Swelling- and TSH-stimulated Taurine Efflux in Rat Thyroid Cells (FRTL-5) Grown on Permeable Membranes

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Abstract

Functions of taurine in animal cells have caught interest for a long time. Its efflux activity has been well-studied during cellular volume regulation, but has also been investigated during stimulation with hormones. In FRTL-5 rat thyroid cells, both cellular swelling and TSH stimulation have been shown to activate taurine efflux. Little is known, however, about taurine efflux in polarized cells, and it was of interest to localize this activity with respect to the apical and the basolateral membrane.

FRTL-5 cells were grown in culture dishes with permeable membranes as bottoms, to establish polarized cell-layers. The cells, preloaded with [^{3}H]taurine, were installed in a perfusion chamber, where two chamber-parts, apical and basolateral, were separated by the cell-layer. Media was perfused through the chamber at a constant flow, and collected separately for counting. Thus the experiments enabled to distinguish between apical and basolateral taurine efflux.

The cells demonstrated polarity in this activity, most pronounced during TSH application on the basolateral side, which lead to a significant activity increase only on the apical side. Hyposmotic exposure stimulated activity on both sides, but was also polarized, as the rate coefficient for apical efflux was two times higher than the basolateral. TSH applied on the apical side induced taurine efflux on both sides, where the difference between the apical and the basolateral activity was not statistically significant. Arachidonic acid metabolites seemed to participate in the swelling-activated efflux, whereas cAMP was not involved. TSH appeared to activate both cAMP-dependent and cAMP-independent taurine efflux. The results indicate that there may be several different types of taurine efflux pathways in polarized thyroid cells. It is also possible that there is more than one type of channel mediating efflux of taurine.

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1. Introduction

Alterations in the environmental osmolyte composition can be critical for animal cells. A difference in osmolality between intracellular compartments and extracellular liquid will result in a net movement of water across the plasma membrane, down the osmotic gradient, which will alter the cell volume. In early evolutionary environments, and also in today's marine habitats, osmolality fluctuations can be great, which is challenging to the marine invertebrates that are isosmotic with their environment.

In vertebrates, the extracellular osmolality is more stable and kept between narrow limits by the kidneys (see Greger R. and Windhorst U, 1996, 2), but metabolic activity can change intracellular osmolality and generate water transport across the plasma membrane (see Lang et al., 1998). Since cellular membranes lack strength to withstand stretch of any magnitude, water influx could lead to lysis and cell death. This is, however, prevented by volume regulatory mechanisms that control the total amount of intracellular solutes and thereby the water content and volume, a process often referred to as regulatory volume decrease (RVD). The counteracting process of the RVD is the regulatory volume increase (RVI), which also is important for the cell volume control, but will not be focused on here (for review see Eveloff and Warnock, 1987; for review see Lang et al., 1998).

A volume increase is supposed to trigger a reflex mechanism which detects the swelling and activates efflux pathways of different osmolytes. These effector-sites appear to be different proteins in the cell membrane with channel properties, which mediate osmolyte efflux down their energy gradient (see Eveloff and Warnock, 1987; see Strange et al, 1996). The molecular identity of the volume sensors is still unknown (see Hoffmann, 2000; see Jacab et al., 2002), whereas the signal transduction systems and channels involved have been revealed to some extent (see Lambert, 1994; see Hoffmann, 2000).

Activation of osmolyte efflux is not seen only in RVD; also certain hormones stimulate solute transport as well (see Dahl et al., 1991; see Armstrong et al., 1992). For instance, in FRTL-5 cells (Fischer rat thyroid cell line), both thyroid stimulating hormone (TSH) and cellular swelling activate efflux of the amino compound taurine (Fugelli, unpublished). It is not known whether the TSH-stimulated efflux is the same as in RVD, but it could possibly be associated with the liquid transport known to occur during TSH stimulation and thyroid hormone secretion.

In this study, FRTL-5 cells were grown on permeable membranes to establish polarized cell-layers, where the aim was to investigate to what side of the cell-layer taurine efflux is localized. It was found that swelling and TSH appear to activate different efflux pathways, with respect to location and signal pathway involved.

1.1. Osmolyte transport and cell volume

1.1.1. Inorganic and organic osmolytes

K^+ and Cl^- channels are transporters that dominate the efflux activity during RVD (see Eveloff and Warnock, 1987; see Lang et al., 1998). There are, however, limits to the extent the intracellular ion concentration can change before it has negative effects on cellular functioning, as it can affect the electric properties of the membrane, change intracellular pH (see Lang et al., 1998), alter enzymatic activity (Combes et al., 1988) and cause macromolecular denaturation (McManus et al., 1995).

The intracellular concentration of organic osmolytes can unlike inorganic ions, change rapidly without having negative effects on the cells (Yancey and Burg, 1989; Garcia-Perez and Burg, 1991; Law, 1991; Kinne, 1993). Since they have the same osmotic properties as inorganic ions, they are well-suited for osmo-regulation and play this role not only in mammalian cells, but also in lower vertebrates and invertebrates, as well as in bacteria, algae and plants (Kirst, 1977; Huxtable, 1992). High intracellular concentrations of organic osmolytes are mainly obtained by active uptake via sodium cotransporters (Kirk, 1997) and by intracellular synthesis (Huxtable, 1992). Exit pathways are partly unknown, but have been proposed to be mediated by anion channels (Kirk et al., 1992), called VSOAC (volume sensitive organic osmolyte anion channel) (Jackson and Strange, 1993; Jackson et al., 1994), which have low substrate specificity and transport several types of anions and organic osmolytes (Strange et al., 1996).

Organic solutes participating in cell volume regulation can be divided into three distinct groups; 1: polyols (for instance sorbitol and myo-inositol), 2: amino acids and their

derivatives (for instance taurine, alanine, and proline) and 3: methylamines (for instance glycerophosphoryl-choline and betaine) (Lang et al., 1998). Several of these have been proposed to have protective effects on the cell in addition of being osmo-effectors, as having stabilizing effects on proteins (Gerlsma, 1968; Arakawa and Timasheff, 1985; Kumazawa and Arai, 1990; Buche et al., 1993; Yancey, 1994) and anti-oxidative properties (Pasantes-Morales and Cruz, 1985; Thomas et al., 1985). In volume regulation, organic osmolytes can to contribute to 30% of the total osmolyte transport (Hoffman and Hendil, 1976).

1.1.2. Taurine

Being one of the most abundant low-molecular-weight organic constituents, taurine (2-B-amino ethane sulfonic acid) plays an important role not only in volume regulation, but in a number of other biological functions as well. It is involved in bile salt formation and apoptosis, it modulates neurotransmitter and hormone release, has anti-oxidative effects, and participates in platelet aggregation, thermoregulation and resistance to anoxia/hypoxia (see Huxtable, 1992). With very few exceptions, taurine appears not to take part in or influence any chemical reactions in mammalian cells. Its zwitterionic nature, with only some few % of the molecules having a net negative charge at physiological pH (Lambert and Hoffman, 1993), its high water solubility and very slow diffusion rate across the lipid membrane, makes it an excellent candidate for osmo-regulation (see Huxtable, 1992).

This amino acid derivative is an end product in sulphur metabolism (Huxtable, 1986), although some mammalian species, for instance cats and humans, are dependent of supplies obtained via food (Gaull, 1986; Wright et al., 1986). The surplus is excreted (see Huxtable, 1992). Its concentration gradient can be high, 7000:1 in HeLa cells (Piez et al., 1958), and its active accumulation is driven by sodium cotransport (Bucuvalas et al., 1987; Huxtable, 1989; Miyamoto Y., et al 1991). Swelling-activated taurine efflux is supposed to be mediated through anion channels (Banderali and Roy, 1992; Jackson and Strange, 1993; Boese et al., 1996; Brès et al., 2000; Schmieder et al., 2002), although chloride and taurine do not show identical efflux pathways during RVD, as shown for instance in HeLa cells (Stutzin et al., 1999), Ehrlich ascites tumor cells (Lambert and Hoffmann, 1993) and EATC cells (Hoffmann, 2000). In addition, swelling-induced taurine efflux can be completely independent of chloride efflux, as shown in Xenopus oocytes (Stegen et al., 2000). These

different results suggest that there could be more than one type of efflux pathway for taurine during RVD, depending on cell-type.

1.1.3. Osmolyte transport in epithelia

Cells in an epithelium are morphologically and functionally polarized, with an apical and a basolateral membrane, which differ by composition of ion-channels, pumps and transporters. Epithelia are often engaged in trans-cellular transport of ions, hormones, nutrients and other solutes, which can alter the cell volume (Reuss and Cotton, 1994). One of the ways for epithelial cells to counteract volume changes is cross-talk communication, which coordinates the activity of the two opposing membranes, a mechanism first described by MacRobbie and Ussing (1961).

1.1.4. Hormone-stimulated osmolyte transport

Hormonal stimulation can initiate net flux of osmolytes, thereby generating liquid transport (see Lang et al., 1998). In hepatocytes, insulin causes swelling, due to activation of both a Na^+/H^+ exchanger and a $Na^+/K^+/2Cl^-$ cotransporter, which lead to uptake of osmolytes and thereby water molecules as well. Glucagon stimulation on similar cells results in shrinking due to osmolyte efflux activation, mediated by ion channels (Dahl et al., 1991). Thyrocytes do also experience alterations in cell volume during hormone stimulation. TSH causes liquid transport in thyroid epithelia, thought to be a result of uptake of ions (Bourke et al., 1987). Absence of TSH causes thyrocytes to shrink (Cauvi et al., 2000).

1.2. Signal transduction pathways involved in RVD

1.2.1. Volume sensors

It is believed that the swelling-induced osmolyte efflux is regulated by intracellular signal pathways that probably differs depending on cell type, and that these pathways are

activated by sensors detecting volume changes. Their molecular identity in mammalian cells are still unknown (see Parker, 1993; see Strange, 1994; see Hoffmann, 2000; see Jakab et al., 2002). Membrane stretch (Jacab et al., 2002) and change of cellular shape which re-organizes the cytoskeleton (Pedersen et al., 2002) have been suggested. Intergrins (Low and Tylor., 1998), change in intracellular ionic strength (Motais et al., 1991; Voets et al., 1998) and the G protein-coupled receptor TSH R (Fugelli, unpublished) are possible candidates as well.

1.2.2. Arachidonic acid metabolites

Eicosanoids, metabolites of arachidonate, are possible participants in volume regulation as intracellular messengers (see Lambert and Hoffmann, 1993; Lambert, 1994) and have been shown to activate both K^+ channels, Cl^- channels and taurine efflux (Hoffmann, 2000). Evidence for the involvement of eicosanoids during swelling-activated taurine efflux in FRTL-5 cells has previously been presented (Kveberg, 1998).

Arachidonic acid (AA) is synthesised from glycerophospholipides, phosphatidyl-choline and phosphatidylethandamine (fig.1) abundant in the plasma membrane, where the phospholipase A_2 enzyme (PLA_2) and DAG-lipase enzyme are central (Lambert, 1994). Once released from the membrane, AA can participate in several intracellular signal pathways. One of them is the cyclo-oxygenase (CO) pathway, which transforms AA into prostaglandins and trombozanes. One of its main end-product, PGE_2, has been shown to inhibit RVD in Ehrlich cells (Lambert, 1987; Lambert et al., 1987). Another pathway transforms AA into leukotrienes and hydroxy-eicosatetraeonoic acid (HETE) by the 5-lipoxygenase enzyme, which plays a central role in swelling-activated osmolyte efflux (see Jackson and Strange, 1993; Lambert, 1987). Two additional main pathways are involved in AA metabolism, but they appear to be less involved in swelling-activated efflux (for review see Lambert, 1994).

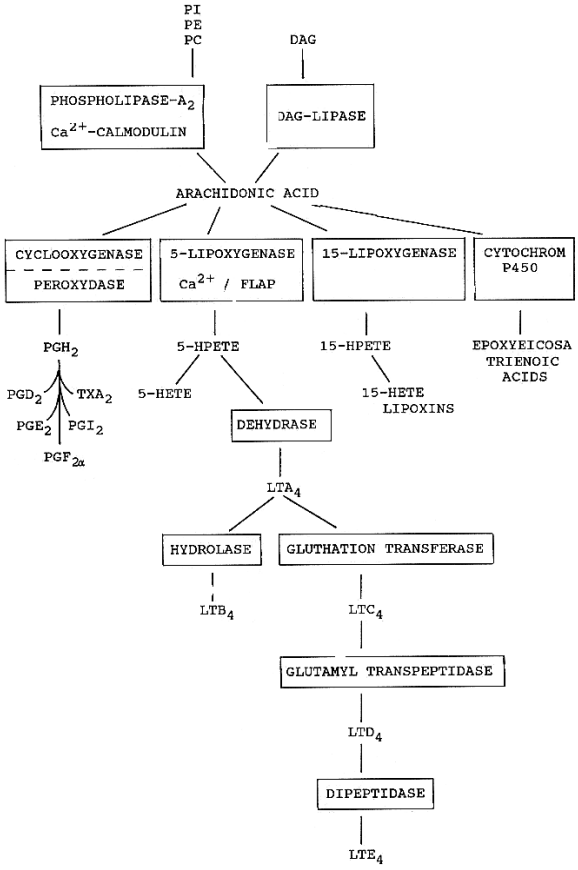

Figure 1: Four main pathways of eicosanoid production from arachidonic acid (AA) are illustrated. AA is liberated from the plasma membrane by phospholipase A_2 or DAG-lipase, when stimulated. Cyclo-oxygenase, 5-lipoxygenase, 15-lipoxygenase and cytochrom P450 are the enzymes transforming A.A. into prostaglandins (PG), leukotrienes (LT), HETE, HETE-lipoxins and epoxyeicosa trienoic acids respectively (Lambert, 1994).

1.2.3. cAMP

cAMP is an additional compound thought to be involved in swelling-activated efflux pathways (Thoroed et al., 1995; Cetani et al., 1996). In FRTL-5, it stimulates liquid transport (Bourke et al., 1987) and activates taurine efflux (Fugelli, unpublished). cAMP has also been shown to induce efflux of other osmolytes, as Cl^- (Armstrong et al., 1992; Bourke et al., 1995; Yoshida et al., 1999) and K^+ (Yoshida et al., 1993), but whether these activities are involved in cell volume regulation, is unknown. Little is also known about the details of cAMP's influence on volume regulatory activities, and its role during RVD in epithelial cells.

1.3. Thyroidea

1.3.1. Structure and function

The thyrocytes are organized as monolayered follicles, and are functionally and morphologically polarized. The follicle interior is called the colloid space, which is where the synthesis of the thyroid hormone, T_3 and T_4, takes place. The function of the thyroid gland is regulated by the pituitary hormone TSH, which stimulates cellular growth, uptake of iodide and synthesis and release of T_3 and T_4 into the blood capillary (for review see Greger and Windhorst, 1996, 1).

1.3.2. The TSH receptor

TSH mediates its effect through interaction with the G protein-coupled receptor TSH R, (Szkudlinski et al., 2002) located on the basolateral side of cultured thyrocytes (Chambard et al., 1983), where it functions as a dimer in the thyrocyte membrane (Loosfelt et al., 1992). It regulates growth and functions of the thyrocytes (Vassart and Dumont, 1992) and stimulates bi-directional trans-epithelial transport of liquid and solutes (Yap et al., 1991; Cauvi et al., 2000).

Stimulating the TSH receptor can activate both the G_s and the G_q protein, thereby activating cAMP production and the phospholipase C pathway respectively (Allgeier et al., 1994). Sho et al. (1991) found that a low concentration of TSH activated the AC/cAMP pathway, while a switch to the PLC/Ca2+ pathway took place at higher TSH concentration. It has also been found that TSH receptor has two different sites with different affinity for TSH (Tramontano and Ingbar, 1986), which could explain how TSH can activate two different signal pathways.

The receptor belongs to a group of G protein-coupled receptors (Bockaert, 2001) whose activities also can be increased without being associated with their respective agonists. This agonist-independent activity is thought to be regulated by the intracellular Na^+ concentration (Gierschik et al., 1989), due to a highly conserved aspartate in the transmembrane structure (Quintana et al., 1993; Martin et al., 1998).

TSH R has been found in to many different cell-types in the mammalian body, as lymphocytes (Pekonen and Weintraub, 1978; Smith et al., 1983), adipocytes, adrenal glands and testicular cells (Trokoudes et al., 1979), but the functional roles of these receptors are unclear.

1.3.3. FRTL-5 cells

The FRTL-5 cell line, originally from rats, has become a popular alternative to primary thyrocyte cultures in physiological studies. The first constructed cell line derived from thyrocytes, able to grow and stay differentiated for long periods, was the Fischer rat thyroid line (FRTL) (Ambesi-Impiombato et al., 1980), and later the FRTL-5 line was established (Ambesi-Impiombato et al., 1982). They grow as monolayers, and do not form three-dimentional follicles in presence of TSH as primary cell cultures do, but have kept many functional properties characteristic for thyrocytes (Bidey et al., 1988). For instance, the TSH receptor is expressed; they show cAMP-induced proliferation and alteration of morphology (Yun et al., 1986) and also TSH-dependent accumulation of iodide (Weiss et al., 1984).

Studies performed on cells grown in petri-dishes with a solid support showed that FRTL-5 cells accumulate taurine (Jhiang et al., 1993), which is released during swelling, as well as during TSH stimulation (Fugelli, unpublished). A preparation more close to in vivo conditions would be to culture the cells on permeable membrane supports to allow the cells to establish polarized cell-layers. This would make it possible to study taurine efflux on each side of the cells separately, and to identify possible differences between the swelling-activated and the TSH-activated efflux.

1.4. Aims

In the present study, I wanted to study the taurine efflux in the polarized FRTL-5 cells, which were grown on permeable membranes. The aims were

a) to identify to which side of the cell-layer the TSH-activated and swelling-activated taurine efflux are located respectively, and

b) to study possible signal pathways involved in taurine efflux in polarized cells.

2. Materials and Methods

2.1. Cell cultures

The experiments were performed on FRTL-5 cells (Fisher rat thyroid cell-line) which were a gift from Aker Medical Hospital (Oslo, Norway). The cells were grown in culture flasks (75 cm^2) with F-12 medium, Coon's modification, added insulin (1.8 μM), transferrin (6.36 mg/l), TSH (0.53 mU/ml) and fetal calf serum (5%), buffered with bicarbonate and phosphate to obtain pH 7.5 ± 0.1 in room temperature, and at osmolality 335 ± 5 mOsm/kg. The flasks were placed in a humidified incubator with 5% CO_2 in air at 37º C. The medium was changed the next day, and subsequently every 2 or 3 days. When the cells had established a confluent layer, they were detached by trypsination, and about 2/3 of the amount transferred to 6 culture dishes with collagen-coated permeable membranes as bottoms (Falcon Cell Culture Inserts, effective diameter: 23.1 mm, pore size: 3.0 micron and pore density: 8.0 x $10^5/cm^2$) (fig. 2). The rest of the cells were transferred to a new culture flask.

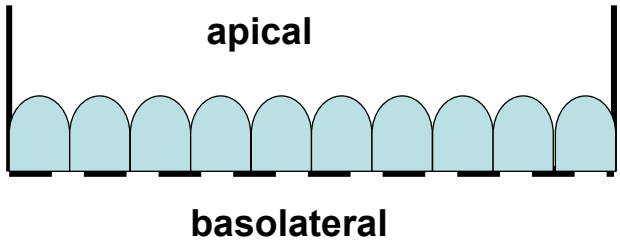

Figure 2: FRTL-5 thyrocytes grown on a permeable membrane with medium-access on both apical and basolateral side of the cell-layer.

The culture dishes were initially kept in a large petridish with medium for 24 hours, to give the membranes a flat support, thereby making the cells spread out as evenly as possible until they were attached. The subsequent day, they were placed in falcon inserts, allowing the cells to form an apical (upper) and a basolateral (lower) membrane. The medium was changed every 2-3 days, on both sides, and kept in an incubator as described. The cells for

tests of effects of TSH were placed in medium deprived of TSH for two days continually before the experiments.

The tests were performed after 4-5 days of incubation. The day before transport studies, the medium was changed and 1.0 μCi [^{3}H]taurine added to each culture dish on the apical side. All handling of the cells before the efflux experiments were performed under sterile conditions.

2.2. Reagents

F-12 medium Coons modification for cell growth, DMSO for freezing cells, TSH, prostaglandin E_2, N^6,2'-o-dibuteryladenosine 3':5'-cyclic monophosphate (db-cAMP), 8-bromoadenosine 3':5'-cyclic monophosphate (8-br-cAMP), indomethacin, insulin and transferrin from Sigma, USA, 2'-5'-dideoxyadenosine and MK-886 from Biomol, USA, fetal calf serum and trypsin EDTA from Bio Whittaker, USA, collagen I from rattail from BD Biosciences, USA, [^{3}H]taurine from Amersham Biosciences, UK, triton x-100 from Koch-Light Laboratories, Eng, TCA and ULTIMA GOLD scintillation fluid from Packard Bioscience, 100% acetic acid from Merck KGaA, Germany, phosphate buffer PBS from EC Diagnostics AB, Sweden, glutaralderhyde and osmium tetra oxide from EMS, USA, and sodium cacodylate buffer (cacodylic acid Na^+ salt trihydrate) from AppliChem, Germany.

Medium for efflux activity studies (in mM): 110 NaCl, 4.1 KCl, 1.12 $CaCl_2$, 0.75 $MgSO_4$, 0.45 KH_2PO_4, 25 HEPES, 10 D-glucose, pH adjusted to 7.5 by adding NaOH and the osmolality adjusted to isosmotic and hyposmotic medium, 335 mOsm/kg or 295 mOsm/kg respectively, by adding choline chloride. The osmolality was measured with a freezing point osmometer (Knauer, Germany).

2.3. Measurement of [^{3}H]taurine efflux activity

After 4-5 days of incubation, the cells had established confluence on 90 % of the membrane surface. The culture dishes were carefully rinsed twice with isosmotic medium,

placed in a special made perfusion chamber and put in a water bath at 37º C. The membrane on which the cells were grown, formed a separating wall between two chamber-parts, apical (upper) and basolateral (lower) side, of 0.5 ml each. The chamber-parts were each connected to a suction pump (Pharmacia Biotech) by silicon tubes, so that each part was perfused separately at a constant flow of fluid at 0.5 ml/min. The perfusates (containing the [^{3}H]taurine released from the cells), were sampled from each chamber-part, into counting vials during one min. periods (fig. 3).

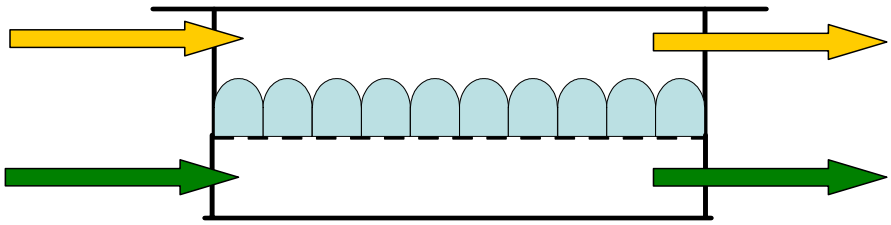

Figure 3: Thyrocytes grown on a porous membrane installed in a perfusion chamber. Media is perfused at a constant rate (0.5 ml/min) through **yellow arrows:** apical, and **green arrows:** basolateral chamber-part separately, and collected in counting vials (1 per min.).

In all the experiments, the cells were first exposed to normal isosmotic medium for 20 minutes to establish a basal activity of [^{3}H]taurine efflux, before exposing the cells to a stimulating medium for 20 min.

Stimulation with hyposmotic medium:

Cells were exposed to hyposmotic medium (295 mOsm/kg) on both sides.
(Cells were also exposed hyposmotic medium without ChCl (260 mOsm/kg) on one side of the cell-layer, and isosmotic medium on the other).

TSH stimulation:

Cells were exposed to isosmotic medium containing TSH, 0.01 IU/ml on one side of the cell-layer, and isosmotic TSH-free medium on the other.

Forskolin (or cAMP analogue) stimulation:
Cells were exposed to isosmotic medium containing 500 μM forskolin (or db-cAMP/8-bromo-cAMP) on both sides. Cells were also exposed forskolin (500 μM) on one side of the cell-layer, and forskolin-free medium on the other.

PGE_2 stimulation:
Cells were exposed to isosmotic medium containing 500 μM PGE_2 on one side of the cell-layer, and isosmotic PGE_2-free medium on the other.

Inhibition of adenylate cyclase with dideoxyadenosine during swelling:
Isosmotic and hyposmotic medium both contained 30 μM DDA and experiments were performed as described with hyposmotic medium. Cells were also exposed to iso- and hyposmotic medium added 100 μM DDA.

Inhibition of adenylate cyclase with dideoxyadenosine during TSH stimulation:
TSH-free and TSH-containing isosmotic medium both contained 30 μM DDA and experiments were performed as described above with TSH stimulation.

Inhibition of 5-lipoxygenase and cyclo-oxygenase with MK-886 and indomethacin respectively during swelling:
Isosmotic and hyposmotic medium were both added MK-886 (2 μM) or indomethacin (10 μM) and experiments were performed as described with hyposmotic medium.

By the end of each experiment, the remaining radioactive taurine in the cells was extracted and isolated. The culture dish membrane was removed from the well with a scalpel and put into 0.5 ml distilled water containing 0.1 % triton and proteins were precipitated with 0.5 ml 10% TCA. The supernatant was isolated by centrifuging and 0.2 ml was transferred to a counting vial. 3 ml of scintillation fluid were added to each counting vial (41 per trial) and the radioactivity was measured in a scintillator counter (Packard). All the samples gave the same counting efficiency.

2.4. Test of the culture dish membranes' diffusion rate

Tests were performed on culture dish membranes without cells. The culture dishes were installed in the perfusion chamber as described earlier, and both chamber-parts were perfused as in experiments with cells. The medium used was the same as described earlier, but without glucose and ChCl. After 5 minutes of perfusion with [^{3}H]taurine-free medium on both sides, the apical chamber-part, or the basolateral, was perfused with medium containing [^{3}H]taurine (1.0 μCi per 30 ml) for 5 min. while no change was made on the opposite side. The perfusates were collected and the amount of radioactivity measured as described earlier. The diffusion rate was found to be 0.096 (n=2).

2.5. Calculations

The rate coefficient for [^{3}H]taurine efflux was calculated for every 1 minute period in each experiment according to the following formula representing the last, n, and second last, n-1, period:

$$k_nA = M_nA(t(0.5M_nA+0.5M_nB+C_n))^{-1}$$
$$k_nB = M_nB(t(0.5M_nA+0.5M_nB+C_n))^{-1}$$

and

$$k_{n-1}A = M_{n-1}A(t(0.5M_nA+0.5M_nB+0.5M_{n-1}A+0.5M_{n-1}B+C_n))^{-1}$$
$$k_{n-1}B = M_{n-1}B(t(0.5M_nA+0.5M_nB+0.5M_{n-1}A+0.5M_{n-1}B+C_n))^{-1}$$

according to the method described by Caldwell and Keynes (1969), where t is the duration of each collecting period, M_n is the amount of radioactive taurine released in the last period n, C_n is the amount of radioactivity in the cells at the end of the experiment, and kn is the rate coefficient for the nth period expressed as 1/min. A and B represents the rate coefficient for efflux from apical and basolateral side of the cells respectively. The values represent the midpoint of each interval.

The increase in rate coefficient, defined to be the difference between maximum value and corresponding basal value, was used to calculate the difference between the apical and

basolateral efflux rate coefficient. Results from experiments performed under identical conditions were compared. For each test, the apical increase value was compared to the basolateral increase value, applying student's paired t-test ($p = 0.05$) in the computer program INSTAT (Mac). To compare between peak values for the rate coefficient with and without inhibitors, student's unpaired test ($p = 0.05$) was used.

The diffusion rate for [^{3}H]taurine across the culture dish membranes without cells was calculated as a ratio between the amount of radioactivity diffused across the membrane each minute, and the radioactivity in chamber-part perfused with [^{3}H]taurine-containing medium. Average value for each test was calculated based on the last four minutes of each test.

2.6. Scanning electron microscopy

Cells grown on permeable membranes in the presence and absence of TSH, respectively, were fixated in 0.1 M Na-cacodylate buffer with 3% glutaralderhyde. The medium on the apical side was removed, and replaced by 2 ml fixation solution in each culture dish for 30 min. This was repeated three times, before the samples were left over night. All handling was done at room temperature.

The medium on both sides was then removed, and the cell-layer was washed with 0.1 M cacodylate buffer, and 1 ml. 1% osmium in 0.1 M buffer was added to each culture dish. These were left for one hour, protected from light, then washed three times with cacodylate buffer and the membranes removed from the wells with a scalpel. The samples were put in metal baskets and dehydrated in 50, 70, 90 and 2 x 96 % alcohol respectively, for 10 min in each concentration, and 3 x absolute alcohol, for 15 min. each time. The cells were further dried at critical point with a Balzers apparatus, attached to stubs with double sided carbon tape and covered with Ag/Pd in Polaron sputter coater, model E5000. The cells were studied in scanning electron microscopy, JEOL JSM 6400, at 5 kV.

3. Results

3.1. Morphology of FRTL-5 cells grown on permeable membrane supports

FRTL-5 thyrocytes grown on permeable membranes in the presence (fig. 4A and C) or absence (fig 4B and D) of TSH were studied in a scanning electron microscopy. They formed a tight epithel-like cell-layer, although the preparation for SEM shrunk the cells and made them detach from each other. Cells grown with TSH, had a high density of microvilli on the apical membrane and had a curved surface. Cells deprived of TSH, had very few microvilli, and had a flat form compared to cells grown with TSH.

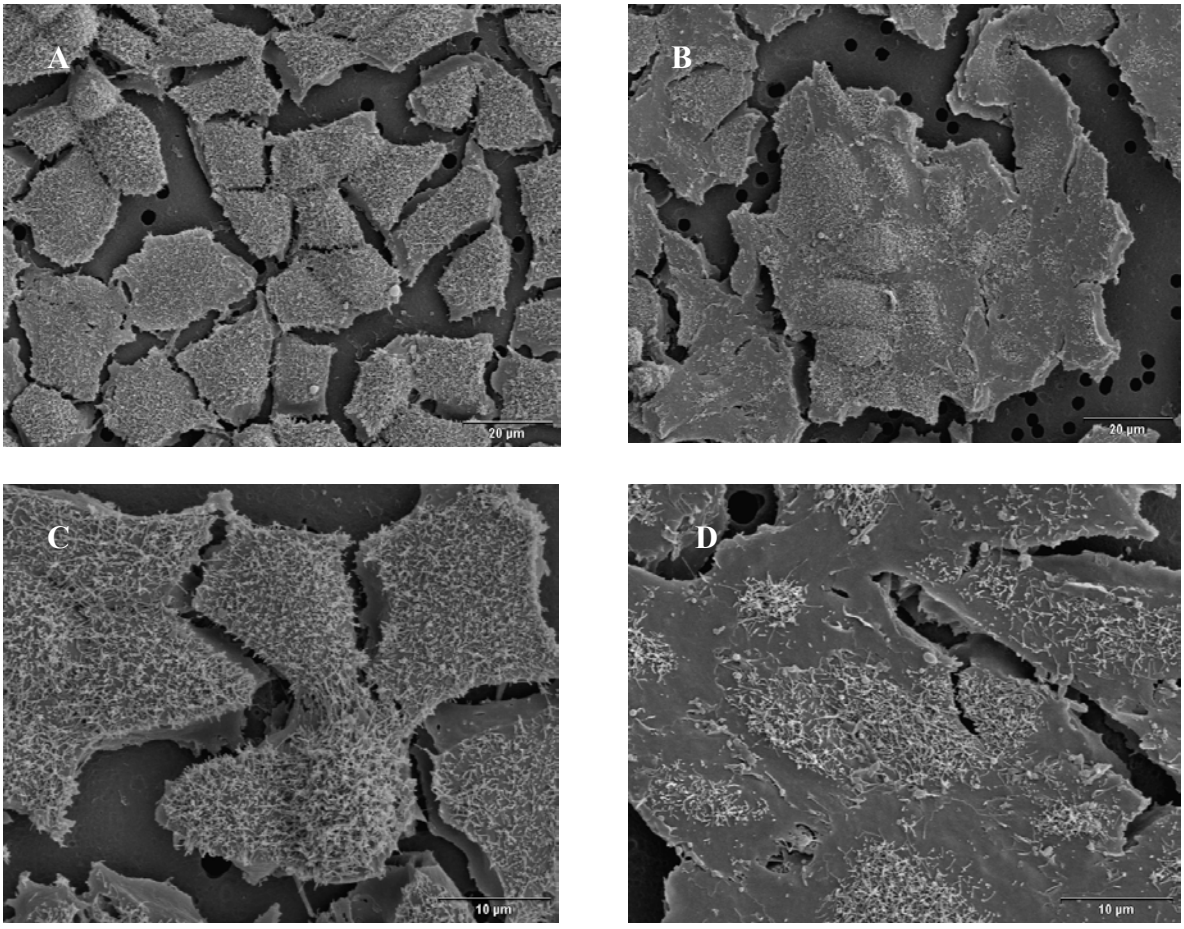

Figure 4: Scanning electron microscopy of FRTL-5 cells on permeable membranes, grown in **A and C:** the presence, and **B and D:** absence of TSH respectively.

3.2. Effect of reduced medium osmolality on rate coefficient for [^{3}H]taurine efflux

FRTL-5 cells, preloaded with [^{3}H]taurine, were initially exposed to isosmotic medium (335 mOsm/kg) on both apical and basolateral membrane (fig.5). The average basal activity for [^{3}H]taurine efflux rate coefficient was low on both membranes, although statistically significantly higher on the apical side. At the arrow indication, the medium was switched to hyposmotic medium (295 mOsm/kg). Rate coefficient for [^{3}H]taurine efflux increased on both membranes, on the basolateral side from 0.0008 ± 0.0003/min to 0.0052 ± 0.0012/min and on the apical side from 0.0017 ± 0.0006/min to 0.0110 ± 0.0024/min. The peak value for the apical rate coefficient was about two times higher than the basolateral. The efflux rate coefficient increased on both sides at osmolality reduction and reached a peak value after about 4 minutes, and was followed by an gradual decrease (fig. 5). During the hyposmotic exposure, the cells lost 12.5 % of their initial [^{3}H]taurine content through the apical side and 6.1% through the basolateral side.

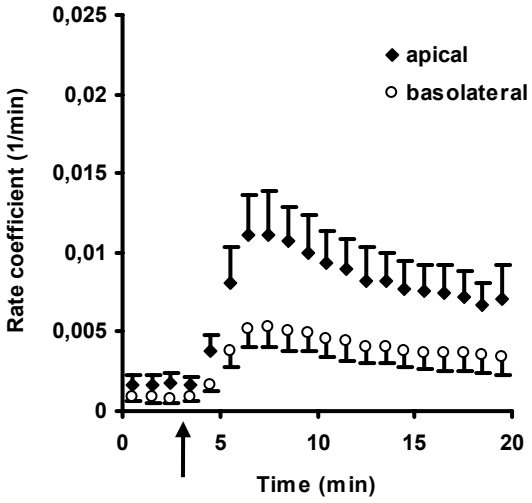

Figure 5: Effect of reduced medium osmolality on the apical and basolateral rate coefficient for [^{3}H]taurine efflux. Cells, preloaded with [^{3}H]taurine, were exposed to isosmotic medium on both membranes and, at arrow indication, to hyposmotic medium (295 mOsm/kg), also on both membranes. Average values are shown ± SD (n=6).

Cells were also exposed to hyposmotic medium, 260 mOsm/kg, on one side of the cell-layer, while no change was made on the other. The activity pattern of [^{3}H]taurine efflux was as seen in experiments with hyposmotic exposure (295 mOsm/kg) on both sides (fig. 5); with an increased rate coefficient on both membranes, significantly higher on the apical side (data not shown).

3.3. Effect of TSH stimulation on rate coefficient for [^{3}H]taurine efflux

FRTL-5 cells, cultured for 2 days in medium deprived of TSH, were initially perfused with isosmotic TSH-free medium as described in 3.2. At the arrow indication, TSH (0.01 IU) was applied to the basolateral (fig. 6A) and the apical (fig. 6B) membrane.

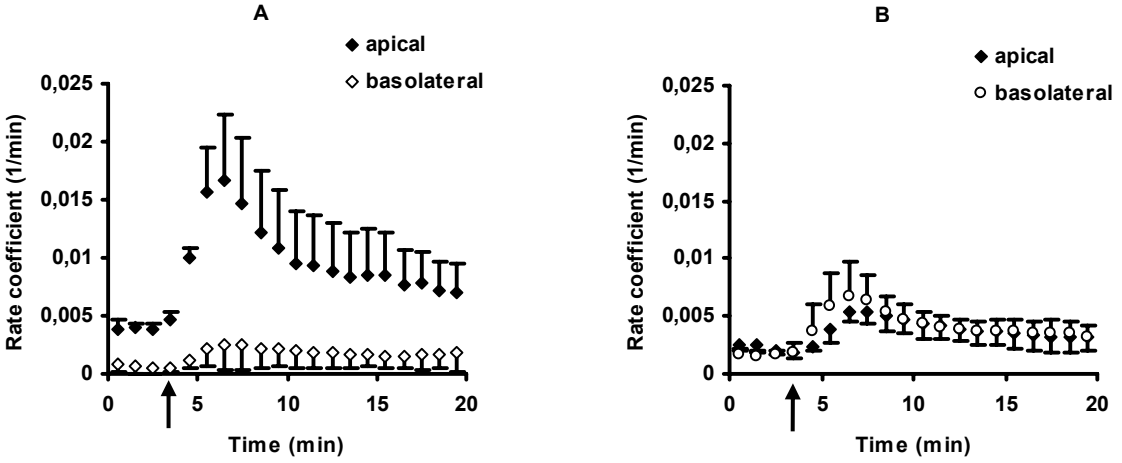

Figure 6: Effect of TSH on the coefficient for [^{3}H]taurine efflux at stimulation on **A:** basolateral and **B:** apical membrane. Cells, preloaded with [^{3}H]taurine, were exposed to isosmotic TSH-free medium on both membranes and, at arrow indications TSH (0.01 IU) was applied on one membrane. Average values for basolateral and apical stimulation are shown ± SD (n= 4 and 5 respectively).

TSH stimulation on the basolateral membrane activated a highly polarized [^{3}H]taurine efflux (fig. 6A), where the apical rate coefficient increased from 0.0039 ± 0.0006/min to 0.0160 ± 0.0051/min. The increase in basolateral rate coefficient, from 0.0006 ± 0.0006/min to 0.0026 ± 0.0022/min, was not statistically significant. The increase in the rate coefficient reached peak values after 4 minutes followed by a decrease (fig. 6A), as seen at exposure to hyposmotic medium (see section 3.2). During basolateral stimulation, the cells lost 15.9 % of their initial [^{3}H]taurine content through the apical side, and 2.8 % through the basolateral side.

Apical TSH application (fig. 6B) increased the basolateral rate coefficient for [^{3}H]taurine efflux from 0.0016 ± 0.005/min to 0.0064 ± 0.0029/min, and the apical from 0.0021 ± 0.004/min to 0.0054 ± 0.0010/min. There was no statistical significant difference between the apical and the basolateral peak values. The pattern of efflux activity was similar to the previous experiments, with peak values reached after 4 minutes, followed by gradual decrease (fig. 6B). During the apical TSH stimulation, the cells lost 6.8 % of their initial [^{3}H]taurine content through the apical side, and 6.7 % through the basolateral membrane.

3.4. Effect of adenylate cyclase activation on rate coefficient for [^{3}H]taurine efflux

cAMP-analogues and the adenylate cyclase activator forskolin have been found to stimulate [^{3}H]taurine efflux in FRTL-5 cells cultured on a solid support (Fugelli, unpublished). The cells grown on permeable membranes showed no increase in the efflux activity when db-cAMP or 8-br-cAMP was added (data not shown), but forskolin caused an increase [^{3}H]taurine efflux (fig. 7). The cells were initially perfused with isosmotic (forskolin-free) medium as described in section 3.2, and at the arrow indication they were exposed to forskolin (500 μM) on both membranes. Basolateral rate coefficient for [^{3}H]taurine efflux increased from 0.0011 ± 0.0002/min to 0.0030 ± 0.0010/min and, the apical rate coefficient from 0.0022 ± 0.0005/min to 0.0066 ± 0.0020/min.

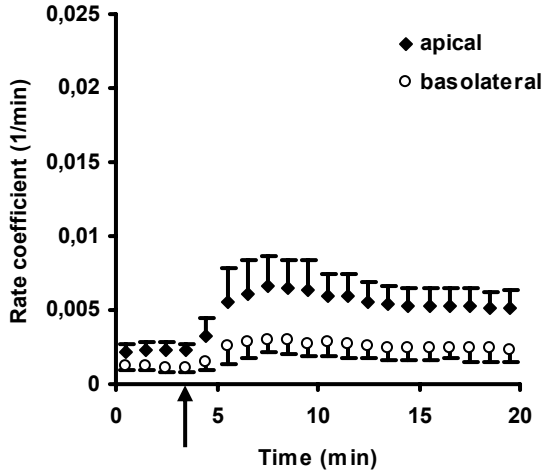

Figure 7: Effect of the adenylate cyclase activator forskolin on apical and basolateral rate coefficient for [^{3}H]taurine efflux. Cells, preloaded with [^{3}H]taurine, were exposed to isosmotic medium on both membranes, and at arrow indications, forskolin (500μM) was applied, also on both membranes. Average values are shown ± SD (n=6).

The apical rate coefficient was higher than the basolateral at all time during the trial. The pattern of [^{3}H]taurine efflux activity was similar to hyposmotic stimulation, reaching peak values after 4 minutes, but the following decline was less prominent.

Similar experiments were repeated with forskolin (500 μM) applied on only one side, apical and basolateral respectively (data not shown). The efflux rate coefficients where similar to those shown in fig. 7.

3.5. Inhibition of adenylate cyclase during stimulation of [^{3}H]taurine efflux

3.5.1. Reduced osmolality

To test the role of cAMP in hyposmotic induced increase of [^{3}H]taurine efflux, experiments with reduced medium osmolality (3.2) were repeated with the AC inhibitor dideoxyadenosine (DDA), (30 μM) added to both isosmotic and hyposmotic medium (fig 8). The peak values of the increase in [^{3}H]taurine efflux rate coefficient during hyposmotic exposure are shown, in the absence (control) and presence of DDA. The inhibitor had no statistically significant effect on the efflux rate coefficient, neither on apical nor basolateral membrane .

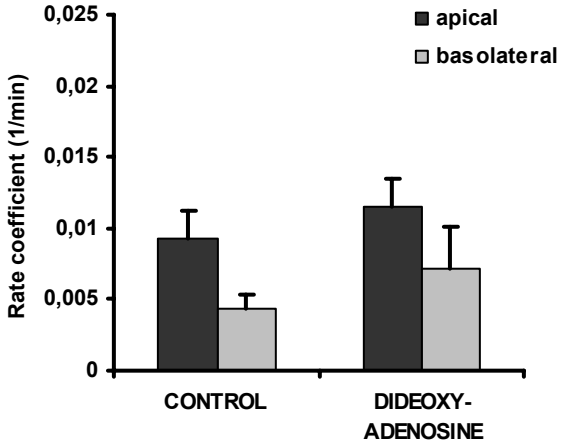

Figure 8: Effect of adenylate cyclase (AC) inhibition on hyposmotic induced [^{3}H]taurine efflux. Cells preloaded with [^{3}H]taurine, were exposed to isosmotic and subsequently to hyposmotic (295 mOsm/kg) medium, on both membranes, where dideoxyadenosine (30 μM), inhibitor of AC, was added to both media. Average peak values during hyposmotic exposure are shown ± SD (n=6 and 4) for control and inhibitor experiments respectively.

Experiments were also performed with a higher concentration of DDA (100 μM). This had no statistically significant effect on the peak values compared to the control experiments without inhibitor (data not shown).

3.5.2. TSH stimulation

Cells were stimulated with TSH as described in section 3.3, where DDA (30 μM) was added to both TSH-free and TSH-containing medium (fig. 9). The peak values of [^{3}H]taurine efflux rate coefficients during TSH application on basolateral (fig 9A) and apical (fig. 9B) membrane are shown, in the absence (control) and presence of DDA, respectively.

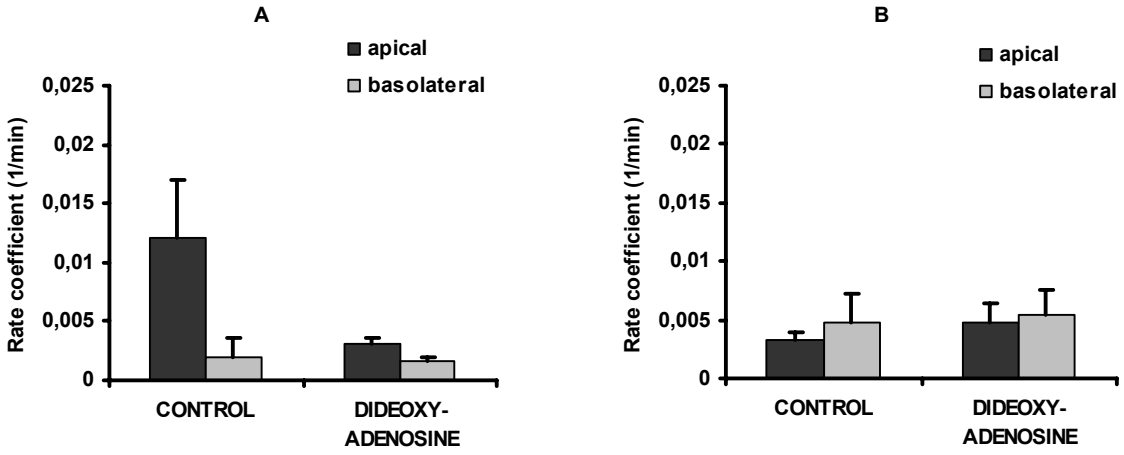

Figure 9: Effect of adenylate cyclase (AC) inhibition on [^{3}H]taurine efflux induced by **A:** basolateral and **B:** apical TSH stimulation. Cells, preloaded with [^{3}H]taurine, were exposed to isosmotic TSH-free medium on both membranes and subsequently to TSH-medium on one membrane, where dideoxyadenosine (30 μM), inhibitor of AC, was added to both media. Average peak values during TSH stimulation for control and dideoxyadenosine experiments are shown ± SD from **A:** basolateral (n= 4, both series) and **B:** apical (n=5 and 4) TSH application, in the order mentioned.

The increased apical [^{3}H]taurine efflux rate coefficient induced by basolateral TSH stimulation, was strongly inhibited by DDA (fig. 9A), but the basolateral activity remained unchanged. The inhibitor had no statistically significant effect on the [^{3}H]taurine efflux rate coefficient induced by apical TSH stimulation, neither on the apical nor the basolateral side (fig 9B).

3.6. Arachidonic acid metabolites involved in [^{3}H]taurine efflux activation

3.6.1. PGE_2 stimulation

Cells, preloaded with [^{3}H]taurine, were initially exposed to PGE_2-free isosmotic medium on both membranes. At the arrow indication, PGE_2 (500 µM) was applied to the basolateral (fig. 10A) and on the apical (fig. 10B) membrane .

Basolateral PGE_2 application caused rate coefficient for [^{3}H]taurine efflux to increase on the stimulated membrane, from 0.0020 ± 0.0011/min to 0.0067 ± 0.0023/min, while no effect was observed on the apical membrane. During the test-period, the cells lost 9.1 % of their initial [^{3}H]taurine content through the basolateral side and 4.7% through the apical side.

Apical PGE_2 application caused the rate coefficient for [3H]taurine efflux to increase on the stimulated side from 0.0037 ± 0.0010/min to 0.0082 ± 0.0025/min, while no effect was observed on the basolateral membrane. During the trial, the cells lost 12.1% of their initial [^{3}H]taurine content through the apical side and 2.8 % through the basolateral side. In the two series of experiments with PGE_2, the efflux activity increase was not followed by any inactivation as seen in previously (see section 3.2., 3.3., and 3.4.).

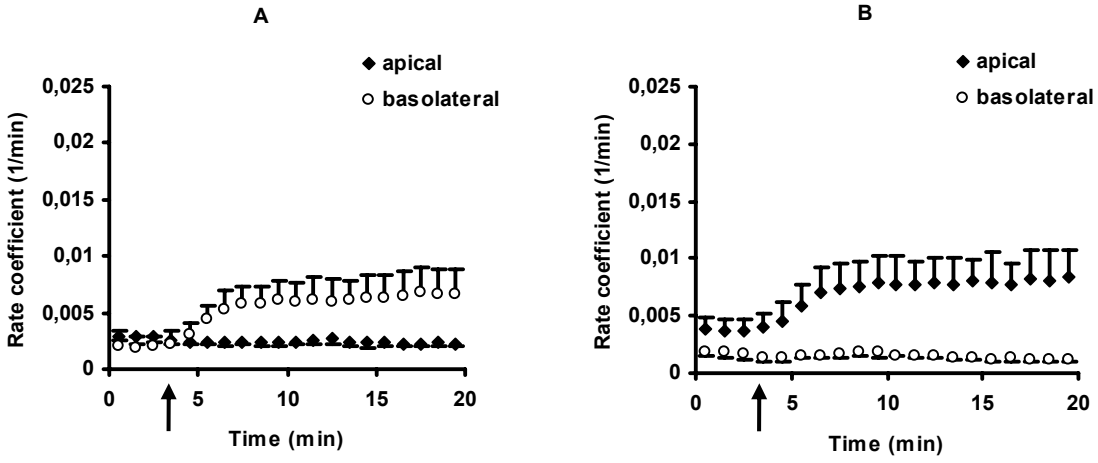

Figure 10: Effect of PGE_2 on the rate coefficient for [^{3}H]taurine efflux at stimulation on **A:** basolateral and **B:** apical membrane. Cells, preloaded with [^{3}H]taurine, were exposed to isosmotic PGE_2-free medium on both membranes, and at arrow indications PGE_2 (500 µM) was applied on one membrane. Average values for basolateral and apical stimulation are shown ± SD (n= 5 and 4 respectively).

3.6.2. Cyclo-oxygenase inhibition

To test the role of the cyclo-oxygenase (CO) pathway in hyposmotic induced [^{3}H]taurine efflux, experiments with reduced medium osmolality (see section 3.2) were repeated with the CO inhibitor indomethacin (10 μM) added to both isosmotic and hyposmotic medium (fig 11A). The relative peak values of [^{3}H]taurine efflux rate coefficient during hyposmotic exposure are shown, in the absence (control) and presence of indomethacin respectively. The inhibitor caused a strong potentiation of the hyposmotic increased efflux activity on the apical side, but no statistically significant effect on the basolateral side.

3.6.3. 5-lipoxygenase inhibition

The experiments in 3.6.2 were repeated, where indomethacin was replaced by the 5-lipoxygenase inhibitor MK-886 (2 μM). The relative peak values of [^{3}H]taurine efflux rate coefficient during hyposmotic exposure are shown, in the absence (control) and presence of MK-886 respectively (fig 11B). The inhibitor caused a statistically significant reduction of the hyposmotic increased efflux activity on both membranes.

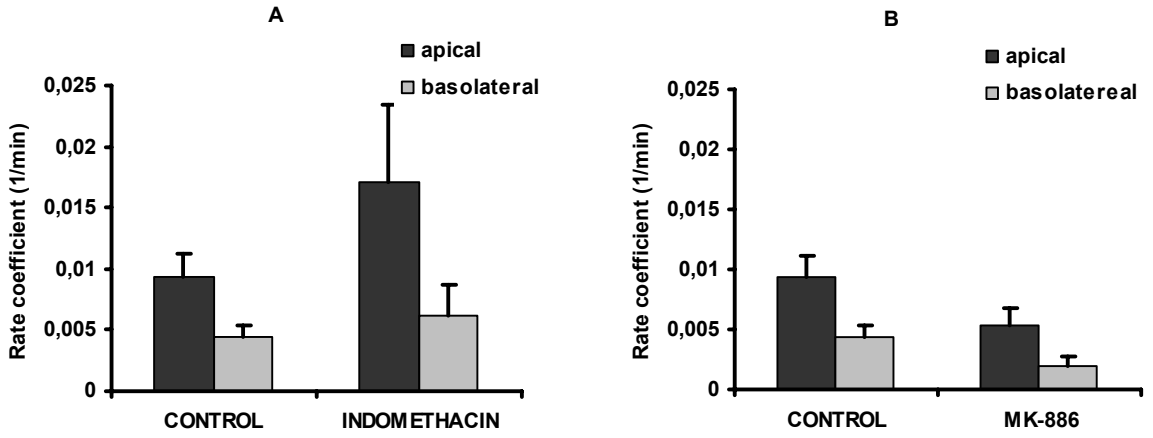

Figure 11: Effect of **A:** cyclo-oxygenase (CO) and **B:** 5-lipoxygenase (5-LO) inhibition on the hyposmotic induced [^{3}H]taurine efflux. Indomethacin (10 μM), inhibitor for CO, and MK-886 (2 μM), inhibitor for 5-LO, were added respectively to isosmotic and hyposmotic (295 mOsm/kg) medium. Cells, preloaded with [^{3}H]taurine, were exposed to the isosmotic medium and subsequently to the hyposmotic medium, on both membranes. Average peak values during hyposmotic exposure are shown ± SD, **A:** for control and indomethacin experiments (n=6, both series) and **B:** for control and MK-886 experiments (n= 6 and 5), in the order mentioned.

4. Discussion

4.1. Morphology of FRTL-5 cells grown on a permeable membrane support.

The scanning electron microscopy (SEM) photos show that the FRTL-5 cells form a morphologically polarized cell-layer, where microvilli appear on the apical side (fig. 4). The cells seemed to form an epithelial-like structure (although the preparation for SEM shrunk the cells and made them detach from each other), but is nevertheless not considered as a true epithelium, since FRTL-5 cells are modified cells and because they were unable to a form a 100 % confluent layer. The cells deprived of TSH, showed a different morphology than cells grown with TSH, as they appeared flatter, had less microvilli and were fewer. This shows the importance of TSH for growth and differentiation of FRTL-5 cells, and is in accordance with earlier studies (see Bidey et al., 1988).

The lack of confluence could potentially interfere with the results, as it could result in leakage of both TSH and PGE_2 and released radioactive taurine across the unoccupied parts of the permeable membrane. The PGE_2 application showed, however, that there was no leakage from one chamber part to another that could have interfered with the results, since the increase in taurine efflux rate coefficient was strictly located to the stimulated side (fig. 10).

4.2. Localization of taurine efflux

Both reduction in medium osmolality (fig. 5) and TSH stimulation (fig. 6) induced taurine efflux rate coefficient in FRTL-5 rat thyroid cells grown on permeable membrane supports. This is in accordance with observations on the same cell-type grown on a solid support where the medium access is restricted to the apical membrane (Fugelli, unpublished).

The present preparation of the FRTL-5 cells made it possible to distinguish between efflux on the apical and the basolateral side of the cell-layer. The cells demonstrated polarity in this activity, most pronounced during TSH application on the basolateral side, which lead

to a strong and significant activity increase only on the apical side (fig. 3A). Hyposmotic exposure stimulated activity on both sides, but was also polarized, as the apical rate coefficient for taurine efflux was two times higher than the basolateral (fig. 5). TSH applied on the apical side induced increase in taurine efflux rate coefficient on both sides, where the difference between the apical and the basolateral activity was not statistically significant (fig. 6B).

Such a distribution of taurine efflux has to my knowledge not been observed earlier, and made it possible to discriminate between activity induced by cellular swelling and TSH respectively. Further, it was also possible to separate the efflux with respect to signal pathways involved.

4.3. TSH stimulation

The apical taurine efflux induced by basolateral TSH application appeared to be mediated by cAMP, since it was inhibited by the adenylate cyclase (AC) inhibitor dideoxyadenosine (fig. 9A). This assumption is supported by earlier studies on FRTL-5 cells, where TSH stimulation has been shown to increased cAMP production (Cauvi et al., 2000), and where application of cAMP analogues have been shown to activate taurine efflux (Fugelli, unpublished).

The AC-activator forskolin activated efflux on both membranes (fig. 7), indicating that taurine efflux could be mediated by cAMP on both the apical and the basolateral side in polarized FRTL-5 cells. It was therefore possible that both the apical and the basolateral efflux seen during apical TSH application (fig 9B), was mediated by AC activation. However, the adenylate cyclase inhibitor had no effect on the apical TSH stimulation, neither on the apical nor the basolateral efflux. This indicates that TSH can induce two different signal pathways in polarized FRTL-5 cells, one that is dependent, and one that is independent, of cAMP, which both increase taurine efflux. The latter is here suggested to be mediated by a phospholipase C (PLC)-dependent signal pathway, since stimulation of the TSH receptor has been shown to stimulate activation of both AC and PLC (Allgeier et al., 1994; Sho et al., 1991), but this needs further investigation.

Apparently the TSH receptor has only been located on the basolateral membrane in primary cultures of thyrocytes (Vassart and Dumont, 1992; Ericson and Nilsson, 2000). The FRTL-5 cells' response to apical TSH stimulation in this study indicates the presence of receptors on the apical membrane surface as well. Whether this is a result of an organisational error in this preparation, or reflects in vivo conditions, remains to be shown. The possibility of TSH diffusing through the membrane on which the cells grow, and thereby activating the receptors located on the basolateral side, is considered unlikely, since the apical and the basolateral TSH application appears to mediate taurine efflux by different signal pathways.

4.4. Reduced osmolality

4.4.1. cAMP

Earlier studies indicate that cAMP is involved in regulatory volume decrease (RVD), as cellular swelling lead to increased production of cAMP (Thoroed et al., 1995). In FRTL-5 cells, inhibition of phosphodiesterase (thereby preventing cAMP degradation), caused potentiation of the swelling-activated taurine efflux (Fugelli, unpublished), suggesting that this taurine efflux pathway is partly mediated through cAMP.

AC inhibition had, however, even at a high concentration, no statistically significant effect on the swelling-activated taurine efflux in the FRTL-5 cells grown on membrane (fig. 8). This indicates that cAMP, in this cell preparation, is not involved in this type of taurine efflux, and that there probably are other signal pathways mediating this activity. An alternative explanation is that the inhibited cAMP-mediated activity is compensated for by a cAMP-independent efflux pathway that normally is inactive or less prominent.

The difference between present and earlier studies on FRTL-5 cells could be explained by the preparation of the cells. A polarized cell-layer could have a different organization of signal pathways than cells cultured on a solid support. cAMP's role during RVD in thyrocytes is still unclear and will need further investigation.

4.4.2. Arachidonic acid metabolites

An alternative signal pathway mediating the swelling-activated taurine efflux, could be the arachidonic acid pathway, since its metabolites have been demonstrated to be involved in RVD (see Lambert, 1994).

MK-886 (inhibitor of the 5-lipoxygenase enzyme), partly inhibited swelling-activated taurine efflux on both sides of the cell-layer (fig. 11A), which indicates that 5-lipoxygenase is involved in the activation of the taurine efflux during swelling. This is in accordance with earlier studies on FRTL-5 cells grown on a solid support (Kveberg, 1998). Several types of leukotrienes, which are end products of the 5-lipoxygenase pathway, could have possible roles as taurine efflux activators in FRTL-5 cells. The present inhibition of 5-lipoxygenase was not complete, which could mean that the inhibitor concentration was too low. It could also indicate that another signal pathway is involved in the swelling-activated taurine efflux.

Inhibiting the cyclo-oxygenase pathway with indomethacin potentiated the swelling-activated taurine efflux on the apical membrane, while there was no statistically significant effect on the basolateral activity (fig. 11B). This could indicate that the cyclo-oxygenase pathway modulates the apical swelling-activated efflux, and when this modulating effect is inhibited, it results in a higher taurine efflux rate coefficient. Since AA is substrate for both cyclo-oxygenase and 5-lipoxygenase, an alternative explanation could be that the inhibition of cyclo-oxygenase results in more AA available for 5-lipoxygenase, which seemed to activate taurine efflux during swelling.

On the contrary to cells grown on permeable membranes, indomethacin showed no influence on swelling-activated taurine efflux in FRTL-5 cells grown on a solid support (Kveberg, 1998). It is therefore possible that the cyclo-oxygenase pathway is involved in a mechanism depending on the polarity of the cells, where communication between the apical and basolateral membrane is necessary.

PGE_2, one of the end products of the cyclo-oxygenase pathway, stimulated taurine efflux (fig. 10A and B), and could therefore be engaged in the cellular liquid transport in FRTL-5 cells. It is unlikely that this is the same type of efflux as seen during swelling, since the cyclo-oxygenase pathway seemed to be a modulator of this taurine efflux (see paragraph above). Also the strong polarization of the PGE_2-stimulated efflux indicates that this is a

differently regulated activity than the one seen during swelling, and seems to have more in common with TSH-stimulated efflux.

It is possible that PGE_2 could be involved in the modulating cyclo-oxygenase effect on swelling-activated taurine efflux, since PGE_2 earlier has been shown to inhibit RVD (Lambert, 1987) and since the inhibitory effects of indomethacin has been shown to be reversed by PGE_2 in thyroid cells (Burch et al., 1986), but its role as activator of taurine efflux is probably related to other cellular functions.

Taurine efflux in FRTL-5 cells grown on permeable membrane supports seems to be activated by different signal pathways. The present results indicate that the arachidonic acid metabolite pathways are central participants in the swelling-activated taurine efflux. The AC/cAMP pathway is also engaged in taurine efflux, but its role during RVD is uncertain with respect to the cells grown on a membrane, and seems be more important during TSH stimulation.

4.5. Physiological significance

The present and former studies indicate that taurine is engaged in the osmolyte efflux during cellular swelling. It is also possible that taurine could participate in regulation of the colloid space volume in the thyroid gland as well, as the apical efflux capacity seems high. Since the swelling induced efflux is stronger on the apical side might indicate a higher distribution of taurine channels on this membrane than on the basolateral, or that the transport is directed towards this side by the intracellular signal system. This could, during RVD, result in a high taurine concentration in the colloid space, however, this has to my knowledge never been analyzed

Both the polarized taurine efflux seen during basolateral TSH application and the bi-directional transport seen at apical TSH application, indicates that taurine could be involved in different types of hormone-stimulated liquid transport as well as in the regulatory volume decrease. The purpose of this TSH-stimulated liquid transport where taurine seems to participate, is unclear. The present results clearly show the ability of the cells to control the

direction of osmolyte efflux and thereby the water movement, which was supported by the PGE_2 stimulation of the cells.

Whether the present preparation reflects the cellular activities in the thyroid gland, is uncertain. The method applied should therefore be considered as a tool for separation of different cellular mechanisms, rather than a simulation of in vivo functions.

4.6. Summary

FRTL-5 cells grown on permeable membrane supports with access to medium on both apical and basolateral side formed functional and morphological polarized cell-layers. With the present preparation it was possible to distinguish between swelling-activated and TSH-activated taurine efflux, which showed different polarity. It was also possible to isolate swelling- and TSH-activated efflux by signal pathways involved, and also to distinguish between two different types of TSH-stimulated activities. The experiments on polarized FRTL-5 cells have thus provided new information about taurine efflux, and have suggested that there could be several efflux pathways. The question of whether there is more than one of them involved in regulatory volume decrease, remains uncertain, as well as to what extent these findings reflect in vivo properties.

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