Extracellular ATP as a Regulator of Intracellular Signaling in Thyroid FRTL-5 Cells

Elina Ekokoski

Department of Biosciences
Division of Animal Physiology
University of Helsinki

Academic dissertation

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Supervised by

Professor Kid Törnquist
Department of Biology
Åbo Akademi University
Turku, Finland

Reviewed by

Professor John Eriksson
Department of Biology
Laboratory of Animal Physiology
University of Turku
Turku, Finland

and

Professor Mika Scheinin
Department of Pharmacology and Clinical Pharmacology
University of Turku
Turku, Finland

Faculty opponent

Professor Karl Åkerman
Department of Physiology
University of Uppsala
Uppsala, Sweden

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To my parents,

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals in the text.

I Ekokoski, E., Dugué, B., Vainio, M., Vainio, P.J. and Törnquist, K. (2000). Extracellular ATP-mediated PLA_2 activation in rat thyroid FRTL-5 cells. Regulation by a G_i/G_o protein, Ca^{2+} and mitogen-activated protein kinase. Journal of Cellular Physiology, 183: 155-162.

II Törnquist, K., Ekokoski, E., Forss, L. and Matsson, M. (1994). Importance of arachidonic acid metabolites in regulating ATP-induced calcium fluxes in thyroid FRTL-5 cells. Cell Calcium, 15: 153-157.

III Ekokoski, E., Forss, L. and Törnquist, K. (1994). Inhibitory action of fatty acids on calcium fluxes in thyroid FRTL-5 cells. Molecular and Cellular Endocrinology, 103: 125-132.

IV Törnquist, K., Ekokoski, E., and Dugué, B. (1996). Purinergic agonist ATP is a comitogen in thyroid FRTL-5 cells. Journal of Cellular Physiology, 166: 241-248.

V Ekokoski, E., Webb, T.E., Simon, J. and Törnquist, K. (2000) Mechanisms of P2 receptor-evoked DNA-synthesis in thyroid FRTL-5 cells. Submitted.

ABBREVIATIONS

AA	arachidonic acid
BAPTA	1,2,-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
4-BPB	4-bromophenylacyl bromide
BCECF	bis-(carboxyethyl)carboxyfluorescein
BSA	bovine serum albumin
BzATP	2'&3'-O-(4-benzoylbenzoyl)adeosine 5'-triphosphate
$[Ca^{2+}]_i$	intracellular concentration of free Ca^{2+}
CaM	calmodulin
cAMP	cyclic AMP
cGMP	cyclic GMP
CICR	Ca^{2+}induced Ca^{2+}release
CIF	Ca^{2+} influx factor
cPLA2	cytosolic phospholipase A2
CRE	cAMP responsive element
DAG	diacylglycerol
DPX	1,3-diethyl-8-phenylxanthine
ECL	enhanced chemiluminescent
EET	epoxyeicosatrienoic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
ETYA	5,8,11,14-eicosatetraynoic acid
FGF	fibroblast growth factor
FRTL-5	Fisher rat thyroid cell line
G protein	GTP-binding protein
GPCR	G protein-coupled receptor
HBSS	Hepes-buffered salt solution
HETE	hydroxyeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
I_{CRAC}	Ca^{2+}-release activated Ca^{2+}current
IEG	immediate early gene
IGF	insulin-like growth factor
IP3	inositol-1,4,5-trisphosphate
LTB4	leukotriene B4
MAPK	mitogen-activated protein kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
αβ–meATP	αβ–methyleneATP
2-MeSATP	2-methylthioATP
MEK	mitogen-activated protein kinase kinase
MEKK	mitogen-activated protein kinase kinase kinase
NDGA	nordihydroguaiaretic acid
OA	oleic acid
PG	prostaglandin
PIA	(-)-N6-(2-phenylisopropyl)adenosine

PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PS	phosphatidylserine
PTX	pertussis toxin
RTK	receptor tyrosine kinase
RT-PCR	reverse-transcriptase polymerase chain reaction
RyR	ryanodine receptor
SH	Src-homology
SOC	store-operated Ca^{2+} channel
SRE	serum-response element
TCA	trichloroacetic acid
TM	transmembrane
TRE	TPA response element
TSH	thyrotropin

INTRODUCTION

ATP, a nucleotide consisting of an adenine base, a ribose sugar and a triphosphate unit, is used as an energy source in muscles, in the movements of cells, and in active transport. ATP also serves as a phosphate donor in biochemical reactions and as a precursor for nucleic acid synthesis. Although first recognized as intracellular molecules, the actions of extracellular adenine nucleotides on various bodily functions have also been known for a long time. In 1929 Drury and Szent-Györgyi published a study showing that adenosine and AMP had biological effects in mammalian heart (Drury and Szent-Györgyi, 1929). In 1934 Gillespie demonstrated that ATP and adenosine had separate effects on blood pressure, and that ATP was more potent than AMP and adenosine in causing contraction of the guinea-pig ileum and uterus (Gillespie, 1934). This was also the first hint of the existence of separate receptors for ATP and adenosine. In 1972 Burnstock raised the possibility that ATP acts as a neurotransmitter, and the term "purinergic" was introduced and purinergic transmission proposed (Burnstock, 1972). In 1978 Burnstock proposed the existence of specific extracellular receptors for adenosine and ATP (Burnstock, 1978). Since then, along with the cloning of the first P2 receptors in the beginning of the 1990's, knowledge of the actions and receptors of extracellular nucleotides has exponentially expanded (Ralevic and Burnstock, 1998).

Today, receptors for ATP and other nucleotides have been found in almost every cell type studied. Extracellular nucleotides have been shown to have a wide physiological involvement both in normal cellular functions and in pathological conditions. While the early investigations focused on ATP's effects on the heart and the vasculature, the effects are now well known in the central nervous system. There are also clear indications that ATP and other nucleotides have regulatory actions in the cells of various endocrine tissues. Investigations in primary thyroid cell cultures from different species and in thyroid cell lines have shown that extracellular ATP is a true agonist in thyrocytes. These studies have focused mainly on the mechanisms of regulation of Ca^{2+} fluxes evoked by ATP (Raspé et al., 1991; Törnquist, 1992; Törnquist, 1993), and some physiological responses such as regulation of iodide fluxes and generation of H_2O_2, events that are essential in thyroid hormone synthesis (Nakamura and Ohtaki, 1990; Raspé and Dumont, 1994). In thyroid cells, ATP activates phospholipase C (PLC), which results in an increase in free intracellular Ca^{2+} concentration, and phospholipase A_2 (PLA_2) and the subsequent release of arachidonic acid (AA). Ca^{2+} is a central second messenger in cells having effects on numerous cellular functions, such as secretion, gene expression and cell proliferation (Carafoli and Klee, 1999). PLA_2 activation with the subsequent release of fatty acids from membranes have effects on e.g. iodide fluxes and proliferation (Burch et al., 1986; Di Girolamo et al., 1991). However, the regulation of PLA_2 activation in response to ATP, and the possible interaction of these two pathways has not been thoroughly investigated in thyroid cells. Furthermore, the mitogenic effect of ATP, which is recognized in many other cell types, has not been investigated other than in thyrocytes of one animal species. Finally, despite many studies showing the effect of ATP and other nucleotides on thyroid cell functions, which may be indicative of the existence of several P2 receptor

subtypes, no studies have been carried out to examine which receptor subtypes are expressed in these cells.

In the present study, we have examined the effects of extracellular ATP in a rat thyroid cell model, the FRTL-5 cell line. We have investigated the signal transduction pathways evoked by ATP: the regulation of PLA_2 activity in response to ATP, and the effect of PLA_2 activity on Ca^{2+} homeostasis. Furthermore, we have examined which subtypes of P2 nucleotide receptors are expressed in these cells, and investigated the effect of ATP and other nucleotides on DNA-synthesis and cell proliferation.

REVIEW OF THE LITERATURE

I EXTRACELLULAR ATP

1. Sources and degradation of extracellular ATP

Cytosolic concentration of ATP in most cells is > 5 mM (Gordon, 1986). ATP can thus be released from any cell into the extracellular space during tissue injury and from dying cells. Cytosolic ATP can also be released by transmembrane transport via specific transporter proteins in response to receptor activation in vascular smooth muscle cells and endothelial cells (Sedaa et al., 1990). ATP may be stored in granules or vesicles from which it is released by exocytosis. Exocytotic release may occur from nerve terminals, where ATP is co-released with classical transmitters and neuropeptides in most major nerve types (Burnstock, 1972), and from platelets. ATP and UTP may also be released from cells by different environmental stressors such as mechanical stress, and recent studies have demonstrated a basal release of nucleotides from resting cells in vitro (Lazarowski et al., 2000). The mechanism of the release is however unresolved (Lazarowski et al., 1995; Pedersen et al., 1999). In the thyroid gland, sympathetic and parasympathetic nerve terminals reach the thyroid follicles and thus could theoretically be the source of ATP (Van Sande et al., 1980). Rat thyroid FRTL-5 cells have also been shown to release adenosine (Vainio et al., 2000).

Once released, extracellular ATP can locally reach biologically active levels from nanomolar to micromolar concentrations (Gordon, 1986; Lazarowski et al., 1995). The half-life of extracellular ATP is short, and ATP is rapidly broken down by ectoenzymes to ADP, AMP and adenosine. Adenosine is further degraded by adenosine deaminase to form inosine. Presently, there is no evidence of carrier-mediated cellular uptake of nucleotides, whereas nucleosides and nucleobases are taken up by several transport systems (Griffith and Jarvis, 1996).

2. Physiological actions of extracellular ATP

Receptors for ATP and other nucleotides have been identified in a remarkable variety of cell types, and extracellular nucleotides have been shown to affect diverse cellular and tissue functions. In the central and peripheral nervous system, ATP acts as a co-transmitter with other classical neurotransmitters in many different nerve types (Burnstock, 1972). It mediates fast synaptic transmission between neurones in autonomic ganglia and the medial habenula (Edwards and Gibb, 1993). A role for P2 receptors in memory and learning (Inoue et al., 1996; Wieraszko et al., 1989; Wieraszko and Seyfried, 1989), as well as in nociception (Burnstock, 1996) has been demonstrated. At neuroeffector junctions ATP regulates the contraction of visceral smooth muscle cells. ATP and adenosine regulate also vascular tone by acting through receptors on endothelial and vascular smooth muscle cells (Ralevic and Burnstock, 1998). ATP has a role also in exo- and endocrine secretion (Abbracchio and Burnstock, 1998). In the testis, extracellular nucleotides have been shown to induce testosterone secretion from Leydig cells, and in pancreas activation of P2Y receptors

results in insulin secretion (Foresta et al., 1996; Petit et al., 1998). In the immune system, ATP has an anti-inflammatory effect (Abbracchio and Burnstock, 1998).

ATP has also long term actions in cells by affecting cell growth and differentiation in both embryonic development and adulthood (Abbracchio and Burnstock, 1998). ATP induces DNA-synthesis and proliferation in several different cell types either on its own, or synergistically with other mitogens (Erlinge et al., 1993; Huang et al., 1989; Huwiler and Pfeilschifter, 1994; Neary et al., 1994; Van Daele et al., 1992; Wang et al., 1992). However, in cells where ATP has been reported to act as an independent mitogen, synergism with other growth factors produced in an autocrine fashion cannot be excluded (Erlinge, 1998). In vascular smooth muscle cells extracellular ATP is probably involved in the development of atherosclerosis and possibly hypertension through induction of cell proliferation (Erlinge et al., 1998). In the kidney, ATP induces cell proliferation and enhances recovery from renal ischemia (Paller et al., 1998). Extracellular nucleotides may contribute to the regulation of chondrocyte function in both bone growth and during trauma and inflammation (Kaplan et al., 1996). Extracellular ATP may also cause cell death in a variety of cells, or it may have protective roles in pathological conditions such as cancer, stress, bone resorption and traumatic tissue damage (Abbracchio and Burnstock, 1998).

ATP stimulates the production of H_2O_2 in porcine and dog thyrocytes in primary culture, and in rat thyroid FRTL-5 cells (Björkman and Ekholm, 1992; Nakamura and Ohtaki, 1990; Raspé et al., 1991). It also regulates iodide efflux in dog thyrocytes and in FRTL-5 cells (Okajima et al., 1988; Raspé and Dumont, 1994; Smallridge and Gist, 1994). ATP evokes Ca^{2+} fluxes in thyroid cells of different species, including human, dog and FRTL-5 cells (Rani et al., 1989; Raspé et al., 1989; Raspé et al., 1991; Schöfl et al., 1995; Törnquist, 1992), and activates phospholipase D (PLD) in dog thyrocytes (Lejeune et al., 1996). In dog thyrocytes, ATP is not a mitogen (Dumont et al., 1992). However, its effect on proliferation has not been tested in the thyrocytes of other species.

3. Receptors for extracellular nucleotides and nucleosides

Extracellular nucleotides interact with receptors located on the plasma membrane. The receptors are divided into P1 and P2 receptor classes, which mediate distinct responses evoked by adenosine and ATP, respectively (Burnstock, 1978; Alexander and Peters, 2000). Within both receptor classes there are several receptor subtypes.

3.1 P2 receptors

The P2 receptors were originally classified into P_{2X} and P_{2Y} receptors based on pharmacological studies on the activity of ATP analogues and antagonists (Burnstock and Kennedy, 1985). New subtypes were then proposed: a platelet P_{2T} receptor selective for ADP, a P_{2Z} receptor, which represented an ATP-activated conductance pore in mast cells, a P_{2D} receptor selective for diadenosine polyphosphates, and a P_{2U} receptor for both ATP and UTP (O'Connor et al., 1991).

Later, it was realized that ATP was acting through two different transduction mechanisms in cells, and along with the cloning of the first receptors, a new classification was proposed: P2X or transmitter-gated ion channel receptors, and P2Y or G protein-coupled receptors (Abbracchio and Burnstock, 1994). Due to the lack of specific agonists and antagonists for P2 receptors, pharmacological knowledge still lag behind the cloning studies, thus making firm conclusions difficult to draw in systems where there are several different P2 subtypes expressed. Also, along with the discovery of more P2 subtypes expressed than expected on the basis of pharmacological studies, some previously published results have to be reevaluated.

3.1.1 P2Y subtypes

The G protein-coupled P2Y receptors typically have seven transmembrane domains (7TM) with an extracellular N-terminus and an intracellular C-terminus (Figure 1). Binding sites for the nucleotides have been localized to the 6th and 7th TM domains (Burnstock, 1997).

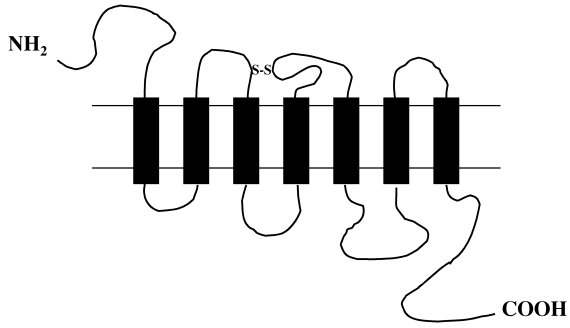

Figure 1. Structure of a P2Y receptor. *S-S indicates a predicted disulfide bridge.*

To date, eight G protein-coupled P2 receptors have been cloned, five from mammalian (human, rodent) sources ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$), two avian receptors ($P2Y_1$ and $P2Y_3$) and one from *Xenopus laevis* (p2y8) (North and Barnard, 1997). The cloned mammalian receptors can be divided into three groups: 1) the purine receptors $P2Y_1$ and $P2Y_{11}$, 2) the pyrimidine receptors $P2Y_4$ (human) and $P2Y_6$, and 3) purine/pyrimidine receptors $P2Y_2$ and $P2Y_4$ (rat). The $P2Y_{11}$ subtype is so far the only ATP-selective P2Y receptor. The distribution and agonist selectivities of the mammalian receptors are summarized in Table 1.

All of the cloned mammalian P2Y receptors are coupled to G_q/G_{11} proteins and activate phospholipase C (PLC) (Ralevic and Burnstock, 1998). However, the $P2Y_1$ receptor may in some native cells couple to the G_i/G_o pathway, and instead of generating IP_3, increases intracellular Ca^{2+} by inhibiting adenylate cyclase (Webb et al., 1996). The $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are also coupled to G_i/G_o proteins (Filippov et al., 1999; Ralevic and Burnstock, 1998). The $P2Y_{11}$ receptor apparently activates both PLC and adenylate cyclase, and is thus unique among P2Y receptors (Communi et al., 1999). The P2Y type receptors in some rat neurones have been shown to be linked to

the activation of a K^+- and Ca^{2+}-channels, and this coupling is G protein-mediated (North and Barnard, 1997).

TABLE 1. Cloned mammalian P2Y receptors.

Subtype	Distribution	Agonists*	References
$P2Y_1$	Wide distribution; brain, spinal cord, pancreas, vascular endothelial cells, platelets	2MeSADP>ADP≥ATPγS> αβmeATP**	Schachter et al., 1996 Ayyanathan et al., 1996 Henderson et al., 1995 Tokuyama et al., 1995 Léon et al., 1997
$P2Y_2$	Wide distribution; epithelial cells, vascular smooth muscle cells, bone	UTP≥ATP>A_{p4}A=ATPγS>> UDP=2MeSATP=αβmeATP	Lustig et al., 1993 Parr et al., 1994 Bowler et al., 1995
$P2Y_4$	Placenta, brain, several peripheral organs	UTP, UTPγS>>ATP (human) UTP =ATP (rat)	Communi et al., 1996 Nguyen et al., 1995 Webb et al., 1998 Bogdanov et al., 1998
$P2Y_6$	Spleen, vascular smooth muscle, wide distribution in brain	UDP>>UTP>2MeSATP, ADP	Communi et al., 1996 Chang et al., 1995
$P2Y_{11}$	Placenta, spleen, granulocytes	ATP>2MeSATP>>ADP	Communi et al., 1997 van den Weyden et al., 2000

* Studies have shown that absolute nucleotide selectivity is likely to vary to a certain degree across species.

** Currently it is uncertain whether or not ATP is an agonist at the $P2Y_1$ receptor (Fagura et al., 1998; Léon et al., 1997).

3.1.2 P2X subtypes

The P2X receptors are transmitter-gated ion channels (Barnard, 1996). They do not exhibit sequence similarity with the other ligand-gated ion channel families and are therefore considered to represent a third major class of these receptors (North, 1996). P2X receptors consist of at least three receptor subunits (Nicke et al., 1998), each of which has two hydrophobic TM segments M1 and M2, short intracellular N- and C-termini, and a large cysteine-rich extracellular loop (Figure 2). Although it is not certain, the conserved cysteines in the extracellular loop could form a binding site for ATP through the formation of a network of disulfide bonds (Barnard et al., 1997). The M2 domain have been demonstrated to be involved, at least in part, in pore formation (Nicke et al., 1998; Rassendren et al., 1997). The channels open within milliseconds, and are permeant to K^+, Na^+ and Ca^{2+} (Humphrey et al., 1995)

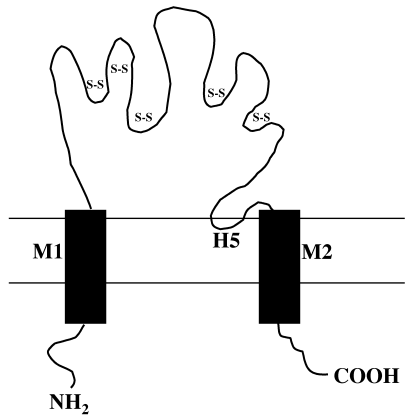

Figure 2. Structure of a P2X receptor subunit. *S-S indicates a predicted disulfide bridge. M1 and M2, membrane-spanning segments; H5, a hydrophopic segment.*

To date, seven P2X receptor subunits designated $P2X_{1\text{-}7}$ have been cloned from mammalian cells. Receptor distribution and agonist selectivities are summarized in Table 2. All P2X receptor subunits can form homomeric channels, but it is likely that in native cells they exist as heteromeric assemblies (Nicke et al., 1998). At least the recombinant $P2X_{2/3}$, $P2X_{1/5}$ and $P2X_{4/6}$ subunits form heteromeric channels when coexpressed in cultured cells (Lé et al., 1998; Lewis et al., 1995; Torres et al., 1998), however not all subunit combinations may be possible (Surprenant, 1996).

TABLE 2. Cloned mammalian P2X receptors.

Subtype	Distribution	Agonists	References
$P2X_1$	Visceral and vascular smooth muscle, sensory ganglia	ATP=2MeSATP≥αβmeATP≥ ATPγS>ADP	Valera et al., 1994 Bo and Burnstock, 1993 Longhurst et al., 1996
$P2X_2$	Autonomic and sensory ganglia, various brain nuclei , retina	ATP≥ATPγS≥2MeSATP> ADP>>αβmeATP	Brake et al., 1994 Lewis et al., 1995 Kidd et al., 1995 Greenwood et al., 1997
$P2X_3$	Nociceptive sensory neurones	2MeSATP≥ATP≥αβmeATP	Chen et al., 1995 Lewis et al., 1995
$P2X_4$	Brain, pancreas, glands, central nervous system	ATP≥ATPγS≥2MeSATP>> ADP≥αβmeATP	Bo et al., 1995 Soto et al., 1996
$P2X_5$	Mesencephalic trigeminal nucleus neurones, sensory nerves	ATPγS≥ATP≥2MeSATP> ADP>>αβmeATP	Collo et al., 1996 Khakh et al., 1997
$P2X_6$	Brain, autonomic ganglia, epithelial cells	2MeSATP≥ATP≥ATPγS> ADP>>αβmeATP	Collo et al., 1996 Soto et al., 1996
$P2X_7$	Macrophages, lymphocytes, mast cells, microglia	BzATP>ATP>2MeSATP> ATPγS>>αβmeATP	Rassendren et al., 1997

*The predominant subunit considered to be involved.

The P2X receptors are principally activated by ATP, with the other nucleotides having very low potency (McLaren et al., 1998; North and Barnard, 1997). The receptors can broadly be divided into three groups. The first group, which is comprised of receptors

consisting of $P2X_1$ and $P2X_3$ subunits, is almost equally activated by ATP and the stable analogue αβ-meATP. The $P2X_3$ is also activated by 2-MeSATP. The second group of $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors is not activated by αβ-meATP, but the $P2X_4$ and $P2X_5$ receptors are activated by ATPγS and 2-MeSATP. The third group is formed by the $P2X_7$ receptor (former P2Z), which is relatively insensitive to ATP, and sensitive to BzATP (MacKenzie et al., 1999).

3.2 P1 receptors

There are four cloned and pharmacologically characterized adenosine P1 receptors: A_1, A_{2A}, A_{2B} and A_3 (Ralevic and Burnstock, 1998). They all belong to the G protein-coupled receptor family. The A_1 receptor subtype couples to G_i/G_o proteins (Freissmuth et al., 1991; Munshi et al., 1991). The most widely recognized signaling pathway for the A_1 receptor is inhibition of adenylate cyclase which results in a decrease in the intracellular cAMP level (Van Calker et al., 1978). The receptor also affects other effector systems, i.e. activation of PLC leading to Ca^{2+} mobilization, inhibition of N-, P- and Q-type Ca^{2+} channels, and opening of K^+ channels (Ralevic and Burnstock, 1998). Both A_{2A} and A_{2B} receptors are positively coupled to adenylate cyclase via G_s proteins and thus increase intracellular cAMP levels (Ralevic and Burnstock, 1998). The A_{2B} receptors may also activate PLC (Yakel et al., 1993), possibly via G_q/G_{11} proteins (Feokistov and Biaggioni, 1997). The A_3 receptor couples to G_i and to a lesser extent to $G_q/_{11}$ proteins (Palmer et al., 1995). The receptor stimulates PLC and inhibits adenylate cyclase (Ralevic and Burnstock, 1998).

3.3 P1 and P2 receptors in thyroid cells

In thyroid cells, no molecular characterization of the different P1/P2 receptor subtypes present has been made. There are, however, functional studies suggesting the presence of several P1 and P2 receptor subtypes in thyroid cells. In human thyroid cells in primary culture, on the basis of intracellular Ca^{2+} experiments, the P_{2U} receptor is suggested to be the predominant P2 receptor type (Schöfl et al., 1995). A UTP-preferring P2 receptor mediating inhibition of Na^+ transport has been proposed in porcine thyroid cells (Bourke et al., 1999). In FRTL-5 cells, one or more receptor types for ATP have been suggested. They are thought to be coupled to distinct signal transduction pathways, that are the activation and inhibition of adenylate cyclase, and the activation of PLC (Okajima et al., 1989; Sato et al., 1992). In FRT thyroid cells, a cell line derived from FRTL-5 cells, the presence of a P2 receptor-operated Ca^{2+} channel has been suggested (Aloj et al., 1993). Of the P1 receptors, the A_1 and A_{2B} subtypes may function in FRTL-5 cells (Vainio et al., 2000; Nazarea et al., 1991). The A_{2B} subtype may be expressed at a lower level than A_1 (Vainio, 2000).

In thyroid as well as in other cell types, P1 receptors may interact with other G protein-coupled receptors, and this may result in potentiation or inhibition of their responses (Okajima et al., 1989; Ralevic and Burnstock, 1998; Tomura et al., 1997). In FRTL-5 cells it has been shown that (-)-N6-(2-phenylisopropyl)adenosine (PIA), an adenosine derivative, which binds to the A_1 subtype, may potentiate e.g. the

effects of thyrotropin (TSH) and noradrenaline, both of which activate the PLC-pathway (Sho et al., 1991). Also, potentiation of the P2 receptor-evoked Ca^{2+}-responses by adenosine or a derivative has been observed (Nazarea et al., 1991; Vainio and Törnquist, 2000). On the other hand, stimulation of the A_1 subtype inhibits the TSH-mediated increase in cAMP levels, which may indicate a physiological feedback mechanism (Vainio et al., 2000).

II THE THYROID GLAND

1. The thyroid gland and thyroid FRTL-5 cells

The thyroid gland is an endocrine organ located just below the larynx. It produces and secretes two hormones, thyroxine (T4) and triiodotyronine (T3). The thyroid gland, through the action of thyroid hormones, increases the overall metabolic rate of the body, has both general and specific effects on e.g. growth, carbohydrate and fat metabolism, the cardiovascular system, respiration, and on the central nervous system.

The thyroid gland consists mainly of thyrocytes (70%), which are arranged in follicles. Other cell types in the thyroid tissue are fibroblasts (10%) and endothelial cells of the capillaries (20%). The follicles are filled with a secretory substance called colloid. The major constituent of colloid is thyroglobulin, a glycoprotein synthetized by the thyroid cells. The follicles are encapsulated by small ramifications of a capillary network. Each of the capillary endothelial cells has hundreds of endothelial fenestrations which may allow the passage of nutrients, signaling factors, and products between the capillaries and the interstitial and cellular compartments (Köhrle, 1990). There are also scarce calcitonin-secreting parafollicular cells located in the periphery of the follicles (Dumont et al., 1992).

Thyroid cells concentrate iodide by actively pumping it from the extracellular fluid at the basal membrane. Iodide ions are oxidized to iodine by thyroid peroxidase and H_2O_2 at the apical membrane. Thyroglobulin, which contains tyrosine residues, is then iodinated by the thyroid peroxidase enzyme. Tyrosine is first iodinated to monoiodotyrosine and then to diiodotyrosine. Two diiodotyrosine residues become coupled to form T4, and a mono- and a diiodotyrosine couple to form T3. The thyroid hormones remain as part of the thyroglobulin molecule until secreted. Upon secretion, colloid is ingested in small portions by thyroid cells by endocytosis, and lysosomes immediately fuse with these vesicles. Lysosomal proteases cleave the peptide bonds between the iodinated residues and thyroglobulin, and T4 and T3 are released into the cytoplasm, from which they diffuse into the blood (Guyton and Hall, 1996).

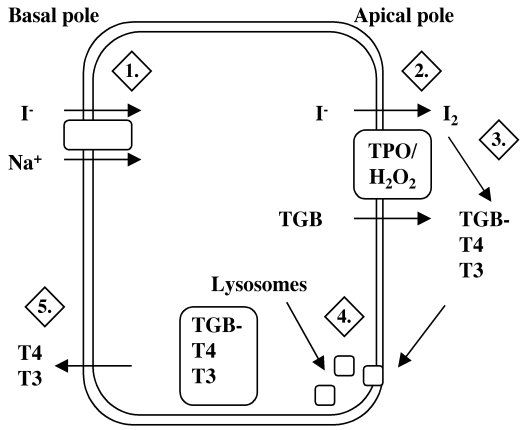

Figure 3. Thyroid cellular mechanism for synthesis of thyroid hormones. *1. Iodide is transported from the blood to the cell by an active pump. 2. Thyroid peroxidase enzyme (TPO) oxidizes iodide (I-) to iodine (I_2), which then 3. binds to thyroglobulin (TGB). 4. Droplets of colloid enter the cell by endocytosis, and merge with lysosomes, thus releasing free T4 and T3. 5. The lipid soluble T4 and T3 diffuse through the plasma membrane to enter the blood.*

The FRTL-5 cell line, derived from Fischer rat thyroid follicular cells (Ambesi-Impiombato et al., 1980), is a model for thyroid cells. The cells maintain many of the thyroidal functions, such as sensitivity to TSH, iodide concentration (Ambesi-Impiombato et al., 1980; Weiss et al., 1984), thyroglobulin synthesis and secretion (Avvedimento et al., 1984; Di Jeso et al., 1993). FRTL-5 cells grow as monolayers and do not form follicles. In addition, they do not synthetize or secrete thyroid hormones. However, when stimulated, they release iodide into the extracellular medium, and iodinate thyroglobulin (Corda et al., 1985; Marcocci et al., 1987).

2. Intracellular signal transduction systems

2.1 G proteins

Guanine nucleotide binding proteins (G proteins) comprise a superfamily of GTPases that couple plasma membrane receptors to their effector molecules. The heterotrimeric G proteins consist of α, β and γ subunits, and are attached to the cytoplasmic surface of the plasma membrane. The activated receptors promote G protein activation by stimulating the release of GDP from an α subunit, which then binds a GTP molecule causing dissociation of the α subunit from the βγ subunit. The dissociated subunits, α-GTP and free βγ, can then both bind to effector molecules and regulate their activity. The α subunit possesses intrinsic GTPase activity, and hydrolysis of GTP to GDP inactivates the G protein, and allows the subunits to reassociate (Wess, 1997).

Generally, the G proteins are named after their α subunits. The G_s proteins stimulate adenylate cyclase, whereas the G_i proteins mediate inhibition of

adenylate cyclase. The $G_{q/11}$ proteins regulate the activity of phospholipase C. The $G_{i/o}$ proteins are sensitive to inhibition by pertussis toxin (PTX), whereas G_s proteins are sensitive to cholera toxin. The Gα subunits G_s (Berg et al., 1994; Laugwitz et al., 1996), G_{i-2} (Laugwitz et al., 1996), G_{i1-3} (Berg et al., 1994), G_o, G_q (Laugwitz et al., 1996; Nikmo et al., 1999), G_{12} and G_{13} (Nikmo et al., 1999) have been identified in FRTL-5 cells.

2.2 Receptor tyrosine kinases

The receptor tyrosine kinases (RTKs) are a family of transmembrane polypeptides with a protein tyrosine kinase domain in their intracellular portion. RTKs are involved in the control of cell growth, differentiation and cell survival. Upon binding their corresponding agonist, of which many are dimeric molecules, the receptors undergo dimerization. This promotes transphosphorylation of the receptor subunits on tyrosine residues, and activation of the catalytic domains. The autophosphorylated dimers recruit cytoplasmic substrates that have an increased affinity for the phosphorylated tyrosine residues. A common feature of many substrate molecules is that they contain Src-homology (SH) domains, e.g. PLCγ, the phosphoinositide 3-kinase and small adaptor proteins such as Shc and Grb2. A number of intracellular signaling pathways have been shown to be activated by RTKs. These include the phosphoinositide 3-kinase, 70 kDa S6 kinase, mitogen-activated protein kinase, phospholipase Cγ, and the Jak/STAT pathways. A single type of RTK can elicit very different biological responses in different cell types (Fantl et al., 1993).

Among the RTK subfamilies there are insulin-like growth factor-I (IGF-I) and II (IGF-II) receptors and insulin receptors, of which the receptors for insulin and IGF-I are closely related (Fantl et al., 1993). Both insulin and IGF-I receptors have been found in thyroid cells, and there is much evidence that insulin and IGF-I may bind to each other's receptors (Eggo and Sheppard, 1994). Other RTKs in thyroid cells are the receptors for fibroblast growth factor (FGF) and epidermal growth factor (EGF) (Eggo and Sheppard, 1994).

2.3 Phospholipase C

Phospholipase C (PI-PLC) specifically hydrolyzes a plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in formation of two second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is released into the cytosol where it binds to specific receptors located in the endoplasmic reticulum (ER) and mediates the release of sequestered Ca^{2+} (Berridge, 1995). DAG remains in the plasma membrane where it binds to protein kinase C (PKC) and promotes its activation (Nishizuka, 1992).

In mammalian cells there are at least ten isoforms of PI-PLC which are divided into β, γ and δ subfamilies. The PLC β isoforms (β1, β2, β3, β4) have been shown to be activated by G protein-coupled receptors, whereas PLC γ is activated by binding to receptor-tyrosine kinases (Rhee and Bae, 1997). In FRTL-5 cells, the PLC β3 have been shown to be the dominant subtype (Laglia et al., 1996).

The α subunits of the G_q subfamily of G proteins have been shown to specifically activate the four PLC β isoforms (Morris and Scarlata, 1997; Singer et al., 1997). All of the PLC β isoforms may also be activated by the βγ subunits, but β2 and β3 are more sensitive than β1 and β4 (Clapham and Neer, 1993; Neer, 1995; Singer et al., 1997). The βγ subunits activate PLC with lower potency than the α subunits (Singer et al., 1997). The activation by βγ subunits suggests that any G protein may stimulate PLC activity. This is thought to be the mechanism by which the G_i proteins mediate PTX-sensitive stimulation of PLC activity (Singer et al., 1997).

2.4 Calcium signaling

Ionized free Ca^{2+} is the most widely used second messenger in organisms (Clapham, 1995). Ca^{2+} signaling involves a rise in the cytosolic Ca^{2+} concentration, which may vary in the magnitude, location and duration (Barritt, 1999). Prolonged elevated Ca^{2+} levels are harmful to cells and therefore cells tightly regulate $[Ca^{2+}]_i$. Resting free cytosolic $[Ca^{2+}]_i$ in cells is maintained at low concentrations (10-200 nM) by the action of intracellular and plasma membrane Ca^{2+} pumps, and by diverse intracellular Ca^{2+}-binding proteins, such as calreticulin and calsequestrin (Clapham, 1995). Cells store Ca^{2+} in the ER and in mitochondria (Hofer et al., 1998, Babcock et al., 1997). A large electrochemical gradient between the cell interior and exterior is the driving force for rapid increases in $[Ca^{2+}]_i$ in response to various signals. The gradient between the cytosol and ER ($[Ca^{2+}]$ in ER is 0.4 mM) is also large, thus enabling rapid Ca^{2+} fluxes also inside the cells.

2.4.1 Ca^{2+} release

Intracellular Ca^{2+} concentrations can be increased by two mechanisms: by release from internal Ca^{2+} stores, and by movement of Ca^{2+} into the cells from the extracellular space. Release from the internal stores in the ER is mediated by IP_3 acting on its receptors (IP_3R). The tetrameric IP_3R functions as a ligand-gated channel and activation by IP_3 leads to the rapid release of Ca^{2+} to the cytoplasm. The IP_3-mediated signal can increase $[Ca^{2+}]_i$ from ~100 nM to ~1 μM (Clapham, 1995). The N-terminal region of the receptor extends to the cytoplasm containing binding sites for e.g. IP_3, Ca^{2+} and ATP (Berridge, 1995). It is thought that during very strong receptor-mediated stimulation, the generated IP_3 may act directly to release Ca^{2+}. However, at normal physiological stimulation levels IP_3 may increase the sensitivity of the IP_3R to Ca^{2+}, resulting in a process called Ca^{2+} induced Ca^{2+} release (CICR). This enables Ca^{2+} released from one IP_3R to excite its neighbors, which causes spontaneous calcium spikes and waves. The Ca^{2+} release is usually transient and fully deactivates within tens of seconds (Parekh and Penner, 1997).

2.4.2 Ca^{2+} influx

Ca^{2+} influx from the extracellular space occurs through channels in the plasma membrane. The Ca^{2+} influx pathways may broadly be divided into voltage-dependent and voltage-independent ones. Voltage-dependent Ca^{2+} channels are

expressed in exitable cells, and are affected by the membrane potential. The voltage-independent influx pathways may consist of numerous channel subtypes, which are activated by different mechanisms. These influx pathways include 1) ligand-gated non-specific cation channels, 2) second messenger-operated channels which are activated by mobile intracellular messengers such as cyclic GMP (cGMP), cyclic AMP (cAMP), IP_3, IP_4, and arachidonic acid or its metabolites, 3) a cytoplasmic Ca^{2+} increase, 4) channels activated by a trimeric G protein, and 5) store-operated Ca^{2+} channels (SOCs; formely called the capacitative Ca^{2+} entry) (Putney and Bird, 1993).

The store-operated Ca^{2+} influx pathway is coupled to the Ca^{2+} content of the store such that the empty store transmits a signal to the plasma membrane to open Ca^{2+} channels (Putney, 1999). The best characterized store-operated Ca^{2+} current is the Ca^{2+}-release-activated Ca^{2+} current (I_{CRAC}) (Hoth and Penner, 1992). The current is activated equally by dialysis with IP_3 via a patch pipette, receptor stimulation or by using thapsigargin, a sesquiterpene lactone which inhibits the ER Ca^{2+}ATPase pump irreversibly (Parekh et al., 1997). It seems likely that refilling of the stores turns off the Ca^{2+} influx, although this has been shown only in a few investigations. However, the mechanism of the inactivation of influx has not been revealed (Parekh and Penner, 1997).

There are two models to explain how the depleted store provokes entry of extracellular Ca^{2+}. In the first model, the empty store releases a diffusible factor which then serves as a messenger to activate the entry pathway. Proposed messengers include Ca^{2+} influx factor (CIF), cGMP, small G proteins, tyrosine kinase, arachidonic acid or its metabolites (Barritt, 1999). In the second model, the emptying of the store induces a reversible coupling of ER with the plasma membrane to activate SOCs. The channel is activated and maintained by contact with the IP_3R, which are moved to the vicinity of SOCs (Berridge, 1995; Ma et al., 2000). In most cells, Ca^{2+} entry after stimulation with agonists can be explained by activation of SOCs, but neither the mechanism nor the molecular nature of the plasma membrane channels mediating Ca^{2+} entry has been definitively established. The possibility that combinations of some or all of these elements regulating the Ca^{2+} entry cannot be excluded. Also, cell type specific mechanisms may also exist.

In many cell types agonists may open more than one type of voltage-independent Ca^{2+} entry pathways (Barritt, 1999). In FRTL-5 cells there are no voltage-operated Ca^{2+} channels (Törnquist, unpublished observation). However, a second-messenger-operated (Törnquist and Ekokoski, 1995), as well as store-operated (Törnquist, 1992) Ca^{2+} entry pathways have been shown to be activated in response to ATP in these cells.

2.4.3 Ca^{2+} as a regulator of cellular functions

Ca^{2+} can alter a tremendous number of cellular processes, e.g. contraction, secretion, metabolism, cell survival, and proliferation (Parekh and Penner, 1997). In thyroid cells, Ca^{2+} signaling has roles in various functions, such as thyroid hormone synthesis through generation of H_2O_2 and iodide efflux (Raspé and Dumont, 1994), gene expression (Saji et al., 1991), and cell proliferation (Burch et al., 1986; Takada et al., 1990).

Ca^{2+} acts by affecting the activity of many intracellular targets, such as protein kinases, phospholipases, phosphatases and ion channels (Clapham, 1995). Ca^{2+} may modify protein function by directly binding to a specific region or domain. For example, enzymes such as protein kinase C, PLC and PLA_2 all contain a Ca^{2+}-binding C2-domain, which target them to substrate membranes (Nalefski et al., 1994; Ron and Kazanietz, 1999). Ca^{2+} ions may also act through the Ca^{2+}-receptor proteins, e.g. calmodulin. The Ca^{2+}-calmodulin complex can modulate functions of different target proteins, including the Ca^{2+}/calmodulin-dependent (CaM) protein kinases (Schulman and Braun, 1999).

In proliferating cells, Ca^{2+} and calmodulin have a regulatory role at the G_1/S boundary of the cell cycle, and it is thought that calmodulin is also required for re-entry of G_0 cells into the cycle, in transition from G_2 to mitosis and in the anaphase-metaphase transition (Santella, 1998). Very likely, many of the actions of calmodulin are mediated by CaM kinase II. Although not much is known about the substrates of CaM kinase II in the cell cycle, one possible target could be the tyrosine phosphatase cdc25 (Means et al., 1999). Ca^{2+} and calmodulin may also regulate gene expression. Both immediate early and late genes have been shown to be Ca^{2+}-inducible (Carafoli and Klee 1999). Ca^{2+} may also affect transcription elongation, the stability of mRNA and its translation (Santella and Bolsover, 1999).

2.5 Protein kinase C

Protein kinase C is a family of serine/threonine kinases which takes part in many cellular functions including receptor functions, ion transport, metabolism and cell proliferation (Toker, 1998). There are several PKC isoforms, which are divided into three subgroups: conventional PKCs (cPKCs: α, β1, β2, γ), which are regulated by DAG, phosphatidylserine (PS) and Ca^{2+}; novel PKCs (nPKCs: δ, ε, η, θ) which are regulated by DAG and PS but are not dependent on Ca^{2+}; and atypical PKCs (aPKCs: ζ, λ) whose regulation has not been clearly established, but are phorbol ester and Ca^{2+}-insensitive (Newton, 1997). In FRTL-5 cells, the PKC isoforms α, βI, βII, γ, δ, ε, ζ and η have been identified (Wang et al., 1996; Wang et al., 1995).

The activation of PKC includes association of the enzyme with PS, which is controlled by Ca^{2+} in the case of the cPKCs. The membrane-associated enzyme can then bind DAG, which leads to conformational change and activation of PKC. The DAG analogues, phorbol esters, such as 12-myristate 13-acetate (PMA), can bind to and activate cPKCs and most nPKC isoforms but not aPKCs (Ron and Kazanietz, 1999). Free fatty acids have been shown to enhance the DAG induced activation of some PKC isoforms (Khan et al., 1993; Shinomura et al., 1991). Recent studies indicate that PKCs may also become phosphorylated by upstream kinases on residues which are usually required for protein kinase activity (Toker, 1998). Phosphoinositide-dependent protein kinase-1 may be the universal PKC upstream kinase (Toker, 1998).

PKC phosphorylates a number of substrates, which are both membrane-associated and soluble targets, indicating that the substrates may diffuse to PKC, and/or PKC may itself relocate. The substrates include receptors, other kinases, ion channels and cytoskeletal proteins (Toker, 1998). A well known target is the Raf-1 kinase of the MAP kinase cascade (Widmann et al., 1999). PKC may also modulate

agonist-evoked Ca^{2+} signals, probably by affecting receptor-G protein coupling or by activating receptor kinases, or by affecting I_{CRAC} (Oppermann et al., 1996; Parekh and Penner, 1995; Pronin and Benovic, 1997).

2.6 Phospholipase A_2

The A_2 phospholipases (PLA_2s) are a family of enzymes that catalyze the hydrolysis of membrane phospholipids at the *sn*-2-position, resulting in liberation of free fatty acids and lysophospholipids (Leslie, 1997). The most common fatty acid present in mammalian phospholipids is the 20-carbon unsaturated arachidonic acid (AA) (Moncada and Higgs, 1988). The major sources of AA are phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Exton, 1994). PLA_2 activation has been implicated in diverse cellular responses, such as signal transduction, host defense, proliferation, blood coagulation and membrane remodeling (Murakami et al., 1997). In human thyroid cells and in FRTL-5 cells, the activation of PLA_2 and the concomitant release of AA are involved in different cellular functions, such as regulation of iodide efflux and proliferation (Burch et al., 1986; Di Paola et al., 1997; Marcocci et al., 1987; Smallridge and Gist, 1994).

PLA_2 enzymes are divided into several groups based on their structure and enzymatic characteristics (Dennis, 1997). Among these are the small (13-15 kDa) secreted forms of PLA_2 ($sPLA_2$s), and high molecular mass (80-85 kDa) PLA_2s, which are cytosolic enzymes and lack sequence homology with the secreted forms of PLA_2 (Clark et al., 1991). Although the $sPLA_2$s and $cPLA_2$ catalyze the same reaction, their catalytic mechanisms are different: millimolar Ca^{2+} is necessary for $sPLA_2$ catalytic activity, whereas submicromolar Ca^{2+} is essential for $cPLA_2$ translocation to membranes, rather than for catalytic activity (Leslie, 1997). Furthermore, $cPLA_2$ can selectively liberate AA from membrane phospholipids (Murakami et al., 1997), whereas $sPLA_2$s does not show any preference for fatty acid at the *sn*-2 position (Mayer and Marshall, 1993). Recent investigations have revealed a coordinated role for some of the $sPLA_2$ and $cPLA_2$ in the release of AA, at least in hematopoietic cells (Balsinde and Dennis, 1996). In this system, $cPLA_2$ activation precedes the subsequent activation of $sPLA_2$, which is responsible for the bulk release of AA. However, also in this system, the $cPLA_2$ has a key regulatory role.

A novel group of PLA_2s are the Ca^{2+}-independent forms of the enzyme ($iPLA_2$s), with molecular masses ranging from 29 to 85 kDa. The $iPLA_2$s are also capable of releasing AA at least in aortic smooth muscle cells (Murakami et al., 1997). There are conflicting reports of the role of $iPLA_2$ in the cells, some describing a role in signal transduction, others suggesting an involvement in membrane phospholipid remodeling (Murakami et al., 1997).

2.6.1 Activation of $cPLA_2$

The $cPLA_2$ may be activated by many growth factors, cytokines, neurotransmitters, hormones and other extracellular signals acting through G protein-coupled receptors, and through receptor tyrosine kinases (Murakami et al., 1997). The activation process of PLA_2 is complex and not completely understood.

Many G protein-coupled receptors activate both PLC and PLA_2 (Cockroft and Stutchfield, 1989), and studies have suggested that the activation of PLA_2 is a consequence of PLC activation. PLA_2 can also be activated independently of PLC through separate G proteins (Burch et al., 1986; Smallridge and Gist, 1994). In some studies a direct activation of PLA_2 by a GTP-binding protein has been suggested (Ando et al., 1992; Xing and Mattera, 1992). However, this type of activation has not finally been established, and some studies suggest an effector between a G protein and the PLA_2 enzyme (Burch et al., 1986; Winitz et al., 1994). Recent studies suggest a role for a $G\alpha_i$-type protein in the regulation of PLA_2 independently of phosphorylation and Ca^{2+} levels (Burke et al., 1997; Murray-Whelan et al., 1995).

Agents that increase intracellular Ca^{2+} concentration have been shown to cause AA release, suggesting that an increase in $[Ca^{2+}]_i$ is essential for the activation of $cPLA_2$ (Kramer and Sharp, 1997). In vitro, purified $cPLA_2$ is active at Ca^{2+} concentrations of 0.1 - 1 μM (Piomelli, 1993). The $cPLA_2$ enzyme contains a Ca^{2+}-dependent phospholipid binding domain in the N-terminal portion (Nalefski et al., 1994). This kind of domain is also found in PKC and PLC, which have been demonstrated to translocate to phospholipid membranes in a Ca^{2+}-dependent manner (Murakami et al., 1997). The $cPLA_2$ translocates to membranes in the presence of submicromolar concentrations of Ca^{2+}, the nuclear envelope and endoplasmic reticulum being the primary target membranes (Clark et al., 1991; Schievella et al., 1995; Yoshihara and Watanabe, 1990). The Ca^{2+}-induced translocation may take place over a small distance not even visible at the ultrastructural level. It has been suggested that the translocation should be considered as a tighter interaction of the enzyme with the membranes in stimulated cells (Bunt et al., 1997).

$cPLA_2$ has been shown to have multiple phosphorylation sites, including Ser-437, Ser-454, Ser-505, Ser-727 (de Carvalho et al., 1996), of these the Ser-505 is thought to be crucial for activation (Qiu et al., 1993; Rao et al., 1994). $cPLA_2$ have been demonstrated to be a substrate for several kinases, e.g. mitogen-activated protein kinase (MAPK), p38 kinase, PKC, CaM kinase II and PKA (Leslie, 1997). The phosphorylation is an independent phenomenon of the Ca^{2+} induced translocation from cytosol to membranes. Furthermore, phosphorylation per se is not sufficient for $cPLA_2$ activation in intact cells (Murakami et al., 1997), but phosphorylation, nevertheless, regulates $cPLA_2$ activity. A scheme for PLA_2 activation is presented in Figure 4.

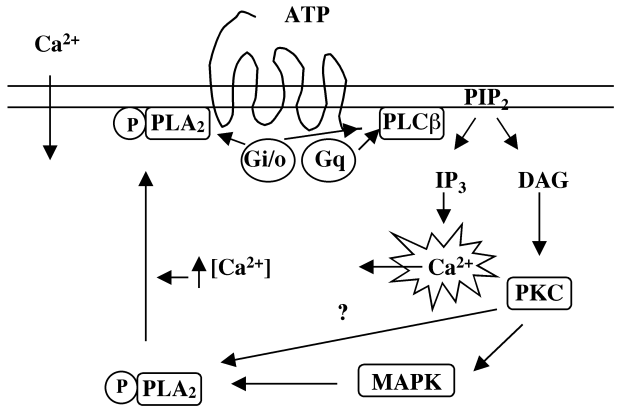

Figure 4. The mechanisms of activation of cPLA$_2$. *Stimulation of a G protein-coupled receptor may activate both G_q and G_i proteins. Both of these may activate PLC, which then leads to activation of PLA_2 through formation of IP_3 and DAG. IP_3 releases Ca^{2+} from intracellular stores and the influx of extracellular Ca^{2+} is also activated. The rise in cytosolic Ca^{2+} induces translocation of PLA_2 to the substrate membranes. DAG activates PKC, which either directly, or indirectly through the MAP kinase cascade, phosphorylates PLA_2. PLA_2 may also be activated independently of PLC, probably through a G_i protein.*

2.6.2 Arachidonic acid metabolism

In addition to PLA_2, AA may also be released by the sequential action of PLC and diacylglyserol lipase. After release, free AA may diffuse out of the cell, it may be reincorporated into phospholipids, or it can be converted to potent lipid mediators, eicosanoids. The eicosanoids are formed by cyclooxygenase, lipoxygenase and cytochrome P-450 enzymes (Fitzpatrick and Murphy, 1989; Moncada and Higgs, 1988). The cyclooxygenase pathway gives rise to stable prostaglandins e.g. PGE_2, PGD_2, PGI_2, PGF_2, prostacyclins and thromboxanes. Leukotrienes A_4, B_4, C_4, D_4, E_4 and 5-, 12- and 15-hydroxyeicosatetraenoic acids are produced by the lipoxygenase pathway. Cis-epoxyeicosatrienoic acids 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET are formed via the epoxygenase pathway, where the cytochrome P-450 monooxygenase enzyme catalyzes the oxidation of AA and conversion into epoxyeicosatrienoic acids, which are then hydrolyzed to corresponding diols by epoxide hydrolase (Fitzpatrick and Murphy, 1989; Piomelli, 1993). Other P450 metabolites are hydroxyeicosatetraenoic acids such as 12-HETE and 15-HETE (Fitzpatrick and Murphy, 1989).

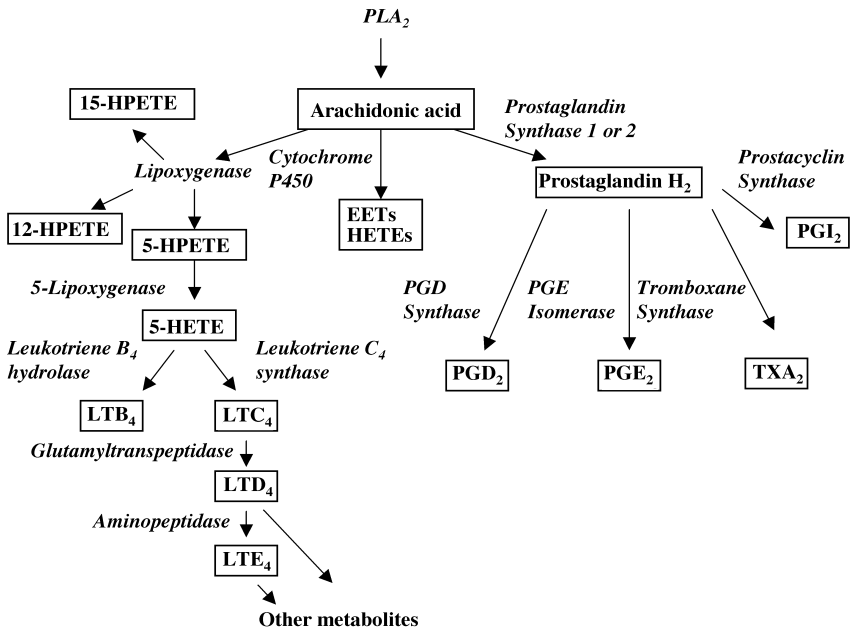

Figure 5. Overview of AA metabolism. *The enzymes are given in italics.*

The eicosanoids may act both as intracellular second messengers and as local mediators, and have a variety of effects both on normal and pathophysiological processes. Besides their action in inflammation, some prostaglandins may increase cell proliferation in a variety of cells, including FRTL-5 cells (Burch et al., 1986). Leukotrienes and cytochrome P-450-derived metabolites may take part in the regulation of cell proliferation (Chen et al., 1998; Harris et al., 1990), but they may also activate and inhibit different ion channels. For example, EETs are considered important regulators of vascular tone (Imig et al., 2000), by activating Ca^{2+}-dependent K^+-channels (Baron et al., 1997) or by enhancing Ca^{2+} influx through voltage-dependent Ca^{2+}-channels (Fang et al., 1999). In cardiac L-type Ca^{2+} channels, EETs act as inhibitors (Chen et al., 1999). Leukotrienes and EETs may also mobilize Ca^{2+} on their own (Luscinskas et al., 1990; Snyder et al., 1986), or enhance the agonist-evoked Ca^{2+} release (Force et al., 1991). They have also been reported to regulate the store-operated Ca^{2+} influx (Hoebel et al., 1997; Mombouli et al., 1999; Rzigalinski et al., 1999).

2.6.3 Free fatty acids

PLA_2 also cleaves other polyunsaturated fatty acids, especially those with three *cis* double bonds between carbons 5 and 6, 8 and 9, and 11 and 12 (Murakami et al., 1997). It has been reported that the order of preference of *sn*-2 fatty acids for PLA_2 is arachinoyl > linolenoyl > linoleoyl > oleoyl > palmitoleyl (Murakami et al., 1997). Free fatty acid concentration may thus locally increase after agonist stimulation.

A variety of fatty acids regulate the activity of specific ion channels. In exitable cells they may activate or inhibit voltage-operated Ca^{2+} channels (Huang et

al., 1992; Shimada and Somlyo, 1992). AA has been shown to activate different types of K^+ channels in cardiac cells and in smooth muscle cells (Kim and Clapham, 1989; Ordway et al., 1989). Polyunsaturated fatty acids block Na^+ channels in neonatal rat cardiac myocytes (Kang and Leaf, 1996). A recent report has shown that polyunsaturated fatty acids activate the Drosophila light-sensitive Ca^{2+} channels TRP and TRPL (Chyb et al., 1999). AA and other unsaturated fatty acids have been reported to promote Ca^{2+} entry (Alonso et al., 1990), or inhibit store-operated calcium influx (Gamberucci et al., 1997). They are also reported to enhance Ca^{2+} extrusion after agonist stimulation, possibly by activating Ca^{2+}ATPase (Randriamampita and Trautmann, 1990). They may affect different cellular functions such as adenylate and guanylate cyclase activation, Na^+/K^+-ATPase activity, and activation of PKC (Piomelli, 1993; Shirai et al., 1998).

2.7 The cAMP-dependent pathway

In thyroid cells the cyclic AMP-protein kinase A pathway is one of the main signaling systems. cAMP is formed from ATP by a membrane-bound enzyme, adenylate cyclase. cAMP activates protein kinase A (PKA), a serine/threonine kinase, which phosphorylates other enzymes and proteins. Depending on the cell type, activation of the cAMP-pathway may result in cell proliferation, differentiation or growth arrest (McKenzie and Pouysségur, 1996).

2.7.1 Adenylate cyclases

Through its interaction with receptors and G proteins, adenylate cyclase activity is regulated by hormones, neurotransmitters and other regulatory molecules (Hanoune et al., 1997). Today nine isoforms of adenylate cyclases have been cloned and characterized from mammalian cells (Ishikawa and Homcy, 1997). All of the isoforms are activated by $G_{s\alpha}$ protein, although probably not equally effectively. Likewise, inhibition by the inhibitory G protein, $G_{i\alpha}$, may not be equal for all the isoforms and may depend on the type of the isoform and the activator of the enzyme (Hanoune et al., 1997). Other potential regulators are $G_{\beta\gamma}$ subunits, Ca^{2+}/calmodulin, divalent metal cations and other kinases. Forskolin is a natural diterpene that is able to activate all isoforms (Hanoune et al., 1997).

2.7.2 Protein kinase A

cAMP activates PKA by binding to the regulatory subunits of the tetrameric enzyme, and facilitates the dissociation of the dimeric catalytic subunit from the regulatory one. The activated free catalytic subunit not only phosphorylates cytoplasmic substrates, but can also migrate into the nucleus, where it can phosphorylate proteins important for the regulation of gene transcription, such as the cAMP response element binding protein (CREB). Free cAMP, but not cAMP bound to the regulatory unit, is rapidly inactivated by cyclic nucleotide phosphodiesterases (PDEs). The catalytic subunits are inactivated by reassociation with regulatory dimers (Hanoune et al., 1997).

2.8 The MAP kinase pathway

The mitogen-activated protein kinases (MAP kinase) comprise several parallel kinase cascades, which are activated by various extracellular stimuli and mediate diverse cellular responses depending on the cell context. There are at least three subgroups of MAP kinases in mammalian cells, differing in substrate specificity and regulation. The core unit of a MAP kinase pathway is a three-member protein kinase cascade. The MAP kinase is the terminal serine/threonine kinase, which is phosphorylated and activated by MAP kinase kinase (MEK) on tyrosine and threonine residues. MEK is in turn phosphorylated by MAP kinase kinase kinases, such as Raf or MEKK, on serine/threonine residues (Widmann et al., 1999).

The first MAP kinase cascade to be characterized in detail was the extracellular signal regulated kinase (ERK) pathway (Cobb et al., 1991). In the literature, the names MAP kinase and ERK are used interchangeably. In the following, this ERK pathway will be referred to as MAP kinase pathway. This pathway regulates different cellular functions such as growth, proliferation and differentiation. MAP kinase occur as two different isoforms p42 and p44 kDa. MAP kinase has been established as an especially important regulator of transcription factors, such as Elk-1, Ets-1 and c-Myc (Widmann et al., 1999). Several cytoplasmic targets have also been implicated, including cytoskeletal proteins (e.g. tau) and microtubule-associated proteins, membrane proteins such as EGF and NGF receptors, $cPLA_2$, and protein tyrosine phosphatase 2C (Widmann et al., 1999).

2.8.1 Coupling of cell surface receptors to the MAP (ERK) kinase cascade

Signaling from the receptor tyrosine kinases (RTKs) to MAP kinase is well established (Fig. 6). A general activation scheme involves the activation of a RTK, which serves as a docking site to recruit SH2 domain containing adaptor proteins, such as Shc and growth factor receptor-bound protein 2 (Grb2). This promotes binding of the guanine nucleotide exchange protein Sos to the SH3 domain of Grb2, leading to recruitment of the GTP binding protein Ras to the signaling complex (Widmann et al., 1999). Ras then binds Raf, a MAPKKK, targeting it to the plasma membrane where Raf activity is increased by a yet unclear, and apparently complex multistep mechanism (Morrison and Cutler, 1997). Raf then phosphorylates MEK 1 and 2 (MAPKKs), thereby activating them. The activated MEK 1,2 then phosphorylate ERK1/ERK2 on threonine and tyrosine residues (Anderson et al., 1990).

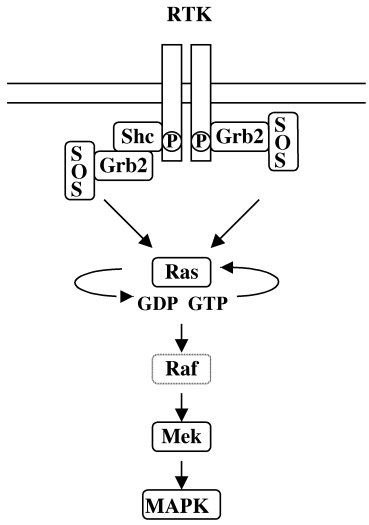

Figure 6. Receptor tyrosine kinase-mediated activation of the MAP kinase cascade. *Binding of the ligand to a receptor tyrosine kinase (RTK) leads to receptor dimerization and autophosphorylation. The receptor then serves as a docking site for adaptor molecules such as Grb2 or Shc. These molecules, either singly or in combinations, link the receptor to the guanine nucleotide exchange protein Sos, which catalyzes GDP release and GTP binding to Ras. Ras then binds Raf and recruits it to the plasma membrane, where the activity of Raf is increased. Raf then phosphorylates Mek1,2 , which in turn phosphorylates the MAP kinases.*

The activation of the MAP kinase cascade by the GPCRs is less clear, and apparently more complex than the RTK signaling system (Figure 7). The cascade becomes activated through $G_{i/o}$ and $G_{q/11}$ proteins (Hawes et al., 1995), and depending on the G protein and also cell type, the signal transduction pathways leading to the MAP kinase cascade may vary (Della Rocca et al., 1997). The α subunit of the $G_{q/11}$ proteins may mediate activation of MAP kinase (Della Rocca et al., 1997), whereas the $\beta\gamma$ subunit may mediate the G_i protein induced activation of MAP kinase (Crespo et al., 1995). Downstream mediators of $\beta\gamma$ might be the tyrosine kinases of the Src-family and the adaptor protein Shc. In contrast, the $G_{q/11}$ coupled receptor activates MAP kinase via a PKC-dependent pathway, which does not involve $\beta\gamma$ and Ras (Hawes et al., 1995). Activation of MAP kinase by PKC activators, such as phorbol esters, have been reported, and this may occur through both Ras-dependent and Ras-independent pathways (Gutkind, 1998). Recent studies have shown that the GPCR-induced activation of MAP kinase requires RTK transactivation. However the mechanism is poorly understood, although a regulatory role for $G\beta\gamma$ subunits, Src kinases, Ca^{2+} and PKC have been suggested (Luttrell et al., 1999; Widmann et al., 1999).

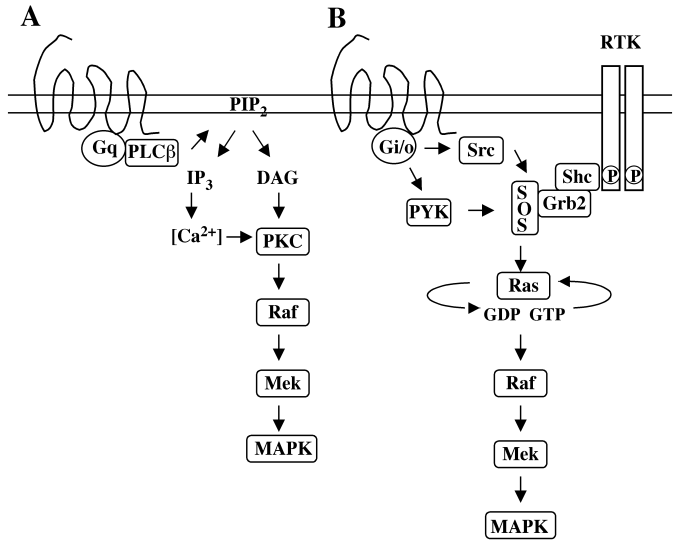

Figure 7. G_q and G_i protein-mediated activation of the MAP kinase cascade. *A) G_q protein activates PLCβ, which hydrolyzes plasma membrane phosphatidylinositol PIP_2, leading to formation of IP_3 and DAG. IP_3 releases Ca^{2+} from intracellular stores, which together with DAG promotes the activation of PKC. PKC then phosphorylates Raf and activates the concomitant MAP kinase cascade. B) Activation of the MAP kinase cascade by a G_i protein most likely occurs through the βγ subunits. A ligand-independent transactivation of a RTK may follow, and the activated RTK then serves as a structural scaffold for assembly of the signaling molecule complex which resembles that formed when the RTK is activated by its own ligand. The mechanism of transactivation is presently not known, although it may involve a Src-family tyrosine kinase or a nonreceptor tyrosine kinase PYK2.*

There have been a number of reports that increasing intracellular cAMP inhibits MAPK activity, via phosphorylation of Raf-1 by protein kinase A. This apparently leads to reduction in the affinity of Raf for Ras (Häfner et al., 1994), and decreases the activity of Raf.

2.9 Immediate early genes c-fos and c-jun

The primary genomic response to a variety of external stimuli is the rapid induction of a set of genes, named immediate-early response genes (IEGs) or primary response genes (Herschmann, 1991). IEGs are functionally defined by their rapid and transient induction in response to a variety of treatments, including growth factor stimulation. IEGs encode transcription factors, which bind to specific target DNA sequences and affect downstream gene expression. Their activation has strongly been related to mitogenesis, but they are also induced in postmitotic cells by various signals.

There are several transcription factor families of which the transcription factor AP-1 is a collection of dimers composed of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, Fra-1, Fra-2, FosB) and ATF families of basic region-leucine zipper DNA binding proteins (Ransone and Verma, 1990). These dimers bind to a common cis acting

element known as the TRE (TPA response element) or the AP-1 site (Angel and Karin, 1991). Jun proteins can form homodimers, and any of the Jun proteins can form heterodimers with c-Fos and Fra-1 proteins (Karin, 1995), whereas Fos proteins can not form homodimers. In many cells stimulation of proliferation is accompanied by an increased expression of c-fos and c-jun. The participation of c-Fos and c-Jun in proliferation has been intensively investigated. However, no unified roles for different mitogens has been found, since some mitogens induce proliferation without inducing AP-1 activity (Columbano et al., 1997; Menegazzi et al., 1997). Also their target genes are largely unknown, although some have been recently identified, such as cyclin A and D (Brown et al., 1998; Sylvester et al., 1998).

In quiescent cells the expression level of c-Fos and c-Jun is low or nondetectable. Enhanced expression of c-fos can be mediated by multiple signal transduction pathways involving cAMP, Ca^{2+}, and PKC, and MAPK (Herschmann, 1991; Karin, 1995). A serum response element (SRE) in the c-fos gene promoter mediates c-fos induction by growth factors and cytokines. CRE or cAMP responding element mediates the effects of Ca^{2+} and cAMP on c-fos. The c-jun promoter is simpler and most of its inducers operate through one major cis element, TRE (Karin, 1995). An increase in $[Ca^{2+}]_i$ has been considered important for IEG expression in several cell types (Cesnjaj et al., 1994; Negulescu et al., 1994; Roche and Prentki, 1994; Werlen et al., 1993). Also, phosphorylation at specific sites enhances the activity of c-Fos and c-Jun (Karin, 1995).

3. Regulation of thyroid cells

The thyroid functions include events that are involved in the synthesis of thyroid hormones and their secretion. The hormone synthesis requires uptake and concentration of iodide, synthesis of thyroglobulin, and an oxidation system to iodinate tyrosyl groups in thyroglobulin and couple them into iodothyronines (Vassart and Dumont, 1992). Thyrotropin (TSH), secreted from the anterior pituitary, is the main regulator of thyroid functions (Dumont et al., 1992). Together with thyroid hormones it forms a negative feedback mechanism: the thyroid cells secrete thyroid hormones that inhibit the secretion of TSH from the pituitary. Whenever the secretion of thyroid hormones decreases, TSH secretion increases, which causes an activation of thyroid functions and growth (Vassart and Dumont, 1992). Various hormones, growth factors and cytokines also regulate the thyroid functions. The thyroid gland is under regulation of sympathetic and parasympathetic nerves, and the nerve terminals also reach the thyroid follicles (Amenta et al., 1978; Melander et al., 1975; Van Sande et al., 1980).

3.1 Regulation of function

TSH regulates the sodium-dependent influx of iodide into thyrocytes in the basal membrane by a cAMP-dependent pathway (Weiss et al., 1984). The production of H_2O_2, an essential oxidant in the peroxidase reactions catalyzed by thyroid peroxidase (Kimura et al., 1995; Nakamura and Ohtaki, 1990), is stimulated in response to activation of the PLC/Ca^{2+}-pathway in all species studied (Björkman and

Ekholm, 1992; Raspé et al., 1991; Raspé and Dumont, 1994). It is activated by the cAMP pathway in only a few species, including rat FRTL-5 cells (Björkman and Ekholm, 1992; Roger et al., 1997). No evidence has been found for acute second messenger regulation of the thyroid peroxidase; the activity depends only on its expression level and the availability of substrates (Vassart and Dumont, 1992). Iodide efflux, which in FRTL-5 cells is proposed to be related to the *in vivo* process by which iodide is transported to the lumen and coupled to thyroglobulin, is stimulated by the PLC-pathway, but not by the cAMP-pathway (Weiss et al., 1984). Studies have shown that in addition to PLC, activation of the PLA_2 pathway is essential in the activation of iodide efflux in FRTL-5 cells (Marcocci et al., 1987; Smallridge and Gist, 1994).

3.2 Regulation of growth

In a developing thyroid, the proliferation of thyrocytes is fast, and dependent on TSH (Dumont et al., 1992). In an adult organism, the thyroid gland cells have a slow constant turnover. However, the cells do retain the capability to grow by hypertrophy and proliferation in response to a stimulus (Dumont et al., 1992). In general, the growth of thyroid cells, including FRTL-5 cells, is regulated by TSH (Dumont et al., 1992). This effect has mainly been attributed to the TSH-evoked elevation of cAMP and the activation of PKA. However, in WRT cells, another rat thyroid cell line, TSH is mitogenic also through a cAMP-independent pathway, which is dependent on Ras, but not on Raf-1 or MEK (al-Alawi et al., 1995; Kupperman et al., 1993).

The thyroid produces autocrine growth factors, e.g. IGF-I, IGF-II, EGF and FGF (Eggo and Sheppard, 1994). Depending on the species, these growth factors have been shown to be mitogenic or comitogenic (Deleu et al., 1999; Dumont et al., 1992). The effect of insulin mainly involves the IGF-I receptor in dog thyrocytes, however at high (5 μg/ml) concentrations it activates both its own receptor and the IGF-I receptor (Deleu et al., 1999). It has been shown *in vitro* and *in vivo* that the action of TSH is in part dependent on other hormones, e.g. insulin and IGF-I and II (Cheung et al., 1996; Deleu et al., 1999; Taton and Dumont, 1995). The synergistic effect of TSH and insulin/IGF-I has been suggested to involve elevation of 1,2-diacylglycerol, and thus activation of PKC in FRTL-5 cells (Brenner-Gati et al., 1988). It has recently been shown that TSH and IGF-I or insulin, separately control DNA-synthesis and cell growth, and complement each other in mitogenesis (Deleu et al., 1999). At least in dog thyrocytes, HGF is the only growth factor which elicits all the events required for cell proliferation, i.e. protein accumulation and DNA-synthesis (Deleu et al., 1999).

The activators of the PLC pathway in dog thyroid cells have been shown to have no or only marginal mitogenic activity (Roger et al., 1997). However, a role for Ca^{2+} in FRTL-5 cell proliferation has been shown (Brenner-Gati et al., 1988). Also, activation of PKC by phorbol esters stimulates thyroid cell proliferation (Lombardi et al., 1988; Roger et al., 1997).

Iodide exerts an endogenous negative feedback control on thyrocyte growth (Dumont et al., 1992). Iodide deficiency increases the sensitivity of the thyroid to the goitrogenic effects of TSH. Iodide has been shown in several thyroid culture systems to regulate transforming growth factor β mRNA levels, with induction at

iodide concentrations inhibitory to thyroid growth and function (Eggo and Sheppard, 1994). Another growth regulating factor in the thyrocytes is adenosine, the effect of which is dependent on other growth factors such as insulin and TSH (Moses et al., 1989; Vainio et al., 1997).

AIMS OF THE STUDY

The aims of the study were to investigate the actions of extracellular ATP in FRTL-5 thyroid cells by examining the ATP-activated signal transduction pathways. Specifically, the following themes were examined:

- The regulation of PLA_2 activity in response to ATP (I)
- The regulation of ATP-evoked Ca^{2+}-fluxes by arachidonic acid metabolites (II)
- The effects of fatty acids on ATP-induced Ca^{2+} signals (III)
- Determination of P2 receptors in FRTL- 5 cells (V)
- The effect of ATP and other nucleotides on FRTL-5 cell DNA-synthesis and immediate early gene expression (IV, V)

METHODS

1. Cell culture

Several batches of the rat thyroid FRTL-5 cells were used in this study. The cells, originally obtained from the Interthyr Foundation, were kindly donated by Dr. Egil Haug (Aakers Hospital, Oslo, Norway) and by Dr. Leonard Kohn (NIH, MD, USA). The cells were cultured in Coon's modified Ham's F 12 medium supplemented with 5% new born calf serum and six hormones (6H medium, Ambesi-Impiombato et al., 1980): insulin, 1 µg/ml; transferrin, 5 µg/ml; hydrocortisone, 10 nM; the tripeptide gly-L-his-L- lys, 10 ng/ml; TSH, 0.3 mU/ml; somatostatin, 10 ng/ml. The cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The medium was changed every 2-3 days.

2. Measurement of intracellular Ca^{2+} concentrations

Cells grown in 6H medium were harvested and loaded with 1 µM Fura 2/AM or with 2 µM Fluo-3 in Hepes buffered saline solution (HBSS: NaCl, 118 mM; KCl, 4.6 mM; $CaCl_2$, 0.4 mM; glucose, 5 mM; Hepes, 20 mM; pH 7.2) for 30 min at 37°C. Fluorescence was monitored with a Hitachi F2000 fluorimeter. The excitation wavelengths were 340 nm and 380 nm and the emission wavelength was 510 nm for Fura 2. When using Fluo-3, the exitation wavelength was 506 nm and the emission wavelength 526 nm. $[Ca^{2+}]_i$ was calculated as described (Grynkiewicz et al., 1985) with a computer program using the K_d-value of 224 nM for Fura 2. For Fluo-3 the $[Ca^{2+}]_i$ values were calculated using the formula: $(F-F_{min}/F_{max}-F)K_d$. The K_d-value for Fluo-3 was 390 nM.

3. Measurement of intracellular Ca^{2+} concentrations in permeabilized cells

The cells were harvested and permeabilized by first washing the cells twice in buffer A (KCl, 125 mM; Hepes, 25 mM; KH_2PO_4, 2 mM; EGTA, 0.25 mM; pH 7.0 and BSA, 1 mg/ml) by centrifugation (80 g, 5 min). The cells were then allowed to equilibrate in buffer A for 5 min at 37 °C, whereafter they were permeabilized with 5 µM digitonin. The efficiency of permeabilization was continuously monitored by trypan blue uptake. After 2-4 min incubation with digitonin, ice-cold buffer B (buffer A without EGTA) was added and the cells were washed twice by centrifugation (80 g, 5 min). The cells were then loaded with 2 µM Fura 2/AM for 20 min at 37 °C. The cells were then washed with buffer B and kept on ice until use. $[Ca^{2+}]_i$ measurements were then carried out as described above.

4. Measurement of intracellular pH

The cells were harvested and loaded with 5 µM BCECF/AM for 35 min at 37 °C. pH_i was determined using excitation wavelengths 440 and 500 nm and emission wavelength 530 nm. The calibration method was as described (Törnquist and Alinen, 1992). Briefly, the cells were lysed with Triton-X 100 to obtain pH_{max}, and

the actual pH was measured with a pH meter. Then HCl was added to obtain pH_{min}, and again actual pH was measured. The actual pH values from the experiments were then calculated using the two known pH values and fluorescence values from the calibration.

5. Measurement of membrane potential

The cells were harvested, and bisoxonol (final concentration 100 nM) was added to the cells and they were allowed to stabilize for 10 min at 37 °C. Membrane potential was assessed using an exitation wavelength of 540 nm and an emission wavelength of 580 nm (Rink et al., 1980). After each experiment, 50 mM KCl was added to enable comparisons between separate experiments.

6. Measurement of $^{45}Ca^{2+}$ fluxes

Calcium fluxes were measured as described by Tan and Tashjian (1981). The cells were grown on 35 mm dishes until confluent. In influx experiments the cells were preincubated in BSS-buffer (Hepes, 18 mM; NaCl, 130.6 mM; KCl, 5.9 mM; $CaCl_2$, 0.4 mM; $MgCl_2$, 1.2 mM; glucose, 11.8 mM; pH 7.2), for 10 minutes at 37 °C. The buffer was replaced with 1 ml of fresh BSS containing the test substances and 1 µCi $^{45}Ca^{2+}$. In efflux experiments the cells were incubated with BSS containing 1 µCi $^{45}Ca^{2+}$ and 5 mM $NaHCO_3$ for 3 h at 37 °C. The cells were then washed twice with warm BSS and the test substances were added in 1 ml of BSS. In both methods, after an appropriate incubation time, the solution was aspirated and the cells were washed rapidly three times with ice-cold BSS. Then 750 µl of 0.1 N NaOH was added and solubilized, and 600 µl was removed and counted for radioactivity in a Rackbeta liquid scintillation counter.

7. ^{3}H-Arachidonic acid release

The cells were incubated with ^{3}H-AA (0.1 µCi/ml) for 24 h in 35 mm dishes. After the incubation, the cells were washed three times (5-min intervals) with release buffer (Hepes, 10 mM; NaCl, 134 mM; KCl, 4.7 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $CaCl_2$, 2 mM; $NaHCO_3$, 2.5 mM; glucose, 5 mM; pH 7.4 and 0.1 % BSA). The cells were then incubated with the appropriate agents in 750 µl of the release buffer at 37 °C. After the appropriate incubation times, 600 µl was removed and counted for radioactivity in a Rackbeta liquid scintillation counter.

8. Measurement of ^{3}H-thymidine incorporation

The cells were plated onto 35 mm-dishes and grown in 6H-medium for 3-4 days. Where indicated, the medium was changed to 5H-medium lacking TSH and allowed to grow for an additional 2 days. The cells were then stimulated in medium containing tritiated thymidine (0.4 µCi/ml) and incubated for 24 h. They were then washed 2 times with ice-cold PBS (in mM: NaCl, 137; KCl, 2.7; Na_2HPO_4, 8; KH_2PO_4,

1.5; pH 7.4) and then with cold 5 % trichloroacetic acid (TCA). The TCA-insoluble precipitate was dissolved in 0.1 N NaOH and the radioactivity was measured.

9. Phorbol ester binding assay

For the phorbol ester binding assay, the cells were plated on microtitration plates and cultured until confluent. After three washes with AA buffer, ^{3}H-phorbol-12,13-dibutyrate (final conc. 10 nM) was added to the cells, and the cells were then stimulated. Non-specific binding was assessed by using PMA (1 μM). The incubations were stopped by washing the cells with ice-cold HEPES-buffered saline. The wells were counted for their radioactivity in a Wallac Microbeta counter.

10. SDS-PAGE and Western blotting

In protooncogene experiments, cells grown in 5H medium or 0H (without hormones or serum) in 35 mm-dishes were stimulated with appropriate agents and incubated for the times indicated at 37 °C. The cells were then washed with ice-cold PBS, scraped from the dishes and lysed in ice-cold lysis buffer (in mM: Tris Base, 20; NaCl, 420; EDTA, 2; Na_3VO_4, 2; pH 8 and NP-40, 3%). In MAP kinase studies the cells were detached and the experiments were carried out in cell suspension in HBSS, and the cells were then lysed in the lysis buffer mentioned above, containing 100 mM of NaCl. The supernatant was mixed with an equal volume of Laemmli sample buffer x2 (Tris, 125 mM, SDS, 4%, glycerol, 20%, 2-mercaptoethanol, 10%, bromophenol blue, 0.02%) and boiled for 2 min. The protein contents of the samples were determined by the Lowry method (Lowry et al., 1951) or using a BCA kit (Rockford, IL, USA). Equal amounts of protein were analyzed on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and probed with a 1:1000 (c-Raf-1, MAP kinase), 1:20 000 (phospho MAPK), 1:500 (c-Fos, c-Myc) or 1:250 (c-Jun) dilution of the antibodies. The proteins were detected using the Enhanced Chemiluminescence (ECL) kit from Amersham.

11. RT-PCR

Total RNA from FRTL-5 cells was prepared using Tryzol (Gibco BRL, Paisley, UK) according to the manufacturer's instructions. First strand cDNA was prepared from 5 μg of total RNA using an oligo(dT) 18 primer and Moloney-murine leukemia virus Reverse Transcriptase (Gibco BRL) according to the manufacturer's instructions. PCR reactions were carried out with the four rat P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$) and all seven P2X subtype specific primer pairs designed to amplify partial cDNAs for each receptor subtype as described previously (Michel et al., 1998; Webb et al., 1998). Briefly, the PCR reactions were performed using 7.5% (v/v) of each first strand cDNA reaction and 400 ng of each primer using 2.5 units of either Biotaq (Bioline, London, UK) or Dynazyme DNA polymerase (Flowgen, Sittingbourne, UK) with the appropriate buffer, 200 μM of each deoxy-nucleotide triphosphate and 1.5 mM $MgCl_2$. Amplification conditions for the P2Y receptors were 1 min at 94 °C, 30 sec at 60 °C, 1 min at 72 °C for 30 cycles and, finally 10 min at 72 °C. Amplification

conditions for the P2X receptors were 1 min at 94 °C, 30 sec at 58 °C for all subtypes other than P2X3 which was annealed at 60 °C, 1 min at 72 °C for 35 cycles and, finally, 10 min at 72 °C. The amplification products were separated electrophoretically and visualised with ethidium bromide fluorescence.

12. Cloning and sequencing

FRTL-5 PCR products were cloned into the pCR 2.1 vector (TA cloning kit; Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions and sequenced completely using an automated DNA sequencer.

RESULTS AND DISCUSSION

1. Regulation of PLA_2 activity and arachidonic acid release by ATP (I)

The activation of PLA_2 and the concomitant release of AA are involved in different cellular functions in FRTL-5 cells, such as regulation of iodide efflux, proliferation and Ca^{2+} fluxes (Burch et al., 1986; Smallridge and Gist, 1994). In general, studies have suggested that two events are essential for the activation process of $cPLA_2$, i.e. the Ca^{2+}-dependent translocation to the membranes, and the phosphorylation of the enzyme (Murakami et al., 1997), although even these together may not be sufficient (Balboa et al., 2000). Another important question has been to what extent do G proteins play a role in the activation process (Burke et al., 1997).

1.1 Roles of G proteins and Ca^{2+}

ATP evoked a concentration- and time-dependent ^{3}H-AA release from prelabelled FRTL-5 cells (I, Fig. 1A, B). Typically, the release was 1-2% of the total incorporated ^{3}H-AA (data not shown). Treating the cells with a G_i/G_o protein inhibitor PTX (100 ng/ml for 24 h) completely inhibited the AA release stimulated by 100 μM ATP (I, Fig. 1C), a result which is consistent with an earlier study by Okajima et al. (Okajima et al., 1989). The ATP-evoked Ca^{2+}-signal was also sensitive to PTX-treatment, but it is decreased only by 29% (V, Table 2). Furthermore, preventing the influx of extracellular Ca^{2+} significantly decreased, but did not abolish, the ATP-evoked AA release. (I, Fig. 2A) Thus, the influx of Ca^{2+} does play an important role in the ATP-evoked PLA_2 activation. In an earlier study Smallridge and Gist (Smallridge and Gist, 1994) stated that the ATP-evoked AA release is Ca^{2+}-independent. However, although ATP evoked a significant release of AA in EGTA-treated cells, it was still significantly smaller than in control cells, and thus the conclusion of these authors seems to be incorrect.

There are numerous examples where Ca^{2+} ionophores evoke AA release, suggesting that an increase in $[Ca^{2+}]_i$ alone is sufficient for PLA_2 activation (Murakami et al., 1997). Also we have shown that the ER Ca^{2+}-ATPase inhibitor thapsigargin evokes release of AA, which is totally blocked in the presence of EGTA (Törnquist et al., 1994), but which is PTX-insensitive (Ekokoski and Törnquist, unpublished observation). Thus, depending on the stimulating agent, there seems to be a different demand for elevated $[Ca^{2+}]_i$ and for involvement of a G protein. A minimum length of stimulation (2-5 min, depending on the cell type) of elevated $[Ca^{2+}]_i$ has been shown to be essential for continuous translocation, and complete activation, of $cPLA_2$ (Hirabayashi et al., 1999). A brief increase in $[Ca^{2+}]_i$ induces the translocation of PLA_2 and a weak binding to the membranes, but the still inactive PLA_2 may readily translocate back. Only a continuous $[Ca^{2+}]_i$ increase evokes stronger binding to membranes and AA release. It is thus possible that the $[Ca^{2+}]_i$ increase evoked by ATP is not sustained enough in FRTL-5 cells and, thus, coupling to a G protein could promote the activation of PLA_2. In embryonic fibroblasts inactivation of the $G\alpha_{i2}$ gene resulted in a decreased Ca^{2+}-dependent translocation of $cPLA_2$ (Mattera et al., 1998), and suggests that the locus for $G\alpha_{i2}$ is downstream of the $[Ca^{2+}]_i$ increase.

How the PTX-sensitive G_i/G_o protein is coupled to activation of PLA_2 is still unclear. None of the studies done so far have clearly established a direct physical coupling of a G protein and the PLA_2 enzyme, and it has been suggested that there may exist intermediate or accessory proteins (Mattera et al., 1998; Winitz et al., 1994).

1.2 Role of PKC and the MAP kinase pathway

Stimulation of the PLC pathway leads to activation of PKC. The PKC activator phorbol ester PMA (200 nM) alone or together with ATP (100 μM) had no effect on the AA release (I, Fig. 2D). However, the PKC inhibitor GF109203X (1 μM), which inhibits the α, βI, βII, γ and ε isoforms of PKC (Toullec et al., 1991), or a prolonged incubation with PMA (2 μM for 24 h), significantly decreased the ATP-evoked AA release (I, Fig. 2D). This is in contrast to a previous study showing that the ATP-evoked AA release is PKC-independent in FRTL-5 cells (Smallridge and Gist, 1994). The reason for this discrepancy is not known. Another GPCR agonist, noradrenaline, which activates PLC and PLA_2 in FRTL-5 cells (Burch et al., 1986), evokes a PKC–dependent AA release (Wang et al., 1996). The ineffectiviness of a direct activation of PKC by PMA on AA release could be explained by the inhibitory effect of PKC on agonist-evoked Ca^{2+} signals (Törnquist, 1993). Activation of PKC may also have other inhibitory effects on signals mediated by G proteins (Boarder and Challiss, 1992).

A role for MAP kinase in the phosphorylation and activation of PLA_2 has been suggested in many studies (Lin et al., 1993; Nemenoff et al., 1993). Also PKC can phosphorylate PLA_2 at least in vitro (Lin et al., 1993), but the effect of PKC may occur through the MAP kinase pathway (Qiu and Leslie, 1994). We found that inhibiting MAP kinase using the MEK inhibitor, PD98059 (30 μM) attenuated the ATP-evoked MAP kinase phosphorylation, as assessed by Western blot (I, Fig. 3A), and significantly decreased, but did not abolish, the ATP-evoked AA release (I, Fig. 3B). We also showed that PMA alone evoked phosphorylation of MAP kinase (V, Fig. 5D). These results clearly indicate that MAP kinase is important for the ATP-evoked AA release. However, activation of MAP kinase alone is not sufficient for PLA_2 activation, since PMA was unable to evoke AA release, yet it phosphorylated MAP kinase.

To establish whether PKC and MAP kinase acted in the same signaling pathway in the AA release, the cells were treated together with GF109203X (1 μM) and PD98059 (30 μM) before stimulation with ATP. In these experiments the ATP-evoked AA release was significantly decreased when compared with cells treated with either inhibitor alone (I, Fig. 3B). Also in Western blot studies inhibition of PKC by down-regulation or by GF109203X attenuated the ATP-induced MAPK phosphorylation (I, Fig. 3C, D), suggesting that PKC lies upstream of MAP kinase. However, the greater inhibition of AA release in GF109203X- and PD98059-treated cells compared with cells treated with either inhibitor alone suggests that PKC may also have a direct effect on PLA_2 independent of MAP kinase. In MDCK-D1 cells both MAP kinase and PKC were necessary for maximal activation of $cPLA_2$, however, part of the action of PKC was independent of MAP kinase (Xing et al., 1997). Our result could also indicate two different routes in the activation of MAP kinase: Raf-dependent and MEKK-

dependent, which both could be activated by PKC (Siow et al., 1997). Use of both inhibitors would result in more efficient inhibition of MAP kinase, since PD98059 inhibits only the Raf-kinase-evoked MEK activation (Alessi et al., 1995). However, it is not known if both of these kinases are activated by ATP in FRTL-5 cells.

The pathway leading to MAP kinase activation may involve a complex network of kinases. The Src-like kinases have been implicated in signalling from both G_i and G_q protein-coupled receptors to MAP kinase through βγ subunits (van Biesen et al., 1995; Luttrell et al., 1996; Igishi and Gutkind, 1998). In our study, inhibiting Src-like kinases by PP1 (10 μM; Hanke et al., 1996) attenuated both the ATP-evoked AA release and MAP kinase phosphorylation (I, Fig. 4), thus suggesting that this kinase could be involved in the activation of MAP kinase and AA release. To investigate whether Src- and MAP kinases acted through the same pathway in the regulation of PLA_2 activity, the cells were treated with both PP1 (10 μM) and PD98059 (30 μM) before stimulation with ATP. In these experiments the ATP-evoked AA release was significantly decreased when compared with cells treated with either inhibitor alone (I, Fig. 4B), suggesting that a Src-like kinase is involved in the phosphorylation of MAP kinase, but could also act independently of MAP kinase in the AA release. This latter finding is novel and further investigations are required to understand whether a Src-like kinase is able to regulate the PLA_2 activity independently of MAP kinase.

1.4 Effect of cAMP

Cyclic AMP and protein kinase A may have a modulatory effect on the MAP kinase activation (Nagasaka et al., 1994; Sevetson et al., 1993). A negative feedback loop between the $P2Y_2$ receptor evoked AA release and increased cAMP level has been suggested in MDKC-D1 cells (Xing et al., 1999). However, although cAMP and PKA form an important signaling pathway in thyroid cells, modulation of intracellular cAMP levels by decreasing the steady-state intracellular cAMP level by culturing the cells without TSH for 2 days (Corda et al., 1987), or by increasing the cAMP level rapidly by addition of TSH (0.5 mU/ml; I, Fig. 5A,B) or dBucAMP (1 mM; data not shown) to the cells cultured without TSH for 2 days, had no effect on the ATP-evoked AA release in FRTL-5 cells. Furthermore, neither TSH (I, Fig. 5C) nor dBucAMP (data not shown) alone evoked, or attenuated the ATP-mediated, phosphorylation of MAP kinase. In T lymphocytes a similar kind of insensitivity of MAP and Raf-1 kinases to cAMP has been described (Hsueh and Lai, 1995).

2. Effects of activation of PLA_2 on Ca^{2+} fluxes (II & III)

Activation of PLA_2 leads to release of AA and other fatty acids from membrane phospholipids. Free fatty acids and eicosanoids, the metabolites of AA, can have regulatory effects on ion fluxes in different cell types (Meves, 1994). We therefore investigated how the metabolism of AA and different fatty acids would affect agonist-evoked Ca^{2+} fluxes in FRTL-5 cells.

2.1 Effect of arachidonic acid metabolism on the ATP-evoked Ca^{2+} fluxes

Preincubation of the Fura-2-loaded cells with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 30 μM), the lipoxygenase/cytochrome P-450 pathway inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA; 30 μM), or the cytochrome P-450 inhibitor econazole (30 μM) for 8-10 min decreased the ATP-evoked Ca^{2+} increase in a Ca^{2+}-containing buffer, whereas the PLA_2 inhibitor 4-bromophenylacyl bromide (4-BPB; 30 μM) did not (II, Fig. 2, 3). All of the inhibitors, except econazole, decreased the ATP-evoked Ca^{2+} release from intracellular stores (II, Fig. 2, 3). The cyclooxygenase inhibitor indomethacin (30 μM) was without an effect on the ATP-evoked Ca^{2+} transient in both Ca^{2+}-containing and -free buffers. Furthermore, econazole, NDGA and ETYA also inhibited the ATP-evoked store-operated Ca^{2+} entry, as addition of Ca^{2+} ions back to the cells incubated in Ca^{2+}-free buffer resulted in a decreased Ca^{2+} entry (II, Fig. 4). These results suggested that some of the non-cyclooxygenase products could take part in the ATP-evoked Ca^{2+}-fluxes. Similar observations have been made in several other cell types, where the AA metabolites release sequestered Ca^{2+} (Luscinskas et al., 1990; Snyder et al., 1986), enhance the agonist-evoked Ca^{2+} release (Force et al., 1991), or regulate the store-operated Ca^{2+} influx (Hoebel et al., 1997). However, in our study we did not detect any Ca^{2+} increase when stimulating the cells with the leukotrienes B_4, C_4, D_4, E_4, 12-HETE, 15(S)-HPETE, 8,9-EET or 14,15-EET either alone or with ATP (data not shown).

We then tested the effect of AA on Ca^{2+} -fluxes. AA (30 μM) alone evoked a small release of Ca^{2+} from intracellular stores and evoked the influx of Ca^{2+} (II, Fig. 6). The release occurred at least in part from IP_3-sensitive Ca^{2+} stores, as in permeabilized cells after repeted additions of IP_3, AA evoked only a small release of Ca^{2+} (II, Fig. 7). Addition of AA together with ATP attenuated the effect of ATP (II, Fig. 6), suggesting that the effect of AA on the ATP-evoked response could be due to emptying the same IP_3-sensitive Ca^{2+} stores. The mechanism by which fatty acids mobilize intracellular Ca^{2+} is not known, but in other cells it is IP_3-independent (Chow and Jondal, 1990; Gamberucci et al., 1997).

Taken together, these results show that the non-cyclooxygenase metabolites of AA may be of importance for the ATP-induced release of sequestered Ca^{2+} and for the entry of Ca^{2+} from extracellular space. However the identity of the metabolite responsible remaines to be investigated. In addition, AA itself may modulate agonist-induced release of sequestered Ca^{2+} in FRTL-5 cells.

2.2 Effect of fatty acids on Ca^{2+} fluxes

Several long chain unsaturated (oleic 18:1, arachidonic 20:4, linoleic 18:2 and linolenic 18:3 acid), long chain saturated (palmitic 16:0 and myristic 14:0 acid) and short chain saturated (heptanoic 7:0 and valproic 8:0 acid) fatty acids were tested for their effects on $[Ca^{2+}]_i$ either alone, or on agonist-evoked changes in $[Ca^{2+}]_i$. Extracellularly added long chain unsaturated fatty acids (30 μM) increased $[Ca^{2+}]_i$ when added alone, while the long chain saturated (30 μM) and short chain saturated (30 μM) fatty acids were without effect (III, Table 1). The long chain fatty acids attenuated also the 100 μM ATP-evoked Ca^{2+} transient, while the short ones did not (III, Table 1).

The effects of oleic acid (OA) and arachidonic acid (AA) were then tested in detail. Both (30 μM) evoked entry of Ca^{2+} from the extracellular space, which was not inhibited by the Ca^{2+}-channel blocker SKF 96365 (data not shown). As with AA, also OA mobilized Ca^{2+} at least in part from the same intracellular stores as did ATP (III, Fig. 2). OA also significantly decreased the ATP-evoked store-operated Ca^{2+} entry (III, Fig. 2). As the ATP-evoked entry of Ba^{2+} was also decreased after OA treatment (III, Fig. 3), we conclude that OA inhibited Ca^{2+} influx from the extracellular space, as Ba^{2+} may enter the cells but cannot be extruded by plasma membrane Ca^{2+}ATPases. The inhibitory effect of OA on increase in $[Ca^{2+}]_i$ was also shown using thapsigargin (III, Fig. 4), which releases Ca^{2+} from intracellular stores and activates store-operated Ca^{2+} entry in cells in an IP_3-insensitive manner (Thastrup et al., 1990). In conclusion, the results indicate that the effect of OA and AA on Ca^{2+} homeostasis was twofold: they released Ca^{2+} from the intracellular stores and decreased the agonist-evoked Ca^{2+} entry.

2.3 Mechanisms of the inhibitory action of oleic acid and arachidonic acid on $[Ca^{2+}]_i$

It has been suggested that fatty acids could activate Ca^{2+} extrusion, probably by activating the plasma membrane Ca^{2+}ATPase (Breittmayer et al., 1993; Randriamampita and Trautmann, 1990). However, we found that OA and AA did not enhance the ATP-evoked $^{45}Ca^{2+}$ efflux from the cells (data not shown).

Fatty acids have been shown to activate PKC (Khan et al., 1993; Shinomura et al., 1991), and activated PKC may inhibit agonist-evoked Ca^{2+} signals (Boarder and Challiss, 1992; Törnquist, 1993). However, inhibition of PKC by staurosporine (200 nM) or by prolonged treatment with PMA (2 μM for 24 h) had no effect on the OA-evoked increase in $[Ca^{2+}]_i$, nor did it restore the ATP-evoked Ca^{2+} transient in OA-treated cells (data not shown). Thus, the effects of OA are not dependent on the activation of PKC, which agree with results obtained in several other cell types (Gamberucci et al., 1997). We cannot, however, exclude the possibility that some fatty acid-sensitive PKC isoform(s) were not inhibited by these treatments. In human platelets, OA has been shown to activate PKC α, βII and δ (Khan et al., 1993), and in cardiac myocytes AA stimulates PKC-ε redistribution (Huang et al., 1997).

OA and AA have been shown to reduce the activity of the plasma membrane Na^+/K^+ATPase (Blanco et al., 1998). Inhibition of this pump leads to accumulation of Na^+ inside the cell, and could thus result in increase in $[Ca^{2+}]_i$ through the activation of the Na^+-Ca^{2+}-exchanger (Törnquist, 1992). However, substitution of Na^+ by choline in the incubation buffer did not change the effect of OA on the amplitude of $[Ca^{2+}]_i$, although the temporal pattern of the signal was changed (III, Fig. 5). The ATP-evoked effect in OA-treated cells was enhanced, probably reflecting the prevention of the Na^+-Ca^{2+}-exchanger in Na^+-free buffer. However, the ATP-evoked response was still smaller than in control cells not treated with OA.

Fatty acids can change plasma membrane potential by hyperpolarization (Nordström et al., 1992). Depending on the cell type, hyperpolarization has been shown to either inhibit or enhance the agonist-evoked changes in $[Ca^{2+}]_i$ (Di Virgilio

et al., 1987; Oettgen et al., 1985). However, in the bisoxonol-loaded cells OA had no effect on the membrane potential (data not shown).

Both OA and AA were found to acidify the FRTL-5 cells rapidly, as assessed using the fluorescent pH-probe BCECF (II, Fig. 8; III, Fig. 7). Thus, the Ca^{2+} increase could be due to H^+-tiggered activation of the Na^+/H^+-exhanger followed by activation of the Na^+/Ca^{2+}-exhanger. Furthermore, the IP_3-induced Ca^{2+} release is pH-dependent (Guillemette and Segui, 1988), and thus acidification of the cytosol could affect the agonist-evoked release of Ca^{2+}. The effects of OA and AA on pH_i were compared with that of nigericin, a known K^+-H^+-ionophore. Addition of 0.3 μg/ml nigericin decreased pH_i to the same extent (an average of 0.2 pH units) or more than OA or AA (II, Fig. 8; III, Fig. 8). To determine whether the fatty acid-induced decrease in pH_i caused the effect on the $[Ca^{2+}]_i$ increase, the cells were acidified with nigericin (0.3 μg/ml) and then stimulated with ATP. Nigericin significantly decreased the ATP-evoked increase in $[Ca^{2+}]_i$ (III, Fig. 8). Thus, the fatty acid evoked acidification of the cells could at least in part lead to attenuated Ca^{2+} responses, probably via the pH-sensitive IP_3-evoked Ca^{2+} release. However, the thapsigargin-evoked Ca^{2+} release was also suppressed by fatty acids and, therefore, the decrease in pH_i cannot totally explain the inhibitory effect of fatty acids. Furthermore, the mechanism by which the acidification by fatty acids occurred is not known. Studies with model membranes have shown that un-ionized fatty acids rapidly move to the inner leaflet of the membrane (flip-flop movement), followed by a release of protons from the fatty acid molecules, which induces a decrease in pH_{in} (Kamp and Hamilton, 1992).

Previous studies have shown that bovine serum albumin (BSA), which has a high affinity for fatty acids, can abolish the effects of fatty acids (Chow et al., 1990; Gamberucci et al., 1997). In our study, addition of BSA (0.2%) after OA rapidly restored the intracellular pH, and also almost restored the ATP- and thapsigargin-evoked Ca^{2+} responses (III, Fig. 6, 7). These results suggest that the inhibitory action of fatty acids occurs also at the plasma membrane level.

Taken together, these results show that unsaturated fatty acids may have effects on the agonist-evoked Ca^{2+} signals both in the cytosol and at the plasma membrane. In the cytosol they release Ca^{2+} from the intracellular stores, and at the plasma membrane level they inhibit Ca^{2+} entry. The effect of unsaturated fatty acids on Ca^{2+} signalling has been shown to be directly correlated with the degree of lipid acyl chain perturbation of the plasma membrane, and correlate with the degree of *cis*-unsaturation of the fatty acids, i.e. arachidonic acid>oleic acid>>palmitic acid (Anel et al., 1993; Gamberucci et al., 1997). In pituitary GH_3 cells it was concluded that *cis*- but not *trans*-unsaturated or saturated fatty acids perturb the function of several integral plasma membrane proteins: PLC, a Ca^{2+} entry channel and a Ca^{2+}-extruding pump by incorporation into the plasma membrane (Pérez et al., 1997). However, Anel et al. (Anel et al., 1993) concluded that the effects of fatty acids were due to an altered interaction between membrane lipids and specific proteins, which only then resulted in alteration in the function of these proteins.

2.4 Interactions between arachidonic acid metabolism and free fatty acids in Ca^{2+} signaling

The relevance of the opposing effects of free fatty acids, and the effect of inhibition of AA metabolism, on Ca^{2+} homeostasis is still unresolved in FRTL-5 cells. In canine cerebellar microsomes, AA and LTB_4 have distinct effects on Ca^{2+} homeostasis, i.e. LTB_4 specifically activates the ryanodine receptor (RyR) whereas AA inhibits the IP_3R. It was suggested that LTB_4 could be an endogenous activator of RyR in non-muscle cells, and that the different regulation of these channels might have consequences in the Ca^{2+} signaling in cells expressing both of these channels. Furthermore, the inhibitory action of AA on IP_3R and Ca^{2+} could represent a feedback inhibition mechanism for the activation of $cPLA_2$ (and thus the production of eicosanoids), since both the translocation and phosphorylation (Ca^{2+}-dependent MAP kinase activation) of $cPLA_2$ are Ca^{2+}-dependent (Striggow and Ehrlich, 1997). In human thymocytes and neutrophils it is speculated that the liberated free fatty acids could antagonize the synthesis of EET (Alonso-Torre and Garcia-Sancho, 1997). In platelets OA may serve as an intracellular messenger, as it is released into the cytosol from the membrane by thrombin stimulation, and it also stimulates the release of Ca^{2+} from intracellular stores (Siafaka-Kapadai et al., 1997). On the other hand, the inhibitory actions of unsaturated fatty acids on Ca^{2+} influx in T cells is reversed by BSA, thus leading to a suggestion that plasma albumin protects the cells from harmful effects of fatty acids on Ca^{2+} homeostasis (Chow et al., 1990). It could be possible that in FRTL-5 cells the ATP-evoked Ca^{2+} signals could be regulated by potentiating or maintaining Ca^{2+} entry by some of the newly produced AA metabolites inside the cells. The Ca^{2+} signal could then be counteracted by free fatty acids (in an auto/paracrine manner) by inhibiting further Ca^{2+} influx.

In the light of recent investigations, some of the noncyclooxygenase inhibitors used here may have effects that are not related to inhibition of AA metabolism (Korn and Horn, 1990; Mason et al., 1993; Vostal and Fratantoni, 1993), and, thus, we cannot exclude the possibility that the inhibitors had also unspecific effects on Ca^{2+} homeostasis. ETYA and NDGA are fatty acid analogues, and thus could act in a similar inhibitory manner as AA and OA (Ekokoski and Törnquist, 1994; Huang et al., 1992). Econazole has been shown to inhibit store-operated Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+}ATPase, but the exact mechanism is not known (Christian et al., 1996; Mason et al., 1993). We (Ekokoski and Törnquist, unpublished observation) and others have observed that econazole increases $[Ca^{2+}]_i$ and evokes Ca^{2+} entry from the extracellular space when added to the cells alone (Jan et al., 1999; Mason et al., 1993). However, even if these inhibitors have effects other than those related to inhibition of AA metabolism, the AA metabolites may still act as regulators of Ca^{2+} fluxes. There are recent reports showing that the AA metabolites such as 5,6-EET are the key signals for evoking Ca^{2+} influx in astrocytes (Rzigalinski et al., 1999). It is possible that there are more than one type of Ca^{2+} entry mechanism in a given cell type (Barritt, 1999).

3. Determination of P2 receptor subtypes in FRTL-5 cells (V)

There are several investigations on the effects of nucleotides on FRTL-5 cells and other thyroid cells from different species, however no thorough studies on different ATP or other P2 receptor types have been made. On the basis of earlier functional studies (Aloj et al., 1993; Okajima et al., 1989; Sato et al., 1992), several P2 receptor subtypes could be expected to be found in these cells.

Using RT-PCR analysis, we showed that the FRTL-5 cells contained mRNA for the G protein coupled $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes, and for the transmitter-gated ion-channel $P2X_3$, $P3X_4$ and $P2X_5$ subunits. (V, Fig. 1). In functional experiments using Fura-2 loaded cells, UTP, ATP, ATPγS, and UDP were the most potent agonists in evoking Ca^{2+} transients, with EC_{50} values of 0.2 μM, 0.5 μM, 0.7 μM and 10.7 μM, respectively (V, Fig. 2A, B). Other nucleotides tested, α,β-meATP, 2-MeSATP and pure ADP were weak agonists. Moreover, the nucleotide-evoked increase in $[Ca^{2+}]_i$ was decreased but not abolished in Ca^{2+}-free buffer (V, Table 2), suggesting that they released Ca^{2+}- from intracellular stores, and that part of the increase was due to Ca^{2+} influx, the magnitude of which was dependent on the nucleotide used. In addition, PTX-pretreatment (100 ng/ml for 24 h) decreased the Ca^{2+} transients evoked by all nucleotides (V, Table 2).

The results from the RT-PCR and Ca^{2+} experiments correlate well in favor of the presence of $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in FRTL-5 cells, since ATP and UTP are equipotent agonists at the rat $P2Y_2$ and $P2Y_4$ receptors, and UDP is a potent agonist at the $P2Y_6$ receptor (Bogdanov et al., 1998; Lustig et al., 1993; Nicholas et al., 1996; Webb et al., 1998). The $P2Y_1$ receptor agonist ADP (Léon et al., 1997), after hexokinase treatment, induced Ca^{2+} transient only with low potency and efficacy, thus confirming the absence of this receptor subtype in these cells.

The presence of the P2X receptor subunits is more difficult to show with functional experiments, as all of the P2X receptor subunits detected in FRTL-5 cells are activated by ATP, and presently there are no specific antagonists available to discriminate between these receptor types (North and Barnard, 1997). 2-MeSATP and αβ-meATP, which are the most potent agonists at the $P2X_3$ receptor, induced Ca^{2+} transients with low potency. Of the $P2X_4$ and $P2X_5$ receptor agonists (ATPγS and 2-MeSATP), only ATPγS gave potent Ca^{2+} responses. Furthermore, also the P2X agonist-evoked Ca^{2+} transients were attenuated by PTX. These results suggest that the P2X receptor transcripts may not be translated into proteins, or if they are translated they do not assemble into functional channels. It is also possible that the method used here is insufficient to detect the responses by P2X receptors. Especially the $P2X_3$ receptor shows rapid desensitization kinetics, and thus the effect through this receptor subtype may remain undetected. Clearly, to establish whether the P2X receptors are functioning in FRTL-5 cells, further experiments are warranted, such as electrophysiological measurements.

Taken together, the six different types of P2 receptor transcripts found in these cells is far more than was expected on the basis of pharmacological experiments. There are no earlier studies on the expression of P2 receptors in native thyroid tissue of any animal species, and therefore we cannot compare the observed expression profile with that of thyroid cells. In a recent study it was shown that pancreatic duct cells, which are polarized epithelial cells, express multiple P2Y and P2X receptors (Luo

et al., 1999). The distribution of the P2 receptor subtypes was different in the luminal and basolateral membranes, however subtypes from both receptor classes were expressed in both membranes. The source of nucleotides activating these receptors is also different, that is, the basolateral receptors are stimulated by ATP secreted from purinergic neurones, and the luminal receptors stimulated by ATP secreted e.g. by acinar cells. Many of the receptors were shown to activate Cl^- channels, suggesting that they might have a physiological role in the regulation of pancreatic duct cell function. In a recent report in polarized porcine thyroid cell culture it was shown that a UTP-preferring receptor (EC_{50} of 0.08 μM for UTP), located on the apical membrane, was connected to regulation of Na^+ absorption (Bourke et al., 1999). It was suggested that this receptor, which on the pharmacological basis might be the $P2Y_4$ subtype, could have a role in the physiological regulation of follicle volume. In this study, also ATP and UDP seemed to act as agonists with EC_{50} values of 6.3 - 6.6 μM, which, although not mentioned in the article, could indicate separate receptors at least for uridine nucleotides. Further experiments are needed to establish how the various P2 receptors work and interact in polarized thyroid cell cultures.

4. The mitogenic effect of ATP (IV & V)

4.1 The ATP evoked enhancement of DNA-synthesis

There are several reports showing that extracellular ATP alone acts as a mitogen (Erlinge et al., 1993; Wang et al., 1992), or acts synergistically with other mitogens in various cell types (Erlinge et al., 1995; Huang et al., 1989; Ishikawa et al., 1994). Here we showed that ATP was able to enhance DNA-synthesis in FRTL-5 cells incubated in medium containing serum and insulin (5H medium) or TSH (6H medium; IV, Fig. 1), but not in quiescent cells (data not shown), or in cells incubated in 4H medium (medium with serum but without TSH and insulin; IV, Fig. 1). The effect of ATP was dose- and time-dependent. Furthermore, ATP increased the cell numbers by 30% in the 5H medium (IV, Fig. 2). These results suggested that ATP acted as a comitogen, rather than as an independent mitogen in FRTL-5 cells. The effect of ATP was not due to the breakdown product adenosine, as adenosine deaminase did not have an effect on the ATP-evoked ^{3}H-thymidine incorporation (data not shown). Furthermore, ATP did not have an effect on the binding of I^{125}-TSH in FRTL-5 cells (data not shown), indicating that ATP did not affect the affinity or the number of the TSH receptors.

4.2 Effects of other nucleotides on DNA-synthesis

We then wanted to investigate whether also other nucleotides could evoke mitogenesis in FRTL-5 cells, and also to determine through which P2 receptor subtype ATP exerted its action. UTP, UDP and ATPγS enhanced the ^{3}H-thymidine incorporation in a concentration-dependent manner (V, Fig. 3A), while the effect of αβ-meATP was very weak, and 2-MeSATP was totally ineffective (data not shown). The effects of ATP and UTP were decreased by pretreatment of the cells with PTX whereas it had no effect on the UDP- or ATPγS-evoked responses (V, Fig. 3B). In

paper IV the effect of ATP was almost totally abolished with PTX-treatment (IV, Fig. 3C), which may be due to the different cell batch used. Taken together, the results suggest the involvement of at least three receptor subtypes in nucleotide-evoked mitogenesis: $P2Y_2$ and/or $P2Y_4$ in response to ATP and UTP; and $P2Y_6$ in response to UDP and ATPγS. In several cell types the P_{2U} receptor ($P2Y_2$ in the new nomenclature) has been shown to take part in the activation of cell division (Kaplan et al., 1996; Malam-Souley et al., 1996; Miyagi et al., 1996). Also a role for the $P2Y_4$ receptor has been suggested in smooth muscle cell division (Harper et al., 1998). However, at present, without specific tools, it is impossible to discriminate between mitogenic P2 receptor subtypes in FRTL-5 cells.

4.3 Interaction between P1 and P2 receptor systems

In addition to P2 receptors, FRTL-5 cells also possess P1 adenosine receptors. Studies have shown that the P2 receptor-mediated responses may be potentiated by the P1 receptors in FRTL-5 cells (Nazarea et al., 1991; Okajima et al., 1989). Okajima et al. (1989) proposed the existence of P_{2p} and P_{2i} receptor types in FRTL-5 cells, which could explain the possible interactions between these two (P1 and P2) receptor systems (Okajima et al., 1989). In their model, the P_{2p} receptor is coupled to a PTX-insensitive G protein and activates PLC, whereas the P_{2i} or A_1 receptor is coupled to a PTX-sensitive G protein, and negatively regulates adenylate cyclase, leading to an enhancement of the signal mediated by the PLC-pathway. According to their model, ATP is able to bind to both receptor types, whereas GTP binds only to the P_{2p} type, and PIA, an adenosine derivative, binds only to the P_{2i} type (Okajima et al., 1989). To test the interaction of P1 and P2 receptor systems in the DNA-synthesis, the cells were stimulated with GTP and PIA. GTP *per se* enhanced the incorporation of ^{3}H-thymidine, but only in the presence of TSH (IV, Fig. 4A). PIA decreased the basal as well as the ATP-induced incorporation of ^{3}H-thymidine in the presence of both insulin and TSH (IV, Fig. 4A, B). This is consistent with studies by Moses et al. (Moses et al., 1989) and Vainio et al. (Vainio et al., 1997), who showed that adenosine has inhibitory effects on TSH, insulin and PMA-evoked DNA-synthesis. Furthermore, when added together, GTP and PIA did not mimic the action of ATP (data not shown), and thus we could not confirm the proposed hypothesis. This may be due to more complex signaling mechanism in evoking DNA-synthesis than in evoking Ca^{2+} transients.

4.4 Mechanisms of the ATP-enhanced DNA-synthesis

We then investigated the mechanism(s) by which ATP enhanced DNA-synthesis. A role for PKC has been suggested in mitogenesis (Lombardi et al., 1988; Roger et al., 1997). In our study, PMA (200 nM) *per se* in the presence of insulin increased, but in the presence of TSH, decreased, the ^{3}H-thymidine incorporation (IV, Fig. 1). The observed mitotic and antimitotic effects of PMA here are consistent with other studies in FRTL-5 cells (Akiguchi et al., 1993; Kraiem et al., 1995), and the antimitotic effect probably reflects an inhibitory action of PMA on the cAMP-mediated response of TSH (Roger et al., 1997). Inhibition of PKC by downregulation

with PMA (2 μM 24 h) did not have a significant effect on the ATP-stimulated ^{3}H-thymidine incorporation (IV, Fig. 3A). Furthermore, addition of ATP and PMA together resulted in an additive response in ^{3}H-thymidine incorporation (IV, Fig. 3B). Similar observations of a minor role of PKC in ATP-induced mitogenesis have been made in other cell types, such as smooth muscle cells (Erlinge et al., 1993). However, we cannot exclude the possibility that some PKC isoforms were not inactivated by PMA-treatment. Such isoforms (PKC ζ and λ) have recently been proposed to have a role in mitogenesis (Toker, 1998). The ζ isoform is not inactivated by downregulation in FRTL-5 cells (Wang et al., 1995).

Ca^{2+} ions play important regulatory functions in the cell cycle (Santella, 1998). Here, the basal as well as the ATP-induced incorporation of ^{3}H-thymidine was dose-dependently decreased by Ni^{2+} (IV, Fig. 7), suggesting that Ca^{2+} is of general importance in cell proliferation. The effect of Ca^{2+} can be mediated through calmodulin and Ca^{2+}/CaM kinase II (Santella, 1998). Inhibition of calmodulin with calmidazolium or phenoxybenzamine, or inhibition of the CaM kinase II with KN-62 dose-dependently decreased the basal and the ATP-induced DNA-synthesis (IV, Fig. 7). These results suggested that these effectors of Ca^{2+} signaling may play a role in FRTL-5 cell proliferation. However, an increase in $[Ca^{2+}]_i$ alone is not sufficient for cells to start proliferation, since the Ca^{2+} mobilizing agents thapsigargin and ionomycin either decreased or were without an effect on ^{3}H-thymidine incorporation (data not shown).

In previous studies of FRTL-5 cells, many of the growth-promoting factors, such as TSH, IGF-I and serum, have been shown to induce the production of cyclooxygenase metabolites (Tahara et al., 1991). Furthermore, noradrenaline has been shown to induce DNA-synthesis via an autocrine PGE_2 production (Burch et al., 1986). In the present study incubation of the cells with indomethacin (30 μM) did not decrease the ATP-induced incorporation of ^{3}H-thymidine (data not shown). Furthermore, addition of AA *per se* or together with ATP, did not affect the ^{3}H-thymidine incorporation (data not shown). Also the noncyclooxygenase metabolites 15(S)-HETE, 15(S)-HPETE, 8,9-EET and 14,15-EET were without an effect on ^{3}H-thymidine incorporation (Ekokoski and Törnquist, unpublished observation).

The MAP kinase pathway is one of the key elements in regulating cell growth in many cell types (Widmann et al., 1999). In FRTL-5 cells, ATP induced a transient phosphorylation of MAP kinase, which was initiated within 0.5 min and lasted for at least 10 min (IV, Fig. 5A). To investigate the mechanism of ATP-evoked MAP kinase phosphorylation, the cells were treated with PTX (50 ng/ml for 24 h). The phosphorylation was partially inhibited by PTX treatment (IV, Fig. 5B), suggesting that it was mediated through both G_i and G_q proteins. The phosphorylation of MAP kinase was a Ca^{2+}-independent process, as it was not inhibited by Ni^{2+} (3 mM; IV, Fig. 5C) or in Ca^{2+}-free buffer (Ekokoski and Törnquist, unpublished observation). Also, we have not been able to detect phosphorylation of MAP kinase in response to thapsigargin (Ekokoski and Törnquist, unpublished observation), suggesting that an increase in Ca^{2+} is not sufficient to phosphorylate the kinase, a result consistent with others who have shown that MAP kinase activation may be Ca^{2+}-independent (Chao et al., 1992). The MEK inhibitor PD98059 (30 μM) inhibited the ATP- and PMA-induced MAP kinase phosphorylation, and also decreased the ATP- and PMA-

induced ^{3}H-thymidine incorporation (V, Fig. 5D, 6), suggesting that MAP kinase has a role in DNA-synthesis. However, inhibition of MEK did not totally block DNA-synthesis, and since ATP-induced MAP kinase phosphorylation was detected in quiescent cells, which do not proliferate, these results suggest that the MAP kinase pathway is not sufficient to alone induce cell proliferation, and suggest that additional factors are needed. The reason why inhibition of PKC and MEK did not produce similar effects on DNA-synthesis, as in MAP kinase phosphorylation experiments (I, Fig. 3A, C, D), is not known, but a possibility exists that some residual MAP kinase activity could remain after PKC inhibition. Furthermore, the effect of PD98059 on ^{3}H-thymidine incorporation was more pronounced in ATP-stimulated cells than in PMA-stimulated cells, which indicates that activation of PKC leads also to MAP kinase independent effects on DNA-synthesis.

4.5 Nucleotide-evoked expression of immediate early gene products c-Fos and c-Jun

Many mitogens activate the expression of immediate early genes (IEGs) (Herschmann, 1991). We wanted to examine whether the nucleotides that induced ^{3}H-thymidine incorporation also could induce the expression of the protooncogenes c-Fos and c-Jun. ATP, UTP, UDP and ATPγS dose-dependently induced the expression of c-Fos and c-Jun proteins as assessed by Western blotting (V, Fig. 4A). The effect of αβ-meATP was weak and 2-MeSATP was totally ineffective in these experiments. The expression of c-Fos and c-Jun peaked at 1 hour, and no expression could be seen after 4 hours (IV, Fig. 6A). The expression of c-Fos and c-Jun induced by ATP, UTP, UDP and ATPγS was decreased in cells pretreated with PTX (V, Fig. 4B).

We then wanted to investigate by which mechanisms ATP induced the expression of c-Fos and c-Jun. The effect of Ca^{2+} was investigated using EGTA (3 mM), BAPTA (20 μM) or Ni^{2+} (4 mM), all of which decreased the ATP-induced expression of c-Fos and c-Jun (V, Fig. 5A). These results suggested that Ca^{2+} is important for c-Fos/c-Jun expression. To investigate the mechanism of Ca^{2+} regulation in expression, the cells were treated with the calmodulin inhibitors calmidazolium (30 μM) and fluphenazine (30 μM), or the CaMK II inhibitor KN-62 (10 μM). However, these treatments did not have an effect on the ATP-induced c-Fos and c-Jun expression (data not shown).

The possible role of PKC in protooncogene expression was then examined. Stimulation of the cells with PMA (200 nM) induced the expression of c-Fos and c-Jun (V, Fig. 5B). Furthermore, the ATP-induced c-Fos and c-Jun expression was decreased by PKC inhibition using the PKC-inhibitors H-7 (30 μM) or GF109203X (0.01-10 μM), or by a prolonged incubation with PMA (2 μM for 24 h; V, Fig. 5B), indicating that PKC was important for the ATP-induced c-Fos/c-Jun expression.

MAP kinase may regulate cell division and AP-1 activity (Karin, 1995). We showed that inhibition of MEK by PD98059 (30 μM) decreased the expression of c-Fos and c-Jun in response to both ATP and PMA (V, Fig. 5E), suggesting that this pathway takes part in protooncogene regulation.

4.6 Correlation between DNA-synthesis and c-Fos and c-Jun expression

The obtained results show that ATP induces ^{3}H-thymidine incorporation and the expression of c-Fos and c-Jun genes, and these effects are in part regulated by the same mechanisms (i.e. an increase in $[Ca^{2+}]_i$, activation of MAPK). However, some findings on the regulation of c-Fos and c-Jun expression are not consistent with the ^{3}H-thymidine incorporation results (i.e. inhibition of PKC, inhibition of calmodulin/CaM kinase II), and challenge the assumption that these phenomena are strictly connected. Clearly, the expression of these genes is not sufficient for the cells to commit to divide, since the expression was also induced in quiescent cells where no ^{3}H-thymidine incorporation occurs in response to ATP.

In dog thyrocytes, carbachol, which activates the phosphatidylinositol-Ca^{2+}-pathway, induced the expression of c-fos and c-myc genes but did not induce DNA-synthesis even in the presence of insulin (Raspé et al., 1992). Also, in dog thyroid cells, mitogens using different pathways regulate the expression of protooncogenes in a differential manner. Whereas the expression of c-jun and jun D were induced by protein tyrosine kinase and PKC, TSH and cAMP downregulated c-jun expression but still exerted a synergistic action on the proliferation together with the other mitogenic pathways (Reuse et al., 1991). In those cells the expression of c-jun does not correlate with cell proliferation, but instead, junB was found to correlate best with the proliferative phase (Roger et al., 1997). However, it was suggested that c-jun could be involved in the negative control of expression of differentiation. On the other hand, in human thyroid cells, an association between cell proliferation and the expression of c-fos and c-jun has been suggested in TSH, PMA and EGF-stimulated cells (Heinrich and Kraiem, 1997). In a previous study of FRTL-5 cells, it was shown that there is no simple correlation between the ability of TSH, insulin, IGF-I, PMA and α_1-agonists to increase c-fos/c-myc expression, increase cell number or induce DNA-synthesis, and at least the insulin/IGF-I evoked protooncogene expression could be coupled to functional responses, such as thyroglobulin gene expression (Isozaki and Kohn, 1987). Whether the c-fos and c-jun gene expression is needed in the ATP-enhanced DNA-synthesis, needs further investigations.

4.7 ATP-evoked comitogenesis: possible mechanisms

The observation that ATP itself is not sufficient to trigger mitogenesis in FRTL-5 cells is in agreement with studies performed in other cell types. A role for ATP as a competence factor has been suggested in cells where ATP has the ability to induce limited progression of the cell cycle from G_0 to G_1 (Malam- Souley et al., 1993). Contradictory conclusions were reached by Migyagi et al. (1996) showing that UTP and ATP act as progression factors rather that competence factors, by their action at a P_{2U} receptor. It was hypothesized that different P2 receptor subtypes could regulate cell cycle at different levels, and the subtypes may be differentially expressed during the cell cycle depending on the culture conditions and comitogens and growth factors (Abbracchio and Burnstock, 1998).

Most proliferative growth factors are capable of activating a number of signaling pathways, one of which is the phosphatidylinositol-Ca^{2+}-pathway. It has been suggested that activation of the phosphatidylinositol-Ca^{2+}-pathway may cause cell division (Berridge, 1995). However, there are also contradictory reports showing

that there is no correlation between the activation of this pathway and increased cell proliferation. In vascular smooth muscle cells, ATP was shown to increase Ca^{2+} concentrations, but the mitogenic response was not mediated through phospholipase C (Erlinge et al., 1993). Carbachol and bradykinin, another activator of the phosphatidylinositol-Ca^{2+}-pathway, were inefficient in inducing DNA-synthesis in dog thyrocytes even in the presence of insulin and serum (Raspé et al., 1992), which, thus, indicates differences between thyroid cells from different species. In conclusion, if a possible mitogen produces Ca^{2+} signals, it does not necessarily mean that Ca^{2+} has a direct role in initiating proliferation. However, Ca^{2+} nevertheless has a general role in regulating various steps in the cell cycle.

The mechanism by which GPCRs regulate cell proliferation remains poorly understood (Gutkind, 1998). Several studies have shown that some GPCRs may stimulate ligand-independent tyrosine-phosphorylation of the PDGF receptor (Duff et al., 1992), the IGF-1 receptor beta subunit (Rao et al., 1995), and the EGF receptor (Daub et al., 1996). It has been suggested that the RTKs may function as a scaffolding structure or as an adaptor protein to which other signaling proteins may be recruited in response to GPCRs signaling (Daub et al., 1996). Whether this could be the mechanism for the effect of ATP in FRTL-5 cells, remains to be examined.

SUMMARY AND CONCLUSIONS

Increasing evidence shows that extracellular ATP is essential for the normal physiology of many cells and tissues, and that it is a potent agonist in endocrine cells. Extracellular nucleotides have been shown to have effects in thyroid cells of different species. The present study deals with the effects of extracellular ATP in FRTL-5 cells, an established rat thyroid cell line. Two main areas were examined: 1) the regulation of PLA_2 activity and the interaction of this pathway with the PLC-signaling pathway, and 2) the mitogenic effect of ATP. It is clear that on the basis of experiments carried out using a cell line, direct conclusions concerning the function of the thyroid cells in vivo cannot be made. This experimental cell model nevertheless serves as a model for the elucidation of intracellular signal transduction pathways in thyroid cells.

We showed that extracellular ATP regulates PLA_2 activation and AA release through a PTX-sensitive $G_{i/o}$ protein-coupled receptor. An increase in $[Ca^{2+}]_i$ and activation of the MAP kinase cascade are also important for the ATP-evoked AA release. Furthermore, the AA release is cAMP-insensitive. The metabolism of AA may be important for the ATP-evoked Ca^{2+} signaling, since blocking of noncyclooxygenase enzymes resulted in an inhibition of the ATP-induced Ca^{2+} transients, and suggests that some AA metabolite(s) regulates the ATP-evoked Ca^{2+} signals. On the other hand, free long chain unsaturated fatty acids, which may also be liberated by the action of PLA_2, inhibit the ATP-evoked Ca^{2+} signals, suggesting a regulatory role for these fatty acids. A summary of the presently reported effects of ATP on PLA_2 and PLC pathways is shown in Figure 8. Although the physiological role of the interaction of PLC and PLA_2 remains to be studied in these cells, both pathways have been shown to take part in the regulation of for example iodide efflux in response to ATP. Thus, ATP could be a regulator of thyroid cell functions through these signaling pathways.

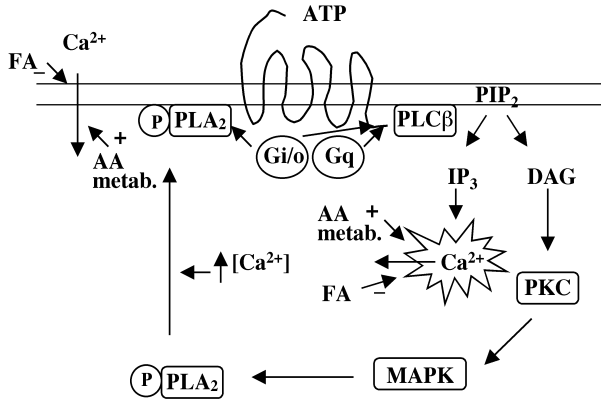

Figure 8. Summary of the interaction between PLC and PLA_2 pathways in response to ATP in FRTL-5 cells.

ATP acts as a co-mitogen by enhancing the effect of insulin and TSH on DNA-synthesis. ATP also induces proliferation in insulin-stimulated cells. The PLC, but not the PLA_2 pathway, seems to take part in the regulation of this co-mitogenic effect, through the action of Ca^{2+}, but independently of PKC. Also, the effect of ATP was shown to be mediated, at least in part, through the MAP kinase pathway. The regulation of the expression of protooncogenes c-fos and c-jun in response to ATP and other nucleotides did not correlate well with the results obtained by measuring DNA-synthesis, therefore no generalization of the involvement of c-fos and c-jun in mitogenesis can be made. A summary of the effect of ATP on mitogenesis is presented in Figure 9. In conclusion, ATP seems to act also as a long term regulator of thyroid cells in terms of cell proliferation.

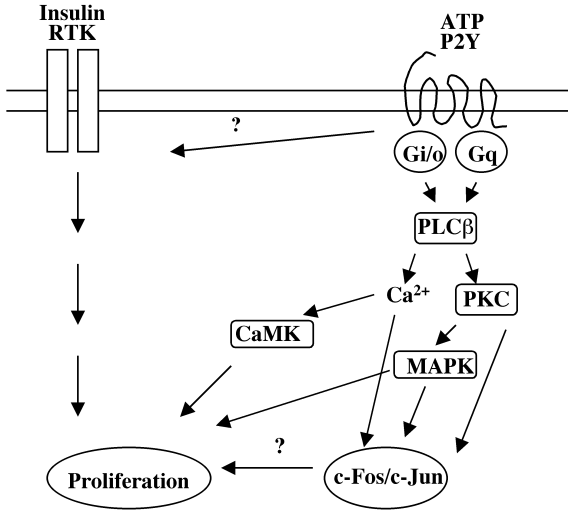

Figure 9. Summary of the co-mitogenic effect of ATP on FRTL-5 cell proliferation.

An unexpected finding was the presence of transcripts for six different P2 receptor subtypes, the G protein-coupled $P2Y_{2/4/6}$ receptors, and the transmitter-gated ion channel $P2X_{3/4/5}$ subunits, which is far more than could have been expected on the basis of previous pharmacological experiments with these cells. It is not possible at present to assign specific roles for each of the P2 receptors in FRTL-5 cells. However, at least the G protein-coupled P2Y receptor subtypes seem to regulate DNA-synthesis and protooncogene expression in these cells. It remains to be examined whether the detected P2X receptor transcripts are translated and form functional receptors. Taken together, this is the first report showing P2 receptor subtypes in thyroid cells. The present results provide a basis for future studies in define the role of the different P2 receptor subtypes in the thyroid gland.

Recent studies have shown that ATP is constitutively released to the extracellular space from resting cells in vitro. This is an interesting phenomenon as it may indicate a constant para/autocrine effect of the nucleotides on the cells. Albeit the source(s) of extracellular nucleotides remains to be examined in the thyroid gland, ATP is clearly an important regulator of thyroid cell functions, and the interaction of ATP with other signaling pathways a challenging subject of future studies.

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