

# Biological activity of activating thyrotrophin receptor mutants: modulation by iodide

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## Abstract

Epidemiological studies have revealed a significantly higher incidence of toxic adenoma (TA) and toxic multi-nodular goitre (TMNG) in regions of iodine deficiency. Fifty to eighty percent of TA and TMNG are caused by activation of the cAMP pathway, mostly by mutations in the thyrotrophin receptor (TSHR).

We aimed to investigate whether iodide could modulate the biological effects of activating TSHR mutations. We have applied an *in vitro* model of TA comprising FRTL-5 cells stably expressing activating TSHR. We have mimicked the *in vivo* situation by examining the effects of prolonged exposure to iodide on the proliferation and signal transduction etc. of these cells.

We observed an iodide-induced 'inhibition of proliferation' which was significant from 10 mM in the presence of serum but from 1 mM in its absence. The inhibition of proliferation was significantly higher in the activating mutant expressing FRTL-5 compared with control Neo or wild-type TSHR, indicating that the effect was mediated via the cAMP cascade. The effect was neither due to hyper-tonicity nor was it the result of an increase in cell death either by apoptosis or necrosis. Prolonged exposure to iodide produces an increase in cells in the G2 and post-G2 phases, indicating that G2/M blockade contributes to the mechanism of inhibition.

The mutant expressing FRTL-5 cells have increased proliferation when chronically exposed to TSH, and this is associated with a reduction in phosphorylated (p) CREB levels. This contrasts with the effect of iodide in which inhibition of proliferation is accompanied by an increase in pCREB.

In conclusion, our studies indicate that the biological effects of activating TSHR mutations vary with the ambient iodide supply and could be masked in regions of high iodine intake.

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## Introduction

Hyperthyroidism is a common disorder, which is caused mainly by pathogenic activation of the thyrotrophin (TSH) receptor (TSHR). This can be achieved by autoantibodies (for references see Paschke & Ludgate 1997) that mimic the action of TSH, the thyroid stimulating antibodies of Graves' disease. The other mechanism involves gain-of-function TSHR mutations (for references see Paschke & Ludgate 1997).

The prevailing pathogenesis underlying hyperthyroidism varies with geographic location. For example, in Jutland, a Danish region of low iodine intake, a significantly higher incidence of hyperthyroidism was reported than in Iceland, which has a high iodine intake. Furthermore, the increased incidence of hyperthyroidism was due to a much higher incidence of toxic multi-nodular goitre (TMNG) and toxic adenoma (TA) in Denmark (Laurberg *et al.* 1991).

In recent years, considerable progress has been made in elucidating the molecular cause of TMNG and TA. Somatic mutations of the TSHR producing a constitutively active receptor and consequent increased basal cAMP, were first described in TA (Parma *et al.* 1993). Subsequently, equivalent mutations in the germline were identified in familial and sporadic cases of non-autoimmune hyperthyroidism (Duprez *et al.* 1994). The latter condition is rare but an impressive spectrum of TSHR mutations has been reported to account for up to 80% of TA cases (Parma *et al.* 1995, Trultsch *et al.* 2001).

Since TSH, via cAMP, regulates the growth and function of the thyroid gland, it was logical to seek activating TSHR mutations in TMNG and there are several reports describing such mutations (Duprez *et al.* 1997, Holzapfel *et al.* 1997, Tonacchera *et al.* 1998). Somatic mutations in the  $\alpha$  subunit of the Gs protein, which abolish its GTPase activity, have also been

described, although they account for only a small proportion of TA and TMNG (Esapa *et al.* 1997). Studies of large series of TA and TMNG have reported that 50–80% of these lesions are the result of mutations in the cAMP signalling pathway, either the TSHR or G $\alpha$  (Parma *et al.* 1997, Trultzsck *et al.* 2001).

Despite the clear epidemiological link between iodine deficiency and thyroid autonomy, the precise pathophysiological mechanism is less clear. It has been suggested that increased generation of hydrogen peroxidase might promote thyroid mutagenesis, including that of the TSHR (Krohn & Paschke 2001). However, it is also possible that iodine intake *per se* might affect the biological outcome of activating TSH receptor mutations.

Iodide has a profound influence on the growth and function of the thyroid (Corvilain *et al.* 1994). Aside from being a component of thyroid hormones, iodide excess has been reported to inhibit its own organification (Corvilain *et al.* 1994) and uptake (Becks *et al.* 1988). Iodide inhibits thyroid adenylate cyclase producing a reduction in TSH- or forskolin-stimulated cAMP production in several species (Van Sande *et al.* 1985). Although this, in itself, would have a negative impact on thyrocyte growth, it is unrelated to the G2/M arrest in the cell cycle of FRTL-5 induced by iodide (Smerdely *et al.* 1993, 1995). Iodide has also been demonstrated to induce apoptosis in a thyroid cell line via a p53 independent mechanism (Vitale *et al.* 2000).

Iodolipids have been identified as the biological mediators of these effects. For example, iodoheptadecanal mimics the inhibition of TSH-induced cAMP production in dog thyroid cells (Panneels *et al.* 1994). In contrast, the inhibition of porcine thyroid growth induced by delta-iodolactone is epidermal growth factor/inositide dependent but cAMP independent (Dugrillon *et al.* 1990).

In a previous study (Fuhrer *et al.* 2003), we have described an *in vitro* model for evaluating the biological activity of gain-of-function TSHR mutations. Stable expression of seven such mutants in FRTL-5 cells enabled us to distinguish differences in their capacity to stimulate proliferation and function. These did not always correlate with the potency of the mutants assessed by basal cAMP generation, but did agree with their activity in human primary thyrocytes. Of particular relevance, FRTL-5 cells are able to trap iodide and we demonstrated differences in the abilities of the mutant TSHR to stimulate this thyroid function, which is central to the evolution of a 'hot-nodule'.

The model provides a means of investigating the prolonged effects of iodide on the proliferation, signal transduction and cell cycle profiles of thyroid cells expressing TSHR gain-of-function mutations. We now report our findings of these investigations.

## Materials and methods

### Cells used and culture conditions

We have studied the SB5 sub-clone of FRTL-5 and low passage number ( $n < 10$ ) pools of these cells stably expressing wild-type (WT) or 3 different gain-of-function TSHR mutants, M453T, L629F and Del613–621, introduced by retroviral infection as previously described (Fuhrer *et al.* 2003). All FRTL-5 cells were maintained in a 2:1:1 mixture of DMEM:Ham's F12:MCDB104 (all from GIBCO Life Technologies, UK) supplemented with 5% calf serum (GIBCO Life Technologies), 10  $\mu$ g/ml insulin, 0.4  $\mu$ g/ml hydrocortisone (Calbiochem, San Diego, CA, USA), 45  $\mu$ g/ml ascorbic acid (Sigma, UK), 5  $\mu$ g/ml transferrin (Calbiochem) and 5 mU/ml bovine TSH (Sigma).

### Investigation of proliferation, signal transduction and cell cycle profiles in mutant-expressing FRTL-5 cells

Mixed pools of FRTL-5 cells expressing the WT, three mutant TSHR and the Neo control, all generated in our previous study, were investigated. The mixed pools were initially plated in the presence of TSH and in serum-containing medium, to allow adherence, but were then maintained without TSH (where indicated) for at least 72 h before all measurements, which were performed at least in duplicate and repeated at least once.

Depending on the parameter being analysed, the mixed pools were then cultured in serum-free or -containing medium, supplemented or not with TSH and in varying concentrations of NaI (or NaCl or methimazole). The cultures remained sub-confluent.

### Direct cell counting

In preliminary experiments, cells were plated at various densities in complete (serum-containing) medium with TSH, switched the next day to serum-free or -containing medium, supplemented or not with 1 mU/ml TSH and counted 3 days later as described below.

Subsequently, cells were plated at various densities, maintained in TSH-containing or switched to TSH-free complete medium for 3 days and then incubated in the same conditions but with 0–100 mM NaI for 2 to 5 days. They were trypsinised on days 3, 4 or 5 (following addition of NaI), resuspended in Isoton II azide-free balanced electrolyte solution and counted using a Beckman Coulter Particle Counter. Results are expressed as total counts per well or as population doubling time (PDT  $\pm$  S.E.M.) calculated as:

$$[\text{total hours in culture/fold increase in cell number}] \times 2$$

In some experiments, 50 mM NaCl was used in the place of NaI, to check for the effects of tonicity.

Experiments were also performed in 50 mM NaI plus methimazole (MMI) at 0.2 and 2 mM, to investigate whether organification was required.

### cAMP pathway

FRTL-5 cell clones for the mutant expressing FRTL-5 and Neo were plated in 12-well plates at a density of  $5 \times 10^4$ /well. Twenty-four hours after plating, they were switched to medium without TSH and 3 days later they were incubated with 50 mM NaI for a further 3 days. At this point cAMP accumulation was measured by incubating the cells for 4 h at 37 °C, in 250  $\mu$ M IBMX alone or supplemented with 1 or 10 mU/ml TSH. Medium was aspirated and the cAMP extracted in 0.1 M HCl. Samples were evaporated to dryness and re-suspended in buffer for measurement of cAMP using an in-house radioimmunoassay (Ashworth *et al.* 1994). Results are expressed as pmoles cAMP per cell, adjusted by direct-cell counting of adjacent wells from the same plates. We then calculated two different stimulation indices for each of the mutant TSHR and Neo pools. (1) cAMP in the presence of TSH/cAMP in the absence of TSH; this gives a measure of TSH responsiveness and how it is modified by iodide. (2) Basal cAMP in the presence of NaI/ basal cAMP in the absence of NaI; this gives a measure of the effect of iodide on the constitutive activity of the gain-of-function mutations.

### Western blotting

The 4 pools of Neo or mutant TSHR expressing FRTL-5 cell clones were propagated in duplicate in 6-well plates in complete medium with/without TSH and in the presence/absence of 50 mM NaI for 3 days. Protein extraction was performed in Laemmli buffer containing 1 mM sodium orthovanadate and 1 mM PMSF. Samples were subjected to standard Western blotting. The gel was electroblotted onto PVDF membrane as previously described (Lewis *et al.* 2002) and probed with the following rabbit polyclonal antibodies: anti phospho (p)-CREB (Ser133, 1:2000 overnight, 4 °C; Cell Signalling Technology, Beverly, MA, USA), anti total-CREB (1:1000, room temperature for 1 h; Santa Cruz, CA, USA), a mix of anti-p44 MAP kinase (MAPK) and p42 MAPK (1:1000, room temperature for 1 h; Santa Cruz) or mouse monoclonal antibodies to phospho-p44/42 MAPK (Thr 202/Tyr 204, 1:2000, overnight, 4 °C; Cell Signalling Technology). Blots were incubated in either an anti-mouse or an anti-rabbit IgG-horseradish peroxidase conjugate (1:5000, 1 h, room temperature; Amersham Biosciences) and visualised by enhanced chemiluminescence (ECL Plus, Amersham Biosciences). Films were analysed using the Alpha Imager 1200 digital imaging system (Alpha Innotech Corp, San Leandro, CA, USA). The blots were

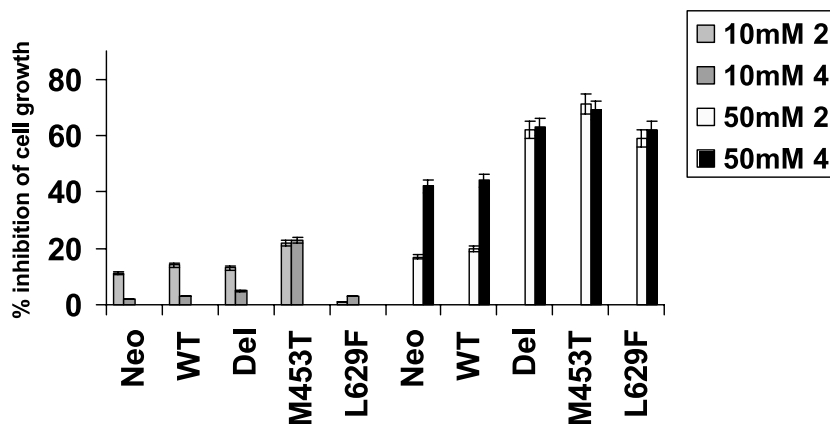
initially probed with the phospho-specific antibodies, they were then stripped and re-probed with antibodies that recognise total p44/42 MAPK or CREB.

### Cell cycle analysis

FRTL-5 cell pools, cultured in complete medium with/without TSH and in the presence/absence of 50 mM NaI were plated in duplicate at a density of  $1 \times 10^5$  cells/well in 12-well plates. After 72 h in these culture conditions, trypsinised cells were washed in PBS and stained with 25  $\mu$ M – 0.5 mM DRAQ 5 (Biostatus Ltd, Shepsted, Leics, UK) for 10 min. Alternatively, the trypsinised cells were fixed in 5 ml ice-cold 70% ethanol at 4 °C overnight, washed, then incubated in 450  $\mu$ l PBS containing 10  $\mu$ l 1 mg/ml ribonuclease for 5 min at room temperature. This was followed by 40  $\mu$ l 0.5 mg/ml propidium iodide (PI; Molecular Probes Europe BV, Leiden, The Netherlands). In each case a minimum of 10 000 events were collected on a FACScan or FACS Vantage flow cytometry (Becton Dickinson UK Ltd, Oxford, Oxon, UK.). Results were analysed using 'WinMDI' and 'Cylchred' software (University of Wales College of Medicine, Cardiff, UK).

### Estimate of Na-induced necrosis and apoptosis

FRTL-5 cell pools were cultured as described above for the cell cycle analysis. Briefly, cell samples ( $4-6 \times 10^5$  cells/ml) were washed with cold PBS and resuspended in  $1 \times$  binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM  $\text{CaCl}_2$ ) at a concentration of  $1 \times 10^6$  cells/ml (Vermees *et al.* 1995). One hundred microlitres per sample were transferred to a polystyrene round bottomed flow tube (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA) to which 5  $\mu$ l Fluorescein-conjugated Annexin V (Annexin V-FITC; Pharmingen, Becton Dickinson UK Ltd) and/or 10  $\mu$ l of 50  $\mu$ g/ml stock PI were added. Control samples were sham-treated as necessary. Samples were gently vortexed, then incubated in the dark for 15 min at room temperature. Four hundred microlitres of  $1 \times$  binding buffer were added to each tube and samples held on ice for a maximum of 1 h prior to analysis using a FACS Vantage flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). This was equipped with a Coherent Enterprise II argon ion laser having 488 nm and multiline UV (351–355 nm) outputs (Coherent, Inc., Santa Clara, CA, USA). The Enterprise II laser power was regulated at 30 mW (monitored on the multiline UV output). CELLQuest software (Becton Dickinson Immunocytometry Systems) was used for signal acquisition and analysis. Forward scatter (FSC) and side scatter (SSC) were acquired in linear mode. FITC and PI fluorescent signals derived from 488 nm excitation were detected in logarithmic mode at FL1/PMT3 and FL2/PMT4, with



**Figure 1** Effect of varying concentrations of NaI on the proliferation of FRTL-5 cells in the absence of TSH. Cells were plated in serum-containing medium plus TSH and switched to serum-containing medium without TSH the next day. Three days later, NaI (0, 10 or 50 mM) was added and the cells were incubated for a further 2 or 4 days (indicated by 2 and 4 in the key). The analyses were performed on days 6 and 8. Results are expressed as percentage inhibition of cell growth derived by direct counting. Del, Del613–621.

FITC 530/30 nm filters and PI 585/42 nm filters respectively and a FL1/2 560 nm SP dichroic SP. Signals for forward and side scatter and fluorescence were collected for 10 000 cells using the forward light scatter parameter as the master signal. Data are expressed as mean fluorescence intensity (FI) values.

### Statistics

Data were expressed as mean  $\pm$  S.E.M. Multiple comparisons were performed using ANOVA with Bonferroni's *post hoc* correction. Skew values of all variables were  $0 \pm 1$ . Correlation between variables was sought calculating Pearson's coefficient. Statistical significance was assumed when the probability ( $P$ ) was less than or equal to 0.05.

### Results

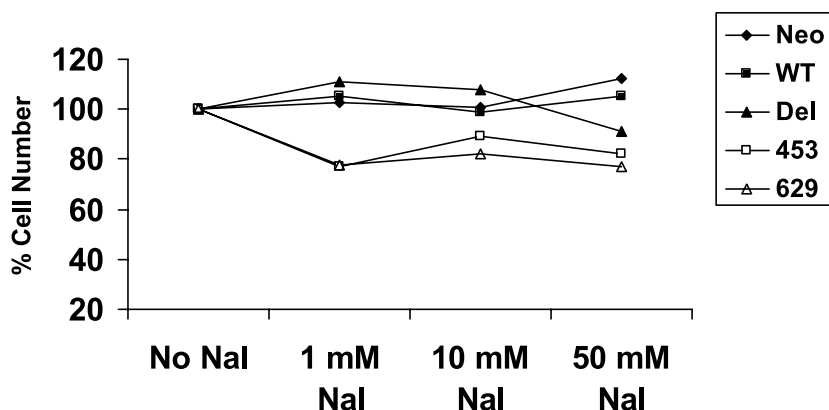
#### Iodide has an inhibitory effect on the proliferation of mutant expressing FRTL-5

In serum-containing medium but without TSH, the control Neo expressing cells had a PDT of  $118.3 \pm 3.0$  h after 4 days of incubation. This is not significantly different from the PDT of the cells expressing the WT TSHR ( $120 \pm 5.0$  h). The proliferative stimulus is provided by growth factors in the serum. The PDT in the presence of 10 mM NaI was not significantly different in either of these cell populations but 50 mM NaI produced a significant increase in PDT (Neo  $208.4 \pm 2.7$  h, WT  $173 \pm 3.2$  h;  $P < 0.001$ ) corresponding to  $43.2 \pm 1.1\%$  and  $44.2 \pm 2\%$  inhibition of cell growth respectively.

We have studied FRTL-5 cells expressing 3 different activating TSHR mutants, M453T which have TSH independent growth, L629F with partial TSH independence and Del613–621 which are TSH dependent (Fuhrer *et al.* 2003). The PDT of the activating mutant TSHR expressing cells in serum-containing medium without TSH after 4 days incubation was  $63 \pm 1.2$  (M453T),  $84 \pm 2.8$  (L629F) and  $110 \pm 0.95$  (Del 613–621) h respectively. In these cells, the proliferative stimulus is provided by serum growth factors and also the increased basal cAMP produced by the constitutively active TSHR (Fuhrer *et al.* 2003 and see below). A significant inhibitory effect was exerted by 10 mM iodide on the PDT of M453T and Del613–621 mutants, producing an inhibition of growth of  $23 \pm 3.3\%$  and  $5 \pm 2.3\%$  respectively. The growth of L629F was also inhibited ( $3 \pm 4.4\%$ ) but this does not reach significance. The magnitude of growth inhibition was increased in the presence of 50 mM NaI:  $69 \pm 11.2\%$  for M453T,  $58 \pm 2.4\%$  for L629F and  $63 \pm 4.5\%$  for Del 613–621 and was significantly greater ( $P < 0.05$ ) for all of the mutants compared with Neo or WT.

Similar results were obtained when using varying initial plating densities and differing exposure times to NaI. We consistently observed significantly lower numbers of TSHR mutant expressing cells in the presence compared with the absence of iodide, which was dose dependent ( $r: 0.81$ ;  $P < 0.001$ ). The results, expressed as percentage inhibition of proliferation, are summarised in Fig. 1.

When the different cell populations were cultured in the presence of TSH, a significant decrease in PDT was observed, compared with growth in the absence of TSH, in the Neo, WT and mutant TSHR expressing cell lines,



**Figure 2** Effect of varying concentrations of NaI on the proliferation of FRTL-5 cells in serum-free medium and in the absence of TSH. Cells were plated in serum-containing medium plus TSH. The next day they were switched to serum-free medium either with or without TSH. Three days later, NaI (0, 1, 10 or 50 mM) was added and the cells were incubated for a further 2 days. The analyses were performed on day 6. Results (replicate counts agree to within 5%) are expressed as percentage of cell number derived by direct counting and taking the 0 mM NaI value as 100%. Del, Del613–621; 453, M453T; 629, L629F.

including M453T. There was no significant difference in the PDT of the five cell lines grown in the presence of TSH, when compared with each other.

NaI significantly inhibited the proliferation of all five cell lines ( $P < 0.01$ ). For example, after four days incubation in 50 mM NaI, inhibition of proliferation was  $63.9 \pm 2.1$  (Neo),  $70.1 \pm 3.2\%$  (WT),  $72.6 \pm 7.9\%$  (M453T),  $66.7 \pm 9.8\%$  (L629F) and  $70.6 \pm 3.6\%$  (Del613–621). However, in contrast to the results obtained in the absence of TSH, there was no significant difference in the magnitude of NaI-induced proliferation inhibition between the mutant or WT expressing and control Neo (data not shown).

In serum-free medium without TSH, FRTL-5 growth depends entirely on endogenous factors within the cell. The PDT of the control Neo expressing cells was  $162 \pm 2.4$  and for WT it was  $165 \pm 4.7$  h after 3 days incubation, NaI from 1–50 mM did not inhibit proliferation of either cell population. The PDT of the activating mutant TSHR expressing cells in serum-free medium without TSH after 3 days incubation was  $78 \pm 2.8$  (M453T),  $98 \pm 4.6$  (L629F) and  $118 \pm 1.8$  (Del613–621) h. In M453T, incubation with 1 mM, 10 mM or 50 mM NaI induced a significant reduction in cell numbers, corresponding to  $20 \pm 2.9$ ,  $12 \pm 3.8$  and  $19 \pm 2.1\%$  inhibition respectively. The highest concentration also exerted an inhibitory effect on Del613–621 and L629F expressing cells and this reached significance in the latter ( $P = 0.04$ ). Results, expressed as a percentage of the 0 mM NaI count because of increased cell numbers in some conditions are summarised in Fig. 2.

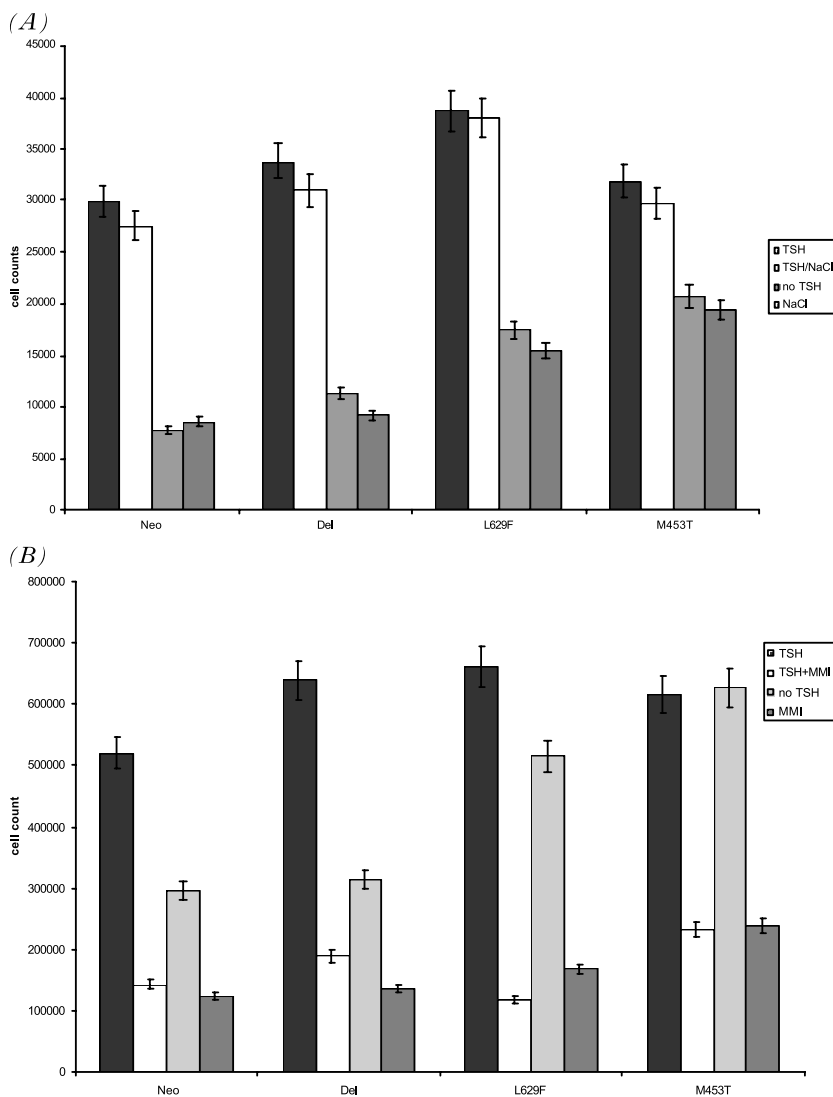
In subsequent experiments, we have compared parameters using 0 and 50 mM NaI (or NaCl) since

this concentration had an inhibitory effect on the proliferation of all 3 mutant TSHR expressing lines. As there was no significant difference in the growth rates or degree of inhibition by NaI in the Neo and WT cell populations, we have used Neo as the control. In view of the opposing effects of TSH and NaI on proliferation of the cells, we have also compared the parameters in the presence and absence of TSH.

#### The inhibitory effect is not due to hypertonicity: MMI alone inhibits proliferation

To investigate the possibility that the hypertonicity of NaI was exerting a toxic effect on the FRTL-5 populations, Neo and the 3 activating mutant TSHR expressing cells were cultured for periods of 2 to 5 days, in serum-containing medium, in the presence and absence of TSH and in 50 mM NaCl. We did not observe any difference in total cell counts when compared with the cells grown in identical conditions but omitting the NaCl. A representative experiment is shown in Fig. 3A.

Growth rates were also examined in the four different pools cultured in 50 mM NaI and with the addition of 2 mM MMI. In control wells containing 2 mM MMI alone, we observed a significant reduction in total cell numbers in all culture conditions, indicating an inhibitory effect of the drug alone (Fig. 3B). We obtained a similar inhibition when using 0.2 mM MMI (data not shown); we were thus unable to conclude whether the NaI required organification in order to exert its negative effect on cell growth.

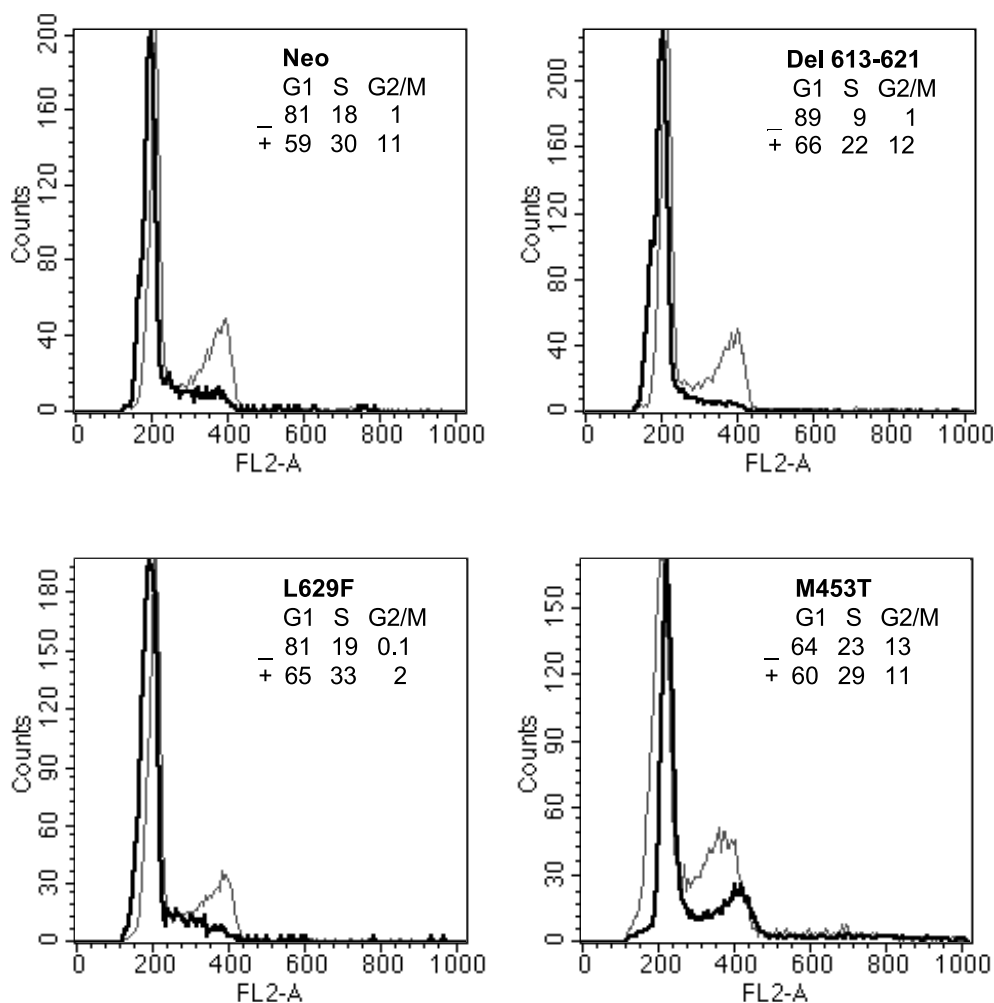


**Figure 3** (A) Effect of 50 mM NaCl on the proliferation of FRTL-5 cells grown in serum-containing or serum-free medium and in the presence or absence of TSH. Cells were plated in serum-containing medium plus TSH. The next day, half the wells were switched to serum-free medium either with or without TSH. Three days later, NaCl (0 or 50 mM) was added and the cells were incubated for a further 2 days. The analyses were performed on day 6. Results are expressed as cell counts. (B) Effect of 2 mM methimazole (MMI) on the proliferation of FRTL-5 cells grown in serum-containing or serum-free medium and in the presence or absence of TSH. Cells were plated in serum-containing medium plus TSH. The next day, half the wells were switched to serum-free medium either with or without TSH. Three days later, MMI (0 or 2 mM) was added and the cells were incubated for a further 2 days. The analyses were performed on day 6. Results are expressed as cell counts. Del, Del613–621.

### Iodide does not increase the percentage of necrotic or apoptotic cells

FRTL-5 cell populations cultured in the continual presence of NaI, which mimics the *in vivo* situation, accumulated fewer cells than their equivalents grown in

the absence of iodide. Having eliminated a direct toxic effect, we investigated whether differences in proliferation between the cell pools in varying culture conditions could be attributable to increased necrosis or apoptosis. We performed Annexin V and propidium iodide staining.



**Figure 4** Cell cycle analysis of FRTL-5 cells in the absence (bold line) and presence (fine line) of 5 mU/ml bovine TSH. Cells were plated in serum-containing medium plus TSH and half the wells switched to serum-containing medium without TSH the next day. The cells were incubated for a further 3 days. The analysis was performed on day 4. Histogram overlays were derived from flow cytometry analysis of propidium iodide stained (FL2-A), ethanol-fixed cells for DNA content. All data were gated on FSC vs SSC plots for exclusion of debris and FL-2 area vs width analysis for exclusion of doublets.

In the 4 cell types cultured in serum-containing medium supplemented or not with TSH and in the presence or absence of NaI, the percentages of necrotic and apoptotic cells ranged from 3.3 to 10.6% and from 1.1 to 4.5% respectively. The differences were associated with cell type rather than culture condition, with the control Neo having the highest percentages.

Contrary to expectations, in the absence of TSH and NaI, Del613-621 and M453T had a similar percentage (~6%) of necrotic+apoptotic cells, indicating that increased cell death does not account for their respective TSH-dependent and -independent growth.

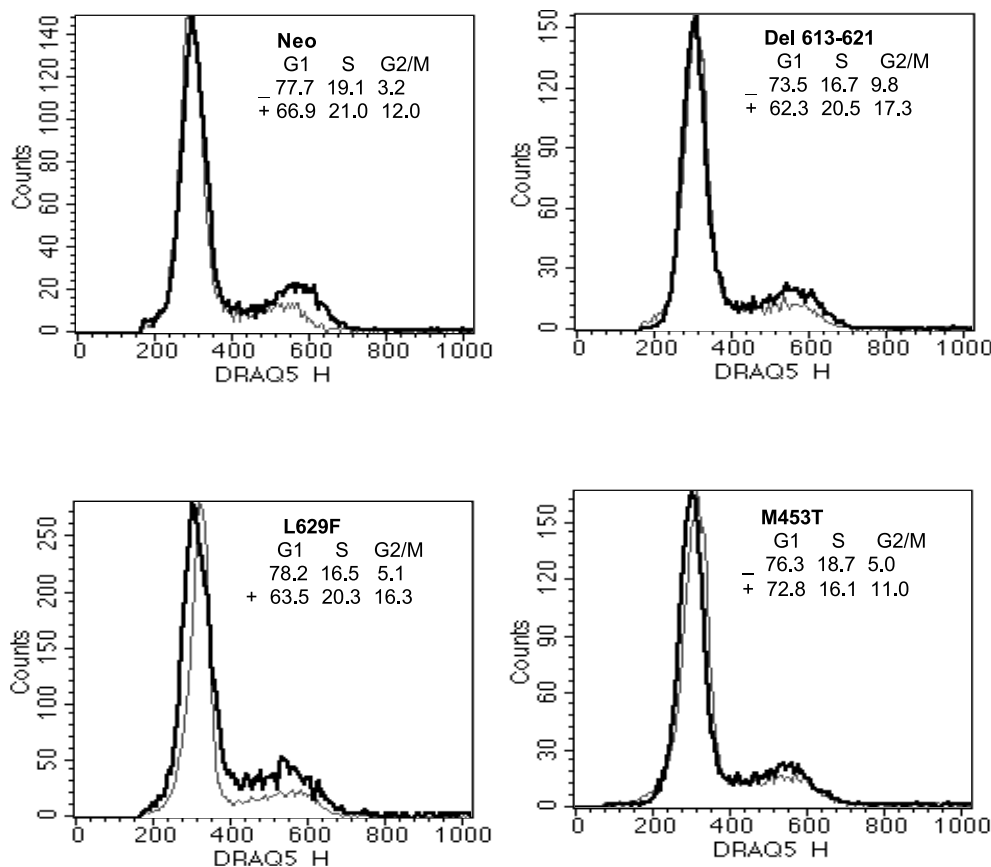
All 4 cell types cultured in the presence of TSH had a small (2-4%) increase in the percentage of

necrotic+apoptotic cells. In contrast, NaI induced no change in the percentages, indicating that increased cell death does not explain the reduced proliferation observed.

#### Cell cycle profiles are modified by iodide and TSH

We then investigated the cell cycle profiles of the 4 cell populations in serum-containing medium, with and without TSH and in the presence or absence of NaI.

There were many similarities between the effects of TSH and NaI on the profiles of the cells, as shown in Figs 4 and 5. In both cases there was a reduction in the percentage of cells in G0/G1 of 4 to 23% and an



**Figure 5** Cell cycle analysis of live FRTL-5 cells in the presence (bold line) and absence (fine line) of 50 mM NaI. Cells were plated in serum-containing medium plus TSH and switched to serum-containing medium without TSH the next day. Three days later, NaI (0 or 50 mM) was added and the cells were incubated for a further 3 days. The analysis was performed on day 7. Histogram overlays were derived from flow cytometric analysis of live DRAQ5 stained cells for DNA content. All data were gated on FSC vs SSC plots for exclusion of debris. Doublets were excluded on height vs width analysis.

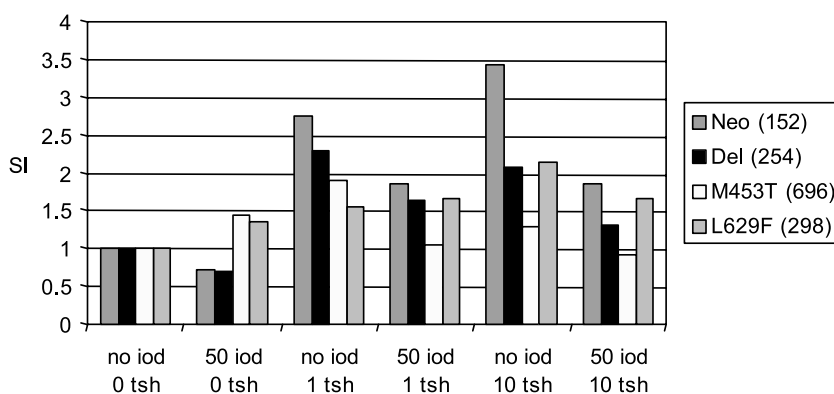
increase in G2/M phase of 2 to 11%. The main difference was the increase in S phase cells, 6 to 14%, induced by chronic exposure to TSH, which did not occur with NaI. Although the changes described were observed in all 4 populations, the profile of M453T, which displays the highest level of TSH-independent proliferation, was minimally altered by TSH. Similarly, these cells, whose proliferation was most inhibited by NaI, displayed a 2.6% reduction in S phase cells when cultured in iodide. In all 4 cell pools, the presence of NaI increased the numbers of polyploid cells due to endoreduplication by two- to threefold, although the overall percentage remained <6%.

#### Iodide inhibits TSH-induced cAMP but not constitutive adenylate cyclase activity

The M453T mutant expressing cells have the highest level of constitutive activity and their proliferation was

most inhibited by, and at the lowest concentration of, NaI (Fuhrer *et al.* 2003 and Fig. 6). This indicates that the reduced proliferation in the presence of NaI depends on TSHR activation. We have compared the basal and TSH-induced cAMP production of the mutant TSHR and Neo pools, in serum-containing medium with and without 50 mM NaI. In cells grown in the presence of NaI, we observed a significant reduction ( $P < 0.05$ ) in cAMP produced in response to acute exposure to 1 and/or 10 mU/ml TSH in the mutant TSHR expressing FRTL-5 and Neo control, as shown in Fig. 6. Of note, the stimulation index for the FRTL-5 populations expressing gain-of-function mutations is lower than that of the Neo control with both 1 and 10 mU/ml TSH, indicating refractoriness in cells experiencing chronic stimulation of adenylate cyclase, exemplified by the elevated un-stimulated cAMP levels. The basal cAMP levels were similarly reduced in the control Neo and Del613-621 FRTL-5 but we found the





**Figure 6** Basal and TSH-induced cAMP in the presence and absence of NaI. Cells were plated in serum-containing medium plus TSH. The next day, they were switched to serum-free medium without TSH. Three days later, NaI (iod; 0 or 50 mM) was added and the cells were incubated for a further 3 days. The analyses were performed on day 7. Results (replicates agree to within 10%) are expressed as a stimulation index (SI) calculated from (cAMP presence TSH/cAMP absence TSH) or (basal cAMP presence NaI/basal cAMP absence NaI). The numbers in parentheses are the basal values for cAMP expressed as pmoles/20 000 cells. Del, Del613–621.

constitutive activity of M453T and L629F to be enhanced by iodide, although this was significant only in M453T.

The cells cultured in the continual presence of TSH were largely unresponsive to the hormone in terms of cAMP production, as expected, and this was not influenced by NaI (data not shown).

#### Phosphorylated:total CREB levels are reduced by TSH but increased by iodide

We then investigated aspects of signal transduction, downstream from the adenylate cyclase, in the mutant expressing cells. Experiments were performed in serum-containing medium.

The ratio of phosphorylated:total CREB was not altered by chronic exposure to TSH in the control Neo population cultured without NaI. In the M453T, L629F and Del613–621 expressing FRTL-5 cell lines, phosphorylated CREB levels were elevated compared with Neo. In contrast to the control cells, TSH produced a reduction in the phosphorylated:total CREB ratio when compared with the same mutant TSHR expressing cells cultured without TSH. The reduction was apparent in these cell pools when grown in the absence (25–55%) or presence (18–65%) of 50 mM NaI. Representative results are shown in Fig. 7A.

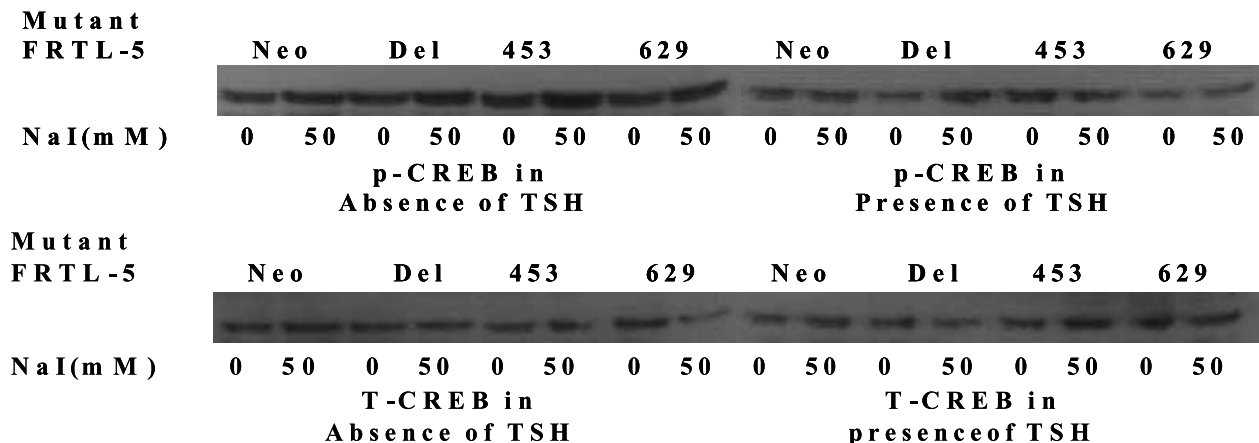
The addition of 50 mM NaI to cells grown in TSH-free medium resulted in an increase in the ratio of phosphorylated:total CREB of at least 22% in the 3 mutant TSHR expressing populations and of 12% in Neo (Fig. 7A).

The ratio of phosphorylated:total MAPK ranges from 31 to 49% in the absence of TSH and was minimally altered in cells cultured in the presence of TSH (range 33 to 46%) in 3 out of 4 of the various FRTL-5 subtypes. Similarly, the addition of 50 mM NaI to cells grown in TSH-free medium produced minimal changes in the ratio of phosphorylated:total MAPK in the 4 cell populations. The exception was Del613–621, the cell type with the greatest dependence on TSH for growth, which displayed the highest (62%) ratio in the absence of TSH and the lowest (19%) ratio in the presence of TSH, but these were not modified by the presence of NaI. The results are shown in Fig. 7B.

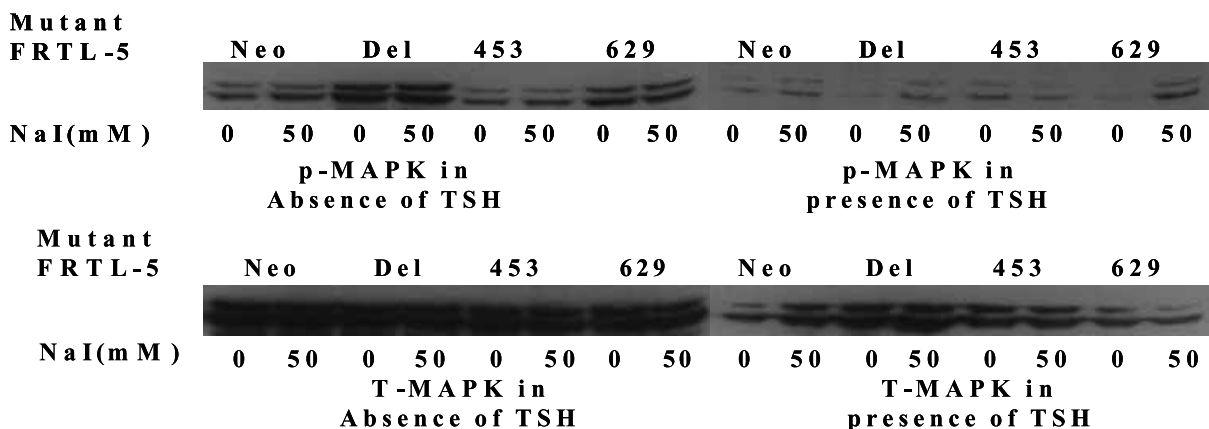
#### Discussion

Individuals with an activating TSHR mutation experience chronic stimulation of the cAMP pathway. Furthermore, depending on the dietary iodine intake, the growth of the thyroid gland will be influenced by the long-term effects of iodide deficiency or excess. Therefore, any study seeking to investigate how iodine intake might influence the biological outcome of activating TSHR mutations requires stable expression of the mutant receptor in a cell type able to trap iodide. We have applied our *in vitro* model of toxic adenoma to address this issue. The model comprises FRTL-5 cells, which permanently express activating human TSHR at levels indistinguishable from the endogenous rat TSHR. FRTL-5 are able to trap iodide in a TSH-dependent manner, and the mutant expressing cells mimic this

(A)



(B)



**Figure 7** Western blot analysis of FRTL-5 cells grown in the presence and absence of 5 mU/ml TSH, with and without 50 mM NaI. Cells were plated in serum-containing medium plus TSH and half the wells were switched to serum-containing medium without TSH the next day. Three days later, NaI (0 or 50 mM) was added and the cells were incubated for a further 3 days. The analysis was performed on day 7. (A) Phosphorylated (p) and total (T) CREB. (B) Phosphorylated (p) and total (T) MAP kinase (MAPK). Del, Del613–621; 453, M453T; 629, L629F.

attribute, but in a TSH-independent manner, to varying degrees (Fuhrer *et al.* 2003).

We have investigated the effects of prolonged exposure to NaI on the proliferation and signal transduction etc. of FRTL-5 thyroid cells expressing activating TSHR mutations. We observed reduced proliferation in the presence of NaI, which was dose dependent and significant from a dose of 10 mM in the presence of serum but from 1 mM in its absence. The difference is probably the result of non-cAMP mediated growth stimulation in the former. The varying sensitivities to NaI of the Neo, WT and different mutant expressing cells most likely reflects the relative abilities of the 5 pools to trap iodide in basal conditions (as

demonstrated in our previous study). This also explains the lack of difference in the degree of 'NaI growth inhibition' when the 5 pools are cultured with TSH.

We then investigated the mechanism responsible for the reduction in cell proliferation in the presence compared with the absence of NaI. Iodide had no influence on the percentages of necrotic or apoptotic cells in any of the FRTL-5 populations. We therefore concluded that NaI does not induce cell death. Of interest, the Neo control had the highest proportion of apoptotic and necrotic cells, suggesting that activating TSHR mutants confer protection from these processes.

TSH and iodide have opposing effects on the proliferation of the FRTL-5 cell pools. It was therefore

rather surprising to observe the similarity of their impact on the cell cycle with both substances producing a decrease in G0/G1 but an increase in G2/M phase cells. However, the proliferation induced by TSH is accompanied by an increase in S phase cells. In contrast the inhibition of proliferation induced by NaI is associated with more cells in the G2 and post-G2 'endoreduplication' populations. These data suggest that cell-cycle blockade (at G2/M) and endocycle escape contribute to the mechanism of inhibition, and are in agreement with studies from Smerdely *et al.* (1993), who demonstrated significant reduction in TSH-induced proliferation by 100 mM NaI. This was attributable to accumulation of the cells both in G0/G1 and G2/M. Furthermore, the abolition of organification by the addition of 0.2 or 2 mM MMI reversed the G2/M but not the G0/G1 blockade. We observed growth inhibition with MMI alone and so were unable to comment on the requirement for organification.

The NaI-induced 'inhibition of proliferation' was more pronounced in the absence of serum and in the FRTL-5 cells expressing the most constitutively active TSHR (M453T). This indicates that the effect is mediated by the cAMP pathway and we have demonstrated that iodide exerted a variety of effects on signal transduction cascades. In agreement with previous studies (Van Sande *et al.* 1985), NaI inhibited TSH-induced cAMP production. We were surprised to observe that iodide enhanced the constitutive activity in M453T and L629F expressing populations whilst Del613–621 behaved like Neo, demonstrating an inhibition of basal levels. These results were further supported by the finding that NaI increased one of the downstream effectors of cAMP, phosphorylated CREB. The opposite effect was demonstrated with TSH in which a reduction in CREB phosphorylation occurred. Thus, lower phosphorylated CREB is associated with proliferation suggesting that chronic elevation of phosphorylated CREB is inhibitory to growth of FRTL-5. This is consistent with the reported IBMX-induced G1/S blockade that occurs in FRTL-5 (Villone *et al.* 1997) but is not consistent with the TSH-independent proliferation of the M453T expressing cells, nor indeed with the evolution of a toxic adenoma. Our work indicates that changes in one signalling pathway are insufficient to explain the biological outcome and that adenoma must overcome the checks and balances imposed on thyrocyte growth to maintain homeostasis, including the down-regulation of protein kinase A during chronic cAMP elevation (Armstrong *et al.* 1995). It seems reasonable to suggest that some mutations, e.g. Del613–621, will be less effective than e.g. T632I (reported in 1 case and 32 cases of hyperthyroidism respectively) at achieving this, as reflected in the frequency of reported mutations (<http://www.uni-leipzig.de/innere/TSH/>) rather than the clinical severity.

In conclusion, our results provide a mechanism to explain the increased incidence of TA and TMNG in regions of iodine deficiency, since adequate or excess iodide intake will counteract the growth-promoting effects of TSHR gain-of-function mutations. Dietary iodine intake may also contribute to the wide range in age of onset of symptoms reported in families harbouring the same activating germline TSHR mutations (Alberti *et al.* 2001).

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