated by flow cytometry of the dispersed cells obtained from islets of healthy rats; (ii) islets from rats with either inherited (GK rats) or acquired (nSTZ rats) NIDDM were isolated. Then, RT-PCR were performed from total RNA extracted from these cells, and the

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expression of Gαolf, AC II, ACIII, AC V, and AC VI was analyzed. Since AC III is known to be stimulated by the calcium-calmodulin complex and that AC V and AC VI are known to be inhibited by calcium, the expression of these AC in B cells where cellular ionized calcium concentrations are of importance in the control of insulin release, may have physiological relevance.

MATERIALS AND METHODS

Animals. Fed adult female Wistar rats (Proefdierencentrum, Heverlee, Belgium were used to obtain purified preparations of normal B and non-B pancreatic cells. Diabetic GK rats were obtained from our Paris colony (13). Diabetic STZ rats were obtained from newborn Wistar rats by a single dose of STZ injected on the day of birth as previously described (14). The diabetic rats were compared to sex- and age-matched Wistar rats raised in parallel. These animals, all adult (13.2 ± 0.1 weeks old; $n = 17$) male rats were given free access to food and water up to the time of killing. The body weight of GK rats (305.8 ± 7.6 g) was significantly ($P < 0.001$) lower and that of STZ rats (374.7 ± 9.6 g) slightly, but not significantly, lower than that of control rats (406±9.7 g). Both GK and nSTZ rats exhibited a higher ($P < 0.001$) plasma glucose concentration (2.02 ± 0.02 and 1.65 ± 0.06 g/liter, respectively) than control animals (1.11±0.04 g/liter).

Islet preparation and isolation of B cells by flow cytometry. Islets were isolated from pancreas by a collagenase digestion technique (15) and subsequently separated from the remaining nonendocrine tissue by handpicking under a stereomicroscope. Hanks' solution saturated with O_2:CO_2 (95%:5%) was used during the isolation procedure. The islets were immediately utilized for experiments. The method used for preparation of purified B and non-B cell suspensions was already described in detail (16). In brief, after islet isolation by the collagenase technique, digestion was conducted in the presence of 0.5 mg/ml dispase (Boehringer-Mannheim, Mannheim, Germany) at 30°C with mechanical disruption of islets through a 17-G gauge needle, the digestion being stopped when about 60% of the islet cells were present as single cells. Cells were suspended in an Earle's Hepes buffer containing 1.8 mM $CaCl_2$, 5.6 mM glucose and 5 mg/ml bovine serum albumin.

Cells were loaded, during 45 min at 30°C, with fluo-3 AM (Molecular Probes, Eugene, OR) at a final concentration of 2 μmol/liter in the presence of 0.3 μg/ml DNAase (Boehringer-Mannheim). Cell sorting was made on a FACSTAR PLUS flow cytometer (Becton–Dickinson, San Jose, CA) equipped with an Enterprise Argon-ion laser (Coherent, Palo Alto, CA). Data collection and processing were accomplished with Lysis II software. The 488 nm blue line of the laser was used for excitation at 50 mW output power; emission was measured through a 530/30 bandpass filter. Two populations of cells with vastly different fluorescence were observed and gated for sorting.

RT-PCR. Total RNAs were isolated from cells by the guanidium isothiocyanate method (17). The RNA pellets were dissolved in sterile distilled water, and quantified by optical density at 260 nm.

cDNAs were synthesized from 0.125 to 1 μg of total RNA. The reaction mixture had a final volume of 20 μl, and contained 75 mM KCl, 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, nuclease free BSA (1 mg/ml), 10 mM DTT, 20 U of RNAsin, 200 U M-MLV reverse transcriptase (Promega), 1 mM of each dNTP, and 128 pmol of random primers (Promega). Annealing and primer extension were performed at 37°C for 1 h. The reaction was then diluted to 100 μl with the same buffer containing 50 pmol of each specific primer, and 5 units of *Taq* DNA polymerase (Eurobio, Les Ulis-Cedex, France). Amplifications were performed as followed - during 40 cycles for Gαolf (throughout sense: 5′-TTG CCT GAT CGG AGT GCG AGC-3′, and antisense primers: 5′-ACT CAC GCT GTC AAT CCT TTC-3′) with an annealing of 60 s at 49°C; during 27–30 cycles for AC of type II (throughout sense: 5′-TTC CTG CTG CTC ATC GTC ATG-3′, and antisense primers: 5′-GCT TGT GGT AGG CTC CCG CCA-3′) with an annealing of 60 s at 56°C; during 27–30 cycles for AC of type III (throughout sense: 5′-CTG GTG GTC TTT GCG GCC CTC-3′, and antisense primers: 5′-ATG ATG CCC ACG ATG ATG GCG-3′) with an annealing of 60 s at 60°C; during 25 cycles for AC V (throughout sense: 5′-ACC ATT GTG CCC CAC TCC CTG TT-3′, and antisense primers: 5′-TCG TCG CCC AGG CTG TAG TTG AA-3′) with an annealing of 60 s at 60°C; during 25 cycles for AC VI (throughout sense: 5′-CAA AGG AAG GGA CGC CGA GAG G-3′, and antisense primers: 5′-TGG GGA CAG ATC ACG GGA CTA GGA-3′), and during 20 cycles for the GAPDH control (throughout sense: 5′-ATC ACC ATC TTC CAG GAG CG-3′, and antisense primers: 5′-CCT GCT TCA CCA CCT TCT TG-3′) with an annealing of 60 s at 59°C. Control reactions were carried out as previously described (18) in a RoboCycler gradient 96 Temperature Cycler (Stratagene, La Jolla, CA).

Southern analysis. Amplified products were analyzed by electrophoresis in 1.2–2.0% agarose gel, visualized by ethidium bromide, transferred to Gene-Screen plus membranes (NEN). The amplified cDNA was hybridized in each case with specific probes labeled with [^{32}P]dCTP using Terminal Deoxynucleotidyl Transferase (Promega) for oligonucleotide probes or Megaprime DNA labeling system (Amersham, UK) for cDNA probes (AC II, AC III, and GAPDH). The Gαolf probe was the cDNA clone 8 of Dr. Hervé (3) prepared from a Bluescript SK plasmid following treatment by *Eco*RI and *Pvu*I to isolate a 3.2-kb specific insert. A 39-mer oligonucleotide probe specific of Gαolf also was used (5′-GCT ATT GTG ACA ATC ATT TCA GCA ATG AGT ACC ATA ATA CCT-3′).

RESULTS AND DISCUSSION

Expression of Gαolf and AC of types II, III, V, and VI in islet B cells separated by flow cytometry. With the use of specific primers and RT-PCR, we were able to unequivocally identified mRNA for Gαolf in the islet B cells, but not in the islet non-B-cells separated by flow cytometry from islets of adult rats (Fig. 1). A strong signal, in Southern blot, as a single band of the expected size (540 bp) was observed in B cells, in islets, and olfactory bulbs used as a positive control, but not in non-B cells. As shown in Fig. 1, the expression of mRNAs for ACII and ACIII, but not Gαolf, was strong enough to be observed in amplicons stained by ethidium bromide. The AC III mRNA was clearly more expressed in non-B cells than in B cells. AC II appeared expressed both in B and non-B cells of the islet. Unfortunately, the GAPDH probe which was used as a control was specifically expressed in the islet B cells (Fig. 1). Moreover, we show for the first time that mRNAs of AC V and AC VI were present both in B and non-B islet cells (Fig. 2). Moreover, AC VI was more expressed in B cells than AC V. This finding was also observed in pancreatic islets where B cells are more abundant than in other cell types. In non-B islet cells, ACV and AC VI mRNAs were equally expressed. We have used newborn rat hearts as a positive control since AC V expression is known to be higher than that of AC VI in the adult (19); in this respect, the pattern of expression of AC V and AC VI mRNAs in islet B cells from adult rats was in contrast to that observed for the rat heart (Fig. 2).

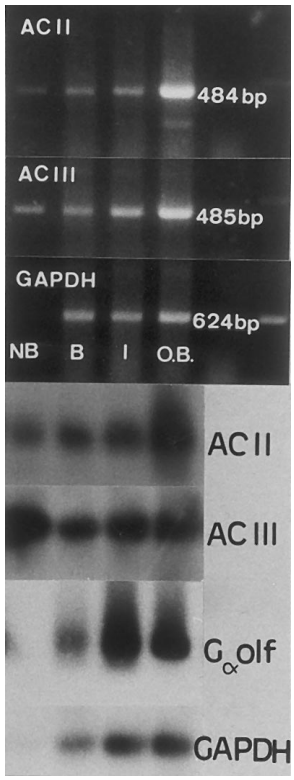

FIG. 1. RT-PCR identification of Gαolf and adenylyl cyclases of type II (AC II) and type III (AC III) in rat pancreatic islets (I), islet B cells (B), and islet non-B cells (NB). Rat olfactory bulbs (O.B.) were used as positive control tissues. At the upper side, products of amplifications were stained by ethidium bromide. At the lower side, Southern blots of the agarose gels seen at the upper side. The GAPDH was a priori used as a control probe.

Expression of Gαolf and AC of types II, III, V, and VI in pancreatic islet cell lines. Our data obtained in normal islet B and non-B cells were extended to pancreatic A and B cell lines. In the mouse βTC3 cell line, the AC of types II, III, V, and VI were expressed (Fig. 3); in agreement with our observations in normal islet B cells, the amount of AC VI mRNA was higher than that of AC V mRNA. The presence of mRNA for Gαolf was also detected by RT-PCR (data not shown) in βTC3

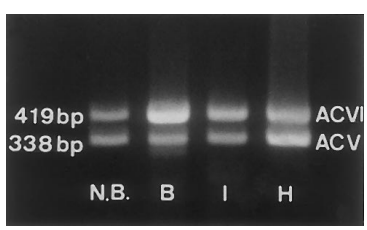

FIG. 2. RT-PCR identification of adenylyl cyclases of type V (ACV) and type VI (AC VI) in rat pancreatic islets (I), islet B cells (B), and islet non-B cells (N.B.). Newborn rat hearts (H) were used as a positive control tissue. The products of amplifications were stained by ethidium bromide after migration in a 2% agarose gel.

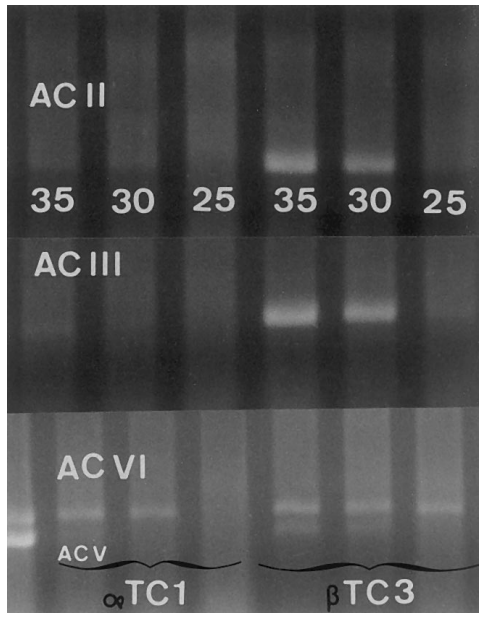

FIG. 3. RT-PCR identification of adenylyl cyclases of type II (AC II), type III (AC III), type V (AC V), and type VI (AC VI) in pancreatic A (αTC1), and B (βTC3) cell lines. The products of amplifications were stained by ethidium bromide. The number of amplification cycles was indicated (25, 30, and 35).

cells. As shown in Fig. 3, the mouse αTC1 cells showed an expression for AC of types II, III, V, and VI, but at a lower level than that found in βTC3 cells. This was in contrast to the findings in normal non-B cells. However, it should be kept in mind that islet non-B cells are not only composed of A cells.

Expression of Gαolf and AC of type II, III, V, and VI in pancreatic islets from GK and nSTZ rats. We found marked alterations in the levels of mRNA coding for Gαolf and AC of types II, and III in the two rat models of NIDDM. In islets of GK rats, Gαolf, AC II, and AC III expressions were greatly increased when compared to control animals (Fig. 4). The specificity of the changes in AC II and AC III mRNAs was assessed by the lack of alterations in AC V and AC VI expressions. In nSTZ rats, no expression of Gαolf was found, even after a long exposure (Fig. 4, right lower side). As shown by amplicons stained by ethidium bromide and by Southern (Fig. 4), mRNA levels for AC II and AC III were increased in nSTZ rats. It has been reported very recently (11) using *in situ* hybridization that AC III expression was increased in pancreatic islets of the GK rat as the result of two point mutations identified in the promoter region of the AC III gene. Our own results for the AC III, using a different approach, are in agreement with those of Abdel-Halim *et al.* (11), but they reveal that other alterations in cellular signaling are also present in GK rats since AC II and Gαolf expressions were increased. In these animals, the B cell mass was reduced by 21% (20) suggesting a specific increase

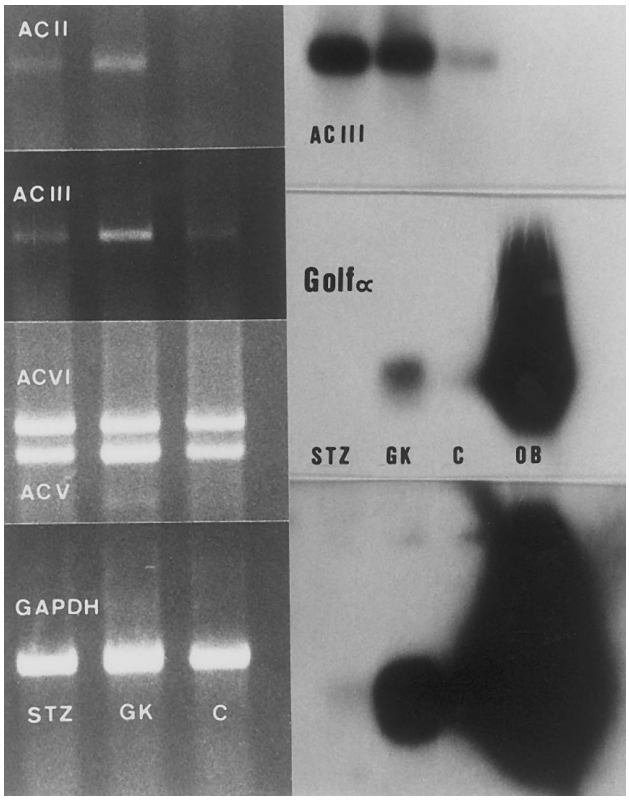

FIG. 4. RT-PCR identification of Gαolf and adenylyl cyclases of type II (AC II), type III (AC III), type V (AC V) and type VI (AC VI) in pancreatic islets of GK rats (GK), and rats treated with streptozotocin (STZ). C: control rats. Rat olfactory bulbs (OB) were used as positive control tissues. On the left, amplifications products were stained by ethidium bromide. On the right, Southern blots of agarose gels. The GAPDH was used as a control probe. Two different times of exposure were shown for Gαolf: a short time in the middle, and a longer time at the lower side.

of ACII and Gαolf in the islet B cell. The elevated mRNA levels for AC II, and AC III found in nSTZ rats in face of half-reduced islet B cell mass (21), may partly reflect the participation of non-B cells in these animals since we have observed that these two AC were expressed in such cells separated by flow cytometry. This increase may argue in favor of a control of both AC II, and AC III expressions by the chronic hyperglycemia prevailing in both GK and nSTZ rats.

In conclusion, alterations in the expression of an heterotrimeric G protein isotype (Gαolf), and two AC isoforms (AC II and AC III) were found in pancreatic islets of diabetic rats; they provide valuable new insights into the role of such cell signaling elements in the control of insulin secretion, and to the pathogenesis of the B cell functional impairment in NIDDM.

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