

# Identification of $\delta$ -Iodolactone in Iodide Treated Human Goiter and its Inhibitory Effect on Proliferation of Human Thyroid Follicles\*

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## Summary

There is evidence that iodoarachidonates are mediators of iodide in thyroid autoregulation, however, their occurrence *in vivo* has not yet been demonstrated. We therefore tried to identify  $\delta$ -iodolactone (5-Hydroxy-6-iodo-8,11,14-eicosatrienoic  $\delta$ -lactone, IL- $\delta$ ) in thyroid tissue from a patient with Graves' disease treated with high doses of iodide. Lipids were extracted from thyroid tissue, purified by reversed phase chromatography and analyzed by gas chromatography – tandem mass spectrometry (GC-MSMS). The retention time in gas chromatography and fragmentation pattern in tandem mass spectrometry were determined with bi-chemically synthesized non-deuterated and deuterated IL- $\delta$ . According to retention time (13.44 min) and specific fragments ( $m/z$  303,  $m/z$  259) the occurrence of IL- $\delta$  could be demonstrated in the extract of iodide treated goiter. *In vitro*, potassium iodide (40  $\mu$ M) as well as IL- $\delta$  (1.0  $\mu$ M) significantly inhibited the proliferation of human thyroid follicular cells induced by phorbol ester TPA (12-O-tetradecanoylphorbol 13-acetate). These results demonstrate for the first time that IL- $\delta$  is present in iodide treated human thyroid. As cell proliferation is under negative control of IL- $\delta$ , a crucial role in thyroid involution following iodide treatment may be possible.

## Key words

Iodide – Iodolactone – GC-MS – Thyroid Growth – Autoregulation

## Introduction

As thyroid growth and function are modified by organified iodide, iodocompounds have been postulated to act as intrinsic mediators of iodine. Thyroid hormones and iodinated amino acids have only little effects on thyroid autoregulation (for review see *Pisarev* 1985 and *Wolff* 1989). Iodinated derivatives of polyunsaturated fatty acids, however, caused inhibitions comparable to the effects of iodide. 5-Hydroxy-6-iodo-8,11,14-eicosatrienoic  $\delta$ -lactone ( $\delta$ -iodolactone, IL- $\delta$ ) as well as other derivatives of the arachidonic acid, iodinated at the  $\omega$ -double bond (e.g. 14-hydroxy-15-iodo-5,8,11-eicosatrienoic  $\omega$ -lactone, IL- $\omega$ ), inhibited iodide uptake and organification in calf thyroid slices and were inhibitors of proliferation in calf thyroid cell line (*Chazenbalk, Valsecchi, Krawiec, Burton, Juvenal, Monteagudo, Chester and Pisarev* 1988; *Pisarev, Bocanera, Chester, Kleiman de Pisarev, Juvenal, Pregliasco and Krawiec* 1992). We recently demonstrated that IL- $\delta$  inhibits epidermal growth factor (EGF)-induced proliferation of isolated porcine thyroid follicles in a 50-fold lower concentration than is required for iodide to exert the same inhibitory effect. This inhibition was even demonstrated in the presence of methimazole (MMI), indicating that no more iodide organification was required (*Dugrillon, Bechtner, Uedelhoven, Weber and Gärtner* 1990). In a first *in vivo*-study, IL- $\delta$  and IL- $\omega$  inhibited MMI-induced goiter formation and decreased thyroidal cyclic adenosine 3',5'-monophosphate (cAMP) content in rat (*Pisarev, Chazenbalk, Valsecchi, Burton, Krawiec, Monteagudo, Juvenal, Boado and Chester* 1988).

The formation of iodolipids in thyroid tissue has been known for a long time, but the chemical structures as well as their physiological role remained unclear. Iodolipids in calf thyroid slices were characterized as iodinated free fatty acids and neutral lipids. Suppression of iodide organification as well as phospholipase A<sub>2</sub> strongly decreased their formation, whereas inhibition of prostaglandin synthesis increased lipid iodination, suggesting a strong correlation to the arachidonic acid metabolism (*Chazenbalk, Pisarev, Juvenal, Kleiman de Pisarev, Mercuri and De Tomás* 1985). The formation of an unipolar iodolipid was observed *in vitro* and identified as 2-iodohexadecanal (*Pereira, Braekman, Dumont and Boeynaems* 1990). In rat thyroid lobes the conversions of arachidonic acid and docosahexaenoic acid into the corresponding  $\delta$ - (IL- $\delta$ ) and  $\gamma$ -iodolactone, respectively, were observed (*Boeynaems and Hubbard* 1980; *Boeynaems, Watson, Oates and Hubbard* 1981).

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as well as the formation of IL- $\delta$  in isolated porcine thyroid follicles (Dugrillon et al. 1990). The occurrence of IL- $\delta$ , however, has only been demonstrated in vitro and in the presence of arachidonic acid, therefore its physiological relevance remained unclear (Dumont, Lamy, Roger and Maenhaut 1992). Therefore, we now tried to demonstrate the occurrence of IL- $\delta$  in human thyroid tissue. The content of endogenous IL- $\delta$  from goiter tissue of a patient suffering from Graves' disease pretreated with high doses of iodide was investigated. For analysis we used the highly specific method of GC-NICI-MSMS (gas chromatography – negative ion chemical ionization – tandem mass spectrometry). Gas chromatography (GC)-retention time, parent and daughter ions were classified with biochemically synthesized non-deuterated and deuterated IL- $\delta$ . Furthermore we tested iodide and IL- $\delta$  on proliferation of thyroid follicles in a previously described in vitro-system (Gärtner, Greil, Stübner, Permannetter, Horn and Pickardt 1985a), now adapted to human thyroid.

## Materials and Methods

### *Synthesis of IL- $\delta$ and tissue extraction*

IL- $\delta$  standards were synthesized by lactoperoxidase catalyzed iodination of arachidonic acid or [5,6,8,9,11,12,14,15- $^2$ H $_8$ ]-arachidonic acid (Biomol, Germany) as previously described (Boeynaems and Hubbard 1980; Dugrillon et al. 1990). Thyroid tissue (15 g) from iodide treated goiter of Graves' disease (15 mg iodide/day for 10 days), was cooled on ice and processed immediately. The tissue samples were minced and homogenized in 35 ml of PBS (pH 7.4), filtered and extracted at 4 °C overnight with 70 ml of ethanol/acetic acid (9:1, v/v). The extracts were centrifuged (1500  $\times$  g, 45 min) and the supernatants were reextracted by adding one volume of each, chloroform and water. The lower phases were evaporated and resolved in 1 ml of chloroform. Silica gel chromatography and high performance liquid chromatography (HPLC) were performed as it were with the in vitro synthesized IL- $\delta$  standards.

### *Derivatization and gas chromatography – negative ion chemical ionization mass spectrometry and tandem mass spectrometry*

To obtain the open form of 5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid the samples were treated with 50  $\mu$ l of water/pyridine/triethylamine (10/10/1, v/v; pH 12.0) for 1 h at room temperature. After esterification with pentafluorobenzylbromide (PFB-Br, Sigma) and trimethylsilylation with BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamid, Pierce, IL, USA) the samples were measured by gas chromatography – mass spectrometry. After purification by HPLC the samples were evaporated and acidified with 200  $\mu$ l of methanol/formic acid (9:1, v/v; pH 3.0) for 1 h at room temperature to obtain quantitatively the lactone of 5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid. GC was performed on a Varian 3400 gaschromatograph (Palo Alto, CA, USA) in the splitless mode using a J&W fused silica capillary column (DB-1; 30 m; 0.25 mm in diameter; coating thickness 0.25  $\mu$ m; ICT, Frankfurt, Germany). For mass spectrometry a Finnigan TSQ 70 (San Jose, CA, USA) was used in the negative ion chemical ionization (NICI)-mode. The conditions for gas chromatography – mass spectrometry-experiments were described earlier (Dugrillon et al. 1990). For the tandem mass spectrometry-experiments argon was used as collision gas. Operating conditions were: source temperature, 130 °C; electron energy, 70 eV; emission current, 0.2 mA; methane chemical ionization gas pressure, 1.0 Torr; electron multiplier, 2000 V; collision cell pressure, 1.8 mTorr; collision energy, 16 eV. For identification of the obtained daughter fragments of IL- $\delta$  the mass spectra of the non-deuterated and the deuterated standard were compared. Fragments of m/z 303/311 and m/z 259/267 were chosen as characteristic ions of non-deuterated and deuterated IL- $\delta$ , respectively.

### *Growth experiments with isolated follicles and cyclic adenosine-3',5'-monophosphate-determination*

Isolated follicles from human paranodular thyroid tissue were prepared according to Gärtner et al. (1985a), with little modifications. Slices (1 mm maximal thickness) were incubated for 90 min at 37 °C in medium containing collagenase (2.5 mg/ml, clostridium histolyticum, Boehringer) and dispase (15 mg/ml, neutral protease, bacillus polymyxa, grade II, Boehringer). The digested tissue was filtered through 500  $\mu$ m-nylon mesh, centrifuged for 10 min at 50  $\times$  g and further processed as described. After a 48 h-reconstitution phase all follicles were closed and single cells as well as follicle fragments were removed by washing the intact follicles twice in fresh medium. About 90% of right-side-right follicles were obtained as verified by phase-contrast microscopy. Vital staining with ethidium bromide/acridine orange revealed over 95% of cells alive.

The proliferation assay was performed according to the method described by Dugrillon et al. (1990). The follicles were distributed into 24-well culture plates (50.000 cells/well, Costar, Germany). After 7 days the medium supernatant was changed together with test substances, and after 14 days the cells were trypsinated (1 h at 37 °C, trypsin 75  $\mu$ g/ml, EDTA 25 mM, Boehringer), resulting in solitary cells as proofed by visualization of the normal distributed cell sizes during determination of cell counts using an adapted cell-counter (Coulter Multisizer, Coulter Electronics, Germany).

For cyclic adenosine-3',5'-monophosphate (cAMP) determination the follicles (30  $\mu$ l follicles/400  $\mu$ l medium/tube) were incubated with 3-isobutyl-1-methyl-xanthine (IBMX, 0.5 mM, Sigma) for 2 min before the test substances were added. The incubation was stopped after 5 min by adding 6% of trichloroacetic acid (TCA) and cooling the tubes on ice. Total cAMP was then extracted and determined by radioimmunoassay (NEN, Germany) as described by Gärtner, Greil, Demhartner and Horn (1985b).

Growth experiments were performed in quadruplicate or triplicate values. Results from independent experiments were pooled after performing ANOVA-analysis of variance and expressed as mean  $\pm$  standard deviation (SD). Significant differences of group means were then calculated according to SCHEFFE at  $p \leq 0.05$ .

## Results

### *Characterization of in vitro-synthesized IL- $\delta$ by gas chromatography – negative ion chemical ionization – mass spectrometry and tandem mass spectrometry*

The GC-retention time of the PFB/TMS (pentafluorobenzyl/trimethylsilyl)-derivative of 5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid was 11.33 min, the lactone of this compound (IL- $\delta$ ) elutes at a GC-retention time of 13.44 min. The equilibrium of the open form and the lactone form depends on the acidity: treatment with acid quantitatively results in the lactone form, whereas treatment with triethylamine mainly leads to the open form of 5-hydroxy-6-iodo-8,11,14-eicosatrienoic acid (data not shown). Measuring the non-deuterated IL- $\delta$  standard by GC-NICI-MSMS fragments of m/z 127, m/z 302 and m/z 303 were obtained (Fig. 1a). The fragment of m/z 303 can be explained by the loss of iodide, the fragment of m/z 302 by the loss of hydrogen iodide. The fragment of m/z 127 goes back to the separated iodide and therefore is characteristic for iodinated compounds. Under analogous gas chromatography – mass spectrometry-conditions the mass spectrum of [5,6,8,9,11,12,14,15- $^2$ H $_8$ ]-IL- $\delta$  shows the corresponding fragments of m/z 311, m/z 310 and m/z 127 in comparable inten-

sities (Fig. 1b). Using the fragments of  $m/z$  303 and  $m/z$  311 as parent ions in the tandem mass spectrometry experiment daughter ions of  $m/z$  285/293 and  $m/z$  259/267 were obtained beside the corresponding parent ions, resp. (Fig. 1c and d). The fragments of  $m/z$  285/293 can be explained by the loss of water, those of  $m/z$  259/267 by the loss of  $\text{CO}_2$ . Beside the remaining parent ions, the latter daughter ions were chosen as characteristic fragments for identification of IL- $\delta$  because of their higher intensity compared with the daughter ions of  $m/z$  285/293.

#### Identification of IL- $\delta$ in human thyroid tissue

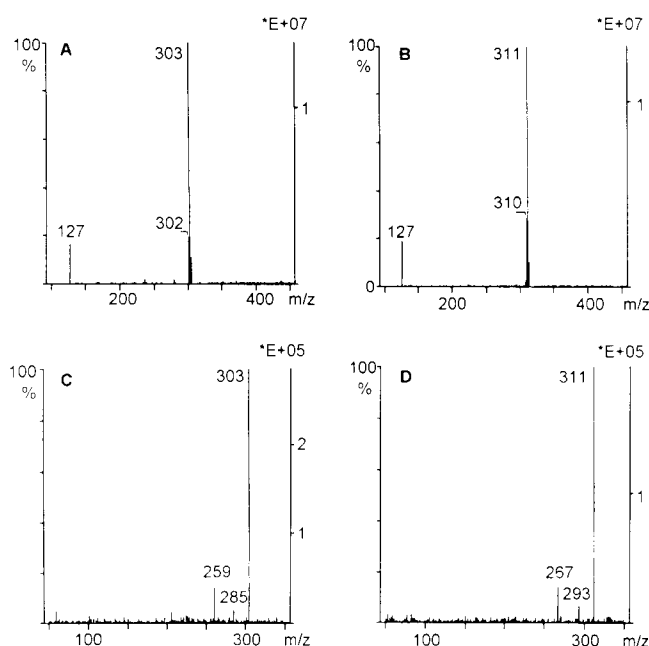
Lipid extracts were prepared from human goiter obtained after surgery. Corresponding to the IL- $\delta$  standard the extract of thyroid tissue pretreated with iodide showed a significant GC-peak at 13.44 min by detecting the specific fragments of  $m/z$  303 and  $m/z$  259 in tandem-mass spectrometry (Fig. 2). These results were obtained in two samples.

#### Growth experiments with isolated human thyroid follicles

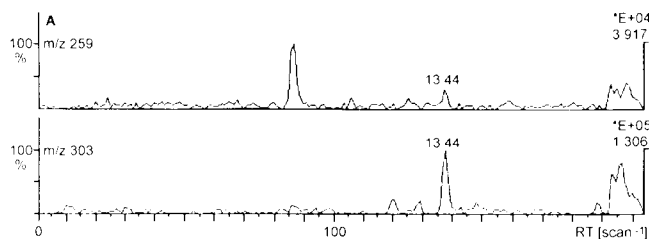
Intact follicles with preserved polarity were seeded into culture wells together with 1% of fetal calf serum and were stimulated with test substances. Thyroid cell monolayers grew within a few days around the mother follicles and cell counts were determined after 14 days. The cell count seeded into the wells was  $41 \pm 7\%$  compared to cell count obtained from basal control after incubation ( $100.0 \pm 8.1\%$ ). EGF (5 ng/ml) significantly enhanced follicle proliferation to  $119.0 \pm 14.0\%$  and the phorbol ester TPA (100 nM) significantly stimulated to  $138.0 \pm 14.1\%$ , whereas TSH (1.0 mU/ml) alone as well as together with EGF did not change growth rates  $92.1 \pm 17.0\%$  and  $124.5 \pm 15.2$ , resp.). IGF-I (100 ng/ml) alone as well as together with TSH also had no effect on follicle cell proliferation ( $97.1 \pm 20.8\%$  and  $91.5 \pm 12.2\%$ ). KI (40  $\mu\text{M}$ ) significantly inhibited TPA (100 nM) stimulated follicle proliferation to  $113.7 \pm 3.5\%$  vs.  $138.0 \pm 14.1\%$ . IL- $\delta$  standard (0.1 and 1.0  $\mu\text{M}$ ) also significantly and dose dependently inhibited TPA stimulated proliferation to  $111.0 \pm 4.4\%$  and  $94.3 \pm 5.0\%$ , resp. (Fig. 3). Although TSH had no effect on thyroid follicle proliferation, stimulation of total cAMP formation in isolated follicles increased from  $0.48 \pm 0.08$  to  $0.68 \pm 0.04$  and  $1.10 \pm 0.08$  pmol cAMP/ $\mu\text{g}$  DNA with 0.1 and 0.5 mU/ml of TSH within 5 min.

#### Discussion

For the first time we were able to demonstrate the occurrence of IL- $\delta$  in human thyroid *in vivo*. The application of a highly specific and highly sensitive method is especially important in the case of determination of endogenously produced, low abundance compounds, such as IL- $\delta$ . Tandem mass spectrometry (MSMS)-detection of daughter ions has several advantages over the mass spectrometry (MS) of parent ions. For every signal appearing in a MSMS-chromatogram at a certain  $m/z$  value the following parameters have to be coordinated to one another: the  $m/z$  value of the parent ion; the  $m/z$  value of the daughter ion; the collision cell pressure; the kinetic energy of daughter ion. Compared with detection in the MS-mode, where only the first of the above conditions has to be met, MSMS-detection produces chromatograms with specific sig-

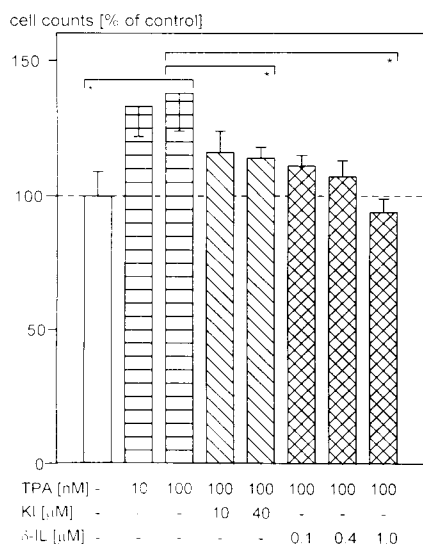


**Fig. 1** Gas chromatography – negative ion chemical ionization – mass spectra (GC-NICI-MS) of the non-deuterated (A) and [5,6,8,9,11,12,14,15- $^2\text{H}_6$ ]-deuterated (B) 5-hydroxy-6-iodo-8,11,14-eicosatrienoic  $\delta$ -lactone (IL- $\delta$ ) at GC-retention time 13.44 min, and the tandem mass spectra (MSMS) of the non-deuterated (C) and deuterated (D) IL- $\delta$  standards, using the fragments of  $m/z$  303 and 311 as parent ions, resolving additional specific daughter fragments at  $m/z$  259 and 267 as well as  $m/z$  285 and 293, respectively.



**Fig. 2** Selected ion monitoring at  $m/z$  259 (upper trace) and  $m/z$  303 (lower trace) of gas chromatography – negative ion chemical ionization – tandem mass spectrometry (GC-NICI-MSMS) of lipid extracts from iodide treated human goiter, with significant peaks at 13.44 min corresponding to 5-hydroxy-6-iodo-8,11,14-eicosatrienoic  $\delta$ -lactone (IL- $\delta$ ).

nals in addition to the compound of interest. The increased selectivity of MSMS therefore leads to more reliable results. In addition, measurement in the negative ion chemical ionization (NICI)-mode has the advantage of the higher sensitivity over the measurement in the EI (electron ionization)-mode: because of the lower energy during the ionization process only few and high intensive parent ions were produced using the NICI-mode, whereas in the high energetic EI-process a lot of fragments of at least lower intensities were produced (Boeynaems and Hubbard 1980). The analysis by MSMS does not allow to distinguish between  $\delta$ - and  $\omega$ -iodolactone. Because of its specific retention time in HPLC and GC, however, which differs from that of other iodocompounds, IL- $\delta$  could be clearly identified in our study.



**Fig. 3** Effects of TPA (12-O-tetradecanoylphorbol 13-acetate) alone and together with iodide as well as 5-hydroxy-6-iodo-8,11,14-eicosatrienoic  $\delta$ -lactone (IL- $\delta$ ) on proliferation of isolated human thyroid follicles. Follicles were stimulated with TPA; KI was added 24 h before TPA; IL- $\delta$  was added together with TPA. Cell counts were determined after 14 days of incubation ( $n = 3$ , Mean  $\pm$  SD, \* $p \leq 0.05$ ).

We focussed on IL- $\delta$ , because arachidonic acid is the major polyunsaturated fatty acid in thyroid membranes (Wolff 1989). Furthermore, IL- $\delta$  is the major product of enzyme catalyzed and thyroidal iodination of arachidonic acid *in vitro* and is decreased by the peroxidase inhibitor MMI (Boeynaems and Hubbard 1980; Dugrillon et al. 1990), whereas  $\omega$ -iodinated compounds occur during chemical iodination and their formation in thyroid has not been found yet. The occurrence of IL- $\delta$  in iodide treated goiter implicates the conversion of iodide into IL- $\delta$  *in vivo*.

The amount of IL- $\delta$  could not be quantified, because no internal standard was employed in our study. The deuterated standard was used as a qualitative external standard and may serve as internal standard in future investigations. The pentafluorobenzyl (PFB)-derivative was made to proof the  $\delta$ -lactonization at low pH and to compare with earlier findings obtained with the PFB-derivative (Dugrillon et al. 1990).

As shown in experiments with porcine thyroid follicles, concentrations in the range of 1  $\mu$ M are sufficient to completely inhibit proliferation (Dugrillon et al. 1990). In FRTL-5 cells 10  $\mu$ M of IL- $\delta$  were necessary to inhibit cell proliferation, while IL- $\omega$  was effective at 1  $\mu$ M. This may contribute to the fact that IL- $\delta$  has no effect on cAMP accumulation at 1  $\mu$ M (Dugrillon et al. 1990), while growth in FRTL-5 cells is cAMP-dependent (Jin, Hornicek, Neylan, Zakarija and McKenzie 1986). The antiproliferative effect of IL- $\delta$  could now be demonstrated in human thyroid follicles. Biochemically synthesized IL- $\delta$  inhibited TPA stimulated proliferation of isolated human thyroid follicles in the same low concentration range as it was observed in porcine follicles before, despite the known low mitotic activity of primary human thyroid follicle cultures. On the assumption that endogenously IL- $\delta$  is produced near the site of action, extremely low concentrations of IL- $\delta$  are estimated to occur under normal iodine intake. Treatment with iodide may increase IL- $\delta$  and therefore may lead to thyroid gland involution. The acute toxic effect of high doses of iodide observed *in vitro*, which is prevented when iodide oxidation or organification is inhibited (Many, Mestdagh, van den Hove and Deneff 1992), may be caused by free radical action and seem not to be caused by IL- $\delta$ , because *in vitro* no signs of cell damage could be observed.

Thyroid autoregulation involves a differentiated network of factors modulated by various iodide effects (for review see Pisarev 1985 and Wolff 1989). The inhibition of cAMP-formation, which also has been attributed to organic iodine (Van Sande, Grenier, Willems and Dumont 1975), should be mediated by another iodocompound, as IL- $\delta$  has been shown not to inhibit cAMP formation (Dugrillon et al. 1990). In isolated porcine thyroid follicles, where cAMP-formation inhibits cell proliferation (Gärtner et al. 1985b; Watanabe, Amino, Tamaki, Iwatani and Miyai 1985), this unidentified iodocompound should increase cell growth. Inhibition of cAMP-production, however, alters the formation of iodocompounds through down-regulation of thyroid peroxidase and iodide transport. Furthermore, high concentrations of iodide, probably through formation of  $I_2$ , directly inhibit the catalytic activity of thyroid peroxidase (summarized in Wolff 1989). These complex interactions lead either to growth inhibition of high doses of iodide (Becks, Eggo and Burrow 1988; Tramontano, Veneziani, Lombardi, Villone and Inghar 1989) or to growth induction (Heldin, Karlsson and Westermark 1987), depending on the culture system used. We previously described a biphasic action of iodide on porcine thyroid follicle growth, increasing at low doses and decreasing at higher concentrations of iodide. We suggested a dose-dependent dual action of iodide by formation of different iodocompounds acting on cAMP-dependent and -independent pathways (Gärtner et al. 1985b; Gärtner, Bechtner, Rafferzeder, Dugrillon, Greil and Pickardt 1988; Dugrillon and Gärtner 1992; Gärtner 1992). A cAMP-independent mechanism of iodide-induced inhibition of thyroid cell proliferation was also suggested by Tramontano et al. (1989) and Becks, Eggo and Burrow (1988). In this hypothesis, IL- $\delta$  may represent the iodocompound, mediating the inhibitory effect on thyroid cell proliferation induced by local growth factors, without affecting cAMP levels.

No data are available on the site of action of IL- $\delta$ . A location of iodolactones at the cell membrane level may be suggested from the site of arachidonic acid release as well as from the inhibition by organic forms of iodine at or near the adenylate cyclase complex and the inhibition of inositol phosphate generation (Filetti and Rapoport 1983; Laurent, Mockel, Takazawa, Erneux and Dumont 1989). Indeed, IL- $\omega$  was found to act at the cell membrane level (Krawiec, Chester, Bocanera, Pregliasco, Juvenal and Pisarev 1991).

For the pathophysiological mechanisms of goiter development these findings may be of great importance because inadequate formation of iodolactones could lead to a predominant proliferative effect of local growth factors. We therefore postulate that iodine deficiency, through a decrease of iodolactones, may cause goiter development *in vivo*.

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