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Inhibition of human thyroid adenylyl cyclase by 2-iodoaldehydes

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Abstract

2-Iodohexadecanal (IHDA), which can be formed upon addition of iodine to the vinyl ether group of plasmalogens, has been identified as a major thyroid iodolipid (Pereira et al. (1990) *J. Biol. Chem.* 265, 17018–17025). In this study, we have investigated the possibility that it would be a mediator of the inhibitory effect of iodide on thyroid adenylyl cyclase. In human thyroid membranes, IHDA inhibited the adenylyl cyclase activity stimulated by thyrotropin (TSH), GTP- γ -S or forskolin (FSK), whereas it did not decrease the specific binding of TSH to its receptors. The inhibitory effect on the cyclase reached a maximum after a 1-h-pre-incubation of the membranes with IHDA at 30°C and was poorly reversible. It was also observed following a 4-h incubation with IHDA at 4°C, a condition in which adenylyl cyclase is protected against heat inactivation. IHDA decreased the V_{\max} of adenylyl cyclase, but had no effect on the K_m for ATPMg²⁻. IHDA also inhibited the FSK-stimulated adenylyl cyclase activity in liver and kidney cortex membranes, but had no effect on the Mg²⁺-ATPase activity of thyroid membranes. The inhibitory effect of IHDA has also been demonstrated in intact cells. As in membranes, IHDA decreased the rise in cAMP induced by TSH in cultured dog thyroid cells and this inhibition was maintained following pretreatment of the cells with pertussis toxin. In order to evaluate the specificity of the IHDA action, various analogs have been synthesized. This study has permitted the identification of two major structural features required for the inhibition of human thyroid adenylyl cyclase; the terminal aldehyde function and an iodine atom at C2, other halogens being ineffective. In conclusion, we have shown that IHDA exerts a direct inhibitory effect at or near adenylyl cyclase; all the properties of this effect characterized so far are identical to those of the adenylyl cyclase inhibition obtained following the exposure of thyroid tissue to iodide.

Keywords: Thyroid; Iodide; Adenylyl cyclase; Iodolipids; 2-Iodohexadecanal

1. Introduction

The metabolism of iodine in the thyroid gland is designed to make the most efficient use of the iodine supply which is often scarce and intermittent. But the thyroid has also adaptation mechanisms which reduce the iodide metabolism when the supply is abundant, in order to avoid thyrotoxicosis. The regulation of thyroid metabolism by iodide involves numerous inhibitory effects (Wolff, 1989). One of them is a decrease in cAMP formation in response to TSH, resulting in an inhibition of all cAMP-mediated stimulatory effects of TSH on the gland. Following its initial discovery by Van Sande and Dumont in dog thyroid slices (Van Sande et al., 1973), this effect has been de-

scribed in several models including: thyroid lobes from hypophysectomized rats receiving a diet rich in iodine (Rapoport et al., 1975; Rapoport et al., 1976), mouse thyroid lobes (Hashizume et al., 1976; Yu et al., 1976; Saddok et al., 1978), slices of horse, beef, sheep (Van Sande et al., 1975), cat (Sherwin, 1978) and human (Van Sande et al., 1980) thyroid, isolated beef thyroid cells (Sherwin and Tong, 1975), cultured dog (Rapoport et al., 1977) and porcine (Heldin et al., 1985) thyroid cells. Several properties of the inhibitory effect of iodide on the cAMP system have been characterized. It is due to a reduction in cAMP generation and not to an accelerated breakdown (Pochet et al., 1977) or cAMP efflux (Cochaux et al., 1986). It is not rapidly reversible after washing slices (Van Sande et al., 1985), has a long duration in culture (Heldin et al., 1985) and recovery requires new protein synthesis (Filetti and Rapoport, 1983). The inhibition can be detected in mem-

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branes prepared from iodide-exposed tissue (Pochet et al., 1977; Heldin et al., 1985; Cochaux et al., 1987), whereas iodide has no direct effect when added to the membranes, probably because of a lack of organification. An inhibition is observed whatever stimulus is used to activate the cyclase: TSH, prostaglandin E₁, FSK or cholera toxin in slices (Filetti and Rapoport, 1983; Van Sande et al., 1985), TSH, GTP- γ -S, fluoride or FSK in membranes (Cochaux et al., 1987). G_i is not the mediator of the inhibition, since it is maintained after treatment with pertussis toxin (Cochaux et al., 1985). These data indicate that the inhibitory effect of iodide involves a stable modification located beyond the receptors, at or near the adenylyl cyclase itself, but not at the level of G_i (Filetti and Rapoport, 1983; Cochaux et al., 1987). Finally, the inhibition results from a decreased V_{max}, with no change in the affinity for ATPMg²⁻ (Cochaux et al., 1987).

The observation that the inhibitory effect of iodide on cAMP is abolished by methimazole (MMI), as well as other blockers of iodide organification, led Van Sande et al. (1975) to suggest that it is mediated by an intracellular iodinated intermediate. So far, this intermediate has not been identified. The hypothesis that the mediator would be T3 or T4 has not been substantiated (Rapoport et al., 1977). More recently there has been a growing interest in the possible role of iodolipids (Wolff, 1989). Since the rat thyroid (Boeynaems and Hubbard, 1980) as well as porcine thyroid follicles (Dugrillon et al., 1990) convert exogenous arachidonic acid into an iodolactone, it was speculated that this iodolactone or related products might be the mediators looked for. A decrease in thyroid cAMP level following injection of 14-iodo-15-hydroxyicosatrienoic acid to rats has been reported (Pisarev et al., 1988) but could obviously represent an indirect effect. Dugrillon et al. (1990) found that the iodo- δ -lactone derived from arachidonic acid inhibited the EGF-stimulated proliferation of porcine thyroid cells, but had no effect on the TSH-induced accumulation of cAMP in these cells. However, there is no evidence that these compounds can be formed in significant amounts in the thyroid in the absence of an exogenous supply of free arachidonic acid. In the meantime, the major iodolipid in the horse and rat thyroid has been identified as 2-iodohexadecanal (IHDA), which can be formed via the addition of iodine to the vinyl ether group of plasmalogens followed by hydrolysis (Pereira et al., 1990). In this study, we have investigated the action of IHDA on the adenylyl cyclase activity in human thyroid membranes and on the accumulation of cAMP in cultured dog thyroid cells.

2. Materials and methods

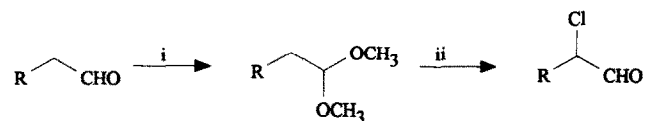
2.1. Material

¹H NMR spectra (250 MHz) were recorded in CDCl₃ on a Bruker WM 250 spectrometer and are reported in ppm from internal TMS on the δ scale. Infrared spectra were taken with a Bruker IFS 25 instrument and the samples

examined as deposited glassy films on NaCl disks or as ground solid in KBr pellets. Electron impact mass spectra were recorded on a VG Micromass 7070 spectrometer. Thin layer chromatography (TLC) analyses were performed on Polygram silica gel SILG/UV254 pre-coated plates (0.25 mm). Column chromatography was performed on silica gel 60 (230–400 mesh) from Merck. Octanal and dodecanal were purchased from Aldrich-Chemie (Steinheim). Collagenase type I, deoxyribonuclease, adenosine 5'-triphosphate disodium salt (ATP) from equine muscle, phosphocreatine, bovine TSH, FSK and GTP- γ -S were provided by Sigma Chemical Co. (St. Louis, MO). Creatine kinase from rabbit muscle, phosphoenolpyruvate, pyruvate kinase, NADH and lactate dehydrogenase were purchased from Boehringer (Mannheim, Germany). The coverslips (Thermanox^R) were provided by Flow Laboratories (Irvine, UK). Rolipram was a gift from the Laboratoires Jacques Logeais, France. The 2-iodoaldehydes, 1-hexadecanol and 2-bromohexadecanal were prepared according to the procedures described previously (Ohayon et al., 1994). The other tested compounds are undescribed derivatives and were prepared using the procedures described below.

2.2. Synthesis of IHDA-related derivatives

2-Chlorohexadecanal. The following sequence of reactions was used to prepare 2-chlorohexadecanal starting from hexadecanal: R = CH₃(CH₂)₁₃-

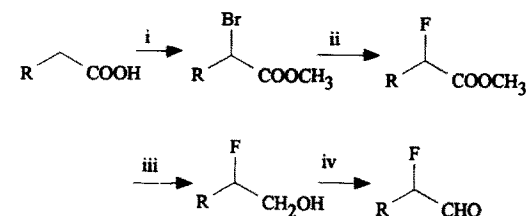


(i) CH₃OH, *p*-toluenesulfonic acid (0.1 equiv.), methylorthoformate (1 equiv.), 2 h, reflux (yield 80%).

(ii) MnCl₂ (0.5 equiv.), MnO₂ (1.2 equiv.), trimethylsilyl chloride (4.8 equiv.), CH₃CN/CH₃OH (1:1), 19 h, room temperature (yield 67%), then trifluoroacetic acid 50%, CH₂Cl₂, 4 h, reflux (yield 71%). Adapted from Bellesia et al. (1992).

Spectral properties of 2-chlorohexadecanal: m.p. 49–50°C, M⁺ at *m/z* 274/276; ¹H NMR: 9.48 (1H, d, *J* = 2.5 Hz), 4.15 (1H, ddd, *J* = 2.5, 5.5, 8.1 Hz). IR: 1738 cm⁻¹.

2-Fluorohexadecanal. The following sequence of reactions was used to prepare 2-fluorohexadecanal starting from hexadecanoic acid: R = CH₃(CH₂)₁₃-



(i) P red (1 equiv.), Br₂ (3.6 equiv.), 80°C, 24 h then CH₃OH, 0°C, 1 h then 80°C, 30 min (yield 67%). Adapted from Pogany et al. (1987).

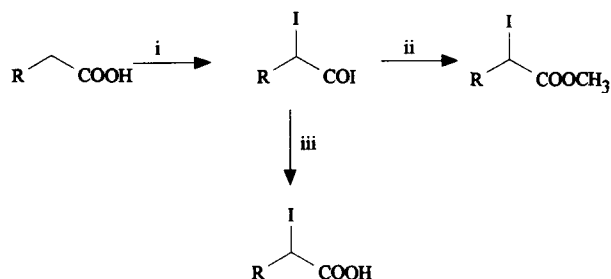
(ii) CH₃CN–H₂O 0.1%, AgF (4.5 equiv.), 80°C, 48 h (yield 50%). Adapted from Pogany et al. (1987).

(iii) H₄LiAl (3 equiv.), ether, reflux, 4 h (yield 83%).

(iv) CH₂Cl₂, (COCl)₂ (3.3 equiv.), DMSO (6.6 equiv.), –40°C (yield 92%). Adapted from Mancuso et al. (1978).

Spectral properties of 2-fluorohexadecanal: m.p. 58–59°C; M⁺ at *m/z* 258; ¹H NMR: 9.76 (1H, dd, *J* = 0.7, *J*_{HF} = 6.2 Hz), 4.81 (1H, dm, *J*_{HF} = 48.8 Hz). IR 1736 cm⁻¹.

Methyl-2-iodohexadecanoate and 2-iodohexadecanoic acid. The following sequence of reactions was used to prepare methyl-2-iodohexadecanoate and 2-iodohexadecanoic acid starting from hexadecanoic acid: R = CH₃(CH₂)₁₃–



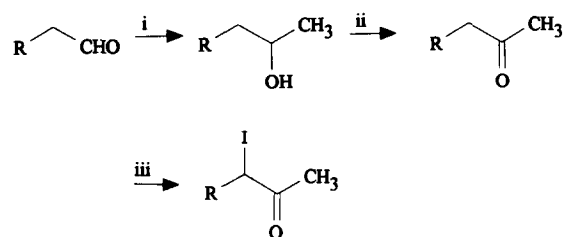
(i) I₂ (0.25 equiv.), chlorosulfonic acid (1 equiv.), 1,2-dichloroethane, 2 h, 80°C.

(ii) CH₃OH.

(iii) H₂O. Adapted from Ogata and Watanabe (1980).

Spectral properties of methyl-2-iodohexadecanoate: M⁺ at *m/z* 396. ¹H NMR: 4.30 (1H, t, *J* = 7.6 Hz), 3.75 (3H, s). IR: 1740 cm⁻¹.

3-Iodo-2-heptadecanone. The following sequence of reactions was used to prepare 3-iodo-2-heptadecanone starting from hexadecanal: R = CH₃(CH₂)₁₃–



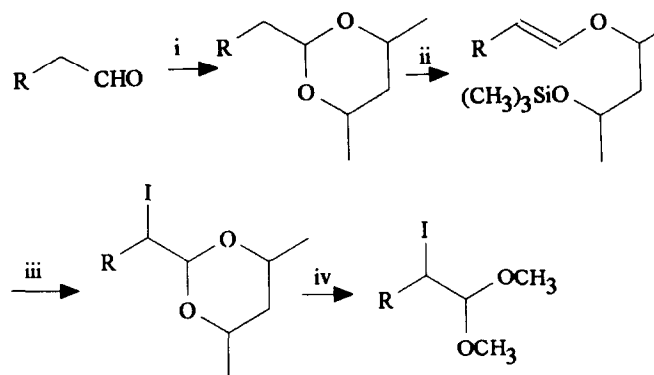
(i) CH₃MgI (3 equiv.), ether, 40 min, room temperature (yield 53%).

(ii) PCC (1.5 equiv.), CH₂Cl₂, 4 h, reflux (yield 64%).

(iii) HgCl₂ (0.5 equiv.), I₂ (1 equiv.), CH₂Cl₂, 4 h, room temperature (yield 48%). Adapted from Barluenga et al. (1986).

Spectral properties of 3-iodo-2-heptadecanone: m.p. 33–34°C; M⁺ at *m/z* 380. ¹H NMR: 4.44 (1H, t, *J* = 7.5 Hz), 2.41 (3H, s). IR: 1713 cm⁻¹.

2-Iodo-hexadecanal-(4R,6R*)-dimethyl-1,3-dioxane and 2-iodohexadecanal dimethylacetal.* The following sequence of reactions was used to prepare 2-iodohexadecanal-(4R*,6R*)-dimethyl-1,3-dioxane and 2-iodohexadecanal dimethylacetal starting from hexadecanal: R = CH₃–(CH₂)₁₃–



(i) *p*-toluenesulfonic acid (0.01 equiv.), (*dl*)-pentan-2,4-diol (1.1 equiv.), anhydrous benzene, reflux, 2 h (yield 85%). Adapted from Denmark and Almstead (1991).

(ii) trimethylsilyl trifluoromethanesulfonate (3 equiv.), diisopropylethylamine (2 × 3.2 equiv.), CH₂Cl₂, 0°C, 4 h (yield 90%). Adapted from Gassman and Burns (1988).

(iii) ICl, anhydrous THF, –75°C, 10 min (yield 89%).

(iv) *p*-toluenesulfonic acid (0.8 equiv.), CH₃OH, 12 h, reflux (yield 70%).

2-Iodo-hexadecanal-(4R*,6R*)-dimethyl-1,3-dioxane was obtained and tested as a mixture of two diastereoisomers [M⁺ at *m/z* 452; UV (hexane): 261 nm (444)].

Spectral properties of M⁺ at *m/z* 412. ¹H NMR: 4.26 (1H, d, *J* = 5.6 Hz), 4.07 (1H, m), 3.43 (3H, s), 3.42 (3H, s).

2.3. Preparation of membranes

Human thyroid membranes were prepared from normal glands (removed for the treatment of a laryngeal cancer, two distinct preparations) or from paranodular tissue obtained from patients undergoing thyroidectomy for autonomous nodules (five distinct preparations). The tissue was minced and rapidly homogenized by hand in a glass/glass homogenizer containing a solution of 250 mM sucrose, 1 mM MgCl₂, 1 mM EGTA, 20 mM Tris–HCl (pH 7.4) (5 ml/g tissue) at 0°C. The homogenate was filtered through two layers of gauze and centrifuged for 10 min at 300 × *g*. The supernatant was centrifuged for 10 min at 30 000 × *g* (Carayon et al., 1979). The resulting supernatant was discarded and the pale part of the pellet was delicately recovered in the buffer. Membranes were stored at –80°C at a protein concentration of ~10 mg/ml of buffer.

2.4. Culture of dog thyroid cells

Minced dog thyroid tissue was digested for 60 min at 37°C by collagenase type I (120 U/ml) and deoxyribonuclease (100 U/ml) in Basal Eagle's Medium (BME) (Roger et al., 1987). The resulting suspension of follicles was filtered through nylon mesh, separated from isolated cells by three centrifugations (2 min at 100 × g) and seeded on coverslips (Thermanox^R) at a density of 200 000 cells/cm² in a mixture of Dulbecco's Modified Eagle's Medium/Ham's F-12 medium/MCDB 104 medium (2:1:1, by vol.) supplemented by 2 mM sodium pyruvate, 5 µg/ml insulin, 40 µg/ml ascorbic acid, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The coverslips were maintained in a water-saturated incubator at 37°C in an atmosphere of 5% CO₂. After 24 h of cell spreading, the medium was replaced by the same mixture supplemented with FSK (5 µM), in order to induce cell differentiation (Roger et al., 1985). The test incubations were made on the fourth day.

2.5. Assay of adenyl cyclase activity in membranes

Thyroid membranes (40 µg protein) were pre-incubated with IHDA or different lipids in 130 µl of Tris 50 mM (pH 7.8) supplemented with rolipram (10 µM), a cyclic AMP phosphodiesterase inhibitor (Beavo and Reifsnnyder, 1990) for 1 h at 30°C or for 4 h at 4°C. Thereafter, a 20-min incubation was started by the addition of ATP (0.5 mM), MgCl₂ (5 mM), an ATP regenerating system consisting of creatine kinase (10 U/ml) and phosphocreatine (10 mM) and an agonist of adenyl cyclase: TSH, FSK or GTP-γ-S in 20 µl. Alternatively, the membranes were directly incubated at 30°C with ATP, MgCl₂, the ATP regenerating system and GTP-γ-S, with or without IHDA, up to 5 h. The reaction was stopped by addition of 1 ml of deionized boiling water and cAMP measurements were made using a radioimmunoassay according to the method of Brooker et al. (1979). Each experimental condition was tested in triplicate and, for each tube, the radioimmunoassay of cAMP was performed in duplicate.

2.6. TSH binding to thyroid membranes

Purified bovine TSH (20 U/mg) from UCB (Braine l'Alleud, Belgium) was labeled with ¹²⁵I by the lactoperoxidase method. The labeled TSH was separated from free radioiodide on a 1.6 × 70 cm Sephacryl 200HR column (Pharmacia, Piscataway, NJ) equilibrated in PBS, EDTA (2 mM), BSA 0.1%. The specific activity of the tracer was 80 µCi/µg TSH. Human thyroid membranes (40 µg protein/150 µl) were pre-incubated with or without IHDA (10 µM) in Tris-HCl 50 mM (pH 7.8) supplemented with rolipram (10 µM) for 60 min at 30°C or for 4 h at 4°C. TSH binding was then measured as described by Costagliola et al. (1992). The membranes (15 µg protein) were mixed with ¹²⁵I-labeled TSH (0.06 µCi/200 µl) and various concentrations of unlabeled TSH (Thyropar, Armour Pharmaceuticals Co, Phoenix, AZ) in a final volume of

200 µl in Tris-HCl (20 mM, pH 7.4), EDTA (1 mM), BSA 0.2%. After 1 h at room temperature, the incubation was stopped by the addition of 1 ml cold buffer and centrifugation for 5 min at 10 000 × g, 4°C. The resulting pellet was counted in a γ-counter.

2.7. Measurement of ATPase activity

Mg²⁺-ATPase activity was measured by the method of Nørby (1988). This procedure is based on the coupling of ATP hydrolysis to the transformation of phosphoenolpyruvate (PEP) into pyruvate by pyruvate kinase (PK). The second reaction is coupled itself with the simultaneous reduction of pyruvate and transformation of NADH, H⁺ to NAD⁺ by a lactate dehydrogenase (LDH). The rate of disappearance of NADH was followed by spectrophotometry (340 nm) and is proportional to the rate of ATP-hydrolysis and the ATPase activity in the mixture. Briefly, human thyroid membranes (60 µg protein) in 150 µl Tris-HCl 50 mM (pH 7.8) were pre-incubated with IHDA or 2-iodooctanal at 30°C. After 60 min, 20 µl of the mixture was added to 500 µl of Tris-HCl (5 mM, pH 7.8), NaCl (130 mM), KCl (20 mM), MgCl₂ (4 mM), ATP (3 mM), PEP (1 mM), NADH (0.2 mM), PK (10 U/ml), LDH (30 U/ml) and histidine (30 mM). Kinetics of NADH disappearance were performed over a period of 15 min at 25°C on a Shimadzu (UV-160) recorder spectrophotometer.

2.8. Assay of creatine kinase activity

Creatine kinase activity was measured by an automated enzymatic assay (activated CK NAC 717, ref. no. 1 273 248 from Boehringer Mannheim). In this assay, the ATP generated by creatine kinase from phosphocreatine and ADP is involved in the phosphorylation of glucose to glucose-6-P by hexokinase. The latter reaction is coupled with the oxidation of glucose-6-P into gluconate-6-P and the reduction of NADP⁺ into NADPH. The rate of formation of NADPH which is detected by spectrophotometry (340 nm) is proportional to the activity of the creatine kinase in the mixture. Briefly, soluble creatine kinase from rabbit muscle (10 U/ml) in Tris-HCl (50 mM, pH 7.8) was pre-incubated with IHDA or different lipids at 30°C. After 60 min, the mixture was diluted 100 times with Tris-HCl (50 mM, pH 7.8) and creatine kinase activity was analyzed.

2.9. cAMP accumulation measurements in cells

Dog thyroid cells on coverslips were pre-equilibrated at 37°C in 11 × 100 mm tubes containing 2.5 ml of the following buffer: 20 mM HEPES (pH 7.4), 1.5 mM Na₂HPO₄, 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose. After 30 min, the coverslips were transferred into fresh buffer supplemented or not with IHDA. After a 30- or 60-min pre-incubation, they were transferred for 1 h in buffer supplemented with IHDA, TSH (1 mU/ml) and rolipram (25 µM). The reaction was quickly stopped by transferring coverslips in tubes containing 3 ml of

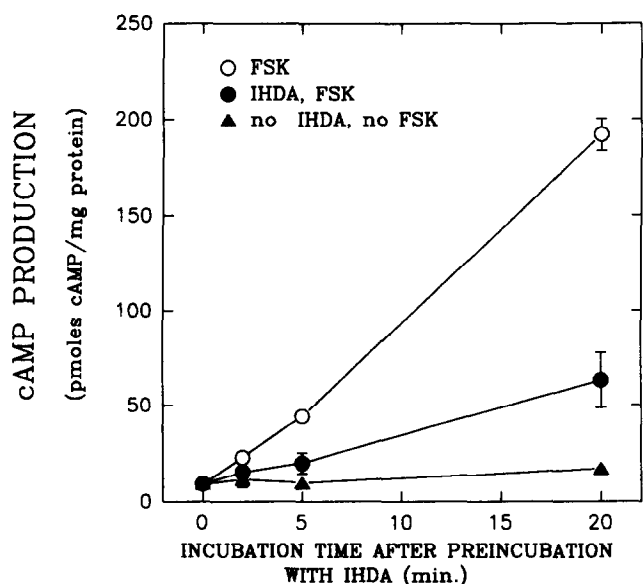


Fig. 1. Inhibitory effect of IHDA on human thyroid membranes adenylyl cyclase. Human thyroid membranes (40 μg protein/tube) were pre-incubated for 1 h at 30°C with or without IHDA (10 μM). Thereafter, ATPMg²⁻ and the ATP regenerating system were added with or without FSK (10 μM). The incubation was stopped at different times: 0, 2, 5 or 20 min. The results represent the mean ± SD of triplicates.

deionized boiling water. Samples were lyophilized and cAMP measurements were made using a radioimmunoassay according to the method of Brooker et al. (1979).

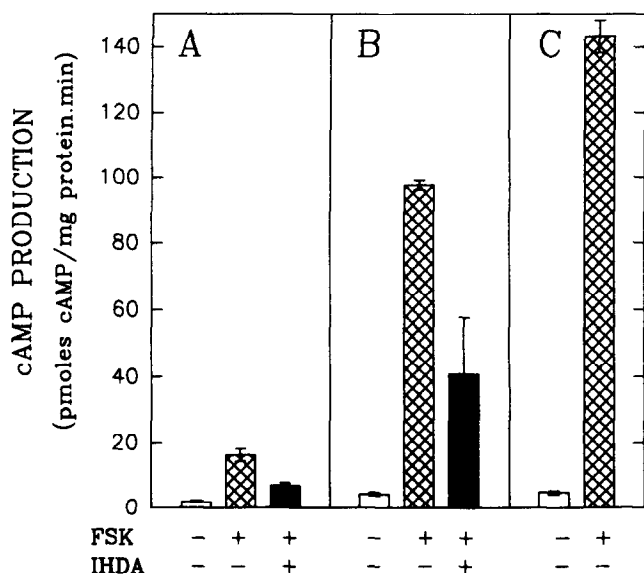


Fig. 2. Temperature-independence of the inhibitory effect of IHDA on FSK-stimulated thyroid adenylyl cyclase. (A,B) Human thyroid membranes (40 μg protein/tube) were pre-incubated for 4 h at 30°C (A) or 4°C (B) with or without IHDA 10 μM. Thereafter, ATPMg²⁻ and the ATP regenerating system were added with or without FSK (10 μM) for 20 min at 30°C. (C) In the same experiment, human thyroid membranes (40 μg protein/tube) were directly incubated for 20 min at 30°C in the presence of ATPMg²⁻ and the ATP regenerating system, with or without FSK (10 μM). The results represent the mean ± SD of triplicates.

Each experimental condition was tested in triplicate and, for each coverslip, the radioimmunoassay of cAMP was performed in duplicate.

2.10. Protein concentration measurement

For cAMP accumulation measurements, results were normalized by measuring protein content sticking on the coverslips according to the method of Lowry et al. (1951). Rapid evaluations of the protein content of the membrane preparations were performed with the Bradford assay (Bradford, 1976).

3. Results

IHDA inhibited the generation of cAMP by FSK-stimulated human thyroid membranes (Fig. 1). A comparable reduction was observed when the ATP regenerating system (creatine kinase + creatine phosphate) was omitted, indicating that it cannot be explained by an artifactual inhibition of creatine kinase (data not shown). Pre-incubation of the membranes at 30°C induced a considerable loss in adenylyl cyclase activity (Fig. 2 compare panels A and C). At 4°C, this heat inactivation, which involves both the cyclase and the Gs (Ross and Gilman, 1977), was largely prevented, whereas the inhibitory action of IHDA was still observed (Fig. 2, panel B); the inhibition of the cyclase by IHDA (10 μM) was comparable at 30°C (60%) and 4°C (43%) (mean of two experiments). The inhibition of FSK-stimulated adenylyl cyclase by IHDA was observed in

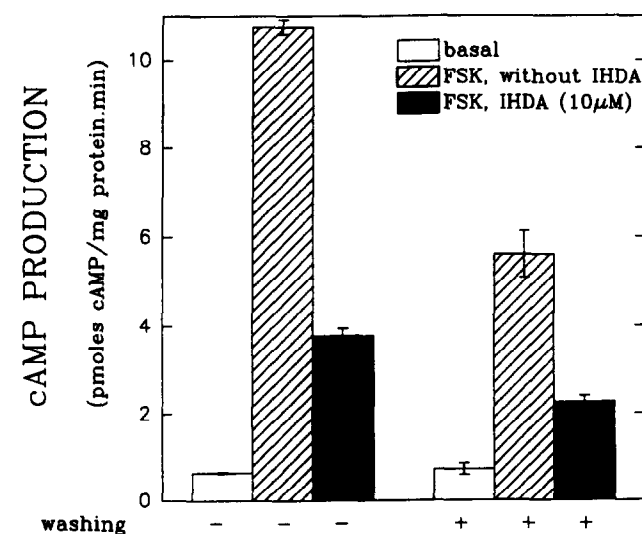


Fig. 3. Lack of reversibility of the inhibitory effect of IHDA on FSK-stimulated thyroid adenylyl cyclase. Human thyroid membranes were pre-incubated at 30°C with or without IHDA (10 μM). After 1 h, 2 ml of BSA (2 mg/ml) in Tris-HCl (50 mM, pH 7.8) at 30°C were added in half the tubes, the mixture was stirred and put on ice. The samples which were washed were centrifuged (10 min at 30 000 × g and at 4°C). The procedure of washing was repeated twice. Thereafter, the membranes (40 μg protein/tube) were incubated with ATPMg²⁻ and the ATP regenerating system supplemented or not with FSK (10 μM) for 20 min at 30°C. The results represent the mean ± SD of triplicates.

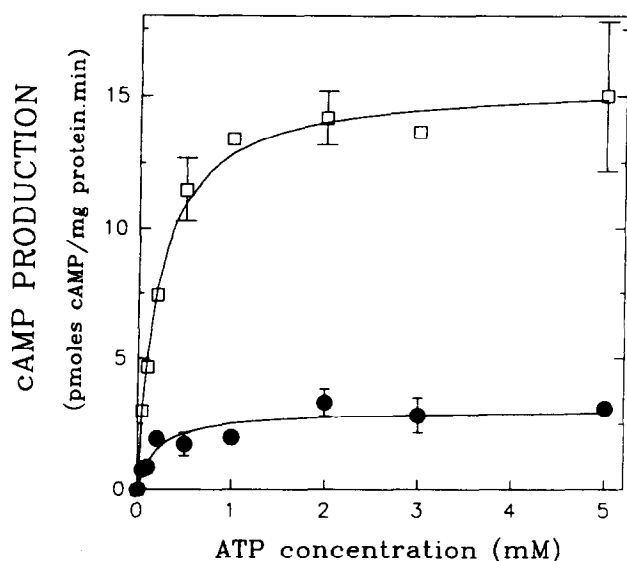


Fig. 4. Effect of IHDA on the substrate-velocity relationship of thyroid adenylyl cyclase. Human thyroid membranes ($40 \mu\text{g}$ protein/tube) were pre-incubated for 1 h at 30°C with (closed circles) or without (open squares) IHDA ($10 \mu\text{M}$). Thereafter, the ATP regenerating system and MgCl_2 (10 mM) were added with FSK ($10 \mu\text{M}$) and various ATP concentrations (0.05 – 5 mM) for 20 min. The results represent the mean \pm SD of triplicates.

seven distinct batches of membranes prepared from different donors; the % of inhibition produced by $10 \mu\text{M}$ IHDA was $47 \pm 18\%$ in the 36 experiments performed (mean \pm

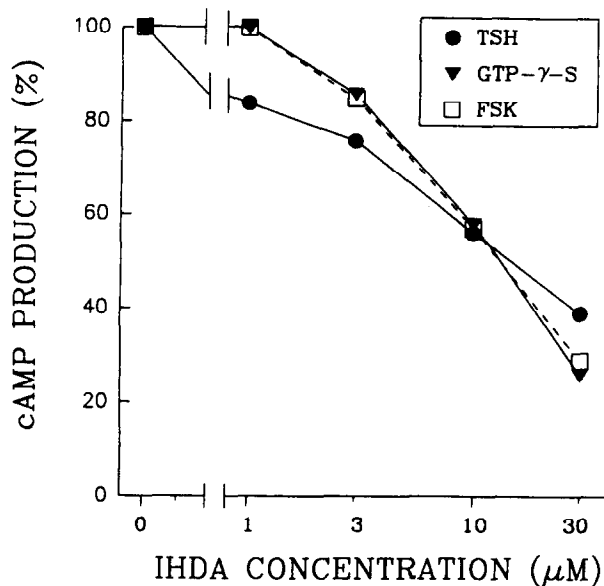


Fig. 5. Effect of IHDA on cAMP production by human thyroid membranes stimulated by TSH, GTP- γ -S or FSK. Human thyroid membranes ($40 \mu\text{g}$ protein/tube) were pre-incubated for 1 h at 30°C with various concentrations of IHDA (1 – $30 \mu\text{M}$). Thereafter, ATPMg $^{2-}$ and the ATP regenerating system were added with TSH (10 mU/ml), GTP- γ -S (0.1 mM) or FSK ($10 \mu\text{M}$) for 20 min. The results are expressed in % of the control value without IHDA. Basal, TSH, GTP- γ -S and FSK-stimulated cAMP production were 1.61 ± 0.013 , 3.47 ± 0.14 , 34.4 ± 1.96 and $26.1 \pm 2.5 \text{ pmol cAMP/mg protein min}$, respectively.

SD). The inhibitory action of IHDA had a slow onset; at 30°C , a maximal effect was reached when the membranes were incubated for 1 h with IHDA before adding ATP and FSK to start the cyclase assay (data not shown). It was not rapidly reversible after washing the membranes (Fig. 3). Washing per se reduced the FSK-stimulated adenylyl cyclase activity, but the % of inhibition by IHDA ($10 \mu\text{M}$) was the same with or without washing: 70% and 69% respectively (mean of two experiments).

As shown in Fig. 4, the pre-incubation of the membranes with IHDA reduced the V_{max} of adenylyl cyclase, but had no significant effect on the K_m for ATPMg $^{2-}$ (207 and $203 \mu\text{M}$, respectively, in IHDA-treated and in control membranes). IHDA also inhibited the adenylyl cyclase activity stimulated by TSH or by GTP- γ -S (Fig. 5). When the membranes were directly incubated at 30°C with ATP, MgCl_2 , the ATP regenerating system and GTP- γ -S, the accumulation of cAMP was linear with time up to 5 h, indicating that the system is protected against inactivation (Kather and Simon, 1976) (Fig. 6). Under these conditions, IHDA, added at the same time as GTP- γ -S, produced an inhibition which had the same magnitude as in other experiments but a slower onset (Fig. 6). At $10 \mu\text{M}$ IHDA, the magnitude of the inhibition was comparable for the three stimuli: TSH, FSK and GTP- γ -S (Fig. 5). At lower concentrations, IHDA had a greater potency on the TSH stimulation: at $1 \mu\text{M}$, the % of inhibition were respectively 32% for TSH, 0% for GTP- γ -S and 4% for FSK (mean of two experiments). This discrepancy cannot be explained by an inhibitory effect of IHDA on the TSH interaction with its receptors (Fig. 7). Indeed, IHDA ($10 \mu\text{M}$) had no effect on

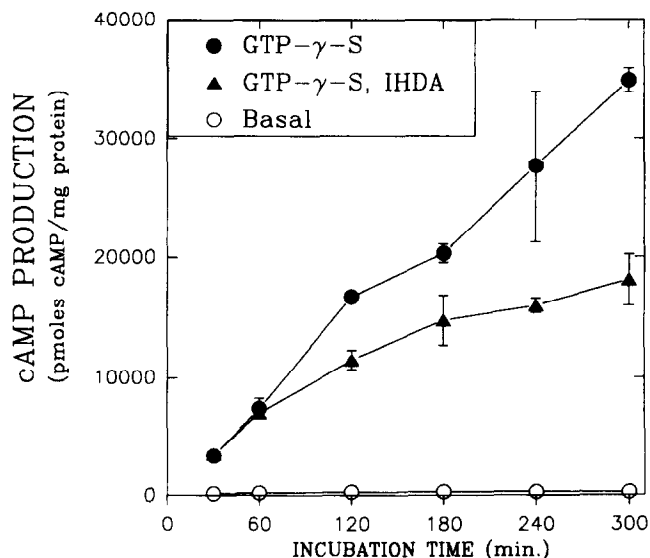


Fig. 6. Kinetics of the inhibitory action of IHDA on thyroid adenylyl cyclase stimulated by GTP- γ -S. Human thyroid membranes ($40 \mu\text{g}$ protein/tube) were incubated at 30°C with or without IHDA ($10 \mu\text{M}$) and GTP- γ -S (0.1 mM), in the presence of rolipram ($10 \mu\text{M}$), ATPMg $^{2-}$ and the ATP regenerating system. The incubation was stopped at different times: 30, 60, 120, 180, 240 and 300 min. The results represent the mean \pm range of duplicates.

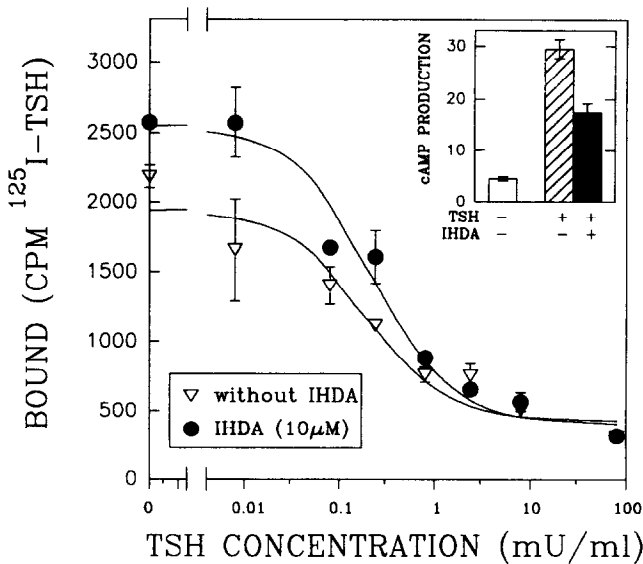


Fig. 7. Lack of inhibitory action of IHDA on TSH binding to thyroid membranes. Human thyroid membranes (40 μg protein/150 μl buffer) were pre-incubated at 30°C with or without IHDA (10 μM). After 60 min, IHDA-treated and untreated membranes (15 μg protein/tube) were stirred for 60 min at room temperature in the presence of ¹²⁵I-labeled TSH and various concentrations of unlabeled TSH (0.01–100 mU/ml). The radioactivity associated with the membranes was counted. Inset: After the pre-incubation, IHDA-treated and untreated membranes were tested in parallel for cAMP production stimulated by TSH (10 mU/ml), in the presence of ATPMg²⁻, and the ATP regenerating system. cAMP production is expressed in pmol cAMP/mg protein min. The results represent the mean ± range of duplicates.

the affinity of the binding (IC₅₀ was 0.17 mU/ml without IHDA and 0.18 mU/ml in the presence of IHDA) and

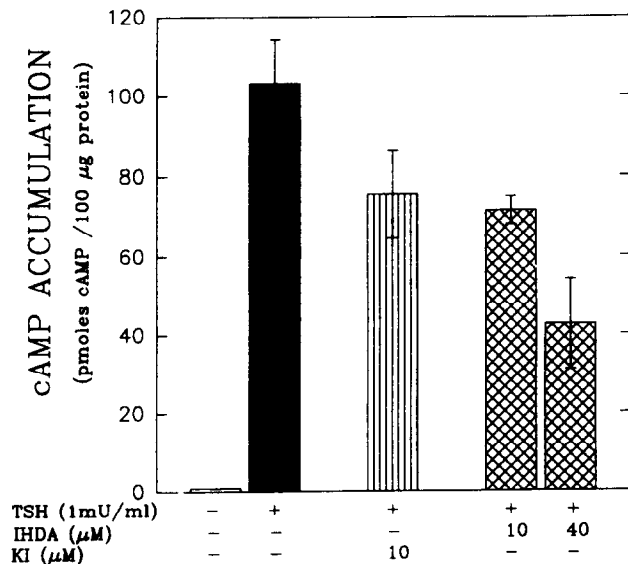


Fig. 8. Inhibitory effect of IHDA on the TSH-stimulated accumulation of cAMP in intact thyroid cells. Dog thyroid cells on coverslips were pre-incubated for 60 min at 37°C in HEPES-buffered medium with KI (10 μM) or IHDA (10 or 40 μM). A 60-min incubation was started by transferring the coverslips in fresh buffer containing IHDA, TSH (1 mU/ml) and rolipram (25 μM). The results represent the mean ± SD of triplicates.

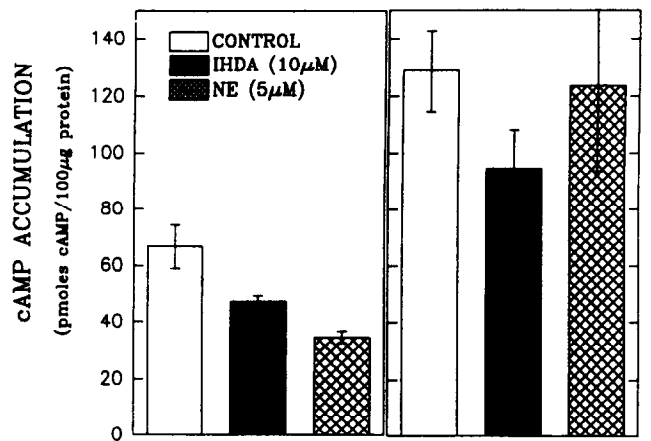


Fig. 9. Effect of pertussis toxin on the inhibition by norepinephrine (NE) and IHDA of cAMP accumulation in TSH-stimulated dog thyroid cells. Cells on coverslips were pretreated with (right panel) or without (left panel) pertussis toxin (20 ng/ml) for the last 18 h of culture. The coverslips were then transferred in HEPES-buffered medium containing IHDA (20 μM) for 30 min at 37°C. A 60-min incubation was started by transferring the coverslips in fresh buffer containing IHDA (20 μM) or NE (5 μM), TSH (1 mU/ml), rolipram (25 μM) and propranolol (100 μM). Propranolol was included in order to antagonize the stimulatory effect of epinephrine on adenylyl cyclase, mediated by β-adrenergic receptors. The results represent the mean ± SD of triplicates.

slightly increased the maximal binding (from 0.13 pmol/mg protein to 0.19); in the same experiments, IHDA inhibited the TSH stimulation of adenylyl cyclase by 56% (mean of two experiments). The inhibitory effect of IHDA has been also tested on intact cells; IHDA inhibited the accumulation of cAMP induced by TSH in cultured dog thyroid cells (Fig. 8). In 10 experiments, the inhibition (mean ± SD) was 32 ± 10% in the case of IHDA (10 μM) and 23 ± 10% for KI (10 μM). The inhibition by IHDA was maintained following blockade of G_i by pertussis toxin (Fig. 9). As expected, the inhibitory effect of norepineph-

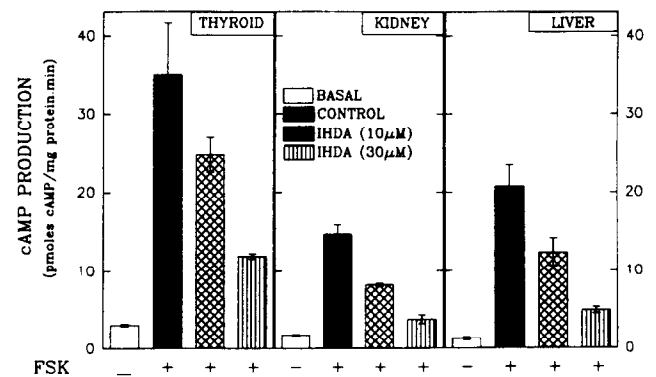


Fig. 10. Lack of tissue-specificity of the action of IHDA on FSK-stimulated adenylyl cyclase. Membranes of various tissues (human thyroid, dog kidney cortex and rat liver) were pre-incubated (40 μg protein/tube) for 1 h at 30°C with or without IHDA (10 or 30 μM). Thereafter, ATPMg²⁻ and the ATP regenerating system were added with or without FSK (10 μM) for 20 min. The results represent the mean ± SD of triplicates.

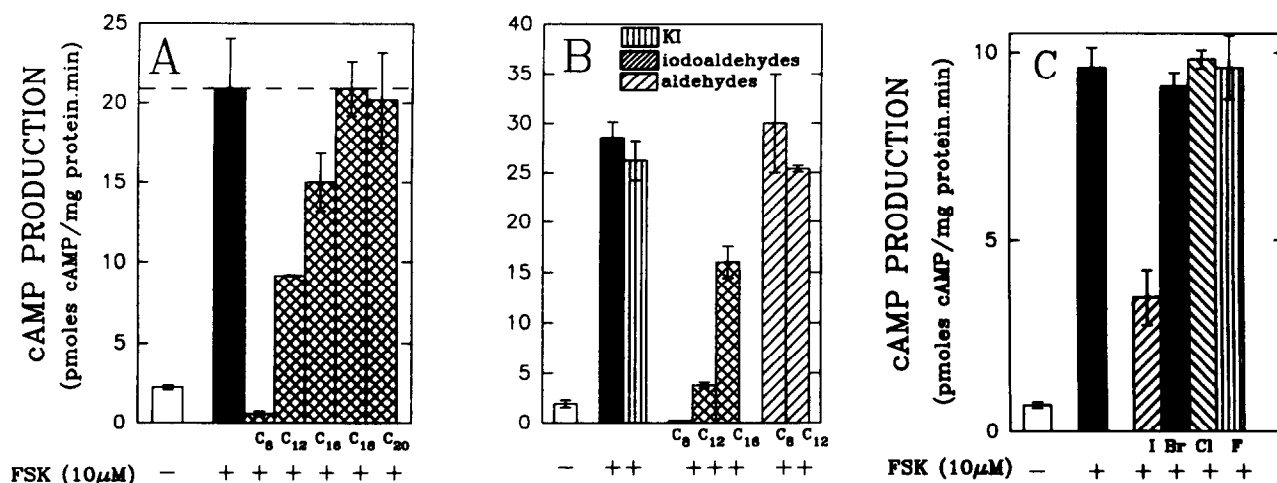


Fig. 11. Comparison of the effects of 2-IHDA and several analogs on human thyroid membranes FSK-stimulated adenylyl cyclase. (A) Influence of hydrocarbon chain length: Human thyroid membranes (40 μ g protein/tube) were pre-incubated for 1 h at 30°C with or without IHDA (C₁₆), 2-iodooctanal (C₈), 2-iodododecanal (C₁₂), 2-iodooctadecanal (C₁₈) or 2-iodoeicosanal (C₂₀) at a concentration of 10 μ M. (B) Comparison of 2-iodoaldehydes with uniodinated aldehydes: Human thyroid membranes (40 μ g protein/tube) were pre-incubated for 1 h at 30°C with or without IHDA (C₁₆), 2-iodooctanal (C₈), 2-iodododecanal (C₁₂), octanal (C₈), dodecanal (C₁₂) at a concentration of 10 μ M or KI (100 μ M). (C) Influence of the nature of the halogen α to the aldehyde function: Human thyroid membranes (40 μ g protein/tube) were pre-incubated for 1 h at 30°C with or without IHDA (I), 2-bromohexadecanal (Br), 2-chlorohexadecanal (Cl), 2-fluorohexadecanal (F) at a concentration of 10 μ M. Thereafter, a 20-min incubation was started by addition of ATPMg²⁺ and the ATP regenerating system, with or without FSK (10 μ M). The results represent the mean \pm SD of triplicates.

rine, which is mediated by α 2-adrenergic receptors coupled to G_i (Cochaux et al., 1985), was abolished by pertussis toxin (Fig. 9). IHDA produced an equivalent inhibition of FSK-stimulated adenylyl cyclase in membranes from human thyroid, rat liver and dog kidney cortex (Fig. 10). As a control, the Mg²⁺-dependent-ATPase activity of the human

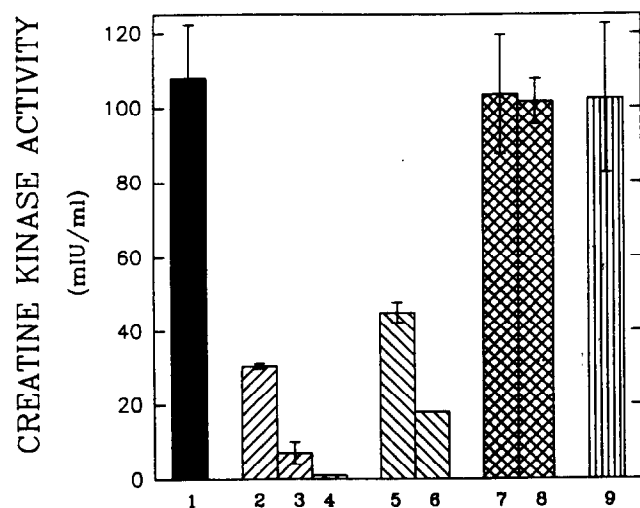


Fig. 12. Effect of different 2-iodoaldehydes and octanal on creatine kinase activity. Creatine kinase from rabbit muscle (10 U/ml) was pre-incubated for 1 h at 30°C without lipid (1), with IHDA (10 μ M (7) or 30 μ M (8)), with 2-iodooctanal (1 μ M (2), 3 μ M (3) or 10 μ M (4)), with 2-iodododecanal (10 μ M (5) or 30 μ M (6)) or with octanal (30 μ M (9)). An aliquot of each mixture was tested for creatine kinase activity as described in Section 2. Each experimental condition was tested in duplicate and, for each tube, the enzymatic assay of creatine kinase activity was performed in duplicate. The results represent the mean \pm SD of these determinations.

thyroid membranes was measured; it was 13.6 U/mg protein and was not affected by IHDA (data not shown).

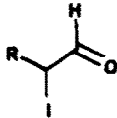
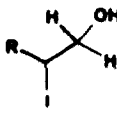
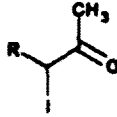
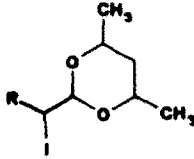
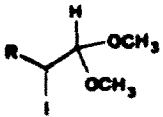
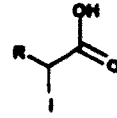
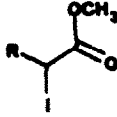
In order to assess the specificity of the inhibitory action of IHDA on the thyroid adenylyl cyclase, various analogs were synthesized and tested. The structural parameters investigated included: the carbon chain length, the nature of the functional group at C1 and the presence and the nature of a halogen atom at C2. As shown in Fig. 11a, the inhibitory potency was inversely related to the carbon chain length between 8 and 20 carbons. If 2-iodooctanal was more inhibitory to the cyclase than IHDA, its action was also less specific, since it also inhibited creatine kinase, on which IHDA had no effect up to 30 μ M (Fig. 12). The replacement of the terminal aldehyde function by other groups (alcohol, carboxylic acid, methyl ester, methyl ketone, acetal) generated inactive compounds (Table 1). Whereas 2-iodooctanal and 2-iodododecanal strongly inhibited the thyroid adenylyl cyclase, the corresponding aldehydes were without effect (Fig. 11b). Hexadecanal was not tested because it trimerizes readily (data not shown). As expected, iodide had no direct effect on adenylyl cyclase (Cochaux et al., 1987). Finally, a comparison between IHDA, 2-bromohexadecanal, 2-chlorohexadecanal and 2-fluorohexadecanal showed that at 10 μ M, only IHDA was able to produce a detectable inhibition of thyroid adenylyl cyclase (Fig. 11c).

4. Discussion

The direct inhibitory effect of IHDA on the adenylyl cyclase activity in human thyroid membranes and dog thyroid cells detected in this study shares many characteristics

Table 1

Role of the aldehyde function in the inhibitory effect of IHDA on the production of cAMP by FSK-stimulated adenylyl cyclase of thyroid membranes

	R = CH ₃ -(CH ₂) ₁₃	pmol cAMP/ mg protein min
1. Control: no lipid		35.8 ± 1.1
2. 2-Iodohexadecanal (IHDA)		12.6 ± 0.5
3. 2-Iodo-1-hexadecanol		32.4 ± 3.5
4. 3-Iodo-2-heptadecanone		33.8 ± 1
5. 2-IHDA-(4 <i>R</i> *,6 <i>R</i> *)-dimethyl-1,3-dioxane		34.5 ± 2.3
6. 2-IHDA-dimethyl acetal		33.9 ± 3
7. 2-Iodohexadecanoic acid		31.0 ± 2
8. Methyl-2-iodohexadecanoate		33.5 ± 1.7

Human thyroid membranes (40 µg protein/tube) were pre-incubated for 1 h at 30°C with IHDA and different analogs, at a concentration of 10 µM. Thereafter, ATPMg²⁻ and the ATP-regenerating system were added with FSK (10 µM) for 20 min. All the results in the table correspond to FSK-stimulated samples (mean ± SD of triplicates). In the absence of IHDA, basal cAMP production was 4 ± 0.2 pmol cAMP/mg protein min.

of the inhibition of cAMP formation by iodide in intact cells or in membranes prepared from thyroid tissue exposed to iodide:

- the inhibition is stable and cannot be reversed by simple washings (Filetti and Rapoport, 1983; Heldin et al., 1985; Van Sande et al., 1985);
- the inhibition is due to a decreased V_{max} , with no effect on the K_m for ATPMg²⁻ (Cochaux et al., 1987);
- all stimuli of adenylyl cyclase (TSH, GTP-γ-S and FSK) are inhibited (Cochaux et al., 1987);
- the inhibition is not mediated by G_i (Filetti and Rapoport, 1983; Cochaux et al., 1985).

The inhibitory action of IHDA has some specificity for adenylyl cyclase, since other enzymes involved in ATP metabolism, such as Mg²⁺-ATPase and creatine phosphokinase, and the TSH receptor were not significantly affected. The inhibitory effect of IHDA is neither restricted to the thyroid nor to one type of receptor, indicating that it acts directly on a component of the adenylyl cyclase system (either the cyclase itself or G_s) and not via a tissue-specific receptor.

The comparison of the activity of IHDA with various analogs has been very informative. The results of these experiments allow us to exclude a non-specific action related either to the hydrophobic chain or to the aldehyde function. Indeed, the two major structural features of IHDA, the aldehyde function and the iodine atom at C2, were both required for biological activity. Replacement of the aldehyde function by alcohol, carboxylic acid, methyl ester, acetal or methylketone groups abolished the biological activity. The presence of iodine at C2 was absolutely required; interestingly other halogens were unable to confer an inhibitory activity. The decrease in biological activity with increasing carbon chain length might be partially related to decreased solubility and micellar aggregation (Vorum et al., 1992). However, it seems that the carbon chain length may modulate the intrinsic activity of the iodoaldehydes, since IHDA had a greater selectivity for adenylyl cyclase than 2-iodooctanal. The structural requirements identified in this study were different from those characterized by Ohayon et al. (1994). They observed that some uniodinated aldehydes inhibited the NADPH oxidase of porcine thyroid membranes to the same degree as the corresponding 2-iodoaldehydes. Nevertheless, the biological activity was clearly modulated by the presence of an iodine atom at C2. On one hand, 2-iodooctanal was inhibitory whereas octanal itself had no effect. On the other hand, the inhibitory effect of aldehydes was reversible after washing whereas the action of 2-iodoaldehydes was not. In another study, we have shown that IHDA inhibits the formation of InsP₃ and H₂O₂ in dog thyroid cells stimulated by carbamylcholine (Panneels et al., 1994). The structural requirements identified in that study were almost identical to those found in the present study, with two exceptions: 2-bromohexadecanal and 3-iodo-2-heptadecanone were able to inhibit the carbamylcholine-stimulated production of H₂O₂, whereas they had no effect on adenylyl cyclase.

One likely mechanism of action of 2-iodoaldehydes is the covalent modification of proteins via formation of

Schiff's bases. The stability of these adducts could vary according to the amino acid microenvironment of a particular protein and might be increased by the presence of an iodine atom at C2. A covalent modification would explain why the inhibitory effect of IHDA on adenylyl cyclase is not rapidly reversible.

In conclusion, we have shown that IHDA inhibits the formation of cAMP in human thyroid membranes and in dog thyroid cells. The characteristics of this inhibition are entirely consistent with the properties of the iodide inhibitory action on the thyroid cAMP system, suggesting that IHDA might be the mediator of that action.

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