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Department of Physiology
University of Göteborg

ADENOSINE AND OVARIAN FUNCTION

Studies on adenosine as substrate and receptor agonist in the rat ovary

HÅKAN BILLIG

Göteborg 1988

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substrate and receptor agonist in the rat ovary

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I Gonadotropin depression of adenosine triphosphate levels and interaction with adenosine in rat granulosa cells.
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Endocrinology, 118:645-652, 1986.

II Gonadotropin-induced inhibition of oxygen consumption in rat oocyte-cumulus complexes: Relief by adenosine.
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III Adenosine receptor-mediated effects by non-metabolizable adenosine analogs in preovulatory rat granulosa cells: A putative local regulatory role of adenosine in the ovary.
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V Adenosine receptor-mediated effects on adenylate cyclase activity in rat luteal tissue. A putative local regulatory role of adenosine in corpus luteum.
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ABSTRACT

BILLIG, H. ADENOSINE AND OVARIAN FUNCTION. Studies on adenosine as substrate and receptor agonist in the rat ovary. pages 1-46
Department of Physiology, University of Göteborg, Box 33031, S-400 33 Göteborg, Sweden.

Adenosine is an indispensable compound in cell energy metabolism, as precursor to cofactors, second messenger and nucleic acids. Adenosine is also an agonist to adenosine receptors. The adenosine receptor can either inhibit (A_1) or stimulate (A_2) adenylate cyclase. Alternatively, in some cells adenosine receptor activation is linked to other cellular events like inhibition of Ca^{2+} fluxes.

In the present study the possible dual effects of adenosine as substrate and adenosine receptor agonist were investigated in rat granulosa cells, cumulus-oocyte complexes, luteal cells and ovarian membranes.

Adenosine was taken up by isolated preovulatory granulosa and luteal cells from PMSG-treated immature rats but follicle stimulating hormone (FSH) decreased the uptake by granulosa cells. Adenosine, but not the non-metabolizable adenosine analogs 5'-(N-ethyl)carboxamido-adenosine (NECA), 2-chloro-adenosine (2-Clado), N^6-(R-phenyl-isopropyl)-adenosine (R-PIA) and N^6-(S-phenyl-isopropyl)-adenosine (S-PIA), increased granulosa cell ATP levels. FSH and luteinizing hormone (LH) decreased granulosa cell ATP levels in the presence and absence of adenosine.

It has previously been shown that FSH and LH decrease oxygen consumption by cumulus-oocyte complexes and increase their lactate production. These effects have been suggested to be due to a competition of cofactors (e.g. ADP) common to glycolysis and respiratory chain. The fact that adenosine reversed the gonadotropin-induced effects on oxygen consumption and lactate production support this theory.

Adenosine and its analogs increased cAMP accumulation in luteal and granulosa cells only in the presence of gonadotropins and this effect was antagonized by the adenosine receptor antagonist 8-phenyl-theophylline (8-PHT). The EC_{50} for NECA on FSH stimulated cAMP accumulation was 40 μM. Adenylate cyclase was stimulated by adenosine analogs in membranes from non-luteinized, luteinized ovarian membranes and in luteal cell homogenate (EC_{50} 0.28-0.65 μM for NECA). The effect of NECA was antagonized by 8-PHT. In the membranes the rank order of potency was NECA > 2-Clado > R-PIA > S-PIA, suggesting adenosine A_2 receptors.

In summary adenosine acts both as a substrate to intracellular metabolism and as an adenosine A_2 receptor agonist in granulosa and luteal cell. A paracrine short loop positive feedback model was proposed where extracellular adenosine, derived from gonadotropin-induced extracellular increases in cAMP and decreases cellular ATP, enhanced exogenous hormone stimulation in granulosa and luteal cells.

Key words: Adenosine, adenosine analogs, A_2 adenosine receptor, adenylate cyclase, ATP, lactate, oxygen consumption, granulosa cells, cumulus-oocyte complex, luteal cells, rat

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LIST OF PUBLICATIONS

The present thesis is based on the papers listed below. These papers will be referred to in the text by their Roman numerals.

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ADA	adenosine deaminase
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BSA	bovine serum albumin
cAMP	cyclic-3',5'-adenosine monophosphate
2-Clado	2-chloro-adenosine
dbcAMP	dibuturyl cyclic-3',5'-adenosine monophosphate
DIP	dipyridamol
EC_{50}	half maximal effective concentration
FAD	flavin adenine dinucleotide
FFA	free fatty acid
FSH	follicle stimulating hormone
GTP	guanosine triphosphate
hCG	human chorionic gonadotropin
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half maximum inhibitory concentration
IU	international units
K_m	Michaelis-Menten constant
LDH	lactate dehydrogenase
LH	luteinizing hormone
NAD	nicotinamide-adenine dinucleotide
NECA	5'-(N-ethyl)carboxamido-adenosine
PBS	phosphate buffered saline
PDE	phosphodiesterase
8-PHT	8-phenyltheophylline
PMSG	pregnant mare's serum gonadotropin
PSOT	8-(p-sulfo)-phenyltheophylline
Q_{10}	relative change in the transport rate constant per 10C increase in temperature
RIA	radioimmunoassay
R-PIA	N^6-(R-phenyl-isopropyl)-adenosine
s.c.	subcutaneous(ly)
SD	standard deviation
SEM	standard error of the mean
S-PIA	N^6-(S-phenyl-isopropyl)-adenosine
V_{max}	maximum velocity

INTRODUCTION

The endocrine regulation of ovarian function is classically considered to be an integrated cyclic interplay between the ovary, in its various functional stages, and the pituitary gland. The ovarian functional cyclicity is interrupted during pregnancy when placental hormones interact with the pituitary secretion of gonadotropins and ovarian steroid production.

Gonadotropins

The gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are secreted from the anterior lobe of the pituitary gland in a cyclic manner during fertile, non-pregnant periods. The gonadotropins meet the classical definition of a hormone: they are produced in certain cells, secreted into and transported by the blood to the target cells where they elicit their effects without being metabolized as a prerequisite for the hormonal effect to occur (Starling, 1905a, b).

On the external surface of ovarian cell membranes specific receptors selectively bind LH or FSH. The hormonal stimulation is mediated in the cells by second messengers. The most well investigated second messenger for gonadotropins in ovarian cells is cyclic-3',5'-adenosine monophosphate (cAMP). Stimulation of the gonadotropin receptor activates membrane adenylate cyclase converting adenosine triphosphate (ATP) to cAMP. Cyclic AMP then binds to protein kinase A (PKA) which phosphorylates specific proteins. These proteins are believed to further mediate the different cellular responses of the gonadotropins. Under certain conditions also other second messenger systems mediate gonadotropin stimulation, e.g. the phosphoinositol turnover activates protein kinase C (PKC).

LH and FSH have different physiological actions. FSH is considered to initiate the recruitment of follicles from a pool of small, preantral follicles which proliferate and differentiate first to antral and later to preovulatory follicles. FSH also stimulates follicular metabolism (e.g. steroid production) and differentiation (e.g. LH receptor development). However, LH also has a role for the differentiation of theca cells and thus for the supply of substrate for aromatization of androgens to estradiol in the granulosa cells. LH stimulates the preovulatory follicle to ovulate and induces maturational changes in the oocyte to make it fertilizable. Following ovulation, the remaining follicle differentiates into a corpus luteum. LH also stimulates luteal steroid production as well as other metabolic processes and supports luteal function.

Even though the classical endocrine regulation of the ovary is precise and well tuned in its magnitude and cyclicity, gonadotropin action cannot alone be accounted for induction of all proliferative, differential and metabolic changes in the ovary. For instance, the first stages of follicular development seem to be independent of FSH (Richards, 1980). Furthermore, of the several millions of primordial follicles present at birth only a minute fraction will develop to preovulatory follicles. The majority of follicles will become atretic and never ovulate despite of the same gonadotropin environment as the subsequently ovulating follicles (Tsafriri and Braw, 1984).

Local intercellular messenger molecules

During the last decade attention has focused on the local regulators of ovarian function (Channing and Segal, 1982; Sharpe, 1984; Tsafriri, 1987). Within the gonads a multitude of different molecules locally produced can influence or regulate the neighboring cells or the producing cell itself (reviewed by Tsafriri, 1987). These factors do not make up one homogeneous group and a variety of effects elicited by these local factors have been registered. Among these factors/hormones in the ovary we find steroids, prostaglandins, growth factors (e.g. EGF, IGF-I, PDGF, TGFβ), catecholamines and others that do not belong to the mentioned groups (e.g. GnRH) or that have not yet been chemically defined.

These local intercellular messenger molecules have been proposed to be intraovarian hormones (cf Tsafriri, 1987), albeit they do not fulfill the criteria for a hormone of the classical endocrine type. The distance between production and release, on the one hand, and the target cell and hormone-induced effects, on the other hand, is minute for "local hormones" as compared to classical hormones such as FSH and LH. Furthermore, the "local hormones" are not necessarily transported via the blood to reach their target cell as the gonadotropins are. Moreover, the production of many of the "local hormones" are modulated by the classical hormones and their effects may be influenced by the "local hormones". The intraovarian hormones resemble the factors described as paracrine (Dockray, 1979) and autocrine hormones (Sporn and Todaro, 1980).

Adenosine as messenger molecule

One substance that could be regarded as an intraovarian hormone is adenosine. In addition to being a precursor to many compounds crucial for life (ATP, RNA, DNA, NAD^+, cAMP) adenosine is an agonist to extracellular receptors in many cells. Activation of adenosine receptors can regulate adenylate cyclase activity (Londos and Wolff,

1977; van Calker et al., 1979) and Ca^{2+} fluxes (Riberio and Sebastiao, 1986).

In general, the adenosine action as receptor agonist, besides being substrate for intracellular metabolism, closely resembles the requirements for paracrine and autocrine action (Sporn and Tordaro, 1980). Adenosine is released locally, affects adjacent cells or the releasing cell itself and the release is locally regulated. Adenosine has also been proposed to be a local hormone (Arch and Newsholme, 1978) or local "retaliatory metabolite" (Newby, 1984).

Several decades ago it was shown that infusion of adenosine or AMP reduced heart rate, blood pressure, inhibited intestinal movements and dilated coronary vessels in a number of animals (Drury and Szent-Györgyi, 1929). Since then adenosine has been shown to elicit a number of effects in adipose, neural, cardiac, hepatic, nephric, skeletal, endocrine and vascular tissue (Burnstock, 1978; Daly, 1983 and references therein). In many instances a paracrine/autocrine relationship between the adenosine-releasing cell and the target cell has been postulated or established.

In blood vessels, adenosine exhibits the well-documented effect of vasodilation (Berne, 1964; Fredholm and Sollevi, 1986). Increase in adenosine is believed to be caused by decreased oxygen tension and, consequently, decreased ATP production. The ATP consumption results in increasing AMP levels and, instead of re-phosphorylation, this will result in an egress of AMP and adenosine from the tissue (Arch and Newsholme, 1978; Newby, 1984). The increase in extracellular adenosine stimulates adenosine receptors and dilates the vessels, thereby restoring the blood flow and oxygenation of the tissue (Newby, 1984; Bruns et al., 1987). Adenosine receptor-mediated effects on cAMP production have been demonstrated in endothelial and smooth muscle cells and also adenosine receptor-mediated inhibition of sympathetic vasoconstrictor nerve activity (Fredholm and Sollevi, 1986). Increased vascular adenosine levels induces dilation in coronary, skeletal, brain and gastrointestinal vessels and increased blood flow and, hence, increased and restored oxygen tension. However, in the kidney adenosine has the opposite effect and induces vasoconstriction (Berne et al., 1983; Fredholm and Sollevi, 1986). These effects in blood vessels are mediated via adenosine receptors (Kusachi et al., 1983).

Adenosine has been shown to be released as a co-transmitter and to have both pre- and post-synaptic adenosine receptor-mediated effects. In neural tissue adenosine receptor activation has been shown to both stimulate and inhibit cAMP accumulation as well as affect Ca^{2+} mobilization (Phillis and Barraco, 1985 and references therein).

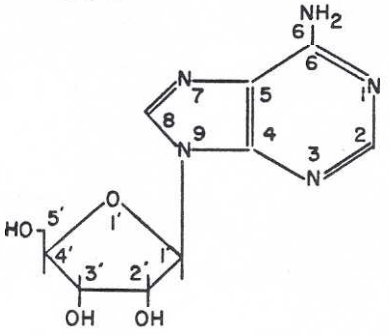
An anti-lipolytic effect by adenosine has been established in adipose tissue. Adenosine receptor activation mediates inhibition of lipolysis and cAMP formation in adipocytes (Fain, 1973; Schwabe et al., 1975; Fredholm, 1978b; Londos et al., 1978).

Also in the gonads effects of adenosine have been observed. For instance, in the testis adenosine receptor binding has been demonstrated and adenosine receptor-mediated effects inhibit FSH-induced cAMP formation and steroidogenesis (Murphy et al., 1983; Monaco et al., 1984; Eikvar et al., 1985). Adenosine causes a number of effects in the ovary such as increase of ATP levels and potentiation of gonadotropin-induced cAMP accumulation and steroidogenesis. These effects of adenosine in the ovary have been claimed to be due primarily to adenosine functioning as a substrate for intracellular metabolism (Behrman et al., 1986). Even though the effects of adenosine in ovarian cells in vitro have been claimed to be due to metabolizable adenosine and not to adenosine-receptor mediated effects, the possibility that adenosine acts as an adenosine receptor agonist in a paracrine/autocrine manner also in the ovary has not been thoroughly investigated.

Adenosine and cell metabolism

When studying the possible role of adenosine in a tissue, e.g. the ovary, knowledge of this molecule and its metabolism is necessary. A short review of some of the current data concerning the general fate and function of adenosine is therefore adequate.

Adenosine is a nucleoside which consist of a nitrogenous purine base (adenine) and a sugar (D-ribose). The nucleotides AMP, ADP and ATP are phosphorylated and derived by estrification of the 5'-hydroxyl group in the pentose moiety of adenosine (fig 1).



Adenosine

Fig 1

Adenosine has a fundamental role in cell metabolism. The fate of adenosine in the cell is complex but essential for cell function. Procaryotic, eucaryotic and plant cells all utilize adenosine in energy transfer within the cell. Adenosine is also one of the precursors for nucleic acids and the synthesis of RNA and DNA. Moreover, coenzymes like FAD and NAD^+ are adenosine products. Cyclic AMP, a second messenger formed from ATP, is the intracellular link between several hormones and their cellular effects. Adenosine also has a role in receptor-mediated intercellular communication, e.g. neural signal transmission, metabolically induced changes in tissue blood flow and as a modulator of cellular metabolism (fig 2).

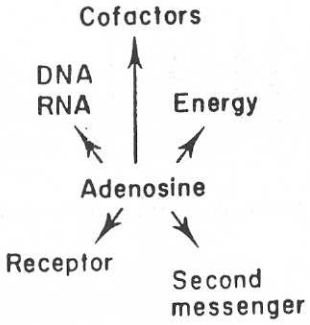


Fig 2

Adenosine metabolism

The formation of adenosine is accomplished through several pathways like de novo synthesis, dephosphorylation of AMP and degradation of S-adenosylhomocysteine (fig 3). AMP is the most likely contributor of the bulk of "recirculating" adenosine within the cell. In general, the net addition of adenosine and purines in the cell is probably not derived from de novo synthesis in the cells but rather a result of uptake from the blood. The most likely local source for adenosine in the peripheral tissues is the erythrocyte. Red blood cells have the ability to take up purines in the liver, which is believed to produce purines and release them into the vessels (Murray et al., 1970). It is also worth noting that adenosine does not seem to have a dietary origin (Salati et al., 1984).

Adenosine deaminase (ADA) degrades adenosine to inosine (fig 3 and 4) and this enzyme has recently been cloned and sequenced (Wiginton et al., 1986). ADA is present in the cytosol of all tissues examined and ADA activity is also

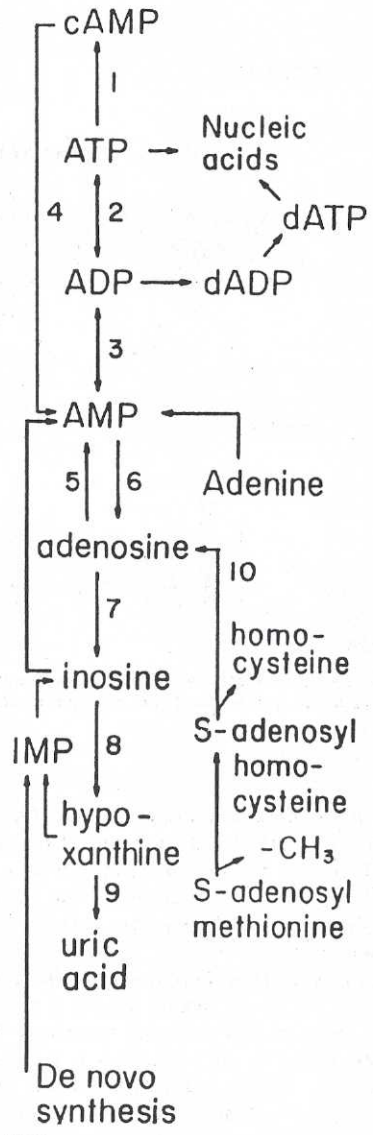


Fig 3

Formation and degradation of adenosine. For further details on localization and action see text.

Nr	Enzyme
1	adenylate cyclase
2	nucleoside diphosphokinase
3	adenylate kinase
4	phosphodiesterase
5	adenosine kinase
6	5'-nucleotidase
7	adenosine deaminase
8	purine nucleoside phosphorylase
9	xanthine oxidase
10	S-adenosylhomocysteine hydrolase

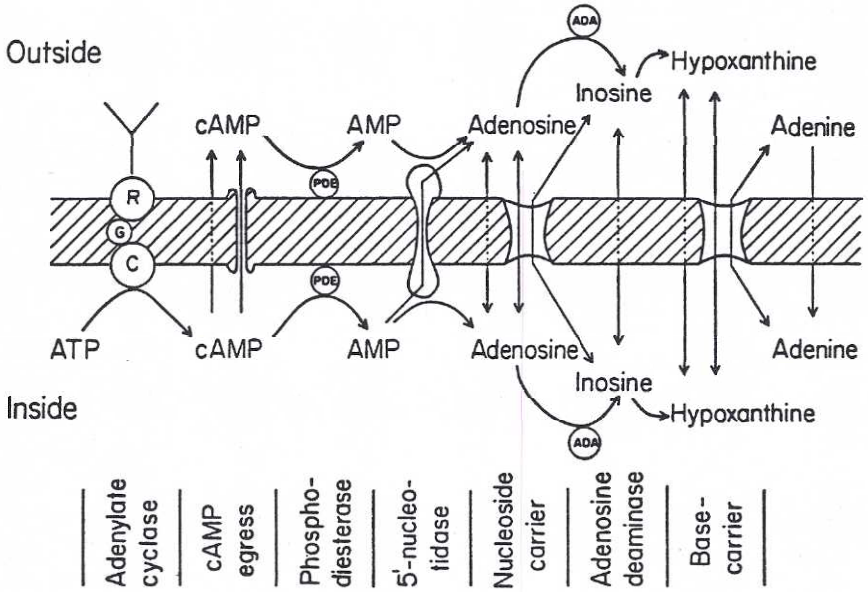


Fig 4

Schematic presentation of adenosine metabolism and possible routes for transmembrane fluxes of adenosine and related compounds. For further details see text.

present in the extracellular space. The K_m for adenosine in this reaction ranges from 35 to 400 μM (Fox and Kelley, 1978) in different tissues. The 5'-hydroxyl group of adenosine seems to be a requirement for the enzyme activity (Nair and Weichert, 1980).

Adenosine is phosphorylated by adenosine kinase to AMP. Adenosine kinase is also present in the cytosol of all tissues examined. The K_m for adenosine in this reaction is 0.4-6μM (Fox and Kelley, 1978). Adenosine kinase accepts a wide range of adenosine analogs, but the enzyme seems to require an intact 5'-hydroxyl group of adenosine (Yamada et al., 1980).

Other enzymes important for adenosine turnover are 5'-nucleotidase and phosphodiesterase (PDE). The enzyme 5'-nucleotidase catalyzes the dephosphorylation of AMP to adenosine. This enzyme is present both in the plasma membrane and in the cytosol and can dephosphorylate both intra- and extracellular AMP (Bruns, 1980; Pearson et al, 1980). In different tissues the K_m for this reaction is reported to be in the range from 10 to 123 μM (Arch and Newsholme, 1978; Fox and Kelley, 1978). Free ADP and ATP in micro-molar concentrations inhibit 5'-nucleotidase. However, almost all ADP and ATP in the cell is complex-bound to Mg^{2+} and these complexes do not seem to inhibit the enzyme activity (Arch and Newsholme, 1978).

AMP is not only a product of ATP and ADP dephosphorylated in cellular energy transfer, but also of degradation of cAMP catalyzed by PDE. It is present both in the cytosol and at the external surface of the plasma membrane and consequently converts both extra- and intracellular cAMP to AMP (Rosberg et al., 1975; Arch and Newsholme, 1978; Barber and Butcher, 1983). In the testis and the ovary the k_m is approximately 2 μM (Conti et al., 1981, 1982, 1983, 1984). In the gonads there are also other PDE enzymes that catalyze the conversion of cGMP to GMP and one form that is calmodulin-calcium dependent (Purvis et al., 1981). Adenosine in milli-molar concentrations has been shown to inhibit PDE to some extent (Gulyassy, 1971). Adenosine (0.2-2 mM) has been demonstrated to augment ACTH-stimulated steroidogenesis in normal rat adrenocortical tissue (Shönbaum et al., 1959) and in isolated rat adrenocortical cells (Cooper and Gleed, 1978). The latter authors concluded that the augmented steroidogenesis was a result of adenosine inhibition of phosphodiesterase since theophylline, a PDE inhibitor, acted synergistically with adenosine.

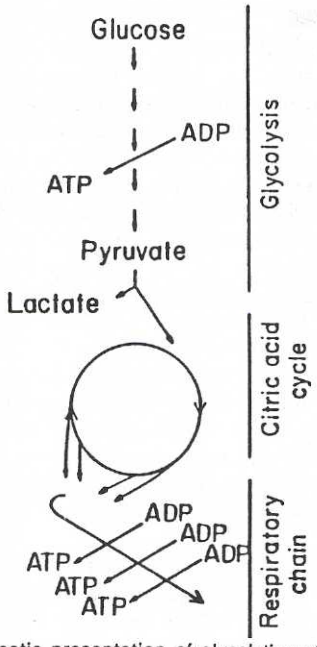


Fig 5

Schematic presentation of glycolytic pathway, citric acid cycle and respiratory chain.

ATP production

Glycolysis and the respiratory chain are pathways where ADP is phosphorylated (fig 5). Glycolysis converts glucose to pyruvate in the cytosol. If sufficient oxygen is present, pyruvate will be further degraded in a series of energy-yielding reactions in the citric acid cycle (Krebs cycle; Krebs and Johnson, 1937) in the mitochondria. The citric acid cycle is the common pathway for the oxidation of glucose, fatty acids and proteins. The NADH and $FADH_2$ transfer energy (i.e. electrons) from the citric acid cycle to the respiratory chain. Electrons are transferred to O_2 from NADH and $FADH_2$ by a series of electron carriers to form ATP and H_2O. This reaction is the process in which the largest amount of ADP is phosphorylated. Pyruvate is converted to lactate when the rate of production of pyruvate exceeds the rate of pyruvate oxidation in the citric acid cycle. In other words, when the NADH production in the glycolysis and citric acid cycle is greater than the oxidation rate in the respiratory chain lactate is formed in the cell.

cAMP formation

Upon hormonal stimulation, adenylate cyclase (fig 4) converts ATP to cyclic AMP (cAMP; Rall et al., 1957). cAMP is an intracellular second messenger to extracellular hormonal stimulation mediating a variety of physiological responses depending on the type of hormone. The activation of adenylate cyclase involves a specific hormone receptor (R) at the external surface of the cell membrane, the catalytic component (C) and the regulatory GTP-binding protein (G). The G component is composed of several subunits and can be either stimulatory (G_s) or inhibitory (G_i) on the C component. Thus, stimulation of adenylate cyclase involves R-G_s-C to increase cAMP production, while inhibition of adenylate cyclase involves G_i instead of G_s (Gilman, 1987). A variety of hormones, among them gonadotropins, activate adenylate cyclase.

Ectoenzymes

Enzymes that are localized at the external surface of the plasma membrane and metabolize extracellular substrates are called ectoenzymes. Both 5'-nucleotidase and PDE are ectoenzymes (fig 4). Other ectoenzymes important in this context are nucleoside triphosphatase and nucleoside diphosphatase which catabolize extracellular ATP and ADP, respectively (Manery and Dryden, 1979; Pearson et al., 1980). Thus, adenosine can be derived from extracellular ATP, ADP, AMP and cAMP when these catalyzing enzymes are present and functional (Rosberg et al., 1975; Selstam and Rosberg, 1976; Arch and Newsholme, 1978; Pearson et al., 1980; Bruns, 1980; Barber and Butcher, 1983).

Uptake of adenosine into the cell

Adenosine and other nucleosides can be transported from the extracellular space across the cell membrane into the cell by facilitated diffusion. At physiological concentrations specific nucleoside carrier proteins transport nucleosides. The carrier proteins seem to be present in all mammalian cells (Arch and Newsholme, 1978; Fox and Kelley, 1978; Young and Jarvis, 1985). Nucleosides can also be transported by simple diffusion. Purine and pyrimidine bases share a common carrier protein distinct from the nucleoside carrier protein (Berlin and Oliver, 1975).

As has been stressed by many authors, the rate of translocation of nucleosides, like adenosine, from the exterior to the interior of the cells is dependent on two processes, the transport mechanism itself and the intracellular metabolization (Berlin and Oliver, 1975). The intracellular concentrations of adenosine are very low, since the nucleoside is rapidly metabolized and a concentration gradient over the cell membrane is maintained. Thus, the translocation of adenosine is dependent on the kinetics of the carrier as well as the

kinetics of the intracellular adenosine kinase and ADA. This combined activity has been referred to as nucleoside *uptake* (Berlin and Oliver, 1975; Arch and Newsholme, 1978). In most studies on adenosine fluxes, nucleoside metabolism has not been inhibited resulting in kinetic data which represent both transport over the cell membrane and metabolism.

Adenosine uptake is reversibly and competitively inhibited by other nucleosides since they use the same carrier. Other compounds, not using the nucleoside carrier for transport but inhibit uptake are dipyridamole (DIP), papaverine, hexobendine and reserpine (Arch and Newsholme, 1978). These latter inhibitors are not solely specific for nucleoside uptake. For instance, in higher concentrations DIP may also inhibit phosphate fluxes. Also other substances not primarily used as transport inhibitors may interfere with the uptake, for example, the commonly used solvent dimethyl sulfoxide (DMSO) (Berlin and Oliver, 1975 and references therein).

Release and removal of adenosine from the cell

There are three principal ways in which adenosine is removed from the cell: deamination, phosphorylation or release to the extracellular space as adenosine or adenosine derived compounds (AMP, ATP, cAMP).

Since adenosine is transported by facilitated diffusion, adenosine efflux is dependent on higher intracellular than extracellular concentrations. Another suggested possibility for efflux of adenosine is the association of 5'-nucleotidase with the plasma membrane and its use of intracellular AMP as substrate for adenosine release into the extracellular space. Thus, 5'-nucleotidase functions both as a catalyzing enzyme and as a carrier for adenosine (Arch and Newsholme, 1978). Both mechanisms may exist and both require increased cellular AMP levels. Such increased levels of AMP may result from decreased re-phosphorylation due to hypoxia. In addition, AMP levels are also increased by increased "cellular work" (e.g. metabolic, biosynthetic, ion pumping, muscle contraction etc) causing higher ATP consumption or by other situations when the relative balance is shifted from phosphorylation to dephosphorylation.

Extracellular adenosine may also be derived from cAMP. Cyclic AMP release is a common phenomenon during hormone stimulation of adenylate cyclase in many cells (Barber and Butcher, 1983).- Fain and coworkers (1972) proposed that cAMP may be the precursor of extracellular adenosine in adipose tissue. The mechanism for deriving adenosine from cAMP involves the presence of extracellular PDE converting cAMP to AMP, and 5'-nucleotidase which degrades AMP to adenosine. This hypothesis was abandoned later when they found no evidence for hormone-induced adenosine release in fat cells (Fain, 1979). However, theoretically the proposal may still be valid for other tissues.

Adenosine levels

Under normal conditions the activity of the adenosine metabolism results in unmeasurable or very low concentrations of adenosine within the cell (Henderson, 1979). Outside the cell detectable amounts of adenosine can usually be found in the intercellular fluid, plasma or, under experimental conditions, in the medium. Under normal conditions the concentrations have been reported to be between 0.03-2.6 μM in mammalian body fluids and 1-30 nmol/g wet weight (approximately 1-30 μM) in tissue (reviewed in Arch and Newsholme, 1978). However, both the treatment of the samples and the sampling procedure are critical since adenosine levels increase dramatically in hypoxic tissue. Adenosine is also subject to rapid degradation by endogenous enzymes present in the tissue such as ADA. As a consequence, a fairly high degree of variation in adenosine levels has been reported.

Considerable species differences have also been reported, the levels of adenosine in plasma ranging from less than 0.015 μM in the cat to 0.52 μM in the rat. There are also strain differences in plasma adenosine levels within the same species (Fredholm, 1980b). Despite this, the estimation of physiological concentrations of extracellular adenosine is of crucial importance for judgment of the validity of experimental data, in particular those evaluating putative adenosine receptor actions. In this context, it is of interest to note that very little adenosine escapes via the general circulation from the organs and cells, where it is produced and released. The quantitatively most important removal of adenosine from the extracellular space is re-uptake by the cell from which it was released or by neighboring cells.

Adenosine receptors

As previously mentioned adenosine is not only a substrate but also exerts action without being metabolized. Adenosine conjugated to large molecules that are thought not to enter cells mimics some of the actions of free adenosine (Olsson et al., 1976, 1977; Schrader et al., 1977; Fain and Shephard, 1979). These data, in combination with the variety of documented cellular effects of adenosine, were the basis for the concept of cell surface receptors for adenosine. Different classes of purine and adenosine receptors have been identified based on agonist specificities and

functional responses.

Purinoceptors

Burnstock (1978) proposed the existence of two groups of membrane purinergic receptor sites based on the relative selective action of agonists (adenosine, ATP, ADP and AMP) and antagonists, in analogy with the classification of adrenergic receptors (α and β receptors) and histamine receptors (H1 and H2). The receptors were named P1 and P2 purinoceptors (fig 6). He found the P1 adenosine purinoceptor predominant in cardiovascular beds, in the trachea and in the brain, while the P2 purine nucleotide purinoceptor was mainly found in the gastrointestinal tract and urogenital system. The P1 purinoceptor was more sensitive to adenosine than to AMP and less sensitive to ADP and ATP (adenosine ≥ AMP > ADP ≥ ATP). The adenosine action on P1 purinoceptors was competitively antagonized by methylxanthines and the P1 purinoceptor activation was coupled to adenylate cyclase activation. At the P2 purinoceptor the agonists have the reversed rank order of potency (ATP ≥ ADP > AMP ≥ adenosine) and was antagonized by quinidine instead of methylxanthine. Furthermore, activation of the P2 purinoceptor did not involve adenylate cyclase activation (Londos et al., 1983).

Receptors with adenosine as agonist

Londos and Wolff (1977) proposed that adenosine acts on receptors or adenosine-sensitive sites to inhibit or stimulate adenylate cyclase activity. Based on which part of the adenosine molecule that proved important for activation of the sites or receptors they were named R-site, the *r*ibose moiety of adenosine being necessary for the activity, or P-site, the *p*urine moiety being necessary for the activity.

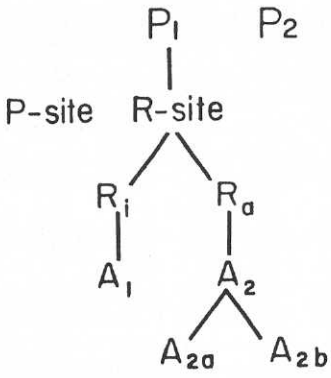


Fig 6

Purine receptor nomenclature

Even though adenosine activated both the R- and the P-sites (fig 6), these sites were distinguished by the use of analogs of adenosine modified in the purine or ribose moiety, respectively. Thus, 2',5'-dideoxyadenosine neither bound nor activated the R-site but was an agonist for the P-site (Haslam et al., 1978; Londos et al., 1983). The P-site was found to be located on the internal surface of the cell membrane (Haslam et al., 1978). It inhibited adenylate cyclase (Londos and Wolff, 1977) and was not antagonized by methylxanthines. The affinity constant for the P-site was in the low to high micro-molar range for adenosine and analogs (table 1). The physiological significance, if any, of the P-site is unknown, since it is unlikely that intracellular concentrations ever reach micromolar concentrations in the living cell (Daly, 1983).

The R-site was located on the external surface of the cell membrane, and was originally claimed only to stimulate adenylate cyclase (Londos and Wolff, 1977). However, van Calker and coworkers (1979) showed that activation of the R-site in cultured mouse brain cells not only stimulated but also inhibited adenylate cyclase depending on the concentration of adenosine. At concentrations of adenosine above the micromolar range, the cAMP levels in the cultures increased, while submicro-molar concentrations inhibited β-adrenoceptor-stimulated cAMP accumulation. Based on these observations they suggested the name A_1 receptor for those R-sites that mediated the inhibition and A_2 receptors for those that mediated stimulation of adenylate cyclase. They also showed that the A_1 receptor-mediated inhibition was not equivalent to the inhibition mediated by the P-site, since the A_1 receptor-mediated effect was dependent on the integrity of the ribose moiety and was antagonized by methylxanthine. Londos and coworkers (1980) confirmed that the R-site did have subclasses by studying adenosine analog stimulation of adenylate cyclase activation in hepatocytes and Leydig tumor cells and inhibition in adipocytes. They designated the subclasses R_i and R_a for the adenosine receptors mediating inhibition and stimulation of adenylate cyclase, respectively. In the literature, A_1 and R_i are used as equivalent entities, and so are A_2 and R_a. The terms A_1 and A_2 will be used in the following discussion (fig 6).

Methylxanthines antagonize adenosine action on the A_1 and A_2 receptors but not adenosine action on the P-site. Adenosine antagonists will be discussed below.

The A_1 and A_2 receptors are not only distinguished by their opposite effects on adenylate cyclase, but also by the ranking of potencies of the different adenosine analogs. Some authors stress that the latter criterion for classification

Table 1

property	Type of receptor		
	A_1	A_2	P-site
Location	outer cell surface	outer cell surface	cytoplasmatic cell surface
Effect on AC	inhibition	stimulation	inhibition
Purine modified adenosine analogs	agonist	agonist	poor effector
Ribose modified* adenosine analogs	poor effector	poor effector	agonist
GTP	required	required	not required
Relative potency	R-PIA>NECA	NECA>R-PIA	-
NECA: EC_{50} range	100-500nM	20nM-2μM	-
R-PIA: IC_{50} range	5-10nM	200nM-100μM	-
Methylxanthine	antagonist	antagonist	no effect

*NECA, in contrast to most ribose-modified analogs, is a potent agonist at the A_2 receptor.
Table modified after Londos et al. (1983)

is more correct since most, but not all, the effects of adenosine receptor are mediated via adenylate cyclase (Fredholm, 1982b; Stone, 1983, 1984; Hamprecht and van Calker, 1985). For the most utilized adenosine analogs, the rank order of potency for the A_1 receptor is R-PIA>2-Clado>-NECA>S-PIA and for the A_2 receptor NECA>2-Clado>R-PIA>S-PIA (Daly, 1983). The half-maximal effect and affinity of the adenosine analogs to the receptors also differ between the A_1 and A_2 receptor. The A_1 receptor is considered to be a high affinity receptor with an IC_{50} in the nanomolar range. The A_2 receptor is described as a low affinity receptor with an EC_{50} in the low micromolar range (Daly, 1983; Londos et al., 1983). However, several groups have noted that the A_2 receptor affinity differs considerably between tissues (Daly et al., 1983, Londos et al., 1983; Elfman et al., 1984; Daly, 1985) and it seems likely that the A_2 receptor can be further divided in two subclasses, one high ($EC_{50}<1$ μM) and one low ($EC_{50}>5$ μM;Daly, 1985) affinity A_2 receptor named A_{2a} and A_{2b}, respectively (Bruns et al., 1986, 1987).

The two functionally and pharmacologically distinct adenosine receptor-mediated effects coupled to adenylate cyclase are GTP-dependent like the effects of other hormones coupled to adenylate cyclase stimulation and inhibition (Londos et al., 1979 ;Rodbell, 1980; Londos et al., 1983; Gilman, 1987). However, not all adenosine receptor effects are mediated by adenylate cyclase. It has also been presented data demonstrating non-adenylate cyclase-mediated effects (e.g.; Wallace et al., 1984; Dix et al., 1985; Fredholm et al., 1986; Challiss et al 1987; Fredholm and Lingren, 1987). For instance, in cardiac muscle (Schrader et al., 1975) and in neural tissue (Phillis and Wu, 1981) it has been shown that adenosine receptor activation is coupled to inhibition of Ca^{2+} fluxes.

Adenosine receptor antagonists

Sattin and Rall (1970) studying adenosine-induced cAMP accumulation in guinea pig brain cortex noted that methylxanthines blocked the effect of adenosine. Methylxanthines like caffeine, theophylline and IBMX are classical phosphodiesterase inhibitors (Amer and Kreighbaum, 1975), but they also are adenosine receptor antagonists blocking both A_1 and A_2 receptors. The effect of methylxanthines on adenosine receptors are separated from their effect on phosphodiesterase. Methylxanthines binds to adenosine receptors at concentrations lower than those required for inhibition of phosphodiesterase (Smellie et al., 1979). Furthermore, some alkylxanthines, such as 8-PHT (Griffith et al., 1981), are weak phosphodiesterase inhibitors, but adequate and potent adenosine receptor antagonists. Unfortunately, methylxanthines do not discriminate between A_1 and A_2 receptors (Daly, 1982), but they are not antagonistic to the P-site or to the P2 receptor.

The widespread effects of methylxanthines in the body have primarily been ascribed to their effects on the phosphodiesterase enzyme. Among these effects are CNS activation, enhanced lipolysis, increased renal blood flow, increased release of catecholamines, increased heart rate and force of contraction and inhibited anaphylactic bronchoconstriction. All these can be elicited by caffeine and theophylline, therapeutically or just from extensive coffee drinking. However, Fredholm

(1980a) suggested that these effects are due to adenosine receptor blockade, since opposite effects could be demonstrated with adenosine and its analogs in vivo or in vitro. This view also sheds some light on the physiological importance of adenosine and adenosine receptors. The reverse, that all effects of theophylline and caffeine are due to adenosine receptor antagonism, is probably not true since other effects of these substances may be operative clinically (Fredholm and Sollevi, 1986).

Adenosine receptor regulation

The mechanism for the regulation of the adenosine receptors is not understood. However, a number of studies have been presented on changes in adenosine receptor responsiveness or agonist binding. One example of up-regulation of the adenosine receptor is prolonged treatment with adenosine receptor antagonist (caffeine and theophylline) which increased the adenosine receptor binding to rat cerebral cortical membranes but did not change the adenosine receptor-mediated effect on cAMP accumulation (Fredholm, 1982a; Murray, 1982).

Hormonal regulation of adenosine receptor number and responsiveness has been suggested. Adenosine receptor binding increased in the rat testis up to two month of age (Monaco and Conti, 1986) and hypophysectomy decreased binding (Murphy et al., 1983). Moreover, smooth muscle contractility in the human oviduct was modulated by adenosine analogs and adenosine receptor responsiveness varied with the menstrual cycle (Wiklund et al., 1986). Differentiation of cells may also change the adenosine receptor expression. Preadipocytes expressed A_2 receptors and when the cells differentiated to adipocytes the A_2 receptors decreased and concomitantly A_1 receptors appeared (Ravid and Lowenstein, 1988).

The molecular synthesis or composition of the adenosine receptor are at the present not known. Recently, a number of reports have been presented regarding the size of the A_1 receptor. The reported size of the A_1 receptor in testis using photoaffinity labelling technique was 42 kD (Stiles et al., 1986a), while the receptor size in fat cells and brain was slightly smaller, 38 kD (Stiles et al., 1986b). Another group has presented data for a receptor size of 34 kD protein (Linden et al., 1986) in cerebral cortex, while still others using other techniques suggested a larger molecular size. They also suggested it to be a dimer sized 79.5 kD (Reddington et al., 1987) or 63 kD (Prez-Reyes et al., 1987). The lack of information on the size of the A_2 receptor may be due to the low binding affinity to the A_2 receptor as compared to the A_1 receptor.

AIM OF THE PRESENT INVESTIGATION

Adenosine can be postulated to be involved in a number of cellular events in the ovary based on the above described effects of adenosine in cells in general. For instance, adenosine is an indispensible compound in energy metabolism and the carbohydrate metabolism in ovarian cells, these having a specialized high glycolytic capacity (Ahrén and Kostyo, 1963; Ahrén et al., 1969, 1973, 1976). For example, as much as 80-90% of metabolized glucose is found as lactate in isolated follicles (Hillensjö, 1976), suggesting that the bulk of follicular ATP is derived from the glycolysis. Furthermore, gonadotropins decrease both oxygen consumption in oocyte-cumulus complexes (Hillensjö et al, 1975) and the ATP content in prepubertal ovaries (Ahrén et al., 1968). Addition of adenosine increases the ATP, cAMP and steroidogenesis levels in ovarian cells and the cellular effects of adenosine in the ovary have been proposed to be due mainly to adenosine metabolism (Behrman et al., 1986).

The testis and the ovary, though differing in functional end products, share many metabolic characteristics, such as cAMP production and steroidogenic responses to gonadotropins (Baker et al., 1976; Richards 1980), as well as the lactate production by supportive cells (i.e. Sertoli and granulosa cells; Mita et al., 1982; Le Gac et al., 1983; Billig et al., 1983) and substrate requirements of the germ cells (i.e. spermatocytes and oocytes; Biggers et al., 1967; Kennedy and Donahue, 1969; Zeilmaker et al., 1974; Jutte et al., 1981; Robinson and Fritz, 1981). In the testis adenosine receptors have been demonstrated (Stiles et al.,1986a) and, when activated, these adenosine receptors exhibit functional responses (Monaco et al, 1984; Eikvar et al., 1985). The considerable similarities between the ovary and the testis suggest the presence of adenosine receptors also in the ovary.

The aim of the present investigation was to study adenosine as substrate for cellular metabolism and as agonist to putative adenosine receptors in the ovary.

METHODS

Animals

Immature female Sprague-Dawley rats (Alab Ltd, Stockholm, Sweden) were kept under standardized conditions with lights on between 0500-1900 h and with 55-60% relative humidity. Water and pelleted food were given ad libitum. The animals arrived 2-6 days prior to the initiation of the experiments.

Granulosa cell and cumulus complex isolation and incubation procedures

The animals were given 10 IU pregnant mare's serum gonadotropin (PMSG, NIDDK), s.c., on day 26 of life to induce follicular growth (Cole, 1936; Fuxe et al., 1972) and were killed by cervical dislocation 48-50 h later, before the endogenous LH/FSH surge (Hillensjö et al., 1974; Bauminger et al.,1978; Ekholm and Hillensjö, 1982). Ovaries were isolated aseptically and placed in incubation medium. Cumulus and mural granulosa cells were obtained by incising and gently squeezing the largest follicles in each ovary. Oocyte-cumulus complexes were separated from the granulosa cells with a micro-pipette under a dissection microscope (40x), washed twice, and approximately 20 cumuli, with intact surrounding corona, from the same rat were incubated together.

Granulosa cells from all rats in an experiment were pooled in one test tube, washed once in fresh medium and centrifuged for 5 min at 200xg. The cell concentration was estimated in a hemacytometer and viability (60-80%) was checked with the trypan blue exclusion test. The granulosa cells were cultured in 400 µl medium in multiwell culture plates (Costar, Cambridge, MA) at 37C in humidified air or 5% CO_2 in humidified air for varying periods of time.

At the end of the incubation period aliquots of medium were frozen for later determinations of lactate, progesterone and cAMP. To determine ATP the incubations were terminated by the addition of 100 µl 50% trichloroacetic acid and sonicated. These terminated incubations were stored at 4C until the ATP assay was carried out later the same day.

Luteal cell preparation and incubation procedure

To obtain heavily luteinized ovaries, rats were injected s.c. with 50 IU of PMSG (NIADDK) at day 26 of life, followed by 25 IU of hCG (Gonadex) 56 h later (Parlow, 1961). The animals were killed by cervical dislocation 2, 5 or 6 days after the hCG injection. The luteal cells were prepared from the heavily luteinized ovaries with an enzymatic digestion method according to Sender Baum and Rosberg (1987). Cell number was estimated with a hemocytometer and viability (95%) was checked with the trypan blue exclusion test. The luteal cells ($2x10^5$ cells/well) were incubated in 500 µl medium in multiwell culture plates (Costar, Cambridge, MA). The incubations were kept at 37C in humidified air for 180 min, unless otherwise indicated.

Incubation medium

Eagle's Minimum Essential Medium (Gibco, Paisley, Scotland) with Earle's salts was used, HEPES (10 mM) and BSA (0.1%). When incubated in 5% CO_2 it was also supplemented with $NaHCO_3$ (2.19 g/l). The medium was stored frozen until the day of experiment.

Hormones and chemicals

Stock solution of ovine FSH (0.1 mg/ml; NIAMDD-oFSH-15, 20 U/mg; contamination LH 0.04 U of NIH-LH-S1/mg and TSH, GH, PRL < 0.1% by weight), human FSH (gift from Dr P Torjesen, Oslo, Norway), ovine LH (0.1 mg/ml; NIAMDD-oLH-24, 2.3 U/mg; contamination GH, PRL < 0.1% by weight and TSH, FSH < 0.5% by weight) and hCG (1000 IU/ml; Gonadex, Leo, Helsingborg, Sweden; 0.1 mg/ml) in sterile PBS with 0.1% BSA and PMSG (400 IU/ml; NIADDK) in 0.9% NaCl were kept frozen at -20C until use. NECA (5'-(N-ethyl)-carboxamido-adenosine), R-PIA (N^6-(R-phenyl-isopropyl)-adenosine) and S-PIA (N^6-(S-phenyl-isopropyl)-adenosine) (Boehringer-Mannheim, Mannheim, FRG) and 2-Clado (2-chloro-adenosine; Sigma, St Louis, MO) were dissolved in the medium by mixing and short sonication. Stock solutions of adenosine deaminase (ADA, Boehringer-Mannheim; 400 U/ml in glycerol), 8-phenyltheophylline (8-PHT, Sigma; 20 mM in 0.1 M NaOH), 8-(p-sulfo)-phenyltheophylline (PSOT, a gift from Dr L Gustafsson, Karolinska Institute, Stockholm, Sweden), Ro 20-1724 (a gift from Hoffman LaRoche, Basel, Switzerland; 500 mM in 95% ethanol) and dipyridamole (DIP, Sigma; 20 mM in 95% ethanol) were diluted in medium. [α-^{32}P]ATP, [^{3}H]cAMP and [1,2,6,7-^{3}H]progesterone were all purchased from Amersham International (Buckinghamshire, UK).

cAMP assay

cAMP was determined in aliquots taken from the medium immediately after the incubation, since the major part of cAMP was found in the medium after stimulation of the cells with FSH both in the absence and in the presence of adenosine analogs and other compounds used. The intracellular cAMP comprised 25 ± 2 % of total cAMP in all groups incubated with FSH in different combinations with adenosine and its analogs, DIP and 8-PHT for 3h. Intracellular cAMP was below

detection limit in groups without FSH. The aliquots of medium were kept frozen at -20C until analysis within one week after the experiment. cAMP was determined according to Gilman (1970) with cAMP-dependent protein kinase as binding protein. Dextran coated charcoal (charcoal 5 g/ml and Dextran 50 μg/ml) was used to separate free and bound cAMP. The sensitivity of the cAMP assay was approximately 0.5 pmol/tube and the coefficient of intra-assay variation was 15%.

Progesterone assay

Progesterone was determined by radioimmunoassay (RIA) and was determined in unextracted aliquots of incubation medium. The progesterone antibody was purchased from ICM Immuno-Chemicals (Tumba, Sweden). High correlation has been found between values obtained with direct RIA compared to values obtained in lipid-extracted medium samples ($r = 0.98$; Hedin, 1984). The intra-assay and inter-assay variations were 11% and 15%, respectively. Cross reaction with other substances used in the experiments and other steroids (estrogen and testosterone) was less than 1%. The standard curve (6.25-800 pmol/tube) was calculated using a logit-log transformation of standards.

Lactate assay

Lactate was measured with a fluorimetric method according to Passonneau (1974) with some modifications. Perchloric acid ($HClO_4$, 3 M) was used to precipitate the proteins. After centrifugation, the supernatant was neutralized with potassium hydrogen carbonate ($KHCO_3$, 3 M). After a second centrifugation, carbonate buffer (0.1 M. pH 9.7; Lowry et al., 1964) was added to the samples taken from the supernatant. Lactate dehydrogenase (LDH, from beef heart, 10 μg) and nicotinamide-adenine dinucleotide (β-NAD, 1.5 μmol) were finally added to the samples. The reaction was carried out at room temperature (20C) for 30 min. The NADH produced was recorded using a spectrophotofluorometer (Aminco-Bowman) at excitation and emission wavelengths of 340 nm and 450 nm, respectively. A standard curve (10^{-9}-10^{-1} M) for lactate was constructed using serial dilutions of a 1 M lactate solution treated in a manner the same manner as the samples.

ATP assay

The ATP assay was based on the luciferin-luciferase enzyme luminescence method (Strehler and Totter, 1952; Lyman and DeVincenzo, 1967). The sonicated and trichloroacetic acid-treated cell suspension (0.5 ml) was extracted three times with 4 ml diethylether. ATP standards (1.5-800 pmol) and blanks were treated identically. The assay was linear at least down to 1.5 pmol ATP. The emitted light from the luciferin-luciferase reaction was recorded using a spectrophotofluorometer with a photon counter (Aminco-Bowman, American Instrument Co., Silver Spring, MD) at 555 nm, with the primary light source disconnected. The buffered luciferin-luciferase (16 mg/ml; ATP bioluminescences, CLS, Boehringer-Mannheim, Mannheim, West-Germany) was dissolved in 10% glycerol-1% BSA in distilled water and kept in a light-shielded bottle. Aliquots (100 μl) of the ether-extracted samples were added to plastic tubes, and the reaction was started by adding 100 μl of the luciferin-luciferase solution (final pH 7.0). The emitted light was stable for several minutes. The coefficient of intra-assay variation was 4.7%.

Adenylate cyclase assay and membrane preparation

Membranes were prepared from preovulatory ovaries isolated 2 days after the PMSG injection (ovarian membranes) and from heavily luteinized ovaries were isolated 5 days after the hCG injection (luteal membranes). The ovaries were trimmed from adnexal tissues and kept frozen at -70C until assayed within 2 weeks. At the time of the assay, the frozen ovaries were homogenized in ice-cold Tris-sucrose buffer (25 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 27% sucrose, pH 7.5) with an all glass Dounce homogenizer. The homogenate was centrifuged for 5 min at 160xg to remove debris, filtered through two layers cheese cloth and centrifuged again for 50 min at 10 000xg. The crude membrane fraction was resuspended in Tris-sucrose buffer and aliquots of this suspension were used in the adenylate cyclase assay.

In the experiments where adenylate cyclase activity in isolated luteal cells was studied, the cells were isolated as described earlier (Sender Baum and Rosberg, 1987), frozen in Tris-sucrose buffer and kept frozen until assayed within 2 weeks. The cells were homogenized as described above, but the homogenate was not centrifuged prior to the adenylate cyclase assay.

The final concentrations of reagents in the adenylate cyclase assay were: 0.1 mM ATP (with approximately $2x10^6$ cpm [α-^{32}P]ATP), 0.1 mM cAMP, 0.05 mM GTP, 5 mM creatine phosphate, 25 U/ml creatine phosphokinase, 5 mM $MgCl_2$ and 1 U/ml ADA in 25 mM Tris-HCl at pH 7.5. The reaction was initiated by the addition of 100 μl membrane suspension to 100 μl assay medium. After 10 min at 37C, the reaction was terminated by the addition of 100 μl stopping solution (5 mM cAMP, 20 mM ATP and 1% Na-dodecylsulfate). The [^{32}P]cAMP formed was isolated by Dowex and alumina column chromatography (Salomon et al., 1974), with added [^{3}H]cAMP for recovery

correction. The eluates from the alumina columns were collected directly into scintillation vials, mixed with scintillation fluid and counted for radioactivity. Protein contents of the membrane aliquots (80-120 μg/sample) were determined with the Lowry method (1951), after precipitation with ice-cold 10% trichloroacetic acid. The described adenylate cyclase assay is essentially the assay presented by Birnbaumer and coworkers (1976) with the omission of myokinase.

The relatively poor adenylate cyclase activation (approximately 30% above basal activity for the adenosine analogs, 60% for FSH and hardly any for LH) might be explained by the low ATP concentration (0.1 mM) in the assay system. The rationale for choosing such a low concentration was to minimize adenosine contamination. However, Birnbaumer and coworkers (1976) have pointed out that membranes contain sufficient nucleoside triphosphate pyrophosphohydrolase activity to convert ATP to AMP without forming ADP, at least luteal membranes. Such mechanism would impair the ATP-regenerating system composed of creatinine phosphate and creatinine phosphokinase from efficiently converting ADP to ATP in the assay cocktail and would thus decrease the substrate availability to the adenylate cyclase. The strategy to circumvent this problem would be to either increase the ATP concentration or add adenylate kinase (myokinase) that converts AMP to ADP (Birnbaumer et al., 1976). However, basal cAMP production was linear over 20 min with and without 0.1 U/ml myokinase and forskolin-stimulated (50 μM) luteal membrane adenylate cyclase to the same extent in the absence and presence of myokinase. These results suggested sufficient ATP in the assay for the amount membranes used.

Adenosine transport

In paper I, adenosine uptake was estimated by incubating granulosa cells in culture medium with [^{3}H]adenosine (0.5-100 μM; 5-10 μCi/tube) at 37C. At specified time intervals, the cells were separated from the medium by filtration on Millipore filters (0.45 μm; HAWP 02500, Millipore Corp, Bedford, MA). The filters were then washed six times with 2 ml ice-cold buffer (150 mM NaCl, 10 mM HEPES, 5 ml $CaCl_2$, and 0.1% BSA, pH 7.4), transferred with cells to scintillation vials, dried over night and dissolved in 2 ml ethyleneglycol-mono-methylether. Ten milliliters of scintillation fluid [2,5-diphenyloxazole (2.5 g/l) and 1,4-bis[2-(5-phenyloxazolyl)] benzene (0.2 g/l) in toluene] were added, and the radioactivity was determined in a liquid scintillation counter.

In paper III, the adenosine uptake was estimated by a modified method. Granulosa cells (10^6 cells per sample), [^{3}H]adenosine (100 μM, 5-10 μCi/sample) and indicated test substances in a total volume of 100 μl were layered on top of 300 μl 7.5% Percoll solution (Pharmacia, Uppsala, Sweden) in 400 μl Beckman microfuge tubes. The tubes were incubated at room temperature for the time indicated (21-23C) and were then centrifuged for 1 min at 8500xg in a Beckman microfuge to sediment the cells through the Percoll layer. The centrifuged tubes were rapidly frozen at -80C. The frozen tips of the tubes, with the sedimented cell layer, were cut off, placed in scintillation vials together with scintillation fluid and counted for radioactivity. Less than 0.05% of the labelled medium, approximately 2% of the radioactivity in control cells, was found in the tip of the tubes.

When calculating the K_m and V_{max} for the uptake kinetics of adenosine by the cells obtained values were fitted to a Michaelis-Menten curve with nonlinear regression. The calculated K_m gave an approximation of the true K_m value for adenosine uptake, since no equilibrium of adenosine was established due to nucleoside metabolism. The calculated values are given as mean ± SD.

Oxygen consumption

Oxygen consumption was determined with a microspectrophotometric method originally developed by Hultborn (1974). Single cumulus-oocyte complexes were placed in a small (12.3 nl), gas-tight, cylindrical cuvette containing culture medium with 20-40 μM oxyhemoglobin. The increase in absorbance at 435 nm, reflecting dissociation of oxyhemoglobin due to the gradual decrease in oxygen tension, was recorded in a thermostat-controlled (37C) Zeiss single-beam microphotometer. The duration of each measurement was 5-15 min, and the respiration was expressed as nl oxygen/h/cumulus-oocyte complex (Magnusson et al., 1977; Magnusson, 1980).

Statistics

Each experiment was repeated three or more times. Resulting values are given as mean ± SEM of triplicate samples, if not otherwise stated. When appropriate, logarithmically transformed data were used to obtain homogeneous variances. Differences between groups were calculated by analysis of variance, followed by Student-Newman-Keuls' multiple range test (Woolf, 1968). For some comparisons, the Wilcoxon signed ranks test for two samples was used (Colquhon, 1971). A p-value less than 0.05 was considered significant. The dose-response curves were fitted with nonlinear regression to 4-parametric dose-response curves (McIntosh and McIntosh, 1980). The calculated EC_{50} and slope values from these curves are given as mean ± SD.

Table 2

Effect of forskolin on ATP levels in granulosa cells.

ADO (μM)	ATP (pmol/10^5 cells) Control	forskolin
0	80 ± 4	51 ± 3
0.1	81 ± 4	48 ± 2
1	87 ± 2	54 ± 1
10	239 ±17	128 ± 7
100	248 ± 7	217 ±15

Granulosa cells ($2*10^5$ cells/well) were incubated in humudified air (37C) for 90 min with adenosine (ADO) in the absence or presence of forskolin (100 μM). Each value represents the mean ± SEM for triplicate samples.

RESULTS AND COMMENTS

In this section the results in paper I-V will be summarized and, in part, commented on. Some additional data is also included.

One specific problem studying adenosine, or for that matter other substances with postulated autocrine/paracrine or local actions, is the unintended interaction between the administered experimental substance and the putative endogenous substance, both produced and present. In the case of adenosine, there is also the difficulty of separately studying intra- and extracellular actions of adenosine. In an effort to bypass some of these problems different cell preparations have been studied. Moreover, adenosine analogs, adenosine transport inhibitor, adenosine receptor antagonists and adenosine deaminase have been used in an attempt to more selectively study intra- and extracellular effects of adenosine. Besides adenylate cyclase activation and cAMP accumulation, oxygen consumption, lactate and progesterone accumulation and changes in cellular ATP levels have been used as functional parameters for adenosine action in ovarian cells and membranes.

Adenosine uptake in ovarian cells (papers I and III)

Adenosine is taken up by granulosa cells, but compared to luteal cells and most other cells from non-gonadal tissue, the uptake of adenosine by granulosa cells was slower (Berlin and Oliver, 1975; Arch and Newsholme, 1978). The K_m was found to be 15.9 ± 4 μM in granulosa cells as compared to 19.4 ± 8 μM in luteal cells. However, the V_{max} was considerably lower in granulosa cells (1.6 ± 0.1 pmol/min/10^5 cells) than in luteal cells (4.1 ± 0.6 pmol/min/10^5 cells). The uptake of adenosine in granulosa cells was slower in the presence of FSH than in the absence (paper I).

The nucleoside transport inhibitor DIP almost completely (90%) inhibited adenosine uptake. Furthermore, both R-PIA and S-PIA inhibited adenosine uptake almost as well as DIP did. Inosine and NECA in concentrations equimolar to adenosine decreased the uptake of adenosine by 25-40% suggesting that NECA and inosine compete for the same carrier as adenosine. The receptor antagonist 8-PHT did not appreciably affect adenosine uptake (paper III).

- Adenosine is taken up by granulosa and luteal cells

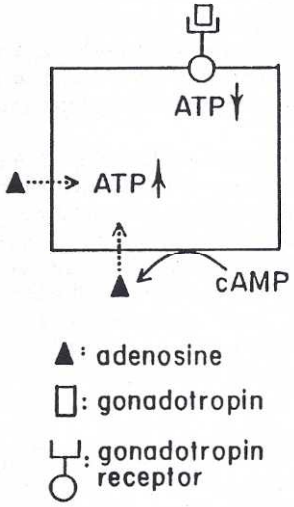
Effects of gonadotropins, adenosine and adenosine-derived compounds on ATP levels in granulosa cells and cumulus-oocyte complexes (papers I, II and III)

In the presence of adenosine, the ATP content in incubated granulosa cells increased for at least 225 min (paper I). In this respect the granulosa cells were slower than luteal cells where a maximum was reached already within 15 min (Behrman et al., 1983b). FSH decreased the adenosine-augmented ATP levels in granulosa cells, an effect which diminished with time. In addition, without adenosine added, FSH suppressed the levels of ATP (paper I). As in granulosa cells, the adenosine-induced increase in ATP levels in cumulus-oocyte complexes were diminished by FSH (paper II). Adenosine was phosphorylated, according to measurements of cellular ATP levels, in a dose-dependent manner in both granulosa cells and cumulus-oocyte complexes. Adenylate cyclase activation seems to be the common denominator for the decrease in ATP levels in granulosa cells since LH (paper I), FSH (papers I and II) and

forskolin (table 2) all induced a decrease in cellular ATP levels.

Thus, FSH depressed both the cellular content of ATP and the uptake of adenosine in granulosa cells. However, the suppressive effect of FSH on cellular ATP levels was probably not a result only of decreased adenosine transport but also of FSH-induced intracellular processes such as adenosine metabolism. This can be appreciated from paper I in which FSH depressed ATP levels in adenosine-preincubated cells where adenosine transportion and phosphorylation were permitted for 90 min before FSH was added (paper I). The adenosine analogs NECA, 2-Clado, R-PIA and S-PIA did not significantly increase ATP levels in the granulosa cells. The decreasing effect of FSH (paper I) was still present with all analogs since they did not increase the ATP levels (paper III). The analogs, in this respect, were considered non-metabolizable.

Inosine dose-dependently increased ATP levels, but the increase was blocked by DIP (paper I). Cyclic AMP added to the incubation medium also increased ATP levels in granulosa cells. The effect was not as fast as the adenosine-induced ATP increases. The ATP increase was dependent on both PDE activity and nucleoside transport since a PDE inhibitor (Ro 20-1724) and DIP both inhibited cAMP-induced ATP increases in the cells (tables 3 and 4). These results suggest that extracellular cAMP is converted to AMP by PDE and further metabolized to adenosine which then is transported into the cell where it is phosphorylated to ATP.



- Adenosine is metabolized and increases cellular ATP levels in granulosa cells and cumulus-oocyte complex
- FSH, LH and forskolin decreases granulosa cell ATP levels
- Adenosine analogs do not increase cellular ATP levels
- Extracellular cAMP is metabolized to adenosine and increases ATP in granulosa cells

Effect of adenosine on oxygen consumption and lactate formation in follicular cells (paper II)

Whole follicles (Nilsson 1974; Tsafriri et al., 1976a), cumulus-oocyte complexes and granulosa cells (Billig et al., 1983; Billig et al., 1984) have a high glycolytic capacity measured as lactate accumulation and the lactate production is stimulated by gonadotropins, in spite of hyper-physiological oxygen tension during the incubation. Furthermore, gonadotropins also decrease oxygen consumption in cumulus-oocyte complexes (Dekel et al., 1976; Magnusson and Hillensjö, 1981). It has been suggested, in earlier works from our laboratory, that this gonadotropin-induced decrease in oxygen consumption and concomitantly increased lactate accumulation could be due to competition for a limited supply of cofactors common to both glycolysis and oxidative phosphorylation.

If adenosine-derived cofactors (AMP, ADP) were limiting factors then addition of adenosine would reverse the above mentioned gonadotropin-induced effects. Addition of low concentrations of adenosine reversed the FSH-induced decrease in oxygen consumption while other nucleosides or an adenosine analog did not. When the adenosine uptake into the cells was blocked by DIP, the reversal of oxygen consumption was abolished. Furthermore, if the limitation of ADP was the cause of diminished respiration and if the added adenosine after phosphorylation increased the ADP pool, then adenosine kinase had to be active. Enzyme activity is temperature-dependent and, when the cumulus complexes were incubated with adenosine at room temperature, the reversal of FSH-inhibited respiratory rate was not seen (paper II).

If adenosine increased a former limited pool of cofactors common, ADP according to the suggestion, for the glycolytic pathway and the respiratory chain, then pyruvate would enter the citric acid cycle at a higher rate and less lactate would accumulate. Indeed, when adenosine was added to the incubated cumulus-oocyte complex, lactate accumulation decreased both in the absence and presence of FSH (paper II). In FSH and LH-stimulated granulosa cells adenosine decreased lactate formation as in cumulus-oocytes complexes (table 5).

Table 3

Effect of cAMP and adenosine on ATP levels in granulosa cells in the presence and absence of DIP.

	ATP ($pmol/10^5$ cells)					
Incubation time (min)	Control		cAMP		ADO	
	-DIP	+DIP	-DIP	+DIP	-DIP	+DIP
15	54 ± 2	59 ± 2	60 ± 2	71 ± 2	100 ± 3	58 ± 1
90	77 ± 4	71 ± 3	93 ± 6	84 ± 1	305 ±16	108 ±12
180	91 ± 3	88 ± 8	127 ± 3	96 ± 5	387 ±20	126 ±15
360	131 ± 4	107 ± 6	280 ±16	137 ± 1	434 ±18	195 ± 7

Granulosa cells ($2*10^5$ cells/well) were incubated in humidified air (37C) for the indicated time in the absence or presence of cAMP (50 µM) and adenosine (ADO, 50 µM) with and without DIP (10 µM). Each value represents the mean ± SEM for triplicate samples.

Table 4

Effect of cAMP in the presence and absence of Ro 20-1724 and DIP, respectively, on ATP levels in granulosa cells.

	ATP ($pmol/10^5$ cells)		
	Control	DIP	Ro 20-1724
Control	39 ± 2	36 ± 2	35 ± 2
cAMP	95 ± 5	36 ± 5	39 ± 3
FSH	27 ± 2	25 ± 2	19 ± 1
FSH + cAMP	66 ± 2	30 ± 1	24 ± 1

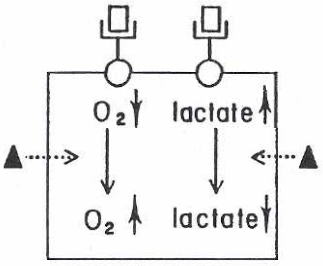
Granulosa cells ($2*10^5$ cells/well) were incubated in humidified air (37C) for 360 min in the absence or presence of cAMP (50 µM), FSH (100 ng/ml), DIP (10 µM) and Ro 20-1724 (1 mM). Each value represents the mean ± SEM of triplicate samples.

Table 5

Effect of adenosine (ADO) on lactate accumulation in granulosa cells in the presence and absence FSH and LH, respectively.

	Lactate ($nmol/10^5$ cells)					
Incubation time (h)	Control		FSH		LH	
	-ADO	+ADO	-ADO	+ADO	-ADO	+ADO
4	12 ± 3	4 ± 1	28 ± 2	20 ± 2	29 ± 1	16 ± 3
24	35 ± 2	12 ± 3	121 ± 7	30 ± 2	103 ± 5	24 ± 2

Granulosa cells ($2*10^5$ cells/well) were incubated in humidified air (37C) for the indicated time in the absence or presence of FSH (100 ng/ml) or LH (100 ng/ml) with and without adenosine (ADO; 50 µM). Each value represents the mean ± SEM, n=6.



- Adenosine reverses FSH-induced decrease in oxygen consumption in cumulus-oocyte complexes
- Adenosine decreases lactate formation in granulosa cells and cumulus-oocytes complexes

Effect of adenosine on cAMP accumulation in ovarian cells (papers I, III and V)

Adenosine (50 μM) slightly increased the basal cAMP levels in the medium during a 90 min incubation of granulosa cells. FSH-stimulated cAMP accumulation was dose-dependently potentiated by adenosine. The maximal adenosine potentiation of maximally FSH-stimulated cAMP accumulation was 5-10 fold. LH-stimulated cAMP accumulation was also potentiated, but not to the same extent as with FSH (paper I). These results could be due to two principally different mechanisms (or a combination of both): adenosine could increase ATP levels and, thus, increase the substrate availability to the adenylate cyclase or adenosine could act as an agonist to the stimulatory adenosine receptor (A_2). To investigate the latter possibility, metabolization of adenosine had to be prevented and for this purpose adenosine analogs (NECA, 2-Clado, R-PIA and S-PIA) were used.

Adenosine A_2 receptors coupled to adenylate cyclase present in ovarian cells should fulfill the following four criteria (see Introduction): 1) Adenosine and adenosine analogs should both stimulate adenylate cyclase activity and cAMP production. 2) The EC_{50} of NECA should be in the low micromolar or high nanomolar range. 3) The effect of NECA should be antagonized by a xanthine like theophylline, IBMX, 8-PHT 4) The rank order of potency of adenosine analogs should be NECA > 2-Clado > R-PIA > S-PIA according to the convention.

In initial experiments with adenosine analogs 2-Clado, in combination with maximally stimulatory concentrations of FSH, inconsistently enhanced cAMP accumulation (paper I). Extended studies showed that the non-metabolizable adenosine analog dose-dependently increased the cAMP accumulation in granulosa cells in spite of no increase in ATP levels. In combination with FSH, adenosine, NECA and 2-Clado elicited a marked dose-dependent potentiation of the cAMP accumulation (paper III). The EC_{50} value for NECA on FSH-stimulated cAMP accumulation was found to be 40 μM. NECA (100 μM) additively increased the cAMP accumulation for the whole dose range of FSH stimulation. Even at maximal stimulation of FSH, NECA increased the cAMP accumulation. In addition, LH-stimulated cAMP was increased (paper III).

Since NECA was considered to be non-metabolizable and still increased cAMP accumulation, the results suggested that the effect was not due to increased substrate availability to adenylate cyclase, but rather due to inhibited PDE or to actual increases of adenylate cyclase activity mediated via A_2 receptors. If this was the case, in spite of the high EC_{50} compared to EC_{50} in other tissues (see Introduction), then the adenosine receptor antagonists should counteract the effect of NECA. However, the adenosine receptor antagonist 8-PHT dose-dependently decreased FSH-stimulated cAMP accumulation. This finding might be explained either by an unspecific effect of 8-PHT or by adenosine receptor antagonistic effect of 8-PHT inhibiting the effects of endogenously produced and released adenosine.

Adenosine deaminase, ADA, was added to the incubations to circumvent the effect of any assumed endogenous adenosine release. ADA degrades adenosine to inosine, which is not an adenosine receptor agonist. However, 8-PHT still decreased FSH-stimulated cAMP accumulation. Moreover, the attenuating effect on FSH action was even further enhanced. This finding may be explained by an unspecific effect of 8-PHT. Alternatively ADA was ineffective in degrading endogenous adenosine or inosine had an effect on FSH-stimulated cAMP accumulation. Indeed, inosine increased ATP levels and potentiated FSH-stimulated cAMP accumulation. The effects were blocked by DIP. The results indicate that the latter possibility seems the most likely, but does not exclude the former.

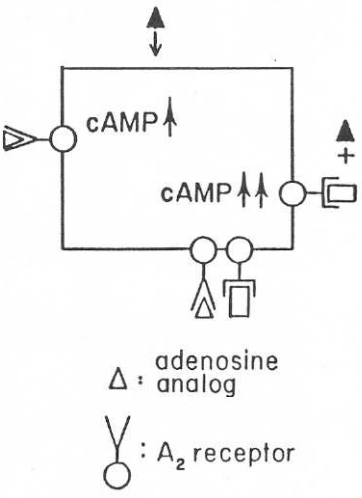
Nucleosides, including inosine, use the same carrier for transport across the cell membrane (see Introduction). Dipyridamole, DIP, a nucleoside transport inhibitor, abolished the effect of inosine on cAMP accumulation. It is likely that inosine was taken up by the cells, converted to adenosine and expressed the same effects as metabolizable adenosine. However, the potentiating effect of both NECA and adenosine on FSH-stimulated cAMP accumulation was still as demonstrable as in the absence of DIP.

When both ADA and DIP were present 8-PHT did not further decrease FSH-stimulated cAMP accumulation. Under these conditions the dose-response curve for NECA was shifted to the right by 8-PHT and it increased the EC_{50} for NECA three-fold (paper III). This suggests a competition

between NECA and 8-PHT for the adenosine receptor.

The adenosine analogs S-PIA and R-PIA, are known to have lower affinities for the stimulatory A_2 receptor than for the inhibitory A_1 receptor. However, these analogs did not potentiate FSH-stimulated cAMP accumulation. On the contrary, both S-PIA and R-PIA inhibited cAMP accumulation in granulosa cells. The precursor to S-PIA and R-PIA, amphetamine, was also tested and it did not elicit inhibitory effects. The inhibitory effect of S-PIA was reversible, indicating that the cells were still viable and responsive to FSH. These results indicate that the effect of PIA analogs was not due to irreversible toxicity of the compounds suggesting the presence of inhibitory A_1 receptors in granulosa cells. However, the effects of S-PIA and R-PIA were not antagonized by 8-PHT. Furthermore, when the cells were incubated under more stringent conditions with respect to endogenous adenosine, i.e. with DIP and ADA, the inhibitory effects of S-PIA and R-PIA on FSH-stimulated cAMP accumulation were almost completely abolished (paper III). Therefore the inhibitory effects are likely to be intracellular and not due to A_1 receptors.

Incubation of isolated luteal cells from both 2 and 5-6 day-old corpora lutea with NECA or S-PIA alone did not increase cAMP accumulation. However, LH-stimulated cAMP accumulation was dose-dependently potentiated by NECA, but not by S-PIA. This effect of NECA was slightly antagonized by 8-PHT, but not to the same magnitude as in the granulosa cells (paper V).



- Adenosine increases gonadotropin-induced cAMP accumulation
- Adenosine analogs increases gonadotropin-induced cAMP accumulation in granulosa and luteal cells with the characteristics of A_2 receptor activation

Effect of adenosine analogs on adenylate cyclase activity in membrane preparations from the ovary (papers III, IV and V)

To exclude the possibility that adenosine analog effects were a result of direct or indirect intracellular events but rather were a result of membrane-associated events, i.e. adenosine receptor activation, adenylate cyclase activity was studied in ovarian membranes. The ovarian membranes were prepared from ovaries of immature PMS-treated preovulatory rats (see Methods). NECA (1 μM) stimulated adenylate cyclase both in the absence and presence of FSH. This stimulating effect of NECA, with and without FSH, was decreased by 8-PHT (paper III).

NECA dose-dependently increased the adenylate cyclase activity in the ovarian membrane preparation. The calculated EC_{50} was found to be 0.28 μM, well below that found in granulosa cells. NECA additively increased FSH-stimulated adenylate cyclase activity over the whole tested dose range of FSH (1-1000 ng/ml). Both in the presence and absence of FSH, NECA dose-dependently increased cAMP production. However, the EC_{50} for NECA in the presence of FSH seemed to be subject to greater variations than in the absence of FSH. Nevertheless, the EC_{50} for NECA in membranes in the presence of FSH was in the same range as without FSH and considerably lower than in whole cells. The maximal response to NECA was approximately half of the maximal response to FSH. However, the absolute response to NECA was relatively small (approximately 30% above the basal activity for the adenosine analogs) and might be explained by the low ATP concentration (0.1 mM) in the assay system (for further discussion see Methods).

The adenosine receptor antagonist 8-PHT shifted the dose-response curve for NECA to the right and increased the EC_{50} almost 10-fold. Also in the presence of FSH the potentiating effect of NECA was antagonized by 8-PHT.

In the ovarian membrane preparation, four different adenosine analogs were tested for their relative potency on adenylate cyclase activation. All four analogs NECA, 2-Clado, R-PIA and S-PIA stimulated adenylate cyclase activity. NECA was found to be the most potent of the four, followed by 2-Clado, R-PIA and S-PIA (paper IV).

In membranes from luteinized ovaries, NECA alone was found to stimulate adenylate cyclase activity by 30% (see above) and the EC_{50} for NECA was 0.65 μM. The dose-dependent effect of NECA was antagonized by 8-PHT and increased the EC_{50} by one order of magnitude. When the

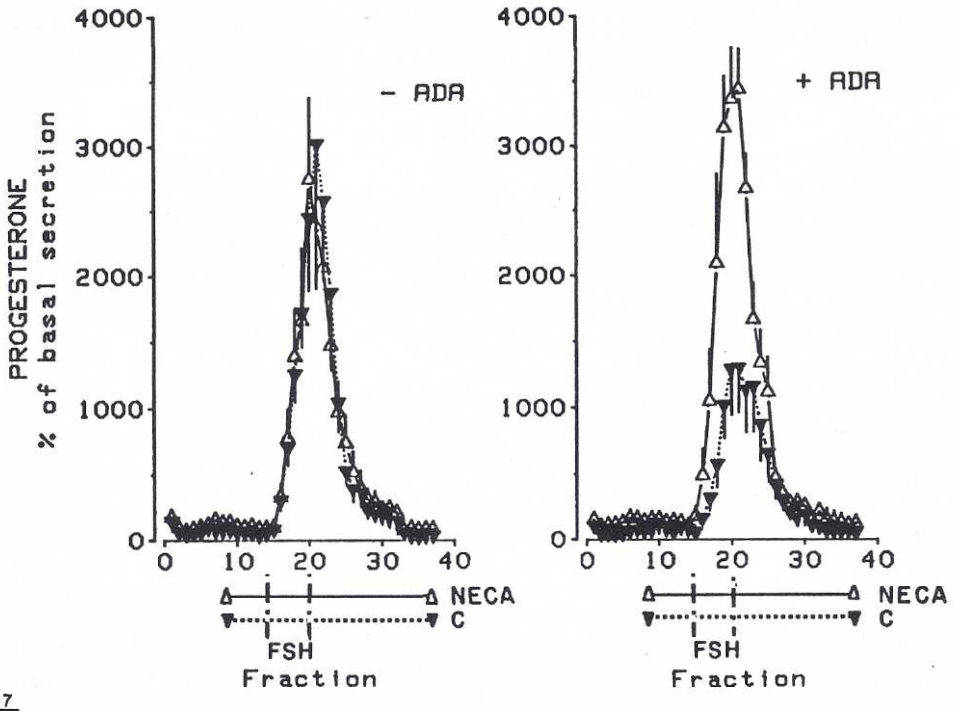


Fig 7

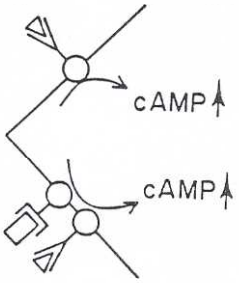
Effect of NECA on FSH-stimulated progesterone production in superfused granulosa cells in the presence and absence (right) of adenosine deaminase (left).

Granulosa cells were superfused for 9h. Medium, 30 µl/min, was sampled in 15 min fractions. FSH (10 ng/ml) was present from fraction 14 to 20 in all superfusions. NECA (open triangles, 100 µM) was introduced from fraction 9 and was present until the end of superfusion.

The basal secretion was set to 100 %, and was the mean of fraction 1-8, i.e. before addition of NECA. The basal secretion in absolute terms was 1.33 ng/fraction/mg protein and 3.31 ng/fraction/mg protein in the absence and presence of adenosine deaminase (ADA), respectively. All subsequent values are expressed as percent of the basal secretion. The data (mean ± SEM) were pooled from two identical experiments with two superfusions for each treatment.

other adenosine analogs were tested for adenylate cyclase activation NECA was found to be the most potent one, with the following order of potency NECA > 2-Clado > R-PIA > S-PIA. Also in luteal cell homogenates, NECA stimulated the adenylate cyclase (EC_{50} 0.53 µM) and the dose-response curve was shifted to the right by 8-PHT (paper V).

These results suggest the presence of A_2 receptors in luteal cells, ovarian and luteal membranes.



- Adenosine analogs stimulate adenylate cyclase in membranes from non-luteinized and luteinized ovaries as well as luteal cell homogenate with the characteristics of A_2 receptor activation

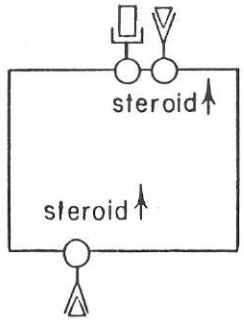
Effect of adenosine on steroidogenesis in ovarian cell (papers I and V)

Progesterone production in luteal cells was stimulated by NECA. The effect was time-dependent and was detectable already after 15 min of incubation. In LH-stimulated cells, a significant additional effect by NECA on progesterone synthesis was only seen after longer incubation times (3 h) (paper V).

In granulosa cell incubations the progesterone secretion was minutely, but significantly, increased in the presence of adenosine. However, adenosine did not significantly affect maximum gonadotropin-stimulated progesterone secretion, nor did it significantly alter the EC_{50} for FSH (paper I). The adenosine analog 2-Clado had no significant change in basal and FSH-stimulated progesterone accumulation in granulosa cells (paper I).

NECA slightly stimulated the progesterone synthesis in incubated granulosa cells. However, the dose-dependent stimulatory effect of NECA on FSH-induced cAMP accumulation in granulosa cells was not reflected in progesterone synthesis. This finding might imply that the enhancing effect of adenosine analogs on adenylate cyclase activity is an in vitro artefact. Alternatively steroidogenesis was maximally stimulated by endogenous adenosine. This possibility is supported by the finding that FSH-stimulated granulosa cells exposed to ADA in a superfusion system (Johanson and Johanson, 1988) responded with higher progesterone production in the presence than in the absence of NECA. In the absence of ADA, NECA did not change FSH-stimulated progesterone production (fig 7). However, the cAMP response to NECA was similar in the presence and in the absence of ADA. This finding indicates that endogenously produced adenosine "auto-stimulates" the cells to increase their steroidogenic response to a submaximal FSH stimulation.

This suggests that only a fraction of the putative adenosine receptors need to be occupied in order to achieve a maximal steroidogenic response.



- Adenosine analogs stimulate progesterone synthesis in luteal cells and, to some extent, in granulosa cells
- Adenosine analogs potentiate submaximal FSH-stimulated progesterone synthesis in granulosa cells

Summary of results

Effects of metabolizable adenosine:

- Adenosine is taken up into granulosa and luteal cells.
- Adenosine is metabolized and increases cellular ATP levels in granulosa cells and cumulus-oocyte complexes.
- Extracellular cAMP is metabolized to adenosine and increases ATP in granulosa cells.
- FSH, LH and forskolin decreases granulosa cell ATP levels.
- Adenosine reverses the FSH-induced decrease in oxygen consumption in cumulus-oocyte complexes.
- Adenosine decreases lactate formation in granulosa cells and cumulus-oocytes complexes.
- Adenosine increases gonadotropin-induced cAMP accumulation.

Effects of non-metabolizable adenosine analogs:

- Adenosine analogs do not increase cellular ATP levels.
- Adenosine analogs increase gonadotropin-induced cAMP accumulation in granulosa and luteal cells with the characteristics of A_2 receptor activation.
- Adenosine analogs stimulate adenylate cyclase in membranes from non-luteinized and luteinized ovaries as well as luteal cell homogenate with the characteristics of A_2 receptor activation.
- Adenosine analogs stimulate progesterone synthesis in luteal cells and, to some extent, in granulosa cells.
- Adenosine analogs potentiate submaximal FSH-stimulated progesterone synthesis in granulosa cells.

In conclusion, these results suggest both a role of adenosine as intracellular substrate as well as agonist to extracellular receptors participating in the activation of ovarian adenylate cyclase system.

GENERAL DISCUSSION

Adenosine and adenosine receptors in endocrine tissues.

Adenosine may be a candidate for autocrine/-paracrine function whereby it acts as a "local hormone". However, adenosine is also a substrate for cellular metabolism. In some metabolic pathways adenosine is a precursor and in others a product, for instance, phosphorylation of adenosine by adenosine kinase to AMP and dephosphorylation of AMP to adenosine. As a result, adenosine can be recirculated within and between adjacent cells. For these reason adenosine cannot, according to definition (see Introduction), be a classical hormone. Classical hormones are degraded and inactivated as a part of the regulation of hormone action. In the presence of adenosine receptors, adenosine may act both as a hormone and as substrate depending on its localization, inside or outside the cell, and on the metabolic demands of the cell. Based on this concept and experimental data, authors have proposed a local action for adenosine in the gonads: as receptor agonist in the testis (Monaco et al, 1984) and via intracellular action in the ovary (Behrman et al., 1986).

Since the knowledge of adenosine action in the ovary is limited it may be adequate to broaden the perspective to other endocrine tissues where adenosine action has been studied, i.e. the pancreas, the adrenals, the thyroid and the testis.

The adrenals, the pancreas and the thyroid

One of the earliest reports introducing the concept of adenosine receptors demonstrated stimulatory effects of adenosine in adrenal tumor cells, both on cAMP production and steroidogenesis (Wolff and Cook, 1977). However, adenosine inhibited cAMP and steroid production in adrenal cortex cells isolated from non-tumor adrenals. ACTH-stimulated cAMP generation and steroidogenesis was inhibited by adenosine analogs, but also dbcAMP-induced steroid production. These findings indicated that, in this cell preparation, adenosine receptors mediated inhibition both prior and subsequent to the adenylate cyclase (Shima, 1986). These opposing effects of adenosine analogs in tumor and normal cells was due to the presence of different adenosine receptors i.e. A_2 and A_1 receptors in the different cell types. Changes in receptor expression induced by tumor transformation or in vitro cell culture adaptation has also been observed in other tissues like the thyroid and the testis (see below).

When adenosine was administered in vivo, it acted synergistically with ACTH on adrenal steroidogenesis (Formento et al., 1975). Adenosine alone stimulated steroid production but this effect was prevented by dexamethasone treatment. This indicated that the target organ for adenosine might be the pituitary or that adrenal adenosine action was markedly potentiated by low concentrations of ACTH. It could not be concluded from the presented data which of the mechanisms that was responsible for the reported results, or if it was due to increased substrate availability to cAMP, since adenosine analogs were not used in the study. Another possible mechanism could involve the adenosine-induced vascular changes.

Adenosine affects the endocrine pancreas in a dual mode having both inhibitory and stimulatory actions on the secretion of insulin and glucagon, respectively. Adenosine and its analogs inhibited glucagon-stimulated adenylate cyclase activity and inhibited glucose-stimulated insulin secretion from islets of Langerhans via A_1 receptors (Ismail et al., 1977; Schutz et al, 1979). The inhibition of insulin release does not seem to involve changes in insulin production or glucose oxidation (Campbell and Taylor, 1982). Interestingly, adenosine mediated not only decreased insulin secretion but also increased glucagon secretion in perfused pancreas (Weir et al., 1975; Bacher et al., 1982; Loubatieres-Mariani et al., 1982; Chapal et al., 1985). Infusion of adenosine analogs in vivo increased glucagon release but also increased glucose levels and decreased FFA levels (Schutz et al., 1978a,b). Hyperglycemia and decreased FFA levels are the expected results from increased glucagon action, although the change in FFA could also be the result of adenosine-receptor mediated anti-lipolytic action in adipocytes (Fain, 1973; Fredholm, 1978a).

Effects of adenosine have also been reported in the exocrine pancreas. Adenosine and ATP alone do not change the exocrine secretion but submaximal secretin stimulation was potentiated by adenosine and this effect was antagonized by theophylline. The potentiating effect of adenosine on secretin-stimulated exocrine secretion was claimed to be mediated via A_2 receptors (Yamagishi et al., 1985, 1986, 1987). Secretin stimulates pancreatic exocrine secretion and this effect is suggested to be, at least in part, cAMP-mediated (Case et al., 1972; Case and Scratchered, 1972).

Both in adrenal cells and in thyroid cells opposing results regarding adenosine responsiveness have been reported in primary cells from normal tissue and in cell lines. In normal guinea pig thyroid tissue adenosine analogs increased cAMP production in a way that was compatible with the presence of A_2 receptors (Fradkin et al., 1982), whereas, in a thyroid cell line, inhibitory A_1 receptor-mediated effects on TSH-stimulated cAMP formation were recently presented (Berman et al., 1986).

Table 6

Adenosine receptor mediated effects in endocrine tissue and interaction with hormone action in endocrine target tissue

A1 receptors

Tissue	species	preparation	assay	analog	IC50	Author	year
pituitary cell line		cell	GH secretion	PIA	30nM	Dorflinger and Schonbrunn	1985
pituitary cell line		cell	GH secretion	ado	.6-3uM	Dorflinger and Schonbrunn	1985
islets of Langerhans	rat	membrane	AC	ado	*<0.1uM	Ismail et al	1977
islets of Langerhans	rat	cell	inhib insulin secretion	ado	*<10uM	Ismail et al	1977
islets of Langerhans	rat	cell	inhib insulin secretion	NECA	0.1-10uM	Schutz et al	1979
islets of Langerhans	rat	cell	cAMP	ado	*<10uM	Campbell and Taylor	1982
islets of Langerhans	rat	cell	cAMP	PIA	*<10uM	Campbell and Taylor	1982
islets of Langerhans	rat	cell	inhib insulin secretion	ado	1-10uM	Campbell and Taylor	1982
islets of Langerhans	rat	cell	inhib insulin secretion	PIA	0.1uM	Campbell and Taylor	1982
pancreas	dog	perfused in vivo	inhib insulin secretion	NECA	*<1nM	Bacher et al	1982
pancreas	dog	perfused in vivo	stim insulin secretion	NECA	*>0.1uM	Bacher et al	1982
thyroid cell line	rat	cell	cAMP	PIA	0.5nM	Berman et al	1986
thyroid cell line	rat	membrane	binding	CHA	0.5nM	Berman et al	1986
Sertoli cells	rat	membrane	binding	CHA	2.1nM	Monaco et al	1984
Sertoli cells	rat	cell	cAMP	PIA+FSH	2.1nM	Monaco et al	1984
Sertoli cells	rat	cell	inhib estogen accumulation	PIA+FSH	3.1nM	Monaco et al	1984
Sertoli cells	rat	cell	cAMP	PIA	0.5-1nM	Eikvar et al	1985
Sertoli cells	rat	cell	cAMP	PIA		Monaco et al	1986
adrenal cortex cells	rat	cell	inhib of steroidogenesis	PIA	2-5nM	Shima	1986
adrenal cortex cells	rat	membrane	AC	PIA	13-64nM	Shima	1986
hepatocytes	rat	cell	preventing glucagon inhib of insulin induced PDE	PIA	6nM	Wallace et al	1984
hepatocytes	rat	cell	inhib of glucagon induced desensitization of AC	PIA+ insulin	4nM	Wallace et al	1984

A2 receptors

Tissue	species	preparation	assay	analog	ED50	Author	year
luteal cells	rat	cells	cAMP	ado+LH	22uM	Hall et al	1981
granulosa cells	rat	cells	cAMP	NECA+FSH	40uM	Paper III	1988
ovary	rat	membrane	AC	NECA	0.28uM	Paper IV	1988
luteinized ovary	rat	membrane	AC	NECA	0.65uM	Paper V	1988
luteal cells	rat	cell homogenate	AC	NECA	0.59uM	Paper V	1988
Leydig tumor cells		membrane	AC	Clado	8uM	Londos and Wolff	1977
Leydig tumor cells		membrane	AC	PIA	40uM	Londos and Wolff	1977
Leydig tumor cells I-10		membrane	AC	Clado	10-100uM	Wolff and Cook	1977
Leydig tumor cells I-10		membrane	AC	NECA	1-10uM	Londos et al	1980
Leydig tumor cells I-10		cells	20a-dihydroprogesterone	NECA	10-100nM	Londos et al	1980
Leydig cells	rat	cell	stim steroid production	PIA	50uM	Rommerts et al	1984
Leydig tumor cells		cells	cAMP	ado+LH	1uM	Dix et al	1985
Leydig tumor cells		cells	cAMP	PIA+LH	*<10uM	Dix et al	1985
Adrenal tumor cells Y-1		cells	20a-dihydroprogesterone	NECA	100 nM	Londos et al	1980
Adrenal tumor cells Y-1		membrane	AC	Clado	10-100uM	Wolff and Cook	1977
thyroid tissue	porcine	pieces	cAMP	NECA	0.7uM	Fradkin et al	1982
pancreas	dog	perfused in vivo	stim glucagon secretion	NECA	*<1nM	Bacher et al	1982
pancreas	rat	perfused in vitro	stim glucagon secretion	NECA	40nM	Chapal et al	1985

*significant effect
ED50 or IC50 not determined

The testis

In the testis several aspects of adenosine receptor action have been documented. Crude homogenates of testis and brain exhibit the highest binding capacity for A_1 receptor agonist of all organs tested (Williams and Risley, 1980; Murphy and Snyder, 1981). In the testis adenosine receptor binding or activation has been claimed to be associated with spermatocytes, Leydig cells and Sertoli cells and some controversy exists as to what testicular cell type the physiological action of adenosine should be attributed.

Binding of adenosine appeared to be associated with the spermatocytes within the seminiferous tubule epithelium (Murphy et al., 1983). However, binding to Sertoli cells with the characteristics of A_1 receptors has convincingly been demonstrated (Monaco et al., 1984; Monaco and Conti, 1986). Furthermore, the binding capacity, not the affinity, of adenosine A_1 agonist increased markedly in rat testis up to 60 days of age (Monaco and Conti, 1986). Testosterone treatment, which suppresses LH secretion in intact animals, did not affect adenosine receptor binding. However, hypophysectomy decreased total binding to rat testis (Murphy et al., 1983), indicating that testicular adenosine receptors needed gonadotropin support, most likely FSH (Baker et al., 1976).

The functional response of adenosine agonists in the normal testis suggests the presence of an A_1 receptor. In Sertoli cells FSH-stimulated adenylate cyclase activity was inhibited by adenosine analogs and so was FSH-stimulated androgen aromatization (Monaco et al., 1984; Monaco and Conti, 1986). Other agents stimulating Sertoli cell adenylate cyclase were also inhibited by adenosine (Monaco et al., 1984; Eikvar et al., 1985). However, when androgen aromatization was stimulated by dbcAMP, adenosine agonists were not inhibitory (Monaco et al 1984) in contrast to the demonstrated A_1 receptor-mediated inhibition of dbcAMP-stimulated steroidogenesis in normal adrenocortical cells (Shima, 1986).

In early reports, adenosine agonists were shown to stimulate adenosine receptor-mediated adenylate cyclase activity in a Leydig tumor cell line and these results were used as part of the basis for the definition of A_2 adenosine receptors (Londos and Wolff, 1977; Wolff and Cook, 1977). A_2 receptors do not seem to be present in the normal testis (Murhpy et al 1983; Monaco et al., 1984; Eikvar et al., 1985; Monaco and Conti, 1986). However, it has been reported that normal Leydig cells developed responsiveness to adenosine during culture and the authors suggested that this A_2 adenosine receptor was a result of in vitro conditions (Rommerts et al., 1984).

A plausible physiological role for adenosine receptor-mediated effects has been presented. It was suggested that Sertoli cell adenosine receptors were part of intercellular communication between the Sertoli and germ cells whereby Sertoli cells monitored the energy balance in germ cells by sensing adenosine, the ATP catalyzate, released from the germ cells (Monaco and Conti, 1986).

Some of the characteristics of adenosine receptors in endocrine tissue are summarized in table 6.

Adenosine as substrate and receptor agonist in the ovary

In the gonads, adenosine has been suggested to exert local action; extracellularly in the testis as a receptor agonist (Monaco et al, 1984) and intracellularly in the ovary as a substrate (Behrman et al., 1982, 1986b). If the action of adenosine in the ovary is autocrine/paracrine then:

1) adenosine has to induce metabolic or other effects in neighboring cells or in the adenosine releasing cell itself
2) adenosine has to be produced and released by ovarian cells
3) the ovarian cells must have a regulatory mechanism for the production, release and/or response to adenosine.

These features may not be shared by all ovarian cells. From a regulatory point of view, it is perhaps more likely that they are not shared by all cells if a local and/or autocrine/paracrine regulatory role exists in the ovary.

Before addressing the question of a possible physiological autocrine/paracrine role for adenosine in ovarian tissue, the possible cellular effects, production, release and regulation of adenosine have to be discussed.

Adenosine augmentation of adenylate cyclase activity and cAMP generation in ovarian cells

Adenosine and its analogs dose-dependently stimulated basal cAMP accumulation and potentiated gonadotropin-stimulated cAMP accumulation in granulosa cells (Polan et al., 1983; Ohkawa et al., 1985; papers I and III), in cumulus-oocyte complexes (Preston et al., 1987) and in luteal cells (Hall et al., 1981; Brennan et al., 1983; Behrman et al., 1983b; Sender Baum and Ahrén, 1986a; paper V). However, the stimulation by adenosine or its analogs alone in ovarian cells was quantitatively small compared to the augmenting effect on gonadotropin-stimulated adenylate cyclase in granulosa cells, in luteal cells and also in Leydig cells (Dix et al., 1985) similar to adenosine potentiation of the secretin-stimulated secretion from the exocrine pancreas (Yamagishi et al., 1985,

1986, 1987).

Adenosine may increase cAMP in two principally different ways, either by increasing substrate availability to adenylate cyclase or by stimulating adenylate cyclase through adenosine receptors.

From experiments where adenosine uptake was inhibited by DIP, Behrman and coworkers (Hall et al., 1981) concluded that the potentiating effect of metabolizable adenosine on LH-induced cAMP accumulation in luteal cells was mainly (80%) due to intracellular and to a minor degree (20%) due to extracellular events. Furthermore, they concluded that the predominant effect of adenosine on gonadotropin-induced cAMP accumulation in granulosa cells was intracellular and that "the action of adenine-derived purines was shown to be linked to an increase in granulosa cell ATP levels" (Ohkawa et al., 1985). This suggested that adenosine acted as a substrate. However, in the same paper they it was shown that the nucleoside transport inhibitor, DIP, blocked adenosine-induced ATP increase, while adenosine still dose-dependently increased FSH-stimulated cAMP accumulation to 80% of that in the absence of DIP (Ohkawa et al., 1985). In a similar experiment with granulosa cells, we confirmed this result. While DIP (10 μM) inhibited 90% of the adenosine (50μM) uptake adenosine potentiated cAMP accumulation in the FSH-stimulated cells to 60-75% of the levels reached in the absence of DIP. Furthermore, NECA in combination with FSH stimulated cAMP both in the presence and absence of DIP. However, DIP alone seemed to have an inhibitory effect on FSH-induced cAMP accumulation and it was decreased to approximately the same extent both in the presence and absence of adenosine and NECA (paper III). This may represent non-specific effects of DIP but it may also be that DIP inhibited uptake of endogenous or exogenous adenosine to an extent that decreased or partially impaired the intracellular nucleoside metabolism.

Considering the papers discussed above, it seems justified to modify the statement that the predominant site of action for adenosine is intracellular (Hall et al., 1981; Ohkawa et al., 1985), at least for granulosa cells.

The effects of adenosine in the presence of DIP suggested an extracellular site of action. Furthermore, since NECA presumably was not metabolized in ovarian cells (paper I), the effects of the analog indicated a non-substrate effect. These indications suggest an extracellular non-substrate action of adenosine, possibly receptor-mediated. If adenosine receptors of the stimulatory A_2 subtype are involved then the adenosine receptor antagonists should reverse the effect or diminish it. The order of potency for the utilized adenosine analogs should be NECA > 2-Clado > R-PIA > S-PIA and the EC_{50} for NECA should be in the low micromolar range (see Introduction).

As with adenosine alone, NECA stimulated basal cAMP production only slightly in granulosa (paper III) and luteal cells (paper V). However, NECA stimulated gonadotropin-induced cAMP accumulation much more prominently (paper III and V). Similar findings have been presented for Leydig cells (Dix et al, 1985). The EC_{50} for NECA in FSH-induced cAMP accumulation was around 40 μM in granulosa cells which was in the same magnitude as for adenosine (paper III). Similar results have been reported for adenosine in LH-stimulated luteal cells where the EC_{50} was reported to be 22 μM (Hall et al. 1981). The stimulatory effect of NECA on cAMP accumulation was antagonized by the receptor antagonist 8-PHT in FSH-stimulated granulosa cells (paper III) and, to a lesser degree, in LH-stimulated luteal cells (paper V).

The EC_{50} values presented above are at least one order of magnitude higher than those reported for most other cells with A_2 receptors (Londos et al, 1981; Daly, 1983). It may for this reason be questioned whether the effect of NECA was mediated via an adenosine receptor, as it has been described in other cells, in spite of the fact that 8-PHT displaced the dose-response curve to the right, at least in granulosa cells, and the other adenosine analog, 2-Clado, showed similar stimulatory characteristics as NECA and adenosine (paper III).

In three different membrane preparations obtained from preovulatory ovaries from PMSG-treated immature rats, luteinized ovaries and luteal cells, NECA alone stimulated adenylate cyclase activity. The EC_{50} values for NECA in these preparations were found to be lower (0.28-0.65 μM) than in granulosa cells and more in the expected range for an A_2 receptor. The effect of NECA was antagonized by 8-PHT and other xanthines. The order of potency for adenosine analogs was found to be that of an A_2 receptor, i.e. NECA > 2-Clado > R-PIA > S-PIA (papers III, IV and V). However, the maximal stimulatory effect of NECA was rather limited, only 30% over basal activity and half that of maximal FSH stimulation (paper III and IV). This limited response may be due to an intrinsic limitation of the energy regenerating system in the assay (see Results and Comments).

If the criteria for an A_2 receptor were not fulfilled with whole cells, the data from the experiments on membrane preparations strongly suggested the presence of A_2 receptors. However, still some results reside un-explained. Why were the EC_{50} values so much higher in the cells than in membranes? Why do not the analogs alone

stimulate cAMP in the cells as well as they stimulate of adenylate cyclase activity in the membranes? It is not easy to give a satisfactory explanation to this but with insufficient data only speculation remains. One explanation may be that endogenously produced adenosine was present in whole cells and displaced NECA from the putative receptor. This explanation is not very likely since approximately the same EC_{50} value for NECA was found in incubated granulosa cells the absence and in the presence of ADA in combination with FSH. However, in granulosa cell superfusion experiments NECA augmented FSH-stimulated steroidogenesis in the presence of ADA (see Results and Comments). Another suggestion could be that NECA interfered with nucleoside transport into cells and, hence, directly or indirectly interfered with the intracellular adenosine metabolism. In support of this was the fact that DIP, a nucleoside transport inhibitor, also decreased cAMP accumulation and, to some extent, also ATP formation in granulosa cells (paper III). In Leydig tumor cells adenosine and its analogs potentiated LH-induced cAMP accumulation, while adenosine inhibited LH-induced desensitization of adenylate cyclase. The effects of adenosine were shown to have the characteristics of A_2 receptor activation. However, adenosine alone did not affect cAMP levels (Dix et al., 1985). The authors suggested that adenosine has potentiating effects through an action at or proximally to the G-protein in LH-stimulated adenylate cyclase. The G-protein is a membrane protein that mediates the stimulation between the LH-receptor and the adenylate cyclase (see Introduction). A similar non-adenylate cyclase stimulatory or inhibitory action of adenosine has been reported in hepatocytes (Wallace et al., 1984). Adenosine in these cells, without affecting adenylate cyclase alone, abolished the effect of glucagon in blocking insulin activation of PDE and glucagon desensitization of adenylate cyclase activity.

In spite of the short-comings mentioned above, the data suggest the presence of A_2 receptors in granulosa and luteal cells. However, it is perhaps wrong to phrase the question whether the action of adenosine is extracellular *or* intracellular. Instead one should ask whether the extracellular receptor-mediated *and* the intracellular substrate actions of adenosine are related to each other and cooperate in the cell.

Steroidogenesis in relation to adenosine in ovarian cells.

Adenosine stimulated basal progesterone synthesis in human and rat granulosa cells (Polan et al., 1983; paper I) and luteal cells (Hall et al., 1981; Polan et al., 1983). Also the adenosine analog NECA markedly stimulated the progesterone synthesis in luteal cells (paper V). The stimulatory effect of adenosine and its analogs on steroidogenesis was limited and not in concordance to the cAMP response, especially not the adenosine augmentation of gonadotropin stimulated cAMP accumulation (papers I, III, V).

It might be that the lack of concordance between the marked increase in cAMP and the very limited increase in progesterone from gonadotropin-stimulated ovarian cells is an in vitro artifact or that not the correct functional parameter was monitored for the adenosine receptor-mediated response. An alternative suggestion is that endogenous adenosine may occupy a fraction of adenosine receptors, small but sufficient for maximal steroidogenic response. In favour of the latter suggestion is the finding that, in superfused granulosa cells where adenosine was removed with ADA, NECA potentiated FSH-stimulated progesterone production (see Results and Comments).

Effects of adenosine on glycolysis and oxygen consumption in ovarian cells.

The preovulatory follicle has a high glycolytic capacity (Nilsson, 1974; Hillensjö, 1976; Tsafriri et al., 1976a) and lactate accumulation is high in gonadotropin stimulated granulosa cells (Billig et al., 1983; Hillier et al., 1985). The lactate accumulation occurred during incubation in atmospheric oxygen concentrations and is not likely to be due to decreased oxygen availability (Billig et al., 1983, 1984). Despite increased metabolic activity, gonadotropins decreased the oxygen consumption in cumulus-oocyte complexes (Hillensjö et al 1975; Dekel et al, 1976; Magnusson and Hillensjö, 1981). The increased lactate accumulation may indicate a decreased rate of pyruvate entering the citric acid cycle (see Introduction). The concomitant decreased oxygen utilization suggests that the increased lactate accumulation is not due to a lack of available oxygen.

It has been suggested from our laboratory (Hillensjö, 1976; Dekel et al., 1976; paper II) that the decreased oxygen consumption in cumulus-oocyte complexes is due to a competition for a limited quantity of cofactors in common for both glycolysis and oxidative phosphorylation. This mechanism was proposed several decades ago (Belitzer, 1936) to be the cause of decreased oxygen consumption after addition of glucose to certain tumor cells (Crabtree, 1929). It has been suggested that a relative lack of ADP (Ibsen, 1961) or inorganic phosphate (Koobs, 1972) would be limiting factors for the decreased rate in the respiratory chain with concomitant decreased oxygen consumption and increased lactate formation.

Addition of metabolizable adenosine abolished

the gonadotropin-induced decrease in oxygen consumption in cumulus-oocyte complexes. Lactate accumulation also decreased, while ATP levels increased (paper II). Addition of adenosine to granulosa cells also decreased lactate accumulation (see Results and Comments) and increased ATP formation (Ohkawa et al, 1985; paper I). The results suggest that, in both gonadotropin-stimulated cumulus-oocyte complexes and granulosa cells, adenosine increased the activity in the respiratory chain leading to increased rate of pyruvate entering the citric acid cycle, while lactate accumulation decreased. However, it has been suggested that lactate production is important for the oocyte, since it cannot utilize glucose as energy substrate but requires lactate and pyruvate (Biggers et al., 1967; Kennedy and Donahue, 1969; Zeilmaker et al., 1974). In analogy, in the testis Sertoli cells produce lactate (Robinson and Fritz, 1981) and the production is stimulated by FSH (Mita et al., 1982; LeGac et al., 1983). Testis germ cells can be supported with lactate, but not glucose (Jutte et al., 1981; Robinson and Fritz, 1981) and oxygen consumption increase in isolated germ cells after addition of lactate (Robinson and Fritz, 1981).

Relatively low concentrations of adenosine, 0.5 μM, abolished the FSH-induced decrease in oxygen consumption in cumulus-oocyte complexes. As will be argued below, the adenosine levels may well be 0.5 μM or higher in the cumulus-oocyte complex. Consequently, the physiological relevance of the decreased respiration and perhaps also the increased lactate accumulation in the presence of gonadotropins may be questioned. However, FSH decreased ATP levels (paper II) and is likely to increase the efflux of adenosine from the cumulus-oocyte complexes, this in turn decreasing the uptake of adenosine, as in granulosa cells (paper I). FSH-stimulated adenylate cyclase and other ATP-requiring enzyme reactions thereby increased the ATP consumption and the demand on ATP production. Thus, the glycolytic activity has to be increased, especially since the activity in the respiratory chain is restricted, to meet the demands for ATP. This may imply that the increased lactate formation is physiological and the decreased oxygen consumption is an in vitro result, since sufficient adenosine to normalize the respiration may be present in vivo but not in vitro due to relatively larger extracellular space (e.g. volume of incubation medium). The lactate concentration being 5-6 times higher in the rat preovulatory follicle than in the serum (Zeilmaker and Verhamme, 1977) supports this contention.

The results support the hypothesis that a relative lack of cofactors (i.e. ADP) common for both glycolysis and the respiratory chain causes the gonadotropin-induced decrease in oxygen consumption and increase in lactate accumulation in cumulus-oocyte complexes. In this regard, it has been reported that ADP was more abundant than AMP and ATP after addition of adenosine to cumulus-oocyte complexes (Downs et al., 1986a) and to luteal cells (Behrman et al., 1983b).

As mentioned above gonadotropins increased lactate accumulation and decreased ATP levels in granulosa cells, while adenosine had the opposite effects as in cumulus-oocyte complexes. There is a lack of data on granulosa cell respiration from the same experimental model from which these data were derived and if the same metabolic restriction reside in granulosa cells as in cumulus-oocyte complexes remains an open question. However, under certain experimental conditions LH-stimulated granulosa cells from prepubertal rat ovaries increased the oxygen consumption in the presence of succinate in hypotonic medium (Hamberger, 1968).

Adenosine and oocyte maturation

The meiotic division of the oocyte is arrested during follicular development and this has to be completed prior to fertilization. In vivo, the oocyte resumes meiotic division prior to ovulation, which is after the stimulatory mid-cycle LH/FSH peak (Lindner et al., 1974). The meiotic division also occurs spontaneously when the oocyte is isolated from the follicle (Pincus and Enzmann, 1935). In vitro oocyte maturation (i.e. meiotic division) is prevented or retarded by agents that increase or maintain cAMP levels within the cumulus-oocyte complex (Cho et al., 1974; Magnusson and Hillensjö, 1977; Hillensjö et al., 1978; Dekel et al., 1984; Ekholm et al., 1984; Törnell et al.,1984; Hanski and Dekel, 1987) and a decrease in oocyte cAMP levels precedes oocyte maturation (Schultz et al., 1983; Vivarelli et al., 1983). Also, follicular fluid and follicular fluid components inhibit spontaneous maturation (Chang, 1955; Tsafriri and Channing, 1975; Tsafriri et al., 1976b). In this context, it is interesting to note that one group has suggested hypoxanthine to be the principal active component in an inhibitory fraction of follicular fluid. The concentrations of hypoxanthine were remarkably high, in the milli-molar range (Downs et al., 1985; Eppig et al., 1985).

Purines inhibit spontaneous oocyte maturation and the inhibition is potentiated by cAMP and cAMP-inducing substances. Guanosine is the most potent of the purines (Downs et al., 1985; Billig et al., 1985). Eppig and coworkers have elegantly shown that the effect of hypoxanthine and guanosine could be attributed to derivatives of these purines, xanthyl and/or guanyl compounds (Downs et al., 1986a,b; Downs and Eppig, 1987).

Adenosine in combination with other purines or FSH also inhibit spontaneous oocyte maturation (Eppig et al., 1985; Downs et al., 1986a; Miller and Behrman, 1986; Petrungaro et al., 1986; Billig et al., 1985). At least three principally different mechanisms could be suggested for the inhibitory action of adenosine on spontaneous oocyte maturation: 1) adenosine being a precursor to xanthyl/guanyl compounds (see above), 2) increased cAMP levels caused by increased substrate availability to adenylate cyclase and 3) increased cAMP levels caused by stimulation of adenosine receptors.

The first possibility is described above. High levels of adenosine (0.1-1 mM) is probably needed for it to be the sole contributor of this effect. Adenosine, in combination with FSH, increases cAMP levels in cumulus-oocyte complexes (Preston et al, 1987). However, the inhibitory effect of adenosine is as effectively elicited by 2-Clado in combination with hypoxanthine (Downs et al., 1986) or FSH (Miller and Behrman, 1986) suggesting a non-substrate receptor-mediated mechanism for adenosine in oocyte maturation. Adenosine receptor-mediated inhibition of Ca^{2+} fluxes have been described in a number of different tissues (Schrader et al., 1975; Phillis and Barraco, 1985; Silinsky, 1986; Riberio and Sebastiao, 1986) and it has also been suggested that the inhibitory effect of adenosine on oocyte maturation could be ascribed to decreased mobilization of Ca^{2+}. Ionophore reversed the inhibitory effect of FSH-inhibited on spontaneous oocyte maturation in isolated cumulus-enclosed oocytes and in the presence of adenosine the effect of ionophore was counteracted (Preston et al., 1987). However, the role of Ca^{2+} in the initiation of oocyte maturation is far from settled (Tsafriri et al, 1988).

Origin and regulation of ovarian adenosine

Adenosine exerts a number of different effects in ovarian tissue but, to meet the requirements for establishing an autocrine/paracrine action, a local regulation of adenosine metabolism, uptake and release must exist.

Adenosine metabolism in ovarian cells

Adenosine increases the ATP level in luteal cells (Behrman et al., 1983a, b; Brennan et al., 1983; Sender Baum and Billig, 1986), in granulosa cells (Knecht et al., 1984; Ohkawa et al., 1985; paper I) and in cumulus-oocyte complexes (paper II). In luteal cells, 90% of the added adenosine was phosphorylated within 15 min to AMP, ADP and ATP. However, most of the adenosine was found as ADP and AMP in luteal cells (Behrman et al., 1983b), instead of ATP (Pearson et al., 1978). In contrast to luteal cells, where the ATP level rapidly reached a maximum 15 min after addition of adenosine, maximum levels in granulosa cells were reached after a longer incubation period (60 min, Ohkawa et al., 1985; 225 min, paper I).

The ATP content in granulosa cells was dose-dependently decreased by FSH (paper I). This was even more evident when the cells were co-incubated with adenosine (Ohkawa et al., 1985; paper I). The same phenomenon was also seen with cumulus-oocyte complexes (paper II). LH also decreased the ATP level in prepubertal ovaries (Ahrén et al., 1968), in luteal cells isolated from young postovulatory corpora lutea (Sender Baum and Billig, 1986), in luteinized ovaries in vivo (Soodak et al., 1988) and in incubated granulosa cells (paper I) but not in cells isolated from corpora lutea from mid-luteal phase (Sender Baum and Billig, 1986; Soodak et al., 1988). Forskolin also decreased ATP levels in granulosa cells (see Results and Comments). Ohkawa and coworkers (1985), on the other hand, reported that LH did not decrease ATP levels in granulosa cells. This discrepancy may be due to the different animal models used in the two papers, hypophysectomized estradiol- and FSH-treated rats being used in the latter report and PMS-treated in the former, since LH receptors may be less developed in estradiol-FSH-treated rats (Richards et al., 1979; Richards, 1980).

The FSH-induced decrease of ATP levels in granulosa cells was not only due to a diminished uptake of adenosine since FSH acutely decreased ATP levels in adenosine-preincubated cells. Furthermore, in granulosa cells incubated in the absence of adenosine FSH still decreased ATP levels.

Uptake of adenosine by ovarian cells

Adenosine is transported into granulosa cells (paper I and III), cumulus-oocyte complexes (Heller and Schultz, 1980; Downs et al., 1986a) and luteal cells (Behrman et al., 1983; paper I). Behrman and coworkers (1983b) reported a K_m of 7.3 μM and a V_{max} of about 2 pmol/min/10^5 luteal cells at 24 C. With a Q_{10} of 2.9 (Behrman et al., 1983b) the V_{max} will be about 6 pmol/min/10^5 luteal cells at 37 C. Although we reported a lower V_{max} of 4 pmol/min/10^5 luteal cells (paper I), the V_{max} in luteal cells was markedly higher than in granulosa cells, where it was found to be 1.6 pmol/min/10^5 cells while the K_m was 15.9 μM (paper I). It is also of interest to note that FSH decreased both the maximal uptake of adenosine and also to some extent its $t_{1/2}$ for uptake (paper I). In contrast, LH does not affect adenosine uptake in luteal cells (Behrman et al, 1983b).

The uptake of adenosine is most likely regulated, not by a limitation of the transmembrane transport,

as such, but rather by the intracellular activity of adenosine kinase and adenosine deaminase and, hence, by the intracellular concentration of adenosine which is likely to be very low due to metabolism as in other cell types (Arch and Newsholme, 1978). If this assumption is valid also for ovarian cells, then, judging by the reported data on adenosine uptake, there seems to be a difference in the intracellular metabolism of adenosine in granulosa and luteal cells.

Origin and putative release of ovarian adenosine

Extracellular adenosine in the ovary may have different origins (see also Introduction):

1) the general circulation
2) ovarian cells
   a) released as co-transmitter from nerves
   b) released as adenosine/AMP as a result of hypoxia
   c) released as adenosine/AMP as a result of decreased levels of cellular ATP not due to hypoxia
   d) released as cAMP/AMP

The blood is the probable source for purine homeostasis and for purines required in tissue metabolism (Murray et al., 1970). However, it is not likely that the blood concentration of adenosine, under normal physiological conditions, would be the regulating factor for adenosine related effects in the ovary since changes in adenosine levels in the blood do not correlate to ovarian function (deSanchez et al., 1983). However, during a period of general hypoxia the adenosine levels in blood are likely to be increased in the ovary as well as all other parts of the body.

The ovary is, of course, innervated (Stefansson et al., 1981) and catecholamines affect luteal function (Selstam et al., 1987). No evidence of adenosine/ATP release from nerve endings in the ovary has been presented. If this was the case, however, this would give a highly selective increase of adenosine in the ovarian compartments.

Adenosine release as a result of hypoxia is a well established mechanism (see Introduction). Behrman and coworkers (Ohkawa et al., 1985; Behrman et al., 1986) have suggested that the granulosa cells may be exposed to relatively high concentrations of adenosine caused by hypoxia, residing as they are in an avascular area of the follicle with the distance to oxygenating blood being relatively large. This suggestion is in analogy with adenosine action in vessels, although the explanations for the decreased oxygen tension required for increase of adenosine efflux differs. In the coronary vessels the explanation is metabolic and in the ovarian follicle it is morphological. If the oxygen tension is low in the follicle, then it is probably at the same low level over many hours or even days. The low oxygen tension is most likely not subject to sudden changes if it is explained by the distance to blood vessels and this distance is a function of follicular development and growth.

It has to be stressed that the increased efflux of adenosine is due to *changes* in oxygen tension. When the oxidative phosphorylating activity in the respiratory chain decreases, due to a relative oxygen shortage, the relative amount of AMP and ADP increases, resulting in an increased efflux of AMP and adenosine (Arch and Newsholme, 1978). If the lower oxygen tension is sustained over a period of time, as assumed in the follicle, then adenosine would not be subject to re-uptake, since the uptake is regulated by the intracellular metabolism (adenosine kinase and ADA, for reference see Introduction). Considering this, a more likely scenario is that, when a new and sustained level of oxygen tension is at hand, this will also result in a new and sustained steady state of adenosine turnover. The excessive extracellular adenosine, due to a previous change in oxygen tension, is likely to be transported by diffusion from that particular part of tissue or degraded by deamination and further metabolized to hypoxanthine before transportation from the tissue. Based on these assumptions, the adenosine level is not likely to be increased in the follicle or newly formed corpus luteum due to low vascularization, even though the oxygen tension may be low, but sustained. Furthermore, the oxygen tension in follicular fluid has been reported to be as high as in venous blood (Shalgi et al., 1972).

The high lactate concentration in follicular fluid (Zeilmaker and Verhamme, 1977) may intuitively indicate carbohydrate metabolism in low oxygen tension. However, incubated follicular cells do produce a considerable amount of lactate even in atmospheric oxygen tension (Billig et al., 1983, 1984). The lactate production may reflect, instead, a specialized metabolism adapted to the environmental requirements and substrate demands of the oocyte, as discussed above. Nevertheless, the low vascularization of the follicle may maintain a given adenosine level in the extracellular space as a result of the low transport capacity of adenosine and its metabolites from the ovarian compartment.

Yet another hypothetical mechanism for adenosine release in the ovary may be considered. A decrease in cellular ATP will increase AMP concentrations and, in the presence of 5'-nucleotidase, also adenosine (Arch and Newsholme, 1978). Gonadotropins decreased both basal (Ahrén et al., 1968, paper I, II) and adenosine-augmented ATP levels (Ohkawa et al., 1985; Sender Baum and

Billig, 1986; paper I,II) in prepubertal ovaries, follicular cells and luteal cells from young corpora lutea. This decrease in ATP levels may be due to increased consumption of ATP, but perhaps also to a decreased re-phosphorylation (paper I); both are mechanisms that would increase AMP and lead to an increased release of adenosine/AMP. Cyclic AMP turnover also consumes ATP and produces AMP by adenylate cyclase and PDE action, respectively. In this context it is interesting to recapitulate that FSH decreased the uptake of adenosine, decreased ATP levels, increased lactate accumulation and decreased oxygen consumption in follicular cells (Dekel et al., 1976; Magnusson and Hillensjö, 1981; Billig et al 1983; Ohkawa et al., 1985; paper I and II). The data suggest a decreased propulsion and a re-localization of adenosine in gonadotropin-stimulated follicular cells that indirectly would increase the extracellular adenosine concentration due to decreased uptake or increased release.

The prerequisites for extracellular adenosine formation from cAMP are that cAMP has to be produced, released and degraded in ovarian cells. Furthermore, the enzymes responsible for degradation of cAMP to adenosine (i.e. PDE and 5'-nucleotidase) have to be present in the same compartment as the cAMP.

Egress of cAMP is well established in a multitude of tissues and has been suggested to be carrier-mediated. Furthermore, cAMP as such is not subject to re-uptake through intact cell membranes before degradation to adenosine or its metabolites (Barber and Butcher, 1983). Incubated whole ovaries (Selstam et al., 1976a), perfused ovaries (Selstam et al., 1976b), incubated follicles (Nilsson et al., 1974), corpora lutea (Herlitz et al., 1974), isolated granulosa (Hamberger et al., 1978) and luteal cells (Sender Baum and Ahrén, 1986b) all released cAMP in response to hormonal stimulation. The physiological significance of the marked outflow of cAMP has remained an enigma (Barber and Butcher, 1983).

In many tissues (Arch and Newsholme, 1978), including the ovary (Rosberg et al., 1975; Knecht and Catt, 1981), extracellular cAMP can be degraded by the ectoenzymes phosphodiesterase and 5'-nucleotidase to AMP and adenosine. cAMP added to the medium increased granulosa cell ATP levels. The ATP increment was inhibited by DIP. Furthermore, a PDE inhibitor prevented cAMP from increasing ATP levels. This indicates that the ATP increase was dependent on PDE activity and nucleoside transport (see section Results and comments). Consequently, cAMP must have been degraded to AMP and adenosine, since cAMP and AMP are not transported into cells across intact plasma membranes (Barber and Butcher, 1983). In this context it is also of interest to note that FSH stimulates both extra- and intracellular PDE activity, thereby increasing both the cAMP production and its degradation to AMP within and between the ovarian cells (Selstam and Rosberg, 1976; Conti et al., 1984). The intracellular PDE is stimulated by FSH with a lag-phase of about 1 h (Conti et al., 1984). Contradictory results have been reported suggesting that FSH inhibits extracellular PDE activity (Knecht and Catt, 1981). However, reevaluation of the latter data suggest that, due to the experimental design, total adenosine metabolism was studied rather than extracellular PDE activity.

Observations in isolated luteal tissue, similar to those mentioned above, have not been presented to my knowledge. However, ovarian PDE activity varies during the estrous cycle in the rat, the activity being increased during metestrus (Schmidtke et al., 1980). Also in the testis, FSH stimulates PDE activity (Conti et al., 1981, 1982, 1983).

It could be concluded that cAMP is released in ovarian tissue and, subsequently degraded extracellularly to adenosine. Therefore, an adenosine recirculation and reutilization may be proposed.

Extracellular adenosine levels

If the above described mechanisms of adenosine formation from cAMP and decreasing levels of cellular ATP in ovarian tissue are correct, then a rough estimate could be made of the cellular contribution to extracellular adenosine. An approximate estimation (see section Appendix) reveals that the cAMP efflux, from FSH-stimulated cells in vitro could increase the concentration in the extracellular space in the follicular wall with as much as 4-5 μM *every minute* and this cAMP could be subject to metabolization to adenosine. Moreover, if the gonadotropin-induced decrease in cellular ATP results in egress of adenosine, as has been suggested for other cells (Arch and Newsholme, 1978), a considerable amount of adenosine would be found in the extracellular space. The FSH-induced decrease of granulosa cell ATP levels would result in an adenosine outflow increasing the extracellular concentration with 18-20 μM/min and in FSH-stimulated cumuli of 12-13 μM/min. Taking in account the estimated uptake of adenosine into granulosa cells (paper I) the FSH-stimulated efflux of cAMP/adenosine would theoretically result in an equilibrium of 1-6 μM in the follicular wall after just minutes (for further details on assumptions and calculation see Appendix).

These calculations are mere estimations and resulting from the assumptions. The calculations are curtailed by the lack of knowledge, for instance, of the local 5'-nucleotidase and PDE

activity, the difficulty in estimating cAMP efflux (Barber and Butcher, 1983), the activity of adenosine deaminase, and the estimation of adenosine uptake kinetics. However, the estimations still support a plausible model that adenosine in ovarian tissue is of ovarian origin. The model also presents the possibility that extracellular concentrations of adenosine are regulated by the cellular activity and that the calculated concentrations of adenosine are in the range of effective adenosine receptor activation. It may be concluded that there is a basis for a local modulation of ovarian cell activity by adenosine.

Thus, the adenosine concentrations in the follicle may be theoretically a few μM. Much higher levels of purines have been reported in porcine follicular fluid (1.41 mM hypoxanthine, Downs et al., 1985) and in murine follicular fluid (0.35-0.70 mM adenosine, 2-4 mM hypoxanthine, Eppig et al 1985). However, these values may be overestimations due to the sampling procedure and the lack of use of degradative enzyme inhibitors. In human follicular fluid collected during surgery and with prompt addition of ADA inhibitor, the values were much lower (0.3 μM adenosine, 3.7 μM hypoxanthine, Billig and Fredholm, unpublished results) and in bovine follicular fluid no purines could be detected (Eppig and Downs, 1987).

A model for para/autocrine action of adenosine in the ovary.

The data presented and referred to on adenosine action in general and in ovarian cells in particular meet many of the requirements for a local and/or paracrine action and reveal that:

- adenosine may be produced in and released from ovarian cells (i.e. the enzymes for adenosine formation are present in the same compartment as the substrate for adenosine formation)
- adenosine may induce metabolic effects as a substrate (e.g. ATP increase, normalization of oxygen consumption, decreased lactate accumulation, increased substrate availability to cAMP formation etc)
- adenosine may induce metabolic effects as a receptor agonist (e.g. adenylate cyclase stimulation, cAMP production)
- adenosine production, release and uptake may be regulated within the ovary (i.e. regulation by gonadotropins)
- adenosine concentrations in the extracellular space, deduced from theoretical calculations, are in the range for efficient uptake and receptor action and are not in conflict with estimated concentrations in other tissues

If these circumstantial pieces of evidence (derived from incubation experiments) for modulation of ovarian cell activity by adenosine prove to be correct, also in vivo, then they favour the adenosine receptor-mediated local regulation rather than regulation via adenosine substrate availability. The net increase in ATP and cAMP due only to adenosine as substrate would require a net increase of ovarian adenosine, and not a relocation of a constant, or decreasing, adenosine content from the intracellular to the extracellular space in the ovarian compartments. Adenosine receptor-mediated effects on cellular metabolism do not require a net increase in adenosine, but just a metabolically induced relocation of adenosine from within the cell to the extracellular space to increase receptor agonist interactions.

This does not imply that adenosine as substrate is of no importance. Adenosine as a substrate seems to be a prerequisite for a number of cellular events and not a regulatory factor like the receptor-mediated events.

A speculative and simplified model for paracrine/autocrine action in the ovary may be proposed based on the discussion above: The adenosine is of ovarian origin and gonadotropin stimulation induces a relocation of adenosine from within the cell to the extracellular space. The relocation is a result of a gonadotropin-induced decrease of cellular ATP levels and subsequent increase in AMP concentration (due to a relative decrease in the phosphorylation/dephosphorylation ratio of phosphorylated adenine nucleotides) and an increased efflux of cAMP. The cAMP is degraded to AMP by PDE. Intra- and extracellular AMP is further degraded to adenosine. Extracellular adenosine bind to and activate adenosine A_2 receptors in an autocrine/paracrine manner. Adenosine receptor activation augments the adenylate cyclase response to gonadotropin and its functional parameters and indirectly increases the adenosine (AMP/cAMP) outflow by a short loop feedback mechanism. Adenosine is also subject to uptake into cells. However, the uptake is diminished gonadotropin stimulated cells.

If the gonadotropin-stimulation in every cell is not equal, e.g. if the receptor occupancy is different due to submaximal levels of hormones or the distribution of receptor is unequal in a group of cells (e.g. part of the corpus luteum, the follicle or the whole follicle), then the gonadotropin response and adenosine uptake would be unequally distributed. The less gonadotropin-stimulated or the unstimulated cells would take up more adenosine than the stimulated ones. The less stimulated cells would then increase their ATP levels and be more responsive to a weak gonadotropin stimula

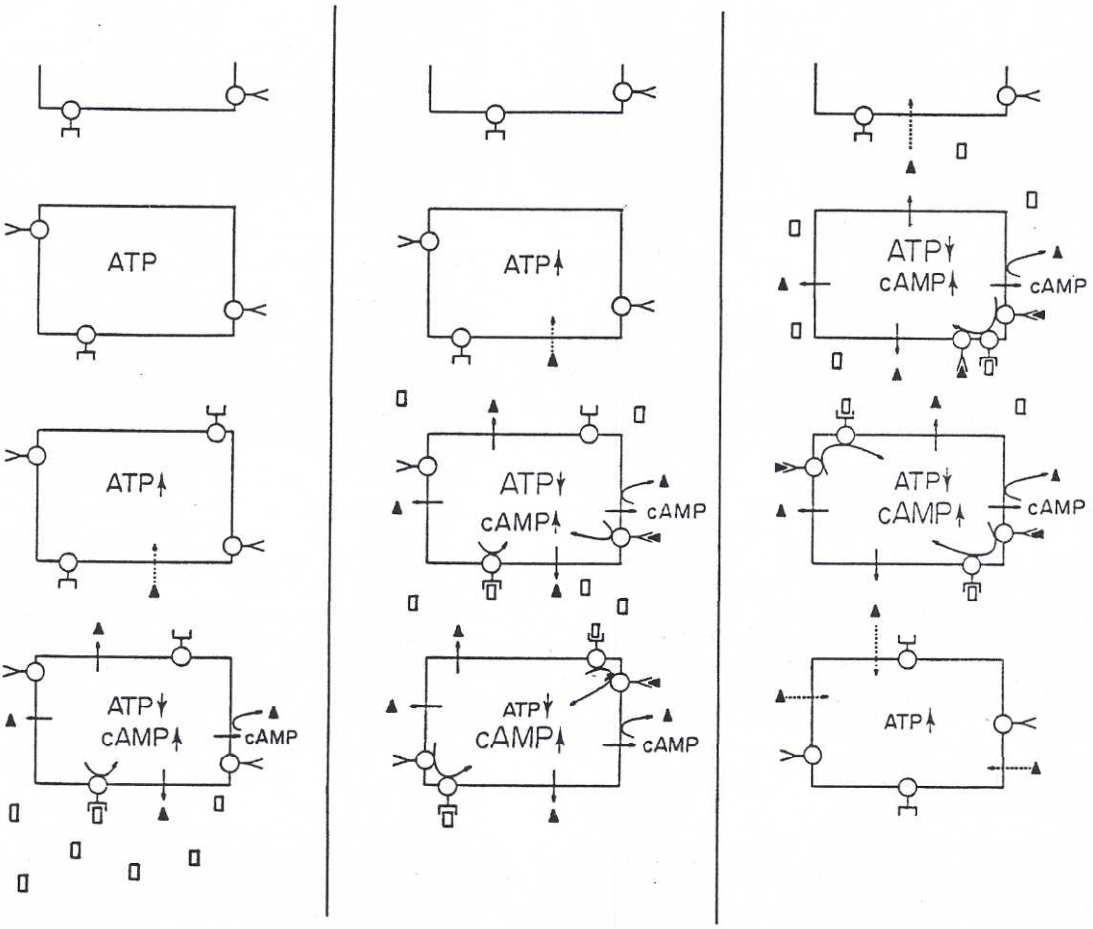


Fig 8

A model for auto/paracrine action of adenosine in ovarian cells. Initiation of gonadotropin stimulation (open squares, left panel) with subsequent adenylate cyclase activation, decrease of cellular ATP, outflow of cAMP and adenosine (closed triangels).

The left panel depicts the initiation of gonadotrophic stimulation of granulosa cells. The panels to the right depict events later on during stimulation. For further details see page 35.

tion at a later stage. When the gonadotropin stimulation is terminated, the remaining augmented levels of extracellular adenosine are decreased by cellular uptake and adenosine receptor activation is decreased (fig 8).

This theoretical model of dual adenosine action as substrate and receptor agonist in the ovary would imply that the gonadotropin response is more equally distributed in, for instance, the preovulatory follicle, than the gonadotropin receptor distribution would suggest (Amsterdam et al., 1975; Uilenbroek and Richards, 1979). The model also delineates a possibility of prolonging the gonadotropin action when hormone levels fall, of mediating a faster response when hormone levels rise, and of equalizing variations in gonadotropin stimulation due to pulsatility or restrains in diffusion of the hormone.

Concluding remarks

The presented study has not established a physiological regulating role for adenosine in ovarian function, but demonstrated potentiating effects of adenosine, both as substrate and as adenosine A_2 receptor agonist, on ovarian cell metabolism. The results suggest a possible paracrine/autocrine role for adenosine but do not estimate the relative importance of adenosine as substrate and as adenosine receptor agonist in

relation to other regulatory endocrine or paracrine hormones in the ovary, such as gonadotropins, prostaglandins, steroids, growth factors etc. Moreover, if a number of questions regarding adenosine and its action have been clarified or become less obscure, initial questions remain and new have appeared. Most of them are unanswered and urging to be resolved.

ALTE DUBITAT QUI CREDIT

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APPENDIX

Theoretical calculations on extracellular adenosine levels

If the above described mechanisms of adenosine formation from cAMP and decreasing levels of cellular ATP in ovarian tissue are correct, then it would be possible to make a rough estimation of the cellular contribution of extracellular adenosine. However, a number of assumptions have to be made and the conclusions could of course not be more accurate than the assumptions (table 7). Let us assume that maximal FSH-stimulated cAMP formation and outflow are linear over 90 min and result in a net efflux of 10 pmol cAMP/10^5 granulosa cells or 110 fmol cAMP/10^5 cells/min (as in paper I). Furthermore, let us assume that the outflow of cAMP is of the same magnitude in the intact follicle in situ and that cAMP is degraded to adenosine. The extracellular space within the follicular wall is assumed to be as large as 30% of total volume or 60% of the intracellular volume, as in prepubertal ovaries (Nilsson and Selstam, 1975) and the granulosa cell diameter is assumed to be 9 μm (Nordenström et al., 1985).

Based on these assumptions, not taking in account the time required for cAMP metabolization in vitro, re-uptake of adenosine etc, the net increase of extracellular cAMP/adenosine concentration would be 4-5 μM *per minute*. The chance for degradation of cAMP in the tissue must be greater than in dispersed cell incubations since the degrading enzymes are cell membrane-associated.

If the same assumptions are made for the maximal FSH induced decrease in ATP levels in granulosa cells (approximately 40% or from 2.5 mM to 1.5 mM cellular ATP, paper I) and the ATP decrease exclusively resulted in a proportional adenosine/AMP outflow, then the adenosine concentration in the extracellular space would increase with 18-20 μM/10^5 cells *every minute* if linear over 90 min. However, judging from the data in paper I the FSH-induced decrease in ATP levels seems to be much more prompt and sustained. Similar calculations made for the FSH-induced ATP decrease in cumulus-oocyte complexes suggest that the addition of adenosine within the cumulus cell mass would be 12-13 μM/cumulus per minute.

The microenvironment surrounding the cells will be the immediate acceptor of the cell-derived adenosine. However, the adenosine will diffuse in the follicle. If the equilibrium volume for adenosine, assumed to be derived from the FSH-induced decrease in ATP levels, would be the whole follicular volume then the increase in adenosine concentration would be 1-2 μM/min.

In this hypothetical model for extracellular adenosine effluxes the effect of adenosine uptake has not been considered. The values for adenosine kinetics presented in paper I (K_m 16 μM and V_{max} 1.6 μM/min/10^5 cells) are used in the calculations. The cAMP outflow from granulosa cells, if promptly converted to adenosine, would result in an equilibrium between release and uptake of adenosine irrespective of the size of the extracellular space. The equilibrium concentration of adenosine is calculated to 1 μM and reached within one minute in an extracellular space of 30% of total volume. If the same assumptions and calculations are carried out for the gonadotropin-induced ATP decrease an equilibrium concentration of 6 μM will be reached within one minute (for further details see table 7).

Table 7

Theoretical calculations on extracellular adenosine derived from follicular cells from gonadotropin induced cAMP outflow and cellular ATP decrease

Assumptions:

cAMP outflow and cellular decrease in ATP is reflected in a prompt and equimolar increase in adenosine

gonadotropin induced cAMP outflow in granulosa cells is assumed to be linear over 90 min

$10\ pmol/10^5$ cells/90 min = $110\ fmol/10^5$ cells/min (data from paper I)

gonadotropin induced decrease ATP level in granulosa cells is assumed to be linear over 90 min

$40\ pmol/10^5$ cells/90 min = $440\ fmol/10^5$ cells/min (data from paper I)

granulosa cell diameter 9 µm (Nordenström et al., 1985)

granulosa cell volume of 10^5 cells (V_{cell})

$V_{cell} = (4\pi/3) * (4.5)^3 * 10^{-9} = 0.038\ \mu l$

extracellular volume between cells (V_{ex}) is assumed to be 60% of cell volume (V_{cell}) (Nilsson and Selstam, 1975)
60% * 0.038 µl = 0.023 µl

Increase of extracellular adenosine concentration derived from cAMP outflow, not taking in account adenosine uptake:

(cAMP efflux/min) / (V_{ex}) = $Flux_{cAMP}$
(110 $fmol/10^5$ cells/min) / (0.023 µl) = 4.8 µM/min

Increase of extracellular adenosine concentration derived from decreased ATP level, not taking into account adenosine uptake:

(ATP decrease/min) / (V_{ex}) = $Flux_{ATP}$
(440 $fmol/10^5$ cells/min) / (0.023 µl) = 19.1 µM/min

Effect of adenosine uptake with Michaelis-Menten kinetics on time-dependent increase ($Flux_{cAMP}$ and $Flux_{ATP}$) of extracellular adenosine concentrations. The extracellular concentration of cAMP- and ATP-derived adenosine (C) will be reach an equilibrium (C_{max}) when the outflow equals the uptake, not taking in account adenosine degradation etc. The rate of gonadotropin-induced increase in extracellular adenosine concentration (V(t)) is described by:

$V(t) = Flux - (V_{max} * C)/(K_m + C)$

$K_m = 16\ \mu M$ $V_{max} = 1.6$ pmol/min (data from paper I)

When $V(t) = 0$ then $Flux = (V_{max} * C)/(K_m + C)$ $C = C_{max}$

$Flux_{cAMP} = Flux = (V_{max} * C)/(K_m + C)$ $Flux_{ATP} = (V_{max} * C)/(K_m + C)$

$C_{max} = 1.2\ \mu M$ $C_{max} = 6.2\ \mu M$

The C_{max} will be reached irrespective of the size of the extracellular space. However, the time required is of course increased with increased equilibrium volume. With the extracellular volume used in the calculations above ($V_{ex} = 0.6 * V_{cell}$), C_{max} will be reached in approximately one minute. If the V_{ex} is increased 10 times C_{max} will be reached in approximately 10 minutes.

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