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Induction of Specific Phosphodiesterase Isoforms by Constitutive Activation of the cAMP Pathway in Autonomous Thyroid Adenomas*

LUCA PERSANI, ANDREA LANIA, LUISELLA ALBERTI, ROBERTO ROMOLI, GIOVANNA MANTOVANI, SEBASTIANO FILETTI, ANNA SPADA, AND MARCO CONTI

Institute of Endocrine Sciences, University of Milan, Istituto Auxologico Italiano IRCCS (L.P., L.A.), and Ospedale Maggiore IRCCS (A.L., R.R., G.M., A.S.), 20145 Milan, Italy; Cattedra di Endocrinologia, Università di Catanzaro (S.F.), 88100 Catanzaro, Italy; and Division of Reproductive Biology, Department of Gynecology and Obstetrics, Stanford University (M.C.), Stanford, California 94305

ABSTRACT

Thyrocytes largely depend on cAMP signaling for replication and differentiation. This pathway may be constitutively activated by mutations of the TSH receptor (TSHR) and $G_s\alpha$ in autonomous thyroid adenomas (ATAs). Because steady state cAMP results from production by adenylyl cyclase and degradation by phosphodiesterases (PDEs), we evaluated PDE activity and expression in ATAs with wild-type and mutant TSHR and $G_s\alpha$. Activating mutations of TSHR and $G_s\alpha$ were identified in 7 and 1 of 18 ATAs, respectively. No difference was observed in the cAMP content in ATAs with or without activating mutants. In the surrounding normal thyroid tissue (NTs), PDE activity was 80% isobutylmethylxanthine sensitive, with the major contribution by PDE1 and a minor contribution by PDE4. No differences were observed in PDE activities between NTs and ATAs with wild-type TSHR and $G_s\alpha$. In contrast, in the presence of mutant TSHRs or $G_s\alpha$, total PDE activity was higher. This increase was primarily due to PDE4 induction (917 ± 116% over NTs), associated with a minor PDE1 increase only in ATAs with mutant TSHR. By RT-PCR, increments of PDE4D and 4C messenger ribonucleic acids were found in the ATAs with mutant TSHR or $G_s\alpha$, whereas messenger ribonucleic acids encoding other cAMP-specific PDEs were not significantly increased. This study provides a characterization of the PDEs expressed in human thyroid and demonstrates a dramatic PDE4 induction in the ATAs bearing mutant TSHR or $G_s\alpha$ genes. The increase in cAMP-degrading activity may represent a marker of constitutive adenylyl cyclase activation and constitutes an intracellular feedback mechanism with significant impact on the phenotypic expression of the activating mutations. (*J Clin Endocrinol Metab* **85:** 2872–2878, 2000)

ACTIVATION of the cAMP pathway is involved in the regulation of cell replication and differentiation in the thyroid gland (1, 2). Intracellular cAMP levels are the result of the steady state of the activity of two different enzymatic systems. Although adenylyl cyclase is the effector enzyme accounting for the cyclic nucleotide production by converting ATP to cAMP, phosphodiesterases (PDEs) account for the cyclic nucleotide degradation by hydrolyzing-cAMP to 5′-AMP (3). PDEs are a large group of isoenzymes encoded by at least 21 different genes and organized into 10 families depending on their biochemical and pharmacological properties, such as substrate affinity (cAMP or cGMP) or sensitivity to specific inhibitors (3–8). Among the more recent discoveries in this field, the database search of expressed sequence tags allowed the identification and cloning of a novel isobutylmethylxanthine (IBMX)-insensitive cAMP-specific PDE with specific thyroid expression, named PDE8B (5).

Studies using *in vitro* experimental systems have provided evidence that some PDE isoforms are regulated by hormones or other stimuli via changes in intracellular cyclic nucleotide levels. In particular, increases in cAMP levels in FRTL-5 cells after TSH treatment result in short-term activation of cAMP-specific PDEs (such as PDE4) via protein kinase A-induced phosphorylation and in long-term activation via gene expression and protein synthesis regulation (9–11). An increase in PDE cAMP-degrading activity also has recently been demonstrated in FRTL-5 cells expressing a constitutively active $G_s\alpha$ protein (12, 13). This finding opens the possibility that the increased cAMP hydrolysis caused by PDE activation, if occurring in human thyroid cells, may have a great impact on the *in vivo* phenotypic expression of natural mutations that constitutively activate the cAMP pathway.

In human thyroid tissue, the ligand-independent activation of the adenylyl cyclase may be caused by mutations affecting two elements of the cAMP pathway, the TSH receptor, or the $G_s\alpha$ protein itself (14, 15). Indeed, somatic mutations in the TSH receptor gene are a frequent finding in autonomous thyroid adenomas (ATAs) (16–21), whereas somatic mutations of the $G_s\alpha$ gene have been identified in a

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Address all correspondence and requests for reprints to: Luca Persani, M.D., Ph.D., Laboratorio di Ricerche Endocrinologiche, Istituto Auxologico Italiano IRCCS, Via Ariosto 13, 20145 Milan, Italy. E-mail: persani@auxologico.it.

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small subset of these tumors (15, 16, 22). However, no phenotypical differences have yet been described between ATAs with or without the constitutive activation of the cAMP cascade.

In this study we investigate the pattern of activity and expression of potentially relevant PDEs in the normal thyroid and in ATAs with and without gain of function mutations of TSH receptors or $G_s\alpha$. Moreover, we provide biochemical and molecular evidence for the induction of specific PDE isoforms in the presence of constitutive activation of the cAMP cascade induced by these oncogenes.

Materials and Methods

Thyroid tissue samples

Eighteen ATAs and the surrounding normal thyroid tissue (NT) were collected at surgery and included in the study. All ATAs were diagnosed on the basis of the patients' clinical hyperthyroid features, suppressed TSH secretion, and borderline high or elevated free thyroid hormone levels, in the absence of antithyroid autoantibodies and in the presence of a single capsulated nodule at ultrasound examination. The diagnosis was confirmed when ^{99}Tc uptake confined to the nodular tissue and suppression of the surrounding parenchyma were seen. After adenomectomy, all tissue was quickly frozen for genetic characterization, determination of cAMP content, and studies of PDE activity and expression. NT obtained from four patients who underwent total thyroidectomy for thyroid carcinoma was also included in the study. Local ethical approval was obtained for all studies.

Analysis of mutations in $G_s\alpha$ and TSH receptor genes

Genomic DNA and total ribonucleic acid (RNA) were obtained from tissue homogenates by acid guanidine thiocyanate-phenol chloroform extraction, as previously described (23). Sequence analyses of $G_s\alpha$ and TSH receptor genes were directly performed by the dideoxynucleotide method and by automatic techniques (ABI Prism 310, Perkin-Elmer Corp., Norwalk, CT) on PCR products obtained from adenomatous tissue genomic DNA, or cDNA. The hot spots of the $G_s\alpha$ gene were amplified using intronic oligonucleotide primers, as previously described (24). The 5th to 10th exons of the TSH receptor gene were amplified either from genomic DNA or cDNA, using the following set of oligonucleotide primers: 5–10U, 5′-TGG ACT TAA AAT GTT CCC T-3′; and 5–10L, 5′-AGG CAT TCA CAG ATT TTC TCT GGC-3′ [annealing temperature (Ta), 50 C]; E9U, 5′-AAG CCA CTG CTG TGC CTT TA-3′; and 1264L, 5′-GGG TTG AAC TCA TCG GAC TT-3′ (Ta, 48 C); 1161U, 5′-CCC AGG AAG AGA CTC TAC AAG-3′; and 1535L, 5′-TGC AAA GAC AGT GAA GAA ACC A-3′ (Ta, 58 C); 1409U, CTG GCC TTT GCG GAT TTC TGC-3′; and 1876L, 5′-TTG TCC CCT GGG TTG TAC TGC G-3′ (Ta, 66 C); 1649U, 5′-AGG CAC GCA TGT GCC ATC ATG-3′; and 2084L, 5′-CCT CTG GAA GGC CTT GGT GAA-3′ (Ta, 64 C); 1876U, CGC AGT ACA ACC CAG GGG ACA A-3′; and 2355L, 5′-TTC CCC TAC CAT TGT GAG-3′ (Ta, 59 C). The intronic oligonucleotide primers for the amplification of 9th and 10th exons had been previously reported by others (25). PCR products were obtained in a 50-μL reaction mixture containing 0.1–1 μg DNA, 0.5–1 U *Taq* polymerase (Perkin-Elmer Corp./Cetus), 10–20 pmol of each primer, 200 mmol/L deoxy-NTPs, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 1.5–2.0 mmol/L $MgCl_2$. Each sequence was checked using either the upstream or downstream primers on two different PCR products.

cAMP content assay

Frozen tissue (~30 mg) was homogenized on ice using a glass-Teflon Potter homogenizer in 6% (wt/vol) trichloroacetic acid. The homogenate was centrifuged at 2000 × *g* for 15 min at 4 C, and subsequently the pellet was discarded. The supernatant was washed four times with 5 vol water-saturated diethyl ether, and the upper ether layer was discarded after each wash. The remaining aqueous extracts were evaporated and resuspended in cAMP kit buffer (0.25 mL). cAMP was measured in nonacetylated homogenate samples, using a commercial RIA kit (NEN Life Science Products, Boston, MA). The minimum detection limit was 0.1 nmol/L for nonacetylated samples; cross-reaction with cGMP was less than 0.001%. The intra- and interassay coefficients of variations were less than 6% and less than 10%, respectively.

PDE assay

ATAs and the corresponding NTs (~30 mg) were homogenized using a glass-Teflon Potter homogenizer in 20 mmol/L Tris-HCl (pH 8) containing 5 mmol/L 2-mercaptoethanol, 10 mmol/L NaF, 1 mmol/L ethylenediamine tetraacetate, 0.2 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 2 mmol/L phenylmethylsulfonylfluoride, 0.2 μg/mL leupeptin, 0.7 μg/mL pepstatin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, and 7.8 mg/mL benzamidine. Tissue and homogenates were always kept in ice, and an aliquot (5 μL) of the fresh homogenate was immediately assayed for PDE activity in the absence or presence of nonspecific (IBMX, 1 mmol/L) or specific [8-methoxy-IBMX (50 μmol/L) for calmodulin (CaM)-dependent PDE1; rolipram (10 μmol/L) for cAMP-specific PDE4] pharmacological inhibitors. The PDE assay was performed as previously described (12, 26) in 200 μL reaction mixture containing 40 mmol/L Tris-0.1% BSA, 10 mmol/L $MgCl_2$, 5 mmol/L 2-mercaptoethanol, 1 μmol/L cAMP, and 1 μmol/L [^{3}H]cAMP (0.1 μCi). After incubation at 34 C for 10 min, the reaction was terminated by adding an equal volume of 40 mmol/L Tris-HCl buffer, pH 7.5, containing 10 mmol/L ethylenediamine tetraacetate followed by heat denaturation for 1 min. To each reaction tube, 50 μg *Crotalus atrox* snake venom were added, and the incubation was continued at 34 C for 15 min. The reaction products were separated by anion exchange chromatography on AG 1-X8 resin (100–200 mesh; Bio-Rad Laboratories, Inc., Hercules, CA), and the amount of radiolabeled adenosine collected was quantitated by scintillation counting. In each case, data were corrected for the amount of protein present in the extracts. Protein content was measured by the bicinchinonic assay (Pierce Chemical Co., Rockford, IL). The contributions of the four different PDE4 enzyme isoforms were assessed by testing the homogenates after immunoprecipitation with specific antibodies (K112, nonselective polyclonal Ab for all PDE4 enzymes; AC55, polyclonal Ab against PDE4A isoforms; K118, polyclonal Ab against PDE4B isoforms; M3S1, monoclonal Ab against PDE4D isoforms); finally, the amount of PDE4C isoforms in one sample was estimated by the following formula: K112/PDE4 − (AC55/PDE4A + K118/PDE4B + M3S1/PDE4D) (9, 27). The antibodies were bound to protein A (polyclonals) or G (monoclonal antibody) Sepharose beads in PBS-0.05% BSA, pH 7.4; the suspension was incubated at 4 C in rotation for 90 min. Then, the PDE-Ab-beads complex was washed twice with fresh buffer, and after resuspension in 40 mmol/L Tris-0.1% BSA, an aliquot (5 μL) of the pellet was immediately tested for PDE activity.

cAMP-specific PDE messenger RNA (mRNA) expression

The mRNA expression of the four PDE4 genes and the PDE8B gene was studied by means of a semiquantitative RT-PCR method, using amplification of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene for the normalization of the data, as previously described (28). Poly(A^+) RNA was prepared from ATAs and surrounding NTs using a commercial kit (Amersham Pharmacia Biotech, Piscataway, NJ) and was subjected to deoxyribonuclease digestion. Specific amplification of the four PDE4 transcripts was obtained by means of primers located at the 3′-end of each gene; these regions encode the C-terminal portion of the proteins downstream from the catalytic domain and are not affected by the various splicing variants of each gene product (3, 4). For each cDNA, preliminary experiments with different sets of primers (at least two sets for each cDNA) were conducted to determine the PCR cycles corresponding to the exponential phase. The sequences of the selected oligonucleotides were: PDE4A: CT0s, 5′-ATC AAT GGC CCA GAT ACC-3′; and CT0as, 5′-ACA GGG ACA GAG GTC TG-3′ (Ta, 54 C; PCR product, 518 bp); PDE4B: CT1s, 5′-ATT CTG AAG GAC CTG AGA-3′; and CT2as, 5′-GTG TCT TGA TCA GAA TCG-3′ (Ta, 54 C; 404 bp); PDE4C: CT2s, 5′-GCC TGA CAG ATT CCA GTT-3′; and CT2as, 5′-ATT CCT AAG TCC TCT GGT T-3′ (Ta, 52 C; 197 bp); PDE4D: CT0s, 5′-TGG TGA GTC AGA CAC GGA-3′; and CT1as, 5′-AGT TTT TGC ACT GTT ACG TG-3′ (Ta, 54 C; 215 bp); and PDE8B: S3, 5′-AAC AGC CCA GTC ACA GTA GC-3′; and AS3, 5′-ACC TTT AAG CCC AGA TAA

ACC A-3′ (Ta, 62 C, 344 bp). Each reaction was preceded by 3 min of denaturation at 94 C, followed by a hot start; amplifications of PDE4A, -4B, and -4C were run for 30 cycles, those of PDE4D, PDE8B, and GAPDH were run for 25, 28, and 23 cycles, respectively (each cycle: 93 C for 1 min, Ta for 45 s; 72 C for 1 min). No product was obtained when the nonretrotranscribed materials were subjected to PCR amplification. The identities of the PCR products were confirmed by direct automatic sequencing analysis. The amount of each amplification product was determined at densitometer analysis (Multianalyst software, Bio-Rad Laboratories, Inc.) on agarose gel stained with ethidium bromide, and the PDE/GAPDH ratios were calculated for each sample.

Statistical analyses

The results are expressed as the mean ± SD. Paired or unpaired two-tailed Student's *t* test was used to detect significance between two series of data. $P < 0.05$ was accepted as statistically significant.

Results

Analysis of mutations in $G_s\alpha$ and TSH receptor genes

Mutations known to cause the constitutive activation of cAMP-dependent pathway were found in 8 of 18 cases. Heterozygote mutations in the TSH receptor gene were found in 7 adenomas (cases 1–7): 6 ATAs had previously reported mutations [the single nucleotide substitution G to A at codon 281 (S281N) in ATA 4; the substitution T to C at codon 453 (M453T) in ATAs 1, 2, and 6; the substitution A to T at codon 486 (I486F) in ATAs 3 and 5], whereas 1 (ATA 7) showed a novel T to A substitution at codon 631 (F631I) in the VI transmembrane domain (Fig. 1). In 3 different experiments, the F631I mutant exhibited a cAMP accumulation 2.0 ± 0.1-fold higher than the wild-type TSH receptor when transfected in COS7 cells (data not shown). One adenoma was found to harbor the $G_s\alpha$ mutant (ATA 8), with the substitution A to G at codon 227 (Q227R) in the heterozygote state.

cAMP content

The cAMP concentrations in the homogenates from normal thyroid tissue obtained at thyroidectomy for thyroid carcinoma and ATAs were largely overlapping. Moreover, no significant differences were found among the cAMP levels in NTs and ATAs regardless of whether they had mutant TSH receptors or $G_s\alpha$, although slightly higher cAMP concentrations were seen in the group of ATAs bearing a mutant TSH receptor [NTs, 3.7 ± 1.2 pmol/mg protein (n = 4); range, 2.3–4.7; ATAs with wild-type TSH receptor or $G_s\alpha$, 3.7 ± 1.1 (n = 4); range, 2.7–5.1; ATAs with mutant TSH receptor, 5.0 ± 1.6 (n = 4); range, 3.3–6.9].

PDE activity

The total PDE activity in unfractionated homogenate of the normal thyroid tissue surrounding the ATAs was 43.2 ± 10.2 (±SD) pmol/min·mg protein (range, 27.8–62.0). This activity was mostly IBMX sensitive (31.3 ± 7.6 pmol/min·mg protein; range, 19.2–47.4); in all cases, however, a significant fraction of total PDE activity was IBMX insensitive (28 ± 10%; range, 14–44%). The activity sensitive to 8-methoxy-IBMX (calcium-CaM-dependent PDE1) was 11.4 ± 4.8 pmol/min·mg protein (range, 3.8–18.2), whereas 7.8 ± 3.3 pmol/min·mg protein (range, 4.9–14.8) were rolipram sensitive (cAMP-specific PDE4); PDE1 accounted for 40 ± 22%, and PDE4 accounted for 26 ± 10% of IBMX-sensitive activity, respectively. A similar pattern of PDE activities was found in the normal tissue removed at surgery for thyroid cancer (data not shown).

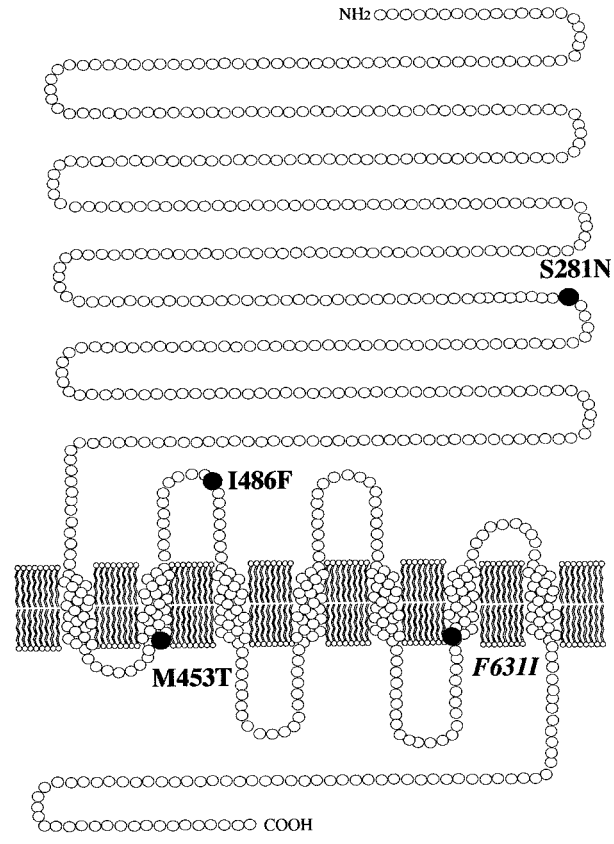

FIG. 1. Schematic representation of TSH receptor with the localization of the gain of function mutations reported in this study. Seven of 18 ATAs were bearing a TSH receptor mutation leading to the constitutive activation of cAMP signaling pathway. One ATA was bearing the S281N in the extracellular domain (21), 3 ATAs were bearing the M453T in the II transmembrane (20), 2 ATAs were bearing the I486F in the I extracellular loop (16), and 1 ATA was bearing the new substitution F631I. Only 1 of 18 adenomas was bearing a mutant $G_s\alpha$ (Q227R).

ATAs lacking activating mutations of cAMP pathway had PDE activities similar to those of their surrounding NTs (Fig. 2). In the ATAs bearing constitutively active mutant TSH receptors, the total PDE activities were always higher than those of surrounding NTs (248 ± 26% over NTs; range, 209–289%). This increase in total PDEs was due to the induction of both IBMX-sensitive (264 ± 37%; range, 214–327%) and insensitive PDE activities (205 ± 74%; range, 116–325%). As far as IBMX-sensitive activity is concerned, in these adenomas we observed a dramatic induction of rolipram-sensitive PDE4 (917 ± 116% over NTs; range, 745-1085%) that was associated with a significant increase in PDE1 activity (238 ± 30%; range, 211–292%; Fig. 2). All of these PDE activities were higher than those observed in the other adenomas without mutant TSH receptors and $G_s\alpha$. Contrary to observations of the adenomas bearing the mutant TSH receptors in the homogenate of the ATA bearing the mutant $G_s\alpha$, the high increase in IBMX-sensitive PDE activity

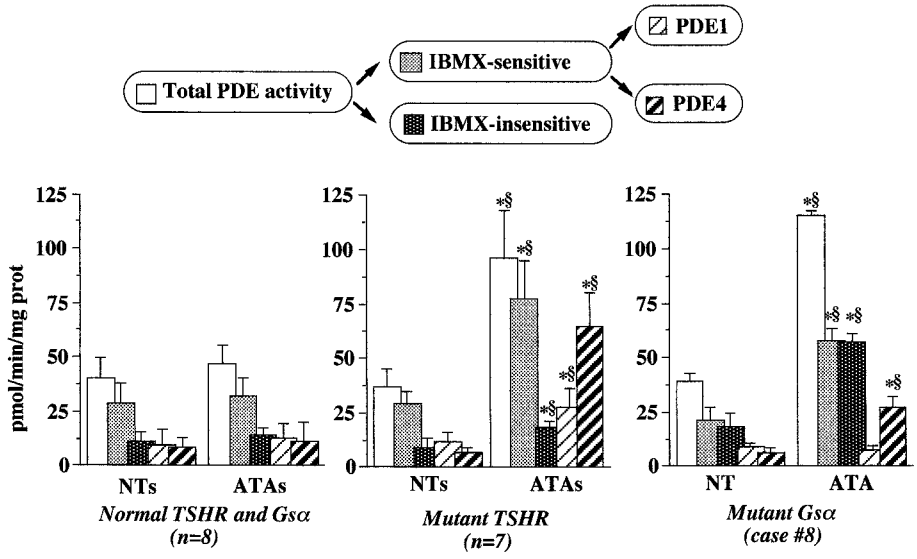

FIG. 2. Pharmacological characterization of cAMP-hydrolyzing activity in tissue homogenates evaluated by means of nonselective (IBMX, 1 mM) or selective inhibitors (8-methoxy-IBMX, 50 μM, for CaM-dependent PDE1; rolipram, 10 μM, for cAMP-specific PDE4). Each homogenate was tested in triplicate, as described in *Materials and Methods*. Data are reported as the mean ± SD in the different groups. *, $P < 0.05$ *vs.* the surrounding normal tissue (paired *t* test analysis); §, $P < 0.05$ *vs.* the adenomas with normal TSH receptor and $G_s\alpha$ (unpaired *t* test analysis).

(293% over NT) was exclusively due to PDE4; the level of PDE1 was similar to that in the homogenate of the normal surrounding tissue (ATA *vs.* NT, 7.5 ± 1.9 *vs.* 8.7 ± 2.0 pmol/min·mg protein; P = NS; Fig. 2).

In cases 1 and 7, the different isoforms contributing to the rolipram-sensitive PDE activity were evaluated after immunoprecipitation by means of specific antibodies (Fig. 3). The PDE4 activity recovered after immunoprecipitation with K112, an antibody recognizing all PDE4 isoforms, confirmed the dramatic increase observed in ATAs bearing the ligand-independent activation of adenylyl cyclase (850% and 466% over NTs, in cases 1 and 7, respectively). The main contribution of PDE4D was documented in both cases by immunoprecipitation with a PDE4D-specific antibody (M3S1; 976% and 523% over NTs), whereas a residual PDE4C activity could be demonstrated only in the adenomatous tissue. In these two ATAs, minor increases in PDE4A and PDE4B activities were also observed (Fig. 3).

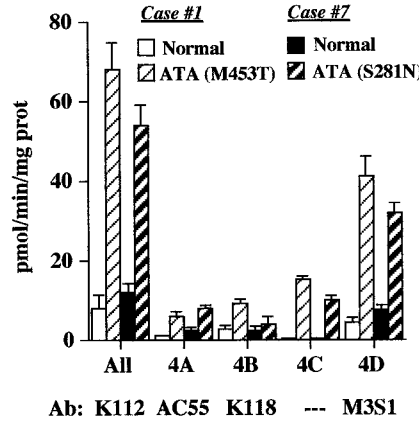

FIG. 3. Molecular characterization of cAMP-specific PDE4 activity in the case of two adenomas with mutant TSH receptor and their surrounding normal tissues. The contributions of the four different PDE4 enzyme isoforms was assessed by testing the homogenates after immunoprecipitation with specific antibodies [K112, nonselective Ab for all PDE4 enzymes (All); AC55, specific Ab for PDE4A isoforms (A); K118, specific Ab for PDE4B isoforms (B); M3S1, specific Ab for PDE4D isoforms (D); the amount of PDE4C isoforms (C) in one sample was then estimated by the following formula: K112/PDE4 − (AC55/PDE4A + K118/PDE4B + M3S1/PDE4D)]. Each *bar* represents the mean ± SD of triplicate determinations.

cAMP-specific PDE mRNA levels

cAMP-specific PDE4 mRNA levels were evaluated by means of densitometric analysis in five cases with mutant TSH receptors (no. 1–5) and in three cases (no. 9, 12, and 15) with wild-type TSH receptors and $G_s\alpha$ (Fig. 4). The data are expressed as the ratio (×1000) between the PDE fragments and GADPH. In the case of the ATAs with normal TSH receptors and $G_s\alpha$, no significant difference (P = NS) was observed between NTs and ATAs for all PDE4 mRNA levels (Table 1). In the cases with mutant TSH receptors, significant differences were observed for 4C and 4D mRNA levels (Table 1). When the statistical analysis was performed between the ATAs with or without mutant TSH receptors, significant differences were found only in the case of PDE4D ($P = 0.008$).

The mRNA levels of cAMP-specific PDE8B have been evaluated in cases 1 and 4, bearing two different mutant TSH receptors (M453T and S281N) in the ATA. No significant differences were detected between the ATAs and surrounding NTs (no. 1: NT, 848; ATA, 881; no. 4: NT, 811; ATA, 864; Table 1).

Discussion

This study describes the activity and expression of the most relevant PDEs in normal and adenomatous human thyroid tissue and provides evidence for a dramatic induction of the cAMP-specific PDE type 4 in the autonomous thyroid adenomas associated with mutations leading to the constitutive activation of the cAMP pathway. Contrary to observations in FRTL-5 cells (12) in which the CaM-dependent PDE1 activity is a minor component, in the normal thyroid the CaM-dependent PDE1 accounts for a large portion of total PDE activities, whereas cAMP-specific PDE4 were minor components of the PDE activity. Moreover, in normal tissue a relevant role is played by

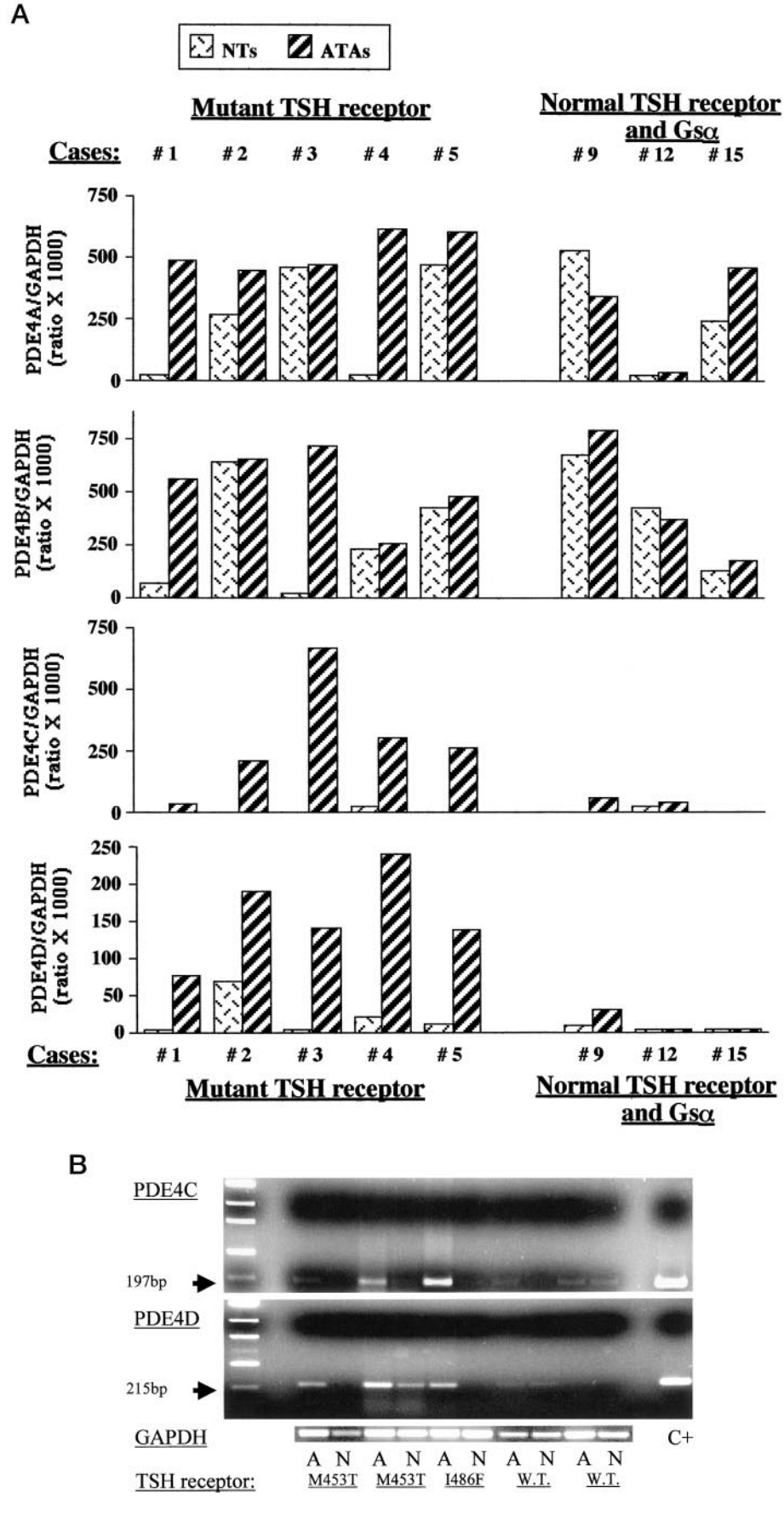

FIG. 4. A, Densitometer analysis of RT-PCR products obtained using oligonucleotides specific for the amplification of PDE4A, PDE4B, PDE4C, PDE4D, and GAPDH mRNAs in eight cases. Each bar represents the ratio × 1000 between the densitometric values (counts per mm^2) of the amplification products of the four PDE4 and GAPDH in eight ATAs and surrounding normal tissues (NT). Five of these ATAs (no. 1–5) were bearing a mutant TSH receptor, whereas normal TSH receptor and $G_s\alpha$ were present in the remaining three (no. 9, 12, and 15). The identity of each RT-PCR product was confirmed by sequencing analysis. B, Agarose gel stained with ethidium bromide of RT-PCR products obtained using oligonucleotides specific for the amplification of PDE4C, PDE4D, and GAPDH mRNAs in five representative adenomas (A) and surrounding normal tissues (N). Sequence analysis revealed the presence of mutant TSH receptor (two with M453T and one with I486F) in three of these adenomas, whereas wild-type TSH receptor and $G_s\alpha$ were present in the remaining two. PDE4C and 4D cDNAs included in expression vectors were used as a positive control (C+).

TABLE 1. Results of densitometric analysis of cAMP-specific PDE mRNA levels in three cases with normal TSH receptor and $G\alpha_s$ and in five cases with mutant TSH receptor

mRNA	Cases with normal TSH receptor (n = 3)		Cases with mutant TSH receptor (n = 5)	
	Normal tissues	ATAs	Normal tissues	ATAs
PDE4A	267 ± 253	280 ± 216[a]	249 ± 220	524 ± 79[b]
PDE4B	817 ± 546	888 ± 627[a]	554 ± 517	1064 ± 359[a]
PDE4C	8 ± 14	35 ± 31[a]	5 ± 11	297 ± 233[c]
PDE4D	3 ± 6	10 ± 17[a]	21 ± 29	157 ± 61[d]
PDE8B[e]			830 ± 26	873 ± 12[a]

Data (mean ± SD) are expressed as the ratio (×1000) between the PDE fragments and GAPDH (see *Materials and Methods*).
[a] $P > 0.1$ *vs.* corresponding normal tissues.
[b] $P = 0.063$ *vs.* corresponding normal tissues.
[c] $P = 0.048$ *vs.* corresponding normal tissues.
[d] $P = 0.004$ *vs.* corresponding normal tissues. $P = 0.008$ *vs.* ATAs with normal TSH receptor.
[e] Evaluated only in cases 1 and 4.

IBMX-resistant PDEs, such as the recently identified thyroid-specific PDE8B (5).

The pattern of PDE activity in the normal thyroid was maintained in the ATAs expressing wild-type TSH receptors and $G_s\alpha$, although it was dramatically modified in the presence of mutations in these genes. Consistent with the high prevalence of mutations in the TSH receptor gene and with the rarity of those located in the two hot spots of the $G_s\alpha$ gene (16, 22), 7 of 18 ATAs of the present series expressed mutant TSH receptors and only 1 mutant $G_s\alpha$. All substitutions had been previously demonstrated to constitutively activate the cAMP cascade (16–21), with the exception of the F631I mutation, which is a new substitution (Ile for Phe) at a codon already reported to be affected by other substitutions (Leu or Cys for Phe) (19). The ligand-independent activity of the F631I mutant was similar to that reported for previous missense substitutions (29). In ATAs with mutant $G_s\alpha$ or TSH receptors, a 2- to 3-fold increase in total PDE activity was observed. This increase was due largely to the dramatic induction of PDE4 activity, with a 10-fold higher activity than that measured in the homogenates of the surrounding normal tissue. The increase in PDE activities is in agreement with data obtained in human GH-secreting pituitary adenomas (24) or FRTL-5 cells (12) expressing mutant $G_s\alpha$. Moreover, PDE up-regulation is likely to have a significant impact on the phenotype of cells expressing these activating mutations, as the increased cAMP degradation may account for the large overlap of cAMP content between normal thyroid samples and the ATAs bearing, or not, the mutant TSH receptor or $G_s\alpha$. The cAMP-dependent stimulation of PDE4 may be the result of a series of phosphorylation processes and/or of the induction of gene transcription (9–11). Semiquantitative RT-PCR experiments suggest that the stimulation of cAMP-specific PDE4 is the consequence of an increased mRNA steady state and protein synthesis, at least in the case of PDE isoforms encoded by the 4C and 4D genes. The PDE4C mRNAs were almost undetectable in the normal tissue and in the ATAs with wild-type TSH receptors and $G_s\alpha$, although the PDE4D transcripts were constantly abundant in the ATAs with mutant TSH receptors or $G_s\alpha$ and were low in the adenomas not expressing these mutants. These results are consistent with data obtained by studying the different isoforms at the protein level. Indeed, in the ATAs with the constitutive activation of adenylyl cyclase, about 60% of the PDE4 cAMP-degrading activity was immunoprecipitated with the Ab specific for the PDE4D isoforms, although the activity attributable to PDE4C isoforms reached about 20%; minor changes were seen for the activities immunoprecipitated with the Abs specific for the PDE4A and -4B isoforms. These results are consistent with the data showing 1) a marked cAMP inducibility of PDE4D expression and the presence of cAMP-responsive elements in the promoter region of PDE4D gene (10, 30), and 2) a modest cAMP effect on the promoter activity of PDE4A and -4B genes in different cell systems (30–32). Furthermore, our data indicate a marked cAMP-dependent inducibility of PDE4C expression in human thyroid cells.

In addition to the induction of PDE4, our data show that ATAs expressing mutant TSH receptors are associated with a significant stimulation of 8-methoxy-IBMX-inhibited, CaM-dependent PDE1 activity, consistent with the constitutive activation of phospholipase C caused by the mutant receptor. Interestingly, in the homogenate of the ATA carrying mutant $G_s\alpha$, no induction of PDE1 activity was observed, consistent with a poor phospholipase Cβ stimulation by the mutant $G_s\alpha$. Although not detected in the functional studies of M453T or S281N TSH receptors expressed in COS-7 cells (20, 21), the ability to stimulate the inositol phosphate cascade has been observed in the case of other mutant TSH receptors (19), including I486F (16). In the present study the stimulation of CaM-dependent PDE1 was similar among the ATAs bearing these different mutant receptors (data not shown). Recently, the possibility has been put forth that the constitutive activation of different intracellular pathways may contribute to the superior oncogenic potential of a mutant TSH receptor (33).

This is the first study evaluating the expression of the cAMP-specific PDE8B in human normal and adenomatous tissue. This PDE isozyme has been recently described to be IBMX insensitive and to have a thyroid-specific expression (5). Our results indicate that this new PDE may account for about 20% of the total activity present in the homogenates of normal tissue. In the presence of the constitutive activation of the cAMP pathway, a significant increase in IBMX-insensitive PDE activity is observed. As no enhancement of the PDE8B mRNA steady state is detected, we can hypothesize that the PDE8B expression is already maximal in normal thyroid tissue and is only marginally regulated at the transcriptional level by cAMP, whereas the reported increase in

IBMX-insensitive PDE activity in the ATAs may be secondary to cAMP-dependent phosphorylation.

Taken all together, the data indicate that the constitutive activation of the cAMP pathway in autonomous thyroid adenomas is constantly associated with the up-regulation of rolipram-sensitive PDE activity (PDE4). This finding would be in accordance with the studies of GH-secreting adenomas and FRTL-5 cells expressing the mutant $G_s\alpha$ (12, 15). Because the ATAs lacking any mutation in the TSH receptor or in the hot spots of the $G_s\alpha$ gene are associated with a normal cAMP-specific PDE4 activity, the biochemical finding of an increased PDE4 activity may represent a marker of a constitutively active cAMP pathway.

Furthermore, the enhanced cAMP-degrading PDE activity constitutes a compensatory mechanism opposing the chronic increase in cAMP production induced by the activating mutation, thus explaining the large overlap of values of cAMP content among the normal and adenomatous samples. There are two possible interpretations of this phenomenon. The increased cAMP degradation by PDEs could represent an intracellular mechanism counteracting the phenotypic expression of these two types of oncogene. This interpretation supports the hypothesis that in addition to TSH receptor or $G_s\alpha$ mutations, other events might be required for tumor progression. Alternatively, some experiments have shown that increases in intracellular cAMP may have biphasic effect on cell growth, with prolonged and sustained increases being associated with the arrest of the cell cycle at the G_1-S border (34). It is conceivable that the enhanced PDE cAMP-degrading activity in the tumors characterized by a constitutively active adenylyl cyclase may be a necessary event for tumor progression by determining intracellular cAMP concentrations that are compatible with the initiation of cell replication.

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