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Reduced protein expression of the phosphodiesterases PDE4A4 and PDE4A8 in *AIP* mutation positive somatotroph adenomas

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ABSTRACT

Type 4 phosphodiesterases (PDE4s) of the large PDE enzyme superfamily have unique specificity for cAMP and may, therefore, be relevant for somatotroph tumorigenesis. Somatotroph adenomas typically overexpress PDEs probably as part of a compensatory mechanism to reduce cAMP levels. The rat PDE4A5 isoform (human homolog PDE4A4) interacts with the AIP protein, coded by a tumour suppressor gene mutated in a subgroup of familial isolated pituitary adenomas (FIPAs). PDE4A8 is the closest related isoform of PDE4A4. We aimed to evaluate the expression of both PDE4A4 and PDE4A8 in GH cells of *AIP*-mutated adenomas and compare their expression with that in GH cells from sporadic *AIP*-mutation negative GH-secreting adenomas, where we had shown previously that both PDE4A4 and PDE4A8 isoforms had been over-expressed. Confocal immunofluorescence analysis showed that both PDE4A8 and PDE4A4 had lower expression in *AIP*-mutated somatotropinoma samples compared to sporadic GH-secreting tumours ($P < 0.0001$ for both). Based on the association of low PDE4A4 and PDE4A8 expression with germline *AIP*-mutations positive samples we suggest that lack of AIP hinders the upregulation of PDE4A8 and PDE4A4 protein seen in sporadic somatotrophinomas. These data point to a unique disturbance of the cAMP-PDE pathway in *AIP*-mutation positive adenomas, which may help to explain their well-described poor response to somatostatin analogues.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) comprise a large enzyme superfamily (PDE1 through PDE11) that breaks down the second messengers cAMP and/or cGMP and thereby regulate signal transduction pathways (Francis et al., 2011). PDE4 enzymes are differentiated from other members of the PDE superfamily by their specificity for cAMP. PDE4 subtypes PDE4A, PDE4B, PDE4C and PDE4D are encoded by four different genes and each of these genes encodes multiple isoforms, via isoform-specific promoters or by alternative splicing (Conti et al., 2003; Houslay and Adams, 2003; Houslay et al., 1998). The PDE4A4 (rat analogue: PDE4A5) isoform is expressed in a variety of tissues, including various brain regions (Bolger et al., 1993, 1994, 1996; McPhee et al., 2001). This isoform has a highly conserved amino-terminal region with 88% similarity with the rat PDE4A5 amino-terminal region (Bolger et al., 1994) and two upstream conserved regions (UCR1 and UCR2) which is also present in PDE4A8 (Mackenzie et al., 2008). The PDE4A8 isoform, expressed in the brain and skeletal muscle, is closely related to PDE4A4, differing only in its amino-terminal region (Mackenzie et al., 2008) and containing the EELD motif in the UCR2, which may be relevant to protein-protein interactions.

When compared to other PDE isoforms, human PDE4A4/A5 is uniquely associated with the AIP protein (aryl hydrocarbon receptor interacting protein) (Bolger et al., 2003), a co-chaperone known to have

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tumour suppressor functions in the pituitary. Loss of function mutations in AIP are typically associated with invasive, difficult to treat somatotroph adenomas occurring in children or young adults (Beckers et al., 2013) either in familial or simplex setting. While wild-type AIP protein interacts in vitro with PDE4A5 (Bolger et al., 2003), mutated AIP protein loses its ability to interact with PDE4A5 (Bolger et al., 2016; Igreja et al., 2010). It is not known how would AIP interact with the PDE4 molecules, but as a co-chaperone it might influence protein levels in the cell.

We have recently demonstrated the expression of both PDE4A8 and PDE4A4 in normal human pituitary tissue, and established that both of these proteins are overexpressed in GH-, ACTH-, PRL- and FSH-positive adenomas (Bolger et al., 2016). Here we studied PDE4A4 and PDE4A8 protein expression in GH-secreting tumours from patients carrying germline *AIP* mutations. We hypothesized that lack of functional *AIP* protein might alter the expression of these phosphodiesterase isoforms.

2. Materials and methods

2.1. Subjects and patients

Samples from pituitary adenomas (n = 11) were obtained at transsphenoidal surgery (Table 1). Patients were diagnosed on the basis of clinical and biochemical findings of acromegaly before surgery, followed by immunohistochemical determination confirming the GH expression: all slides utilized from GH-secreting tumours showed more than 90% tumour tissue, as confirmed by the haematoxylin and eosin technique. Autopsy pituitary samples (n = 3) were collected within 24-h of death from adult patients without evidence of any endocrine abnormality and were taken as controls. Pituitary architecture was evaluated by haematoxylin and eosin and reticulin staining. Slides from both normal pituitary tissue and GH-secreting tumours were obtained through consecutive cuts; in some samples we had limited tissue available. Written informed consent was obtained from all pituitary patients, and the protocol was approved by the institutional Research Ethics Committee.

All patients with acromegaly (Table 1) included in the study showed clinical and biochemical signs of acromegaly and tumour samples were positive for GH immunohistochemistry. Patients (2 males and 2 females) with *AIP* mutations (F269_H275dup, R304*, Q164*, and E222*) had macroadenomas with a median age of diagnosis at 25.5 years (range 23–32). Sporadic patients (4 males and 3 females) with somatotroph macroadenomas had a median age of diagnosis of 47.5 years (range 35–70). As expected, patients harbouring *AIP* mutations were characterized by early onset of disease (age of diagnosis, P < 0.01 when compared with sporadic cases). None of the patients with acromegaly were treated before the surgery with somatostatin analogues or dopamine agonists.

Table 1
Clinical characteristics of the patients included in the study.

Patient Number	Sex	Diagnosis	Size	AIP mutation	Age at diagnosis
Patient AIP_1	M	Gigantism	macro	p.F269_H275dup	24
Patient AIP_2	F	Acromegaly	macro	p.R304*	27
Patient AIP_3	M	Acromegaly	macro	p.E222*	32
Patient AIP_4	F	Acromegaly	macro	p.Q164*	23
Patient control_1	F	Acromegay	macro	negative	53
Patient control_2	F	Acromegaly	macro	negative	42
Patient control_3	F	Acromegaly	macro	negative	40
Patient control_4	M	Acromegaly	macro	negative	NA
Patient control_5	M	Acromegaly	macro	negative	70
Patient control_6	M	Acromegaly	macro	negative	53
Patient control_7	M	Acromegaly	macro	negative	39

2.2. Immunoblotting, immunofluorescent staining, and confocal microscopy

To generate specific antibodies against human PDE4A4 and PDE4A8, COS7 cells were transfected with the plasmids containing pcDNAN46VSV (including a fragment of a vesicular stomatitis virus (VSV) epitope at their carboxy terminus) to express human PDE4A4, or with pcDNA4A8VSV to express human PDE4A8, or with vector pcDNA3 (Life Technologies), as previously described (McPhee et al., 1999; Mackenzie et al., 2008; Christian et al., 2010). Incubation with the primary PDE4A4 and PDE4A8 (dilution 1:500) and with VSV (Sigma, USA, Kreis, 1986) antibodies were performed for 1 h in Tris-buffered saline with 0.1% Tween-20, followed by 2 washes in the same buffer. Secondary antibody incubations (SC-5099, Santa Cruz Biotechnology, USA, 1:10,000) were performed in the same buffer, followed by 2 washes in the same buffer. Signal development was performed using enhanced chemiluminescence (Pierce-ThermoFisher, Waltham, MA USA).

For human PDE4A4 staining we used the mouse monoclonal antibody 4A4-1 generated against the specific amino-terminal region of PDE4A4 (SAERAERERQPHRPIERADA) (GenBank L20965) (Bolger et al., 1993, 1994, 2016; Mackenzie et al., 2008). For human PDE4A8 staining we used a mouse monoclonal antibody 4E1.16, that was generated using the unique amino-terminal region of PDE4A8 sequence GDERSRETPESDRAN (GenBank AY593872) (Bolger et al., 2016; Mackenzie et al., 2008). All monoclonal antibodies were purified by limiting dilution and then grown in culture. Antibodies were then purified from culture supernatants with a protein G-affinity matrix (Pierce-ThermoFisher), and there was no cross reactivity between the antibodies.

Immunostaining for GH, PDE4A4 and PDE4A8 was performed as previously described (Bolger et al., 2016), using fat tissue as negative control. We performed confocal immunofluorescence analysis of GH-secreting adenomas, with and without *AIP* mutations, and normal pituitary tissue. In brief, after deparaffinization and application of pre-warmed retrieval solution (Dako, California, USA, 40 ml of stock into 360 mL water) sections were incubated in blocking solution (1% BSA and 0.1% Tween 20) at room temperature for 1 h and then incubated overnight at 4 °C with one of the primary antibodies. In order to co-localize the selected proteins in somatotroph cells, goat anti-GH (L20; 1:50) from Santa Cruz, were also added to the incubation buffer. Secondary antibodies were applied for 1 h with anti-goat Alexa 546 (1:100, Invitrogen, USA), anti-rabbit Alexa 488 (1:400, Invitrogen) or anti-mouse DyLight 488 (Pierce-ThermoFisher), according to the primary antibody previously used. Images were captured through confocal microscope (Leica TCS SP5), 63× objective, and 630× original magnification).

All confocal settings were determined at the beginning of the imaging session and remained unchanged. For quantitative analysis, images were captured at eight bits and analysed in grey scale, using the protocol we have published previously (Bolger et al., 2016; Ribeiro-Oliveira et al., 2008). Three to four images were captured randomly from each sample and three measurements were obtained for each image, with about 30–50 cells also chosen randomly in each analysed sample. ImageJ (NIH, Bethesda, USA) software was used to quantify fluorescence intensity and area intensity, as well as area of each individual cell. The background fluorescence and the energy intensity threshold were then subtracted from the region of interest in the same level for all examined pictures. The relative fluorescence corresponded to the unit ‘grey level’, varying from zero (black) to 255 (white), as an average of the area for each cell (sum of grey value of all pixels divided by the number of pixels/area).

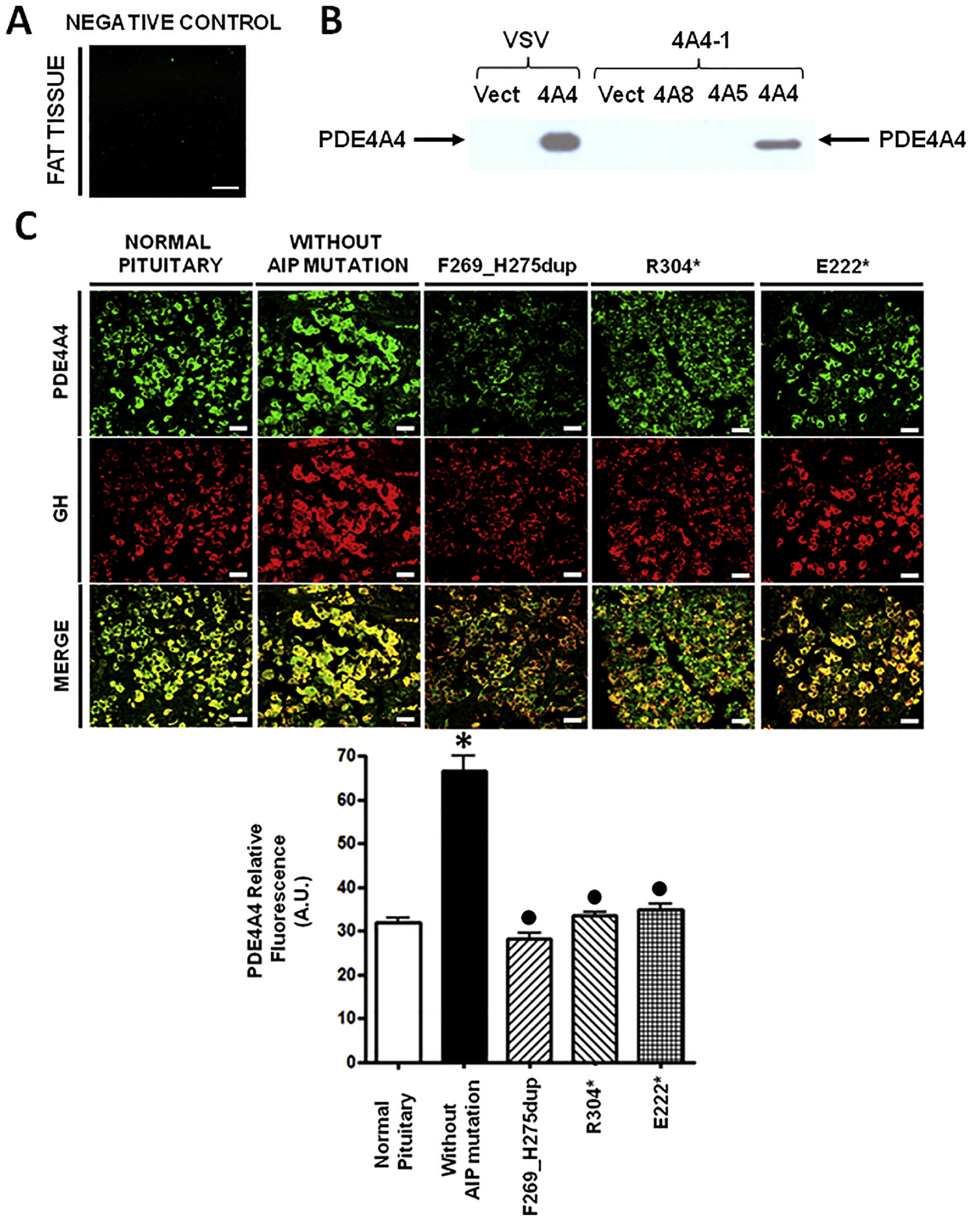

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Fig. 1. Expression of PDE4A4 in GH-secreting tumours with different *AIP* mutations.
(A) Immunofluorescence with the 4A4-1 antibody in fat tissue, as a negative control.
(B) Immunoblotting with the 4A4-1 antibody. COS7 cells were transfected to express human PDE4A4 (4A4) or vector (Vect). All PDE constructs also encoded a VSV epitope at the carboxyl-terminus of the protein. LDS-PAGE and immunoblotting with an antibody against VSV, or with 4A4-1, were performed as described in the 'Materials and methods' section. The PDE4A4 protein migrated at 97 kDa.
(C) Double immunofluorescent staining using monoclonal 4A4-1 (green staining) and polyclonal GH antibody (red staining) in normal pituitary (representative example from the 3 samples), in GH-secreting tumour without *AIP* mutation (representative example from the 7 samples), and in GH-secreting tumour samples with *AIP* mutations [(F269_H275dup, R304*, E222*)]. The bar graphs below the images provide semi-quantitation of the relative fluorescence of PDE4A4 in the 3 normal pituitaries, 7 sporadic adenomas and the 3 individual *AIP* mutation positive samples. Scale bar: 25 µm. All data are shown as mean ± standard error; AU, arbitrary units; *P < 0.0001 in relation to normal GH pituitary cells, and ●P < 0.0001 in relation to non-mutated GH-secreting tumour cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.3. Statistical analysis

Variables were first checked for normal distribution through the Shapiro-Wilk test. Data were tested for statistical significance with the non-parametric Mann-Whitney test or the Kruskal-Wallis test, followed by Dunn's multiple comparisons test using GraphPad Prism 6.0 (La Jolla, USA). Significance was taken as $P < 0.05$. Data are shown as mean ± standard error.

3. Results

3.1. The expression of PDE4A4 in GH-secreting tumours with AIP mutations

We found overexpression of PDE4A4 in the sporadic GH-secreting tumour cells when compared to GH cells in normal pituitary ($P < 0.0001$, Fig. 1), confirming results that we have seen previously in a different set of samples (Bolger et al., 2016). On the other hand, GH-secreting tumour cells with *AIP* mutations showed a significant decrease in the expression of PDE4A4 when compared to those without an *AIP* mutation ($P < 0.0001$, for all), with expression levels similar to those observed in GH cells of normal pituitary.

3.2. The expression of PDE4A8 in GH-secreting tumours with AIP mutations

There was an increased expression of PDE4A8 in somatotroph tumour cells from patients without *AIP* mutations when compared to normal pituitary GH cells ($P < 0.0001$, Fig. 2), confirming results that we have published previously (Bolger et al., 2016). However, somatotroph adenomas from patients with *AIP* mutations showed a significant decrease in the expression of PDE4A8, when compared to tumour cells without an *AIP* mutation ($P < 0.0001$). For two of the analysed mutants, the expression level of PDE4A8 was also decreased when compared to normal pituitary.

3.3. PDE4A4 and PDE4A8 expressions in AIP mutation negative GH-secreting tumours

We compared the expression of PDE4A4 to the expression of PDE4A8 in *AIP* mutation negative GH-secreting tumours. We expressed the level of these two PDE isoforms in the *AIP* negative tumours based on the level seen in somatotroph cells of the normal pituitary and then compared the relative expression of PDE4A4 to that of PDE4A8 ($P < 0.0001$, Fig. 3) in *AIP* mutation negative GH-secreting tumours. We found a higher level of PDE4A4 expression than PDE4A8 (Fig. 3), and also higher PDE4A4 was seen in the *AIP* mutation positive samples than PDE4A8 (Figs. 1 and 2), suggesting that this isoform plays a key role in the compensatory rise of PDEs in somatotrophinomas.

4. Discussion

Changes affecting the cAMP pathway are known to predispose to somatotroph adenomas, as patients with somatic *GNAS* mutations leading to overactive adenylyl cyclase or with germline *PRKAR1A* mutations leading to disinhibited protein kinase A often develop pituitary hyperplasia or adenoma (Hernández-Ramírez et al., 2018). PDEs play a key role in cAMP signalling, by catalysing the breakdown of cAMP to AMP, thus influencing downstream regulatory effectors (Francis et al., 2011; Maurice et al., 2014). Expression of overall PDE4A mRNA was found to be variable in somatotroph tumours previously (Persani et al., 2001), independently of gsp mutation status, while PDE4A subtypes were not studied at the protein level. We have recently established that PDE4A8 and PDE4A4 are both consistently over-expressed in sporadic somatotroph adenomas (Bolger et al., 2016). In this study, however, we have found that both isoforms are significantly reduced in somatotrophinomas with *AIP* mutations. This loss of PDE4A4 and PDE4A8 protein might be due to the loss of functional *AIP* protein. As a co-chaperone, AIP may help proteins to keep their normal protein conformation and therefore stability. Indeed, AHR, a well-known binding partner of *AIP*, have reduced levels in *AIP*-mutated pituitary adenomas (Jaffrain-Rea et al., 2009).

The samples used in this study had either a nonsense mutation or a segmental duplication. The nonsense mutations lead either to nonsense mediated decay and therefore no protein at all (Q164* and E222*) or to very rapid degradation of the shortened protein (R304*) (Hernández-Ramírez et al., 2016) which anyway lacks part of the crucial C-terminal alpha-helix, therefore not representing functional protein. We have recently shown that the segmental duplication also leads to a very rapid degradation of the protein (Salvatori et al., 2017), while the duplicated region theoretically disrupts a protein segment important for partner binding. Therefore all these mutations result in complete lack of AIP function.

When samples are compared for the presence of the different phosphodiesterases isoforms, we noted that PDE4A4 expression increases more than PDE4A8 in *AIP* mutation negative adenomas, suggesting that PDE4A4 compensatory increase is higher for this isoform. PDE4A4/5 is known to bind to *AIP*, and the EEDL motif is also present in PDE4A8, suggesting that theoretically this protein might also bind *AIP*. However, no experimental data are available regarding this due to the poor expression of PDE4A8 in *in vitro* experimental settings (Bolger et al., 2016). As *AIP* is a co-chaperone and one of its roles is to protect proteins from degradation, we hypothesise that lack of *AIP* may lead to accelerated PDE4A4/8 degradation in *AIP*-mutation positive tumours.

Overactivation of the cAMP pathway is known to increase PDE levels (Peverelli et al., 2014), possibly as a compensatory mechanism. Indeed, we have observed increased PDE4A4 and PDE4A8 protein expression in sporadic adenomas both in a previous (Bolger et al., 2016) and in the current study. However, in adenomas with *AIP* mutations, the decreased expression of PDE4A4 and PDE4A8 in the mutation samples suggests that these tumours have an altered balance of the cAMP-PDE pathway which may play a role in the tumorigenesis. We note that while both PDE4A4 and PDE4A8 are showing changes into the same direction, there is absolutely no cross reactivity between these antibodies (Bolger et al., 2016; Mackenzie et al., 2008). In addition, the higher expression of PDE4A4 in GH-secreting adenomas without *AIP* mutation compared to PDE4A8 possibly explains why the expression of PDE4A4 in the mutants have not reached lower levels than in normal pituitary, as observed for two mutants with PDE4A8. We see some variability of the reduced PDE4A8 expression, probably related to the

A NEGATIVE CONTROL

FAT TISSUE

B VSV 4E1.16

Tx Vect Tx Vect P1 P2

PDE4A8 PDE4A8

C NORMAL PITUITARY | WITHOUT AIP MUTATION | F269_H275dup | R304* | Q164*

PDE4A8

GH

MERGE

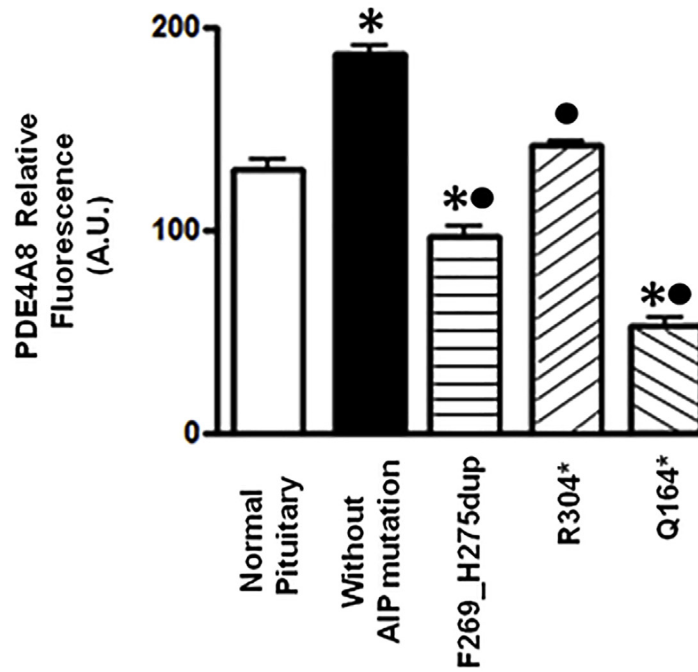

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Fig. 2. Expression of PDE4A8 in GH-secreting tumours with different AIP mutations.
(A) Immunofluorescence with the 4E1.16 antibody in fat tissue, as a negative control.
(B) Immunoblotting with the 4E1.16 antibody. Extracts from COS7 cells transfected to express PDE4A8-VSV or with vector (Vect) were immunoblotted with an antibody against VSV or with 4E1.16. On separate gels, PDE4A8-VSV extracts were immunoblotted with 4E1.16 and the peptide used as immunogen (P1), or an unrelated peptide (P2). The PDE4A8 protein migrated at 102 kDa.
(C) Double immunofluorescent staining using monoclonal 4E1.16 (green staining) and polyclonal GH antibody (red staining) in normal pituitary (representative example from the 3 samples), in GH-secreting tumour without AIP mutation (representative example from the 7 samples), and in GH-secreting tumour samples with AIP mutations (F269_H275dup, R304* and Q164*). The bar graphs below the images provide semi-quantitation of the relative fluorescence of PDE4A8 in the 3 normal pituitaries, 7 sporadic adenomas and the 3 individual *AIP* mutation positive samples. Scale bar: 25 µm. All data are shown as means ± standard error; AU, arbitrary units; *P < 0.0001 for comparisons with normal GH pituitary cells, and ●P < 0.0001 for comparisons with non-mutated GH-secreting tumour cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

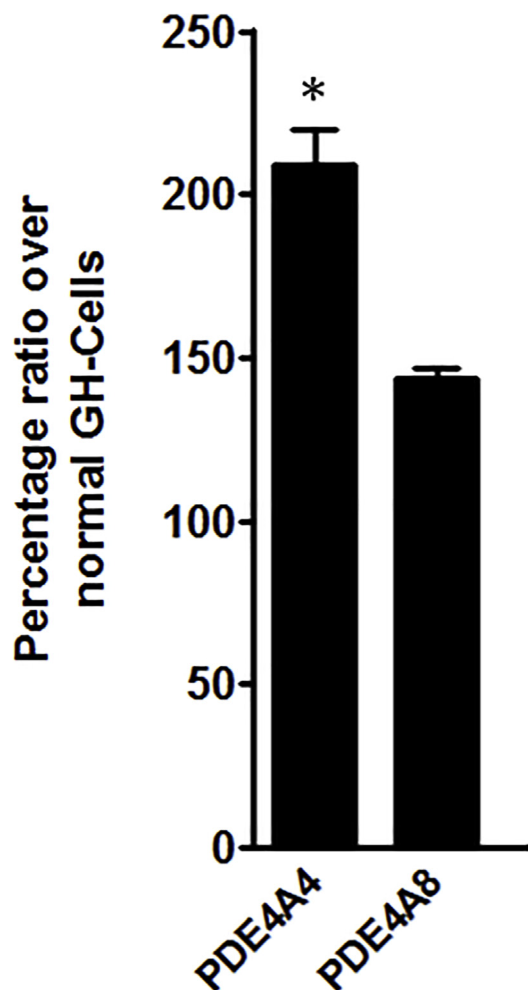

Fig. 3. Comparison of the expression ratio of PDE4A4 and PDE4A8 in GH-secreting tumours over normal GH cells. *P < 0.0001 for comparison.

small sample size.

In vitro data using *AIP* overexpression in GH3 cells lead to increased cAMP levels but this was not altered by general phosphodiesterase inhibitor IBMX or PDE4 inhibitor rolipram, suggesting that PDEs do not have a role in the *AIP*-induced reduction of cAMP levels in this experimental setup (Formosa et al., 2013). Lack or low level of *AIP* leads to reduced expression of inhibitory G protein $G_{alpha1-2}$, both in vitro and in human samples (Ritvonen et al., 2017; Tuominen et al., 2015), but it is unclear how these data relate to the enzymatic activity of phosphodiesterases.

This study carries some limitations. We have a small sample size for *AIP* mutation positive patients due to the rarity of the disease and scarcity of the available high-quality *AIP* mutant tissue blocks. We used a different sample for PDE4A4 and PDE4A8, but as both used samples were nonsense mutations, they should be similar in terms of disease mechanism. However, even with small sample size we were able to see significant changes in pituitary PDE expression as well as to show concordance of expression whenever samples were tested for both isoforms. Furthermore, the double immunostaining we have used in this study is the gold standard method to co-localize protein expression in neoplastic and normal somatotroph tissues from patient samples. In vitro studies are needed to further investigate the suggested mechanism.

In conclusion, there is reduced expression of PDE4A4 and PDE4A8 in *AIP*-mutated pituitary adenomas compared to sporadic somatotrophinomas which have a, probably compensatory, elevation of these cAMP-degrading PDE isoforms. These changes could lead to upregulation of the cAMP-pathway and support tumorigenesis while possibly contributing to somatostatin receptor ligand-resistance of patients with *AIP* mutations. Further studies should investigate these pathways in the light of the data presented here.

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